Submitted to
Food Standards Australia New Zealand (FSANZ)

Date
August 2019

APPLICATION TO AMEND THE AUSTRALIA NEW ZEALAND FOOD STANDARDS CODE: JENNEWEIN 2'-FL IN INFANT AND TODDLER FORMULAS

2'-FUCOSYLLACTOSE FOR USE AS A FOOD INGREDIENT IN INFANT FORMULA AND TODDLER FORMULA

STATUTORY DECLARATION

I, [redacted]

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

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

[redacted]

[redacted]

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ABBREVIATIONS

2'-FL	2'-Fucosyllactose
2-FT	2-Fucosyltransferase
3-FL	3-Fucosyllactose
3'-SL	3'-Sialyllactose
6'-SL	6'-Sialyllactose
6'-SLN	6'-Sialyl-N-acetyllactosamine
aacC1	Genes responsible for the production of the enzyme gentamycine 3'-acetyltransferase conferring resistance to gentamycin
aad1	Genes responsible for the production of the enzyme aminoglycoside adenyltransferase conferring resistance to streptomycin
aadA	aminoglycoside resistance protein, conferring streptomycin/ spectinomycin resistance
acrB	multidrug efflux system protein
ADME	Absorption, distribution, metabolism and excretion
AMU	Atomic mass unit
ATCC	American Type Culture Collection
ble	phleomycin/bleomycin binding protein, conferring zeocin resistance
BVL	Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (German Federal Office of Consumer Protection and Food Safety)
bw	Body weight
C	Celsius
cat	chloramphenicol acetyl transferase conferring resistance to chloramphenicol
CBEC	Cross Border Ecommerce
CCI	Confidential commercial information
CoA	The Parliament of the Commonwealth of Australia
CFDA	China Food and Drug Administration
CF	Control formula
cfu	colony-forming units
CID	collision-induced dissociation
CIP	The Collection of Institut Pasteur
cscA	Genes responsible for the production of the enzyme sucrose-6-phosphate hydrolase
cscB	Genes responsible for the production of sucrose transporter
cscK	Genes responsible for the production of fructokinase enzyme
cscR	Genes responsible for the production of the enzyme transcriptional regulator
d	day
Dhfr	Genes responsible for the production of the enzyme dehydrofolate reductase conferring resistance to trimethoprim
DNA	Deoxyribonucleic Acid
DoH	Australian Department of Health
DOL	Day of life
EC	European Commission
E. coli	*Escherichia coli*
ECCB	Exclusive Capturable Commercial Benefit
EF	Experimental formula
EFSA	European Food Safety Authority
F-	Denotes a bacterial strain that does not carry the F (fertility) plasmid
Fab	Fragment of the monoclonal antibody
FAO	Food and Agricultural Organisation
FOS	Fructo-oligosaccharide

FFDCA	Federal Food and Drug and Cosmetic Act
FSFYC	Formulated Supplementary Foods for Young Children
Fuc	L-fucose
fucI	Fucose-isomerase
fucK	Fuculose-kinase
FSANZ	Food Standards Australia New Zealand
g	Gram(s)
galE	Genes responsible for the production of the enzyme UDP-galactose-4-epimerase
galT	Genes responsible for the production of the enzyme galactose-1-phosphate uridylyltransferase
galK	Genes responsible for the production of Galactokinase
galM	Genes responsible for the production of the enzyme galactose mutarotase
Gas	Galactose
GOS	Galactooligosaccharide
G-CSF	Granulocyte Colony Stimulating Factor
GDP	Guanosine diphosphate
Glc	D-galactose
GlcNAc	*N*-acetylglucosamine
Glu	D-glucose
GLP	Good laboratory practice
GMM	Genetically Modified Microorganism
gmd	Genes responsible for the production of the enzyme GDP-mannose-4,6-dehydratase
GRAS	Generally Recognized As Safe
GRN	GRAS Notification
HBGA	Histo-Blood Group Antigen
Himar	Himar transposase
HMO	Human Milk Oligosaccharide
HPAEC-PAD	High-performance anion-exchange chromatography with pulsed amperometric detection
Ig	Immunoglobulin
IGF	Insulin-like Growth Factor
IL	Interleukin
IPTG	isopropyl ß-D-1-thiogalactopyranoside
ISO	International Organization for Standardization
JECFA	Joint FAO/WHO Expert Committee on Food Additives
Jennewein	Jennewein Biotechnologie GmbH
Kg	Kilogram
KGF	Keratinocyte Growth Factor
L	Litre(s)
LNFP	Lacto-*N*-fucopentaose
LNnT	Lacto-*N*-*neo*tetraose
lacY	Genes responsible for the production of the enzyme lactose permease
lacZ	Genes responsible for the production of the enzyme β-galactosidase
lacZα	Genes responsible for encoding the LacZ αprotein, a 43 N-terminal amino acid fragment of the LacZ β-galactosidase
lacZΩ	Genes responsible for the production of encoding the LacZ Ω fragment of LacZ β-galactosidase
LC	Liquid chromatography
LDFT	Lactodifucotetraose or difucosyllactose
LNP	Lactase non-persistence

manB	Phosphomannomutase
manC	Mannose-1-phosphate guanosyltransferase
mg	milligram
mL	millilitre
mol	Mole
mM	Milli molar
MRM	multiple reaction monitoring
MRSC	Mean rank stool consistency
MS	Mass spectrometer
MW	Molecular Weight
m/z	mass-to-charge ratio
Neu5Ac	sialic acid N acetylneuraminic acid
NDA	Dietetics, Product Distribution and Allergies
NHMRC	Australian National Health and Medical Research Council
NMR	Nuclear Magnetic Resonance
NOAEL	No-observed-adverse-effect level
NTG	*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine
Nts	nucleotides
nptII	Neomycin-phosphotransferase II conferring kanamycin resistance
NZ MoH	New Zealand Ministry of Health
OECD	Organization for Economic Co-operation and Development
PBMC	Peripheral blood mononuclear cells
PCE	Polychromatic erythrocytes
ppm	Part per million
pgbA	p-guanidinobenzoic acid
qPCR	quantitative Polymerase-Chain-Reaction
RNA	Ribonucleic Acid
RSV	Respiratory syncytial virus
SCFA	Short-chain fatty acids
Sh ble	bleomycin resistance protein conferring resistance to zeocin
SLN	Sialyl-*N*-acetyllactosamine
Tn5	Tn transposase
TNF	Tumour necrosis factor
µg	Micro gram
UDP	Uridine diphosphate glucose
UNICEF	United Nations Children's Fund
US FDA	United States Food and Drug Administration
UV	Ultra Violet
UV/P	UV followed by penicillin selection
VEGF	Vascular Endothelial Growth Factor
wbgl	a novel bacterial α1,2-fucosyltransferase
wcaG	Genes responsible for the production of the enzyme GDP-L-fucose synthase
wbgl	Genes responsible for the production of the enzyme 2 fucosyltransferase
WHO	World Health Organisation

3.1.1 GENERAL REQUIREMENTS

B. Applicant Details

Table 1: Applicant Details

Requirement	Details
Applicant Name	Jennewein Biotechnologie GmbH
Name of Contact Person	Dr Stefan Jennewein
Address	Maarweg 32 D-53619 Rheinbreitbach, Germany
Telephone Number	+49 (0) 2224 989 4501 / +49 (0) 173 159 0918 (mobile)
Email Address	Stefan.jennewein@jennewein-biotech.de
Nature of Applicant's Business	Research and development. Manufacture of rare functional sugars for a range of applications including nutritional, pharmaceutical and cosmetic products.
Details of other individuals, companies or organisations associated with the application	Ramboll US Corporation Dr Gavin Thompson (+1 602 734 7704) gthompson@ramboll.com

C. Purpose of Application

The application **must** contain a statement regarding the purpose of the application. To the extent possible, the application should identify existing food regulatory measure(s) that need to be varied to achieve the intended purpose of the application. For applications that relate to a matter dealt with under Chapters 3.2–3.7, the purpose of the application relevant to any particular guidelines **must** be provided.

Jennewein Biotechnologie GmbH (Jennewein) is seeking permission to amend Schedule 26 (Food produced using gene technology) of the Australia New Zealand Food Standards (FSANZ) Code (the Food Standards Code) to include Jennewein's 2'-Fucosyllactose (2'-FL) substance for use as an ingredient in infant formula (from birth to <12 months of age), follow-on formula (from 6 months to <12 months of age), infant formula products for special dietary use, and formulated supplementary foods for young children (FSFYC) i.e. toddler formula (1 – 3 years of age). Toddler formula for children aged 1 – 3 years meets the definition of FSFYC as stated in FSANZ Standard 2.9.3 Division 1[1]). Jennewein 2'-FL meets the definition of a "food produced using gene technology" as it is "a food which had been derived or developed from an organism which has been modified by gene technology" as described in Section 1.1.2-2 of Standard 1.1.2 of the Australia New Zealand Food Standards Code.[2] The organism in this case is an *E. coli* strain engineered to synthesise 2'-FL. As the commodities currently listed in Schedule 26 (as of August 2019) do not apply to 2'-FL produced via microbial fermentation, it is possible that a new category would need to be added to Schedule 26 to accommodate this type of food derived from a genetically modified processing aid. Jennewein intends to market 2'-FL as an ingredient to be added to infant formula and follow-on formula at a level of 2.0 g/L, as consumed, in both liquid and powdered form (**Table 2**).

1 http://www.foodstandards.govt.nz/code/Documents/2.9.3%20Supp%20foods%20v157.pdf
2 http://www.foodstandards.gov.au/code/Documents/1.1.2%20Definitions%20v159.pdf

Table 2: Proposed Uses and Use Levels for Jennewein 2'-FL in Australia and New Zealand

Category in the FSANZ Code	Infant Formula Category	Use level in Formula, 3As Consumed
Standard 2.9.1 – Infant formula products	Infant formula, powder or liquid	2 g/L
	Follow-on formula, powder or liquid	2 g/L
	Infant formula for special dietary use	2 g/L
Standard 2.9.3 – Formulated meal replacements and formulated supplementary foods Division 4 – Formulated supplementary foods for young children	Toddler formula, powder or liquid	2 g/L

To achieve this purpose, we anticipate that the existing food regulatory measures that may need to be varied:

- Standard 2.9.1 (Infant formula products)
- Standard 2.9.3, Division 4 (Formulated supplementary foods for young children)
- Schedule 3 (Identity and purity)
- Schedule 26 (Food produced using food technology)
- Schedule 29 (Special purpose foods)

In this Application, the Jennewein substance will be referred to as "Jennewein 2'-FL" to distinguish it from naturally occurring 2'-FL.

The full chemical name of 2'-FL is α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranoside. This is often abbreviated as:

1. α-L-Fuc-(1→2)-β-D-Gal-(1→4)-D-Glc; or
2. Fuc-α-1,2-Gal-β-1,4-Glc; or
3. 2'-FL

The naturally occurring substance is also commonly known as 2'-fucosyllactose or 2'-fucosyl-D-lactose.

This application is made under a number of Guidelines outlined in the Food Standards Australia New Zealand Application Handbook (1 March 2016, updated July 2019), in order to provide all of the required information to enable FSANZ to carry out its assessment of Jennewein's 2'-FL:

- **Section 3.1.1 General Requirements** (all Subsections)
- **Section 3.3.3 Substances Used for a Nutritive Purpose** (Subsections A, B, C, D, F, G)
- **Section 3.5.1 Foods Produced by Gene Technology** (Subsections A, B.1, B.3, B.5, C, D)
- **Section 3.6.2 Special Purpose Food – Infant Formula Products** (all Subsections)
- **Section 3.6.3 Special Purpose Food – Other Foods**

D. Justification for the Application

The application **must** provide information to indicate why a food regulatory measure is proposed. Such information may, depending on the purpose of the application as outlined according to requirements in Section C of this Guideline (3.1.1), include:

(a) the need for the proposed change

(b) the advantages of the proposed change over the status quo, taking into account any disadvantages.

The application must also contain details of the status of similar applications made in other countries by the applicant, if applicable.

a. The need for the proposed change

Based on consultation with FSANZ, Jennewein understands that 2'-FL is defined according to legislation in Australia and New Zealand as a food produced by gene technology and as a nutritive substance.

As demonstrated in this Application, Jennewein's 2'-FL substance:

- is chemically and structurally identical to the naturally occurring 2'-FL found in human milk (**Section 3.3.3 A.1**) which has shown to provide optimum nutrition and immunity benefits and has a history of safe consumption by infants (**Section 3.3.3 A.1**);
- the processing aid used to manufacture Jennewein's 2'-FL is demonstrated to be non-toxigenic and non-pathogenic lacking the genes necessary for invasion, adhesion and the enterotoxins necessary for pathogenicity (**Section 3.5.1** and **Appendix S**) and the *E. coli* B strain has a history of 100 years' of safe use (**Section 3.5.1 A.2**); and
- has been used in infant formula products sold in numerous countries since 2016; with Jennewein 2'-FL levels up to 2 g/L per serving (**Section 3.3.3 D.5**);
- is subjected to a purification process to eliminate the *E. coli* bacteria, which removes virtually all traces of endotoxins, recombinant DNA, host proteins and other carbohydrates **(Section 3.3.3 B.5**);
- has been shown to be non-toxic and non-genotoxic (**Section 3.3.3 C.2**); and
- has been shown to provide health benefits including the physiological, biochemical and functional benefits have been demonstrated (**Section 3.3.3 A.1** and **C.1**).

Therefore, it is anticipated that approval of Jennewein 2'-FL as a food produced using gene technology and a nutritive substance to be used in infant formula, follow-on-formula, infant formula products for special dietary use and formulated supplementary foods for young children (i.e. toddler formula) will benefit consumers and industry in Australia and New Zealand by providing a superior quality infant formula product that more closely aligns with the composition of human milk. The benefits of 2'-FL are hypothesized to extend past infancy into toddlerhood and thus Jennewein 2'-FL will be beneficial in toddler formula.

Jennewein, therefore, requests amendment to the Food Standards Code to include Jennewein 2'-FL.

b. The advantages of the proposed change over the status quo

Breastfeeding is the preferred method for infant nutrition and is supported and promoted by many Australian and New Zealand organisations and government bodies including:

- The Australian and New Zealand Food Regulation Ministerial Council
- Australian National Health and Medical Research Council (NHMRC, 2012)
- The Australian Breastfeeding Association
- The New Zealand Ministry of Health
- The Parliament of the Commonwealth of Australia (CoA, 2007)
- The Australia & New Zealand Infant Nutrition Council

Human milk contains hundreds of compounds including oligosaccharides. While oligosaccharides are a large component of human milk, they occur, if at all only at very low concentrations in cow's (bovine) milk, the most common milk used for infant formula in Australia and New Zealand.

Most infants and young children have been exposed to 2'-FL because it is a naturally occurring oligosaccharide found in human milk. Naturally occurring 2'-FL is one of the most abundant human milk oligosaccharides (HMOs), present at mean quantities of approximately 2 – 3 grams (g) per litre (L) and making up approximately 20 – 30% of total HMOs (Coulet, 2014; Kunz *et al*, 2000). 2'-FL is an α-1→2 fucosylated lactose derivative represented by the empirical formula of $C_{18}H_{32}O_{15}$ and a molecular weight (MW) of 488.44 g / mole (mol).

The intended benefit of adding Jennewein 2'-FL to infant formula, follow-on formula, and toddler formula is as a nutrient necessary for the body's nutritional and metabolic processes. Consistent with naturally occurring HMOs, Jennewein 2'-FL does not undergo any significant digestion in the upper gastrointestinal tract and serves as a prebiotic for commensal gut bacteria. These bacteria metabolise prebiotics into short-chain fatty acids (SCFA), which are used by colonocytes in energy production and as a stimulant for sodium and water absorption. Refer to **Section 3.3.3 A.1** for further information relating to the positive infant health benefits from consumption of 2'-FL and other HMOs.

The *Australian New Zealand Food Regulation Ministerial Council's* regulation on infant formula products (endorsed 6 May 2011) states that "*the composition of breast milk should be used as a primary reference for determining the composition of infant formula and follow-on formula*" (ANZFRMC, 2011).

Furthermore, the Codex *Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants* (Codex STAN 72-1981, revision 2007) states that "*…other ingredients may be added in order to provide substances ordinarily found in human milk 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 Codex also states that "*The formula shall contain sufficient amounts of these substances to achieve the intended effect, taking into account levels in human milk.*" Therefore, the addition of Jennewein 2'-FL to infant formula and follow-on formula is consistent with these efforts to produce infant formula products with ingredients that match the naturally occurring nutrient composition of human milk. As human milk is also given to toddlers who continue to breastfeed after their first year of life, toddler formula intended to complement other foods would ideally have ingredients that match the naturally occurring nutrient composition of human milk (e.g. Jennewein 2'-FL) as well.

c. Disadvantages of the proposed change over the status quo

There is no disadvantage of the proposed change over the status quo to consumers.

It should be noted that Jennewein's 2'-FL substance will not be manufactured within Australia or New Zealand, and only the finished product (liquid or powder form) will be imported with the intention that infant formula manufacturers will use Jennewein's 2'-FL in their products.

d. Similar applications made in other countries

Jennewein has made similar successful applications in the United States, the European Union, Canada, and other countries. Information regarding these applications is provided below.

United States

In 2014, Ramboll (formerly known as ENVIRON and Ramboll ENVIRON), on behalf of Jennewein, convened a panel of experts that are suitably qualified by scientific training and experience to evaluate the safety of substances added to food (known as the "Expert Panel"). The Expert Panel independently and critically evaluated the identity, manufacturing process, specifications, estimated dietary exposure and published information supporting the safety of Jennewein's 2'-FL. This information was provided in a 'Safety Dossier' prepared by ENVIRON (ENVIRON, 2014a). Based on this review, the Expert Panel concluded that 2'-FL produced in accordance with good manufacturing practices is self-determined to be "Generally Recognized as Safe" (GRAS) under the conditions of intended use as an ingredient in infant and toddler formulas (ENVIRON, 2014b). The proposed maximum target use of Jennewein's 2-FL in infant and toddler formulas is 2 g/L of formula as consumed; which was considered to correspond to the mean concentration of 2'-FL naturally found in human milk.

The same Expert Panel also independently and critically evaluated the safety and GRAS status for Jennewein's 2'-FL for use as an ingredient in foods for all ages in milk-based beverages and specific cereals, at a target daily intake of 10 g of Jennewein's 2'-FL per kilogram of body weight per day. The Expert Panel unanimously concluded that 2'-FL produced in accordance with good manufacturing practices is "Generally Recognized as Safe" (GRAS) under the conditions of intended use as an ingredient in foods for all ages in milk-based beverages and specific cereals

(ENVIRON, 2015). A copy of the Expert Panel's review and conclusions are provided in **Appendix A**.

Furthermore, in September 2015 the Expert Panel unanimously concluded that the proposed uses of Jennewein 2'-FL in medical foods and as dietary ingredients in supplements are self-determined to be GRAS following an independent evaluation of information provided in a Safety Dossier prepared by Ramboll Environ (2015a, b). A copy of the Expert Panel's review and conclusions are provided in **Appendix A**.

The 'Safety Dossier' and Expert Panel's review for use of Jennewein's 2'-FL as a food ingredient in infant and toddler formulas were provided to the United States Food and Drug Administration (US FDA) in March 2015. Following a review of this information, the US FDA designated the notice as GRAS Notice No. GRN 000571, and concluded that "*the agency has no questions at this time regarding Jennewein's conclusion that 2'-FL is GRAS under the intended conditions of use*". A copy of US FDA's letter, dated 11 June 2015, is provided in **Appendix A**.

To date, the Expert Panel's review and 'Safety Dossier' for use of Jennewein 2'-FL in all ages milk-based beverages, specific cereals, medical foods and dietary supplements have not been submitted to the US FDA for review.

European Union

Analyse & Realise GmbH was engaged by Jennewein to prepare a 'Safety Dossier' for the pre-market approval of Jennewein's 2'-FL as an ingredient in infant formula at concentrations of up to 2 g/L (Analyse & Realise, 2014). On 4 August 2014, this information was presented to the competent authority of the Netherlands to request the use of Jennewein's 2'-FL in powder and liquid form on the European Union market as novel food ingredient for consumption by the infant population. On 3 June 2016, the Committee on Safety Assessment of Novel Foods in the Netherlands concluded that Jennewein's 2'-FL powder and liquid concentrate meets the criteria for novel food (NFU, 2016). This document was subsequently provided to the other Member States for their review on 13 June 2016.

Following a review of the information and discussions regarding the intake levels of 2'-FL proposed by Jennewein, the European Food Safety Authority (EFSA) authorised the placing on the market of Jennewein's 2'FL produced with *Escherichia coli* strain BL21 (DE3) as a novel food ingredient under Regulation No 258/97 of the European Parliament and of the Council. The authorised food category was "*Infant formulae and follow-on formulae*", and the authorised maximum level was "*1.2 gram 2'-fucosyllactose per litre final product ready for use marketed as such or reconstituted as instructed by the manufacturer*." A copy of the European Union authorisation letter is provided in **Appendix B**.

Canada

Under Division 28 of Part B of the *Food and Drug Regulations*, manufacturers or importers of novel foods in Canada are required to submit a pre-market notification to Health Canada regarding the product in question so that a determination can be made with respect to the product's safety prior to sale. To determine that foods containing 2'-FL are safe at the proposed use levels, Jennewein engaged Ramboll ENVIRON to conduct a safety assessment and prepare a safety dossier for 2'-FL for these proposed uses (Ramboll ENVIRON, 2017a,b).

In 2017, Ramboll Environ, on behalf of Jennewein, submitted a Novel Food Notification (NFN) to Health Canada (Health Products Food Branch) for 2'-FL for use in infant formulas for term infants and toddler formulas and in milk-based beverages and foods and specific cereals. Health Canada assigned the notification as Submission File #4000740.

For use in infant formulas for term infants and toddler formulas, the proposed use was up to 1.2 grams Jennewein 2'-FL per litre of formula. Based on this intended use, the highest estimated mean intake of Jennewein 2'-FL occurs in infants of 0-5 months and was 1.1 g/day and the estimated intake at the 90th percentile is 1.5 g/d. A small number of infants consume formulas after the first year of life, and formula intakes are lower than intakes by infants 0-5 or 6-11

months of age. The estimated mean and 90th percentile 2-day average intakes of 2'-FL by toddlers, the target consumers of toddler formulas, are 0.7 and 1.2 g/d, respectively.

Health Canada issued a Letter of No Objection to the use of 2'-FL for *E. coli* BL21 (DE3) strain #1540 as an ingredient in formula for term infants at a maximum level of 1.2 g 2'-FL. A copy of the Health Canada authorisation letter is provided in **Appendix C**.

Israel
A petition for Jennewein 2'-FL as a novel food to be used in infant formula was submitted to Israel's Ministry of Health in August 2016 and approved for use in the country in April 2017 at use level up to 2 g/L in infant formulas.

Philippines
An application for Jennewein 2'-FL was submitted to the Food and Drug Administration of the Philippines in March 2017. 2'-FL was registered for use up to 1.2 g/L in infant formula with the FDA in May 2017.

Singapore
An application for Jennewein 2'-FL was submitted to Singapore's Agri-Food and Veterinary Authority in April 2017 and approved for use up to 1.2 g/L in infant formulas in Singapore in August 2017.

Other Applications
An application for Jennewein 2'-FL is currently under review by the Malaysian Ministry of Health, Food Safety and Quality Division for use up to 1.2 g/L in infant formula in Malaysia.

Other Applications in Australia and New Zealand
At the time this Application was prepared, it is our understanding that no infant formula or follow-on formula products on the Australia and New Zealand market contain the oligosaccharide 2'-FL. We are however aware of Glycom's application (Application A1155) to FSANZ on 14 November 2017 to amend The Food Standards Code (Food Standard 1.5.1 Novel Foods) to include their 2'-FL compound and lacto-*N*-*neo*tetraose (LNnT).

e. Any consumer choice issues related to the proposed change

There are no anticipated impediments of consumer choice related to the proposed change.

As stated by DoH (2012), the Australian consumer market has greater access to information and has higher expectations towards product quality and protection. Consumers also have increased scepticism towards marketers and company profits which have led to greater demands for product safety. These expectations have been accompanied by greater health consciousness and concerns about food composition. Consequently, given the improved health benefits infant formula will have with the addition of Jennewein's 2'-FL (refer to **Section 3.3.3 A.1** of this Application), we anticipate that there will be positive benefits to consumer choice relating to infant formula products.

f. Any public health and safety issues related to the proposed change including details of target groups and population groups that may be adversely affected

No public health and safety issues are anticipated related to the proposed change.

g. Any evidence that the food industry generally or other specific companies have an interest in, or support, the proposed change

There has been significant interest from infant formula researchers, clinicians, and nutritionists in the incorporation of human milk oligosaccharides into infant formula for a variety of reasons. Potential customers (IF manufacturers and marketers worldwide) have expressed interest in the use of Jennewein's 2'-FL ingredient in their infant formula products on the Australian and New Zealand (ANZ) markets. IF manufacturers and marketers also may use Australia and/or New Zealand as a manufacturing base for infant formula products containing 2'-FL.

D.1 Regulatory Impact Information

The application must include current information and data on the following costs and benefits:

D.1.1 Costs and benefits of the application
This may include:
(a) the cost and benefits to the consumer e.g. health benefits
(b) the costs and benefits to industry and business in general, noting any specific effects on small businesses e.g. savings in production costs
(c) the costs and benefits to government e.g. increased regulatory costs.
D.1.2 Impact on international trade

D.1.1 Costs and Benefits of the Application

Australia and New Zealand Infant Formula Market Overview
Table 3 presents the companies that manufacture, import and/or export infant formula into Australia based on information obtained from the Australian Government Department of Health (DoH, 2012), FSANZ (2011) and publicly available information current as of May 2018.

Table 3: Australian Importers and Manufacturers of Infant Formula

Company	Manufacture in Australia	Importer	Exporter	Product(s)
Abbott Australasia Pty Ltd	X	√	X	Similac
Bayer Australia Ltd	X	√	X	Novalac
H J Heinz Company Australia Ltd	X	√	X	Nurture
Aspen Nutritionals Australia	X	√	X	Alula, S-26, S-26 Gold, SMA
Nestle Australia Ltd	X	√	X	NAN
Nutricia Australia Pty Ltd (Danone)	X	√	X	Karicare, Aptamil
Bellamy's Organic Formula	√	X	X	Bellamy's Organic
Murray Goulburn Cooperative Co Ltd	√	X	√ (China)	Natrastart
Milk Powder Solutions (Golden Dairies)	√	X	√ (China)	Baby Beauty
Formula One Gold	√	X	√ (China)	Formula One
Infant Formula Australia	√	X	√ (Asia and Middle East)	No brand
Victorian Dairy Pty Ltd	√	X	√ (China and Hong Kong)	No brand
Snowbrand	√	X	√ (Japan)	No brand

Company	Manufacture in Australia	Importer	Exporter	Product(s)
Tatura	√	X	√ (Global)	Produces infant formula on specification to other companies such as OzCare
Pinnacle Laboratories	√	X	√ (Global)	Pinnacle Infant Formula
Nutura	√	X	X	Nutura
RoyalAustNZ	√	X	√ (Global)	ROYALAUSNZ premium Gold infant formula
Munchkin Inc	X	X	√ (Global)	Grass fed milk-based infant formula
Nataplex	√	X	√ (Global)	Nataplex Infant Formula
OzCare (Australian Dairy Park)	√	X	√ (Global)	Ozcare infant formula
Goat Nutrition Australia	√	X	√ (New Zealand and China)	Oli Infant formula
Baby Gro	√	X	√ (China)	Baby Gro infant formula
Sunwild farm	√	X	X	Nutrawiz infant formula
The A2 Milk Company	X	√	X	A2 Platinum

Table 4 presents the companies that manufacture, import and/or export infant formula into New Zealand based on information obtained from publicly available information current as of May 2018 and Coriolis (2014). It is noted that there are a "…*huge number (100+) of smaller "pure play" firms selling and marketing infant formula from New Zealand*" (Coriolis, 2014) that are not included on **Table 4.**

Table 4: New Zealand Manufacturers and Exporters of Infant Formula

Company	Manufacture in NZ	Exporter	Product
Fontera	√	√ (Australia and global markets)	Annum
Dairy Goat Co-operative	√	√ (Australia, Asia, Europe)	Karicare, DG, Karihome, Capri care, Golden Goat, Nanny Care, Bambichen
Danone Nutricia	√	√ (Australia and global markets)	Karicare, Aptamil
Ocean Dairy (Yili)	√	√ (produced for export to Yili in China)	Bes-Kido, Pro-Kido, Pure-Nutra and Tofer (Yili products)
Synlait	√	√ (Australia & global market)	Produces infant formula on specification to companies such as A2 Milk Company
Tatura	√	√	Tatura (private label)
Westland Milk Products	√	√ (Australia, Asia)	Westpro Nutrition

Company	Manufacture in NZ	Exporter	Product
NIG Nutritionals (New Image)	√	√ (Global)	Baby Steps, Symbiotics (goat milk formula)
Yashili	√	√ (Primarily China)	Infant formula base powder, Ambery, Besdom, Newwit, Ewell, Super α-Golden.

As indicated in **Section 3.1.1, D.g** of this Application, other infant formula manufactures have expressed interest in the Australian and New Zealand markets. Of those companies listed on **Table 3** and **Table 4,** Abbott, Bayer, HJ Heinz, Nestlé, Nurticia and Pfizer are all multinational, diversified companies that collectively account for the majority of infant formula sales in Australia and New Zealand (DoH, 2012).

In 2011, FSANZ published the *Regulatory Impact Statement: Policy Guideline for the Regulation of Infant Formula Products* (FSANZ, 2011). Results from the market analysis undertaken valued the domestic consumption of infant formula products in 2008/2009 in Australia to be AUD$133 million and in New Zealand to be NZ$40 million. The value of imports into New Zealand in 2008 was approximately NZ$4.3 million (value for duty) and of exports approximately NZ$192 million. Similar data were not available for Australia, but it was reported that the majority of infant formula products are imported to Australia from Europe or from New Zealand.

Costs and Benefits Analysis

A costs and benefits analysis of Jennewein's 2'-FL Application is provided in **Table 5**.

Table 5: Costs and Benefits Analysis

Benefits	Costs
CONSUMER	
Due to advances in food technology, Australian manufacturers are increasingly adding new substances to infant formula with the intent of generating additional health benefits or making the composition more similar to breast milk (FSANZ, 2012; DoH, 2012). The addition of Jennewein 2'-FL to infant formula products in Australia aligns with the aim of producing infant formula that contains components similar to that of human breast milk. Consumers (infants and toddlers) are likely to benefit due to availability of such infant formula that contain Jennewein 2'-FL due to parents' and infant care providers' consumer inclinations toward infant and toddler formula which contains nutritional substances that closely align with human milk (refer to **Section 3.1.1 D.(e)** of this Application). Infants and toddlers who consume formula with Jennewein 2'-FL will receive positive health benefits (such as physiological, biochemical and functional effects) as demonstrated in **Sections 3.3.3 C.1 and 3.3.3 A.1** of this Application.	Most infant and toddler formula brands have both a 'standard' and 'premium' range of infant formula products. The typical difference between brands is that 'premium' products contain added optional ingredients such as lutein and omega-3 fatty acids, which in turn command a higher price for the product (FSANZ, 2011). Based on recent advertisements, the price of 'standard' infant formula is approximately AUS$20/800g and 'premium' infant formula is approximately AUS$24 to AUS$30/800g.* Therefore, consumers (parents and care providers who then pass onto the ultimate consumer, infants and toddlers) who do not have the financial means to purchase 'premium' products that more closely align with breast milk may likely to be disadvantaged. Jennewein 2'-FL as an ingredient in infant formula products is not expected to have any negative impact on consumers. The safety of the ingredient for this use is demonstrated (**Section 3.3.3 C**) and Jennewein 2'-FL is currently being used for this purpose in other countries (**Section 3.3.3 D.5**).

Benefits	Costs
INDUSTRY	
It is anticipated that inclusion of Jennewein 2-FL in the Food Standard Code will positively benefit the financial returns of manufacturing companies which contain Jennewein 2'-FL due to consumer demand for products that more closely align with human milk. As stated by FSANZ (2011), there is no established process to substantiate the significance of additional substances, such as Jennewein 2'-FL, in the normal growth and development of the infant formula market. Within Australia and New Zealand, supermarkets and pharmacies comprise the primary distribution channels for infant formula. Such retailers actively use discount incentives for selling infant formula products which further impedes the ability to estimate the financial benefits of 'premium' formula products (DoH, 2012). The addition of 2'-FL to infant and toddler formula will diversify infant and toddler formula products and encourage innovation and growth in manufacturing infant and toddler formula products that better reflect the composition of human breast milk to ultimately better serve the consumer.	Infant and toddler formula manufacturers uninterested in incorporating Jennewein 2'-FL into their product may not be able to expand their product line as effectively as manufacturers selling infant formula products with Jennewein 2'-FL substance.
GOVERNMENT	
As mentioned in **Section 3.1.1 D.g**, several multinational infant and toddler formula companies have expressed interest in Jennewein 2'-FL and/or using Australia/New Zealand as a manufacturing base. There is likely to be an increase in potential economic gains to Australia and New Zealand because of an increase in infant formula product innovation on the domestic market. In addition, international demand for infant and toddler formula products that closely align with breast milk is likely to increase export duty from manufacturers in Australia and New Zealand. The Cross-border e-commerce (CBEC) import channel allowing products, such as infant formula, to be sold directly to online consumers attracting government tax revenue.	As stated in **Section 3.1.1 (I)** of this Application "*…Jennewein intends to pay the full cost of processing this Application*" to amend the Food Standard Code to include Jennewein's 2'-FL. Therefore, no costs to the Australian or New Zealand governments are expected from the addition of Jennewein's 2'-FL in the Food Standard Code. The incorporation of Jennewein 2'-FL into infant formula products should have no substantial impact on agencies.

Note: *Accessed online (May 13, 2019): https://www.amcal.com.au/brands/our-nestle-nan-range.

D.1.2 Impact on International Trade

According to Kent (2015), there has been little growth in formula sales in high-income countries in recent years with the major growth seen in so-called "emerging economies" where the middle- and high-income sectors are growing. Coriolis (2014) states that China is driving world growth for baby food/infant nutrition sales, growing at +50% more than the rest of the world combined.

Chinese demand for high-quality infant formula has increased substantially over the past 10 years owing to China's large population (>1.3 billion) and the melamine tainting incident in 2008 which caused the Chinese population to become wary of Chinese-made formula. Wu and Chen

(2016) identified that Chinese mothers aged between 22-35 years are more concerned with the quality and regulatory environment of infant formula in China. Consequently, the Chinese demand for imported formula product increased particularly from countries such as Australia and New Zealand, which require a rigorous food registration process to ensure public safety and that the nutritional benefits (physiological, biochemical and functional effects) of '*optional*' ingredients are sufficiently demonstrated. In 2015, China imported approximately 300 thousand tons of milk powder, accounting for 40% of the total Chinese market (Chen and Wu, 2016).

Therefore, the addition of Jennewein 2'-FL to infant and toddler formula manufactured in Australia and New Zealand is anticipated to have a positive impact on international trade, particularly to the Chinese market where demand for high-quality infant formula is high.

In particularly, positive growth for international trade is anticipated for New Zealand. Fonterra is based in New Zealand and is considered to be the largest exporter of dairy products which supplies the basic milk powder for most infant formula (Coriolis, 2014). According to Fonterra, their dairy products account for 25% of New Zealand's exports and they have over 30 manufacturing sites across New Zealand.

Furthermore, it is understood that three of the four largest dairy companies in China (Yili (greenfields), Mengniu (greenfields) and Bright (Synlait)), have invested in infant formula plants in New Zealand. Coriolis (2014) states that "*There has been significant investment in the* [New Zealand] *industry over the past decade – in the order of $900m to $1b – and exports continue to grow.*"

The addition of Jennewein 2'-FL to the Food Standards Code is therefore anticipated to positively benefit international trade, particularly from New Zealand which is the manufacturing base for many infant formula companies which will have the option to include food ingredients such as Jennewein 2'-FL to their infant and toddler formulas.

E. Data Requirements

On 15 December 2017, Ramboll and Jennewein met with FSANZ in Canberra to discuss the draft application requirements. Based on the information received at that meeting, Ramboll prepared a draft application that was submitted to FSANZ for review in July 2017. FSANZ subsequently received Glycom's application (A1155) to approve 2'-FL and LNnT in November 2017. FSANZ published a 1st Call for submissions for A1155 in November 2018 and a 2nd Call for submissions in July 2019. Ramboll and Jennewein had a teleconference call with FSANZ in December 2018 and an in-person/teleconference meeting in July 2019 to further discuss the information requirements for the final application. Based on these discussions and the information presented in the 1st and 2nd Call for submissions papers for A1155, as well as A1155 itself, it is our understanding that the information to meet the requirements specified in the following guidelines of the FSANZ Application Handbook is necessary:

- Guideline 3.1.1 General requirements (all Subsections)
- Guideline 3.3.3 Substances used for a nutritive purpose (all Subsections)
- Guideline 3.5.1 Foods produced using gene technology (all Subsections)
- Guideline 3.6.2 Special purpose food, infant formula products (all Subsections)
- Guideline 3.6.3 Special purpose food, other foods (all Subsections)

In addition, Ramboll also has provided the following information as supporting documents:

- Appendix A – Information relating to Jennewein's US FDA GRAS application for 2'-FL
- Appendix B – Information relating to Jennewein's ESFA application for 2'-FL
- Appendix C – Information relating to Jennewein's Health Canada application for 2'-FL
- Appendix D – Certificate of analyses for E. coli BL21 (DE3) competent cells from Novagen
- Appendix E – Evaluation of antibiotic resistance genes by qPCR (CONFIDENTIAL)
- Appendix F – Product description and composition of Jennewein 2'-FL concentrate and powder
- Appendix G – Specifications of reference human milk 2'-FL
- Appendix H – Identification of 2'-FL by NMR
- Appendix I – Comparison of Jennewein 2'-FL to 2'-FL from human milk
- Appendix J – Patents
- Appendix K – Manufacturing process (CONFIDENTIAL)
- Appendix L – *E. coli* production medium composition
- Appendix M – Batch Data and Residuals and Contaminants Analyses
- Appendix N – Certificates of analysis for Jennewein 2'-FL concentrate in powder
- Appendix O – Stability testing results and protocols
- Appendix P – Toxicological studies with Jennewein 2'-FL
- Appendix Q – Certificates of analysis for the 2'-FL used in the Marriage *et al*. (2015) study
- Appendix R – *E. coli* genome constructs (CONFIDENTIAL)
- Appendix S – Genetic modification details to create processing aid for the production of 2'-FL (CONFIDENTIAL)
- Appendix T – Gene sequences of genome constructs (CONFIDENTIAL)
- Appendix U – Genetic stability study of *E. coli* strain (CONFIDENTIAL)
- Appendix V – Gene sequences of *E. coli* strains (CONFIDENTIAL)
- Appendix W – Stability of 2'-FL in infant formula (CONFIDENTIAL)
- Appendix X – References

A literature review was undertaken to provide supporting information for this Application. The United States National Library of Medicine National Institutes of Health Pubmed database was searched on August 6, 2019, limited to publications since 1990, as part of this review.

Key search words for the database search included (but were not limited to):

- 2'-fucosyllactose
- 2'-fucosyllactose AND formula
- 2'-fucosyllactose AND fed infants
- 2'-fucosyllactose AND breastfed
- 2'-fucosyllactose AND infants
- 2'-fucosyllactose AND infant
- 2'-fucosyllactose AND adult
- 2'-fucosyllactose AND toddler
- 2'-FL

In total, 189 citations were identified from these searches and additional citations were retrieved from targeted searches. The full reference list of articles identified from the desktop literature review is provided in "Bibliography of Additional Literature." Articles and reports referred to in this Application are listed in the Reference list at the end of the Application and copies of the articles are provided as **Appendix X**.

F. Assessment Procedure

If related to a variation to the Code, the application **must** provide details as to what an applicant considers is the appropriate procedure to be adopted in assessing the application i.e. general, minor, major or high-level health claim variation. This is a requirement under paragraph 22(2)(e) of the FSANZ Act. As a matter of practice, FSANZ has regard to the applicant's suggestion, but makes its own determination on the process to be adopted. The process to be adopted by FSANZ will be communicated to FSANZ in accordance with Section 27(c) of the FSANZ Act.

Based on feedback provided by FSANZ during a meeting held at their Canberra office on 15 December 2017, it is understood that this Application is likely to fall under the 'Major Category'.

G. Confidential Commercial Information (CCI)

Any information that the applicant considers to be CCI must be identified as CCI.

Jennewein would like to keep information confidential relating to the metabolically engineered *E. coli* (BL21) DE3 which is used as a processing aid in the manufacture of 2'-FL. This information is provided in **Appendix E, Appendix K, Appendix R, Appendix S, Appendix T, Appendix V,** and **Appendix W** of this Application.

Please note that **Appendix D** referenced **in Section 3.5.1 A.3** of this Application is not considered CCI.

Section 4 of the *Food Standards Australia New Zealand Act 1991* defines CCI 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."*

The information contained within **Appendix E**, **Appendix K, Appendix R, Appendix S, Appendix T, Appendix U, Appendix V, and Appendix W** of this Application is considered to be CCI as defined by the FSANZ Act 1991 since Jennewein invested considerable time and financial resources to develop a patented technology (**Appendix J**) to manufacture the chemically equivalent 2'-FL substance.

H. Other Confidential Information

Applications **must** identify all non-CCI information that the applicant wishes to be treated as confidential.

Jennewein does not wish to identify any part of this application as confidential information other than as stated in **Section 3.1.1 G**.

I. Exclusive Capturable Commercial Benefit (ECCB)

The applicant should indicate whether or not the application is expected to confer an exclusive capturable commercial benefit. The applicant should provide a justification for their assertion to assist FSANZ in making a decision.

As discussed with FSANZ on 15 December 2017, this Application, if approved, is likely to result in an amendment to the Food Standards Code that provides Exclusive Capturable Commercial Benefits (ECCB) to Jennewein. Therefore, Jennewein intends to pay the full cost of processing this Application. The justification for providing ECCB is provided in **Table 6** below.

Table 6: Justification for ECCB Application

Consideration	Justification
Why are you making this application? What are you hoping to get out of its approval?	As outlined in **Section 3.1.1 D**, there is a large market in Australia and New Zealand for the manufacture of infant formula products both for use in Australia and New Zealand and for export to other countries, most notably China. Increasingly, parents of babies that consume infant formula are seeking to buy higher quality products that more closely resemble the composition of human milk. Australia and New Zealand manufacturers of infant formula are generally perceived and recognised as producing higher quality products that are attractive to the Chinese market, as well as the local market. The conferring of ECCB will assist Jennewein to establish early access to the Chinese market relative to other competitors that may result in greater returns on the investment Jennewein has committed to developing the new ingredient, Jennewein's 2'-FL.
How will you benefit from the approval of your application?	If ECCB is conferred on Jennewein 2'-FL, then that potentially provides Jennewein the capability to market to more infant formula manufacturers in Australia and New Zealand and gain early access to the Chinese market.
Who besides you, will benefit from the approval of your application? How and why will they benefit?	The manufacturers that purchase Jennewein's 2'-FL will be able to market their products as higher quality, premium products that attract higher returns and potentially greater market shares in the local and export markets, particularly China.
If your application is approved, whose permission will be required before anyone can derive a benefit from that approval?	The technology used to synthesise Jennewein's 2'-FL is patented by Jennewein (**Appendix J**) and, therefore, other parties will need to enter into a commercial agreement with Jennewein for access to the technology or a supply agreement to import Jennewein's 2'-FL into Australia and New Zealand.
Who holds the intellectual property in the subject matter of your application?	Jennewein holds the intellectual property rights for the information relating to Jennewein's 2'-FL that is presented in this application. Ramboll holds the intellectual property rights for the report.

J. International and Other National Standards

J.1 International Standards

The application **must** contain details of any Codex Alimentarius Commission (Codex) Standards relevant to the application.

Relevant Codex Alimentarius Commission Standards relevant to this Application include the *FAO/WHO Codex STAN 72-1981 Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants. Revision 2007*.

In comparison to Food Standard Code (Standard 2.9.1 – Infant Formula Products; and Standard 29 – Special Purpose Foods), FSANZ has identified several inadequacies in Codex STAN 72-1981

(FSANZ 2016) for linoleic acid, iron and selenium, and WHO (2017) for sugars and salt. However, these inadequacies do not relate to 2'-FL or the principal components of infant formula.

Of relevance to this Application are the following Sections from Codex STAN 72-1981:

- Section 3.2.1 (Optional Ingredients) "*…other 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 the outcomes of populations of breastfed babies*."
- Section 3.2.2 (Optional ingredients) "*The suitability for the particular nutritional uses of 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*".

In addition, the *FAO/WHO Codex CXS 156-1987 Standard for Follow-Up Formula, Revision 2017* (FAO/WHO, 1987) is of relevance to this Application, in particular requirements of Section 3.3.2 (Optional ingredients):

- Section 3.3.2.1 "*In addition to the vitamins and minerals listed under 3.2.4 to 3.2.6, other nutrients may be added when required to ensure that the product is suitable to form part of a mixed feeding scheme intended for use from the 6th month on*."
- Section 3.3.2.2 "*The usefulness of these nutrients shall be scientifically shown*."
- Section 3.3.2.3 "*When any of these nutrients is added, the food shall contain significant amounts of these nutrients, based on the requirements of infants from the 6th month on and young children*".

The *FAO/WHO Codex CAC/GL 8-1991 Guidelines on Formulated Complementary Foods for Older Infants and Young Children, Revision 2017* (FAO/WHO, 1991) is of relevance to this Application regarding FSFYC:

- Section 4.2 (Other Ingredients): "*Other ingredients… may be used to improve the nutritional quality and/or acceptability of the Formulated Complementary Foods provided that they are readily available and have been proven to be suitable and safe for their intended purpose*"

J.2 Other National Standards or Regulations

The application should contain details of relevant standards or regulations in other countries with comparable regulatory processes, where available.

Of the infant formula products available in Australia and New Zealand, a significant proportion originate from companies based in the EU (FSANZ, 2011). This Application contains the details of US and EU regulations which have comparable regulatory processes; and an overview of the new Chinese registration process which came into effect in October 2016.

United States

In the United States, infant formula is regulated under Section 412 of the Federal Food and Drug and Cosmetic Act (FFDCA) and the US Food and Drug Administration's (FDA) implementing regulations in Title 21 of the Code of Federal Regulations (21 CFR). Special purpose infant formulas are defined in Section 4.12(f)(1) of the Infant Formula Act and are regulated by 21 CFR 107 subpart C. Relevant parts of 21 CFR are:

- 106 Infant formula requirements pertaining to current good manufacturing practice, quality control procedures, quality factors, records and reports, and notifications.
- 107 Infant formula
- 170 Food additives

Substances added to infant formula need to be approved as either a food additive or regarded by the US FDA as GRAS. For a substance to be recognised as GRAS there must be a consensus among qualified experts that the scientific data and information support the safety of the substance under the conditions of intended use. Manufacturers apply for GRAS status by

providing comprehensive supporting documentation of safety to the US FDA for their evaluation and decision of no objections which is usually given as "no further questions at this time".

The notification to US FDA needs to include evidence that the formula will provide adequate nutrition for infants to thrive.

Section 3.1.1 D.d of this Application, outlines the process Jennewein undertook to obtain GRAS status for the Jennewein 2'-FL oligosaccharide.

European Union

In European Union (EU) Member States, infant formula products are regulated under national legislation giving effect to European Commission (EC) Directive 2006/141/EC (EC Directive, 2006). For 'optional ingredients', Article 5 of the EC Directive requires that *'suitability for the particular nutritional use by infants…. be established by generally accepted scientific data.'*

Furthermore, the EC Directive provides that *'such suitability shall be demonstrated through a systematic review of the available data relating to expected benefits and to safety considerations as well as, where necessary, appropriate studies, performed following generally accepted expert guidance on the design and conduct of such studies.'*[3]

In practice, the EC Directive requires that the consumption of a substance by infants must be demonstrated to have a clear link to a specific health benefit for infants if it is to be approved for use in infant formula products in the EU (FSANZ, 2012).

The EC Directive also establishes that "*it is appropriate to set out specific conditions for the use of nutrition and health claims concerning infant formulae*" and notes that it is "*necessary … to define the conditions under which nutrition and health claims are authorised, and to establish a list of authorised claims.*"[4] A list of authorised nutrition and health claims is set out in Annex IV of the Directive.

China

On 6 June 2016, the China Food and Drug Administration (CFDA) announced the *Administrative Measures for the Registration of Recipes for Formula Powder Products for Infants and Young Children (CFDA Decree 26)*, which entered into force on 1 October 2016. The Measures provide requirements and procedures for registration of infant formula recipes. An unofficial translation of the CFDA Decreed 26 is provided by Clever and Ma Jie (2016), which identified the following items in the relevant to this Application:

- The Measures will be used to strictly regulate the registration of recipes for formula powder products for infants and young children to ensure quality and safety of such products.
- The registration of recipes for infant formula produced and distributed in China and imported into China are subject to provisions of these Measures.
- Registration of infant formula recipes should be science-based, strict, open, fair and just
- Chapter VI "Supplementary Provisions", Article 48 states that the "recipe" mentions in this Measure "*…refers to food materials and food additives and their dosage used in producing formula powder for infants and young children, and contents of nutrients in the product*".
- An application form for the registration of infant formula recipes is completed and submitted to the Acceptance Agency of the Chinese Food and Drug Administration (CFDA). The application form contains the following information:
  - Information about the applicant
  - Applicant's credential documents
  - Quality and safety standards for raw materials and auxiliary materials
  - Reports about research and development of the recipe
  - Description of production process
  - Test report of the product
  - Evidencing documents of capacities for R&D, production and testing

3 EC Directive 2006/141/EC, Article 5. Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:401:0001:0033:EN:PDF

4 EC Directive 2006/141/EC, (26)

- Other documents evidencing science basis and safety of the recipe

The CFDA will make their conclusions based on the registration application, an on-site verification report, and a product testing report.

K. Statutory Declaration

The application **must** contain a signed statutory declaration that includes the following statements:
1. The information provided in this application fully sets out the matters required.
2. The information provided in this application is true to the best of my knowledge and belief.
3. No information has been withheld that might prejudice this application, to the best of my knowledge and belief.

