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## **Supporting document 1**

**Safety, technical and health effects assessment – Application  
A1155**

**2'-FL and LNnT in infant formula and other products**

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### **Executive summary**

The application is requesting a variation to the *Australia New Zealand Food Standards Code* (the Code) to permit two human milk identical oligosaccharides (HiMO), 2'-O-fucosyllactose (2'-FL) and lacto-N-neotetraose (LNnT) derived by microbial fermentation from genetically modified (GM) *Escherichia coli* K-12. These two substances are found naturally in human milk. The applicant (Glycom) has requested permission for the addition of 1.2 g/L of 2'-FL alone or with an additional 0.6 g/L of LNnT (i.e. totalling 1.8 g/L) in infant formula products (includes infant formula, follow-on formula, and infant formula products for special dietary use) and formulated supplementary foods for young children (FSFYC or 'toddler milks').

The stated purpose for adding 2'-FL, alone or in combination with LNnT, to infant formula products or FSFYC is to better reflect the oligosaccharide composition of human milk. These HiMOs are stated by the applicant to confer functional benefits to infants and young children (also referred to as 'toddlers' in this report), consistent with the human milk oligosaccharide (HMO) fraction of human milk, with three specified health effects: (1) a bifidogenic effect, (2) an anti-infective effect against pathogens, and (3) immune modulation, improved intestinal barrier function and alleviation of allergic responses.

The safety, technical and health effects assessment includes: (i) a food technology assessment of 2'-FL and LNnT; (ii) a safety assessment to identify potential adverse effects associated with 2'-FL and LNnT; (iii) a dietary intake assessment to estimate the total dietary intake of 2'-FL and LNnT for breastfed infants and intake resulting from the addition of 2'-FL and LNnT to infant formula products and FSFYC; and (iv) an assessment of the stated health effects.

The food technology assessment concluded that the applicant's 2'-FL and LNnT are chemically and structurally identical to the naturally occurring oligosaccharides in human milk and to chemically synthesised oligosaccharides, using appropriate methods of analysis. The shelf-life and specifications are appropriate for addition to infant formula products and FSFYC.

The GM safety assessment concluded that no public health and safety concerns have been identified for 2'-FL and LNnT derived from genetically modified *E. coli* K-12, production strains SCR6 and MP572, respectively.

Based on an assessment of the available toxicological and clinical evidence for 2'-FL and LNnT, it was concluded that there were no public health and safety concerns associated with the addition of 2'-FL, alone or in combination with LNnT, to infant formula products and formulated supplementary foods for young children, at the levels requested by the applicant and at the estimated levels of dietary intake based on 2.4 g/L of 2'-FL and 0.6 g/L of LNnT. Since the applicant's 2'-FL and LNnT are structurally and chemically identical to the forms of these substances present in human milk, no differences in pharmacokinetics between naturally occurring and manufactured forms of 2'-FL and LNnT are expected. Overall, the available data indicated that intestinal absorption is limited, and a significant proportion of HMOs including 2'-FL and LNnT reach the large intestine where they are fermented by the microbiota or excreted unchanged in the faeces.

Both 2'-FL and LNnT produced by microbial fermentation were not genotoxic in *in vitro* bacterial mutagenicity assays or in *in vitro* micronucleus assays in human lymphocytes. No adverse effects were observed in subchronic oral toxicity studies with 2'-FL or LNnT in juvenile rats at doses up to 5000 mg/kg bw/day. In human studies, infant formula supplemented with 2'-FL and LNnT was well tolerated with age-appropriate increases in body weight and other growth measures, and no significant increases in adverse events. 2'-FL and LNnT were also well tolerated in studies with obese children aged 5