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

L. Checklist

The application **must** contain completed checklists for all relevant guidelines with regard to format and information requirements relevant to the application (see Appendix 1).

A completed checklist is provided in on **Page 7** of this Application.

3.3.3 SUBSTANCES USED FOR A NUTRITIVE PURPOSE

A. Information on the use of the nutritive substance

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

The application **must** state all of the purpose(s) of the use of the nutritive substance in food. If such a substance has multiple purposes or functions then these must all be briefly described.
When the purpose for using a nutritive substance in food (including special purpose foods) relates to a nutritional purpose to deliver a potential beneficial physiological or health-related outcome, the application **must**:
(a) Include a brief description of all of the physiological or health-related function(s) of the substance at the proposed level
(b) Be stated in a way that can be measured i.e. as an outcome in clinical studies

Introduction

Humans are exposed to fucosylated oligosaccharides such as 2'-FL and 3-FL (3'-fucosyllactose) while nursing as infants. Statements provided by the Australian National Health and Medical Research Council (NHMRC) indicate breastfeeding is important for the nutrition, immunological protection, growth, and development of infants and toddlers, and notes breastfeeding is the normal and unequalled method of feeding infants (NMHRC, 2012). The same group recommends breastfeeding until 12 months of age and to continue as long past one year of age as the mother and child wish (NMHRC, 2012). The World Health Organization (WHO) and United Nations Children's Fund (UNICEF) recommend exclusive breastfeeding for the first six months of age and continued breastfeeding with complementary foods up to two years of age (WHO/UNICEF, 2003). Human milk is recommended as the first food for infants because it provides optimum nutrition and immunity benefits and reduces instances of disease later in life such as asthma, type 1 diabetes, and childhood leukemia (Agostoni *et al*., 2009; Field, 2005; Kunz *et al*., 1999; van Rossum *et al*., 2005). Many epidemiological studies also suggest that infants who are exclusively breastfed have improved cognition later in childhood (Deoni *et al*., 2013). Other studies indicate that breastfeeding influences the gene expression profile in the infant intestine, and it is likely that dietary factors provided by the mother's milk contribute to this differential gene expression (Chapkin *et al*., 2010).

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

The basic composition of human milk is summarised in **Table 7**.

Table 7: Composition of human milk 3 days and 36 days post-partum (Kunz et al., 1999)

Time	Protein (%)	Fat (%)	Lactose (%)	Oligosaccharides (%)	Total Solids (%)
Colostrum (3 days)	1.0	3.0	5.5	2.4	-
Mature (36 days)	1.0	3.0	6.8	1.3	12.1

Human Milk Oligosaccharides

More than 100 years ago, a bifidogenic effect or bifidus factor in human milk was identified as an oligosaccharide fraction, which is now known as the human milk oligosaccharide (HMO) fraction.

The number of species of bifidogenic bacteria in the faeces of infants correlates with the content of oligosaccharides in human milk. The faecal microbiota of infants fed on breast milk with a high HMO content is much more diverse compared to infants fed on milk with a lower HMO content (Coppa *et al*., 2004; Wang *et al.*, 2015). The microbiota of exclusively breast-fed infants comprise up to 90% bifidobacteria, whereas formula-fed infants develop a microbial community comprising approximately 50% bifidobacteria, but also bacteria from the genera Bacteroides, Clostridium, Enterococcus and Staphylococcus (Donovan *et al*., 2012). It is likely that the neutral HMO fraction plays a pivotal role in the development and maintenance of the microbiota typical of breast-fed infants.

Over 200 different HMOs have been identified and the structures of at least 85 have been characterised (Goehring *et al*., 2014; Thurl *et al*., 2010; Barile and Rastall, 2013). HMOs bearing sialyl groups are classified as acidic oligosaccharides, whereas the unmodified core structures and fucosylated HMOs are neutral. In human milk, 80-85% of the oligosaccharides are neutral, and 15-20 % are acidic (Thurl *et al*., 2010). 35-50% of HMOs are fucosylated, 12-14% are sialylated, and 42-55% are nonfucosylated neutral HMOs (Smilovitz *et al.*, 2014 via Hegar *et al.*, 2019). HMOs are saccharide-based polymers consisting of the following monomers:

- D-glucose (Glu),
- D-galactose (Glc),
- *N*-acetylglucosamine (GlcNAc),
- L-fucose (Fuc) and
- the sialic acid (*N*-acetylneuraminic acid (Neu5Ac)).

The synthesis of HMOs follows a basic blueprint (Bode and Jantscher-Krenn, 2012). All HMOs feature a lactose molecule at the reducing end. The simplest HMOs are based on a single lactose molecule, and the galactose residue can be fucosylated (α1–2 linkage) to form 2'-FL or the glucose residue can be fucosylated (α1–3 linkage) to yield 3-FL (**Figure 1**). Alternatively, the galactose can be sialylated (N-acetylneuraminic acid) with either an α2-3 or α2-6 linkage, to generate 3'-sialyllactose or 6'-sialyllactose, respectively.

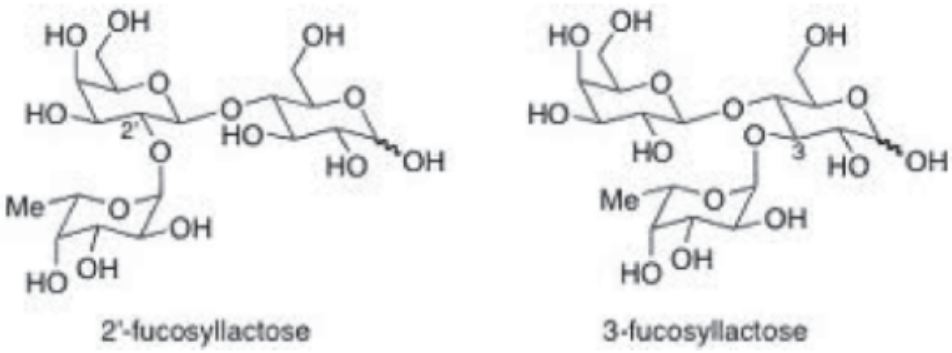

Figure 1: Chemical structures of 2'-FL and 3-FL

The non-reducing end of lactose (galactose) can be elongated and branched with lacto-N-biose or N-acetyl-lactosamine units by β1-3 or β1-4 linkages, respectively, to produce an array of complex oligosaccharides that are classified according to the added carbohydrate structure:

- Type I: lacto-N-biose units (Galβ1-3GlcNAc)
- Type II: N-acetyl-lactosamine units (Galβ1-4GlcNAc).

Complex HMOs may comprise more than 12 of these disaccharide units, comprising 32 monomeric units, which can be connected by 12 different types of glycosidic bonds (Bode and Jantscher-Krenn, 2012; Kunz, 2012; Kunz *et al.*, 2000). Thus far, 13 so-called HMO core structures have been identified, which are the substrates for various fucosyltransferases and sialyltransferases expressed in the mammary gland. The HMO backbone can be modified with one or more fucosyl residues using α1-2, α1-3 and/or α1-4 linkages, or with one or more sialic acid residues using α2-3 or α2-6 linkages (**Figure 2**).

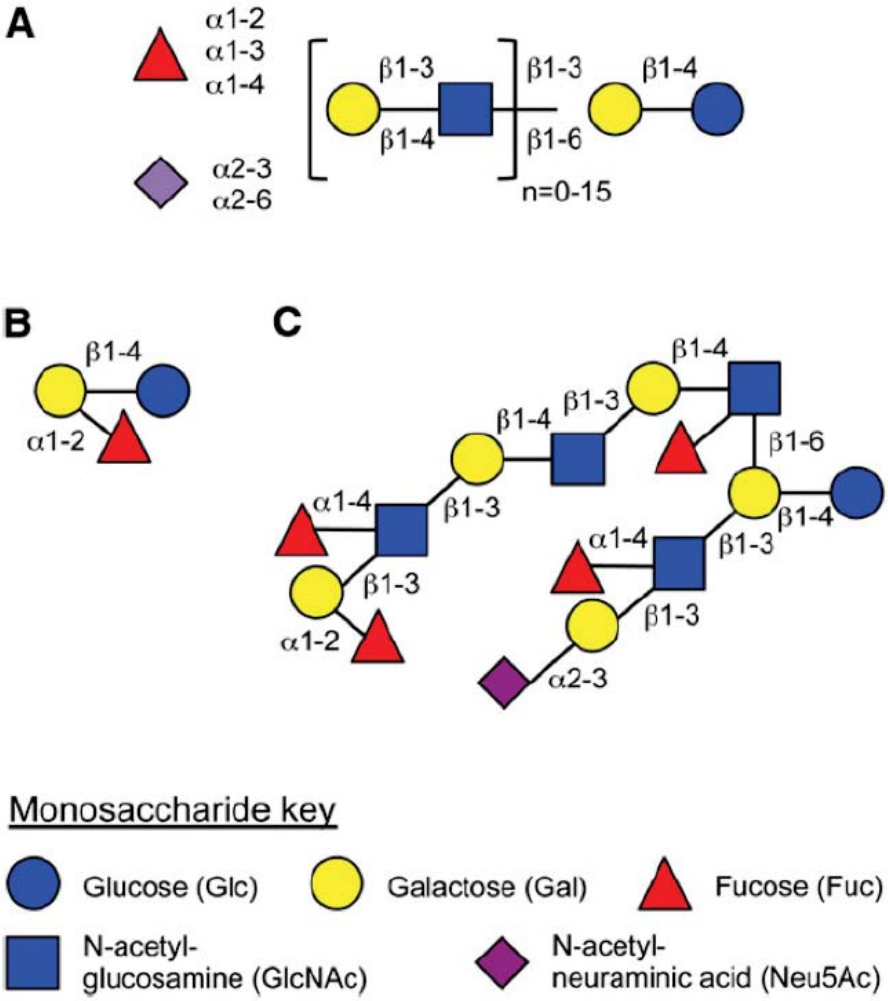

Figure 2: Basic blue print of human milk oligosaccharides (Bode and Jantscher-Krenn, 2012)

A: the elements of HMOs: lactose, lacto-N-biose (Galβ1-3GlcNAc), fucose and sialic acid (N-acetyl neuraminic acid, Neu5Ac).

B: The core structure lactose (*n* = 0) is fucosylated or sialylated, to form simple HMOs such as 2'-FL.

C: Lactose is branched (*n* > 0) and fucosylated and sialylated, to form more complex HMOs such as isolacto-N-decaose

Milk Oligosaccharides from Other Mammalian Species

Besides humans, 2'-FL is also found in the milk of other species of the family Hominidae, which includes chimpanzee, bonobo and orangutans (Castanys-Munoz et al., 2013), as well as other mammals; domestic goat, sheep, and pig milk contains very small amounts of the neutral oligosaccharide though at lower concentrations than human milk (Albrecht et al., 2014) (**Table 8**). Therefore, 2'-FL is likely a very old milk oligosaccharide that has been present throughout the history of humankind.

Table 8: Distribution of 2'-FL in Milk in Animals

Subclass	Order	Family	Common Name
Prototheria	Monotremes	Tachyglossidae	Echidna
Eutheria	Artiodactyla	Bovidae	Domestic goat
		Suidae	Domestic pig
	Carnivora	Ursidae	Ezo brown bear
			Japanese black bear
			Polar bear
		Procyonidae	White-nosed coati
		Canidae	Dog
		Phocidae	Hooded seal
			Artic Harbour seal
			Bearded seal
			Crabeater seal
		Felidae	African lion
		Hyaenidae	Spotted hyena
	Cetacea	Balaenopteridae	Minke whale
		Hominidae	Chimpanzee
			Bonobo
			Orangutan
			Human

Source: based on information presented in Castanys-Munoz *et al*. (2013)

Only 9% of the total oligosaccharide peak area in bovine milk accounts for neutral oligosaccharides, 14 % in sheep milk and 18 % in porcine milk. Of 12 different neutral oligosaccharide structures detected in bovine milk only 3 are fucosylated (Castanys-Munoz *et al*., 2013). This proportion is similar in the milk of other animals such as goat (3/12), sheep (3/12), pig (3/19), horse (2/17) or camel (7/27).

2'-fucosyllactose was found in very small amounts in goat and sheep milk, and in traces in pig and camel milk, but is virtually absent in bovine and horse milk (Albrecht *et al*., 2014). The sialic acid in human milk is always N-acetylneuraminic acid (Neu5Ac), whereas bovine oligosaccharides may also be modified with N glycolylneuraminic acid (Neu5Gc). Therefore, infant formulas based on cow milk or goat milk are devoid of neutral milk oligosaccharides, do not provide substantial amounts of fucosylated oligosaccharides and contain different types of sialic acid (**Table 9**).

Table 9: Main differences between human and bovine milk oligosaccharides

Property	Human Milk	Bovine Milk
Concentration in colostrum	22-23 g/L	1 g/L
Concentration in mature milk	12-15 g/L	10 times less than in bovine colostrum
Structure	Complex	Less complex
Number of known structures	>200 (Garrido *et al*., 2011)	35 (Albrecht et al., 2014)
Dominant type	Type I	Type II
Dominant fraction	Neutral oligosaccharides	Acid oligosaccharides
Main modification	Fucosylation	Sialylation
Sialyl group	Neu5Ac (N-acetylneuraminic acid)	Neu5Ac and Neu5Gc (N-glycolylneuraminic acid)

Endogenous 2'-FL

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

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

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

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

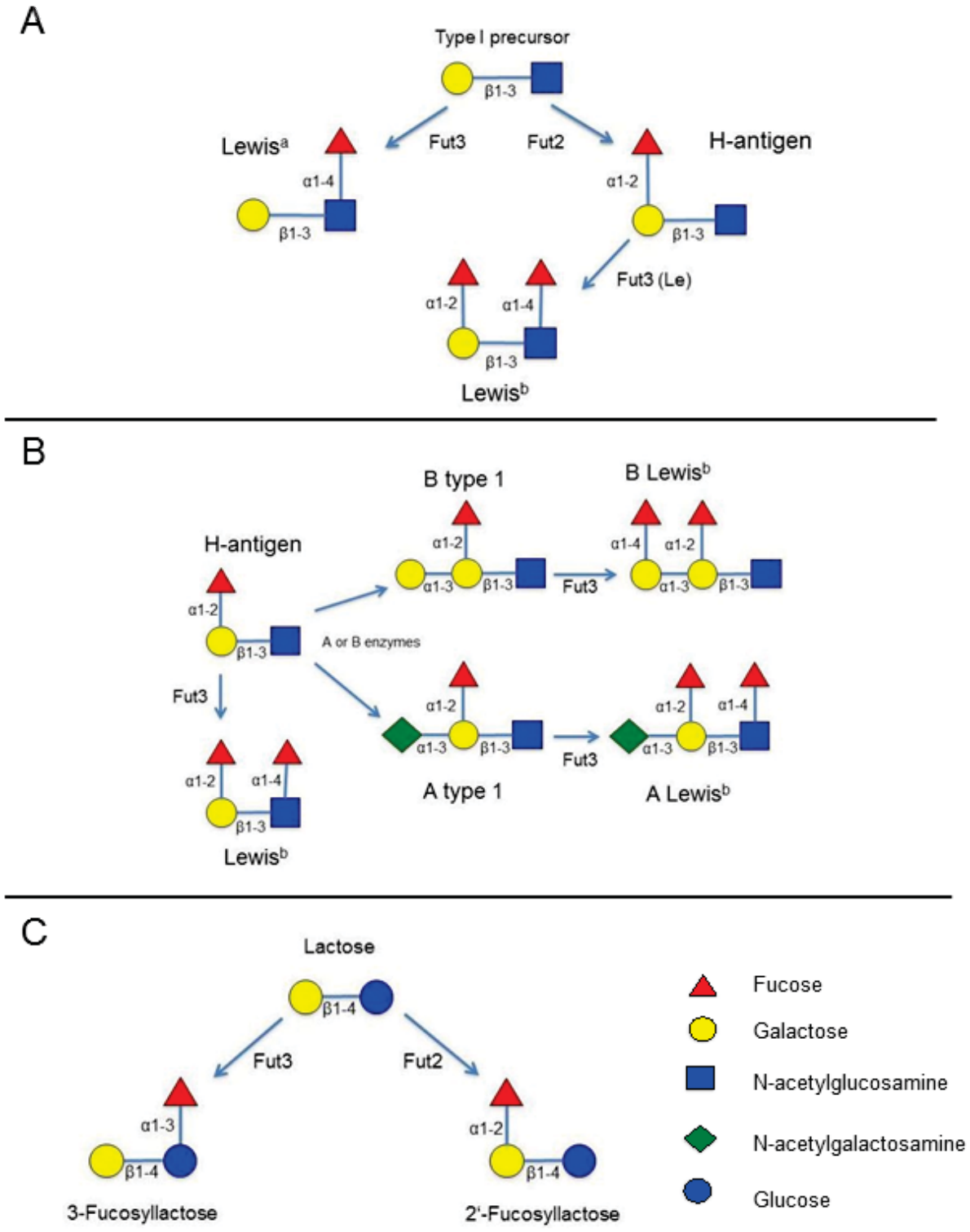

Figure 3: Major biosynthetic pathway of fucosylated ABH and Lewis antigens based on the type 1 precursor disaccharide (adapted from Le Pendu, 2004)

A. Secretor (Fut2) and Lewis-type (Fut3) human blood group antigen (HBGA) based on precursor Type 1.

B. ABO-Type HGBA based on H-type.

C. Synthesis of 2'-fucosyllactose and 3-fucosyllactose from lactose.

The Role of HMOs and Gut Microflora in Infants

A new-born infant has a sterile gastrointestinal tract and breastfeeding is one way the sterile gastrointestinal (GI) tract is colonised by its first microflora (Mackie *et al*., 1999). The gut microflora requires a nutritional substrate to propagate. The substrates are usually oligosaccharides that are not digestible by the upper gastrointestinal tract of the infants. While oligosaccharides are a large component of human milk, they occur only at very low concentrations in cow's milk, the most common milk used for infant formula in Australia (NHMRC, 2012). HMO concentrations vary from over 20 g/L in colostrum to 12 g/L in mature milk (Thurl *et al*., 2010). 2'-FL is present at mean quantities of approximately 2-3 g/L in human breast milk (Coulet *et al.,* 2014), ranging from 1.1 g/L to 3.4 g/L (McGuire *et al.,* 2017) and varies over the course of lactation (Thurl *et al.,* 2017). Because breastfed infants consume at least 0.44 L of breast milk daily, they ingest at minimum several grams of HMO per day (Kent *et al*., 2006). In contrast, bovine milk used in infant formulas contains less than 1 g/L of oligosaccharides (Coppa *et al*., 2004). Compared to formula-fed infants, breastfed infants have a higher concentration of gut bifidobacteria, likely due to the oligosaccharide content in human milk (Coppa *et al*., 2004; Donovan *et al*., 2012; Wang *et al.,* 2015). In the United States, there are numerous publicly-available notifications to the US FDA of plant-derived oligosaccharides such as fructo-oligosaccharides (FOS), galacto-oligosaccharide (GOS), and inulin detailing the role of prebiotics and gut microflora in human nutrition (GRNs 44[5], 236[6], 285[7], 286[8], 334[9], 477[10]).[11] In Canada, Health Canada concluded that Vita Fiber™ isomalto-oligosaccharide derived from plants poses no food safety concerns (Health Canada, 2012).

Intestinal microbiota are responsible for the complex metabolism of HMOs into short-chain fatty acids used for energy by colonocytes, and stimulate sodium and water absorption (Rodricks *et al*., 2007, Engfer *et al*., 2000). The composition of faecal oligosaccharides varies based on the milk group and lactation stage (Albrecht *et al*, 2011a). When human milk serves as the primary food for newborns, neutral and acidic HMOs are present in significant amounts in the faeces of these infants. The 2'-FL concentration and HMO concentration in general varies based on milk stage and the individual mother (Kunz and Rudloff, 2008; Austin *et al.,* 2019; Thurl *et al.*, 2017). The composition of intestinal microflora and faecal HMOs change as the infant transitions to formula or solid foods (Albrecht *et al*, 2011a; Albrecht *et al*., 2011b; Mackie *et al*., 1999). In comparing the fermentation of HMOs of breastfed and formula-fed infants through faecal inoculum, *in vitro* data suggests that 2'-FL and another oligosaccharide, lacto-*N*-*neo*tetraose are fermented rapidly (Vester Boler *et al*., 2013).

For the initial acquisition of microflora, a natural birth provides vaginal and faeces microflora from the mother with some influence of the surrounding environment, whereas infants born via Caesarean section tend to have microflora related to the hospital and attending hospital personnel. Infants that are delivered vaginally have higher concentrations of bifidobacteria as compared to C-section delivered infants (Penders *et al*., 2006). This eventually leads the flora of formula-fed infants to resemble an adult gut containing bacteroides, clostridia, bifidobacteria and a few others (Rodricks *et al*., 2007). After two years of age the gut microflora is typically comparable to that of an adult and may continue to develop as an individual continues through life (Hopkins *et al*., 2002).

5 https://wayback.archive-it.org/7993/20171031033407/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm154122.htm

6 https://wayback.archive-it.org/7993/20171031031448/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm153910.htm

7 https://wayback.archive-it.org/7993/20171031013659/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm185689.htm

8 https://wayback.archive-it.org/7993/20171031035213/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm186158.htm

9 https://wayback.archive-it.org/7993/20171031025954/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm233093.htm

10 https://wayback.archive-it.org/7993/20171031024349/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm361097.htm

11 http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices

Prebiotic effect of 2'-FL and other HMOs

There is consensus that HMOs are prebiotics in infants (Bode, 2006; Boehm and Stahl, 2007; Coppa *et al.*, 2004; Kunz and Rudloff, 2006). Roberfroid (2007) bases the definition of prebiotics on the following criteria:

- resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption;
- fermentation by intestinal microflora; and
- selective stimulation of the growth and/or activity of intestinal bacteria associated with health and wellbeing.

HMOs, including 2'-FL, resist degradation by pancreatic and brush border membrane enzymes (Engfer et al., 2000; Gnoth et al., 2000). One of the most common prebiotics in human milk is the neutral trisaccharide 2'-FL which helps encourage growth of beneficial bacteria (e.g., bifidobacteria) in the infant's intestine (Engfer *et al*., 2000; Marcobal and Sonnenburg, 2012). Among secretor mothers, 2'-FL constitutes almost 30% of the total HMOs among secretor mothers. Categorized as non-secretor mothers, about 20% of mothers secrete lower amounts of HMOs than secretor mothers (Hegar *et al.,* 2019).

In infants, intact HMOs are transported into the lower parts of the intestine where they are either metabolised by the microbiota in the colon or excreted with the faeces. Intestinal commensal bacteria such as bifidobacteria and *Bacteroides* species possess an extensive set of glycoside hydrolases and other specific enzymes that are not present in the neonate intestine, enabling them to metabolise specific types of HMOs (Marcobal and Sonnenburg, 2012). Most studies have focused on the effects on infants due to the presence of 2'-FL in human milk, though others have demonstrated that adult intestines also contain bifidobacteria and *Bacteroides* species (Hopkins *et al*., 2002).

In infants, HMOs provide a substrate for saccharolytic bacteria such as bifidobacteria and lactobacilli, allowing them to dominate the intestinal microbiota and create a more acidic environment that is favourable to the growth of beneficial microbiota (Zivkovic *et al*., 2011). Numerous publications discuss the consumption of 2'-FL by bifidobacteria (Thongaram *et al.*, 2017; Lewis *et al.,* 2015; Yu *et al.*, 2013a; 2013b). The anaerobic incubation of infant faeces-derived bacterial cultures with HMOs as a sole carbon source selectively promoted the growth of bifidobacteria (Yu *et al*., 2013a). The lactate concentration after incubation for 48 hours was higher in cultures with HMOs or FOSs compared to those incubated in basal medium, and the pH was lower (HMOs reduced the pH more than FOS). After 48 hours, the faecal bacteria had consumed more than 90% of the 2'-FL and difucosyllactose (LDFT) and 53% of the 3-FL. *Bifidobacterium infantis (B. infantis)* and a strain of *Bifidobacterium infantis (B. longum)* showed increased growth rates when incubated with HMOs.

In another experiment, Yu *et al.* (2013b) cultivated 25 different intestinal bacteria that were isolated from infant faeces with the most abundant HMOs including 2'-FL, 3-FL, LDFT, 3'-Sialyllactose (3'-SL) and 6'-Sialyllactose (6'-SL). All but one of ten *B. longum* strains tested, and all three *Bacteroides* strains tested, grew on 2'-FL as sole carbon source and consumed more than 40% of the 2'-FL. In some of the *Bifidobacterium* and all tested *Bacteroides* strains, supplementation with 2'-FL resulted in the induction of α(1,2)-fucosidase. Both *Bifidobacterium* species and *Bacteroides* species reduced the pH upon fermentation and growth, but *Bacteroides* species produced less lactate and less SCFA than most of the *Bifidobacterium* species. *Lactobacillus delbrueckii, Enterococcus faecalis* and *Streptococcus thermophilis* showed only modest growth and used only 10-40% of the 2'-FL in the medium. In all 25 bacteria in the test, changes in the fucosidase activity significantly correlated with changes in growth, and changes in organic acid production correlated with changes in pH (Yu *et al*., 2013b).

Marcobal *et al*. (2010) demonstrated that the infant-derived *B. longum* and some strains of *Bifidobacterium breve* (*B. breve)* grow on HMOs as a sole carbon source but less efficiently than *B. bifidus* and *B. infantis,* whereas *Bifidobacterium adolescentis (B. adolescentis)* and some strains of *B. breve* cannot proliferate on HMOs at all. Similarly, HMOs are metabolized by

Bacteroides fragilis (B. fragilis) and *B. vulgatus,* but *B. fragilis* did not use fucosylated HMOs efficiently (Marcobal *et al*., 2010).

The degradation of 2'-FL requires the presence of α(1,2)-fucosidases. Early studies have shown that strains of *Ruminococcus* and *Bifidobacteria*, which were isolated from faeces of healthy adults, produce α-glycosidases with the appropriate specificity to degrade the ABH blood group antigens in mucin. The complete loss of blood group H antigen titers upon incubation with these strains indicates that the bacteria possess α(1,2)-fucosidases, which are also necessary to degrade 2'-FL (Hoskins *et al*., 1985). Katayama *et al*. (2004) cloned an α(1,2)-fucosidase from *Bifidobacterium bifidum* (*B. bifidum)* JCM1254 in *E. coli* and elucidated the enzyme structure and specificity. The enzyme exhibits the highest activity towards 2'-FL, followed by blood group substance H (Fuc-α(1,2)-Gal-β(1,4)-GlcNAc) and Lacto-*N*-fucopentaose I (Fuc-α(1,2)-Gal-β(1,3)-GlcNAc-β(1,3)-Gal-β(1,4)-Glc) (Katayama *et al*., 2004). In addition to these experiments, the presence of α(1,2)-fucosidase in other members of the taxon *B. bifidum* was confirmed by genetic analysis (Turroni *et al*., 2014). *Bacteroides thetaiotaomicron*, a species that appears in the colon at the end of the lactation period is also known to express α(1,2)-fucosidases (Hooper *et al*., 2002). With the complexity of the human microbiota, it is possible that other, yet unidentified species, can hydrolyse the Fuc-α(1,2)-Gal linkage.

In a clinical trial study where 100 adults consumed 2'-FL and/or LNnT daily up to 20 g for 2 weeks along with their regular diet, a dose-dependent increase in bifidobacteria was observed. The increase in relative abundance of bifidobacteria was greater than 25% in some individuals. More details of the study are provided in **Section 3.3.3 C.2** (Elison *et al.*, 2016).

Additional studies show that human milk containing 2'-FL and other HMOs is correlated with high bifidobacterial-dominated gut microbiota in the breast-fed human infant (reviewed in Vandenplas *et al.,* 2018; Lewis *et al.,* 2015; Borewicz *et al.,* 2019). 2'-FL has a demonstrated bifidogenic effect and, in the proposed uses of this Application, would confer this effect on the target population of infants and toddlers consuming formula supplemented with 2'-FL.

Adhesion of pathogens and the anti-infective effect of 2'-FL

Bifidobacteria, especially *Bifidobacterium infantis*, contain gene clusters linked to oligosaccharide metabolism that are only expressed when the bacteria are cultivated with HMOs as a carbon source. This substrate-specific gene expression makes *B. infantis* more adhesive towards undifferentiated cultured intestinal epithelial cells (NT-29) and induces the expression of P-selectin glycoprotein ligand-1. Cultivation in lactose abrogates this effect. Furthermore, both *B. infantis* and *B. bifidum* induce the expression of specific proteins associated with the integrity of the tight junctions in NT-29 and Caco-2 cells, but only if the bacteria are cultivated on HMOs in the absence of lactose (Chichlowski *et al*., 2012).

The intestinal mucosa is covered with complex glycans, glycoproteins, glycolipids, mucins and other carbohydrate-associated structures. To overcome this barrier, bacteria and viruses must bind to specific glycans on the mucosal surface. Bacterial adhesion is a receptor mediated interaction between structures on the bacterial surface and complementary (carbohydrate) ligands on the mucosal surface of the host (Kunz and Rudloff, 2008). Approximately 70% of all human pathogens use cell-surface oligosaccharides as receptors or co-receptors to initiate infection.

HMOs modified with fucose or sialic acid share structural motifs with the glycans found on the surface of the infant intestinal epithelium. Thus, HMOs are thought to act as decoys, by providing soluble homologs or analogs of epithelial cell-surface carbohydrates and pathogen receptors, thus protecting infants against disease (**Figure 4**).

This hypothesis is supported by *in vitro* and *in vivo* observations and reviewed by Vandenplas et al. (2018). The most common cause of bacterial diarrhoea is infection with Campylobacter jejuni, which binds to the intestinal mucosa via the H2-antigen (Ruiz-Palacios et al., 2003). The antigen contains α1,2-fucosylated carbohydrate structures, and binding was inhibited by human milk in an in vitro model (Ruiz-Palacios et al., 2003). Another study demonstrated that 2'-FL in particular

affected *Campylobacter jejuni*-induced inflammation in two human epithelial cell lines and mouse intestinal mucosa (Yu et al., 2016).

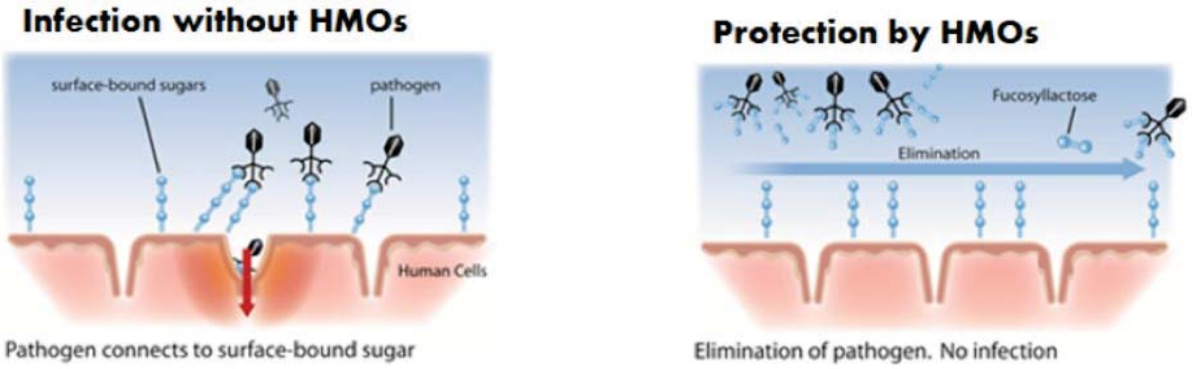

Figure 4: Proposed mode of action of HMOs to prevent infections by bacteria and viruses and to protect cells from toxins after (Bode et al., 2004).

In a study performed by Weichert *et al*. (2013), Caco-2 cells were incubated with bioengineered 2'-FL and 3-FL manufactured by the applicant, human milk, or lactose prior to infection with a number of pathogenic bacteria. At 10 mg/mL, 2'-FL inhibited the adhesion of *C. jejuni*, enteropathogenic *E. coli*, *Salmonella enterica serovar fyris* and *Pseudomonas aeruginosa* by 26%, 18%, 12% and 17%, respectively; and the adhesion of *P. aeruginosa* to the human lung epithelial cell line A549 by 24%. The study demonstrated that bioengineered 2'-FL is capable of interacting with cell and bacteria surfaces, thereby inhibiting pathogen adhesion. When the sugar contents were matched, 2'-FL (10 mg/mL) inhibited the adhesion of *C. jejuni* to differentiated Caco-2 cells to a similar extent as human milk.

The preventative effect of α1-2-fucosylated oligosaccharides was confirmed *in vivo*. The incidence of Campylobacter-associated diarrhoea was investigated in a study with breast-fed infants (*n* = 93, age 0-2 years). The concentration of α1,2-fucosyloligosaccharides in milk collected 1-5 weeks post-partum varied between 0.8 and 20.8 mM (50-92% of the milk oligosaccharides). Infants whose mothers produced higher concentrations of α1,2-fucosylated oligosaccharides suffered significantly fewer episodes of moderate to severe diarrhoea. Campylobacter-associated diarrhoea occurred significantly less often in the infants fed on breast milk containing high levels of 2'-FL, and a lower incidence of calicivirus infections was associated with high levels of lacto-*N*-difucohexaose, another 2-fucosyl oligosaccharide (Morrow *et al*., 2004).

Additional Beneficial Properties of 2'-FL and HMOs

There have been a number of animal studies that have further investigated the beneficial properties of 2'-FL. These studies have shown that oral administration of 2'-FL had a positive impact in animal models of food allergy (Castillo-Courtade *et al*., 2015), necrotising enterocolitis (Autran *et al*., 2016), and recovery after intestinal resection (Mezoff *et al*., 2016). Interestingly, 2'-FL has also been shown to have beneficial impacts on learning and memory (Vazquez *et al*. 2015; 2016; Oliveros *et al*. 2016). An animal study reported that 2'-FL supplementations could have an effect on rotavirus-induced diarrhea (Azagra-Boronat *et al.*, 2019a). Other *in vivo* studies in rodents reported that 2'-FL supplementations correlated with increases in markers of immunomodulatory effect (Azagra-Boronat *et al.*, 2019b) and immune responsiveness in a vaccine model (Xiao *et al.*, 2018; van den Elsen *et al.*, 2019).

Other mechanistic studies *in vitro* and *ex vivo* have been conducted to further elucidate the beneficial effects of HMOs and 2'-FL. This research has demonstrated the immune-modulating potential of HMOs, and includes functional studies using *in vitro* models that have demonstrated that 2'-FL can quench the inflammatory response in cultured human intestinal cells (He *et al*., 2016) and enhance innate immunity to respiratory syncytial virus (Duska-McEwen *et al*., 2014).

In cell culture models, 2'-FL has also shown to be able to inhibit the adhesion of pathogenic bacteria (Coppa *et al.*, 2006; Weichert *et al*., 2013), rotavirus (Laurcirica *et al.*, 2017) and

norovirus virus-like particles (Weichert *et al*., 2016). Additionally, 2'-FL was shown to positively impact the maturation of cultured intestinal cells (Holscher *et al*., 2014) and gut motility (Bienenstock *et al*., 2013).

Stepans *et al*. (2006) conducted a pilot trial in the United States on 49 mother-infant pairs which tested the relationship between HMO consumption, oligosaccharide content of faeces and subsequent disease in breastfed infants. The study concluded that the consumption of HMOs through breastfeeding, represented by lacto-*N*-fucopentaose II (a major milk oligosaccharide), was associated with less reported respiratory and gastrointestinal illness in infants. Kuhn *et al.* (2014) conducted a nested case-cohort analysis of mortality among HIV-exposed, uninfected children in Zambia. The study reported that breastfeeding with higher maternal breast milk concentrations of 2'-FL and other HMOs was protective against mortality to 2 years of age.

FSANZ previously reviewed evidence and opined on the health effects of 2'-FL for the same uses proposed in this Application. FSANZ concluded that the evidence "supports the plausibility of an anti-infective health effect against invasive *C. jejuni* infection and a bifidogenic effect." However, FSANZ concluded that the evidence for health effects associated with immune modulation, improved intestinal barrier function or alleviation of allergic responses was insufficient to make a conclusion supporting those effects (FSANZ 2018).

A.2 General data requirements for supporting evidence

The nutritive substance assessed should be representative of the commercial product on which approval is sought. A statement to that effect **must** be made in the application. If this situation is not the case for any of the relevant studies, then a justification and explanation **must** be provided.
Studies provided as evidence to support an application **must** contain sufficient detail to enable an independent assessment of the methods and results to confirm the study conclusions. The scientific evidence for a potential beneficial physiological or health-related-outcome **must**:
(a) Be based on studies conducted on human subjects
(b) Be based on foods or food groups which contain the nutritive substance rather than the use of the substance alone
(c) Relate to normal use by the target population group and the foods must contribute to the demonstrated nutritional role relevant to that target population

Jennewein confirms that the Jennewein 2'-FL substance is representative of the commercial product on which approval is sought. The scientific evidence for potential beneficial physiological or health-related outcomes presented in the Application is based on human studies and infant formula containing Jennewein 2'-FL, with the focus on infants, being the target population group (see **Section 3.3.3 A.1** for details).

B. Technical information on the use of the nutritive substance

B.1 Information to enable identification of the nutritive substance

This includes the chemical name (according to both Chemical Abstracts (CA) and the International Union for Pure and Applied Chemistry (IUPAC)); structural formula; common name and synonyms; manufacturers' code; marketing name; and CAS registry number. For biologically-derived nutritive substances, the source should be provided.

The Chemical Abstracts Service Registry Number (CASRN) for 2'-FL is 41263-94-9.

The full chemical name is of 2'-FL is α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranoside. This is often abbreviated as:

- α-L-Fuc-(1→2)-β-D-Gal-(1→4)-D-Glc; or
- Fuc-α-1,2-Gal-β-1,4-Glc; or
- 2'-FL

The substance is also commonly known as 2'-fucosyllactose or 2'-fucosyl-D-lactose.

The product will be marketed under the brand name "Jennewein 2'-FL™".

Chemical Structure of 2'-FL
The chemical structure of 2'-FL is identified in **Figure 5**.

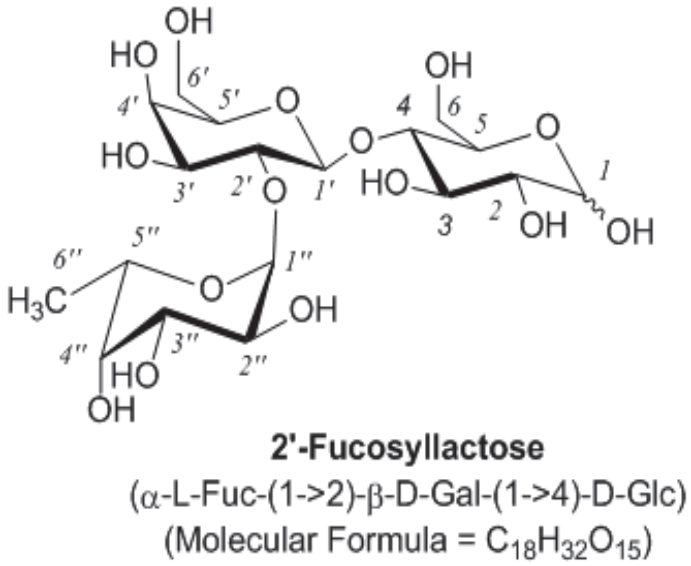

Figure 5: Chemical Structure of 2'-FL

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

This includes detailed chemical and physical properties of the nutritive substance that are important for understanding how the substance is incorporated into the requested food matrices. Specifically, information and data must be provided on how the substance is incorporated in a uniform manner into the food matrices. Studies on the stability of the incorporated substance in particular detailing losses during food processing and storage to the end of shelf life must be provided for the different food matrices.

Jennewein 2'-FL is to be added to infant formula (for infants from birth to <12 months), follow-on formula (for infants from 6 months to <12 months), and toddler formula intended for young children 1 to 3 years of age. Infant and toddler formula manufacturers incorporate Jennewein 2'-FL into infant and toddler formula in one of two ways: wet blending or dry blending. During the wet blending method, 2'-FL is added to a mixture containing all ingredients for the formula and stirred in a tank heated to 60°C so all microorganisms cannot survive. The homogeneous mixture is then spray-dried and packaged as formula powder product or reconstituted to a liquid product. In the dry-blending method, 2'-FL in powder form is added after all other ingredients are mixed and spray-dried. Extensive mixing of the components of formula is employed to ensure that an entire production batch of formula meets the nutritional needs of those consuming it. According to an infant and toddler formula manufacturer currently using Jennewein 2'-FL in their infant formula product, 2'-FL is exceptionally stable. The reconstitution process has no significant effect on 2'-FL levels and the product passes stability tests showing no appreciable losses of 2'-FL (see **Appendix W** for reports).

Jennewein 2'-FL was determined to be equivalent to 2'-FL derived from breast milk. Analytical grade human milk 2'-FL from Sigma-Aldrich (≥ 98% purity; by HPAEC/PAD) was used as the reference material. The chemical and physical properties and specifications for the Sigma-Aldrich human milk isolate 2'-FL product are provided in **Table 10** and **Appendix G**.

The chemical and physical properties of Jennewein 2'-FL are identified in **Table 10**. The product is available as a liquid concentrate and as a spray dried powder. The information contained in **Table 10** and **Table 11** demonstrate the equivalence of Jennewein 2'-FL chemical and physical properties to 2'-FL obtained from human milk.

Table 10: Chemical and physical properties of 2'-Fucosyllactose

Property	Sigma-Aldrich Product	Method
Chemical		
Molecular weight	488.44 AMU	N/A
Exact mass	488.43768 g/mol	N/A
Carbohydrate separation analysis	≥ 98%	HPAEC-PAD
Structural analysis	Conforms to Structure	Proton NMR spectrum
Solubility (Turbidity) 2 mg in 0.2 mL of water	Clear	Visual
Physical		
Appearance (Form)	Powder	Visual
Appearance (Colour)	White	Visual
Solubility in water (Colour)	Colourless	Visual

Sources: Sigma-Aldrich Product Number F0393; PubChem, https://pubchem.ncbi.nlm.nih.gov/

Abbreviations: AMU = atomic mass unit; g = gram(s); HPAEC-PAD = High-performance anion-exchange chromatography with pulsed amperometric detection; L = litre(s); mol = mole; N/A = not available; NMR = Nuclear Magnetic Resonance.

Table 11: Chemical and physical properties of Jennewein 2'-Fucosyllactose powder and liquid concentrate

Property	Powder Product	Concentrate Product	Method
Chemical			
Molecular weight	488.44 AMU	488.44 AMU	N/A
Solubility in water	min. 500 g/L (25 °C)	N/A	Visual
Stability (25 °C/65% humidity)	2 years from production date	6 months from production date	N/A
Physical			
Appearance (Form)	Fine, hygroscopic spray-dried powder	Liquid; clear solution	Visual
Appearance (Color)	White to ivory	Colorless to slightly yellow solution	Visual
Taste	Lactose-like	Lactose-like	N/A
Smell	Neutral	Neutral	N/A

Source: Jennewein Biotechnologie, GmbH

Abbreviations:

°C = degrees Celsius; AMU = atomic mass units; g = gram(s); L = litre(s); N/A = Not Available

Chemical equivalence of Jennewein 2'-FL to naturally occurring 2'-FL

The chemical structure of 2'-FL is identified in **Figure 5**. Absolute and comparative methods were used to confirm the identity of the powdered Jennewein 2'-FL, which is identical in chemical composition and structure to the concentrate product. To confirm that the Jennewein 2'-FL product was comparable in structure to human milk 2'-FL isolate (source: Sigma-Aldrich), proton and carbon nuclear magnetic resonance (NMR) spectroscopy was conducted demonstrating that the Jennewein 2'-FL is substantially chemically equivalent to human milk-derived 2'-FL. Additionally, complete 13C and 1H NMR spectra assignments were made for 2'-FL. **Appendix H** contains the analytical conditions and results of the original NMR spectra. Optical rotation comparisons to published optical rotations from human milk 2'-FL isolates were also conducted. Conclusions and results of the NMR analyses performed on five different batches of Jennewein 2'-FL are presented in **Appendix I-2**.

Analytical data obtained from the analysis of ten independently manufactured batches of 2'-FL powder and 45% concentrate 2'-FL showed that the fermentation process invented by Jennewein for the manufacture of 2'-fucosyllactose, consistently produced 2'-FL (**Appendix M**). To further confirm that the Jennewein 2'-FL product is equivalent to 2'-FL isolated from human milk, Jennewein performed NMR spectroscopic analysis to determine the chemical structure of their 2'-FL product using five independently manufactured batches (**Appendix H**).

LC-MS/MS comparisons between human milk 2'-FL isolates (Sigma-Aldrich) and five lots of Jennewein 2'-FL products were conducted using a Hypercarb chromatography column and are described in **Appendix I-2**. In the analysis using negative electro spray ionisation (ESI) in the multiple reaction monitoring (MRM) mode, 2'-FL samples lost a proton to form the negative molecular ion ($[M-H]^-$) with a 487.2 mass-to-charge ratio (m/z) and a retention time of 15.4 min. Selection and analysis of precursor ions occurred in quadrupole 1. Fragmentation of the molecules occurred in the collision cell, using argon as the collision-induced dissociation (CID) gas, and selection of the fragmentation ions was performed in quadrupole 3.

To illustrate the identical specific fragmentation pattern of five batches of Jennewein 2'-FL in comparison to human milk 2'-FL isolate, a product ion scan of the main compounds was performed in a separate experiment. As shown in **Appendix I-5**, the chromatographic separation performed using the Hypercarb chromatography column in negative ESI MRM mode yielded identical retention times for the comparison between the five batches of Jennewein 2'-FL powdered product and the human 2'-FL reference. Results of corresponding MS/MS spectra in negative ionisation mode also confirmed the equivalence of the negative ions formed by Jennewein 2'-FL powder to that of the reference 2'-FL from breast milk. Results similar to the observations from the fragmentation patterns produced by the precursor ions in the MS/MS spectra were observed in the MRM analysis presented in **Appendix I-6** and in the product ion scan (**Appendix I-1**), respectively. Observations from these analyses confirmed that the main constituent (≥90%) of the Jennewein 2'-FL powder was equivalent in molecular mass (488.17 g) and chemical structure to the major component (≥98%) of the reference 2'-FL from breast milk.

HPAEC-PAD was also used to confirm the identity of Jennewein 2'-FL in comparison to a human milk 2'-FL isolate. A detailed description of the HPAEC-PAD method used is presented in **Appendix I-3**. The chromatogram revealed comparable retention times for the Jennewein 2'-FL product in comparison to the reference 2'-FL (4.83 min) vs. (4.87 min) respectively, an indication that Jennewein 2'-FL mimics or is identical to 2'-FL from human milk (**Appendix I-4**).

Jennewein 2'-FL also contains small amounts of other oligosaccharides. A comparison of the retention times of the peaks of these trace oligosaccharides to commercially available references suggests that these compounds are 3-FL, LDFT, lactose, fucose and galactose, all of which are natural constituents of human milk, and fucosylgalactose (Asakuma *et al*., 2008; Thurl *et al*., 1996). Fucosylgalactose is a naturally occurring breakdown product of 2'-FL that is formed when the glucose is cleaved from the lactose portion of 2'-FL. Fucosylgalactose is an epitope associated with the H-antigen in humans and has been observed in the urine of humans after oral ingestion of galactose or lactose and after intravenous injection of galactose (Chester *et al*., 1979).

The identity and presence of these trace oligosaccharides was confirmed by LC-MS/MS analysis. **Appendix I-7** provides a description of the chromatographic and MS-detector conditions used in the comparison of Jennewein 2'-FL (lot number 2FL-2014-19-3738) to reference samples of related oligosaccharides (3-FL, fucosylgalactose, LDFT) and human milk 2'-FL isolate reference sample (Sigma-Aldrich) using MRM with positive ESI. A LC triple-quadrupole MS detection system was used to perform the characteristic fragment ion detection mass analysis; precursor ions were selected and analyzed in quadrupole 1 (Q1), with nitrogen as the CID gas used for fragmentation in the collision cell, fragment ion selection occurred in quadrupole 3 (Q3).

A precursor ion of 506.0 m/z (MW. 488 + 18 (NH_4^+)) was used for 2'-fucosyllactose, because carbohydrate adduct formation occurs in the presence of an ammonium ion (NH_4^+), leading to a mass shift of +18. Following the adduct formation, fragment ions fucose (-H) (163.3 m/z), Fuc-Gal (-OH) (309.2 m/z), and lactose (-OH) (325.3 m/z) were obtained by fragmenting the 2'-fucosyllactose ion in the collision cell. Partial fragmentation during ionization resulted in the detection of fucosylgalactose (Fuc-Gal (-OH)), a degradation product of 2'-FL. The retention times, molecular mass of the selected precursor ions, and characteristic fragment ions of the LC-MS/MS analysis for 2'-FL, 3-FL, LDFT and fucosylgalactose with positive ESI in Jennewein 2'-FL, human milk 2'-FL isolate, and the reference standard mixtures are shown in **Table 12**.

Table 12: MRM Analysis for Jennewein 2'-FL, Human milk 2'-FL and Reference Standards

Precursor	LC retention time (minutes)	Q1 Mass [m/z] (Precursor Ion)	Fragment	Q3 Mass [m/z] (Fragment Ion)
2'-FL	14.2	506.0	Fucose (-H)	163.3
			Lactose (-OH)	325.3
			Fuc-Gal (-OH)	309.2
3-FL	13.0	506.0	Fucose (-H)	163.3
			Lactose (-OH)	325.3
			Fuc-Glc (-OH)	309.2
LDFT	14.2	653.0	Fucose (-H)	163.3
			Lactose (-OH)	325.3
			Fuc-Gal / Fuc-Glc (-OH)	309.2
Fuc-Gal	14.0	344.0	Fucose (-H)	163.3

Source: Jennewein Biotechnologie, GmbH

Abbreviations: 2'-FL = 2'-fucosyllactose; 3-FL = 3-fucosyllactose; Fuc = fucose; Gal = galactose; LDFT = difucosyllactose; min = minutes; Q = quadrupole.

Retention time [min] and molecular mass [m/z] of precursor ions and fragment ions as found in MRM analysis with positive ESI in Jennewein 2'-FL, human milk 2'-FL (Sigma-Aldrich) as well as the mixture of reference standards

The 2'-FL samples were compared to a reference standard mixture composed of equal amounts of 3-FL, fucosylgalactose and LDFT. Trace amounts of LDFT were detected in the Jennewein 2'-FL sample and the human milk 2'-FL sample (**Appendix M**). Trace amounts of fucosylgalactose at 14.2 min in the Jennewein 2'-FL originate from the degradation of small amounts of 2'-FL during spray drying. An analysis of 3-FL in all three aforementioned samples resulted in similar characteristic fragment ions; 2'-FL and 3-FL were detected together in all the samples; however, the two fucosyllactoses could be differentiated based on their unique retention times: 3-FL at 13.0 minutes and 2'-FL at 14.2 minutes (**Appendix M**).

Jennewein performed additional analyses to quantify the other oligosaccharides in Jennewein 2'-FL via HPAEC-PAD chromatograms. Calibration curves were established for 2'-FL, 3-FL, LDFT, fucosylgalactose, glucose, fucose and lactose, using raffinose as the internal standard. Although the individual response factors of each available carbohydrate in the HPAEC-PAD were taken into account, the intended specificity of the method was not achieved as intended due to a number of limitations. Notable among these limitations was the unavailability of the moisture content of the reference sugars, due to the scarcity in commercial availability of the reference sugars, hence the manufacturers are unable to provide exact moisture data, and this lack of moisture content data resulted in inaccurate calibration of the reference. Data from the 2'-FL analysis revealed only a very small difference between the concentrations obtained via the specific method in comparison to the direct observations from the area under the curve; however, due to the very small concentration of the other oligosaccharides, their analyses were limited in precision (**Appendix M**). For this reason, the individual sugars were only quantified based on the area under the curve.

Optical rotation analysis was also performed to confirm the equivalence of Jennewein 2'-FL to human milk 2'-FL isolate. An optical rotation [α]λ,T of -57.1° was observed for Jennewein 2'-FL. This observation closely mimics the reported rotation (-57°) from the initial structural determination of the human 2'-FL by Kuhn *et al*. (1955).

2'-FL was identified as the main constituent of the Jennewein 2'-FL product (≥90%) by 13C and 1H NMR. Results of various other analyses performed, also confirmed the chemical equivalence of Jennewein 2'-FL to human milk 2'-FL isolate from Sigma-Aldrich:

- In a comparison between the two aforementioned samples via HPAEC-PAD analysis, identical retention times were observed for the main fraction or sugar component;
- Identical mass spectra, and identical negative and positive fragmentation patterns were observed for the comparison of the main component of the Jennewein 2'-FL and human milk 2'-FL isolate from Sigma-Aldrich; and
- The Jennewein 2'-FL product exhibited the same optical rotation properties as initially reported by Kuhn *et al*. (1955) for human milk 2'-FL isolates.

The physiological equivalence of the bioengineered Jennewein 2'-FL product in comparison to human milk oligosaccharide isolates was illustrated by Weichert *et al*. (2013) through substantiated experiments.

B.3 Information on the impurity profile

This includes details on the nature and amounts (by weight) of all impurities, including isomers and manufacturing by-products, present in the nutritive substance preparation. Where possible, impurities should be identified by their CA or IUPAC names.

The modified *E. coli* is not present in Jennewein 2'-FL, therefore the potential for the *E. coli* to colonise the gut and to transfer genetic material to other gut flora does not exist. Use of Jennewein 2'-FL as a prebiotic does impact intestinal microflora development and function.

Lipopolysaccharides (LPS), also known as lipoglycans or endotoxins, are formed when a lipid molecule adheres to a polysaccharide molecule via covalent bonding. LPS is released after bacterial cell death and lysis. They are extremely immunogenic compounds capable of stimulating the manufacture of endogenous pyrogen interleukin-1 and tumour necrosis factor in animal cells and activating macrophages. LPS are a major component of the cell walls of certain bacterial cells, especially gram-negative bacteria such as *E. coli*, *Klebsiella* sp., *Salmonella* sp., and other *Enterobacteriaceae*. LPS assist in the structural stabilisation of the cell membrane and provide the entire cell membrane with protection from certain chemical exposures/effects. Manufacturing processes such as heat treatment, osmotic stress, chemical additions or irradiation, which could typically lead to the deactivation of live bacterial cells, may not necessarily inactivate LPS in a product. Hence a significant amount of LPS could still remain in a product even after the product is declared free of live bacterial cells.

In the Jennewein 2'-FL product, the presence of LPS would be indicative of substantial bacterial cell wall contamination of the product originating from the *E. coli* BL21 (DE3) strain. The European Union mandates manufacturers to keep the levels in their products as low as possible. Jennewein set an internal standard/specification of ≤ 0.3 endotoxin units (EU)/mg (≤ 300 EU/g) for the Jennewein 2'-FL product, but the LPS content of the product was found to be consistently less than 5 EU/g (**Appendix M**). The unit of EU was established by the USFDA (USFDA Inspections Technical Guides (ITG) Bacterial Endotoxins/Pyrogens[12]) to quantify the pyrogenic effect of an endotoxin in relation to the dose of endotoxin to which a person is exposed. Australia does not currently have limits or regulations governing bacterial endotoxins or pyrogens in infant formula, although it had recommended tests and limits for these substances for the pharmaceutical industry[13]. **Appendix M** contains the batch data for the determination of the LPS content of five independently manufactured batches of Jennewein 2'-FL powder.

The LPS IUPAC name is: (2R,5R)-2-[(2R,5R)-2-[(2R,5R)-5-[(2R,3R,5R)-4-[(2R,3R,5R)-4-[(2R,5R)-4-[(2R,5R)-3-[(2R,5S)-3-[(2R,3S,5S)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-5-[(2R,5S)-4-[(3R,6S)-5-[(2R,5R)-4-[(2R,5R)-3,5-dihydroxy-6-methyloxan-2-yl]oxy-5-hydroxy-6-(hydroxymethyl)-3-[(2S,5R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyoxan-2-yl]oxy-3,4-dihydroxy-6-methyloxan-2-yl]oxy-3,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-4-hydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-4,5-dihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-3,5-dihydroxy-6-[[(2S,5R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxymethyl]oxan-2-yl]oxy-6-[2-[(2S,3R,5S)-5-(1,2-dihydroxyethyl)-3,4,5-trihydroxyoxan-2-yl]oxy-1-hydroxyethyl]-3-hydroxy-5-phosphonooxyoxan-2-yl]oxy-5-[[2-aminoethoxy(hydroxy)phosphoryl]oxy-hydroxyphosphoryl]oxy-6-(1,2-dihydroxyethyl)-3-hydroxyoxan-2-yl]oxy-2-carboxy-6-(1,2-dihydroxyethyl)-2-[[(3S,5S,6R)-5-(3-dodecanoyloxytetradecanoylamino)-6-[[(3S,4R,6S)-3-hydroxy-4-(3-hydroxytetradecanoyloxy)-5-pentadecyl-6-(phosphonomethyl)oxan-2-yl]methoxy]-3-phosphonooxy-4-(3-tetradecanoyloxytetradecanoyloxy)oxan-2-yl]methoxy]oxan-4-yl]oxy-2-carboxy-6-(1,2-dihydroxyethyl)-5-hydroxyoxan-4-yl]oxy-6-(1,2-dihydroxyethyl)-4,5-dihydroxyoxane-2-carboxylic acid.

B.4 Manufacturing process

This includes a description of the method of manufacture of the nutritive substance.

Jennewein 2'-FL is manufactured by Jennewein Biotechnologie, GmbH, Maarweg 32 D-53619 Rheinbreitbach, Germany. Jennewein developed a commercially produced biosynthesised, purified, fermentation product 2'-FL manufactured using a genetically engineered strain of *E. coli* BL21 (DE3) as a processing aid in the production of 2'-FL. Jennewein owns the patents for the manufacture of 2'-FL via fermentation (**Appendix J**). Only food- or pharmaceutical-grade chemicals, solvents, and processing aids (e.g., ion exchange resins, activated carbon and filtration membranes) are used in the manufacture of Jennewein 2'-FL (e.g., ion exchange resins, activated carbon and filtration membranes). No antibiotics, other inhibitors, or inductors are used in the production process. Details of the manufacturing process can be found in confidential **Appendix K**. Jennewein deposited one of the *E. coli* BL21 (DE3) strains (strain #1540) at Leibnitz Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) – German Collection of Microorganisms and Cell Cultures. Other strains will also be deposited at the Collection in the near future.

Jennewein 2'-FL will be produced under International Organization for Standardization food safety management standard ISO 22000. The manufacturing flow scheme describing the fermentation process used to produce Jennewein 2'-FL is presented in **Appendix K**.

The *E. coli* BL21 (DE3) production strains used as a processing aid during the fermentation process in the manufacture of 2'-FL are genetically engineered strains of the commensal

12 https://www.fda.gov/iceci/inspections/inspectionguides/inspectiontechnicalguides/ucm072918.htm

13 https://www.legislation.gov.au/Details/F2007B01293/Download

bacterium *E. coli* BL21 (DE3) (refer to **Section 3.5.1** and **Appendix S** of this Application). The *E. coli* BL21 (DE3) cells were genetically modified by the introduction of genes necessary to achieve the import of lactose and enhance GDP-fucose production, the synthesis of 2'-FL followed by its export into the medium.

The Jennewein 2'-FL manufacturing process begins with a fermentation operation. Batch fermentation is performed in a chemically-defined, salt-based, minimal medium that excludes inhibitors or antibiotics, with glycerol as the only carbon source and lactose as the substrate. Jennewein engineered the *E. coli* BL21 (DE3) production strains to produce large amounts of the nucleotide-activated L-fucose donor GDP-L-fucose. This is the substrate acted upon by the recombinant enzyme 2'-fucosyltransferase in the synthesis of 2'-FL from lactose. This *E. coli* strains also expresses a sugar efflux exporter which aids in the recovery of the synthesised oligosaccharide from the medium by exporting the synthesised oligosaccharide, 2'-FL, into the medium. 2'-FL is then purified from the medium by separation from the cells (biomass) by filtration. The sequential filtration and chromatographic purification procedures result in a 2'-FL product devoid of traces of the bacteria, endotoxins, recombinant DNA, and host proteins.

B.5 Specification for identity and purity

This includes a specification from one of the published sources identified in Schedule 3 – Identity and purity. If a published specification is not available, a detailed specification should be provided.

To ensure that a consistent food-grade product is produced, Jennewein has established specifications for their 2'-FL product. The chemical, physical and microbiological specifications of the product are presented in **Table 15** and **Appendix F**. As a published specification is not available, Schedule 3 "Identity and purity" of the Food Standards Australia New Zealand Act may need to be amended, as stated in **Section 3.1.1 C**.

Nine batches of product were analysed with regard to the chemical and microbiological parameters listed in the specifications. All tested batches for both the powdered product and concentrate met the established specifications demonstrating that the Jennewein 2'-FL product complies with appropriate specifications for food-grade materials and that a consistent product can be and is produced (**Appendix M**). The corresponding certificates of analysis for both products are in **Appendix N**. The methods employed by Jennewein to classify its 2'-FL product, determine and quantify other constituents including degradation products and contaminants of the product are described in this section and associated appendices.

Due to the sensitivity of the method, HPAEC-PAD was used to determine the sugar composition of the Jennewein 2'-FL product. Residual components were also identified in the chromatogram and the individual peaks confirmed with LC-MS. A detailed description of the analytical system conditions is provided in **Appendix I-4**.

Heavy metal analysis conducted on five independently manufactured batches of each of the product versions (powdered and concentrate product), by the Institute for Product Quality (IfPQ), Berlin, Germany, demonstrated compliance with the current Codex general standards for contaminants and toxins in foods (CODEX STAN 193-1995) concerning heavy metal content of foods.[14] In Australia and New Zealand, the maximum level of contaminants and natural toxicants permitted in food products are specified in the Food Standards Code in:

- Schedule 19 (Maximum levels of contaminants and natural toxicants) for total arsenic (0.5-1.0 mg/kg), inorganic arsenic (1-2 mg/kg), cadmium (0.05-2.5 mg/kg), mercury (0.1-1.5 mg/kg) and tin (250 mg/kg), for a range of food products. This schedule states that the maximum permissible concentration of lead in infant formula products is to be 0.02 mg/kg. Maximum permissible concentrations for other metals in infant formula products is not specified.

[14] http://www.codexalimentarius.org/download/standards/17/CXS_193e.pdf

- Standard 2.9.1 (Infant formula products) for aluminium in infant formula products at a maximum permissible level of 0.05 mg/100 mL (lower levels are prescribed for pre-term formula and soy-based formula). The ratio of zinc to copper must be no more than 15:1 for infant formula and 20:1 for follow-on formula.

The heavy metal content of the Jennewein 2'-FL product is below the threshold levels specified in Schedule 19 and Standard 2.9.1 of the FSANZ Foods Standards Code (**Appendix M**).

No difference in 2'-FL concentrations of Jennewein 2'-FL powder was detected in a comparison between the same batches of product tested under standard and accelerated conditions (**Table 13** and **Table 14**). A description of the assay and the study results are provided in **Appendix O**. These observations confirm that the Jennewein 2'-FL powder is stable for at least 104 weeks when stored under standard conditions of 25 °C and 60% humidity, and for no less than six months (26 weeks) at 40 °C and 75% humidity.

A broad range of microbiological analyses were conducted to demonstrate that the Jennewein 2'-FL powdered product meets specifications. Batch analyses are presented in **Appendix M** and Certificates of Analyses are presented in **Appendix O**. Due to the limited production quantities of the concentrate product only two batches were available for analysis. The concentrate 2'-FL product is a direct precursor to the powdered products and is sterile filtered just before packaging. Therefore, it is considered at low risk of process-related microbial contamination. A description of control measures applied to prevent microbial contamination is provided in **Section 3.3.3 B.4** of this Application.

Presence of Naturally Occurring Toxins

The growth of certain moulds results in the production of toxic by-products including a group of chemically related toxins known as aflatoxins. FSANZ specifies the maximum level of aflatoxins in peanuts and tree nuts of 0.015 mg/kg (Food Standards Code, Schedule 19 Maximum levels of contaminants and natural toxicants); but no level is prescribed for infant formula products.

Aflatoxins are classified as B1, B2, G1, and G2 and are recognised liver toxins and carcinogens (USFDA CPG Sec. 527.400 USFDA).[15] The most potent of the group is the aflatoxin designated B1, a common contaminant of corn. Aflatoxin M1, a metabolite of aflatoxin B1 with similar chemical properties, is naturally produced when animals ingest aflatoxin B1. Though less potent than aflatoxin B1, aflatoxin M1 has been associated with liver cancer in certain animals. For this reason, nine unique batches of Jennewein 2'-FL powder were tested for aflatoxin M1 and all batches were <0.025 ug/kg for aflatoxin M1 and <5 Endotoxin Units/g for endotoxins (**Appendix M**).

Allergenic Potential and Lactose Intolerance

The batch data and other analyses presented in **Section 3.3.3 B.5** of this Application demonstrate that Jennewein 2'-FL is consistently devoid of proteins, bacteria or bacterial endotoxins, residual recombinant DNA, antibiotics, and chemical sensitisers including metals, or that they are well below levels of concern. Therefore, the potential allergenicity of Jennewein 2'-FL is extremely low.

Jennewein 2'-FL does contain residual lactose and batch analysis reported up to 0.5% lactose total solids (n = 9) (**Appendix M**). Furthermore, Jennewein 2'-FL may produce lactose when metabolised by gut microbiota.

It should be noted however, that lactose is the primary carbohydrate found exclusively in breast milk. Although rare, lactose intolerance can occur among infants with acute diarrheal disease. The age of lactose intolerance onset and its prevalence differ among various populations with approximately 20% of Hispanic and Asian children younger than five years showing evidence of lactase deficiency and lactose malabsorption; whereas Caucasian children typically do not develop symptoms of lactose intolerance until after four or five years (Heyman, 2006).

[15] http://www.fda.gov/iceci/compliancemanuals/compliancepolicyguidancemanual/ucm074482.htm

Table 13: Specifications of Jennewein 2'-Fucosyllactose powder and liquid concentrate

Parameter	Powder Product Specification	Concentrate Product Specification	Method
Chemical			
Solids content	N/A	45% w/v (± 5% w/v) dry matter in water	Dry weight after freeze-drying
Water content	≤ 9.0%	N/A	Karl-Fischer titration
Protein content	≤ 100 µg/g	≤ 100 µg/g freeze-dried matter	Nanoquant (modified Bradford)
Total Ash	≤ 0.5%	≤ 0.5% freeze-dried matter	ASU L 06.00-4 (a)
Arsenic	≤ 0.2 mg/kg	≤ 0.2 mg/kg freeze-dried matter	ASU L 12.00-06 (a)
Cadmium	≤ 0.1 mg/kg	≤ 0.1 mg/kg freeze-dried matte	ASU L 00.00-19/3 (a)
Lead	≤ 0.02 mg/kg	≤ 0.02 mg/kg freeze-dried matter	ASU L 00.00-19/3 (a)
Mercury	≤ 0.5 mg/kg	≤ 0.5 mg/kg freeze-dried matter	ASU 00.00-19/4 (a)
Aflatoxin M_1	≤ 0.025 µg/kg	N/A	DIN EN ISO 14501
Endotoxins	≤ 300 EU/g	N/A	Ph. Eur. 2.6.14
GMO detection	negative	N/A	qPCR
Carbohydrate content			
2'-Fucosyllactose	≥ 90% (Area)	≥ 90% (Area)	HPAEC-PAD
Lactose	≤ 5% (Area)	≤ 5% (Area)	HPAEC-PAD
3-Fucosyllactose	≤ 5% (Area)	≤ 5% (Area)	HPAEC-PAD
Difucosyllactose	≤ 5% (Area)	≤ 5% (Area)	HPAEC-PAD
Fucosyl-Galactose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Glucose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Galactose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Fucose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Microbiology analysis			
Standard Plate Count	≤ 10000 cfu/g	≤ 5000 cfu/g	ISO 4833-2
Yeast and Mold	≤ 100 cfu/g	≤ 50 cfu/g	ISO 21527-2
Coliform / Enterobacteriaceae	absent in 11 g	absent in 22 ml	ISO 4832 / ISO 21528-2
Salmonella	absent in 100 g	absent in 200 ml	ISO 6579
Cronobacter sakazakii	absent in 100 g	absent in 200 ml	ISO/TS 22964

Source: Jennewein Biotechnologie, GmbH

Abbreviations:

ASU = Official collection of determination methods according to § 64 LFGB; cfu = colony-forming units; DIN EN ISO 14501 = German Institute for Standardization Milk and milk powder - Determination of aflatoxin M1 content - Clean-up by immunoaffinity chromatography and determination by high-performance liquid chromatography; HPAEC-PAD = High-performance anion-exchange chromatography with pulsed amperometric detection; ISO = International Organization for Standardization; ISO/TS = International Organization for Standardization Technical Specifications; LFGB = German Code on food and feed; N/A = Not Available; Ph. Eur = European Pharmacopoeia; qPCR = quantitative polymerase chain reaction.

Table 14: Stability of Jennewein 2'-FL under normal storage conditions*

Duration	Lot 2'-FL-2011-29-05		Lot 2'-FL-2011-29-07	
	Mean (%)	% of Baseline	Mean (%)	% of Baseline
Baseline	87.4	100	86.5	100
Week 1	85	97.3	87.1	100.7
Week 4	90.1	103.1	89.3	103.2
Week 8	91.3	104.5	89.9	103.9
Week 13	92	105.3	91.2	105.4
Week 26	91.1	104.2	90.3	104.4
Week 39	90.8	103.9	84.7	97.9
Week 56	90.1	103.1	84.0	97.1
Week 81	88.5	101.3	85.0	98.3
Week 104	92.8	106.2	92.2	106.6
Mean	89.9	102.9	88.0	101.8
Standard Deviation	2.3	2.7	2.9	3.4

Source: Jennewein Biotechnologie, GmbH

*Temperature = 25 °C; relative humidity = 60%; concentration on a dry weight basis.

Table 15: Stability of Jennewein 2'-FL under accelerated storage conditions*

Interval	Lot 2FL-2011-29-05		Lot 2FL-2011-29-07	
	Mean (%)	% of Baseline	Mean (%)	% of Baseline
Baseline	87.4	NA	86.5	NA
Week 1	85.6	97.9	87.4	101.0
Week 4	88.8	101.6	87.5	101.2
Week 8	87.5	100.1	86.5	100.0
Week 13	90.4	103.4	88.5	102.3
Week 26	89.9	102.9	89.5	103.5
Mean	88.3	101.2	87.7	101.6
Standard Deviation	1.8	2.2	1.2	1.3
Maximum	90.4	103.4	89.5	103.5
Minimum	85.6	97.9	86.5	100

Source: Jennewein Biotechnologie, GmbH

*Temperature = 40 °C; relative humidity = 75%; concentration on a dry weight basis.

B.6 Analytical method for detection

This includes a method for detection and quantification of the nutritive substance or its degradation products in the foods in which it is proposed to be used. The application must include a robust analytical method suitable for analytical laboratories to determine compliance of any limits prescribed in the Code.

The analytical methods for detection of the Jennewein 2'-FL substance and other potential components is detailed in **Section 3.3.3 B.2** of this Application. The analytical method for detection of the Jennewein 2'-FL substance in infant formula was provided by an infant formula manufacturer and is enclosed in **Appendix W**.

B.7 Information of the proposed food label

This includes details of the proposed labelling statements relating to the presence of the nutritive substance in the food.

No change to the infant formula and follow-on formula labelling requirements is anticipated due to the addition of Jennewein 2'-FL to formula sold in Australia or New Zealand. The proposed labelling of infant formula and follow-on formula containing Jennewein 2'-FL will be in accordance with Standard 2.9.1 of the FSANZ Food Standard Code.

Jennewein 2'-FL will be identified on the ingredient list as "2'-fucosyllactose" in all categories of infant formula and follow-on formula products, including infant formula for special dietary use. Jennewein 2'-FL will also be listed on the ingredient list of FSFYC products, i.e. toddler formula, as "2'-fucosyllactose".

C. Information related to the safety of the nutritive substance

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

For an application for use of a new nutritive substance, this includes published reviews or individual study reports on the metabolic fate of the nutritive substance and, if necessary, its degradation products and major metabolites.

2'-FL Toxicokinetics – Absorption, Distribution, Metabolism and Excretion

Several studies have evaluated the absorption, distribution, metabolism and excretion (ADME) of human milk components, including human milk oligosaccharides, though no studies were identified that evaluated the ADME of isolated, non-maternal 2'-FL. The focus on infant formula oligosaccharides has been as substrates for intestinal microflora, though studies have demonstrated that certain HMOs can be absorbed into the blood stream through the intestinal wall and excreted by the kidneys intact (Rudloff *et al*., 2012; De Leoz *et al*., 2013; Goehring *et al*., 2014; Ruhaak *et al*., 2014).

After administering nursing mothers a one-time oral bolus of ^{13}C-labeled galactose, Rudloff *et al*. (2012) observed that it was rapidly transferred into oligosaccharides in the mothers' milk and ultimately detected in the infants' urine for a period of 36 hours after the bolus. Rudloff *et al*. (2012) calculated that 1-2% of the total ^{13}C-labeled HMOs ingested by the infants were available systemically and excreted in urine intact or only slightly metabolised. Rudloff *et al*. (2012) also observed cleavage products of HMO, where the glucose on the reducing end had been cleaved, in the urine of infants receiving the ^{13}C-containing milk. Fucosylgalactose is an example of such a cleavage product. Fucosylgalactose is an epitope associated with the H-antigen in humans and has been observed in the urine of humans after oral ingestion of galactose and lactose and after intravenous injection of galactose (Chester *et al*., 1979).

Goehring *et al*. (2014) demonstrated that less than 5% of ingested 2’-FL is absorbed intact into the circulatory system of breastfed infants resulting in 0.1% in plasma and 4% in urine. 2’-FL was not the only HMO absorbed; the authors also detected other HMOs in urine including 3-FL, LNnT, Lacto-N-fucopentaose (LNFP) I, II, and III, 6’-SL and 6’-Sialyl-N-acetyllactosamine (6’-SLN). The concentrations of 2’-FL, 3-FL and LNnT in urine and plasma correlated with the levels of these oligosaccharides in human milk and the mother’s secretor status (Goehring *et al*., 2014). The authors did not find these HMOs in the urine of formula-fed infants. No studies were identified that evaluated whether HMOs such as 2'-FL are systemically available in adults. If 2’-FL is systemically available it may be excreted via urine as was demonstrated for fucosylgalactose (Chester *et al*., 1979). Hallgren and Lundblad (1977) reported that 2’-FL and other HMOs were present in the urine of pregnant and lactating women that supports the excretion of systemically available 2’-FL via urine.

In a pilot study conducted to determine if human milk oligosaccharides appear in amniotic fluid, the urine and amniotic fluid of pregnant women were collected at birth, as well as their milk at 4 days postpartum (Wise *et al.,* 2018). Results indicated 2’-FL was present in all three tissues and was the most abundant oligosaccharide identified. The presence of 2’-FL in the amniotic fluid indicates infants are exposed to 2’-FL *in utero.* Austin *et al.* (2019) reported that while human milk oligosaccharides are generally comparable in the milk of mothers giving birth preterm and term, significantly lower concentrations of 2’-FL were detected in preterm milk during the first month of lactation compared to term milk. Likewise, De Leoz et al. (2012), reported an inconsistent present of 2’-FL across lactation of several mothers that delivered preterm. The decrease in 2’-FL in milk from mothers delivering preterm suggests an increased susceptibility of infection for breastfeeding premature infants.

Bode *et al*. (2004) demonstrated that sialylated HMO reduced adhesion of monocytes, lymphocytes, and neutrophils isolated from human peripheral blood to tumor necrosis factor (TNF)-α-activated endothelial cells by 50%, *in vitro*. Therefore, certain HMOs may influence leukocyte endothelial cell or platelet interactions (Kunz and Rudloff, 2008; Rudloff *et al*., 2012). Though these effects have been demonstrated *in vitro*, they have not been demonstrated *in vivo* or for neutral HMOs such as 2’-FL.

Like other oligosaccharides such as inulin and FOS, HMOs resist degradation by the salivary glands, the pancreas, and the gastric and small intestinal epithelium brush border and arrive intact in the colon and are metabolised by resident microbiota or excreted with the faeces (Engfer *et al*., 2000; Gnoth *et al*., 2000). HMOs in the neonate intestine provide a substrate for bacteria including *Bifidobacterium* and *Bacteroides* spp. which are capable of metabolising the HMOs using glycoside hydrolases and other specific enzymes (Marcobal and Sonnenburg, 2012; El-Hawiet *et al.*, 2015). In infants, HMOs clearly exhibit prebiotic properties. Regarding adults, there are indications that the microbiota in the mature intestine can metabolize 2’-FL (Hoskins *et al*., 1985) and α-L-fucosidase activity, an enzyme that can metabolise 2’-FL, has been reported in adult faecal samples (Rhodes *et al*., 1985; Hoskins and Boulding, 1981). Hopkins *et al*. (2002) reported that concentrations of bifidobacteria in faeces were highest in infants and lowest in elderly subjects though this may be due to the lack of a substrate such as 2’-FL.

These studies indicate that at least 95% of ingested 2’-FL is directly available to gut microbiota and less than 5% is absorbed intact by infants. 2’-FL absorbed by infants enters the circulatory system and excreted in urine intact or minimally metabolised. Gut bacteria common in adult colons are able to use 2’-FL as a substrate *in vitro*. Gut microbiota readily metabolise the 2’-FL into short-chain fatty acids (Newburg 2013).

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

For an application for the use of a new nutritive substance, this includes published reviews or detailed reports of all in vitro and in vivo studies conducted in animals or humans to examine the toxicity of the nutritive substance and, where necessary, its metabolites or degradation products. The following categories of studies need to be considered:
(a) acute toxicity
(b) short-term toxicity
(c) long-term toxicity and carcinogenicity
(d) reproductive toxicity
(e) developmental toxicity
(f) genotoxicity
(g) special studies such as neurotoxicity or immunotoxicity.
Where data are not available or are not considered relevant to the safety assessment of the nutritive substance, an explanatory statement should be provided.

In vivo *and* in vitro *toxicity studies*

The following categories of animal studies have been carried out to assess the toxicity of synthetic 2'-FL (details below, including a summary in **Table 20**):

- Acute toxicity
  - Pilot study of oral toxicity in rats using Jennewein 2'-FL (see **Appendix P-3**)
  - Pilot study of oral toxicity in rats using 2'-FL not synthesized by Jennewein Biotechnologie (Coulet *et al.* 2014)
- Short-term toxicity
  - Repeated dose study of oral toxicity in rats using Jennewein 2'-FL (see **Appendix P-4** for the study evaluating Jennewein 2'-FL; see Coulet et al. 2014, van Berlo et al. 2018, Penard et al. 2015 as described in GRN 650, and Phipps et al. 2018 for studies evaluating 2'-FL not produced by Jennewein).
  - Pre-clinical toxicity study in piglets using Jennewein 2'-FL (Hanlon & Thorsrud 2014, **Appendix P-5**)
- Long term toxicity and carcinogenicity
  - No studies have been conducted on long term toxicity or carcinogenicity in animals due to the lack of toxicity in acute and short-term tests.
- Reproductive toxicity
  - We are not aware of studies investigating reproductive toxicity. The lack of toxicity of 2'-FL has been demonstrated in acute and short-term toxicity tests.
- Developmental toxicity
  - We are not aware of studies investigating developmental toxicity. The lack of toxicity of 2'-FL has been demonstrated in acute and short-term toxicity tests.
- Genotoxicity
  - One micronucleus test in rat bone marrow cells showed that Jennewein 2'-FL is not mutagenic (see **Appendix P-1**). One micronucleus test using cultured human peripheral lymphocytes showed that Jennewein 2'-FL is not genotoxic (see **Appendix P-6**).
  - Four mammalian cell micronucleus tests testing non-Jennewein 2'-FL produced by chemical synthesis or microbial fermentation showed no evidence of mutagenicity or cytotoxicity (Verbaan 2015a and Verbaan 2015b as cited in GRN 650, Phipps et al. 2018, and van Berlo et al. 2018 for studies evaluating 2'-FL not produced by Jennewein).
  - Multiple bacterial reverse mutation tests in *S. typhimurium* show no evidence of cytotoxicity nor mutagenicity of 2'-FL produced by chemical synthesis or microbial fermentation (see **Appendix P-2** for the study evaluating Jennewein 2'-FL; see Coulet et al. 2014, Verspeek 2015 as cited in GRN 650, Phipps et al. 2018, and van Berlo et al. 2018 for studies evaluating 2'-FL not produced by Jennewein).One gene mutation test in mouse

lymphoma cells (Coulet *et al.* 2014) showed that chemically synthesized 2'-FL was not mutagenic at the TK-locus of mouse lymphoma L5178Y cells in the presence or absence of metabolic activation.

- Immunotoxicity or neurotoxicity
  - 2'-FL is a naturally occurring substance found in human milk. It is not expected to have immunotoxic nor neurotoxic effects thus the lack of studies investigating these two topics.

Genotoxicity and Mutagenicity Studies with Jennewein 2'-FL

In a rat bone marrow micronucleus assay conducted in accordance with OECD 474 (**Appendix P-1**), Jennewein 2'-FL was evaluated for its potential to damage the chromosomes or the mitotic apparatus following a single oral administration. The dose levels were selected based on a preliminary oral acute toxicity study using one animal per sex and dose. Dosages of 500, 1000 or 2000 milligram (mg) Jennewein 2'-FL/kg bw were administered by oral gavage. These levels caused no signs of systemic toxicity, and were, therefore, used for the main study.

For the main study, doses of 500, 1000 or 2000 mg Jennewein 2'-FL per kg bw were administered by oral gavage to groups of five male and five female rats (Crl:CD(SD)). A control group received the vehicle (0.8% aqueous hydroxypropylmethylcellulose) and a positive control group received cyclophosphamide. Bone marrow smears were prepared immediately after sacrificing the animals at post-administration times of 24 and 48 hours. Two thousand (2000) erythrocytes were evaluated per animal.

No signs of systemic toxicity were reported through the highest dose level of 2000 mg Jennewein 2'-FL per kg bw. Jennewein 2'-FL did not increase the incidence of micronucleated polychromatic erythrocytes (PCEs) at any of the three tested dose levels. The combined incidence of PCEs for male and female animals was 0.6 micronuclei per 1000 PCEs for the samples collected 24 and 48 hours post-administration. The corresponding value for vehicle control (negative reference) was 0.3 or 0.5 micronuclei per 1000 PCEs (samples collected after 24 and 48 h, respectively). The positive control (cyclophosphamide) significantly increased the number of micronuclei to 13.3 per 1000 PCEs.

Conclusion: Jennewein 2'-FL did not produce signs of systemic acute toxicity and did not increase the incidence of micronucleated polychromatic erythrocytes at any of the concentrations tested.

Jennewein sponsored a mutagenicity study that was conducted in accordance with OECD Test Guideline 471 and Good Laboratory Practice (GLP) guidance. In a bacterial reverse mutation study (**Appendix P-2**), Jennewein 2'-FL was examined for mutagenic activity using the histidine-requiring *Salmonella typhimurium* (*S. typhimurium)* strains TA 98, TA 100, TA 102, TA 1535, and TA 1537 in the absence and presence of metabolic activation. Concentrations up to 5000 µg/plate of Jennewein 2'-FL were used. No signs of cytotoxicity were reported. No increase in revertant colony numbers was reported in plate incorporation and pre-incubation tests compared with control counts for Jennewein 2'-FL at concentrations up to 5000 µg/plate, in any of the five test strains in two independent experiments with Jennewein 2'-FL (cytotoxicity and mutagenicity), with and without metabolic activation. The positive control tests showed a significant increase in the number of revertant colonies for each of the corresponding test strains and confirmed the validity of the test conditions and the sensitivity of the test system.

Conclusion: Jennewein 2'-FL is not cytotoxic or mutagenic under the conditions of this study.

Jennewein sponsored a genotoxicity study that was conducted according to OECD Test Guideline 487 and GLP guidance. In a micronucleus assay (**Appendix P-6**), Jennewein 2'-FL was evaluated for clastogenicity and aneugenicity in cultured human peripheral lymphocytes in the presence and absence of metabolic activation by a rat liver post-mitochondrial fraction (S9 mix). Concentrations from 62.5 up to 5000 µg/mL medium of Jennewein 2'-FL. No signs of cytotoxicity were noted up to the highest concentration of Jennewein 2'-FL tested. The positive control tests (Mytomycin C and colchicine in the presence and absence of cyclophosphamide) induced significant chromosomal damage and significant damage to the cell division apparatus, respectively, confirming the validity of the test conditions and the sensitivity of the test system.

Conclusion: Jennewein 2'-FL showed no indications of chromosomal damage in the *in vitro* micronucleus test. Jennewein 2'-FL is not genotoxic under the conditions of the study.

Oral Toxicity of Jennewein 2'-FL

The oral toxicity studies described below were performed in accordance with OECD test guideline 408. OECD 408 is a general toxicity study guideline intended to provide health hazard information related to oral administration of the test substance and to provide an estimate of a no-effect level.[16]

7-Day Dietary Toxicity Pilot Study of Jennewein 2'-FL

A pilot study was conducted in preparation for the 90-day oral toxicity study as recommended in OECD guideline 408. Ten female rats (Crl:CD(SD)) were fed *ad libitum* for 7 days with a typical rat diet or a typical rat diet supplemented with 10% Jennewein 2'-FL. During the pilot study period, none of the animals died or showed changes in behaviour or appearance. Food consumption and body weight were comparable between the test and placebo groups. The study report is provided in **Appendix P-3**.

90-Day Dietary Toxicity Study of Jennewein 2'-FL

To evaluate the toxicity of Jennewein 2'-FL, rats were fed *ad libitum* with a typical rat diet (ssniff-R/M-H V1530) or a typical rat diet supplemented with 10% Jennewein 2'-FL (the test substance) for 90 days in accordance with OECD test guideline 408 as a limit test and GLP. The detailed study report is provided in **Appendix P-4**.

Materials and Methods: Male and female CD® Crl:CD(SD) rats (n=40) were randomly assigned to groups of 10 males and 10 females and received either the control diet (ssniff-R/M-H V1530) or the Jennewein 2'-FL-supplemented diet. Additional groups of three and nine animals per sex (n=24) were included in the control and treatment groups, respectively, and used exclusively for blood sampling.

The supplemented diet was freshly prepared on a weekly basis. To ensure a consistent diet, samples of the fortified diet were taken from different areas in the feed containers on weeks 1 and 13, and evaluated for 2'-FL concentration, homogeneity and stability (n = 12). Food was provided *ad libitum* and consumption was monitored weekly.

Results: The ingestion of Jennewein 2'-FL over a period of 13 weeks did not affect the body weight or body weight gain in either male or female rats compared with the controls. No significant treatment related effects on food consumption or drinking water intake were reported in the treated rats compared to controls. Based on food consumption the mean test substance intake was 7.66 ± 2.21 g/kg/day in male rats and mean 8.72 ± 1.9 g/kg/day in female rats.

No adverse effects related to the test substance were reported (clinical signs, behaviour, haematology, clinical biochemistry, urinalysis or ophthalmological examination). None of the animals died during the course of the study. The post-mortem macroscopic analysis did not reveal any differences in organ weights or gross pathological findings. There were no reported treatment-related histopathological (including bone marrow) findings. The only test substance related effects reported were pale stools in 7 of 10 males and 4 of 10 females in the treatment group, between days 9 and 69 of the study, which was reported to be due to the amount of undigested test item excreted in the faeces and was not considered adverse.

Conclusion: The ingestion of Jennewein 2'-FL was safe at average doses of at least 7.66 g/kg/d in male and 8.72 g/kg/d in female rats (Jennewein 2'-FL was fed as a 10% dietary admixture).

Oral Toxicity of Jennewein 2'-FL, Pre-Clinical in Piglets

In 2014, Abbott Nutrition (Columbus, OH) conducted a pre-clinical, oral administration study of Jennewein 2'-FL in neonatal piglets (Hanlon and Thorsrud, 2014, **Appendix P-5**). The objective of the study was to investigate the effect of the Jennewein 2'-FL on the health and development of the piglets. Because the first three weeks of piglets' lives share many similarities with the first

[16] http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en

three months of development of human infants, including similarities in digestive enzymes, nutrient absorption, gut closure, gut transit time, dietary requirements, and microbial population, they are suitable models to study the impact of dietary compounds on the development of infants (Guilloteau *et al*., 2010; Reeds & Odle, 1996; Helm *et al*. 2007).

This model has recently been endorsed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). In 2014, the JECFA committee stated "*The neonatal pig and minipig are appropriate models for the young human infant up to at least 12 weeks of age, for whom infant formula may be the sole source of nutrition*" (JECFA, 2015). Data from the piglet model demonstrated a lack of adverse effects at 2 g 2'-FL/L (as-fed) which indicates the ingredient, at these concentrations, is safe and suitable for infants.

The purpose of the safety study in neonatal piglets is designed specifically to address the safety of the test article in an experimental model, relevant to young infants, with an emphasis on the gastrointestinal tract. This is especially important for substances that are poorly absorbed, such as 2'-FL, and therefore most likely to produce adverse effects in the GI tract, if they were to produce adverse effects.

The concentrations of 2'-FL in the formula administered to the neonatal piglets were selected to be representative of the concentration of 2'-FL in formula to which a human infant would be exposed. The concentration of 2'-FL in the product (the same between the piglet formula and the proposed amount in human infant formula) is representative of the concentration that the cells of the gastrointestinal tract would be exposed to. Thus, in this case, there is not a need to scale the concentration between piglets and humans to compensate for likely differences in systemic exposure to the 2'-FL.

Material and Methods: A total of 27 male and 21 female domestic farm pigs (Domestic Yorkshire Crossbred Swine) were received two days after birth. During the first two days after birth, the piglets nursed from the sow. Piglets received injections of iron supplement and antibiotics according to standard practices. The piglets were housed in mobile stainless-steel cages, one animal per cage, in an environmentally controlled room. The piglets received supplemental heating as needed from heating pads in the cages or a heating lamp outside the cage. The piglets were allowed to socialise within their respective study group each morning before feeding by allowing at least two piglets per cage.

The animals were assigned to the test and control diet groups to match number per sex, weight and age of the animals as closely as possible. The test vehicle consisted of a typical milk replacer (ProNurse® Specialty Milk Replacer). The diets contained either vehicle alone (control), or the vehicle and 200, 500 or 2000 mg Jennewein 2'-FL/L, for the low, mid and high concentration treatment groups, respectively. The diets were administered via a feeding bowl that was filled six times per day at a volume of 500 mL/kg/d for 20 consecutive days. The highest concentration in the study corresponds to the suggested use level for 2'-FL in infant nutrition.

The study authors evaluated morbidity, mortality, and injury twice daily, and conducted detailed clinical examinations twice per week. The authors reported daily body weights for the first week and every other day thereafter during the study. Blood samples were collected at study days 7 and 21 (without fasting prior to blood collection). Gross necropsies were performed on all animals at terminal sacrifice on study day 22. Organ weights of brain, heart, kidney, large intestine (cecum, colon, rectum), liver, small intestine (duodenum, jejunum, ileum) and spleen were obtained. The pH of the intestinal contents in cecum and colon was recorded. Histopathological examination was conducted on all organs listed above, and on all gross lesions, the eyes including the optic nerve, gall bladder, stomach, lung with bronchi, mesenteric lymph nodes, pancreas and Peyer's patches. The statistical analysis is described in Hanlon and Thorsrud (2014).

Results: All piglets survived to scheduled necropsy on Day 22. There were no reported treatment related adverse clinical findings during the dosing period. Both male and female piglets showed good growth based on body weight gain and food efficiency. Based on the 200, 500, and 2000

mg 2'-FL/L concentrations, the consumption corresponded to doses of 29.4, 72.2, and 292 mg/kg bw/d, respectively in males, and 29.3, 74.3, and 299 mg/kg bw/d, respectively in females.

There were no reported treatment-related adverse effects on the clinical pathology parameters evaluated, including haematology, clinical chemistry, coagulation and urinalysis. There were no reported treatment-related adverse macroscopic and microscopic findings, including intestinal pH. Microscopic findings reported by the authors to be not related to treatment included mild to moderate inflammation within the keratinised portion of the squamous epithelium in the non-glandular part of the stomach of one male and one female in the highest dose group and in one female in the 500 mg/L group. The male in the 2000 mg/L group also showed focal loss/thinning in the keratinized portion of the squamous epithelium, associated with inflammation but without ulceration. There were no macroscopic findings associated with the observation.

The lack of appetite that was observed in the piglet study was determined not to be toxicologically relevant, nor test-article related. While there were three piglets in the 2 g/L treatment group (1 male and 2 females) that exhibited lack of appetite for a single day, there was also a female in the 0.2 g/L treatment group that exhibited lack of appetite for two days. All of these observations occurred on day 6 of the treatment (and days 5 and 6 for the female in the 0.2 g/L treatment group), and did not affect the final body weight of any of the piglets. The single male piglet in the 2 g/L treatment group had a final body weight of 6.8 kg, which was similar to the body weight of all male piglets in the study (6.8 ± 1.0 kg). The three females that had an incidence of inappetence also did not exhibit reduced body weight because of this, as their final body weights (8.1, 8.7, and 8.2 kg) were not reduced in comparison with all female piglets in the study (6.9 ± 1.3 kg). Therefore, due to the low incidence, short duration, lack of correlation to final body weight, and lack of clear dose-dependence, these incidences were deemed not toxicologically relevant, or associated with the test article.

While watery faeces was observed in the piglet study (Hanlon and Thorsrud, 2014, **Appendix P-5**), the conclusion of this study was that this observation was not related to 2'-FL because there was no clear dose response relationship and there were no other findings that could be attributed to 2'-FL (such as changes in clinical chemistry or pathological observations). Despite a difference in concentration of 10-fold between the 200 and 2000 mg/L treatment groups, there was only an increase by a single animal in the number of animals that had observed watery faeces. While no instances of watery faeces were observed in the control group of this study, this observation is common in control animals in this model. Evaluation of data from control animals from 6 other studies conducted at this facility shows that 8 of 36 males and 12 of 36 females in those studies exhibited watery faeces at least once during the study, occurring sporadically over the course of three weeks. This occurrence rate of approximately 25% overall is consistent with the rate of occurrence in the current study (observed in 12 of the 48 animals), even though no animals in the control group of the current study had observed instances of watery faeces.

The watery faeces that were observed, in all but one instance, had a duration of two days or less (**Table 16**), with nine of the 12 animals having the observation only on a single day. The occurrence of watery faeces also does not increase with the duration of exposure to 2'-FL, with the observation occurring during the first five days of treatment in eight of the 12 animals.

The observation of watery faeces is also not a concern because of the lack of correlation between the occurrences of watery faeces and any other signs of gastrointestinal toxicity. Microscopic evaluation of the non-glandular stomach did identify three animals with an increased magnitude of inflammation (subacute to chronic) within the keratinised portion of the stratified squamous epithelium (animals #228, 239 and 243). However, only one of these three animals (animal #228) had any observed watery faeces. Inversely, of the 12 animals with observed watery faeces in at least one day, only one animal exhibited the increased inflammation.

Inflammation of the non-glandular stomach was a common observation in all of the animals in this study, with the majority of control animals and the animals in all dose groups having variable, minimal to mild, focal acute inflammation of the non-glandular stomach (**Table 17**). Erosion/ulceration and hyperkeratosis (increased thickness of the keratinised portion) of the non-

glandular portion of the stomach are commonly observed in swine (Embaye *et al*., 1990; Krakowka and Ellis, 2006) and are thought to be related to diet, gastric acidity and/or the presence of commensal bacteria.

Occurrence of inflammation of the non-glandular stomach was not dose dependent, with incidence rates of some inflammation in the control, 0.2 g/L, 0.5 g/L, and 2 g/L treatment groups being 58, 67, 42, and 50%, respectively. Because the majority of control animals and animals at all dose concentrations had variable, minimal to mild, focal acute inflammation within the keratinised portion of the non-glandular stomach, a definitive test article-related effect could not be determined pertaining to the increased magnitude of inflammation. Similar observations have been documented in age and species matched historical control data at the test facility. While the incidence rate is variable between studies, this is a common observation. In a review of five other studies conducted at the Abbott Nutrition facility, the incidence rate of some inflammation of the non-glandular stomach in control piglets was 0, 2, 2, 4, and 7% (personal communication). While the incidence rate of inflammation was higher in this study, the incidence rate was higher in all groups including the control group, demonstrating that this result was not test-article related.

The results from the piglet study demonstrated no test article related findings. The cumulative results from all the safety studies conducted and historical evidence of infant exposure to the same chemical compound via human milk demonstrate the safety of the Jennewein 2'-FL ingredient. This includes the lack of gastrointestinal findings in the rat study in which animals were administered a diet consisting of 10% 2'-FL for 90 days (details in **Appendix P-4**), and the human infant clinical studies with a lack of adverse effects (detailed in a subsection "Clinical Studies in Humans" later in this section) related to adverse gastrointestinal effects supports the safety of 2'-FL. Together, the weight of the evidence demonstrates that these effects are an artefact of the piglet model, not test article-related, and are highly unlikely to be present in human infants consuming product containing 2'-FL. All other microscopic findings were considered incidental and were within the range of typical observations in swine of this age and strain as reported by Abbott Nutrition.

Conclusion: The authors concluded that the daily dietary administration of Jennewein 2'-FL in Purina ProNurse® milk replacement formula to neonatal piglets for three weeks following birth at concentrations up to 2000 mg 2'-FL/L/d was well tolerated and did not produce any adverse treatment-related effects on growth and development. There were no reported adverse effects on body weight and, food efficiency. No mortalities were reported. There were no Jennewein 2'-FL-related adverse effects reported on clinical pathology findings, gastrointestinal pH, and macroscopic and microscopic findings at terminal necropsy.

Table 16: Detailed clinical observations, 3-week piglet study

Animals number	Sex	Day(s) watery faeces observed
200 mg/L group		
Animal 220	Male	1, 2
Animal 221	Male	9
Animal 213	Female	2
Animal 215	Female	1
500 mg/L group		
Animal 231	Male	11
Animal 226	Female	5
Animal 227	Female	5, 10
2000 mg/L group		
Animal 243	Male	1, 6, 7, 9, 10, 14, 15
Animal 244	Male	1
Animal 248	Male	3
Animal 237	Female	5
Animal 238	Female	5

Table 17: Summary of non-glandular stomach, microscopic observations, 3-week piglet study

	Concentration of Jennewein 2'-FL			
	0	**200**	**500**	**2000**
Male				
Within normal limits	1/6	2/8	5/7	3/6
Acute inflammation				
Minimal	5/6	6/8	2/7	2/6
Mild	0/6	0/8	0/7	0/6
Subacute/chronic inflammation				
Mild	0/6	0/8	0/7	0/6
Moderate	0/6	0/8	0/7	1/6
Female				
Within normal limits	4/6	2/4	2/5	3/6
Acute inflammation				
Minimal	1/6	1/4	2/5	2/6
Mild	1/6	1/4	0/5	0/6
Subacute/chronic inflammation				
Mild	0/6	0/4	1/5	0/6
Moderate	0/6	0/4	0/5	1/6

14-Day Oral Toxicity Pilot Study of Chemically Synthesised 2'-FL

Coulet *et al*. (2014) studied the oral toxicity of chemically synthesised 2'-FL by its repeated administration to juvenile rats. The 2'-FL evaluated by Coulet *et al*. (2014) is not the Jennewein 2'-FL product and was produced by an entirely different manufacturing process with different residual impurities and by-products. This served as a dose range-finding study for the subsequent 90-day toxicity study.

After acclimatisation, Wistar IGS:Crl:WI Han rats (five male, five female per group) were administered the test item by oral gavage, starting on day seven after birth until day 20. Rats received either a vehicle or the synthetic 2'-FL at doses of 2000, 5000 or 7500 mg/kg bw for 14 consecutive days. A fifth group received 7500 mg/kg bw/day of a reference compound, fructooligosaccharide (FOS) which is currently considered safe and approved for use in infant formula.

The test item was well tolerated up to a dose of 2000 mg/kg bw/d. At higher doses, limited and transient effects such as liquid faeces and a slightly lower body weight gain were reported. This was apparent in the 5000 mg/kg bw/d group and more severe in the groups receiving 7500 mg/kg bw/d of synthetic 2'-FL or the same dose of FOS. Two animals in the highest 2'-FL dose group died, one on day six and one on day 12. Coulet *et al*. (2014) reported no macroscopic abnormalities at necropsy and did not report the cause of death.

Conclusion: Based on the mortalities at the 7500 mg/kg bw/d dose, the high dose for the subchronic study was reduced to 6000 mg/kg bw/d for both chemically synthesised 2'-FL and FOS.

90-Day Oral Toxicity of Chemically Synthesised 2'-FL

Coulet *et al*. (2014) evaluated the sub-chronic oral toxicity study in juvenile rats, based on OECD test guideline 408. Doses of 0, 2000, 5000, or 6000 mg 2'-FL/kg bw/d and 6000 mg FOS/kg bw/d were administered by oral gavage to groups of 10 male and 10 female rats (Wistar IGS:Crl:WI Han) for 90 consecutive days with a recovery period of 28 days.

One male and one female receiving the highest dose of 6000 mg/kg bw/d of 2'-FL were reported dead on day two. Two males administered the FOS at a dose of 6000 mg/kg bw/d of FOS were reported dead: one on day 12 and one on day 13. One female from the 6000 mg 2'-FL/kg bw/d group was reported dead during the recovery period on day 108. The cause(s) of the deaths could not be determined, and the authors concluded the deaths were not treatment related. The reported findings from the necropsies of the 2'-FL animals that died were uncollapsed lungs and a dark focus on the urogenital skin and in one of the deceased males from the 6000 mg FOS/kg bw/d group a pale liver. No other macroscopic abnormalities were reported. Decreased lymphoid follicle development in the spleen was reported for all the animals found dead. Coulet *et al*. (2014) reported that this finding may have been due to normal immaturity or a secondary effect of poor health. The reported histological findings for the female from the 6000 mg/kg bw/d 2'-FL group found dead on day 108 were decreased lymphoid follicle development in the mandibular lymph node and spleen, slight necrosis in the liver, haematopoiesis in the spleen, granulocytic hyperplasia in the bone marrow and vaginal epithelial mucification.

Diarrhoea was reported in "*a few*" (actual number not reported) of the animals receiving 2000 mg/kg bw/d 2'-FL and in all of the animals administered 5000 and 6000 mg 2'-FL/kg bw/d and all of the animals administered 6000 mg FOS/kg bw/d. Coulet *et al*. (2014) reported that these findings were associated with erythema in the urogenital area of "*most*" animals (exact numbers not reported) in both of the 6000 mg/kg bw/d groups. Additionally, hyper-salivation was reported in half of the animals of both sexes in the mid-dose group and in almost all animals in the high-dose groups (exact numbers not reported). Coulet *et al*. (2014) report that hyper-salivation occurred "*mainly on days 57 and 71*." The mean body weight gains were transiently but statistically significantly lower in 5000 and 6000 mg/kg bw/d 2'-FL groups and in the FOS group in both males and females between day 0 and day 3. This reportedly resulted in transient but statistically significantly lower mean body weights in the male 6000 mg/kg bw/d 2'-FL group

on days 3-10 and females in the 5000 and 6000 mg/kg bw/d 2'-FL groups on day 3 and the FOS group males (days 3-7) and females (days 3-10). At the end of the treatment period, small but not statistically significant deviations in body weight were reported compared with the control groups. Females in all 2'-FL and FOS dose groups had the same or a slightly higher mean body weight than the controls, whereas males in the high-dose 2'-FL group had a slight but not statistically significant lower mean body weight than the controls, and the males receiving FOS had a slightly higher mean body weight than the controls. Food consumption was similar in all groups throughout the treatment and recovery periods.

No treatment-related changes in coagulation were found. Minor differences were observed in some clinical chemistry parameters but were considered to be not related to the test substance. A small and dose-dependent reduction in urine specific gravity was reported in all males in all the 2'-FL dose groups and also females in the 5000 and 6000 mg/kg bw/d 2'-FL groups. The change was considered toxicologically irrelevant. Organ weights did not differ among the groups, except the relative adrenal weight was significantly lower in the highest 2'-FL group. Because the lower weight was not associated with histological abnormalities, the findings were considered toxicologically irrelevant. The histological evaluation revealed a statistically significant increase in the incidence of cytoplasmic vacuolation in the corticular tubular epithelium of kidneys in females receiving 5000 mg/kg bw/d of 2'-FL and 6000 mg/kg bw/d of FOS. Because this was not observed in males, was not dose-dependent and was not accompanied by other changes, it was not considered an adverse effect.

The results of the Coulet *et al*. (2014) oral toxicity studies are inconsistent with other studies of FOS, the reference substance which has generally recognised as safe status based on GRN 44. Coulet *et al*. report mortalities in the 6000 mg FOS/kg bw/d group; however, studies in GRN 392 reported no mortalities or other adverse effects at much higher doses of FOS (approximately 15,000 mg/kg bw/d for a subchronic study in Wistar rats). The same research group at the same test facility performed oral toxicity studies for a different oligosaccharide again using FOS as a reference material and reported mortalities for 5000 mg FOS/kg bw/d groups (Coulet *et al*., 2013). Cause of death was reported to be a possible intubation error or trauma during dosing (Coulet *et al*., 2013). The mortalities in the Coulet *et al*. (2014) reference group suggest causes of mortality that are not due to the FOS and 2'-FL test substances.

Conclusion: Coulet *et al*. (2014) concluded that the chemically synthesised 2'-FL product is "*well tolerated and does not elicit any adverse effects in male and female rats following oral administration at dose levels up to 5000 mg/kg bw/d for exposure periods of up to 90 days from postnatal day 7*." Based on these studies, the authors established the no-observable adverse effect level (NOAEL) of chemically synthesised 2'-FL at 5000 mg/kg bw/d and considered the substance safe for the intended use as an ingredient in infant formulas (Coulet *et al*., 2014).

90-day Oral Toxicity of 2'-FL Produced by Microbial Fermentation

The oral toxicity of 2'-FL produced by microbial fermentation was studied in male and female rats exposed to 2'-FL in the diet for 90 days in a study conducted according to OECD testing guideline 408 and GLP (van Berlo *et al*. 2018). The 2'-FL used in this study was produced using a different manufacturing process than that of Jennewein 2'-FL and has different residual impurities. Male and female Wistar Han IGS rats (Crl:WI(Han)), 10 per sex per group, were fed diets containing 2'-FL concentrations of 0, 3, 6, or 10% (w/w) for 13 consecutive weeks. Diets were analyzed for stability, homogeneity and concentration of 2'-FL throughout the study.

Food intake was reported to decrease with increasing age of the rats; therefore, the intake of 2'-FL per kilogram body weight decreased in all groups during the study. The overall mean 2'-FL intake was 2.17, 4.27, or 7.25 g/kg/day for males and 2.45, 5.22, or 7.76 g/kg/day for females. Results following dietary intake of 2'-FL for 13 weeks produced no exposure-related changes in mortality of clinical signs in any of the treated groups. Results of functional observational battery and motor activity assessment did not indicate any neurotoxic potential for 2'-FL. No significant differences were noted between controls and treated groups. No changes in food consumption in male rats was reported; however, food consumption in the high-dose females was significantly

decreased. Hematology results indicated a significant increase in thrombocytes in the high-dose females; however, this finding was determined by the authors to be a chance finding because the difference from controls was only slight and occurred in one sex only. No other hematological or clinical chemistry changes were noted in the treated groups.

Results of renal concentration tests showed a significantly decreased specific gravity in female in the high dose group. The authors attributed the change to a higher urinary excretion volume and the change was not considered toxicologically significant. Relative liver weight was significantly increased in the high dose males and absolute and relative weights of the filled and empty cecum were significantly increased in the mid- and high-dose group in male and female rats. In addition, the absolute weight of the filled cecum was significantly increased. No significant macroscopic or microscopic changes related to treatment were reported in any of the treatment groups.

Conclusion: The authors concluded that ingestion of 2'-FL for 13 weeks produced no treatment related changes in male and female rats. The authors reported a no observed adverse effect level (NOAEL) at the highest concentrations tested, corresponding to ≥7.25 g/kg/day in male rats and ≥7.76 g/kg/day in female rats.

Penard *et al.* (2015) as described in GRN 650 (2016) also conducted a 90-day oral toxicity study in rats following OECD testing guideline 408 modelled on a 90-day toxicity study (Coulet *et al*. 2014) described previously in GRN 571. For 90 to 91 days with 28-day recovery, rats were administered 0, 2000, 4000, or 5000 mg 2'-FL/kg bw/day and a reference group was administered FOS at 5000 mg/kg bw/day.

Conclusion: In this study, the authors reported no exposure-related mortalities, no significant changes in body weight, weight gain, organ weight, or food consumption as well as in other parameters including serum biochemical parameters. The authors reported no treatment-related changes to support evidence for toxic effects of 2'-FL in a 90-day oral toxicity study and concluded a NOAEL of 5 g/kg/day, the highest dose tested.

Phipps *et al.* (2018) conducted a 90-day repeated dose oral toxicity test with 2'-FL/DFL in male and female Sprague-Dawley rats following OECD Guideline Test No. 408. An 8:1 ratio mixture of 2'-FL and difucosyllactose (DFL) was administered via oral gavage to neonatal rats daily at 0, 1000, 3000, and 5000 mg/kg bw/day of 2'-FL/DFL for 90 days followed by a 28-day recovery period. No mortality or exposure-related clinical signs were observed. Mean body weight and food consumption did not differ significantly between treatment groups and vehicle. Furthermore, the authors reported no treatment-related adverse effects with a dose-response relationship were observed for development and maturation, behavioral endpoints, clinical pathology, organ weights, or histopathology.

Conclusion: The results of the 90-day oral toxicity test conducted by Phipps *et al.* (2018) similarly show that 2'-FL (in a mixture of 2'-FL and DFL at an 8:1 ratio) produced no treatment-related effects in male and female rats. The authors concluded a NOAEL at 5,000 mg/kg bw/day, the highest dose tested.

Summary of Toxicological Information for Jennewein 2'-FL

The toxicological studies performed with Jennewein 2'-FL demonstrate the safety of the substance. Jennewein 2'-FL was neither mutagenic nor genotoxic.

The ingestion of a diet fortified with up to 2000 mg Jennewein 2'-FL/L by neonatal farm piglets for a period of three weeks was well tolerated and no differences in the development of the piglets between control and test groups or any substance-related adverse events were observed. Dietary administration to piglets for a period of three weeks is a common animal model to study the early infant metabolism during weaning.

In a 90-day dietary toxicity study conducted in accordance with OECD test guidelines, Jennewein 2'-FL was safe at average doses of 7.66 g/kg/d in male and 8.72 g/kg/d in female rats.

The results from these studies confirm that Jennewein 2'-FL, a product of bioengineering that is substantially chemically equivalent to 2'-FL isolated from human milk, is safe and suitable for its

proposed uses. Furthermore, numerous toxicological studies evaluating 2'-FL produced by chemical synthesis or microbial fermentation from other sources also demonstrate the safety of manufactured 2'-FL.

A summary of the toxicological data regarding 2'-FL is presented in **Table 20**.

CLINICAL STUDIES WITH HUMANS

Several clinical studies (Marriage *et al.* 2015, Goehring *et al.* 2016, Elison *et al*. 2016; Kajzer *et al.* 2016 via Reverri *et al*. 2018; Puccio *et al.* 2017; Storm *et al*. 2019; Nowak-Wegrzyn *et al.*, 2019) demonstrating the nutritional safety and tolerance of synthesized 2'-FL in humans have been conducted and are summarized below and in Table 20 "Toxicological and Clinical Data Summary of 2'-Fucosyllactose".

Clinical studies with infants

Five published studies, of which one is a sub-study of a full clinical trial, with human infants demonstrating the nutritional safety and tolerance of synthesized 2'-FL were identified and summarized below (Marriage *et al.* 2015; Goehring *et al.* 2016; Puccio *et al.* 2017; Kajzer *et al.* 2016 via Reverri *et al*. 2018; Storm *et al*. 2019). One study tested the hypoallergenicity of a formula containing 2'-FL and is also summarized below (Nowak-Wegrzyn *et al.*, 2019).

Growth and Tolerance Test of Chemically Synthesised 2'-FL in Infants (Marriage *et al*., 2015)

Marriage *et al*. (2015) conducted a 119-day study to examine growth and tolerance by infants fed infant formulas of chemically-synthesized 2'-FL supplemented with a caloric density approximating human milk and to study the uptake of the 2'-FL. Healthy, full-term infants of singleton birth were enrolled by the fifth day of life from 28 sites throughout the United States. Infants were randomly assigned to groups with one of four diets: formula with no 2'-FL, formula containing 0.2 g 2'-FL/L, formula containing 1.0 g 2'-FL/L, or human milk.

The formulas also contained galactooligosaccharides (GOS) to bring the prebiotic concentrations up to 2.4 g/L. A total of 304 infants completed the study, with about 80 infants per group. The authors measured growth using weight, length and head circumference; tolerance was measured by average stool consistency, number of stools per day, and percent of feedings associated with spit-up or vomit; and uptake was measured by levels of 2'-FL in infant plasma and urine in a subset of infants at day of life 42 and 119 and from the human milk of the breast-feeding mothers at day of life 42.

The certificates of analysis for the 2'-FL used in this study are provided in **Appendix Q**.

The authors reported no significant differences between groups for weight, length or head circumference. The authors also reported that all tolerance metrics were comparable, and that 2'-FL was present in the plasma and urine of infants fed 2'-FL though the concentration fed did not result in a significant difference in 2'-FL uptake between groups, including the human milk-fed infants. The percent of feedings with spit-up or vomit within 1 hour of feeding was significantly higher ($p \leq 0.05$) in the group fed formula without 2'-FL (17.5%) and the groups fed formula containing 2'-FL (0.2 g/L: 21.5%; 1.0 g/L: 18%) compared to the breastfeeding group (10.5%) from enrolment through the first month (day of life 28). However, spit-up frequency did not appear to be dose dependent and after the first month of life there were no differences among the groups.

The authors reported that the breastfeeding group exhibited a significantly higher mean number of stools per day than the formula groups from enrolment through the first month of life and that the mean rank stool consistency (1=watery, 5=hard) was not significantly different among the three formula groups but was significantly greater ($p<0.05$) compared to the breastfed group after the first month of life.

The authors reported no safety concerns with any of the formulas containing 2'-FL. The authors also reported no significant differences in adverse events between the experimental groups and the control group based on percentages. The group fed formula containing 0.2 g 2'-FL/L had fewer reported adverse events with respect to "*infections and infestations*" compared to the other

formula-fed groups. The types of adverse events included upper respiratory tract symptoms, otitis media, viral infections, and oral candidiasis. The group fed formula without 2'-FL also reported five incidents of eczema, while the groups fed formula containing 2'-FL reported none. A table presenting information related to the number of parents that exited the study is provided (**Table 18**).

In regard to absorption and excretion of 2'-FL, the mean plasma concentrations at day of life 42 were significantly different for each treatment group through the relative absorption of 2'-FL was comparable at 0.7% among infants fed formula containing 0.2 g 2'-FL/L, 0.05% among infants fed formula containing 1.0 g 2'-FL/L, and 0.05 among infants fed human milk. By day of life 119, the mean plasma concentrations between the two groups fed formula containing 2'-FL were not significantly different (data was not collected for the human milk-fed group at this time point). The plasma concentrations of 2'-FL decreased significantly for the 0.2 g 2'-FL/L formula group, the 1.0 g 2'-FL/L formula group, and the human milk groups ($p = 0.017$, 0.008 and 0.015, respectively) for day of life 42 to 119 and urine concentrations decreased significantly for the human milk-fed group ($p=0.018$) but did not change significantly for the groups fed formula containing 2'-FL. The authors reported that mean urine concentrations were significantly different among the groups, but relative excretion was similar among the groups fed human milk or formula containing 2'-FL: 1.35%, 1.50% (formula containing 0.2 g 2'-FL/L) and 1.26% (formula containing 1.0 g 2'-FL/L), respectively.

Conclusion: Chemically-synthesized 2'-FL is well-tolerated in human infants at concentrations up to 1.0 g 2'-FL/L (the highest exposure concentration tested in this study) and for up to four months of oral consumption with reported adverse effects at levels not significantly different from controls.

Table 18: Exit due to adverse event (Marriage *et al.*, 2015)

Treatment group	Exit reason	Nature	Severity*	Frequency
Experimental Formula 0.2 g 2'-FL/L	Parent exited	Infantile colic	AE, Mild	Sporadic/Intermittent
	Parent exited	Spitting up	AE, Mild	Sporadic/Intermittent
		Constipation	AE, Mild	Single Episode
	Parent exited	Diarrhoea	AE, Moderate	Sporadic/Intermittent
	Parent exited	Constipation	AE, Mild	Single Episode
		Vomiting	AE, Mild	Sporadic/Intermittent
	Parent exited	Vomiting	AE, Mild	Sporadic/Intermittent
	Investigator exited	Convulsion	SAE†	Single Episode
Experimental Formula 1.0 g 2'-FL/L	Parent exited	Constipation	AE, Mild	Sporadic/Intermittent
	Parent exited	Flatulence	AE, Moderate	Sporadic/Intermittent
		Crying	AE, Moderate	Sporadic/Intermittent
		Constipation	AE, Mild	Sporadic/Intermittent
	Investigator exited	Diarrhoea	AE, Moderate	Sporadic/Intermittent
	Parent exited	Vomiting	AE, Moderate	Sporadic/Intermittent
Human Milk	Investigator exited	Gastric Reflux	AE, Severe	Sporadic/Intermittent
Control Formula	Parent exited	Crying	AE, Moderate	Sporadic/Intermittent

Notes: *AE: Adverse Event; SAE: Serious Adverse Event. † Serious adverse event was determined by physician as not due to study product. Source: Abbott Nutrition.

Influence of Formula Containing Synthesised 2'-FL on Immune Function Biomarkers in Healthy Infants (Goehring *et al*., 2016)

Goehring *et al*. (2016) investigated the effects of feeding formula supplemented with synthesised 2'-FL on biomarkers of immune function in healthy term infants. This study represents a sub-study of the clinical trial reported by Marriage *et al*. (2015), described above. The trial was registered at clinicaltrials.gov as NTC01808105. Of the 424 infants enrolled in the original clinical trial (described above for Marriage *et al*., 2015), 315 infants participated in the sub-study where urine, stool and blood samples were collected from these infants as described in **Table 19**. Stools were analysed for concentration of IgA, microbiota composition and characterisation of biological factors influential to gastrointestinal health.

Table 19: Participant details of the sub-study

	Control Formula	Experimental Formula 0.2 g 2'-FL/L	Experimental Formula 1.0 g 2'-FL/L	Human Milk
Enrolled in sub-study	75	76	78	86
Exited sub-study	6	9	6	5
Stool & urine collection only	27	22	30	35
Blood specimens collected & analysed	39	37	37	42
Number of stool, urine or blood samples either lost or unable to analyse	5	6	7	11

At six weeks of age, 2-3 mL non-fasting venous blood was collected and analysed for blood cellular phenotyping, *ex vivo* phytohemagglutinin-stimulated cytokines and plasma cytokines; which are biomarkers of immune function. Peripheral blood mononuclear cells (PBMCs) were isolated for cellular phenotyping and stimulated ex vivo with phytohaemagglutinin for proliferation and cell cycle progression or respiratory syncytial virus (RSV). Cytokine concentrations were measured in plasma and in *ex vivo*-stimulated culture supernatants.

Breastfed infants and infants fed either of the experimental formulas containing 2'-FL were not different but had 29-83% lower concentrations of plasma inflammatory cytokines than did infants fed the control formula. In *ex vivo* RSV-stimulated PBMC cultures, breastfed infants were no different than either of the groups fed formula with 2'-FL, but they had lower concentrations of inflammatory biomarkers than did infants fed the control formula.

Conclusion: The data indicated that infants fed formula supplemented with synthesised 2'-FL exhibited lower plasma and *ex vivo* inflammatory cytokine profiles, similar to those of a breastfed reference group.

Gastrointestinal Tolerance of Infants Consuming Formula with 2'-FL (Kajzer *et al*., 2016 via Reverri *et al*., 2018)

Kajzer *et al*. (2016) conducted a prospective, randomized, multi-center, double blinded, controlled, tolerance trial in 131 healthy term infants 0 to 8 days old. The results of this study were published as an abstract (Kajzer *et al.*, 2016) and further discussed in a review article by Reverri *et al.* (2018). The study was performed to assess gastrointestinal tolerance of infants fed formula supplemented with 0.2 g 2'-FL/L and 2.0 g short-chain fructooligosaccharide (scFOS)/L, compared to infants fed formula without 2'-FL/scFOS or infants fed breast milk for 35 days. There were no statistically significant differences in sex, ethnicity, race, gestational age, weight, length,

or age at enrollment among the three groups, nor were there differences in anthropometric measures. The infants were exclusively fed the assigned formula or breast milk for the duration of the study. The authors reported no significant differences in mean rank stool consistency, formula intake, anthropometric measures, or percent feedings with spit-up or vomit associated with feedings across the three groups at 35 days of age.

Conclusion: The authors concluded that formula supplemented with up to 0.2 g/L 2'-FL and 0.2 g/L scFOS was safe and well tolerated in infants.

Effects of Infant Formula with Human Milk Oligosaccharides on Growth and Morbidity (Puccio *et al*., 2017)

As reported in GRN 735, Puccio *et al.* (2017) conducted a double-blind, randomized, controlled clinical trial in 175 healthy, full-term infants between October 2012 and July 2013 at two hospitals in Italy and Belgium. The study aimed to evaluate the effects of infant formula supplemented with two HMOs (2'-FL and lacto-*N*-*neo*tetraose (LNnT)) on infant growth, tolerance and morbidity. One hundred and seventy-five (175) infants aged 0 to 14 days old were randomly assigned to either a treatment group receiving formula containing a combination of 2'-FL (1 – 1.2 g/L) and lacto-N-neotetraose (LNnT) (0.5 – 0.6 g/L) (n = 88) or formula that did not contain either oligosaccharide (n = 86) for up to 6 months. All infants received standard follow-up formula without HMOs from six to 12 months of age.

The primary endpoint was weight gain through four months; secondary endpoints included additional anthropometric measures, gastrointestinal tolerance, behavioural patterns, and morbidity through 12 months of age. The mean daily formula intake was 908 mL in the test group (formula containing 2'-FL and LNnT) and 929 mL in the control group (standard formula not containing either oligosaccharide). Weight gain was similar for both the control and test groups. Digestive symptoms and behavioural patterns were also similar between the groups; exceptions included softer stools and fewer night time wake-ups in the test group at two months. Infants fed formula containing 2'-FL and LNnT had significantly fewer parental reports of bronchitis through four and 12 months, antipyretics use through four months, lower respiratory tract infection through 12 months, and antibiotics use through six and 12 months.

Conclusion: The authors concluded that infant formula containing synthesised 2'-FL and LNnT is safe, well-tolerated and supports age-appropriate growth. Secondary outcome findings showed associations between consuming 2'-FL and LNnT-supplemented formula and lower parent-reported morbidity (particularly bronchitis) and medication use (antipyretics and antibiotics).

Safety and Tolerance of Infant Formula Supplemented with 2'-FL (Storm *et al*., 2019)

Storm *et al.* (2019) conducted a randomized, controlled multicentre study at seven sites in the United States from September 2017 through February 2018. Healthy infants 14 days of age were randomized into two groups and fed formula made from partially hydrolyzed, 100% whey protein, with (test group) or without (control group) the addition of 0.25 2'-FL g/L for six weeks. Caregivers and investigators were blinded as to the identity of the study formulas. During the initial visit at enrollment, the Infant Gastrointestinal Symptom Questionnaire (IGSQ) was administered and anthropometric measurements were taken. After 42 days of feeding the subject and caregivers returned for a second visit after completing a formula intake, stooling, spit-up, and vomit for 2 days prior to the visit. Adverse events were recorded throughout the study and assessed by the site investigator.

Results indicated IGSQ scores were similar at baseline and visit 1 at 6 weeks exposure. Stool frequency and consistency were similar among the test and control groups throughout the study. More stools were reported to be difficult to pass in the control subjects compared to the test group ($p = 0.04$); however, the number of infants with difficulty passing stools did not differ between groups. Crying, fussing duration and vomiting frequency were similar between the two groups. Average formula intake, body weight, and body lengths did not differ between the control and test groups. There were more subjects with spit-up noted as frequent in the test

group compared to controls. More subjects reported infections in the control group compared to the test group.

Conclusion: The authors concluded that the 100% whey, partially hydrolyzed infant formula with or without the addition of 0.25 g 2'-FL/L formula is safe and well tolerated based on the results of this study.

Hypoallergenicity Test of Infant Formula Supplemented with 2'-FL and LNnT (Nowak-Wegrzyn *et al.*, 2019)

A double-blind, placebo-controlled food challenge hypoallergenicity study was performed in infants and children between 2 months and 4 years of age with documented cow's milk protein allergy (Nowak-Wegrzyn et al. 2019). The test infant formula was 100% whey-based extensively hydrolyzed formula (EHF) supplemented with 1.0 g/L 2'-FL and 0.5 g/L lacto-N-neotetraose (LNnT). The control infant formula was a commercially available EHF without HMOs confirmed to be hypoallergenic. In a blinded, cross-over fashion, the test and control formulas were given in a randomized order, with the first challenge occurring 3 to 28 days after enrolment and the second challenge with 2 to 7 days of the first session. Subjects that passed both sessions of the double-blind, placebo-controlled food challenge were given a one-week open challenge with the test formula to assess tolerance and confirm the absence of any delayed allergic reactions. Thirty-six subjects received the test formula first and control formula second, 31 subjects received the control formula first and the test formula second. Hypoallergenicity was accepted if at least 90% of the subjects tolerated the test formula. Of the 67 children that began the study 61 completed the study. Results indicated they was one allergic reaction to the test formula and one allergic reaction to the control formula.

Conclusion: The authors concluded the whey-based EHF with 2'-FL and LNnT met the clinical hypoallergenicity criteria.

Clinical study with adults

Safety and Tolerance of 2'-FL in the Diets of Adults (Elison *et al.* 2016)

In a double-blind, parallel, randomized, placebo-controlled study, Elison *et al.* (2016) evaluated the effects of supplementing the diets of 100 healthy adults (19 – 57 years old) with 2'-FL (provided by Glycom A/S) and/or lacto-N-neotetraose (LNnT) for up to two weeks. Study participants (51 males and 49 females) received 5, 10 or 20 grams of either 2'-FL, LNnT or 2'-FL with LNnT (2:1 mass ratio) or 2 g of glucose as the placebo each day at breakfast. The authors requested that participants not alter their current diet and considered participants compliant if they took the treatment for at least 12 of the 14 days of the intervention.

Participants completed a self-administered form reporting on abdominal pain, indigestion, reflux, diarrhea, and constipation which are ranked from 1 (no discomfort) to 7 (very severe discomfort) (gastrointestinal symptom rating scale (GSRS)). Participants completed the form at screening, at entry to the study, and at the end of the intervention period. Participants also recorded bowel movement frequency and stool consistency. Blood samples were collected at screening and the end of the intervention and were analyzed for hemoglobin, erythrocytes, hematocrit, leucocytes, thrombocytes, creatinine, sodium, potassium, alanine aminotransferase, alkaline phosphatases, coagulation factor II, VII and X, bilirubin, albumin, C-reactive protein and glucose as well as HbA1c, apoA1, apoB, transferrin, progesterone, cortisol, estradiol, interleukin-10, interleukin-6, tumor necrosis factor-α, blood urea nitrogen, iron, TAG, HDL-cholesterol, LDL-cholesterol, total free fatty acids, insulin, lysozyme, testosterone and glucagon. Fecal samples were collected prior to study entry and at the end of the intervention and analyzed for calprotectin, secretory IgA, and short-chain fatty acid levels as well as fecal microbiota composition.

Elison *et al.* (2016) stated that 44 participants reported a total of fifty-six adverse events that the author judge as mild. No participants dropped from the study. The adverse events were usually a combination of symptoms including flatulence, bloating, and constipation. The participants taking the highest doses of 2'-FL and LNnT reported the most adverse events. Flatulence was the most commonly reported adverse event followed by stomach pain, diarrhea or loose stool, and

"rumbling." The reports of bloating and gas were significantly higher in the 20 g 2'-FL and LNnT groups. The 20 g 2'-FL group also reported increased rumbling. The mean GSRS scores were low (mean score of < 3 which is mild discomfort or below) and those subjects receiving the highest dosages did not have statistically significant changes in their GSRS. Changes in the average number of daily bowel movements were small, though statistically higher in the 20 g 2'-FL, 20 g LNnT, and 5 g LNnT groups (increase of 0.3 movements per day compared to baseline) but the authors deemed this as clinically irrelevant. The authors stated that participants receiving 20 g 2'-FL reported softer stools as compared to baseline. Because many of the participant reported events are common gastrointestinal symptoms, the authors state that it is difficult to determine if they were due to treatment or normal variation and increased participant awareness of gastrointestinal symptoms during the study period. The authors also reported that all measured clinical chemistry and hematology parameters remained within normal ranges.

Conclusion: Elison *et al.* (2016) concluded that 2'-FL is safe and well tolerated at concentrations up to 20 g 2'-FL per day for 14 days in healthy adults.

POST-MARKET REPORTS

Based on an infant formula supplier's post-market surveillance of products containing Jennewein 2'-FL, no indications of issues with Jennewein 2'-FL have been observed or reported.

Table 20: Toxicological and clinical data summary of 2'-Fucosyllactose.

Study Type	Cell type or Species/ Sex / Number or Population	Route of Exposure; Dose – Duration	Observed Effects	Reference	Conclusionary Remarks
Jennewein 2'-FL					
Mutagenicity OECD 474	Rat bone marrow cells	Oral; 500, 1000 or 2000 mg/kg bw – 24 and 48 h post-administration	No signs of acute systemic toxicity; no mutagenic effects at any dose.	Appendix P-1	Under the test conditions, Jennewein 2'-FL is not genotoxic and is not acutely toxic.
Mutagenicity OECD 471	*S. typhimurium* (TA98, TA100, TA102, TA1535 and TA1537)	Up to 5000 µg/plate; with & without metabolic activation	No cytotoxicity; no increase in revertant colonies; no mutagenic effects.	Appendix P-2	Under the test conditions, Jennewein 2'-FL is not mutagenic or cytotoxic.
Genotoxicity OECD 487	Cultured human peripheral lymphocytes	Up to 5000 µg/mL medium; with & without metabolic activation	No genotoxicity; no indications of chromosomal damage.	Appendix P-6	Under the test conditions, Jennewein 2'-FL is not genotoxic.
Oral Toxicity Pilot Study	Rat / f / 10	Oral – dietary; 10% in feed, ad libitum; 7 days	No mortalities; no change in behaviour or appearance; no difference in food consumption or body weight from controls.	Appendix P-3	Under the test conditions, Jennewein 2'-FL did not induce toxic effects after ingestion by female rats.
Oral Toxicity, Repeated Dose OECD 408	Rat / mf / 64	Oral – dietary; 10% in feed, ad libitum (Mean = 7700 mg/kg bw in m; 8700 mg/kg bw in f); 90 days	No mortalities; no change in behaviour or appearance; pale faeces observed in approximately half of 2'-FL animals; no difference in food consumption or body weight from controls.	Appendix P-4	Under the test conditions, Jennewein 2'-FL did not induce toxic effects after repeated ingestion by rats.

3.3.3 Substances Used for a Nutritive Purpose

Study Type	Cell type or Species/ Sex / Number or Population	Route of Exposure; Dose – Duration	Observed Effects	Reference	Conclusionary Remarks
Oral Toxicity, Pre-clinical	Pigs, neonatal farm / mf / 48	Oral – dietary; 0, 200, 500, and 2000 mg/L; 21 days	Mortalities: None. **Clinical signs:** No treatment related effects. **Body weight:** No treatment related effects. **Necropsy:** Microscopic findings: 1 m and 1 f in high-dose group, 1 f in mid-dose group exhibited mild to moderate inflammation within the keratinized portion of the squamous epithelium of the non-glandular part of the stomach. Another m of high-dose group exhibits focal loss/thinning of this area, but no ulceration. The authors considered these effects incidental and typical.	Hanlon & Thorsrud 2014	Under the test conditions, Jennewein 2'-FL did not induce toxic effects after repeated ingestion by piglets.
2'-FL Produced by Chemical Synthesis or Microbial Fermentation from Other Sources					
Mutagenicity OECD 471	*S. typhimurium* (TA98, TA100, TA102, TA1535 and TA1537)	up to 5000 µg/plate; with & without metabolic activation	No cytotoxicity; no increase in revertant colonies; no mutagenic effects.	Coulet *et al.*, 2014	Under the test conditions, 2'-FL is not mutagenic or cytotoxic.
Mutagenicity OECD 471	*S. typhimurium* (TA1535, TA1537, TA98, and TA100); *E. coli* strain WP2 *uvrA*	Up to 5000 µg/plate with & without metabolic activation	No toxicity to any of the strains tested; no significant or dose related increase in revertant colonies; no mutagenic effect.	Van Berlo *et al.*, 2018	Under the test conditions, 2'-FL is not mutagenic.
Mutagenicity OECD 471	*S. typhimurium* (TA98, TA100, TA1535, and TA1537); *E. coli* WP2 *uvr*A	Up to 5000 ug/plate with & without metabolic activation	No significant differences in the mean number of revertant colonies in the presence or absence of metabolic activation between control and exposed groups.	Phipps *et al.*, 2018	Under the test conditions, 2'-FL/DFL is not mutagenic.
Mutagenicity OECD 471	*S. typhimurium* (TA1535, TA1537, TA98, and TA100); *E. coli* strain WP2 *uvrA*	Up to 5000 µg/plate with & without metabolic activation	No cytotoxicity to any of the strains tested; no significant or dose related increase in revertant colonies; no mutagenic effect.	Verspeek-Rip *et al.*, 2015 as cited in GRN 650 and GRN 735	Under the test conditions, 2'-FL is not mutagenic or cytotoxic.

3.3.3 Substances Used for a Nutritive Purpose

Study Type	Cell type or Species/ Sex / Number or Population	Route of Exposure; Dose – Duration	Observed Effects	Reference	Conclusionary Remarks
Mutagenicity OECD 487	Human peripheral blood lymphocytes	Up to 2000 ug/mL (3 hours with and without metabolic activation or 20 hours without metabolic activation)	No biologically relevant differences in the percentage of micronucleated cells between control and exposed groups. No evidence of clastogenicity or aneugenicity.	Phipps *et al.*, 2018	Under the test conditions, 2'-FL/DFL is not mutagenic.
Mutagenicity OECD 487	Cultured binucleated human lymphocytes	Up to 2000 µg/mL with & without metabolic activation (4 hours treatment/20 hours recovery or 20 hours treatment and no recovery)	No cytotoxicity observed at any concentrations tested with or without metabolic activation; no significant dose-dependent increase in the number of binucleated cells containing micronuclei; no mutagenic effect	Van Berlo *et al.*, 2018	Under the test conditions, 2'-FL is not mutagenic or cytotoxic.
Mutagenicity OECD 487	Peripheral human lymphocytes	Up to 2000 µg/mL with & without metabolic activation	No significant increase in the number of micronucleated cells in the presence or absence of metabolic activation.	Verbaan *et al.*, 2015a as cited in GRN 650 and 735	Under the test conditions, 2'-FL is not mutagenic.
Mutagenicity OECD 487	Peripheral human lymphocytes	Up to 2000 µg/mL with & without metabolic activation for 3 hours with a 27-hour harvest time or for 24 hours with a 24-hour harvest time	No significant increase in cytotoxicity or in the number of micronucleated cells in the presence or absence of metabolic activation.	Verbaan *et al.*, 2015b as cited in GRN 650 and 735	Under the test conditions, 2'-FL is not mutagenic or cytotoxic.
Mutagenicity OECD 476	Mouse lymphoma cells (TK-locus)	up to 5000 µg/mL; with metabolic activation (4 h) & without (4 h, 8 h)	No cytotoxicity; no increase in mutant frequency.	Coulet *et al*., 2014	Under the test conditions, 2'-FL is not mutagenic or cytotoxic.
Oral Toxicity Pilot Study	Rat / mf / 50	Oral – gavage; 0, 2000, 5000, 7500 mg/kg bw & FOS at 7500 mg/kg bw – 14 days	**Mortalities:** 2 f in 7500 mg/kg bw group. **Clinical signs:** Yellow, liquid feces (erythema in urogenital area) in 2'-FL 5000 & 7500 mg/kg bw groups (from days 1,3 up to 9, 11) and in FOS group. **Body weight:** Lower in females of high dose 2'-FL (-7.7%) & FOS groups (-6.3%). **Necropsy:** 1 f 2'-FL mid-dose had herniation between right & left median liver lobes; 1 m 2'-FL low dose had small right testis. Findings considered unrelated to the 2'-FL by the authors.	Coulet *et al*., 2014	The results are consistent with other indigestible carbohydrates.

3.3.3 Substances Used for a Nutritive Purpose

Study Type	Cell type or Species/ Sex / Number or Population	Route of Exposure; Dose – Duration	Observed Effects	Reference	Conclusionary Remarks
Oral Toxicity, Repeated Dose OECD 408	Rat / mf / 100	Oral – gavage; 0, 2000, 5000, 6000 mg/kg bw & FOS at 6000 mg/kg bw – 90 days	**Mortalities:** 1 m and 1 f of high dose 2'-FL on day 2. 2 m of FOS group on days 12 & 13; 1 f of FOS group on day 108. Could not demonstrate relationship to treatment. **Clinical signs:** Diarrhea in all high-, mid-dose 2'-FL, & FOS animals, & several low-dose 2'-FL. Erythema in urogenital area high dose & FOS groups. Hyper-salivation in most high-dose 2'-FL & FOS groups, half of mid-dose 2'-FL group. **Body weight:** Transient lower weights in high-dose and FOS groups, not significant by study end. **Histopathology:** Higher incidence of minimal cortical tubular epithelial cytoplasmic vacuolation in kidneys of the mid- & high-dose 2'-FL and FOS groups. Not dose-dependent, not associated with clinical pathology changes, degeneration. Findings considered unrelated to the 2'-FL by the authors.	Coulet *et al.*, 2014	The results are consistent with other indigestible carbohydrates.

3.3.3 Substances Used for a Nutritive Purpose

Study Type	Cell type or Species/ Sex / Number or Population	Route of Exposure; Dose – Duration	Observed Effects	Reference	Conclusionary Remarks
90-day Oral Toxicity OECD 408	Rat/mf/40 per sex	0, 3, 6, or 10% (w/w) added to feed for 13 weeks	**Mortalities**: No exposure related mortalities were observed. **Clinical signs**: No exposure related clinical signs were observed. **Body weight**: No significant or treatment related changes were observed. **Organ weights:** Relative liver weight was significantly increased in males in the high-dose group. Absolute and relative filled and empty cecum weights significantly increased in the mid- and high-dose males and females **Haematology and Clinical chemistry**: No treatment related changes in haematology or clinical chemistry. **Neurotoxicity**: Functional observational battery and motor activity assessment did not indicate any neurotoxicity. **Histopathology:** No treatment-related macroscopic or microscopic changes were reported. **NOAEL:** Highest level tested of ≥7.25 g/kg body weight/day in males and ≥7.76 g/kg body weight/day in females.	Van Berlo *et al.*, 2018	Under the test conditions 2'-FL did not induce toxic effects after repeated ingestion by rats.

3.3.3 Substances Used for a Nutritive Purpose

Study Type	Cell type or Species/ Sex / Number or Population	Route of Exposure; Dose – Duration	Observed Effects	Reference	Conclusionary Remarks
90-day Oral Toxicity OECD 408	Rat/NR/NR	Oral gavage; 0, 2000, 4000, or 5000 mg 2'-FL/kg bw/day & FOS at 5000 mg/kg bw/day; 90 to 91 days with 28-day recovery	**Mortalities:** No exposure related mortalities were observed. **Clinical Signs:** Liquid faeces in mid- and high-dose groups and reference groups; soiled urogenital areas in mid- and high-dose groups; hypersalivation, abnormal foraging and/or pedaling in mid- and high dose group and reference group. **Body weights and organ weights**: No significant changes in body weight, body weight gain, or food consumption reported; no changes in organ weights. **Other:** No toxicological effects noted in tibia length, reflex and physical development, time to sexual maturation, learning capacity memory, motor activity, exploratory behaviour, or general movement. **Haematology and clinical chemistry**: No significant haematological changes; decreased triglyceride concentrations in the mid- and high-dose males; decreased cholesterol concentrations in all treated males and mid- and high-dose females; individual urea concentrations increased in high-dose group. Changes were within historical control ranges and were not observed following the recovery period. **Urinalysis:** No treatment related changes. **Macroscopic and histopathological evaluation:** No treatment related effects reported. **NOAEL:** The highest dose tested of 5000 mg/kg/day.	Penard *et al.*, 2015 as cited in GRN 650	Under the test conditions 2'-FL did not induce toxic effects after repeated ingestion by rats.

3.3.3 Substances Used for a Nutritive Purpose

Study Type	Cell type or Species/ Sex / Number or Population	Route of Exposure; Dose – Duration	Observed Effects	Reference	Conclusionary Remarks
90-day Oral Toxicity OECD 408	Neonatal rats/mf/10 per sex per group	Oral gavage; 0, 1000, 3000, or 5000 mg 2'-FL/DFL (2'-fucosyllactose and difucosyllactose in an 8:1 ratio)/kg bw/day for 90 days with 28-day recovery	**Mortalities**: No mortalities were observed. **Clinical signs**: No exposure related clinical signs were observed. **Body weight**: No significant or treatment related changes were observed. **Organ weights:** Relative kidney and seminal vesicle weight were significantly increased in males in the low-dose group. Thymus weight was significantly increased for all male treatment groups, but no dose-response observed. Relative pituitary weights were significantly increased in females in the high-dose group at the end of the recovery period. **Sexual maturation and development** No exposure-related differences in the age or body weight at which the males and females attained physical signs of sexual maturation. The mean age for balano-preputial skinfold separation was slightly higher in highest-exposed males compared with vehicle controls. No differences in age of attainment of reflexes, startle response test, and mean ulna growth **Haematology and Clinical chemistry**: No test item-related or dose-responsive changes in haematology or clinical chemistry. **Neurotoxicity**: Functional observational battery and motor activity assessment did not indicate any neurotoxicity. **Histopathology:** No treatment-related macroscopic or microscopic changes were reported. **Urinalysis:** No biologically relevant or test item-related differences. **NOAEL:** Highest level tested of 5000 mg/kg bw/day	Phipps *et al.*, 2018	Under the test conditions 2'-FL did not induce toxic effects after repeated ingestion by rats.

3.3.3 Substances Used for a Nutritive Purpose

Study Type	Cell type or Species/ Sex / Number or Population	Route of Exposure; Dose – Duration	Observed Effects	Reference	Conclusionary Remarks
Clinical studies in infants					
Randomized, controlled, double-blind multicenter study	Infants age 14 ± 5 days; 30 infants in study population; 33 infants in control population	Ingestion of formula containing 0 or 0.25 g/L 2'-FL; 42 days	*Outcomes based on questionnaires completed by caregivers* **Primary Outcome** **Infant Gastrointestinal Symptom Questionnaire (IGSQ) score:** No significant differences reported in IGSQ scores at baseline or at visit 1 at 42 weeks exposure. **Secondary Outcomes** **Stool frequency, consistency, and ease of passing:** No significant differences in stool frequency or consistency between the control and test group. Significantly more stools reported difficult to pass in the control groups compared to the test group. However, the number of infants with stool difficult to pass did not differ significantly between groups. **Spit up, Vomiting, Crying, and Fussing:** No differences in the occurrences of crying and fussing and vomiting frequency between groups. Proportion of infants to have any spit up did not differ between groups; however, in the infants reported to spit up, significantly more were reported to spit up > 5 times per day in the test groups compared to the control. **Formula intake**: Average intake of formula did not differ significantly between groups. **Adverse Events:** No serious adverse events reported. Spit up as an adverse event occurred in more test subjects compared to controls. Significantly more infections and infestations reported in the control group than in the treated groups.	Storm *et al.*, 2019	Formula containing 2-FL was tolerated well based on a comprehensive tolerance assessment tool.

Study Type	Cell type or Species/ Sex / Number or Population	Route of Exposure; Dose – Duration	Observed Effects	Reference	Conclusionary Remarks
Randomised, double-blind and controlled study.	Healthy full-term singleton infants were enrolled by five days of age; 424 originally enrolled; 304 infants completed the study (79 Control formula, 70 Experimental formula 1, 72 Experimental formula 2, and 83 Human milk)	Ingestion of formula containing 0, 0.2, 1.0 g/L 2-FL or human breast milk for 119 days	No significant differences reported among any groups for weight, length or head circumference; 2'-FL was present in the plasma and urine of infants fed 2'-FL, and there were no significant differences in 2'-FL uptakes relative to the concentration fed; growth and 2'-FL uptakes were similar to those of breast-fed infants.	Marriage *et al.*, 2015	Formula containing 2-FL was tolerated well.
Sub-study nested within the Marriage *et al.* (2015) study; and included the same study groups.	Healthy full-term singleton infants were enrolled by five days of age; 315 of the 424 originally enrolled in Marriage *et al*. (2015); 155 infants completed the study (39 Control formula, 37 Experimental formula 1, 37 Experimental formula 2, and 42 Human milk)	Ingestion of formula containing 0, 0.2, 1.0 g/L 2'-FL or human breast milk for 119 days	Infants fed formula supplemented with 2'-FL exhibited lower plasma and *ex vivo* inflammatory cytokine profiles, similar to those of a breastfed reference group. These findings indicate that 2'-FL supports aspects of immune development and regulation similar to that in a breastfed reference group.	Goehring *et al.*, 2016	Formula containing 2-FL was tolerated well.
Multi-center, randomized, double-blind trial of two parallel groups	175 healthy full-term infants; 0 to 14 days old	Ingestion of formula containing 0 or 1 to 1.2 g/L 2'-FL for 6 months	No significant differences in weight gain between the test and control groups; mean weight, length, head circumference and body mass index (BMI) for all infants through age 4 months were comparable with the WHO standard growth curves; no changes in stool endpoints or composition of the microbiota in infants in the test group compared to controls; significantly lower incidences of bronchitis and antibiotic use in treated infants compared to infants in the control group.	Puccio *et al*., 2017	Formula containing 2-FL was tolerated well

Study Type	Cell type or Species/ Sex / Number or Population	Route of Exposure; Dose – Duration	Observed Effects	Reference	Conclusionary Remarks
Prospective, randomized, multi-center, double-blinded, controlled tolerance trial	131 Healthy term infants	Infant formula containing 0 (n = 30) or 0.2 g/L 2'-FL and 2 g/L short-chain fructooligosaccharide (scFOS) (n = 35) or human breast milk (n = 36); 35 days	No significant differences in stool consistency, formula intake, anthropometric measures, or percent feedings with spit-up/vomit associated with feeding among the three groups at 35 days of age; breast milk fed infants had a greater number of stools/day than formula fed infants.	Kajzer *et al.*, 2016 as cited in Reverri *et al.*, 2018	No significant differences in gastrointestinal tolerance between infants fed formula containing 2'-FL and infants fed human breast milk were reported.
Double blind, placebo-controlled food challenge	67 children with cow's milk protein allergy	Extensively hydrolyzed infant formula containing 1 g/L 2'-FL and 0.5 g LNnT or control formula; one challenge 3-28 days after enrolment and a second challenge 2-7 days after the first followed by a one-week open challenge with test formula	One allergic reaction to the test formula and one allergic reaction to the control formula.	Nowak-Wegrzyn *et al.*, 2019	The authors concluded that the formula containing 2'-FL and LNnT met the clinical hypoallergenicity criteria.
Clinical study in adults					
Double-blind, parallel, randomized, placebo-controlled study	100 healthy adults age 19 to 57 years (51 males and 49 females).	Diets supplemented with 5, 10 or 20 g of either 2'-FL, LNnT or 2'-FL+LNnT (2:1 mass ratio) or 2 g of glucose as the placebo each day at breakfast	44 participants reported a total of 56 mild adverse events usually a combination of symptoms including flatulence, bloating, and constipation. The most adverse events were reported by participants receiving the highest doses of 2'-FL and LNnT with flatulence being the most commonly reported adverse event followed by stomach pain, diarrhea or loose stool, and "rumbling"; reports of bloating and gas were significantly higher in the 20 g 2'-FL and LNnT groups; mean GSRS scores were low and participants receiving the highest dosages did not have statistically significant changes in their GSRS; participants receiving 20 g 2'-FL reported softer stools as compared to baseline; all measured clinical chemistry and haematology parameters remained within normal ranges.	Elison *et al.*, 2016	No adverse health effects were noted in adults consuming diets supplemented with up to 20 g 2'-FL per day.

Abbreviations: 2'-FL = 2'-fucosyllactose; bw = body weight; d = day; f = female; FOS = fructo-oligosaccharide; h = hour(s); kg = kilogram(s); m = male; mg = milligram(s); mL = milliliter(s); OECD = Organization for Economic Cooperation and Development; µg = microgram(s)

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

This includes safety assessment reports prepared by the WHO or by other national or supranational agencies responsible for food safety or public health.

A safety assessment that was prepared by the Committee of Safety Assessment of Novel Foods in the Netherlands and presented to the European Union (NFU, 2016) concluded that, based on Jennewein's application, 2'-FL can be safely used as an ingredient in infant formula and follow-on formula as described in the dossier.

In addition to GRN 571 in the United States, five other GRNs (546, 571, 650, 735, and 749) were submitted for 2'-FL intended for similar use and use levels those applied for in this Application. All five GRNs received letters of no objection from the FDA. A safety assessment conducted by the European Food Safety Authority in 2015 (EFSA 2015) conservatively concluded that 2'-FL in combination with lacto-N-neotetraose in infant formula at concentrations up to 1.2 g/L was safe for infants up to one year old.

We are unaware of safety assessment reports on the 2'-FL substance prepared by WHO or by other national supranational agencies responsible for food safety or public health.

However, the nutritional benefits of human milk, which contains 2'-FL, has been identified by many health agencies including the following Australian agencies/organisations (refer also to **Section 3.3.3 A.1** of this Application for the nutritional benefits of human milk):

- The Australian and New Zealand Food Regulation Ministerial Council
- Australian National Health and Medical Research Council (NHMRC, 2012)
- The Australian Breastfeeding Association
- The New Zealand Ministry of Health
- The Parliament of the Commonwealth of Australia (CoA, 2007)
- The Australia & New Zealand Infant Nutrition Council

Also refer to **Section 3.1.1 D.d** for information on the assessment of similar applications for Jennewein 2'-FL in the United States, the European Union, Canada and other countries.

D. Information on dietary intake of the nutritive substance

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

This includes information about the nutrient content of foods to which the use of the nutritive substance is proposed such as total fat and saturated fat, total sugars, sodium, and energy content.

Jennewein 2'-FL is proposed to be used as a food ingredient in infant formula (for infants 0 to <12 months), follow-on formula (for infants 6 months to <12 months of age), infant formula products for special dietary use, and FSFYC, i.e. toddler formula (for children 1 to 3 years of age). Infant formula (for infants 0 to <12 months), follow-on formula (for infants 6 months to <12 months of age), and infant formula products for special dietary use are listed in Standard 2.9.1 of the Australia New Zealand Food Standards Code "Infant Formula Products" (under review as of November 2017). Standard 2.9.3 Division 4 "Formulated Supplementary Foods for Young Children" lists requirements for toddler formula. Infant formula standards have been put forth by the Food

and Agriculture Organization of the United Nations[17] as well as other entities including the United States Food and Drug Administration[18], European Commission[19], and Health Canada[20].

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

This includes information on the proposed levels of the use in food, as well as naturally-occurring levels in foods.

Intended uses of Jennewein 2'-FL

Jennewein intends to use Jennewein 2'-FL as an ingredient in infant formula (0 – 12 months), follow-on formula (6 – 12 months), and toddler formula (1 – 3 years).

The target daily intake of 2'-FL in infant formula is a maximum of 2 g of 2'-FL per litre of formula (as consumed), which corresponds to the mean concentration of 2'-FL normally found in human milk (Chaturvedi *et al*., 2001). Refer to the discussion below for further information relating to infant dietary exposure to naturally occurring sources of 2'-FL.

Infant dietary exposure to naturally occurring sources of 2'-FL

Most infants have been exposed to 2'-FL because it is a naturally occurring component of human milk. HMO is the third largest component of breast milk solid matter after lactose and lipids and 2'-FL is the most abundant glycan in human milk (Castanys-Munoz *et al*., 2013; Coppa *et al*., 2004).

A study conducted by Erney *et al*. (2000) collected 549 breast milk samples from 435 mothers residing in 10 countries: Chile, France, Germany, China (Hong Kong), Italy, Mexico, the Philippines, Singapore, Sweden and the United States. Of the samples collected, information regarding lactation stage was obtained for 492 samples (90%) which permitted the samples to be categorised into the following groups:

- the first two days after birth (*n*= 23);
- days 3 through 10 after birth (*n*=77);
- days 11 through 30 after birth (*n* = 222); and
- more than 30 days after birth (*n* = 170).

All samples represented the entire content of one or both breasts and were frozen immediately following collection. This study revealed that 2'-FL was the most abundant sugar (2.38 g/L) in the breast milk samples which was identified in 85% of the samples; with concentrations ranging between 0.06 to 4.65 g/L. A summary of the results relating to detection of 2'-FL in breast milk samples is provided in **Table 21, Table 22** and **Table 23**.

17 http://www.fao.org/fao-who-codexalimentarius/sh-proxy/fr/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCODEX%2BSTAN%2B72-1981%252FCXS_072e.pdf

18 https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=107.100

19 https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32006L0141

20 https://laws-lois.justice.gc.ca/eng/regulations/C.R.C.,_c._870/page-85.html?txthl=b.25.059#s-B.25.047

Table 21: Percent of mothers producing 2'-FL reported in the Erney *et al.* (2000) study

Country	Percent of Mothers Producing 2'-FL	Number of Samples Analysed
Chile (44)	84%	44
China (32)	78%	32
France (22)	91%	22
Germany (18)	83%	18
Italy (29)	86%	29
Mexico (156)	100%	156
Philippines (22)	46%	22
Singapore (26)	72%	26
Sweden (7)	100%	7
United States (79)	68%	79

Table 22: Average concentration of 2'-FL in human milk by region (Erney *et al.*, 2000)

Region	Concentration of 2'-FL (g/L)	Number of Samples Analysed
Asia	2.07	80
Europe	2.58	68
Latin America	2.47	197
United States	1.99	36
All Samples	2.38	381

Table 23: Average concentration of 2'-FL in human milk by postpartum interval and region (Erney *et al.*, 2000)

Postpartum Interval	Concentration of 2'-FL (g/L)	Number of Samples Analysed
3-10 days		
Asia	2.26	25
Europe	2.69	14
Latin America	2.79	19
United States	2.78	4
11-30 days		
Asia	2.36	20
Europe	2.38	21
Latin America	2.61	129
United States	2.56	8
31-452 days		
Asia	1.5	24
Europe	2.36	25
Latin America	1.91	49
United States	1.69	24

A subsequent study found that the average concentration of 2'-FL over a lactation period of 50 weeks was 2.43 ± 0.26 g 2'-FL/L of breast milk (Chaturvedi *et al*., 2001).

Not all infants are exposed to 2'-FL, however, because not all women produce breast milk containing 2'-FL. The fucosylation of glycans depends on the mother's blood group status: Lewis (+)/(-) and Secretor/non-Secretor (**Table 24**).

Table 24: Enzyme activity by blood group in the general population

Blood groups	Enzyme activity	% Population	Major HMOs
Secretor (Group 1)	α1-2 fucosyltransferase	70	2'-FL (Fucα1-2Galβ1-4Glc), Lacto-N-fucopentaose (Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc)
Lewis (a+b-) (Group 2)	α1-4 fucosyltransferase	20	Lacto-N-fucopentaose II (Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc)
Lewis (a-b+) (Group 3)	α1-2 fucosyltransferase α1-4 fucosyltransferase	70	Lacto-N-difucohexaose I (Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc)
Lewis a-b- (GROUP 4)	α1-3 fucosyltransferase	10	Lacto-N-fucopentaose)III (Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc)

Source: adapted from Kunz and Rudloff (2008)

Glc: glucose; Gal: galactose; FUC: L-fucose; GlcNac: N-acetylglucosamine

The Secretor can synthesise 2'-FL in the mammary gland (Castanyz-Munoz *et al*., 2013). About 70% of women are Secretors due to the presence of α1-2 fucosyltransferases (FUT2) in their milk (Kunz *et al*., 1999). The breast milk of non-Secretor women does not contain FUT2 but another fucosyltransferase, α-1,3/4-fucosyltransferase (FUT3), which links Fuc to subterminal GlcNAc in α1-4 linkages (Bode and Jantscher-Krenn, 2012). Secretor Lewis (+) women have the most complex HMO composition while non-Secretor Lewis (-) women have the least complex (Bode and Jantscher-Krenn, 2012). Because the majority of women are Secretors, infants receiving milk from donor human milk programs are likely to be ingesting 2'-FL. Therefore, infants born to Secretor and non-Secretor mothers are routinely exposed to 2'-FL.

Thurl *et al*. (2010) found that 2'-FL concentration decreased from day 3 to day 90 of the lactation period from 4.1 to 2.6 g/L, respectively. Though the concentration of 2'-FL declines as lactation continues, the volume of breast milk consumed increases as the infant develops, therefore the amount of 2'-FL remains fairly constant throughout the nursing period (Asakuma *et al*., 2008; Thurl *et al*., 2010). As a result, an infant up to three months of age born to a Secretor mother may ingest from two to three grams of 2'-FL per day.

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

This includes any consumption information for food groups not included in the most recent Australian or New Zealand NNSs which relate to the application. Data distinguishing likely consumption levels among target and non-target groups are preferred.

Note: Information on likely consumption can be based on proposed levels of consumption (grams per day) or on consumption data for these foods from a similar market in another country. The most recent NNSs are the 2011–12 National Nutrition and Physical Activity Survey (NNPAS) component of the 2011–13 Australian Health Survey (2 years and above), the 2008–09 New Zealand NNS (15 years and above) and the 2002 New Zealand Children's NNS (5–14 years).

NHMRC/NZ MoH (2006) and EFSA NDA Panel (2013, 2014) report that the mean volume of formula intake for infants aged 0-<6 months is approximately 0.8 L/day (rounded up from 0.78 L/day). For infants aged 6-<12 months, the mean volume of follow-on formula intake is approximately 0.6 L/day (NHMRC and NZ MoH, 2006) which assumes intake of complementary foods in addition to follow-on formula. The reference bodyweights are 7 kg for infants 2-6 months of age, and 9 kg for infants 7-11 months of age. These daily estimates of breast milk/formula intake are commonly used in Australian dietary exposure estimates for infants aged 0-<12 months (FSANZ, 2016). In FSANZ's "Proposal P306 for the addition of inulin/FOS and GOS to Food", a theoretical estimate of formula intake was prepared for children aged 1-3 years due to lack of food consumption data from children aged <2 years in Australia and <5 years in New Zealand (FSANZ, 2008). The estimates were derived from the Australian National Nutrition Survey for Australian children and from the New Zealand Total Diet Study for New Zealand children. FSANZ determined that the range of toddler formula consumption was between 0.3 L/day (rounded up from 0.285 L/day) and 0.4 L/day (rounded down from 0.425 L/day) for young children between the ages of 1 and 3 years.

Published scientific literature demonstrates that 2'-FL used at a level of 2 g/L in infant formula corresponds to the mean concentration of 2'-FL normally found in breast milk (refer to **Section 3.3.3 D.2** of this Application). Therefore, the proposed maximum target use of Jennewein 2-FL in infant and follow-on formula is 2 g/L of formula as consumed.

Table 25 presents the estimates of Jennewein 2'-FL intake per user per day assuming a concentration of 2 g/L in infant formula, follow-on formula, and toddler formula.

Table 25: Estimated daily intake of Jennewein 2'-FL from infant formula, follow-on formula, and toddler formula (Australia and New Zealand)

Population	Concentration of 2'-FL (g/L)	Mean Formula Intakes Per User (L/d)		Jennewein 2'-FL Intakes per User (g/d)	
Infants, 0-<6 months (infant formula)[a]	2	0.8		1.6	
Infants, 6-<12 months (follow-on formula)[a]	2	0.6		1.2	
Toddlers, 1 – 3 years (toddler formula)[b]	2	0.3	0.4	0.6	0.8

Source: [a]NHMRC and NZ MoH, 2006; [b]FSANZ 2008; Jennewein Biotechnologie, GmbH

Abbreviations: 2'-FL – 2'-fucosyllactose; d – day; g – gram(s); L – litre(s); NHMRC – National Health and Medical Research Council; NZ MoH – New Zealand Ministry of Health

Some infants and toddlers, however, consume a combination of human milk and infant or toddler formula (commonly known as complimentary feeding). Because the proposed use level of 2 g/L is equivalent to the mean concentration of 2'-FL normally found in breast milk, the overall intake of 2'-FL by combination breast milk/formula fed infants and toddlers is unlikely to differ significantly than the estimates provided in this Application.

As described in **Section 3.1.1 D.d**, several countries/jurisdictions have accepted applications regarding Jennewein 2'-FL. Estimated daily intake values were calculated using each jurisdiction's exposure data for the target population of infants and toddlers.

Based on the proposed use of up to 2 grams Jennewein 2'-FL/L in non-exempt infant formula for term infants and toddler formula and exposure data from the U.S. Department of Health and Human Service's 2009-2010 National Health and Nutrition Examination Survey, the highest estimated mean intake of Jennewein 2'-FL is 1.8 g/d and occurs in infants 0-5 months of age. See **Table 26** below for details. The estimates of potential ingredient intake by infants and toddlers were based on a survey population of non-breastfeeding infants. Some infants, however, consume a combination of human milk and infant formula. Because the use level of 2 g/L corresponds to the mean concentration of 2'-FL normally found in human milk, the overall intake of 2'-FL by combination human milk/formula-fed infants would likely be comparable to the intake of 2'-FL by infants receiving human milk only.

Table 26: Estimated daily intake of Jennewein 2'-FL from infant formula, follow-on formula, and toddler formula (U.S.)

Population	Concentration of 2'-FL (g/L)	Mean Formula Intakes Per User (L/d)	Jennewein 2'-FL Intakes per User (g/d)
Infants, 0 – 5 months (infant formula)	2	0.9	1.8
Infants, 6 – 11 months (follow-on formula)	2	0.8	1.6
Toddlers, 12 – 35 months (toddler formula)	2	0.5	1.1

Source: GRN 571 (Jennewein Biotechnologie, GmbH) (Appendix A)

Abbreviations: 2'-FL – 2'-fucosyllactose; d – day; g – gram(s); GRN – Generally Recognized As Safe Notice; L – litre(s)

Jennewein 2'-FL received a Letter of No Objection from Health Canada stating no objection to the use of 2'-FL at a maximum level of 1.2 g 2'-FL/L infant formula for term infants. This was similarly based on the U.S. Department of Health and Human Service's 2009-2010 National Health and Nutrition Examination Survey. Estimated daily intake was requested to be calculated on a 1.0 g/L concentration of 2'-FL. Based on a concentration of 1.0 g Jennewein 2'-FL per L formula, the highest estimated mean intake of Jennewein 2'-FL is 0.9 g/d and occurs in infants 0-5 months of age (**Table 27**).

Table 27: Estimated daily intake of Jennewein 2'-FL from infant formula, follow-on formula, and toddler formula (Canada)

Population	Concentration of 2'-FL (g/L)[a]	Mean Formula Intakes Per User (L/d)[b]	Jennewein 2'-FL Intakes per User (g/d)
Infants, 0 – 5 months (infant formula)	1.0	0.9	0.9
Infants, 6 – 11 months (follow-on formula)	1.0	0.8	0.8
Toddlers, 12 – 35 months	1.0	0.5	0.5

Source: [a]Health Canada NFN4000740 (Jennewein Biotechnologie, GmbH) and Health Canada Letter of No Objection, December 2018 (Appendix C); [b]NHANES 2009-2010

Abbreviations: 2'-FL – 2'-fucosyllactose; d – day; g – gram(s); L – litre(s); NFN – Novel Food Notification

In the EU, the maximum intended use level of Jennewein 2'-FL in infant formulas is 2 g/L as consumed. Consumption data for infant formulas are difficult to obtain, and in the Application to the EU, the product information on commercial infant formulas was used. The anticipated intake of Jennewein 2'-FL was extrapolated under the assumption that infants would be exclusively formula-fed and that only formula fortified with Jennewein 2'-FL would be used. As the infant gets older, the number of meals decreases as the size of the meal increases. Thus, the anticipated intake of Jennewein 2'-FL at the maximum intended use level of 2 g/L as consumed is only slightly lower in infants older than 6 months compared with infants 6 months or younger (see **Table 28**).

Table 28: Estimated daily intake of Jennewein 2'-FL from infant formula and follow-on formula (European Union):

Population	Concentration of 2'-FL (g/L)	Formula Intakes Per User (L/d)	Jennewein 2'-FL Intakes per User (g/d)
Infants, 0 – 6 months (infant formula)	2	0.5	0.98
Infants, >6 months (follow-on formula)	2	0.5	0.92

Source: EFSA 2017 (Appendix B); Jennewein Biotechnologie, GmbH

Abbreviations: 2'-FL – 2'-fucosyllactose; d – day; g – gram(s); L – litre(s)

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

This includes information based on projected uptake of the use of the nutritive substance in foods or market share data for foods to which the use of the nutritive substance is likely. This could be based on a similar market in another country.

At this stage, the percentage of infant and toddler formula products on the Australian and New Zealand markets that will contain the Jennewein 2'-FL oligosaccharide is unknown. However, expressions of interest have been received from three major infant formula manufactures (refer to **Section 3.1.1 D.g** of this Application) to include the Jennewein 2'-FL oligosaccharide in their infant formula product. The market share is estimated to be 25-50%.

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

This includes information on the foods or food groups in which the nutritive substance is used, the use levels and consumption amounts in other countries.
This information provides an indication of the range of foods in Australia and New Zealand that might contain the used nutritive substance.

Jennewein 2'-FL can be found in infant and toddler formula internationally. Jennewein has launched the product in almost 30 countries with applications for Jennewein 2'-FL currently under review in several others. More details on these applications can be found in **Section 3.1.1 D.d**.

Table 29: Countries/Regions in which Jennewein 2'-FL has been launched

Country/Jurisdiction	Product launch date
United States	September 2016
Europe (EFSA)	Projected second half of 2019
Mexico	September 2017
Hong Kong	November 2017
CACM (Costa Rica, El Salvador, Guatemala, Honduras, and Nicaragua)	January 2018
Saudi Arabia	February 2018
Colombia	March 2018
Ecuador	March 2018
Peru	March 2018
Israel	April 2018
Singapore	May 2018
Kuwait	May 2018
Oman	May 2018
UAE	May 2018
Qatar	May 2018
Vietnam	June 2018
Cambodia	June 2018
Philippines	July 2018
Myanmar	July 2018
Chile	August 2018
Russia	August 2018

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

This includes any consumption information for foods where there has been a significant change in consumption since the most recent Australian and New Zealand NNSs which relate to the application. This can be based on market share data or sales data or on a similar market in another country.

It is not anticipated that the consumption of infant formula, follow-on formula, and toddler formula will change from that experienced in recent years. **Section 3.3.3 D.3** of this Application contains information about the consumption of these two foods.

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

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

This includes data to demonstrate the nutritive substance is consistent with its proposed purpose as described in subsection A.1 in this Guideline (3.3.3) and must include:
(a) the target population(s) be clearly stated
(b) data to demonstrate that specific food(s) containing the form and amount of the nutritive substance can contribute to the nutritional purpose in the target population(s) at the anticipated level of intake. The total amount should include naturally-occurring and added amounts.
(c) data to demonstrate that the nutritional composition of the specified substitute food can be aligned with the reference food.

Jennewein 2'-FL is proposed to be added as an ingredient in infant formulas for infants aged 0-12 months, follow-on formulas for infants aged 6-12 months, and toddler formulas for young children aged 1-3 years. The proposed maximum target use of Jennewein 2'-FL in these foods is 2 g/L of formula as consumed. This use level corresponds to the mean concentration of 2'-FL naturally found in breast milk, as reported in published scientific literature (see **Section 3.3.3 D.2** of this Application). Information supporting the potential beneficial effects to infants from consumption of naturally occurring 2'-FL at levels similar to the proposed use levels of Jennewein 2'-FL are detailed in **Section 3.3.3 A.1** of this Application. Clinical studies supporting the nutritional purpose and tolerance of synthesized 2'-FL added to infant formulas are described in **Section 3.3.3 C.2** of this Application. Lastly, evidence supporting substantial chemical equivalence of naturally occurring 2'-FL in human milk and Jennewein's 2'-FL is reported in **Section 3.3.3 B.2** of this Application. Therefore, consumption of infant formula or follow-on formula containing Jennewein 2'-FL will not cause a nutritional imbalance in the diet of infants. Incorporating 2'-FL into infant formula provides a nutritional component present in human milk that is not present in infant formula otherwise.

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

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

It is not expected that the majority of the Australian or New Zealand consumers will have an understanding of the natural composition of human milk, in particular the presence of naturally occurring 2'-FL and the positive health benefits that result from consumption of 2'-FL by infants (as outlined in **Section 3.3.3 A.1** of this Application).

Australian and New Zealand health advisories and breastfeeding associations have increased awareness of the positive health benefits that breastfeeding provides to infants via marketing campaigns and publicly available information (e.g. NHMRC, 2012). Thus, it is anticipated that the majority of parents/care providers in Australia and New Zealand will be aware of the positive health benefits from breastfeeding. According to the *2010 Australian National Infant Feeding Survey* (AIHW, 2011), 96% of mothers initiate breastfeeding and thereafter, exclusive breastfeeding rates decline. Less than half (39%) of infants are exclusively breastfed to three months of age, and less than one quarter (15%) are exclusively breastfed to five months.

Therefore, although it is not expected that Australian and New Zealand consumers are aware of the naturally occurring 2'-FL oligosaccharide, it is expected that consumers are aware and understand the positive health benefits that result from consumption of human milk that contains the 2'-FL substance. As described in **Section 3.3.3 D.5**, Jennewein 2'-FL has been on the market

in the United States and other countries. Consumer survey data is not available for this Application however adverse events are reported and monitored in the U.S. and the Applicant is not aware of any adverse event reports.

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

This includes information such as changes in consumption behaviour and changes in health and diet behaviour.

According to the 2010 Australian *2010 Australian National Infant Feeding Survey* (AIHW, 2011), 94% of mothers breastfed because it was "*healthier for the child*"; and of those mothers who did not breastfeed 26% reportedly said that infant formula was "*as good as breast milk*". These survey data indicate that the majority of the Australian population recognise breastfeeding as the ideal food substance for infants due to the positive health benefits that breast milk provides to infants.

Consumer trend towards the purchase and consumption of healthier food products has been observed for organic infant formula products sold in Australia and New Zealand which are perceived as a superior product by consumers compared to non-organic formula products. According to 'Market Research Future' – Global Organic Infant Formula Report, the market for organic infant formula in Australia and New Zealand is expected to grow from $218 million in 2017 to $400 million by 2023.

Therefore, it is anticipated that consumers will choose infant formula products that contain Jennewein's 2'-FL oligosaccharide since this compound is naturally occurring in breast milk and provides positive health benefits (refer to **Section 3.3.3 A.1** of this Application).

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

Note: Consumption behaviour changes include substitution, addition or avoidance. Health and diet behaviour changes relate to the potential impacts of the food in the context of not promoting patterns inconsistent with nutrition and physical activity policies or guidelines for Australia and New Zealand. The extent of the impact of the use of a nutritive substance to food on consumer behaviour will vary depending on:

(a) the nature of the nutritive substance and the food(s) to which it will be added
(b) the projected consumption levels for the food(s) containing the nutritive substance including amount consumed and how often it will be consumed
(c) whether currently used foods may be substituted for food(s) containing the nutritive substance
(d) whether there is a claim.

Thus, the amount of information necessary to address the impact on consumer behaviour will depend on the level of the impact. This will need to be considered in addressing the points above. Information to support subsections G.1–3 in this Guideline (3.3.3) could include:

(a) a literature review of the available evidence from Australia and New Zealand, or internationally (where appropriate)
(b) robust quantitative or qualitative empirical research (where appropriate) assessing consumer responses to the proposed change e.g. studies assessing the Australian and New Zealand general population; findings broken down by population subgroups, including target and nontarget population groups. Where there is insufficient information on Australian and New Zealand consumer responses (or potential responses), as specified in Section F in this Guideline (3.3.3), FSANZ may request the applicant to conduct empirical research to address these points. FSANZ can provide guidance here.

All infant formula products sold in Australia and New Zealand are regulated by the *Australia New Zealand Food Standards Code* and contain adequate nutrients for infants including added iron and vitamins (including A, B group, C, D, E and K) (4). Through *Standard 2.9.1 (Infant Formula Products)* and *Standard 2.9.3 Division 4 (Formulated Supplementary Foods for Young Children)* of the Food Standards Code, infant formula and toddler formula quality, composition and labelling is regulated for all formula product sold in Australia and New Zealand. The projected consumption levels (estimated daily intake levels) for 2'-FL in infant formula, follow-on formula, and toddler formula are described in **Section 3.3.3 D.3** of this Application.

As stated in **Section 3.3.3 G.1** of this Application, only 15% of infants in Australia are exclusively breastfed to five months of age. NHMRC (2012) states that "*if an infant is not breastfed or is partially breastfed, commercial infant formulas should be used as an alternative to breast milk until 12 months of age. Health workers should provide families with all of the information and support they need to prepare, store and use feeds correctly".* As Jennewein 2'-FL is chemically equivalent to the human milk oligosaccharide 2'-FL found in human milk, infant and toddler formula products containing Jennewein 2'-FL better mimic human milk than infant and toddler formula products not containing Jennewein 2'-FL. It is expected that a certain percentage of infant and toddler formula consumed by the Australian and New Zealand markets will be replaced with infant and toddler formula containing Jennewein 2'-FL. See **Section 3.3.3 D.4** for specific market information.

Therefore, providing the infant formula is prepared and stored according to the guidance provided by NHMRC (2012) and toddler formula is similarly handled, infant and toddler formula products containing Jennewein 2'-FL is not expected to adversely affect any population groups in Australia or New Zealand.

3.5.1 FOODS PRODUCED BY GENE TECHNOLOGY

Applications to vary the Code are required to approve the use of new foods produced using gene technology. Approved genetically modified (GM) foods are specified in Schedule 26 – Food produced using gene technology.
The following information is required to support an application for a new genetically modified food. This information is in addition to that specified in Guideline 3.1.1 – General requirements.

A. Technical information on the food produced using gene technology

A.1 Nature and identity of the genetically modified food

This must include all of the following:
(a) a description of the GM organism from which the new GM food is derived. The description must include the nature and purpose of the genetic modification
(b) the name, line number and OECD Unique identifier of each of the new lines or strains of GM organism from which the food is derived
(c) the name the food will be marketed under (if known).

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

The production of Jennewein 2'-FL involves a processing aid in the form of a genetically modified strain of the bacterium *E. coli*. Thus, this Application includes safety and technical information on the *E. coli* BL21 (DE3) production strains.

Production strain from which Jennewein's 2'-FL was originally derived
The naturally occurring oligosaccharide 2'-FL is only found in the milk of a small group of mammals, which includes humans (Castanys-Munoz *et al.*, 2013). It is not produced by any non-mammalian species, and, specifically, there is no known microorganism which is capable of synthesising this molecule. However, 2'-FL can be produced by fermentation using genetically modified microorganisms. See **Appendix S** for information on the nature of the genetic modification.

Jennewein 2'-FL is produced using genetically modified strains of the bacterium *E. coli*. The production strains were derived by genetic modification of the starting strain *E. coli* BL21 (DE3), which is a derivative of the *E. coli* B strain. The *E. coli* BL21 (DE3) strain was purchased by Jennewein from Novagen (now part of Merck Millipore, Darmstadt, Germany), and a certificate of analysis is provided in **Appendix D**.

The *E. coli* B strain BL21 (DE3) is safe and well-characterised in terms of genetics and biochemistry, and benefits from the availability of genome engineering tools. In addition, Jennewein observed significantly better functional expression of bacterial glycosyltransferases in *E. coli* BL21 (DE3) compared to *E. coli* K-12 (JM109) for unknown reasons.

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

Taxonomic information of the production strain

Taxonomic information on the production strain *E. coli* is presented in **Table 30**.

Table 30: Taxonomic Information of the Production Strain

Taxonomy	Classification
Domain	Bacteria
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gamma-Proteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	Escherichia
Species	*Escherichia coli*
Strain	*Escherichia coli* BL21 (D3)
Commercial name	The *E. coli* BL21 (DE3) strain was purchased from Novagen (now part of Merck Millipore, Darmstadt, Germany) (certificate of analyses is provided in **Appendix D**)

Natural occurrence of E. coli strain

E. coli B strain is a ubiquitous inhabitant of the mammalian colon and was first isolated from the stool of a healthy breastfed infant (Escherich, 1885). This work not only launched the field of prebiotics and probiotics in the nutritional sciences but also ensured that *E. coli* became the best-characterised laboratory microorganism and a model organism for genetics and biochemistry. The ancestor of the *E. coli* B strain was derived from apparently normal commensals of the human gut by d'Herelle at the Institut Pasteur, Paris, in 1918 (Daegelen *et al*., 2009). Together with the closely related *E. coli* K strain, the *E. coli* B strain is a model of bacterial physiology, metabolism and genomics, and has thus been widely used for metabolic engineering. The *E. coli* B strain is also a well-established microorganism for the commercial production of therapeutic proteins. The first biopharmaceutical product produced in genetically modified cells was manufactured by the fermentation of an engineered *E. coli* B strain (recombinant insulin, marketed as Humulin, approved in 1982).

Laboratory strains of *E. coli* were among the first organisms to have complete genome sequences published and they are widely used models of bacterial physiology, genetics, biochemistry, molecular biology and now systems biology (Blattner *et al*., 1997; Hayashi *et al*., 2006).

The *E. coli* B strain has been used in the laboratory for almost 100 years and was developed for T7 RNA polymerase-based gene expression by DE3 prophage integration, UV treatment and selection. The resulting *E. coli* BL21 (DE3) strain is commercially available and has become established as a host for recombinant protein expression worldwide.

b. Name, line number and OECD unique identifier of each of the new lines or strains of GM organism from which the food is derived

The OECD unique identifiers[21] are assigned to transgenic plants. *E. coli* B strain is a bacterium found in the mammalian colon and widely used for metabolic engineering. Thus, *E. coli* BL21 (DE3), the GM organism used as a processing aid in the manufacture of 2'-FL, has neither a line number nor an OECD unique identifier.

c. The name the food will be marketed under (if known).

The Jennewein 2'-FL substance will be commercially available in two versions with identical sugar compositions: as a spray-dried lyophilised powder and as a liquid concentrate comprising 45% 2'-FL content (**Appendix F**).

The products will be marketed as "Jennewein 2'-FL™ powder" and "Jennewein 2'-FL™ concentrate."

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

The common and scientific names of host and donor organisms must be stated. Where information relating to an organism has been included in previous safety assessments prepared by FSANZ, it is not necessary to provide any further information. Where an organism has not been considered previously by FSANZ, the following information must be provided.

(a) For the donor organism(s) from which the genetic elements are derived:
  (i) any known pathogenicity, toxicity or allergenicity of relevance to the food;
  (ii) history of use of the organism in the food supply or history of human exposure to the organism through other than intended food use (e.g. as a normal contaminant).

(b) For the host organism into which the genes were transferred:
  (i) its history of safe use for food
  (ii) the part of the organism typically used as food
  (iii) the types of products likely to include the food or food ingredient
  (iv) whether special processing is required to render food derived from the organism safe to eat.

The *E. coli* B strain BL21 (DE3) was chosen for the metabolic engineering of 2'-FL production because it is safe, well-characterised in terms of genetics and biochemistry, and benefits from the availability of genome engineering tools (details in **Section 3.5.1 A.1**). Jennewein 2'-FL produced using *E. coli* BL21 (DE3) is included in products in other countries (**Section 3.3.3 D.5**). Please refer to **Section 3.5.1 A.1** for the background on *E. coli* B strain and **Appendix S** for details regarding the pathogenicity and toxicity of the *E. coli* BL21 (DE3) production strains. The certificate of analysis for *E. coli* BL21 (DE3) cells is provided in **Appendix D** of this Application.

The exact inserted genes are considered confidential commercial information (CCI) and are described in confidential **Appendix S**. Most genes used in the metabolic engineering of the fermentation strains originate from *E. coli* strains at risk level 1 (according to German Biosafety regulations); other inserted genes from different species range between risk level 1 and 2. The genes that originate from species at higher risk levels encode proteins or enzymes with well-characterised functions. The donor organisms are not widely consumed in foods and do not have a history of consumption. All genes used to engineer the production strain were specifically synthesized by polymerase chain reaction meaning no other genomic material of the donor strains is implemented in the 2'-FL production strains. Therefore the putative risks for donor organisms are not present; no genetic material has been transferred into the host organism nor into the product as no genomic DNA of donor organisms is ever used for construction of the production

21 https://www.oecd-ilibrary.org/environment/safety-assessment-of-foods-and-feeds-derived-from-transgenic-crops-volume-1/unique-identifier-for-transgenic-plants_9789264180147-5-en

strains. There are no toxic products resulting from reactions catalysed by those enzymes, nor do the enzymes display toxicity. Therefore, the resulting strains are considered risk level 1 according to German Biosafety regulations[22]. Risk level 1 means that there is no risk for humans and the environment. The strains are handled at safety level 1 (lowest level) according to the German regulations for genetically modified organisms[23] (no risk for human health and environment according to the current knowledge).

The *E. coli* BL21 (DE3) strains engineered by Jennewein produce 2'-Fl highly efficiently. The production strains are closely related. For example, strain #1540 was created by further development of strain #1242, engineered to be able to degrade lactose that remains at the end of the fermentation process in a temperature-dependent manner. Details are provided in **Appendix S**.

Use of the strain in food enzyme production

E. coli (and particularly the *E. coli* B strain) is a well-established microorganism for the commercial production of therapeutic proteins. The first biopharmaceutical product produced in genetically modified cells was manufactured by the fermentation of an engineered *E. coli* B strain (recombinant insulin, marketed as Humulin, approved in 1982). *E. coli* BL21 (DE3) is probably the most widely used bacterial strain for the overexpression of heterologous and homologous recombinant proteins. Almost 30% of recombinant proteins approved by biopharmaceuticals are expressed in *E. coli* B strain (Ferrer-Miralles *et al*., 2009).

Table 31 lists some examples of therapeutic proteins manufactured using recombinant *E. coli* that have been granted marketing authorisation through the centralised European procedure (Regulation EC 726/2004), including some like granulocyte colony-stimulating factor (marketed as Grastofil, approved in 2012), which are manufactured using *E. coli* BL21 (DE3).

Table 31: Recombinant Proteins for Human use Manufactured Using *E. coli* as a Production Host Source: www.vfa.de/gentech

Recombinant Protein	Name of the active ingredient	Indication
Insulin analog (long-acting)	Insulin Glargin	Diabetes
Insulin analog (fast acting)	Insulin Lispro	Diabetes
Human insulin	Insulin	Diabetes
Interleukin-1 (IL-1) receptor antagonist	Anakira	Rheumatoid arthritis
Monoclonal antibody to tumour necrosis factor alpha (TNF-α)	Certolizumab pegol	Crohn's disease, Rheumatoid arthritis
Chimeric monoclonal antibody against tumour necrosis factor alpha (TNF-α)	Infliximab	Autoimmune diseases
Granulocyte macrophage colony-stimulating factor	Molgramostim	Adjuvant cancer therapy
Granulocyte colony-stimulating factor (G-CSF) analogue	Filgastrim	Neutropenia
Interferon alpha-2a	Interferon alfa 2a	Cancer, hepatitis B/C
Interferon gamma-1b	Interferon gamma 1b	Immune stimulant
PEGylated interferon alpha-2b	Peginterferon alfa-2a	Hepatitis C

[22] Verordnung über Sicherheit und Gesundheitsschutz bei Tätigkeiten mit Biologischen Arbeitsstoffen (Biostoffverordnung - BioStoffV) § 3 Einstufung von Biostoffen in Risikogruppen

[23] Gesetz zur Regelung der Gentechnik (Gentechnikgesetz - GenTG) § 7 Sicherheitsstufen, Sicherheitsmaßnahmen

Recombinant Protein	Name of the active ingredient	Indication
PEGylated interferon beta-1b	Peginterferon beta-1b	Multiple sclerosis
Recombinant human insulin-like growth factor 1 (IGF-I)	Mecasermin	IGF-1-dependent growth failure
Parathyroid hormone	Teriparatide	Osteoporosis
Truncated human recombinant keratinocyte growth factor (KGF)	Palifermin	Oral mucositis
Recombinant porcine-like uricase.	Pegloticase	Gout
Growth hormone receptor antagonist	Pegvisomant	Acromegaly
Fragment of the monoclonal antibody (Fab) against vascular endothelial growth factor A (VEGF-A).	Ranibizumab	Age-related macular degeneration
Non-glycosylated form of human tissue plasminogen activator	Reteplase	Thrombolytic
Fusion protein analogue of thrombopoietin	Romiplostim	chronic idiopathic (immune) thrombocytopenic purpura
Growth hormone	Somatropin	Growth disorders
Tumor necrosis factor TNFα-1	Tasonermin	Cancer
Glucagon-like peptide-1 analogue	Teduglutid	Short bowel syndrome
Parathyroid hormone.	Teriparatid	Osteoporosis
Interleukin IL-2	Aldesleukin	Cancer

In Australia, *Schedule 18 (Processing aids)* of the Food Standards Code permits the production of the enzyme Chymosin (Enzyme Commission No. 3.4.23.4) using the processing aid *E. coli* K-12 strain GE81, for use in food applications.

E. coli strains have also been approved to manufacture a number of food ingredients including:

- On April 15, 2014, US FDA responded favourably to a GRAS determination by Clasado Inc. notifying US FDA of Clasado's intended use of a beta-galactosidase enzyme preparation produced using a recombinant *E. coli* BL21 (DE3) strain. The US FDA had no questions.[24]
- EFSA concluded that Glycom A/s 2'-FL produced by chemical synthesis is safe for infants up to one year of age when added to infant and follow-on formulas, safe for young children older than one year of age when added to follow-on and young-child formulas, and safe when added to various other foods (EFSA, 2015).
- The US FDA has issued a "*no questions at this time*" letter regarding Glycom's determination that use of Glycom 2'-FL is GRAS when used in term infant formulas and various other foods (GRN 542).

E. coli BL21 (DE3) is classified as a risk group 1 organism, the lowest possible risk group according to the German Federal Office of Consumer Protection and Food Safety (BVL).[25] Several

[24] http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm400632.htm

[25] https://www.bvl.bund.de/DE/Home/homepage_node.html

comprehensive studies have demonstrated the safety of *E. coli* BL21 (DE3), which is why it is ideal for the production of 2'-FL.

- In an acute oral toxicity study, endotoxins isolated from *E. coli* BL21 (DE3) were evaluated in mice. Female and male mice were administered single doses of isolated endotoxin (100000, 500000 and 1000000 EU/animal) followed by observation for 14 days. Even at the highest dose of 1000000 EU (3.3 mg/kg body weight), no toxic effects were observed (Harper *et al*., 2011). These results provide convincing evidence that *E. coli* BL21 (DE3) is not toxic.
- *E. coli* BL21 (DE3) does not carry the well-recognised pathogenic components required by *E. coli* strains that cause the majority of enteric infections. *E. coli* BL21 (DE3) is therefore considered to be non-pathogenic and unlikely to survive in host tissues or to cause disease (Chart *et al*., 2000).
- The genome sequence of *E. coli* BL21 (DE3) revealed the absence of genes encoding invasion factors, adhesion molecules and enterotoxins associated with virulence (Jeong *et al*., 2009). The safety of *E. coli* BL21 (DE3) is further supported by its inability to express long-chain lipopolysaccharides and its susceptibility to the effects of serum complement. The strain was unable to survive in selected tissues of a BALB/c mouse model, or to persist in the gut (Chart *et al*., 2000). *E. coli* is a commensal bacterium not typically used as food. **Section 3.5.1 A.2** and **Appendix E** include details of the process through which the final product 2’-FL is rendered free from any process-related contaminants including bacterial DNA, endotoxins and proteins as well as individual analysis results. *E. coli* BL21 (DE3) is used as a processing aid for the manufacture of Jennewein’s 2'-FL in a contained process. No food is derived from *E. coli* itself. The final product 2’-FL is rendered free from any process-related contaminants. After the batch fermentation process is completed, the bacterial cells are separated from the medium and inactivated. The medium is sterile filtered and further processed to isolate the product. In order to prove the absence of recombinant DNA, Jennewein conducted qPCR (real-time quantitative polymerase-chain-reaction) analysis for five independent batches and found no bacterial DNA. The assay was specifically developed for the strains used and utilized specific antibiotic resistance genes as templates. After several purification steps, the 2'-FL product is a pure carbohydrate free from any process-related contaminants including bacterial DNA, endotoxins and proteins. (see **Appendix S** for more information). A detailed method validation report and the individual analysis results are provided in **Appendix E**.

A.3 The nature of the genetic modification

This must include all of the following:
(a) a description of the method used to transform the host organism
(b) a description of the construct and the transformation vectors used, including:
  (i) the size, source and function of all the genetic components including marker genes, regulatory and other elements
  (ii) a detailed map of the location and orientation of all the genetic components contained within the construct and vector, including the location of relevant restriction sites.
(c) A full molecular characterisation of the genetic modification in the new organism, including:
  (i) identification of all transferred genetic material and whether it has undergone any rearrangements
  (ii) a determination of the number of insertion sites, and the number of copies at each insertion site
  (iii) full DNA sequence of each insertion site, including junction regions with the host DNA
  (iv) a map depicting the organisation of the inserted genetic material at each insertion site
  (v) details of an analysis of the insert and junction regions for the occurrence of any open reading frames (ORFs).
(d) A description of how the line or strain from which food is derived was obtained from the original transformant (i.e. provide a family tree or describe the breeding process) including which generations have been used for each study.
(e) Evidence of the stability of the genetic changes, including:
  (i) the pattern of inheritance of the transferred gene(s) and the number of generations over which this has been monitored
  (ii) the pattern of inheritance and expression of the phenotype over several generations and, where appropriate, across different environments.
(f) an analysis of the expressed RNA transcripts, where RNA interference has been used.

a. Description of the method used to transform the host organism

The highly stable microbial production host of *E. coli* BL21 (DE3) strain was used to establish a scalable fermentation process for the multi-ton production of 2'-FL for infant food applications. Jennewein developed *E. coli* strains for this purpose that are able to grow in a chemically defined, salt-based medium, with fermentation possible without antibiotics or other inhibitors to select for 2'-FL production. The *E. coli* strains were always transformed by electroporation; no other methods such as chemical transformation, transfection or conjugation were used. Several genetic modifications were necessary to ensure that the *E. coli* cells were able to synthesize 2'-FL efficiently. Details of the gene construction and modification of *E. coli* BL21 (DE3) strain to yield the specific strains used in for the synthesis of 2'-FL are considered CCI and enclosed in **Appendix S**.

b. A description of the construct and the transformation vectors used, including:

i. The size, source and function of all the genetic components including marker genes, regulatory and other elements

E. coli is a facultative anaerobic enterobacterium that can grow in the presence and absence of oxygen. The genotype of *E. coli* BL21 DE3 is F^- *ompT* $hsdS_B$ ($r_B^- m_B^-$) *gal dcm* (DE3).

The *E. coli* BL21 (DE3) strain was developed for T7 RNA polymerase-based gene expression. Wild-type *E. coli* BL21 (DE3) lacks antimicrobial selection markers and is therefore sensitive to antibiotics such as ampicillin, chloramphenicol, kanamycin, streptomycin, tetracycline, zeocin and trimethoprim. See **Appendix S** for more information.

All genetic components are described in **Appendix S**.

ii. A detailed map of the location and orientation of all the genetic components contained within the construct and vector, including the location of relevant restriction sites.

A genome map of the *E. coli* BL21 (DE3) strains is provided in **Appendix S** along with descriptions of all genetic components contained within the construct and vector. Details of the constructs are considered CCI and are found in confidential **Appendix R** and **Appendix T**.

c. A full molecular characterization of the genetic modification in the new organism, including:

i. Identification of all transferred genetic material and whether it has undergone any rearrangements

The full genomes of the final *E. coli* BL21 (DE3) strains used for the production of Jennewein 2'-FL with annotation of the insertion sites and flanking regions can be found in confidential **Appendix V.** No unexpected genomic rearrangements leading to a potential risk for the product were identified. Furthermore, the genomes of bacterial strains resulting from several integration events have been fully sequenced; no rearrangement of the inserted DNA sequences was ever observed compared to the DNA sequence used for genomic insertion.

ii. A determination of the number of insertion sites, and the number of copies at each insertion site

The number of insertion sites and the number of copies at each insertion site are detailed in **Appendix S** and listed in **Table S-3** in **Appendix S.**

iii. Full DNA sequence of each insertion site, including junction regions with the host DNA

The genomes of the constructs are in **Appendix T** and the full gene sequences of the final *E. coli* BL21 (DE3) strains with annotation of the insertion sites and flanking regions are enclosed in **Appendix V.** Details of the insertion sites of synthetic constructs can be found in **Appendix S**.

iv. A map depicting the organization of the inserted genetic material at each insertion site

The genome maps of *E. coli* BL21 (DE3) strains used in the production of Jennewein 2'-FL are found in **Figures S-4 and S-5** in **Appendix S.**

v. Details of an analysis of the insert and junction regions for the occurrence of any open reading frames (ORFs).

The full gene sequences of the *E. coli* BL21 (DE3) strains with annotation of the insertion sites and flanking regions are in **Appendix V** and show no unexpected open reading frames within the sequence.

d. A description of how the line or strain from which food is derived was obtained from the original transformant (i.e. provide a family tree or describe the breeding process) including which generations have been used for each study.

Genealogy of the E. coli BL21 (DE3) strain

Figure 6 illustrates the genealogy of *E. coli* BL21 (DE3) strain from *E. coli* and the relationships among strains leading to, derived from, or closely related to the *E. coli* B of Delbrück and Luria, as described above. Also shown are lines of descent from K-12 to W3110 (the source of DNA for P1 transduction of B and Bc) and MG1655, whose complete genome sequences are known, and the first *E. coli* strain isolated and studied in the laboratory, by Escherich in 1885. Entries contain the name of the strain, the person associated with the strain, and the approximate year the strain was received, constructed, or described in a publication. Note that the Hershey strains R, S, and H; the Doermann strain S/6 (derived from S); and coli Bordet are not descended from the B of Delbrück and Luria but derived from progenitors of B. The strain S. Lederberg referred to as Bc25182 was actually a P1 transductant of B itself, not Bc, as described in the text, and is designated (Bc251)* in the figure. P1 transductions and treatments with 1-methyl-3-nitro-1-nitrosoguanidine, UV, or UV followed by penicillin selection (UV/P) are indicated in **Figure 6**, as is the integration of DE3 into BL21 to produce BL21(DE3). The mal^+ λ^S genotype of strains obtained by P1 transduction is retained by their descendants but not annotated in the figure, and the Levin strain, REL606, and

BL21(DE3) have the genotype of the strain above them. The accession number, depositor, and year of deposit are given for five strains deposited initially in the UK National Collection of Type Cultures, the Collection of Institut Pasteur (CIP), or the American Type Culture Collection(Daegelen *et al*., 2009).

Jennewein sequenced the genome of the parental *E. coli* BL21 (DE3) strain used to generate their production strains (which serves as the processing aid) and found only a single point mutation difference compared to the published *E. coli* BL21 (DE3) genome sequence (GenBank NC_012971; Jeong *et al*., 2009). The point mutation was found at position 453255, corresponding to a silent mutation (195G>A I65I) in the coding region of the *acr*B gene which encodes a multidrug efflux system protein (GenBank ECD_00413).

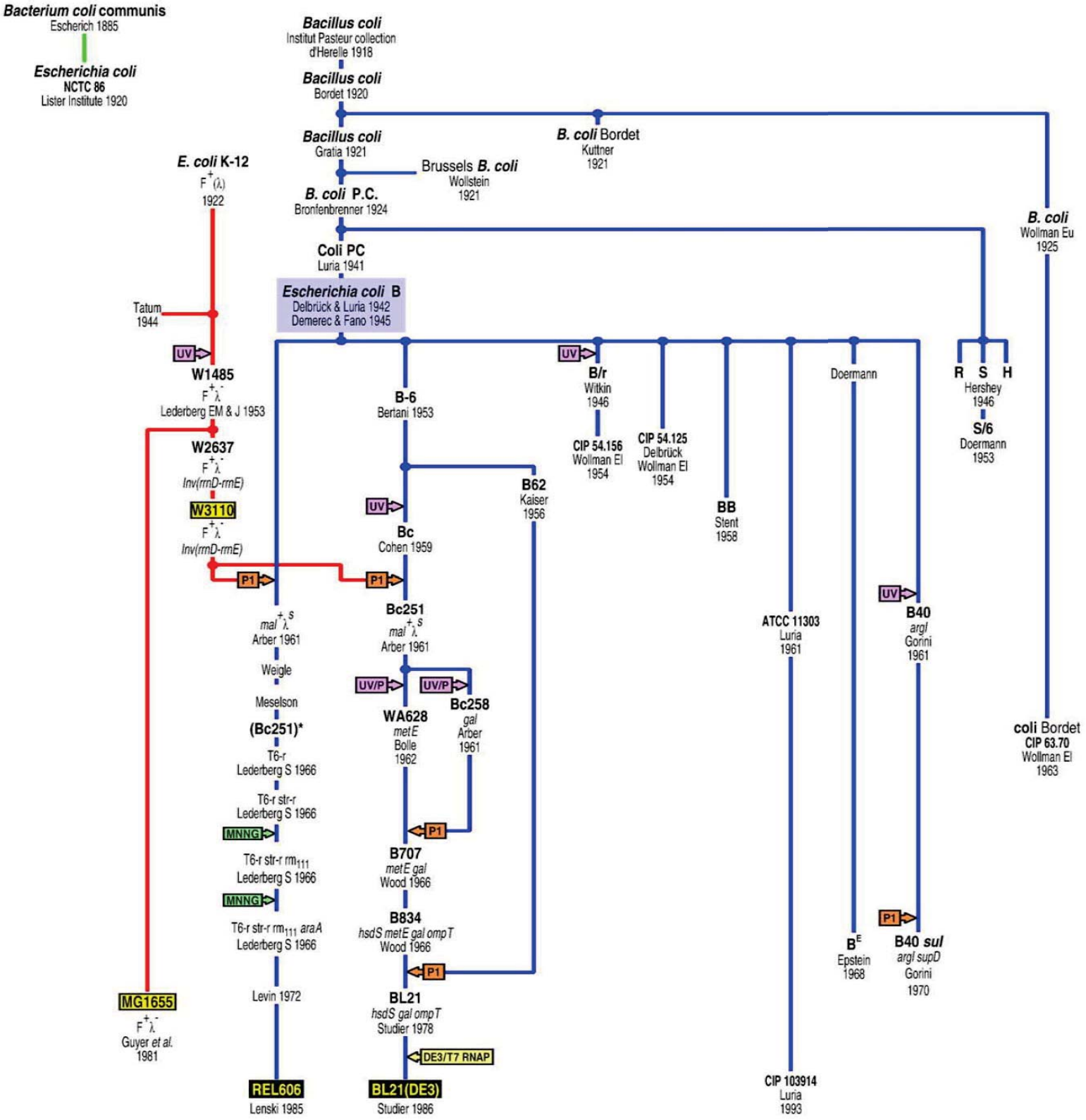

Figure 6. Genealogy of *E. coli* BL21 (DE3) (source: Daegelen *et al.*, 2009).

e. Evidence of the stability of the genetic changes, including:

(i) The pattern of inheritance of the transferred gene(s) and the number of generations over which this has been monitored

Multiple genomic integrations of several of the required genetic elements resulted in a highly stable fermentation strain. Details of the gene modifications are enclosed in **Appendix S**. To demonstrate genetic stability of the strains, one of the *E. coli* BL21 (DE3) strains was sequentially grown five times to exponential growth in fermentation processes. The genomic sequences from five clones obtained after five rounds of fermentation were compared and all genetic elements introduced into the host genome were found to be present and unmodified in all five clones. See **Appendix U** for the full study methods and results.

In regard to the genetic stability of the source organism, *E. coli* BL21 (DE3) is widely used in laboratories around the world and experience has shown that the strain is genetically highly stable. The re-sequencing of the entire genome of Jennewein's purchased *E. coli* BL21 strain and a comparison with the genome sequence published in 2009 (GenBank NC_012971; Jeong et al., 2009) revealed a single point mutation representing a silent mutation in the acrB gene encoding a multidrug efflux system protein.

E. coli BL21 (DE3) does not contain plasmids or other episomal vectors and is not capable of conjugation, i.e. genes necessary for the genetic transfer of DNA are missing from the *E. coli* BL21 (DE3) genome. The microbial fermentation process is conducted in a contained sterile environment and the fermentation strain is inactivated after the completion of batch fermentation.

In order to exclude the potential carryover of even the smallest amounts of antibiotics or similar inhibitors, fermentations are run without antibiotics or any other inhibitors. This required the development of extremely stable 2'-FL production strains expressing the genes required for 2'-FL synthesis and export. In addition, the production of 2'-FL from lactose and glycerol by fermentation requires a strictly sterile working environment, because any contaminating microbes would quickly overgrow the fermentation by metabolising the substrates as well as the product. The necessary genetic stability was achieved by introducing the corresponding genes directly into the *E. coli* BL21 (DE3) genome, with some constructs integrated as multiple copies at different sites to enhance the stability of the introduced production traits even further.

Whole genome sequencing was performed for the final production strains and several progenitor strains were sequenced to prove the genome's integrity. From these results, one can see that the genomic insertions are still there in the same place, and that the initial number (of those of the same kind) did not change over the process of further strain development.

(ii) The pattern of inheritance and expression of the phenotype over several generations and, where appropriate, across different environments

Based on the stability of the genetic modifications made in the *E. coli* BL21 (DE3) strain #1540, a test showing the pattern of inheritance and expression of the phenotype over several generations was not conducted. However, as the genetic elements were present in *E. coli* BL21 (DE3) strain #1540 after five rounds of the fermentation process (details provided in **Appendix U**), the phenotype is likely to be similarly stable. 2'-FL production of the five clones obtained after five rounds of fermentations were very similar.

f. An analysis of the expressed RNA transcripts, where RNA interference has been used

RNA interference was not used in the production of Jennewein 2'-FL.

B. Characterisation and safety assessment of new substances

B.1 Characterisation and safety assessment of new substances

This must include all of the following:
(a) a full description of the biochemical function and phenotypic effects of all new substances (e.g. a protein or an untranslated RNA) that are expressed in the new GM organism, including their levels and site of accumulation, particularly in edible portions
(b) information about prior history of human consumption of the new substances, if any, or their similarity to substances previously consumed in food.
(c) information on whether any new protein has undergone any unexpected post-translational modification in the new host
(d) where any ORFs have been identified (in subparagraph A.3(c)(v) of this Guideline (3.5.1)), bioinformatics analyses to indicate the potential for allergenicity and toxicity of the ORFs.

Section 3.5.1 A.3 and **Appendix S** detail the phenotypic traits and biochemical characterisation of the final production strains of *E. coli* BL21 (DE3). The *E. coli* BL21 (DE3) strains are genetically engineered to export the 2'-FL into the fermentation medium during the manufacturing process of Jennewein 2'-FL (details in **Section 3.3.3 B.4**). The new substance 2'-FL is chemically and structurally identical to naturally occurring 2'-FL found in human milk (see **Section 3.3.3 A.1**) and has the same nutritional and immunity potential in infants. Furthermore, 2'-FL has a history of safe consumption by infants (see **Section 3.3.3 A.1**). No open reading frames with potential for allergenicity and toxicity have been identified (full analyses in confidential **Appendix T**).

B.3 Other (non-protein) new substances

If other (non-protein) substances are produced as a result of the introduced DNA, information must be provided on the following:
(a) the identity and biological function of the substance
(b) whether the substance has previously been safely consumed in food
(c) potential dietary exposure to the substance
(d) where RNA interference has been used:
(i) the role of any endogenous target gene and any changes to the food as a result of silencing that gene
(ii) the expression levels of the RNA transcript
(iii) the specificity of the RNA interference

Section 3.3.3 A.1 explains the biological function of HMO 2'-FL and how infants and toddlers are exposed to naturally occurring 2'-FL in human milk through normal, safe consumption. The structural and chemical similarities between Jennewein 2'-FL and human 2'-FL are detailed in **Section 3.3.3 B.2**. Please refer to **Section 3.3.3 D.2** for a discussion on potential dietary exposure to Jennewein 2'-FL and refer to **Section 3.3.3 D.5** for a list of countries where products containing Jennewein 2'-FL have been launched.

RNA interference was not employed. Jennewein 2'-FL is not genetically modified, so no changes as a result of silencing an endogenous target gene in the processing aid *E. coli* BL21 (DE3) would have a change to the food.

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

This must include all of the following:
(a) the levels of relevant key nutrients, toxicants and anti-nutrients in the food produced using gene technology compared with the levels in an appropriate comparator (usually the non-GM counterpart). A statistical analysis of the data must be provided.
(b) information on the range of natural variation for each constituent measured to allow for assessment of biological significance should any statistically significant differences be identified
(c) the levels of any other constituents that may potentially be influenced by the genetic modification, as a result, for example, of downstream metabolic effects, compared with the levels in an appropriate comparator as well as the range of natural variation.

The addition of Jennewein 2'-FL to infant formula, follow-on formula, and toddler formula is consistent with the Codex Alimentarius International Food Standards' direction to, as much as possible, produce infant formula products with ingredients that match the naturally occurring nutrient composition of human milk (see **Section 3.1.1 D.b**). The structural and chemical similarities between Jennewein 2'-FL and human 2'-FL are detailed in **Section 3.3.3 B.2** with further supporting information in **Appendix I**. Jennewein 2'-FL is substantially chemically equivalent to naturally occurring 2'-FL. Jennewein 2'-FL has demonstrated purity via a purification process that eliminates the genetically modified *E. coli* bacteria and removes virtually all traces of endotoxins, recombinant DNA, host proteins and other carbohydrates. Furthermore, it is manufactured consistently without the use of antibiotics, inhibitors, organic solvents or other toxic substances. As a result of this highly monitored production, unintended effects due to the genetic modification of the processing aid are highly unlikely (see **Section 3.3.3 B.4** for more details). The proposed use of Jennewein 2'-FL is up to 2 grams per litre of formula, which is equivalent to the mean concentration of 2'-FL normally found in breast milk (see **Section 3.3.3 B.2** for more information).

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

The application must contain the following information if the compositional analysis indicates biologically significant changes to the levels of certain nutrients in the food produced gene technology compared to the non-GM counterpart food:
(a) data are required on the anticipated dietary intake of the GM food in relation to the overall diet, together with any information which may indicate a change to the bioavailability of the nutrients from the GM food
(b) where the GM food contains an intended nutritional change, information, such as clinical trial data, must be provided to determine the nutritional impact of the GM food.

Jennewein 2'-FL is substantially chemically equivalent to naturally-occurring 2'-FL in human milk (see **Section 3.3.3 B.2** and **Appendix I**) and no biologically significant changes are expected in 2'-FL produced by microbial fermentation compared to naturally-occurring 2'-FL. Clinical studies with healthy term infants testing infant formula supplemented with 2'-FL show that the formula is well-tolerated (see **Section 3.3.3 C.2**).

D. Other information

There is no requirement to conduct animal feeding or whole food toxicity studies on the food produced using gene technology. However, if a 90-day (or longer) whole food toxicity study in rodents has been provided to satisfy the data and information requirements of another jurisdiction, this should also be provided to FSANZ as additional supporting information.

Refer to **Section 3.3.3 C.2** for details on toxicity studies conducted in animals. **Appendix P-4** contains the study report of a 90-day dietary toxicity study using Jennewein 2'-FL.

3.6.2 SPECIAL PURPOSE FOOD – INFANT FORMULA PRODUCTS

A. Information related to composition

A.1 Purpose of the compositional change

> The application must state the purpose of the compositional change to infant formula products.
>
> This includes a brief description of all of the technological, nutritive or health-related function(s) of the substance at the proposed level in the relevant infant formula product(s). Where an added substance or compositional change has multiple purposes or functions, then these must be specified. This includes information on the target infant population(s) e.g. healthy term infants aged 0–12 months, or infants older than 6 months.

Section 3.3.3 A.1 of this Application outlines the purpose of adding Jennewein 2'-FL to infant formula and follow-on formula. **Section 3.3.3 D.2** of this Application provides information relating to the proposed level of Jennewein 2'-FL in infant formula and follow-on formula (as consumed).

A.2 General data requirements

> This includes the general evidential requirements whereas A.3 includes the specific information required for the assessment of nutritional safety and efficacy.
>
> Studies provided as evidence to support an application must contain sufficient detail to enable an independent assessment of the methods and results to confirm the study conclusions.
>
> An application must include human studies as supporting evidence for nutritional safety, tolerance and the efficacy of the proposed compositional change. This can include published studies, detailed reports of unpublished studies and systematic reviews (with underlying studies also provided). It may be acceptable in certain cases not to include human studies. In this situation, safety and efficacy must be demonstrated by relevant data (as specified elsewhere in this Handbook); and the application must include an explanation of why human studies are not applicable.

Please refer to the following sections of this Application which provide information relating to:

- The benefits of naturally occurring 2'-FL in human milk (**Section 3.3.3 A.1**)
- Evidence of the chemical equivalence of Jennewein 2'-FL to naturally occurring 2'-FL (**Section 3.3.3 B.2**)
- Clinical studies demonstrating the health benefits of infants fed formula with synthesised 2'-FL (**Section 3.3.3 C.2**)
- Toxicity studies of the Jennewein 2'-FL demonstrating safety of the ingredient (**Section 3.3.3 C.2**).

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

This describes evidential requirements that **must** be addressed for a proposed change to the composition of infant formula products and it is divided into two components depending on the category of compositional change.
An application that relates to addition or changing the level of a nutritive substance (including energy or macronutrient), novel food or novel food ingredient must address the requirements listed in subsection A.3.1 of this Guideline (3.6.2).
An application that relates to a food additive or processing aid must address the requirements listed in component in subsection A.3.2 of this Guideline (3.6.2).

A.3.1 Nutritive substance (including energy or macronutrient), novel food, or novel food ingredient

a. Characterisation of proposed substance or the comparable substances in breast milk

Refer to **Section 3.3.3 D.2** of this Application which provides information relating to the mean concentration and range of naturally occurring 2'-FL in human milk from mothers in countries with similar dietary patterns to Australia and New Zealand.

These studies analysed the concentration of 2'-FL in breast milk obtained from mothers in Chile, France, Germany, China, Italy, Mexico, the Philippines, Singapore, Sweden and the United States; and were reported in studies conducted by Erney *et al*. (2000), Chaturvedi *et al*. (2001), Castanyz-Munoz *et al*. (2013) and Thurl *et al*. (2010). The breast milk samples were collected from colostrum samples (the first two days after birth) up to 452 days after birth.

Data from these studies supports the proposed maximum target use of Jennewein's 2-FL in infant formula and follow-on formula of 2 g/L of formula as consumed; which is considered to correspond to the mean concentration of 2'-FL naturally found in human milk.

b. Nutritional safety and tolerance of the proposed change

Refer to **Section 3.3.3 C.2** of this Application which provides information relating to four clinical studies and one sub-study of a full clinical trial conducted with healthy term infants to assess the tolerance of synthesised 2'-FL in infant formula at concentrations up to 1.0 g/L of 2'-FL. These studies were conducted by Marriage *et al*. (2015), Goehring *et al*. (2016), Puccio *et al*. (2017), Kajzer *et al.* (2016) as cited in Reverri *et al.* (2018), and Storm *et al.* (2019). **Table 32** presents a summary of reported clinical studies which support the nutritional safety and tolerance of the addition of Jennewein's 2'-FL to infant formula and follow-on formula products. In addition to the five clinical studies with infants, another study tested the hypoallergenicity of an extensively hydrolysed formula containing 2'-FL among infants, toddlers, and young children with cow's milk protein allergy and concluded that the formula containing 2'-FL was safe and suitable for hypoallergenic use (Nowak-Wegrzyn *et al.*, 2019). Further detail of these studies is provided in **Section 3.3.3 C.2** of this Application.

Although the maximum level of 2'-FL added to infant formula products in these clinical studies was 1.0 g/L, the proposed level of Jennewein 2'-FL of 2.0 g/L more closely represents the level naturally found in human milk (refer to **Section 3.3.3 D.2**). Furthermore, 2'-FL has a history of safe human exposure in infants as 2'-FL is naturally present in human milk in a range of concentrations.

Table 32: Summary of clinical studies demonstrating the nutritional safety and tolerance of synthesised 2'-FL

FSANZ Study Requirements	**Marriage *et al.* (2015) study**	**Goehring *et al.* (2016) study**	**Puccio *et al.* (2017) study**	**Kajzer *et al.* (2016) as cited in Reverri *et al.* (2018)**	**Storm *et al.* (2019) study**
Human infant study	Healthy full-term singleton infants were enrolled by five days of age. Gestational age was 37-42 weeks and birth weight was ≥ 2490 g.	This study was a sub-study nested within the Marriage *et al*. (2015) study.	Healthy infants aged from birth to 12 months old were assessed.	Healthy full-term infants 0-8 days of age at enrolment and followed until 35 days of age.	Healthy infants aged 14±5 days at enrolment.
Infant formula product containing 2'-FL at proposed level (i.e. 2 g/L)	Two test formulas containing either 0.2 or 1.0 g/L were included in the study.	Two test formulas containing either 0.2 or 1.0 g/L were included in the study.	Test formula was an intact protein cow's milk based, whey-predominant, formula with 1.0 g of 2'-FL added per litre.	The test formula was a formula containing 0.2 g 2'-FL per litre.	The test formula contained 0.25 g 2'-FL per litre.
Study interval of 3-4 months beginning no later than 1 month of age	Infants were enrolled by five days of age and were assessed until 119 days of age (approximately four months of age).	Blood samples were drawn from infants at six weeks of age (*n* = 31-42 / group).	Infants completed a baseline visit at ≤ 14 days of age, followed by visits at 1, 2, 3, 4, 6 and 12 months.	This study was a tolerance study that followed infants until 35 days of age; this criteria does not meet FSANZ requirements but does provide information to supplement other studies regarding the nutritional safety and tolerance of 2'-FL.	The study followed infants for 42 days; this criteria does not meet FSANZ requirements but does provide information to supplement other studies regarding the nutritional safety and tolerance of 2'-FL.

FSANZ Study Requirements	**Marriage *et al*. (2015) study**	**Goehring *et al*. (2016) study**	**Puccio *et al*. (2017) study**	**Kajzer *et al.* (2016) as cited in Reverri *et al.* (2018)**	**Storm *et al.* (2019) study**
Reported growth measures must include infant length and weight	Reported growth measures included weight, length and head circumference. Other secondary variables were also reported.	This study was a sub-study nested within the Marriage *et al*. (2015) study; which analysed blood samples collected. The peripheral blood mononuclear cells were isolated for cellular phenotyping and stimulated ex vivo with phytohemagglutinin for proliferation and cell cycle progression or respiratory syncytial virus (RSV). Cytokine concentrations were measured in plasma and in ex vivo-stimulated culture supernatants.	Information including weight, length, head circumference, digestive tolerance, behavioural patterns and formula intake were recorded at each visit.	Weight and length were assessed at enrolment and two visits during the study.	Weight and length were assessed at enrolment and after 6 weeks.
Include control group, an exposure group and breastfed group	The study included a) control group receiving formula containing no 2'-FL, b) test formula with the addition of 0.2 g/L 2'-FL, c) test formula with the addition of 1.0 g/L 2'-FL, d) exclusively breast-fed group.	This study was a sub-study nested within the Marriage *et al*. (2015) study; and included the same study groups.	Study included a control formula group and a test formula group containing 2'-FL. No breastfed group was included in the study.	The study had three groups: one with a control formula without 2'-FL and scFOS supplementation, one test formula containing 2'-FL and scFOS, and a breastfed group.	The study included a control formula group and a test formula group containing 2'-FL. A breastfed group was not included in the study.

FSANZ Study Requirements	**Marriage *et al*. (2015) study**	**Goehring *et al*. (2016) study**	**Puccio *et al*. (2017) study**	**Kajzer *et al.* (2016) as cited in Reverri *et al.* (2018)**	**Storm *et al.* (2019) study**
Present information on the study design, methodology and study population	Randomised, double-blind and controlled study. Refer to **Section 3.3.3 C.2** for further information regarding the methodology and study population.	This study was a sub-study nested within the Marriage *et al*. (2015) study. Refer to **Section 3.3.3 C.2 f**or further information regarding the methodology and study population.	Multi-centre, randomized, double-blind trial of two parallel groups (formula control and formula with 2'-FL added). Refer to **Section 3.3.3 C.2** for further information regarding the methodology and study population.	Prospective, randomized, multicentre, double-blinded, controlled tolerance trial. Refer to **Section 3.3.3 C.2** for further information regarding methodology and study population.	Randomized, controlled, double-blind multicentre study. Refer to **Section 3.3.3 C.2** for further information regarding methodology and study population.
Study limitations reported	The authors reported one limitation: the study did not include infants fed formula with the standard caloric density and therefore could not compare growth and intake between infants fed formula with the standard versus lower caloric density.	Three limitations were reported by the authors: 1) small group size limited the interpretation of the results, 2) the secretor status of the mothers was not determined, and 3) data was provided at only one point in time (i.e. 6 weeks of age).	A limitation of the study was reported to be the absence of growth and secondary outcome data from a breastfed reference group. In addition, the gastrointestinal tolerance data were based on parent reporting which may be susceptible to over- or under-reporting.	Limitations were not reported by the authors or by the review article (Reverri *et al.* 2018).	The authors reported one limitation: the tolerance assessment was limited to a level of 2'-FL at the lower range of what has been observed in human milk.
Evidence to demonstrate there is no risk of nutrient imbalances	There were no significant differences among any groups for weight, length or head circumference. 2'-FL was present in the plasma and urine of infants	The authors concluded that infants fed formula supplemented with 2'-FL exhibited lower plasma and ex vivo inflammatory cytokine profiles, similar to those of a breastfed reference group.	Authors concluded that infant formula supplemented with 1 g of 2'-FL per litre was safe and well tolerated which supported normal age-	The authors concluded that the test formula containing 2'-FL was safe and well-tolerated in infants with no significant differences in stool consistency, formula intake,	The authors concluded that the test formula containing 2'-FL was tolerated well based on a comprehensive tolerance assessment tool.

FSANZ Study Requirements	Marriage *et al.* (2015) study	Goehring *et al.* (2016) study	Puccio *et al.* (2017) study	Kajzer *et al.* (2016) as cited in Reverri *et al.* (2018)	Storm *et al.* (2019) study
	fed 2'-FL, and there were no significant differences in 2'-FL uptakes relative to the concentration fed. The authors concluded that growth and 2'-FL uptakes were similar to those of breast-fed infants.	These findings indicate that 2'-FL supports aspects of immune development and regulation similar to that in a breastfed reference group.	appropriate infant growth.	percent feedings with spit-up or vomit, and reported adverse effects compared to infants in the other two groups.	

c. Efficacy of the proposed compositional change

Information supporting the potential beneficial effects to infants from consumption of a) naturally occurring 2'-FL is provided in **Section 3.3.3 A.1** of this Application, and b) synthesised 2'-FL added to infant formula products (as assessed in clinical studies) is provided in **Section 3.3.3 C.2** of this Application.

A.3.2 For a food additive or processing aid

Compositional changes involving a food additive or processing aid must meet the respective safety requirements of Guidelines 3.3.1 and 3.3.2.

Refer to **Section 3.5.1 A**. of this Application which provides the safety information relating to the *E. coli* strain used to manufacture Jennewein 2'-FL.

In addition, the following must be provided:
(a) Tolerance of the proposed compositional change
Evidence to support tolerance must include appropriate human studies. This includes an explanation of the way in which this evidence relates to infants.

Refer to **Section 3.3.3 C.2** of this Application which provides information relating to four clinical studies and one sub-study of a full clinical trial conducted with healthy term infants to assess the tolerance of synthesised 2'-FL in infant formula at concentrations up to 1.0 g/L of 2'-FL. These studies were conducted by Marriage *et al*. (2015), Goehring *et al*. (2016), Puccio *et al*. (2017), Kajzer *et al.* (2016) as cited in Reverri *et al.* (2018), and Storm *et al.* (2019).

(b) Efficacy of the proposed compositional change
If the food additive also provides a nutritive or health-related function, the information requirements listed in component (I) for efficacy of proposed change must be met. If the function is purely technological, there are no further requirements in this section.

Please refer to the following sections of this Application which provide information relating to:

- The benefits of naturally occurring 2'-FL in human milk (**Section 3.3.3 A.1**);
- Evidence of the chemical equivalence of Jennewein 2'-FL to naturally occurring 2'-FL (**Section 3.3.3 B.2**); and
- Clinical studies demonstrating the health benefits of infants fed formula with synthesised 2'-FL (**Section 3.3.3 C.2**).

B. Information related to the dietary intake or dietary exposure

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

> The application **must** meet the information requirements for the dietary exposure of a food additive, processing aid, novel food or novel food ingredient, or dietary intake of a nutritive substance (including energy or macronutrient), as outlined in these application guidelines. The information provided must have a focus on infants.

Refer to **Section 3.3.3 D.3** of this Application for information relating to the likely dietary exposure of Jennewein 2'-FL in infant formula and follow-on formula. These calculations estimated a daily consumption of 1.6 g of Jennewein 2'-FL for infant formula and 1.2 g of Jennewein 2'-FL for follow-on formula.

B.2 Data on the recommended level of formula consumption for the target population

> The application must contain the following information:
> (i) the capacity of the product scoop (in grams of product)
> (ii) the number of scoops required per feed
> (iii) the volume of water required per feed
> (iv) total volume of the made-up feed
> (v) recommended number of feeds per day relevant to each age group in the relevant target population.

The Jennewein 2'-FL is intended to be an ingredient in infant formula and follow-on formula products in Australia and New Zealand at a level of 2 g of 2'-FL per litre of formula (as consumed); which represents the natural level of 2'-FL in human milk. Therefore, no changes to the formula preparation instructions are proposed (e.g. capacity of the product scoop, volume of water required per feed), as consumers will be required to follow the manufacturers' preparation instructions.

B.3 Information relating to the substance

> The application should also contain information or references on the levels (naturally occurring or naturally occurring and added) of the proposed substance in other foods that infants are likely to consume.

Apart from human milk, naturally occurring 2'-FL is not anticipated to be in other foods that infants are likely to consume because it is an oligosaccharide specific to human milk. Refer to **Section 3.3.3 A.1** of this Application for information relating to the presence of 2'-FL in human milk. Furthermore, to our knowledge the inclusion of synthesised 2'-FL is not currently permitted by FSANZ to infant food products such as infant cereals. Therefore, it is not anticipated that Australian or New Zealand infants will be exposed to 2'-FL in other foods that they are likely to consume (with the exception of breast milk if complimentary feeding is occurring).

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

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

The application **must** include information to support the proposed labelling change. For example, the inclusion of (or change to) a warning or advisory statement, directions for use, or conditions.

No change to the infant formula and follow-on formula labelling requirements is anticipated due to the addition of Jennewein 2'-FL to formula sold in Australia or New Zealand.

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

This should include consumer research information to demonstrate the anticipated consumer response to the proposed change, or data obtained from an overseas market where the proposed labelling is already in place.

The extent of the impact of a labelling change on consumer understanding and behaviour will vary depending on:
(a) the nature of the labelling change; and
(b) the foods to which it will apply.
Thus the amount of information necessary to address the impact on consumer understanding and behaviour will depend on the level of impact. Consultation with FSANZ may be necessary to examine the expected level of impact.

No change to the infant formula and follow-on formula labelling requirements is anticipated due to the addition of Jennewein 2'-FL to formula sold in Australia or New Zealand. Jennewein 2'-FL meets the exception listed under Standard 1.5.2-4(a) in that Jennewein 2'-FL "has been highly refined where the effect of the refining process is to remove novel DNA or novel protein" and is not listed in subsections S26-3(2) and (3).

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

The application must include information demonstrating the level of consistency with internationally recognised standards, codes of practices, recommendations or guidelines such as Codex and the WHO, relating to the manufacture and labelling of infant formula products.

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

3.6.3 SPECIAL PURPOSE FOODS – OTHER FOODS

A. Information related to general compositional requirements

The application **must** contain the following information if it relates to a change to the general compositional requirements.

A.1 Information on the identity and physical physiological need of the target population

The application **must** include a description of the target population for the special purpose food. It **must** also include a description of the physical and physiological need of specific life stages e. g. infancy, physical disease, disorder and disability of the target population; or physical and physiological need of the target population that require altered energy or nutrient intake.

Jennewein intends to use Jennewein 2'-FL in FSFYC suitable for children from 1 to 3 years of age, i.e. and heretofore referred to as 'toddler formulas'. Statements provided by the Australian National Health and Medical Research Council (NHMRC) indicate breastfeeding is important for the nutrition, immunological protection, growth, and development of infants and toddlers, and notes breastfeeding is the normal and unequalled method of feeding infants (NMHRC, 2012). The World Health Organization (WHO) and United Nations Children's Fund (UNICEF) recommend exclusive breastfeeding for the first six months of age and continued breastfeeding with complementary foods up to two years of age (WHO/UNICEF, 2003). Young children aged 1 – 3 years would benefit from 2'-FL in toddler formula as the same mechanism, effects, and health benefits are expected to occur past infancy; however, clinical studies of the effects for this population are not currently available. Studies have shown that adult intestines also contain bifidobacteria and *Bacteroides* species (Hopkins *et al*., 2002). See **Section 3.3.3 A.1** for more details regarding 2'-FL in human milk and its role in optimal development of infants and young children.

A.2 Purpose of the compositional change

The application **must** include a brief description of all of the nutritive or health-related function(s) of the substance at the proposed level in the relevant food product(s). Where an added substance or compositional change has multiple purposes or functions, then these **must** be specified.

The postulated mode of action based on the scientific literature is the same in toddlers as in infants, that is that 2'-FL, as a major HMO, has a beneficial, bifidogenic effect in the gut by aiding in the development and maintenance of bifidobacteria in the gut. Numerous other health effects of 2'-FL have been postulated, e.g., anti-infective benefit, immunomodulation. (See **Section 3.3.3. A.1**).

A.3 Information related to the safety of the proposed compositional change

The application **must** include information related to the safety of a food additive, processing aid, novel food or novel food ingredient, or nutritive substance for the target population (Information to demonstrate safety is also requested elsewhere in Part 3).

The details of 2'-FL and HMO existing in naturally occurring human milk are described in **Section 3.3.3 A.1.** The safety of Jennewein 2'-FL as demonstrated in toxicological and clinical studies as well as safety assessment reports prepared by other agencies is detailed in **Section 3.3.3 C**. For

example, a clinical study reported that children aged 2 months to 4.75 years (mean age 24.5 months) tolerated an extensively hydrolysed formula supplemented with 2'-FL and LNnT well (Nowak-Wegrzyn *et al.*, 2019). Furthermore, a study of 2'-FL supplementation in the diets of 100 adults showed that it was well tolerated and observed a bifidogenic effect (Elison *et al.*, 2016).

A.4 Information related to the nutritional impact or performance impact of the proposed compositional change

This demonstrates how the compositional change would contribute to achieving the intended purpose of the special purpose food.

The application **must** include clinical studies that examine the nutritional suitability of the food, for the target population.

2'-FL is naturally occurring in human milk and a substantial body of evidence supports the nutritional benefits of 2'-FL in infants (more details in **Section 3.3.3 A.1**) The chemical equivalence of Jennewein 2'-FL to naturally occurring 2'-FL is demonstrated in **Section 3.3.3 B.2, B.3,** and **B.5**. Furthermore, the safety of Jennewein 2'-FL is supported by toxicological studies and by clinical studies (detailed in **Section 3.3.3 C.1** and **C.2**).

B. Information related to the dietary intake or dietary exposure

The application **must** contain the following information if it relates to a change to the general compositional requirements

B.1 Dietary intake or exposure of target population

This include information on the dietary exposure of a food additive, processing aid, novel food or novel food ingredient, or dietary intake of a nutritive substance (as indicated elsewhere in these Applications guidelines for the target population).

Table 25: Estimated daily intake of Jennewein 2'-FL from infant formula, follow-on formula, and toddler formula (Australia and New Zealand) in **Section 3.3.3 D.3** presents the estimates of Jennewein 2'-FL intake per user per day assuming a concentration of 2 g/L in infant formula, follow-on formula, and toddler formula. These intake estimates are based on FSANZ guidance (detailed in **Section 3.3.3 D.3** of this Application). These calculations estimated a daily consumption of 0.6 – 0.8 g of Jennewein 2'-FL in toddler formula.

B.2 Level of consumption of the special purpose food for the target population

Information relating to the recommended number of serves per day and the size of each recommended serve should be provided for relevant special purpose foods for the target population.

2'-FL in human milk varies greatly by individual. There is evidence that 2'-FL is present in human milk past 365 days postpartum. Thus, toddlers who breastfeed continue to be exposed to 2'-FL. See **Section 3.3.3 D.3** of this Application. The addition of Jennewein 2'-FL to toddler formula mimics the nutritional composition of human mother's milk for a toddler. As 2'-FL exists in very low or no levels in other foods, young children aged 1-3 years would only be exposed to significant amounts of 2'-FL via human milk or toddler formula containing 2'-FL. The expected amount of Jennewein 2'-FL in toddler formula consumed daily is 0.6 – 0.8 g as described in

Section 3.3.3 D.3. Toddler formulas are available in Australia and New Zealand. One of these formulas recommends 32 g as a serving in 0.23 L.[26]

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

The application **must** contain the following information if it relates to a change to labelling requirements.

C.1 Safety or nutritional impact of labelling change

This includes information to support the proposed labelling change e.g. the inclusion of (or change to a warning of advisory statement, directions for use, or claim conditions.

No change to FSFYC labelling requirements is anticipated due to the addition of Jennewein 2'-FL to formula sold in Australia or New Zealand.

C.2 Demonstrated consumer understanding of labelling change

This includes consumer research information to demonstrate the anticipated consumer response to the proposed change, or data obtained from an overseas market where the proposed labelling is in place.

For example, information to demonstrate how the proposed label change will assist consumer understanding of the specific nature of the food, the intended population group or the intended special purpose of the food.

Note: A proposed labelling change will only be relevant to consumers for those special purpose foods which are available for retail sale.

No change to the FSFYC labelling requirements is anticipated due to the addition of Jennewein 2'-FL to formula sold in Australia or New Zealand. Jennewein 2'-FL meets the exception listed under Standard 1.5.2-4(a) in that Jennewein 2'-FL "has been highly refined where the effect of the refining process is to remove novel DNA or novel protein" and is not listed in subsections S26-3(2) and (3).

D Internationally recognised codes of practice and guidelines on labelling

The application must contain information demonstrating the extent to which the application is consistent with internationally recognised standards and codes of practices. These include Codex and the WHO recommendations and guidelines, relating to the composition and labelling of special purpose foods.

Infant formula and FSFYC (i.e. toddler formula) containing Jennewein 2'-FL will adhere to relevant standards or guidelines. This includes the WHO International Code of Marketing of Breast-milk Substitutes, the Codex Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants, Codex Guidelines for Formulated Supplementary Foods for Older Infants and Young Children. Refer to **Section 3.1.1 D** and **Section 3.1.1 J.1** of this Application which provide information relating to internationally recognised standards and recommendations.

[26] https://www.nestle.com.au/brands/baby-toddler-nutrition/nan-toddler

REFERENCE LIST

[*an electronic copy of the references cited in this Application has been provided separately in Appendix X*]

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Yu ZT, Chen C, Kling DE, Liu B, McCoy JM, Merighi M, Heidtman M, Newburg DS (2013a) The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. *Glycobiology*, 23(2): 169-177.

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.

Yu, Z. T., Nanthakumar, N. N., & Newburg, D. S. (2016). The human milk oligosaccharide 2'-fucosyllactose quenches campylobacter jejuni–induced inflammation in human epithelial cells HEp-2 and HT-29 and in mouse intestinal mucosa. The Journal of nutrition, 146(10), 1980-1990.

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BIBLIOGRAPHY OF ADDITIONAL LITERATURE

Title	Year	Citation	Study Objective
Kilogram scale chemical synthesis of 2'-fucosyllactose	2019	Agoston, K., Hederos, M. J., Bajza, I., & Dekany, G. (2019). Kilogram scale chemical synthesis of 2’-fucosyllactose. Carbohydrate Research, 71–77. https://doi.org/10.1016/j.carres.2019.03.006	This study presents the results of a scalable synthetic procedure to high quality 2'-fucosyllactose, the most abundant oligosaccharide in human breast milk, designed and validated in kilogram scale.
Human Milk Oligosaccharides in the Milk of Mothers Delivering Term versus Preterm Infants.	2019	Austin, S., De Castro, C. A., Sprenger, N., Binia, A., Affolter, M., Garcia-Rodenas, C. L., ... & Fischer Fumeaux, C. J. (2019). Human Milk Oligosaccharides in the Milk of Mothers Delivering Term versus Preterm Infants. Nutrients, 11(6), 1282.	This study aimed to determine if the HMO composition of preterm milk differed from that of term milk at equivalent stage of lactation and equivalent postmenstrual age.
Correlating Infant Faecal Microbiota Composition and Human Milk Oligosaccharide Consumption by Microbiota of One-Month Old Breastfed Infants.	2019	Borewicz, K., Gu, F., Saccenti, E., Arts, I. C. W., Penders, J., Thijs, C., … Smidt, H. (2019). Correlating Infant Fecal Microbiota Composition and Human Milk Oligosaccharide Consumption by Microbiota of 1-Month-Old Breastfed Infants. Molecular Nutrition and Food Research, 63(13). https://doi.org/10.1002/mnfr.201801214	This study examined a link between HMOs in maternal milk and infant faecal microbiota composition and investigated the role of microbiota in degrading HMOs within the GI tract of healthy, breastfed, one-month old infants.
Mechanisms by which sialylated milk oligosaccharides impact bone biology in a gnotobiotic mouse model of infant undernutrition.	2019	Cowardin, C. A., Ahern, P. P., Kung, V. L., Hibberd, M. C., Cheng, J., Guruge, J. L., ... & Barratt, M. J. (2019). Mechanisms by which sialylated milk oligosaccharides impact bone biology in a gnotobiotic mouse model of infant undernutrition. Proceedings of the National Academy of Sciences, 116(24), 11988-11996.	To characterize interactions among the gut microbiota, human milk oligosaccharides (HMOs), and osteoclast and osteoblast biology, young germ-free mice were colonized with cultured bacterial strains from a 6-mo-old stunted infant and fed a diet mimicking that consumed by the donor population.
Amino-2’-fucosyllactose inhibits biofilm formation by Streptococcus agalactiae.	2019	Craft, K. M., & Townsend, S. D. (2019). 1-Amino-2’-fucosyllactose inhibits biofilm formation by Streptococcus agalactiae. Journal of Antibiotics, 72(6), 507–512. https://doi.org/10.1038/s41429-019-0151-6	This study evaluated the potential of 2’-FL to serve as an antibacterial agent against Group B Streptococcus (GBS).

Title	Year	Citation	Study Objective
Creating an in vivo bifunctional gene expression circuit through an aptamer-based regulatory mechanism for dynamic metabolic engineering in Bacillus subtilis	2019	Deng, J., Chen, C., Gu, Y., Lv, X., Liu, Y., Li, J., … Liu, L. (2019). Creating an in vivo bifunctional gene expression circuit through an aptamer-based regulatory mechanism for dynamic metabolic engineering in Bacillus subtilis. Metabolic Engineering, 55, 179–190. https://doi.org/10.1016/j.ymben.2019.07.008	The authors deveop an aptamer-based synthetic regulatory circuit to dynamically upregulate and downregulate the expression of target genes in response to the ligand thrombin at transcriptional and translational levels, respectively, and further use the system to dynamically engineer the synthesis of 2'-fucosyllactose (2'-FL) in Bacillus subtilis.
Analysis of Fucosylated Human Milk Trisaccharides in Biotechnological Context Using Genetically Encoded Biosensors	2019	Enam, F., & Mansell, T. J. (2019). Analysis of Fucosylated Human Milk Trisaccharides in Biotechnological Context Using Genetically Encoded Biosensors. JoVE (Journal of Visualized Experiments), (146), e59253.	The authors demonstrate here, a genetically encoded bacterial biosensor for the high-throughput, linkage-specific detection and quantification of the fucosylated HMO structures, 2'-fucosyllactose and 3-fucosyllactose, which was achieved via heterologous expression of fucosidases.
Breast milk oligosaccharides: effects of 2’-fucosyllactose and 6’-sialyllactose on the adhesion of Escherichia coli and Salmonella fyris to Caco-2 cells.	2019	Facinelli, B., Marini, E., Magi, G., Zampini, L., Santoro, L., Catassi, C., ... & Coppa, G. V. (2019). Breast milk oligosaccharides: effects of 2’-fucosyllactose and 6’-sialyllactose on the adhesion of Escherichia coli and Salmonella fyris to Caco-2 cells. The Journal of Maternal-Fetal & Neonatal Medicine, 32(17), 2950-2952.	The aim of the present study is to evaluate the anti-adhesive effect of the above oligosaccharides on Escherichia coli and Salmonella fyris.
Restraint stress induced gut dysmotility is diminished by a milk oligosaccharide (2’-fucosyllactose) in vitro.	2019	Farhin, S., Wong, A., Delungahawatta, T., Amin, J. Y., Bienenstock, J., Buck, R., & Kunze, W. A. (2019). Restraint stress induced gut dysmotility is diminished by a milk oligosaccharide (2’-fucosyllactose) in vitro. PloS one, 14(4), e0215151.	This study tested whether 2'FL could benefit the dysmotility of isolated jejunal and colonic segments from animals subjected to prior acute restraint stress.
The Role of Two Human Milk Oligosaccharides, 2’-Fucosyllactose and Lacto-N-Neotetraose, in Infant Nutrition.	2019	Hegar, B., Wibowo, Y., Basrowi, R. W., Ranuh, R. G., Sudarmo, S. M., Munasir, Z., ... & Manoppo, C. (2019). The Role of Two Human Milk Oligosaccharides, 2’-Fucosyllactose and Lacto-N-Neotetraose, in Infant Nutrition. Pediatric gastroenterology, hepatology & nutrition, 22(4), 330-340.	This review summarizes the health benefits of 2'-FL and LNnT and effect on infant nutrition.

Title	Year	Citation	Study Objective
Engineering two species of yeast as cell factories for 2'-fucosyllactose	2019	Hollands, K., Baron, C. M., Gibson, K. J., Kelly, K. J., Krasley, E. A., Laffend, L. A., … Rothman, S. C. (2019). Engineering two species of yeast as cell factories for 2'-fucosyllactose. Metabolic Engineering, 52, 232–242. https://doi.org/10.1016/j.ymben.2018.12.005	This study presents results that demonstrate the yeast species Saccharomyces cerevisiae and Yarrowia lipolytica both can be engineered to produce 2'-fucosyllactose (2'FL), which is the most abundant oligosaccharide in human breast milk, at high titer and productivity.
Evidence of human milk oligosaccharides in maternal circulation already during pregnancy: a pilot study	2019	Jantscher-Krenn, E., Aigner, J., Reiter, B., Köfeler, H., Csapo, B., Desoye, G., … van Poppel, M. N. M. (2019). Evidence of human milk oligosaccharides in maternal circulation already during pregnancy: A pilot study. American Journal of Physiology - Endocrinology and Metabolism, 316(3), E347–E357. https://doi.org/10.1152/ajpendo.00320.2018	In a pilot study, the authors investigated individual and temporal variations in HMO composition and concentration in maternal serum at gestational weeks 10-14 (visit 1), 20-24 (visit 2), and 30-35 (visit 3) (V1, V2, and V3, respectively) and associations with maternal body composition.
Enhanced production of 2'-fucosyllactose from fucose by elimination of rhamnose isomerase and arabinose isomerase in engineered Escherichia coli	2019	Jung, S. M., Chin, Y. W., Lee, Y. G., & Seo, J. H. (2019). Enhanced production of 2'-fucosyllactose from fucose by elimination of rhamnose isomerase and arabinose isomerase in engineered Escherichia coli. Biotechnology and Bioengineering. https://doi.org/10.1002/bit.27019	In this study, deletion of the genes (araA and rhaA) coding for arabinose isomerase (AraA) and rhamnose isomerase (RhaA) was attempted in engineered Escherichia coli for improving 2-FL production by using fucose, lactose, and glycerol.
Modulation of Intestinal Epithelial Glycocalyx Development by Human Milk Oligosaccharides and Non-digestible Carbohydrates.	2019	Kong, C., Elderman, M., Cheng, L., de Haan, B. J., Nauta, A., & de Vos, P. (2019). Modulation of Intestinal Epithelial Glycocalyx Development by Human Milk Oligosaccharides and Non-digestible Carbohydrates. Molecular nutrition & food research, 1900303.	The effects of hMOs and NDCs on human gut epithelial cells (Caco2) are investigated by quantifying thickness and area coverage of adsorbed albumin, heparan sulfate (HS), and hyaluronic acid (HA) in the glycocalyx. The study concludes that 2'-FL and 3-FL and inulins stimulate glycocalyx development in a structure-dependent fashion.

Title	Year	Citation	Study Objective
Recognition of early and late stages of bladder cancer using metabolites and machine learning.	2019	Kouznetsova, V. L., Kim, E., Romm, E. L., Zhu, A., & Tsigelny, I. F. (2019). Recognition of early and late stages of bladder cancer using metabolites and machine learning. Metabolomics, 15(7), 94.	The goal of this research was to elucidate the biomarkers including metabolites and corresponding genes for different stages of BCa, show their distinguishing and common features, and create a machine-learning model for classification of stages of BCa.
Human milk oligosaccharide composition is associated with excessive weight gain during exclusive breastfeeding–an explorative study.	2019	Larsson, M. W., Lind, M. V., Laursen, R. P., Yonemitsu, C., Larnkjær, A., Mølgaard, C., ... & Bode, L. (2019). Human milk oligosaccharide composition is associated with excessive weight gain during exclusive breastfeeding–an explorative study. Frontiers in pediatrics, 7, 297.	The purpose of this exploratory study was to evaluate if HMO composition was different in milk fed to infants with excessive weight gain compared to infants with normal weight gain. Furthermore, we aimed to examine if HMO composition was associated with growth velocity and change in body composition and if there were maternal determinants of HMO composition.
L-Fucose production by engineered Escherichia coli	2019	Liu, J. J., Lee, J. W., Yun, E. J., Jung, S. M., Seo, J. H., & Jin, Y. S. (2019). L-Fucose production by engineered Escherichia coli. Biotechnology and Bioengineering, 116(4), 904–911. https://doi.org/10.1002/bit.26907	Th authors modified the strain genome to eliminate endogenous L-fucose and lactose metabolism, produce 2'-fucosyllactose (2'-FL), and to liberate L-fucose from 2'-FL.
Label-free targeted LC-ESI-MS(2) analysis of human milk oligosaccharides (HMOS) and related human milk groups with enhanced structural selectivity	2019	Mank, M., Welsch, P., Heck, A. J., & Stahl, B. (2019). Label-free targeted LC-ESI-MS 2 analysis of human milk oligosaccharides (HMOS) and related human milk groups with enhanced structural selectivity. Analytical and bioanalytical chemistry, 411(1), 231-250.	The authors developed a negative ion mode LC-ESI-MS(2) approach featuring straightforward sample preparation, environmentally friendly EtOH gradient elution, and enhanced, semiquantitative characterization of distinct native HMOS by multiple reaction monitoring (MRM).

Title	Year	Citation	Study Objective
Expeditious Synthesis of C-Glycosyl Barbiturate Ligands of Bacterial Lectins: From Monomer Design to Glycoclusters and Glycopolymers	2019	Portier, F., Imberty, A., & Halila, S. (2019). Expeditious Synthesis of C-Glycosyl Barbiturate Ligands of Bacterial Lectins: From Monomer Design to Glycoclusters and Glycopolymers. Bioconjugate Chemistry, 30(3), 647–656. https://doi.org/10.1021/acs.bioconjchem.8b00847	The study offers an approach for straightforward and efficient access to beta- C-glycosyl barbiturate ligands, spanning from glycomimetics to multivalent C-neoglycoconjugates, with the aim of deciphering structural parameters impacting the binding to pathogenic lectins.
Development of a quantitative assay for 2 -fucosyllactose via one-pot reaction with alpha1,2-fucosidase and l-fucose dehydrogenase	2019	Seydametova, E., Shin, J., Yu, S. H., Kim, C., Kim, H., Park, Y. J., ... & Ban, C. (2019). Development of a quantitative assay for 2′-fucosyllactose via one-pot reaction with α1, 2-fucosidase and l-fucose dehydrogenase. Analytical biochemistry, 582, 113358.	In this study, two genes, namely alpha1,2-fucosidase from Xanthomonas manihotis and l-fucose dehydrogenase from Pseudomonas sp. no. 1143, were identified, cloned and overexpressed in E. coli. The recombinant enzymes were produced as 6xHis-tagged proteins and were purified to homogeneity using Ni(2+) affinity chromatography.
Search for bacterial alpha1,2-fucosyltransferases for whole-cell biosynthesis of 2'-fucosyllactose in recombinant Escherichia coli	2019	Seydametova, E., Yu, J., Shin, J., Park, Y., Kim, C., Kim, H., ... & Kweon, D. H. (2019). Search for bacterial α1, 2-fucosyltransferases for whole-cell biosynthesis of 2′-fucosyllactose in recombinant Escherichia coli. Microbiological research, 222, 35-42.	In this study, 10 alpha1,2-FT genes from bacteria of biosafety level one were identified, and the main features of the deduced amino acid sequences were characterized.Four codon-optimized alpha1,2-FT genes were synthesized and introduced into Escherichia coli DeltaL M15 strain containing the plasmid pBCGW encoding guanosine 5'-diphosphate-l-fucose biosynthetic enzymes. Among the four genes, 2'-FL was produced only by the alpha1,2-FT from Thermosynechococcus elongatus (Te2FT).
Human Milk Oligosaccharides: Factors Affecting Their Composition and Their Physiological Significance. In Human Milk: Composition, Clinical Benefits and Future Opportunities	2019	Sprenger, N., Binia, A., & Austin, S. (2019). Human Milk Oligosaccharides: Factors Affecting Their Composition and Their Physiological Significance. In Human Milk: Composition, Clinical Benefits and Future Opportunities (Vol. 90, pp. 43-56). Karger Publishers.	This review summarizes the clinical, observational, and basic research data available on early-life microbiota composition and immune protection

Title	Year	Citation	Study Objective
2'-Fucosyllactose Is Well Tolerated in a 100% Whey, Partially Hydrolyzed Infant Formula With Bifidobacterium lactis: A Randomized Controlled Trial.	2019	Storm, H. M., Shepard, J., Czerkies, L. M., Kineman, B., Cohen, S. S., Reichert, H., & Carvalho, R. (2019). 2'-Fucosyllactose Is Well Tolerated in a 100% Whey, Partially Hydrolyzed Infant Formula With Bifidobacterium lactis : A Randomized Controlled Trial . Global Pediatric Health, 6, 2333794X1983399. https://doi.org/10.1177/2333794x19833995	We evaluated feeding tolerance of the human milk oligosaccharide 2'-fucosyllactose (2'FL) in a 100% whey, partially hydrolyzed infant formula with the probiotic Bifidobacterium animalis ssp lactis strain Bb12 (B lactis; Test) as compared with the same formula without 2'FL (Control) in a randomized controlled trial of healthy infants enrolled at 2 weeks of age (±5 days).
Validation and application of a method for the simultaneous absolute quantification of 16 neutral and acidic human milk oligosaccharides by graphitized carbon liquid chromatography - electrospray ionization - mass spectrometry.	2019	Tonon, K. M., Miranda, A., Abrão, A. C. F. V., de Morais, M. B., & Morais, T. B. (2019). Validation and application of a method for the simultaneous absolute quantification of 16 neutral and acidic human milk oligosaccharides by graphitized carbon liquid chromatography – electrospray ionization – mass spectrometry. Food Chemistry, 274, 691–697. https://doi.org/10.1016/j.foodchem.2018.09.036	This paper describes a simple HMOs extraction and analysis for the simultaneous and absolute quantification of neutral and acidic HMOs by graphitized carbon liquid chromatography–electrospray ionization–mass spectrometry and was validated and applied to analyze HMOs in the human milk obtained from 10 women.
Prebiotic oligosaccharides in early life alter gut microbiome development in male mice while supporting influenza vaccination responses.	2019	van den Elsen, L. W. J., Tims, S., Jones, A. M., Stewart, A., Stahl, B., Garssen, J., ... & van't Land, B. (2019). Prebiotic oligosaccharides in early life alter gut microbiome development in male mice while supporting influenza vaccination responses. Beneficial microbes, 10(3), 279-291.	Mice were supplemented with the prebiotic milk oligosaccharide 2'-fucosyllactose (2'FL) as well as a complex mixture of immune modulatory prebiotic short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides (scGOS/lcFOS) from different stages in early life and the development of the gut microbiota and antibody-mediated vaccine responses were followed over time after vaccination with trivalent influenza vaccine.

Title	Year	Citation	Study Objective
The Combination of 2’-Fucosyllactose with Short-Chain Galacto-Oligosaccharides and Long-Chain Fructo-Oligosaccharides that Enhance Influenza Vaccine Responses Is Associated with Mucosal Immune Regulation in Mice.	2019	Xiao, L., Engen, P. A., Leusink-Muis, T., van Ark, I., Stahl, B., Overbeek, S. A., ... & Folkerts, G. (2019). The Combination of 2’-Fucosyllactose with Short-Chain Galacto-Oligosaccharides and Long-Chain Fructo-Oligosaccharides that Enhance Influenza Vaccine Responses Is Associated with Mucosal Immune Regulation in Mice. The Journal of nutrition, 149(5), 856-869.	Dietary supplementation with a combination of 2'FL and prebiotic short-chain (sc) galacto-oligosaccharides (GOS) and long-chain (lc) fructo-oligosaccharides (FOS) was employed to examine human milk oligosaccharide effects on immune responsiveness, within a murine influenza vaccination model.
Novel Genes and Metabolite Trends in Bifidobacterium longum subsp. infantis Bi-26 Metabolism of Human Milk Oligosaccharide 2’-fucosyllactose.	2019	Zabel, B., Yde, C. C., Roos, P., Marcussen, J., Jensen, H. M., Salli, K., … Morovic, W. (2019). Novel Genes and Metabolite Trends in Bifidobacterium longum subsp. infantis Bi-26 Metabolism of Human Milk Oligosaccharide 2’-fucosyllactose. Scientific Reports, 9(1), 7983. https://doi.org/10.1038/s41598-019-43780-9	To understand the relationship between bifidobacteria utilizing HMOs and how the metabolites that are produced could affect the host, this study investigated the metabolism of HMO 2’-fucosyllactose (2’-FL) in Bifidobacterium longum subsp. infantis Bi-26.
Determination of 2’-Fucosyllactose and Lacto-N-neotetraose in Infant Formula	2018	Austin, S., Cuany, D., Michaud, J., Diehl, B., & Casado, B. (2018). Determination of 2’-Fucosyllactose and Lacto-N-neotetraose in Infant Formula. Molecules, 23(10), 2650.	The authors developed two different approaches for analysis of 2'-FL and LNnT in formula; high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and hydrophilic interaction liquid chromatography with fluorescence detection (HILIC-FLD).
Human Milk Oligosaccharide Concentrations Are Associated with Multiple Fixed and Modifiable Maternal Characteristics, Environmental Factors, and Feeding Practices	2018	Azad, M. B., Robertson, B., Atakora, F., Becker, A. B., Subbarao, P., Moraes, T. J., ... & Bode, L. (2018). Human milk oligosaccharide concentrations are associated with multiple fixed and modifiable maternal characteristics, environmental factors, and feeding practices. The Journal of nutrition, 148(11), 1733-1742.	The aim of the study was to identify modifiable and nonmodifiable factors associated with HMO concentrations.

Title	Year	Citation	Study Objective
Supplementation With 2'-FL and scGOS/lcFOS Ameliorates Rotavirus-Induced Diarrhea in Suckling Rats.	2018	Azagra-Boronat, I., Massot-Cladera, M., Knipping, K., Van't Land, B., Stahl, B., Garssen, J., … Pérez-Cano, F. J. (2018). Supplementation With 2'-FL and scGOS/lcFOS Ameliorates Rotavirus-Induced Diarrhea in Suckling Rats. Frontiers in Cellular and Infection Microbiology, 8, 372. https://doi.org/10.3389/fcimb.2018.00372	This study aimed to evaluate the potential protective role of a specific human milk oligosaccharide, 2'-fucosyllactose (2'-FL), a mixture of the prebiotic short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides 9:1 (GOS/FOS) and their combination (2'-FL+GOS/FOS) on RV-induced diarrhea in suckling rats.
Enzyme and microbial technology for synthesis of bioactive oligosaccharides: an update	2018	Chen, R. (2018, April 1). Enzyme and microbial technology for synthesis of bioactive oligosaccharides: an update. Applied Microbiology and Biotechnology. Springer Verlag. https://doi.org/10.1007/s00253-018-8839-2	Using examples of galactose-containing oligosaccharides, this review analyzes the pros and cons of two forms of biocatalysts and provides an updated view on the status of biocatalysis in this important field.
Human Milk Oligosaccharides in Colostrum and Mature Milk of Chinese Mothers: Lewis Positive Secretor Subgroups.	2018	Elwakiel, M., Hageman, J. A., Wang, W., Szeto, I. M., van Goudoever, J. B., Hettinga, K. A., & Schols, H. A. (2018). Human milk oligosaccharides in colostrum and mature milk of Chinese mothers: Lewis positive secretor subgroups. Journal of agricultural and food chemistry, 66(27), 7036-7043.	To study the variability in human milk oligosaccharide (HMO) composition of Chinese human milk over a 20-wk lactation period, HMO profiles of 30 mothers were analyzed using CE-LIF.
TCA cycle-powered synthesis of fucosylated oligosaccharides	2018	Guan, N., Shin, H. D., Long, L., Azadi, P., & Chen, R. (2018). TCA cycle-powered synthesis of fucosylated oligosaccharides. Glycobiology, 28(7), 468–473. https://doi.org/10.1093/glycob/cwy047	The authors demonstrated a new approach to overcome the challenges related to microbioal synthesis of fucosylated oligosaccharides by directly tapping into the cellular "power house," the TCA cycle, to provide the cellular energy for synthesis.

Title	Year	Citation	Study Objective
Synthesis of a Fucosylated Trisaccharide Via Transglycosylation by alpha-L-Fucosidase from Thermotoga maritima	2018	Guzmán-Rodríguez, F., Alatorre-Santamaría, S., Gómez-Ruiz, L., Rodríguez-Serrano, G., García-Garibay, M., & Cruz-Guerrero, A. (2018). Synthesis of a Fucosylated Trisaccharide Via Transglycosylation by α-l-Fucosidase from Thermotoga maritima. Applied Biochemistry and Biotechnology, 186(3), 681–691. https://doi.org/10.1007/s12010-018-2771-x	In this work, the effect of an acceptor substrate (lactose) and the donor substrate 4-nitrophenyl-alpha-L-fucopyranoside (pNP-Fuc) on the synthesis of a fucosylated trisaccharide was studied in a transglycosylation reaction using alpha-L-fucosidase from Thermotoga maritima.
Biosynthesis of a Functional Human Milk Oligosaccharide, 2'-Fucosyllactose, and l-Fucose Using Engineered Saccharomyces cerevisiae	2018	Liu, J. J., Kwak, S., Pathanibul, P., Lee, J. W., Yu, S., Yun, E. J., … Jin, Y. S. (2018). Biosynthesis of a Functional Human Milk Oligosaccharide, 2’-Fucosyllactose, and l -Fucose Using Engineered Saccharomyces cerevisiae. ACS Synthetic Biology, 7(11), 2529–2536. https://doi.org/10.1021/acssynbio.8b00134	In this study, an alternative route to produce 2-FL via a de novo pathway using a food-grade microorganism, Saccharomyces cerevisiae, has been devised.
Simulation and modeling of dietary changes in the infant gut microbiome	2018	Medina, D. A., Pinto, F., Ortuzar, V., & Garrido, D. (2018). Simulation and modeling of dietary changes in the infant gut microbiome. FEMS Microbiology Ecology, 94(9). https://doi.org/10.1093/femsec/fiy140	The study authors simulated the impact of a dietary switch from fructooligosaccharides (FOS) to 2-fucosyllactose (2FL) in a continuous culture containing a consortium of species of the infant gut microbiome.
The oligosaccharides 6’-sialyllactose, 2’-fucosyllactose or galactooligosaccharides do not directly modulate human dendritic cell differentiation or maturation.	2018	Perdijk, O., van Neerven, R. J., van den Brink, E., Savelkoul, H. F., & Brugman, S. (2018). The oligosaccharides 6’-sialyllactose, 2’-fucosyllactose or galactooligosaccharides do not directly modulate human dendritic cell differentiation or maturation. PloS one, 13(7), e0200356.	In this study the authors investigated the effect of the HMOs 6'-sialyllactose (6'SL) and 2'-fucosyllactose (2'FL) as well as prebiotic galactooligosaccharides (GOS) on DC differentiation and maturation. Isolated CD14+ monocytes were cultured for six days in the presence of GM-CSF and IL-4 with or without 6'SL, 2'FL, GOS, VitD3 or TGFbeta.

Title	Year	Citation	Study Objective
Safety evaluation of a mixture of the human-identical milk oligosaccharides 2’-fucosyllactose and difucosyllactose.	2018	Phipps, K. R., Baldwin, N., Lynch, B., Flaxmer, J., Šoltésová, A., Gilby, B., … Röhrig, C. H. (2018). Safety evaluation of a mixture of the human-identical milk oligosaccharides 2’-fucosyllactose and difucosyllactose. Food and Chemical Toxicology, 120, 552–565. https://doi.org/10.1016/j.fct.2018.07.054	Safety assessment of 2'-FL/DFL included conduct of in vitro genotoxicity tests and a subchronic oral toxicity study.
Human milk oligosaccharides, milk microbiome and infant gut microbiome modulate neonatal rotavirus infection.	2018	Ramani, S., Stewart, C. J., Laucirica, D. R., Ajami, N. J., Robertson, B., Autran, C. A., ... & Ferreon, J. C. (2018). Human milk oligosaccharides, milk microbiome and infant gut microbiome modulate neonatal rotavirus infection. Nature communications, 9(1), 5010.	Using multidisciplinary approaches, the study shows a complex interplay between HMOs, milk microbiome, and infant gut microbiome impacts neonatal rotavirus infections.
Review of the Clinical Experiences of Feeding Infants Formula Containing the Human Milk Oligosaccharide 2’-Fucosyllactose.	2018	Reverri, E., Devitt, A., Kajzer, J., Baggs, G., & Borschel, M. (2018). Review of the Clinical Experiences of Feeding Infants Formula Containing the Human Milk Oligosaccharide 2’-Fucosyllactose. Nutrients, 10(10), 1346.	he purpose of this narrative review was to summarize the clinical experiences of feeding infant formula supplemented with the HMO, 2'-FL.
A Simple Enzymatic Method for Quantitation of 2'-Fucosyllactose	2018	Seydametova, E., Shin, J., Yu, J., & Kweon, D. H. (2018). A Simple Enzymatic Method for Quantitation of 2'-Fucosyllactose. Journal of microbiology and biotechnology, 28(7), 1141-1146.	The authors developed a simple method for quantifying 2'-FL in a microplate format. The method involves two steps: (1) release of L-fucose from 2'-FL by alpha-(1-2,3,4,6)-L-fucosidase and (2) measurement of NADPH formed during the oxidation of L-fucose by L-fucose dehydrogenase. This method enables measurement of up to 5 g/l 2'-FL in 50 min using a 96-well microplate.
Chemical structures of oligosaccharides in milk of the raccoon (Procyon lotor)	2018	Urashima, T., Yamaguchi, E., Ohshima, T., Fukuda, K., & Saito, T. (2018). Chemical structures of oligosaccharides in milk of the raccoon (Procyon lotor). Glycoconjugate journal, 35(3), 275-286.	In this study on milk saccharides of the raccoon (Procyonidae: Carnivora), free lactose was found to be a minor constituent among a variety of neutral and acidic oligosaccharides, which predominated over lactose.

Title	Year	Citation	Study Objective
Safety assessment of biotechnologically produced 2'-Fucosyllactose, a novel food additive.	2018	van Berlo, D., Wallinga, A. E., van Acker, F. A., & Delsing, D. J. (2018). Safety assessment of biotechnologically produced 2'-Fucosyllactose, a novel food additive. Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association, 118, 84.	This study assessed the safety of 2'-FL in juvenile rats for 25 days and concluded that 2'-FL was nontoxic.
Human milk oligosaccharides: 2’-fucosyllactose (2’-FL) and lacto-n-neotetraose (LNnT) in infant formula. Nutrients.	2018	Vandenplas, Y., Berger, B., Carnielli, V. P., Ksiazyk, J., Lagström, H., Luna, M. S., … Wabitsch, M. (2018, September 1). Human milk oligosaccharides: 2’-fucosyllactose (2’-FL) and lacto-n-neotetraose (LNnT) in infant formula. Nutrients. MDPI AG. https://doi.org/10.3390/nu10091161	The authors reviewed the published evidence on the presence of oligosaccharides in human milk and their benefits in in vitro and in vivo studies and clinical trials
Infants Are Exposed to Human Milk Oligosaccharides Already in utero	2018	Wise, A., Robertson, B., Choudhury, B., Rautava, S., Isolauri, E., Salminen, S., & Bode, L. (2018). Infants are exposed to human milk oligosaccharides already in utero. Frontiers in pediatrics, 6.	In this pilot study the authors aimed to determine whether or not HMOs also appear in amniotic fluid.
Human milk oligosaccharide 2’-fucosyllactose improves innate and adaptive immunity in an influenza-specific murine vaccination model	2018	Xiao, L., Leusink-Muis, T., Kettelarij, N., van Ark, I., Blijenberg, B., Hesen, N. A., ... & van’t Land, B. (2018). Human milk oligosaccharide 2’-fucosyllactose improves innate and adaptive immunity in an influenza-specific murine vaccination model. Frontiers in immunology, 9, 452.	Aim: To determine the effect of 2'FL on vaccination responsiveness (both innate and adaptive) in a murine influenza vaccination model and elucidate mechanisms involved.
Engineering of α-1, 3-fucosyltransferases for production of 3-fucosyllactose in Escherichia coli.	2018	Yu, J., Shin, J., Park, M., Seydametova, E., Jung, S. M., Seo, J. H., & Kweon, D. H. (2018). Engineering of α-1, 3-fucosyltransferases for production of 3-fucosyllactose in Escherichia coli. Metabolic engineering, 48, 269-278.	In this study, the effect of truncated version of 1,3-FTs structural components on the production of 3-FL in Escherichia coli was evaluated through systematic truncation and elongation of the C-terminal regions of three 1,3-FTs from Helicobacter pylori.

Title	Year	Citation	Study Objective
Production of a human milk oligosaccharide 2'-fucosyllactose by metabolically engineered Saccharomyces cerevisiae	2018	Yu, S., Liu, J. J., Yun, E. J., Kwak, S., Kim, K. H., & Jin, Y. S. (2018). Production of a human milk oligosaccharide 2′-fucosyllactose by metabolically engineered Saccharomyces cerevisiae. Microbial Cell Factories, 17(1). https://doi.org/10.1186/s12934-018-0947-2	In this study, 2-FL was produced alternatively by using a yeast Saccharomyces cerevisiae, which may have advantages over E. coli.
Human Milk Oligosaccharides Attenuate Antigen–Antibody Complex Induced Chemokine Release from Human Intestinal Epithelial Cell Lines	2018	Zehra, S., Khambati, I., Vierhout, M., Mian, M. F., Buck, R., & Forsythe, P. (2018). Human Milk Oligosaccharides Attenuate Antigen–Antibody Complex Induced Chemokine Release from Human Intestinal Epithelial Cell Lines. Journal of food science, 83(2), 499-508.	The authors demonstrate that the HMO, 6'-sialyllactose (6'SL) inhibited chemokine (IL-8 and CCL20) release from T-84 and HT-29 cells stimulated with antigen-antibody complex, TNFalpha or PGE2 ; an effect that was PPARgamma dependent and associated with decreased activity of the transcription factors AP-1 and NFkappaB.
Substrate specificity and transfucosylation activity of GH29 alpha-l-fucosidases for enzymatic production of human milk oligosaccharides	2018	Zeuner, B., Muschiol, J., Holck, J., Lezyk, M., Gedde, M. R., Jers, C., … Meyer, A. S. (2018). Substrate specificity and transfucosylation activity of GH29 α-L-fucosidases for enzymatic production of human milk oligosaccharides. New Biotechnology, 41, 34–45. https://doi.org/10.1016/j.nbt.2017.12.002	this study investigates transfucosylation catalysed by retaining alpha-l-fucosidases as a new route for manufacturing biomimetic HMOs.
Improved production of 2'-fucosyllactose in engineered Escherichia coli by expressing putative alpha-1,2-fucosyltransferase, WcfB from Bacteroides fragilis	2017	Chin, Y. W., Kim, J. Y., Kim, J. H., Jung, S. M., & Seo, J. H. (2017). Improved production of 2′-fucosyllactose in engineered Escherichia coli by expressing putative α-1,2-fucosyltransferase, WcfB from Bacteroides fragilis. Journal of Biotechnology, 257, 192–198. https://doi.org/10.1016/j.jbiotec.2016.11.033	In the present study, the wcfB gene coding for alpha-1,2-fucosyltransferase from Bacteroides fragilis was screened from eleven candidates of putative alpha-1,2-fucosyltransferase
α1, 2-Fucosyllactose does not improve intestinal function or prevent Escherichia coli F18 diarrhea in newborn pigs.	2017	Cilieborg, M. S., Sangild, P. T., Jensen, M. L., Østergaard, M. V., Christensen, L., Rasmussen, S. O., ... & Bering, S. B. (2017). α1, 2-Fucosyllactose does not improve intestinal function or prevent Escherichia coli F18 diarrhea in newborn pigs. Journal of pediatric gastroenterology and nutrition, 64(2), 310-318.	2'-FL inhibited in vitro adhesion of E coli F18 to epithelial cells, but had limited effects on diarrhea and mucosal health in newborn pigs challenged with E coli F18.

Title	Year	Citation	Study Objective
Dietary human milk oligosaccharides but not prebiotic oligosaccharides increase circulating natural killer cell and mesenteric lymph node memory T cell populations in noninfected and rotavirus-infected neonatal piglets.	2017	Comstock, S. S., Li, M., Wang, M., Monaco, M. H., Kuhlenschmidt, T. B., Kuhlenschmidt, M. S., & Donovan, S. M. (2017). Dietary human milk oligosaccharides but not prebiotic oligosaccharides increase circulating natural killer cell and mesenteric lymph node memory T cell populations in noninfected and rotavirus-infected neonatal piglets. The Journal of nutrition, 147(6), 1041-1047.	The authors measured the effects of HMOs and prebiotic oligosaccharides on immune cell populations from noninfected and rotavirus-infected pigs, hypothesizing tha dietary HMOs would modulate systemic and gastrointestinal immunity.
Analytical characterization of human milk oligosaccharides–potential applications in pharmaceutical analysis	2017	Grabarics, M., Csernák, O., Balogh, R., & Béni, S. (2017). Analytical characterization of human milk oligosaccharides–potential applications in pharmaceutical analysis. Journal of pharmaceutical and biomedical analysis, 146, 168-178.	This review highlights the potential applications of HMOs in the (bio)pharmaceutical industry, also summarizes the analytical methods available for the characterization of HMOs. An overview of the structure and function of HMOs along with their determination methods in complex matrices are provided.
Metabolic engineering of Escherichia coli for the production of 2'-fucosyllactose and 3-fucosyllactose through modular pathway enhancement	2017	Huang, D., Yang, K., Liu, J., Xu, Y., Wang, Y., Wang, R., … Feng, L. (2017). Metabolic engineering of Escherichia coli for the production of 2'-fucosyllactose and 3-fucosyllactose through modular pathway enhancement. Metabolic Engineering, 41, 23–38. https://doi.org/10.1016/j.ymben.2017.03.001	In this study, a synthetic biology approach was devloped to promote the efficient biosynthesis of 2'-FL and 3-FL in engineered Escherichia coli.
Human norovirus inhibition by a human milk oligosaccharide.	2017	Koromyslova, A., Tripathi, S., Morozov, V., Schroten, H., & Hansman, G. S. (2017). Human norovirus inhibition by a human milk oligosaccharide. Virology, 508, 81-89.	This study identified the HBGA binding pocket for an emerging GII genotype 17 (GII.17) variant using X-ray crystallography and found that 2'-fucosyllactose (2'FL) blocked both the GI.1 and GII.17 noroviruses from binding to HBGAs.

Title	Year	Citation	Study Objective
Milk oligosaccharides inhibit human rotavirus infectivity in MA104 cells.	2017	Laucirica, D. R., Triantis, V., Schoemaker, R., Estes, M. K., & Ramani, S. (2017). Milk oligosaccharides inhibit human rotavirus infectivity in MA104 cells. The Journal of nutrition, 147(9), 1709-1714.	In this study, the authors determined the effect of specific and abundant milk oligosaccharides on the infectivity of 2 globally dominant human rotavirus strains.
Prebiotics mediate microbial interactions in a consortium of the infant gut microbiome.	2017	Medina, D. A., Pinto, F., Ovalle, A., Thomson, P., & Garrido, D. (2017). Prebiotics mediate microbial interactions in a consortium of the infant gut microbiome. International Journal of Molecular Sciences, 18(10). https://doi.org/10.3390/ijms18102095	The study authors investigated the transformation of prebiotics by a consortium of four representative species of the infant gut microbiome, and how their interactions changed with dietary substrates.
Regulatory Aspects of Human Milk Oligosaccharides	2017	Salminen, S. (2017). Regulatory Aspects of Human Milk Oligosaccharides. In Intestinal microbiome: functional aspects in health and disease (Vol. 88, pp. 161-170). Karger Publishers.	In regulatory sense human milk oligosaccharides are classified as novel foods or novel food ingredients requiring safety assessment. In addition, if any health messages are intended to be used also health claim regulations apply. This chapter reviews the regulatory settings and studies human milk oligosaccharides are required to fulfill to be able to enter markets in European Union or United States or elsewhere.
Production of human milk oligosaccharides by enzymatic and whole-cell microbial biotransformations	2017	Sprenger, G. A., Baumgärtner, F., & Albermann, C. (2017). Production of human milk oligosaccharides by enzymatic and whole-cell microbial biotransformations. Journal of Biotechnology, 258, 79–91. https://doi.org/10.1016/j.jbiotec.2017.07.030	The authors review approaches to HMO preparation by (chemo-)enzymatic syntheses or by whole-cell biotransformation with recombinant bacterial cells.

Title	Year	Citation	Study Objective
FUT2-dependent breast milk oligosaccharides and allergy at 2 and 5 years of age in infants with high hereditary allergy risk	2017	Sprenger, N., Odenwald, H., Kukkonen, A. K., Kuitunen, M., Savilahti, E., & Kunz, C. (2017). FUT2-dependent breast milk oligosaccharides and allergy at 2 and 5 years of age in infants with high hereditary allergy risk. European journal of nutrition, 56(3), 1293-1301.	The authors studied whether FUT2-dependent breast milk oligosaccharides are associated with allergic disease in breast-fed infants later in life.
Human milk oligosaccharide consumption by probiotic and human-associated bifidobacteria and lactobacilli.	2017	Thongaram, T., Hoeflinger, J. L., Chow, J. M., & Miller, M. J. (2017). Human milk oligosaccharide consumption by probiotic and human-associated bifidobacteria and lactobacilli. Journal of Dairy Science, 100(10), 7825–7833. https://doi.org/10.3168/jds.2017-12753	Using a high-throughput, low-volume growth determination, the study evaluated the ability of 12 lactobacilli and 12 bifidobacteria strains, including several commercial probiotics, to ferment HMO and their constituent monomers.
Major human milk oligosaccharides are absorbed into the systemic circulation after oral administration in rats	2017	Vazquez, E., Santos-Fandila, A., Buck, R., Rueda, R., & Ramirez, M. (2017). Major human milk oligosaccharides are absorbed into the systemic circulation after oral administration in rats. British Journal of Nutrition, 117(2), 237-247.	The aim of this study was to evaluate the absorption and urine excretion of HMO in rats.
Temporal Change of the Content of 10 Oligosaccharides in the Milk of Chinese Urban Mothers	2016	Austin, S., De Castro, C., Bénet, T., Hou, Y., Sun, H., Thakkar, S., ... & Wang, P. (2016). Temporal change of the content of 10 oligosaccharides in the milk of Chinese urban mothers. Nutrients, 8(6), 346.	In this cross-sectional observational study, the HMO composition of milk from Chinese mothers was studied to determine the impact of stage of lactation, mode of delivery and geographical location.
Sialylated galacto-oligosaccharides and 2’-fucosyllactose reduce necrotising enterocolitis in neonatal rats	2016	Autran, C. A., Schoterman, M. H., Jantscher-Krenn, E., Kamerling, J. P., & Bode, L. (2016). Sialylated galacto-oligosaccharides and 2’-fucosyllactose reduce necrotising enterocolitis in neonatal rats. British Journal of Nutrition, 116(2), 294-299.	The authors assessed the in vivo efficacy of 2'FL, as well as of GOS that we enzymatically sialylated (Sia-GOS).

Title	Year	Citation	Study Objective
Structural characterisation of human galectin-4 N-terminal carbohydrate recognition domain in complex with glycerol, lactose, 3’-sulfo-lactose, and 2’-fucosyllactose.	2016	Bum-Erdene, K., Leffler, H., Nilsson, U. J., & Blanchard, H. (2016). Structural characterisation of human galectin-4 N-terminal carbohydrate recognition domain in complex with glycerol, lactose, 3’-sulfo-lactose, and 2’-fucosyllactose. Scientific reports, 6, 20289.	The study presents the X-ray structures of human galectin-4 N-terminal CRD (galectin-4N) bound to different saccharide ligands.
Fucosyllactose and L-fucose utilization of infant Bifidobacterium longum and Bifidobacterium kashiwanohense.	2016	Bunesova, V., Lacroix, C., & Schwab, C. (2016). Fucosyllactose and L-fucose utilization of infant Bifidobacterium longum and Bifidobacterium kashiwanohense. BMC Microbiology, 16(1), 1–12. https://doi.org/10.1186/s12866-016-0867-4	The aim of this study was to characterize HMOs utilization by rare bifidobacterium species taken from stool of six-month-old Kenyan infants
Metabolic engineering of Escherichia coli to produce 2'-fucosyllactose via salvage pathway of guanosine 5'-diphosphate (GDP)-l-fucose.	2016	Chin, Y. W., Seo, N., Kim, J. H., & Seo, J. H. (2016). Metabolic engineering of Escherichia coli to produce 2’-fucosyllactose via salvage pathway of guanosine 5’-diphosphate (GDP)-l-fucose. Biotechnology and Bioengineering, 113(11), 2443–2452. https://doi.org/10.1002/bit.26015	This study describes the way in which the salvage guanosine 5’-diphosphate (GDP)-l-fucose biosynthetic pathway from fucose was employed in engineered Escherichia coli BL21star(DE3) for efficient production of 2-FL.
Minimal short-term effect of dietary 2'-fucosyllactose on bacterial colonisation, intestinal function and necrotising enterocolitis in preterm pigs	2016	Cilieborg, M. S., Bering, S. B., Østergaard, M. V., Jensen, M. L., Krych, Ł., Newburg, D. S., & Sangild, P. T. (2016). Minimal short-term effect of dietary 2'-fucosyllactose on bacterial colonisation, intestinal function and necrotising enterocolitis in preterm pigs. British Journal of Nutrition, 116(5), 834-841.	Preterm pigs were used to test whether infant formula enriched with alpha1,2-fucosyllactose (2'-FL, the most abundant oligosaccharide in human milk) would benefit gut microbial colonisation and NEC resistance after preterm birth.
Human milk oligosaccharides influence neonatal mucosal and systemic immunity	2016	Donovan, S. M., & Comstock, S. S. (2016). Human milk oligosaccharides influence neonatal mucosal and systemic immunity. Annals of Nutrition and Metabolism, 69(Suppl. 2), 41-51.	This review will focus on the role human milk oligosaccharides (HMO) play in neonatal gastrointestinal and systemic immune development and function.

Title	Year	Citation	Study Objective
Oral supplementation of healthy adults with 2'-O-fucosyllactose and lacto-N-neotetraose is well tolerated and shifts the intestinal microbiota.	2016	Elison, E., Vigsnaes, L. K., Rindom Krogsgaard, L., Rasmussen, J., Sorensen, N., McConnell, B., … Bytzer, P. (2016). Oral supplementation of healthy adults with 2’-O-fucosyllactose and lacto-N-neotetraose is well tolerated and shifts the intestinal microbiota. British Journal of Nutrition, 116(8), 1356–1368. https://doi.org/10.1017/S0007114516003354	This study provides the first set of data on safety, tolerance and impact of HMO on the adult gut microbiota.
Similar to those who are breastfed, infants fed a formula containing 2’-fucosyllactose have lower inflammatory cytokines in a randomized controlled trial	2016	Goehring, K. C., Marriage, B. J., Oliver, J. S., Wilder, J. A., Barrett, E. G., & Buck, R. H. (2016). Similar to those who are breastfed, infants fed a formula containing 2’-fucosyllactose have lower inflammatory cytokines in a randomized controlled trial. The Journal of nutrition, 146(12), 2559-2566.	The objective was to investigate the effects of feeding formulas supplemented with the HMO 2'-fucosyllactose (2'-FL) on biomarkers of immune function in healthy term infants.
The human milk oligosaccharide 2’-fucosyllactose attenuates the severity of experimental necrotising enterocolitis by enhancing mesenteric perfusion in the neonatal intestine.	2016	Good, M., Sodhi, C. P., Yamaguchi, Y., Jia, H., Lu, P., Fulton, W. B., ... & Ma, C. (2016). The human milk oligosaccharide 2’-fucosyllactose attenuates the severity of experimental necrotising enterocolitis by enhancing mesenteric perfusion in the neonatal intestine. British Journal of Nutrition, 116(7), 1175-1187.	The authors hypothesise that an abundant human milk oligosaccharide (HMO) in breast milk, 2'-fucosyllactose (2'FL), protects against NEC by enhancing intestinal mucosal blood flow, and sought to determine the mechanisms underlying this protection.
Human milk components modulate toll-like receptor–mediated inflammation	2016	He, Y., Lawlor, N. T., & Newburg, D. S. (2016). Human milk components modulate toll-like receptor–mediated inflammation. Advances in Nutrition, 7(1), 102-111.	Aberrant expression of TLRs is found in neonatal inflammatory diseases. Several bioactive components of human milk modulate TLR expression and signaling pathways, including soluble toll-like receptors (sTLRs), soluble cluster of differentiation (sCD) 14, glycoproteins, small peptides, and oligosaccharides.

Title	Year	Citation	Study Objective
The human milk oligosaccharide 2'-fucosyllactose modulates CD14 expression in human enterocytes, thereby attenuating LPS-induced inflammation	2016	He, Y., Liu, S., Kling, D. E., Leone, S., Lawlor, N. T., Huang, Y., ... & Newburg, D. S. (2016). The human milk oligosaccharide 2'-fucosyllactose modulates CD14 expression in human enterocytes, thereby attenuating LPS-induced inflammation. Gut, 65(1), 33-46.	This study tested whether human milk oligosaccharides (HMOSs) influence pathogenic Escherichia coli-induced interleukin (IL)-8 release by intestinal epithelial cells (IECs), identified specific proinflammatory signalling molecules modulated by HMOSs, specified the active HMOS and determined its mechanism of action.
Novel alpha-L-Fucosidases from a Soil Metagenome for Production of Fucosylated Human Milk Oligosaccharides	2016	Lezyk, M., Jers, C., Kjaerulff, L., Gotfredsen, C. H., Mikkelsen, M. D., & Mikkelsen, J. D. (2016). Novel α-L-fucosidases from a soil metagenome for production of fucosylated human milk oligosaccharides. PLoS ONE, 11(1). https://doi.org/10.1371/journal.pone.0147438	This paper describes the discovery of novel alpha-L-fucosidases and evaluation of their potential to catalyse the transglycosylation reaction leading to production of fucosylated human milk oligosaccharides.
A key genetic factor for fucosyllactose utilization affects infant gut microbiota development.	2016	Matsuki, T., Yahagi, K., Mori, H., Matsumoto, H., Hara, T., Tajima, S., … Kurokawa, K. (2016). A key genetic factor for fucosyllactose utilization affects infant gut microbiota development. Nature Communications, 7. https://doi.org/10.1038/ncomms11939	This study analyzed the gut microbiota develpoment of 27 infants during the first month of life
The human milk oligosaccharide 2'-fucosyllactose augments the adaptive response to extensive intestinal.	2016	Mezoff, E. A., Hawkins, J. A., Ollberding, N. J., Karns, R., Morrow, A. L., & Helmrath, M. A. (2016). The human milk oligosaccharide 2'-fucosyllactose augments the adaptive response to extensive intestinal. American Journal of Physiology - Gastrointestinal and Liver Physiology, 310(6), G427–G438. https://doi.org/10.1152/ajpgi.00305.2015	This study tested the hypothesis that the major noncaloric human milk oligosaccharide 2'-fucosyllactose (2'-FL) contributes to the adaptive response after intestinal resection using a previously described murine model of intestinal adaptation.
Porcine Milk Oligosaccharides and Sialic Acid Concentrations Vary Throughout Lactation	2016	Mudd, A. T., Salcedo, J., Alexander, L. S., Johnson, S. K., Getty, C. M., Chichlowski, M., ... & Dilger, R. N. (2016). Porcine milk oligosaccharides and sialic acid concentrations vary throughout lactation. Frontiers in nutrition, 3, 39.	In this study, we hypothesized that OS and sialic acid (SA) composition of porcine milk would be influenced by stage of lactation.

Title	Year	Citation	Study Objective
Human DC-SIGN binds specific human milk glycans.	2016	Noll, A. J., Yu, Y., Lasanajak, Y., Duska-McEwen, G., Buck, R. H., Smith, D. F., & Cummings, R. D. (2016). Human DC-SIGN binds specific human milk glycans. Biochemical Journal, 473(10), 1343-1353.	Human milk glycans (HMGs) are prebiotics, pathogen receptor decoys and regulators of host physiology and immune responses. Mechanistically, human lectins (glycan-binding proteins, hGBP) expressed by dendritic cells (DCs) are of major interest, as these cells directly contact HMGs. The authors screened many C-type lectins and sialic acid-binding immunoglobulin-like lectins (Siglecs) expressed by DCs for glycan binding on microarrays presenting over 200 HMGs.
Oral supplementation of 2'-fucosyllactose during lactation improves memory and learning in rats.	2016	Oliveros, E., Ramirez, M., Vazquez, E., Barranco, A., Gruart, A., Delgado-Garcia, J. M., ... & Martin, M. J. (2016). Oral supplementation of 2'-fucosyllactose during lactation improves memory and learning in rats. The Journal of nutritional biochemistry, 31, 20-27.	The present study aimed to determine whether oral 2'-FL has an effect on the development of newborn brain, contributing to enhance cognitive skills later in life.
Characterization of porcine milk oligosaccharides during early lactation and their relation to the fecal microbiome	2016	Salcedo, J., Frese, S. A., Mills, D. A., & Barile, D. (2016). Characterization of porcine milk oligosaccharides during early lactation and their relation to the fecal microbiome. Journal of dairy science, 99(10), 7733-7743.	The composition of porcine milk oligosaccharides (PMO) was analyzed during early lactation and their relation to piglet gut microbiome was investigated.
Dietary 2'-fucosyllactose enhances operant conditioning and long-term potentiation via gut-brain communication through the vagus nerve in rodents.	2016	Vazquez, E., Barranco, A., Ramirez, M., Gruart, A., Delgado-Garcia, J. M., Jimenez, M. L., ... & Rueda, R. (2016). Dietary 2'-fucosyllactose enhances operant conditioning and long-term potentiation via gut-brain communication through the vagus nerve in rodents. PloS one, 11(11), e0166070.	This study had two aims: (1) determine if the effect of ingested 2 -FL on the modulation of CNS function is dependent on the integrity of the molecule; and (2) confirm if oral 2 -FL modified hippocampal LTP and associative learning related skills in rats submitted to bilateral subdiaphragmatic vagotomy.

Title	Year	Citation	Study Objective
Structural Basis for Norovirus Inhibition by Human Milk Oligosaccharides.	2016	Weichert, S., Koromyslova, A., Singh, B. K., Hansman, S., Jennewein, S., Schroten, H., & Hansman, G. S. (2016). Structural Basis for Norovirus Inhibition by Human Milk Oligosaccharides. Journal of Virology, 90(9), 4843–4848. https://doi.org/10.1128/jvi.03223-15	The study showed that two human milk oligosaccharides, 2'-fucosyllactose (2'FL) and 3-fucosyllactose (3FL), could block norovirus from binding to surrogate HBGA samples. 2'FL and 3FL bound at the equivalent HBGA pockets on the norovirus capsid using X-ray crystallography.
The human milk oligosaccharide 2’-fucosyllactose quenches campylobacter jejuni–induced inflammation in human epithelial cells HEp-2 and HT-29 and in mouse intestinal mucosa.	2016	Yu, Z. T., Nanthakumar, N. N., & Newburg, D. S. (2016). The human milk oligosaccharide 2’-fucosyllactose quenches campylobacter jejuni–induced inflammation in human epithelial cells HEp-2 and HT-29 and in mouse intestinal mucosa. The Journal of nutrition, 146(10), 1980-1990.	This study investigated the effects of 2'-FL on the cell invasion central to *C. jejuni* pathogenesis by treated human epithelial cells infected with virulent *C. jejuni* strain and the degree of infection and inflammatory response was measured.
Determination and quantification of 2'-O-fucosyllactose and 3-O-fucosyllactose in human milk by GC-MS as O-trimethylsilyl-oxime derivatives	2015	Balogh, R., Szarka, S., & Béni, S. (2015). Determination and quantification of 2’-O-fucosyllactose and 3-O-fucosyllactose in human milk by GC–MS as O-trimethylsilyl-oxime derivatives. Journal of pharmaceutical and biomedical analysis, 115, 450-456.	This study reports a fit for purpose GC-MS method for the quantification of the TMS ether oxime derivatives of 2'-O-fucosyllactose and 3-O-fucosyllactose, the two most abundant trisaccharides in human milk.
Structural characterization of human galectin-4 C-terminal domain: elucidating the molecular basis for recognition of glycosphingolipids, sulfated saccharides and blood group antigens.	2015	Bum-Erdene, K., Leffler, H., Nilsson, U. J., & Blanchard, H. (2015). Structural characterization of human galectin-4 C-terminal domain: elucidating the molecular basis for recognition of glycosphingolipids, sulfated saccharides and blood group antigens. The FEBS journal, 282(17), 3348-3367.	Structural characterization of the complex with 2'-fucosyllactose, a carbohydrate with similarity to the H antigen, and molecular dynamics studies highlight structural features that allow specific recognition of A and B antigens, whilst a lack of interaction with the 2'-fucose of blood group antigens was revealed.
Attenuation of food allergy symptoms following treatment with human milk oligosaccharides in a mouse model	2015	Castillo-Courtade, L., Han, S., Lee, S., Mian, F. M., Buck, R., & Forsythe, P. (2015). Attenuation of food allergy symptoms following treatment with human milk oligosaccharides in a mouse model. Allergy, 70(9), 1091-1102.	The authors assess the effect of two HMOs, 2'-fucosyllactose and 6'-sialyllactose, on symptomatology and immune responses in an ovalbumin-sensitized mouse model of food allergy.

Title	Year	Citation	Study Objective
The sweet branch of metabolic engineering: cherry-picking the low-hanging sugary fruits	2015	Chen, R. (2015, December 9). The sweet branch of metabolic engineering: Cherry-picking the low-hanging sugary fruits. Microbial Cell Factories. BioMed Central Ltd. https://doi.org/10.1186/s12934-015-0389-z	A short review that focuses on the most recent development in metabolic engineering, with emphasis on the synthesis technology for glycoprotein, polysaccharide, and oligosaccharide.
Enhanced production of 2'-fucosyllactose in engineered Escherichia coli BL21star(DE3) by modulation of lactose metabolism and fucosyltransferase	2015	Chin, Y. W., Kim, J. Y., Lee, W. H., & Seo, J. H. (2015). Enhanced production of 2'-fucosyllactose in engineered Escherichia coli BL21star(DE3) by modulation of lactose metabolism and fucosyltransferase. Journal of Biotechnology, 210, 107–115. https://doi.org/10.1016/j.jbiotec.2015.06.431	In this study, E. coli BL21star(DE3) was engineered through deletion of the whole endogenous lactose operon and introduction of the modified lactose operon containing lacZ big up tri, openM15 from E. coli K-12.
Recognition of human milk oligosaccharides by bacterial exotoxins	2015	El-Hawiet, A., Kitova, E. N., & Klassen, J. S. (2015). Recognition of human milk oligosaccharides by bacterial exotoxins. Glycobiology, 25(8), 845-854.	The affinities of the most abundant oligosaccharides found in human milk for four bacterial exotoxins (from Vibrio cholerae and pathogenic Escherichia coli) were quantified for the first time.
Screening of enzymatic synthesis and expression of Lewis determinants in human colorectal carcinoma.	2015	Fernández-Briera, A., Cuevas, E., & Gil-Martín, E. (2015). Screening of enzymatic synthesis and expression of Lewis determinants in human colorectal carcinoma. Revista Española de Enfermedades Digestivas, 107(10), 598-607.	This study is an attempt to characterize the terminal fucosylation steps responsible for the synthesis of mono-Le(a)/Le(x)- and difucosylated -Le(b)/Le(y)- Lewis antigens in healthy and tumour CRC tissue.
In vitro impact of human milk oligosaccharides on Enterobacteriaceae growth	2015	Hoeflinger, J. L., Davis, S. R., Chow, J., & Miller, M. J. (2015). In vitro impact of human milk oligosaccharides on Enterobacteriaceae growth. Journal of agricultural and food chemistry, 63(12), 3295-3302.	In this study, the in vitro growth of purified HMOs and other related carbohydrates was evaluated using individual strains of Enterobacteriaceae and an Enterobacteriaceae consortia enriched from piglet feces.

Title	Year	Citation	Study Objective
Maternal fucosyltransferase 2 status affects the gut bifidobacterial communities of breastfed infants.	2015	Lewis, Z. T., Totten, S. M., Smilowitz, J. T., Popovic, M., Parker, E., Lemay, D. G., … Mills, D. A. (2015). Maternal fucosyltransferase 2 status affects the gut bifidobacterial communities of breastfed infants. Microbiome, 3(1). https://doi.org/10.1186/s40168-015-0071-z	This study investigated the effects of maternal secretor status on the developing infant microbiota with a special emphasis on bifidobacterial species abundance.
Infants fed a lower calorie formula with 2' FL show growth and 2' FL uptake like breast-fed infants.	2015	Marriage, B. J., Buck, R. H., Goehring, K. C., Oliver, J. S., & Williams, J. A. (2015). Infants fed a lower calorie formula with 2' FL show growth and 2' FL uptake like breast-fed infants. Journal of pediatric gastroenterology and nutrition, 61(6), 649.	The aim of the present study was to examine the growth and tolerance of infants fed infant formulas with a caloric density closer to human milk (HM) supplemented with human milk oligosaccharides (HMOs) and to study uptake of the HMOs.
The human milk oligosaccharide 2'-fucosyllactose augments the adaptive response to extensive intestinal.	2015	Mezoff, E. A., Hawkins, J. A., Ollberding, N. J., Karns, R., Morrow, A. L., & Helmrath, M. A. (2015). The human milk oligosaccharide 2'-fucosyllactose augments the adaptive response to extensive intestinal. American Journal of Physiology-Gastrointestinal and Liver Physiology, 310(6), G427-G438.	We tested the hypothesis that the major noncaloric human milk oligosaccharide 2'-fucosyllactose (2'-FL) contributes to the adaptive response after intestinal resection: weight gain, energy availability through microbial community modulation, histological changes consistent with improved adaptation.
Effects of a human milk oligosaccharide, 2'-fucosyllactose, on hippocampal long-term potentiation and learning capabilities in rodents.	2015	Vázquez, E., Barranco, A., Ramírez, M., Gruart, A., Delgado-García, J. M., Martínez-Lara, E., ... & Prieto, P. (2015). Effects of a human milk oligosaccharide, 2'-fucosyllactose, on hippocampal long-term potentiation and learning capabilities in rodents. The Journal of nutritional biochemistry, 26(5), 455-465.	This study evaluated the role of the most abundant HMO, 2'-fucosyllactose (2'-FL), in synaptic plasticity and learning capabilities in rodents.
Fecal microbiota composition of breast-fed infants is correlated with human milk oligosaccharides consumed.	2015	Wang, M., Li, M., Wu, S., Lebrilla, C. B., Chapkin, R. S., Ivanov, I., & Donovan, S. M. (2015). Fecal microbiota composition of breast-fed infants is correlated with human milk oligosaccharides consumed. Journal of pediatric gastroenterology and nutrition, 60(6), 825.	This study tested the hypothesis that the fecal bacterial genera of breast-fed (BF) and formula-fed (FF) infants differ and that human milk oligosaccharides (HMOs) modulate the microbiota of BF infants.

Title	Year	Citation	Study Objective
Structure and substrate specificity of a eukaryotic fucosidase from Fusarium graminearum	2014	Cao, H., Walton, J. D., Brumm, P., & Phillips, G. N. (2014). Structure and substrate specificity of a eukaryotic fucosidase from Fusarium graminearum. Journal of Biological Chemistry, 289(37), 25624-25638.	The secreted glycoside hydrolase family 29 (GH29) alpha-L-fucosidase from plant pathogenic fungus Fusarium graminearum (FgFCO1) actively releases fucose from the xyloglucan fragment. We solved crystal structures of two active-site conformations, i.e. open and closed, of apoFgFCO1 and an open complex with product fucose at atomic resolution.
Fecal metabolomics of healthy breast-fed versus formula-fed infants before and during in vitro batch culture fermentation	2014	Chow, J., Panasevich, M. R., Alexander, D., Vester Boler, B. M., Rossoni Serao, M. C., Faber, T. A., … Fahey, G. C. (2014). Fecal metabolomics of healthy breast-fed versus formula-fed infants before and during in vitro batch culture fermentation. Journal of Proteome Research, 13(5), 2534–2542. https://doi.org/10.1021/pr500011w	Nontargeted metabolomics analyses were used (1) to compare fecal metabolite profiles of healthy breast-fed (BF) and formula-fed (FF) infants before and during in vitro fermentation in batch culture and (2) to evaluate fecal metabolomics in assessing infant diet.
Select human milk oligosaccharides directly modulate peripheral blood mononuclear cells isolated from 10-d-old pigs	2014	Comstock, S. S., Wang, M., Hester, S. N., Li, M., & Donovan, S. M. (2014). Select human milk oligosaccharides directly modulate peripheral blood mononuclear cells isolated from 10-d-old pigs. British Journal of Nutrition, 111(5), 819-828.	The direct effects of human milk oligosaccharides (HMO) on immune cells were analysed ex vivo.
Pre-clinical safety evaluation of the synthetic human milk, nature-identical, oligosaccharide 2'-O-fucosyllactose (2' FL).	2014	Coulet, M., Phothirath, P., Allais, L., & Schilter, B. (2014). Pre-clinical safety evaluation of the synthetic human milk, nature-identical, oligosaccharide 2'-O-fucosyllactose (2' FL). Regulatory Toxicology and Pharmacology, 68(1), 59-69.	To investigate the toxicological profile in a model representative of the intended target population, 2'FL was administered via gavage in a juvenile adapted sub-chronic rat study at dose levels of 0, 2000, 5000 and 6000 mg/kgbw/day

Title	Year	Citation	Study Objective
WbgL: a novel bacterial alpha1,2-fucosyltransferase for the synthesis of 2'-fucosyllactose	2014	Engels, L., & Elling, L. (2014). WbgL: A novel bacterial α1,2-fucosyltransferase for the synthesis of 2'-fucosyllactose. Glycobiology, 24(2), 170–178. https://doi.org/10.1093/glycob/cwt096	The present paper introduces a novel bacterial alpha1,2-FucT of the glycosyltransferase family 11 encoded by the gene wbgL in the E. coli O126 genome, which only displays 25-30% homology to previously published alpha1,2-FucTs. A tailor made cloning and expression strategy allowed the successful production of active soluble enzyme in the cytoplasm of E. coli BL21(DE3) and E. coli JM109(DE3), respectively.
Direct evidence for the presence of human milk oligosaccharides in the circulation of breastfed infants	2014	Goehring, K. C., Kennedy, A. D., Prieto, P. A., & Buck, R. H. (2014). Direct evidence for the presence of human milk oligosaccharides in the circulation of breastfed infants. PLoS One, 9(7), e101692.	The authors determined the relative fractions of several ingested HMOs in infant urine and plasma. Plasma from formula-fed infants was used as a control.
A 3-week pre-clinical study of 2'-fucosyllactose in farm piglets.	2014	Hanlon, P. R., & Thorsrud, B. A. (2014). A 3-week pre-clinical study of 2'-fucosyllactose in farm piglets. Food and Chemical Toxicology, 74, 343–348. https://doi.org/10.1016/j.fct.2014.10.025	A neonatal piglet model was used to evaluate the tolerability of 2'-FL to demonstrate the suitability of this compound for human infants under 12 weeks of age.
Human milk oligosaccharides influence maturation of human intestinal Caco-2Bbe and HT-29 cell lines.	2014	Holscher, H. D., Davis, S. R., & Tappenden, K. A. (2014). Human milk oligosaccharides influence maturation of human intestinal Caco-2Bbe and HT-29 cell lines. The Journal of nutrition, 144(5), 586-591.	The objective of this study was to assess the impact of 3 predominant HMOs on multiple aspects of enterocyte maturation in vitro.
Oligosaccharide composition of breast milk influences survival of uninfected children born to HIV-infected mothers in Lusaka, Zambia.	2014	Kuhn, L., Kim, H. Y., Hsiao, L., Nissan, C., Kankasa, C., Mwiya, M., ... & Bode, L. (2014). Oligosaccharide composition of breast milk influences survival of uninfected children born to HIV-infected mothers in Lusaka, Zambia. The Journal of nutrition, 145(1), 66-72.	Using a nested case-cohort analysis in the context of an early weaning trial, the authors investigated whether HMO composition influences survival to 2 y of age in HIV-infected and HIV-exposed, uninfected (HEU) children during and after breastfeeding.

Title	Year	Citation	Study Objective
Can an ancestral condition for milk oligosaccharides be determined? Evidence from the Tasmanian echidna (Tachyglossus aculeatus setosus)	2014	Oftedal, O. T., Nicol, S. C., Davies, N. W., Sekii, N., Taufik, E., Fukuda, K., … Urashima, T. (2014). Can an ancestral condition for milk oligosaccharides be determined? Evidence from the Tasmanian echidna (Tachyglossus aculeatus setosus). Glycobiology, 24(9), 826–839. https://doi.org/10.1093/glycob/cwu041	Saccharides were characterized from milk of the Tasmanian echidna Tachyglossus aculeatus setosus.
Ultra high performance liquid chromatography–tandem mass spectrometry method for the determination of soluble milk glycans in rat serum	2014	Santos-Fandila, A., Zafra-Gomez, A., Vazquez, E., Navalon, A., Rueda, R., & Ramírez, M. (2014). Ultra high performance liquid chromatography–tandem mass spectrometry method for the determination of soluble milk glycans in rat serum. Talanta, 118, 137-146.	The main objective of the present work was to develop and validate a multicompound method to measure soluble milk glycans (SMGs) in biological fluids such as serum.
Selective proliferation of intestinal Barnesiella under fucosyllactose supplementation in mice.	2014	Weiss, G. A., Chassard, C., & Hennet, T. (2014). Selective proliferation of intestinal Barnesiella under fucosyllactose supplementation in mice. British Journal of Nutrition, 111(9), 1602-1610.	To determine the specific effect of fucosyllactose exposure on intestinal microbiota in mice, in the present study, we orally supplemented newborn mice with pure 2-fucosyllactose and 3-fucosyllactose. Exposure to 2-fucosyllactose and 3-fucosyllactose increased the levels of bacteria of the Porphyromonadaceae family in the intestinal gut, more precisely members of the genus Barnesiella as analysed by 16S pyrosequencing. The ability of Barnesiella to utilise fucosyllactose as energy source was confirmed in bacterial cultures.
Construction of Escherichia coli strains with chromosomally integrated expression cassettes for the synthesis of 2'-fucosyllactose	2013	Baumgärtner, F., Seitz, L., Sprenger, G. A., & Albermann, C. (2013). Construction of Escherichia coli strains with chromosomally integrated expression cassettes for the synthesis of 2'-fucosyllactose. Microbial Cell Factories, 12(1). https://doi.org/10.1186/1475-2859-12-40	The study reports findings of the construction of the first selection marker-free E. coli strain that produces 2'-FL from lactose and glycerol.

Title	Year	Citation	Study Objective
2′-Fucosyllactose: an abundant, genetically determined soluble glycan present in human milk.	2013	Castanys-Muñoz, E., Martin, M. J., & Prieto, P. A. (2013). 2′-Fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. Nutrition reviews, 71(12), 773-789.	This review summarizes the attributes of 2'-FL in terms of its occurrence in mammalian phylogeny, its postulated biological activities, and its variability in human milk.
Human milk oligosaccharides inhibit rotavirus infectivity in vitro and in acutely infected piglets.	2013	Hester, S. N., Chen, X., Li, M., Monaco, M. H., Comstock, S. S., Kuhlenschmidt, T. B., ... & Donovan, S. M. (2013). Human milk oligosaccharides inhibit rotavirus infectivity in vitro and in acutely infected piglets. British Journal of Nutrition, 110(7), 1233-1242.	The anti-RV activity of oligosaccharides was tested in an established in vitro system for assessing cellular binding and viral infectivity/replication, and also tested in a newly developed, acute RV infection, in situ piglet model.
"Amide resonance" in the catalysis of 1,2-alpha-L-fucosidase from Bifidobacterium bifidum.	2013	Liu, J., Zheng, M., Zhang, C., & Xu, D. (2013). Amide resonance in the catalysis of 1,2-α-l-fucosidase from bifidobacterium bifidum. Journal of Physical Chemistry B, 117(35), 10080–10092. https://doi.org/10.1021/jp402110j	We present here the detailed simulation of the enzymatic hydrolysis of 2'-fucosyllactose catalyzed by 1,2-alpha-L-fucosidase from Bifidobacterium bifidum using the combined quantum mechanical and molecular mechanical approach.
The human milk metabolome reveals diverse oligosaccharide profiles.	2013	Smilowitz, J. T., O'sullivan, A., Barile, D., German, J. B., Lönnerdal, B., & Slupsky, C. M. (2013). The Human Milk Metabolome Reveals Diverse Oligosaccharide Profiles. The Journal of Nutrition, 143(11), 1709–1718. https://doi.org/10.3945/jn.113.178772	To determine the effect of maternal phenotype and diet on the human milk metabolome, milk collected at day 90 postpartum from 52 healthy women was analyzed by using proton nuclear magnetic resonance spectroscopy.
Neutral and acidic milk oligosaccharides of the striped skunk (Mephitidae: Mephitis mephitis).	2013	Taufik, E., Sekii, N., Senda, A., Fukuda, K., Saito, T., Eisert, R., ... & Urashima, T. (2013). Neutral and acidic milk oligosaccharides of the striped skunk (M ephitidae: M ephitis mephitis). Animal Science Journal, 84(7), 569-578.	The authors investigated milk oligosaccharides of the striped skunk (Mephitis mephitis) and compared these results to other species of the clade Mustelida.

Title	Year	Citation	Study Objective
In vitro fermentation characteristics of select nondigestible oligosaccharides by infant fecal inocula.	2013	Vester Boler, B. M., Rossoni Serao, M. C., Faber, T. A., Bauer, L. L., Chow, J., Murphy, M. R., & Fahey Jr, G. C. (2013). In vitro fermentation characteristics of select nondigestible oligosaccharides by infant fecal inocula. Journal of agricultural and food chemistry, 61(9), 2109-2119.	This study sought to determine the fermentation potential of human milk oligosaccharides by mixed cultures of fecal microbiota from breast-fed (BF; n = 4) and formula-fed (FF; n = 4) infants.
Bioengineered 2'-fucosyllactose and 3-fucosyllactose inhibit the adhesion of Pseudomonas aeruginosa and enteric pathogens to human intestinal and respiratory cell lines.	2013	Weichert, S., Jennewein, S., Hüfner, E., Weiss, C., Borkowski, J., Putze, J., & Schroten, H. (2013). Bioengineered 2'-fucosyllactose and 3-fucosyllactose inhibit the adhesion of Pseudomonas aeruginosa and enteric pathogens to human intestinal and respiratory cell lines. Nutrition Research, 33(10), 831–838. https://doi.org/10.1016/j.nutres.2013.07.009	The purpose of the study was to evaluate 2'-fucosyllactose (2'-FL) and 3-fucosyllactose (3-FL) synthesized by whole-cell biocatalysis for their biological activity in vitro.
Utilization of major fucosylated and sialylated human milk oligosaccharides by isolated human gut microbes.	2013	Yu, Z. T., Chen, C., & Newburg, D. S. (2013). Utilization of major fucosylated and sialylated human milk oligosaccharides by isolated human gut microbes. Glycobiology, 23(11), 1281–1292. https://doi.org/10.1093/glycob/cwt065	This study assessed the influence of specific HMOS on the growth and metabolic products of individual microbiota bacteria. Most Bifidobacteria spp. and Bacteroides spp. grew, induced α-L-fucosidase activity, and produced abundant lactate or short-chain fatty acids (SCFAs) when fed 2'-fucosyllactose (2'-FL), 3-FL, and lactodifucotetraose (LDFT).
The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota	2013	Yu, Z. T., Chen, C., Kling, D. E., Liu, B., McCoy, J. M., Merighi, M., … Newburg, D. S. (2013). The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. Glycobiology, 23(2), 169–177. https://doi.org/10.1093/glycob/cws138	This study assessed major human milk oligosaccharides (HMOS) for their ability to promote growth of bifidobacteria and to acidify their environment, key features of prebiotics.

Title	Year	Citation	Study Objective
Functional in vivo delivery of multiplexed anti-HIV-1 siRNAs via a chemically synthesized aptamer with a sticky bridge.	2013	Zhou, J., Neff, C. P., Swiderski, P., Li, H., Smith, D. D., Aboellail, T., ... & Rossi, J. J. (2013). Functional in vivo delivery of multiplexed anti-HIV-1 siRNAs via a chemically synthesized aptamer with a sticky bridge. Molecular Therapy, 21(1), 192-200.	The authors chemically synthesized the gp120 aptamer with a 3' 7-carbon linker (7C3), which in turn is attached to a 16-nucleotide 2' OMe/2' Fl GC-rich bridge sequence.
Lacto-N-tetraose, fucosylation, and secretor status are highly variable in human milk oligosaccharides from women delivering preterm	2012	De Leoz, M. L. A., Gaerlan, S. C., Strum, J. S., Dimapasoc, L. M., Mirmiran, M., Tancredi, D. J., … Underwood, M. A. (2012). Lacto-N-tetraose, fucosylation, and secretor status are highly variable in human milk oligosaccharides from women delivering preterm. Journal of Proteome Research, 11(9), 4662–4672. https://doi.org/10.1021/pr3004979	In this study the authors present the first detailed mass spectrometric analysis of the fucosylation and sialylation in HMOs in serial specimens of milk from 15 women delivering preterm and 7 women delivering at term using nanohigh performance liquid chromatography chip/time-of-flight mass spectrometry.
Whole cell biosynthesis of a functional oligosaccharide, 2'-fucosyllactose, using engineered Escherichia coli	2012	Lee, W. H., Pathanibul, P., Quarterman, J., Jo, J. H., Han, N. S., Miller, M. J., … Seo, J. H. (2012). Whole cell biosynthesis of a functional oligosaccharide, 2'-fucosyllactose, using engineered Escherichia coli. Microbial Cell Factories, 11.	The study was designed to construct a 2-FL producing Escherichia coli through overexpressing genes coding for endogenous GDP- l-fucose biosynthetic enzymes and heterologous fucosyltransferase.
Structural characterization of neutral and acidic oligosaccharides in the milks of strepsirrhine primates: greater galago, aye-aye, Coquerel's sifaka and mongoose lemur.	2012	Taufik, E., Fukuda, K., Senda, A., Saito, T., Williams, C., Tilden, C., … Urashima, T. (2012). Structural characterization of neutral and acidic oligosaccharides in the milks of strepsirrhine primates: Greater galago, aye-aye, Coquerel's sifaka and mongoose lemur. Glycoconjugate Journal, 29(2–3), 119–134. https://doi.org/10.1007/s10719-012-9370-9	The structures of milk oligosaccharides were characterized for four strepsirrhine primates to examine the extent to which they resemble milk oligosaccharides in other primates.
Comprehensive profiles of human milk oligosaccharides yield highly sensitive and specific markers for determining secretor status in lactating mothers	2012	Totten, S. M., Zivkovic, A. M., Wu, S., Ngyuen, U., Freeman, S. L., Ruhaak, L. R., … Lebrilla, C. B. (2012). Comprehensive profiles of human milk oligosaccharides yield highly sensitive and specific markers for determining secretor status in lactating mothers. Journal of Proteome Research, 11(12), 6124–6133. https://doi.org/10.1021/pr300769g	In this study, HMOs were extracted from the milk of 60 women from The Gambia, Africa with various Lewis and secretor blood types.

Title	Year	Citation	Study Objective
The predominance of type I oligosaccharides is a feature specific to human breast milk.	2012	Urashima, T., Asakuma, S., Leo, F., Fukuda, K., Messer, M., & Oftedal, O. T. (2012). The Predominance of Type I Oligosaccharides Is a Feature Specific to Human Breast Milk. Advances in Nutrition, 3(3), 473S-482S. https://doi.org/10.3945/an.111.001412	The chemical structures of >100 human milk oligosaccharides (HMO) have been characterized to date. The authors determined the concentrations of 10 neutral and 9 acidic colostrum HMO collected during the first 3 d of lactation by using reverse phase HPLC after derivatization with 2-aminopyridine or 1-methyl-3-phenyl-5-pyrazolon.
Bi- to tetravalent glycoclusters: synthesis, structure-activity profiles as lectin inhibitors and impact of combining both valency and headgroup tailoring on selectivity.	2012	Wang, G. N., André, S., Gabius, H. J., & Murphy, P. V. (2012). Bi-to tetravalent glycoclusters: synthesis, structure–activity profiles as lectin inhibitors and impact of combining both valency and headgroup tailoring on selectivity. Organic & biomolecular chemistry, 10(34), 6893-6907.	We herein tested a panel of bi-, tri- and tetravalent compounds against two plant agglutinins and adhesion/growth-regulatory lectins (galectins).
Development of biosensor-based assays to identify anti-infective oligosaccharides.	2011	Lane, J. A., Mehra, R. K., Carrington, S. D., & Hickey, R. M. (2011). Development of biosensor-based assays to identify anti-infective oligosaccharides. Analytical biochemistry, 410(2), 200-205.	This study describes a number of biosensor-based methods to achieve a quick and sensitive method for detecting the binding of microorganisms to milk oligosaccharides
Utilization of natural fucosylated oligosaccharides by three novel α-l-fucosidases from a probiotic Lactobacillus casei strain.	2011	Rodríguez-Díaz, J., Monedero, V., & Yebra, M. J. (2011). Utilization of natural fucosylated oligosaccharides by three novel α-l-fucosidases from a probiotic Lactobacillus casei strain. Appl. Environ. Microbiol., 77(2), 703-705.	Three putative alpha-L-fucosidases encoded in the Lactobacillus casei BL23 genome were cloned and purified. The proteins displayed different abilities to hydrolyze natural fucosyloligosaccharides like 2'-fucosyllactose.

Title	Year	Citation	Study Objective
Determination of sialyl and neutral oligosaccharide levels in transition and mature milks of Samoan women, using anthranilic derivatization followed by reverse phase high performance liquid chromatography.	2010	Leo, F., Asakuma, S., Fukuda, K., Senda, A., & Urashima, T. (2010). Determination of sialyl and neutral oligosaccharide levels in transition and mature milks of samoan women, using anthranilic derivatization followed by reverse phase high performance liquid chromatography. Bioscience, Biotechnology and Biochemistry, 74(2), 298–303. https://doi.org/10.1271/bbb.90614	An improved analytical method using reverse-phase high performance liquid chromatography following anthranilic acid derivatization for the measurement of each oligosaccharide level in transition (5 to 10 d lactation) and mature (21 to 155 d lactation) milks of sixteen Samoan women is described. The method was applied for the measurement of sialyl as well as neutral oligosaccharide levels.
Chemical characterization of milk oligosaccharides of an African lion (Panthera leo) and a clouded leopard (Neofelis nebulosa)	2010	Senda, A., Hatakeyama, E., Kobayashi, R., Fukuda, K., Uemura, Y., Saito, T., … Urashima, T. (2010). Chemical characterization of milk oligosaccharides of an African lion (Panthera leo) and a clouded leopard (Neofelis nebulosa). Animal Science Journal, 81(6), 687–693. https://doi.org/10.1111/j.1740-0929.2010.00787.x	In this study, lactose was found to be the dominant saccharide in the milk or colostrum of two species of Feloidea, namely the African lion (Panthera leo) and the clouded leopard (Neofelis nebulosa).
Two distinct alpha-L-fucosidases from Bifidobacterium bifidum are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates.	2009	Ashida, H., Miyake, A., Kiyohara, M., Wada, J., Yoshida, E., Kumagai, H., … Yamamoto, K. (2009). Two distinct α-L-fucosidases from Bifidobacterium bifidum are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates. Glycobiology, 19(9), 1010–1017. https://doi.org/10.1093/glycob/cwp082	This study identified two genes from a bifidobacterium bifidum that utilizes 2'-FL
Improved determination of milk oligosaccharides using a single derivatization with anthranilic acid and separation by reversed-phase high-performance liquid chromatography	2009	Leo, F., Asakuma, S., Nakamura, T., Fukuda, K., Senda, A., & Urashima, T. (2009). Improved determination of milk oligosaccharides using a single derivatization with anthranilic acid and separation by reversed-phase high-performance liquid chromatography. Journal of Chromatography A, 1216(9), 1520-1523.	An improved analytical scheme for human milk neutral oligosaccharides determination was developed, in which, the oligosaccharides were pooled in two fractions (pools 1 and 2) after gel filtration, and then were quantitatively derivatized with a single fluorescent reagent, 2-anthranilic acid.

Title	Year	Citation	Study Objective
Multivalent human blood group ABH and Lewis glycotopes are key recognition factors for a Lfuc>Man binding lectin from phytopathogenic Ralstonia solanacearum.	2009	Wu, A. M., Wu, J. H., Singh, T., Singha, B., Sudakevitz, D., & Gilboa-Garber, N. (2009). Multivalent human blood group ABH and Lewis glycotopes are key recognition factors for a LFuc> Man binding lectin from phytopathogenic Ralstonia solanacearum. Biochimica et Biophysica Acta (BBA)-General Subjects, 1790(4), 249-259.	In this study, recognition factors of RSL were comprehensively examined with natural multivalent glycotopes and monomeric ligands using enzyme linked lectin-sorbent and inhibition assays.
Fucosylated glycan inhibition of human hepatocellular carcinoma cell migration through binding to chemokine receptors.	2009	Wu, L. H., Shi, B. Z., Zhao, Q. L., & Wu, X. Z. (2009). Fucosylated glycan inhibition of human hepatocellular carcinoma cell migration through binding to chemokine receptors. Glycobiology, 20(2), 215-223.	SMMC-7721 hepatocellular carcinoma cells (HCC) were incubated with fucosylated glycoproteins that had been isolated from retinoic acid-treated cells by affinity chromatography. HCC migration was significantly inhibited by AAL- and LCA-glycoproteins.
Variation of major neutral oligosaccharides levels in human colostrum	2008	Asakuma, S., Urashima, T., Akahori, M., Obayashi, H., Nakamura, T., Kimura, K., … Sanai, Y. (2008). Variation of major neutral oligosaccharides levels in human colostrum. European Journal of Clinical Nutrition, 62(4), 488–494. https://doi.org/10.1038/sj.ejcn.1602738	The aim of this present study was to determine the concentration of each of the major neutral oligosaccharide for three consecutive days from the start of lactation.
1,2-alpha-l-Fucosynthase: a glycosynthase derived from an inverting alpha-glycosidase with an unusual reaction mechanism.	2008	Wada, J., Honda, Y., Nagae, M., Kato, R., Wakatsuki, S., Katayama, T., ... & Yamamoto, K. (2008). 1, 2-α-l-Fucosynthase: A glycosynthase derived from an inverting α-glycosidase with an unusual reaction mechanism. FEBS letters, 582(27), 3739-3743.	The authors present a new route for synthesizing a Fucalpha1,2Gal linkage by introducing glycosynthase technology into 1,2-alpha-l-fucosidase.
Structural basis of the catalytic reaction mechanism of novel 1,2-alpha-L-fucosidase from Bifidobacterium bifidum.	2007	Nagae, M., Tsuchiya, A., Katayama, T., Yamamoto, K., Wakatsuki, S., & Kato, R. (2007). Structural basis of the catalytic reaction mechanism of novel 1,2-α-L-fucosidase from Bifidobacterium bifidum. Journal of Biological Chemistry, 282(25), 18497–18509. https://doi.org/10.1074/jbc.M702246200	This study identified x-ray crystal structures of the AfcA catalytic (Fuc) domain in unliganded and complexed forms with deoxyfuconojirimycin (inhibitor), 2'-fucosyllactose (substrate), and L-fucose and lactose (products) at 1.12-2.10 Å resolution.

Title	Year	Citation	Study Objective
Oligosaccharides in colostrum of Italian and Burkinabe women	2006	Musumeci, M., Simpore, J., D'Agata, A., Sotgiu, S., & Musumeci, S. (2006). Oligosaccharides in colostrum of Italian and Burkinabe women. Journal of Pediatric Gastroenterology and Nutrition, 43(3), 372–378. https://doi.org/10.1097/01.mpg.0000228125.70971.af	Oligosaccharides were identified and characterized in the colostrum from 53 Burkinabe women and 50 Italian women that spontaneously delivered at term.
Immunomodulation of fucosyl-lactose and lacto-N-fucopentaose on mononuclear cells from multiple sclerosis and healthy subjects.	2006	Sotgiu, S., Arru, G., Fois, M. L., Sanna, A., Musumeci, M., Rosati, G., & Musumeci, S. (2006). Immunomodulation of fucosyl-lactose and lacto-N-fucopentaose on mononuclear cells from multiple sclerosis and healthy subjects. International journal of biomedical science: IJBS, 2(2), 114.	The authors investigated the possible immune function of human 2'-FL and LNFP-I in vitro on LPS-activated mononuclear cells (MNC) from 12 patients with multiple sclerosis (MS) and 20 matched health controls (HC).
Interactions of the fucose-specific Pseudomonas aeruginosa lectin, PA-IIL, with mammalian glycoconjugates bearing polyvalent Lewisa and ABH blood group glycotopes.	2006	Wu, A. M., Wu, J. H., Singh, T., Liu, J. H., Tsai, M. S., & Gilboa-Garber, N. (2006). Interactions of the fucose-specific Pseudomonas aeruginosa lectin, PA-IIL, with mammalian glycoconjugates bearing polyvalent Lewisa and ABH blood group glycotopes. Biochimie, 88(10), 1479-1492.	In order to delineate the structures of these lectin receptors, its detailed carbohydrate recognition profile was studied both by microtiter plate biotin/avidin-mediated enzyme-lectin-glycan binding assay (ELLSA) and by inhibition of the lectin-glycan interaction.
An unusual anti-H lectin inhibited by milk from individuals with the Bombay phenotype.	2005	Joshi, S. R., Vasantha, K., & Robb, J. S. (2005). An unusual anti-H lectin inhibited by milk from individuals with the Bombay phenotype. Immunohematology, 21(1), 1-4.	An anti-H lectin, extracted from the seeds of the plant Momordica dioica Roxb. ex willd., was tested for its hemagglutination and inhibition properties, using standard serologic methods and panel RBCs, serum, saliva, milk, and oligosaccharides purified from milk.
The fucose-binding lectin from Ralstonia solanacearum. A new type of beta-propeller architecture formed by oligomerization and interacting with fucoside, fucosyllactose, and plant xyloglucan.	2005	Kostlánová, N., Mitchell, E. P., Lortat-Jacob, H., Oscarson, S., Lahmann, M., Gilboa-Garber, N., ... & Imberty, A. (2005). The Fucose-binding Lectin from Ralstonia solanacearum A NEW TYPE OF β-PROPELLER ARCHITECTURE FORMED BY OLIGOMERIZATION AND INTERACTING WITH FUCOSIDE, FUCOSYLLACTOSE, AND PLANT XYLOGLUCAN. Journal of Biological Chemistry, 280(30), 27839-27849.	In the present study, surface plasmon resonance experiments conducted on a series of oligosaccharides show a preference for binding to alphaFuc1-2Gal and alphaFuc1-6Gal epitopes.

Title	Year	Citation	Study Objective
Purification and characterization of extracellular 1,2-alpha-L-fucosidase from Bacillus cereus.	2005	Miura, T., Okamoto, K., & Yanase, H. (2005). Purification and characterization of extracellular 1, 2-α-L-fucosidase from Bacillus cereus. Journal of bioscience and bioengineering, 99(6), 629-635.	Bacillus cereus isolated from a soil sample, inductively produced alpha-L-fucosidase in culture medium containing porcine gastric mucin (PGM).
Molecular cloning and characterization of Bifidobacterium bifidum 1,2-alpha-L-fucosidase (AfcA), a novel inverting glycosidase (glycoside hydrolase family 95).	2004	Katayama, T., Sakuma, A., Kimura, T., Makimura, Y., Hiratake, J., Sakata, K., … Yamamoto, K. (2004). Molecular cloning and characterization of Bifidobacterium bifidum 1,2-α-L-fucosidase (AfcA), a novel inverting glycosidase (glycoside hydrolase family 95). Journal of Bacteriology, 186(15), 4885–4893. https://doi.org/10.1128/JB.186.15.4885-4893.2004	A genomic library of Bifidobacterium bifidum constructed in Escherichia coli was screened for the ability to hydrolyze the alpha-(1-->2) linkage of 2'-fucosyllactose, and a gene encoding 1,2-alpha-l-fucosidase (AfcA) was isolated.
Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants.	2004	Morrow, A. L., Ruiz-Palacios, G. M., Altaye, M., Jiang, X., Guerrero, M. L., Meinzen-Derr, J. K., ... & Newburg, D. S. (2004). Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants. The Journal of pediatrics, 145(3), 297-303.	To determine the association between maternal milk levels of 2-linked fucosylated oligosaccharide and prevention of diarrhea as a result of Campylobacter, caliciviruses, and diarrhea of all causes in breast-fed infants.
Crystal structures of Erythrina cristagalli lectin with bound N-linked oligosaccharide and lactose.	2004	Turton, K., Natesh, R., Thiyagarajan, N., Chaddock, J. A., & Acharya, K. R. (2004). Crystal structures of Erythrina cristagalli lectin with bound N-linked oligosaccharide and lactose. Glycobiology, 14(10), 923-929.	This study reports the crystal structures of native and recombinant forms of the lectin in three new crystal forms, both unliganded and in complex with lactose.
Characterization of oligosaccharides in milk of bearded seal (Erignathus barbatus).	2004	Urashima, T., Nakamura, T., Nakagawa, D., Noda, M., Arai, I., Saito, T., ... & Kovacs, K. M. (2004). Characterization of oligosaccharides in milk of bearded seal (Erignathus barbatus). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 138(1), 1-18.	Carbohydrates were extracted from milk of a bearded seal, Erignathus barbatus (Family Phocidae and oligosaccharide structures were determined by 1H-NMR spectroscopy.

Title	Year	Citation	Study Objective
Lectinochemical studies on the affinity of Anguilla anguilla agglutinin for mammalian glycotopes.	2004	Wu, A. M., Wu, J. H., Singh, T., Liu, J. H., & Herp, A. (2004). Lectinochemical studies on the affinity of Anguilla anguilla agglutinin for mammalian glycotopes. Life sciences, 75(9), 1085-1103.	The authors analyzed the detailed carbohydrate specificity of AAA by enzyme-linked lectinosorbent assay (ELLSA) with an extended glycan/ligand collection and lectin-glycan inhibition assay.
Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants	2003	Newburg, D. S., Ruiz-Palacios, G. M., Altaye, M., Chaturvedi, P., Meinzen-Derr, J., Guerrero, M. D. L., & Morrow, A. L. (2003). Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. Glycobiology, 14(3), 253-263.	To test the hypothesis that human milk fucosyloligosaccharides are part of an innate immune system, the authors addressed whether their expression (1) depends on maternal genotype and (2) protects breastfed infants from pathogens.
Studies of oligosaccharide specificity of perch roe fucolectin, using gold labeled neoglycoproteins.	2003	Piskarev, V. E., Evstigneeva, R. P., & Iamskov, I. A. (2003). Studies of oligosaccharide specificity of perch roe fucolectin, using gold labeled neoglycoproteins. Prikladnaia biokhimiia i mikrobiologiia, 39(1), 105-109.	The specificity of perch (Perca fluviatilis) roe fucolectin was studied using the protein dot blot technique, followed by detection with colloidal gold-labeled neoglycoproteins bearing human milk polysaccharides.
Determination of each neutral oligosaccharide in the milk of Japanese women during the course of lactation	2003	Sumiyoshi, W., Urashima, T., Nakamura, T., Arai, I., Saito, T., Tsumura, N., … Kimura, K. (2003). Determination of each neutral oligosaccharide in the milk of Japanese women during the course of lactation. British Journal of Nutrition, 89(1), 61–69. https://doi.org/10.1079/bjn2002746	Using reverse-phase HPLC after pyridylamination, the concentrations of major neutral oligosaccharides were quantified in the milk of sixteen Japanese women collected at 4, 10, 30 and 100 d postpartum.
Differences in oligosaccharide pattern of a sample of polar bear colostrum and mid-lactation milk	2003	Urashima, T., Nagata, H., Nakamura, T., Arai, I., Saito, T., Imazu, K., ... & Wiig, O. (2003). Differences in oligosaccharide pattern of a sample of polar bear colostrum and mid-lactation milk. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 136(4), 887-896.	Although the concentrations of carbohydrate in the colostrum and in the mid-lactation milk of polar bear (Ursus maritimus) were similar, the oligosaccharide patterns differed.

Title	Year	Citation	Study Objective
Chemical characterization of the oligosaccharides in milk of high Arctic harbour seal (Phoca vitulina vitulina)	2003	Urashima, T., Nakamura, T., Yamaguchi, K., Munakata, J., Arai, I., Saito, T., … Kovacs, K. M. (2003). Chemical characterization of the oligosaccharides in milk of high Arctic harbour seal (Phoca vitulina vitulina). Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology, 135(4), 549–563. https://doi.org/10.1016/S1095-6433(03)00130-2	Carbohydrates were extracted from high Arctic harbour seal milk, Phoca vitulina vitulina (family Phocidae). Free neutral oligosaccharides were separated by gel filtration and preparative thin layer chromatography, while free sialyl oligosaccharides were separated by gel filtration and then purified by ion exchange chromatography, gel filtration and high performance liquid chromatography.
Cloning of a rat gene encoding the histo-blood group A enzyme: Tissue expression of the gene and of the A and B antigens.	2002	Cailleau-Thomas, A., Le Moullac-Vaidye, B., Rocher, J., Bouhours, D., Szpirer, C., & Le Pendu, J. (2002). Cloning of a rat gene encoding the histo-blood group A enzyme: Tissue expression of the gene and of the A and B antigens. European journal of biochemistry, 269(16), 4040-4047.	The complete coding sequence of a BDIX rat gene homologous to the human ABO gene was determined.
High-resolution crystal structures of Erythrina cristagalli lectin in complex with lactose and 2'-alpha-L-fucosyllactose and correlation with thermodynamic binding data.	2002	Svensson, C., Teneberg, S., Nilsson, C. L., Kjellberg, A., Schwarz, F. P., Sharon, N., & Krengel, U. (2002). High-resolution crystal structures of Erythrina cristagalli lectin in complex with lactose and 2′-α-L-fucosyllactose and correlation with thermodynamic binding data. Journal of molecular biology, 321(1), 69-83.	The primary sequence of Erythrina cristagalli lectin (ECL) was mapped by mass spectrometry, and the crystal structures of the lectin in complex with lactose and 2'-alpha-L-fucosyllactose were determined at 1.6A and 1.7A resolution, respectively

Title	Year	Citation	Study Objective
Chemical characterization of the oligosaccharides in beluga (Delphinapterus leucas) and Minke whale (Balaenoptera acutorostrata) milk.	2002	Urashima, T., Sato, H., Munakata, J., Nakamura, T., Arai, I., Saito, T., ... & Lydersen, C. (2002). Chemical characterization of the oligosaccharides in beluga (Delphinapterus leucas) and Minke whale (Balaenoptera acutorostrata) milk. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 132(3), 611-624.	Carbohydrates were extracted from the milk of a beluga, Delphinopterus leucas (family Odontoceti), and two Minke whales, Balaenoptera acutorostrata (Family Mysticeti), sampled late in their respective lactation periods. Free oligosaccharides were separated by gel filtration and then neutral oligosaccharides were purified by preparative thin layer chromatography and gel filtration, while acidic oligosaccharides were purified by ion-exchange chromatography, gel filtration and high performance liquid chromatography (HPLC).
Synthesis of the milk oligosaccharide 2'-fucosyllactose using recombinant bacterial enzymes	2001	Albermann, C., Piepersberg, W., & Wehmeier, U. F. (2001). Synthesis of the milk oligosaccharide 2'-fucosyllactose using recombinant bacterial enzymes. Carbohydrate research, 334(2), 97-103.	The enzymatic synthesis of GDP-beta-L-fucose and its enzymatic transfer reaction using recombinant enzymes from bacterial sources was examined.
Structural characterization of fucose-containing oligosaccharides by high-performance liquid chromatography and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry	2001	Suzuki, M., & Suzuki, A. (2001). Structural characterization of fucose-containing oligosaccharides by high-performance liquid chromatography and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Biological chemistry, 382(2), 251-257.	Eight pyridylamino (PA) derivatives of fucose-containing oligosaccharides, which occur as free oligosaccharides in human milk and also are derived from glycosphingolipids, have been analyzed by high-performance liquid chromatography (HPLC) on normal-phase and reversed-phase columns, and by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Title	Year	Citation	Study Objective
Chemical characterisation of the oligosaccharides in hooded seal (Cystophora cristata) and Australian fur seal (Arctocephalus pusillus doriferus) milk	2001	Urashima, T., Arita, M., Yoshida, M., Nakamura, T., Arai, I., Saito, T., ... & Lydersen, C. (2001). Chemical characterisation of the oligosaccharides in hooded seal (Cystophora cristata) and Australian fur seal (Arctocephalus pusillus doriferus) milk. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 128(2), 307-323.	Carbohydrates were extracted from hooded seal milk, free oligosaccharides were separated by gel filtration and then purified by ion exchange chromatography, gel filtration and preparative thin layer or paper chromatography and their structures determined by 1H-NMR.
Comparison of oligosaccharides in milk specimens from humans and twelve other species	2001	Warren, C. D., Chaturvedi, P., Newburg, A. R., Oftedal, O. T., Tilde, C. D., & Newburg, D. S. (2001). Comparison of oligosaccharides in milk specimens from humans and twelve other species. In Bioactive components of human milk (pp. 325-332). Springer, Boston, MA.	In this study a comparison was made of the major individual oligosaccharides in milk specimens from a variety of species, including the great apes.
Variability of human milk neutral oligosaccharides in a diverse population	2000	Erney, R. M., Malone, W. T., Skelding, M. B., Marcon, A. A., Kleman–Leyer, K. M., O'Ryan, M. L., ... & Prieto, P. A. (2000). Variability of human milk neutral oligosaccharides in a diverse population. Journal of pediatric gastroenterology and nutrition, 30(2), 181-192.	This report describes a study in which a large number of human milk samples were analyzed for the presence and content of nine neutral oligosaccharides. The resultant data were used to probe for distribution trends by donor groups and stage of lactation.
A lectin from an edible mushroom Pleurotus ostreatus as a food intake-suppressing substance.	2000	Kawagishi, H., Suzuki, H., Watanabe, H., Nakamura, H., Sekiguchi, T., Murata, T., ... & Ito, K. (2000). A lectin from an edible mushroom Pleurotus ostreatus as a food intake-suppressing substance. Biochimica et Biophysica Acta (BBA)-General Subjects, 1474(3), 299-308.	In hemagglutination inhibition assays, Me-alphaGalNAc was the most potent inhibitor among the monosaccharides tested. Among all the sugars tested, 2'-fucosyllactose (Fucalpha1-->2Galbeta1-->4Glc) was the strongest inhibitor and its inhibitory potency was five times greater than that of Me-alphaGalNAc.

Title	Year	Citation	Study Objective
Structural basis of carbohydrate recognition by lectin II from Ulex europaeus, a protein with a promiscuous carbohydrate-binding site.	2000	Loris, R., De Greve, H., Dao-Thi, M. H., Messens, J., Imberty, A., & Wyns, L. (2000). Structural basis of carbohydrate recognition by lectin II from Ulex europaeus, a protein with a promiscuous carbohydrate-binding site. Journal of molecular biology, 301(4), 987-1002.	This study determined the structural basis of carbohydrate recognition by lectin II from Ulex europaeus.
Chemical characterization of milk oligosaccharides of the polar bear, Ursus maritimus	2000	Urashima, T., Yamashita, T., Nakamura, T., Arai, I., Saito, T., Derocher, A. E., & Wiig, Ø. (2000). Chemical characterization of milk oligosaccharides of the polar bear, Ursus maritimus. Biochimica et Biophysica Acta - General Subjects, 1475(3), 395–408. https://doi.org/10.1016/S0304-4165(00)00103-3	Two trisaccharides, three tetrasaccharides, two pentasaccharides, one hexasaccharide, one heptasaccharide, one octasaccharide and one decasaccharide were isolated from polar bear milk samples by chloroform/methanol extraction, gel filtration, ion exchange chromatography and preparative thin-layer chromatography. The oligosaccharides were characterized by 1H-NMR.
Occurrence of an unusual lactose sulfate in dog milk.	1999	Bubb, W. A., Urashima, T., Kohso, K., Nakamura, T., Arai, I., & Saito, T. (1999). Occurrence of an unusual lactose sulfate in dog milk. Carbohydrate research, 318(1-4), 123-128.	The milk of a beagle dog (Canis familiaris) was extracted and fractionated to yield, inter alia, beta-D-Galp3S-(1-->4)-D-Glc (lactose 3'-sulfate).
Isolation and characterization from porcine serum of a soluble sulfotransferase responsible for 6-O-sulfation of the galactose residue in 2′-fucosyllactose: Implications in the synthesis of the ligand for L-selectin.	1999	Huynh, Q. K., Shailubhai, K., Boddupalli, H., Hong, H. Y., Broschat, K. O., & Jacob, G. S. (1999). Isolation and characterization from porcine serum of a soluble sulfotransferase responsible for 6-O-sulfation of the galactose residue in 2′-fucosyllactose: Implications in the synthesis of the ligand for L-selectin. Glycoconjugate journal, 16(7), 357-363.	A soluble sulfotransferase from porcine serum which catalyzes the transfer of sulfate from adenosine 3'-phosphate 5'-phosphosulphate (PAPS) to 2'-fucosyllactose (2'-FL) was purified 36,333-fold using a combination of conventional and affinity chromatographic steps.

Title	Year	Citation	Study Objective
Purification and characterization of a lymph node sulfotransferase responsible for 6-O-sulfation of the galactose residues in 2’-fucosyllactose and other sialyl LewisX-related sugars.	1999	Shailubhai, K., Huynh, Q. K., Boddupalli, H., Hong, H. Y., & Jacob, G. S. (1999). Purification and characterization of a lymph node sulfotransferase responsible for 6-O-sulfation of the galactose residues in 2’-fucosyllactose and other sialyl LewisX-related sugars. Biochemical and biophysical research communications, 256(1), 170-176.	A microsomal galactose-6-O-sulfotransferase (Gal-6-O-Stase) from porcine lymph nodes, able to transfer the sulfate group from adenosine 3'-phosphate 5'-phosphosulphate (PAPS) onto 2'-fucosyllactose (2'-FL) and other sialyl LewisX (sLex)-related sugars, has been purified and characterized.
Chemical characterization of milk oligosaccharides of the Japanese black bear, Ursus thibetanus japonicus	1999	Urashima, T., Sumiyoshi, W., Nakamura, T., Arai, I., Saito, T., Komatsu, T., & Tsubota, T. (1999). Chemical characterization of milk oligosaccharides of the Japanese black bear, Ursus thibetanus japonicus. Biochimica et Biophysica Acta - General Subjects, 1472(1–2), 290–306. https://doi.org/10.1016/S0304-4165(99)00134-8	Two trisaccharides, two tetrasaccharides, one penta-, one hexa-, two hepta-, one deca- and two undeca-saccharides were isolated from several Japanese black bear milk samples by chloroform/methanol extraction, gel filtration and preparative thin-layer chromatography and characterized.
Identification of fucose alpha(1-2) galactose epitope-containing glycoproteins from rat hippocampus.	1998	Smalla, K. H., Angenstein, F., Richter, K., Gundelfinger, E. D., & Staak, S. (1998). Identification of fucose α (1-2) galactose epitope-containing glycoproteins from rat hippocampus. Neuroreport, 9(5), 813-817.	The stuy authors raised antibodies against the plasticity-relevant fucalpha(1-2)gal epitope and used them to determine the distribution of the epitope in adult rat hippocampus. To identify proteins bearing fucalpha(1-2)gal glycostructures antibodies against known synaptic fucoglycoproteins were used in combination with the fucalpha(1-2)gal antibodies.

Title	Year	Citation	Study Objective
Milk oligosaccharide profiles by reversed-phase HPLC of their perbenzoylated derivatives	1997	Chaturvedi, P., Warren, C. D., Ruiz-Palacios, G. M., Pickering, L. K., & Newburg, D. S. (1997). Milk oligosaccharide profiles by reversed-phase HPLC of their perbenzoylated derivatives. Analytical biochemistry, 251(1), 89-97.	To investigate qualitative and quantitative individual variation of human milk oligosaccharides, a sensitive method for routine identification and quantification of intact milk oligosaccharides was developed and applied to milk samples from 50 donors.
Localized agglutinin staining in muscle capillaries from normal and very old atrophic human muscle using winged bean (Psophocarpus tetragonolobus) lectin	1997	Kirkeby, S., Singha, N. C., & Surolia, A. (1997). Localized agglutinin staining in muscle capillaries from normal and very old atrophic human muscle using winged bean (Psophocarpus tetragonolobus) lectin. Histochemistry and cell biology, 107(1), 31-37.	WBA I staining was inhibited by p-nitrophenyl alpha-galactopyranoside and N-acetylgalactosamine, whereas 2'-fucosyllactose and preincubation with an antibody against type-1 chain H abolished capillary staining with WBA II. The study demonstrates the usefulness of WBA as a marker of capillaries in human muscle.
Multiple fucosyltransferases and their carbohydrate ligands are involved in spermatogenic cell-Sertoli cell adhesion in vitro in rats	1997	Raychoudhury, S. S., & Millette, C. F. (1997). Multiple Fucosyltransferases and their Carbohydrate Ligands are Involved in Spermatogenic Cell-Sertoli Cell Adhesion in Vitro in Rats1. Biology of Reproduction, 56(5), 1268–1273. https://doi.org/10.1095/biolreprod56.5.1268	Using fluorescence laser scanning cytometry, the study reports that multiple fucosyltransferases are implicated in germ cell-Sertoli cell adhesion in vitro.
Identification of 2'-fucosyllactose in milk of the crabeater seal (Lobodon carcinophagus)	1997	Urashima, T., Hiramatsu, Y., Murata, S., Nakamura, T., & Messer, M. (1997). Identification of 2′-fucosyllactose in milk of the crabeater seal (Lobodon carcinophagus). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 116(3), 311-314.	This work represents the first identification of an oligosaccharide, other than lactose, in the milk of a pinniped.

Title	Year	Citation	Study Objective
Immobilized Lotus tetragonolobus agglutinin binds oligosaccharides containing the Le(x) determinant.	1997	Yan, L., Wilkins, P. P., Alvarez-Manilla, G., Do, S. I., Smith, D. F., & Cummings, R. D. (1997). Immobilized Lotus tetragonolobus agglutinin binds oligosaccharides containing the Lex determinant. Glycoconjugate journal, 14(1), 45-55.	A defined set of oligosaccharides and glycopeptides containing alpha-linked fucose were used to examine the specificity of the immobilized fucose-binding lectin Lotus tetragonolobus agglutinin (LTA1), also known as lotus lectin.
Thermodynamics of monosaccharide and disaccharide binding to Erythrina corallodendron lectin	1996	Surolia, A., Sharon, N., & Schwarz, F. P. (1996). Thermodynamics of monosaccharide and disaccharide binding to Erythrina corallodendron lectin. Journal of Biological Chemistry, 271(30), 17697-17703.	Isothermal titration calorimetry measurements of the binding of 2'-fucosyllactose, lactose, N-acetyllactosamine, galactopyranose, 2-acetamido-2-deoxygalactopyranoside, methyl alpha-N-dansylgalactosaminide (Me-alpha-DNS-GalN), methyl alpha-D-galactopyranoside, methyl beta-D-galactopyranoside, and fucose to Erythrina corallodendron lectin (ECorL), a dimer with one binding site per subunit, were performed at 283-286 and 297-299 K.
Characterization of Biomphalaria alexandrina-derived lectins recognizing a fucosyllactose-related determinant on schistosomes.	1995	Mansour, M. H., Negm, H. I., Saad, A. H., & Taalab, N. I. (1995). Characterization of Biomphalaria alexandrina-derived lectins recognizing a fucosyllactose-related determinant on schistosomes. Molecular and biochemical parasitology, 69(2), 173-184.	Two novel lectins that bind selectively to a schistosome-associated fucosyllactose-related determinant have been characterized and purified from the hemolymph of Biomphalaria alexandrina, the snail vector of Schistosoma mansoni.

Title	Year	Citation	Study Objective
Remodeling of mouse milk glycoconjugates by transgenic expression of a human glycosyltransferase	1995	Prieto, P. A., Mukerji, P., Kelder, B., Erney, R., Gonzalez, D., Yun, J. S., … Kopchick, J. J. (1995). Remodeling of mouse milk glycoconjugates by transgenic expression of a human glycosyltransferase. Journal of Biological Chemistry, 270(49), 29515–29519. https://doi.org/10.1074/jbc.270.49.29515	This study was designed to explore the factors regulating the production and biosynthesis of specific glycoconjugates in the mammary gland using a fusion gene containing a cDNA encoding the human alpha 1,2-fucosyltransferase (alpha 1,2FT), which generates the H-blood group antigen, flanked by the murine whey acidic protein promoter and a polyadenylation signal.
Glycosidic specificity of fucosyltransferases present in rat epididymal spermatozoa	1995	RAYCHOUDHURY, S. S., & MILLETTE, C. F. (1995). Glycosidic specificity of fucosyltransferases present in rat epididymal spermatozoa. Journal of andrology, 16(5), 448-456.	This study identifies and partially characterizes the glycosidic linkage specificity of FTs present in spermatozoa from caput and cauda epididymides.
Lactose as affinity eluent and a synthetic sulfated copolymer as inhibitor, in conjunction with synthetic and natural acceptors, differentiate human milk Lewis-type and plasma-type alpha-L-fucosyltransferases.	1994	Chandrasekaran, E. V., Rhodes, J. M., Jain, R. K., & Matta, K. L. (1994). Lactose as affinity eluent and a synthetic sulfated copolymer as inhibitor, in conjunction with synthetic and natural acceptors, differentiate human milk Lewis-type and plasma-type α-L-fucosyltransferases. Biochemical and biophysical research communications, 198(1), 350-358.	Human milk Lewis-type (alpha 1,3/4) fucosyltransferase (FT) was separated from the plasma-type by chromatography on bovine IgG glycopep-Sepharose using lactose as the selective eluent and further purified on a column of Sephacryl S-100 HR.
Purification and characterization of alpha-L-fucosidase from Chinese hamster ovary cell culture supernatant	1994	Gramer, M. J., Schaffer, D. V., Sliwkowski, M. B., & Goochee, C. F. (1994). Purification and characterization of α-L-fucosidase from Chinese hamster ovary cell culture supernatant. Glycobiology, 4(5), 611–616. https://doi.org/10.1093/glycob/4.5.611	In this study, alpha-L-fucosidase from Chinese hamster ovary (CHO) cell culture supernatant was purified 11 200-fold to apparent homogeneity to assess the rate of fucose hydrolysis from oligosaccharide and glycoprotein substrates.

Title	Year	Citation	Study Objective
Fucose and fucose-containing sugar epitopes enhance hippocampal long-term potentiation in the freely moving rat.	1994	Krug, M., Wagner, M., Staak, S., & Smalla, K. H. (1994). Fucose and fucose-containing sugar epitopes enhance hippocampal long-term potentiation in the freely moving rat. Brain research, 643(1-2), 130-135.	Male Wistar rats were intrahippocampally injected with L-fucose and the sugar epitope 2'-fucosyl-lactose prior to induction of long-term potentiation (LTP).
Presence of multiple fucosyltransferases in rat Sertoli cells and spermatogenic cells	1994	Raychoudhury, S. S., & Millette, C. F. (1994). Presence of Multiple Fucosyltransferases in Rat Sertoli Cells and Spermatogenic Cells1. Biology of Reproduction, 51(5), 1006–1013. https://doi.org/10.1095/biolreprod51.5.1006	To determine the glycosidic linkage specificity of FTs present in cultured Sertoli cells and in germ cells, the authors quantified FT activities by thin-layer chromatography using both high and low molecular weight acceptors in the presence of GDP-[14C]-L-fucose.
Purification and properties of the alpha-3/4-L-fucosyltransferase released into the culture medium during the growth of the human A431 epidermoid carcinoma cell line.	1993	Johnson, P. H., Donald, A. S., & Watkins, W. M. (1993). Purification and properties of the α-3/4-L-fucosyltransferase released into the culture medium during the growth of the human A431 epidermoid carcinoma cell line. Glycoconjugate journal, 10(2), 152-164.	A soluble alpha-3/4-fucosyltransferase secreted into the growth medium of the human A431 epidermoid carcinoma cell line has been purified 700,000 fold by a series of steps involving chromatography on Phenyl Sepharose 4B, CM-Sephadex C-50 and GDP-hexanolamine Sepharose 4B.
Assay of α1, 3 N-acetyl-d-galactosaminyl transferase by affinity chromatography	1992	Dakour, J., Zopf, D., & Lundblad, A. (1992). Assay of α1, 3 N-acetyl-d-galactosaminyl transferase by affinity chromatography. Analytical biochemistry, 204(1), 210-214.	A high-performance liquid affinity chromatography column that contains immobilized anti-A monoclonal antibody specifically retards blood group A-active oligosaccharides and can be used to detect the product(s) of the reaction catalyzed by alpha-1,3-N-acetyl-D-galactosaminyltransferase

Title	Year	Citation	Study Objective
Variations in human liver fucosyltransferase activities in hepatobiliary diseases.	1992	Jezequel-Cuer, M., Dalix, A. M., Flejou, J. F., & Durand, G. (1992). Variations in human liver fucosyltransferase activities in hepatobiliary diseases. Liver, 12(3), 140-146.	This study determined the activities of alpha 3, alpha 2 and alpha 3/4 F.T. in 35 liver biopsy samples from patients with fatty liver, alcoholic or post-hepatic liver cirrhosis, primary or secondary biliary cirrhosis, acute hepatitis or a normal liver.
Purification of the Lewis blood-group gene associated alpha-3/4-fucosyltransferase from human milk: an enzyme transferring fucose primarily to type 1 and lactose-based oligosaccharide chains.	1992	Johnson, P. H., & Watkins, W. M. (1992). Purification of the Lewis blood-group gene associated α-3/4-fucosyltransferase from human milk: An enzyme transferring fucose primarily to Type 1 and lactose-based oligosaccharide chains. Glycoconjugate journal, 9(5), 241-249.	A soluble Lewis blood-group gene associated alpha-3/4-L-fucosyltransferase has been purified from human milk by a series of steps involving hydrophobic chromatography on Phenyl Sepharose 4B, ion exchange chromatography on CM-Sephadex C-50, affinity chromatography on GDP-hexanolamine Sepharose 4B and gel filtration on Sephacryl S-200.
Thermodynamic analysis of ligand binding to winged bean (Psophocarpus tetragonolobus) acidic agglutinin reveals its specificity for terminally monofucosylated H-reactive sugars	1990	Acharya, S., Patanjali, S. R., Sajjan, S. U., Gopalakrishnan, B., & Surolia, A. (1990). Thermodynamic analysis of ligand binding to winged bean (Psophocarpus tetragonolobus) acidic agglutinin reveals its specificity for terminally monofucosylated H-reactive sugars. Journal of Biological Chemistry, 265(20), 11586-11594.	The sugar-specific binding of N-dansylgalactosamine to WBA II (n = 2; Ka = 5.6 x 10(3) M-1; delta H = -21 kJ.mol-1; delta S = -21.3 J.mol-1.K-1) was utilized in substitution titrations for evaluating the association constants for the interaction of sugars with the lectin.
Complete purification and characterization of alpha-3-N-acetylgalactosaminyltransferase encoded by the human blood group A gene	1990	Takeya, A., Hosomi, O., & Ishiura, M. (1990). Complete purification and characterization of α-3-N-acetylgalactosaminyltransferase encoded by the human blood group A gene. The Journal of Biochemistry, 107(3), 360-368.	A modified procedure in the Sepharose 4B step was developed by batch adsorption and desorption experiments. Cibacron Blue F3G-A, the chromophore of Blue Dextran, was found to bind to the enzyme. UDP is an effective inhibitor of this binding.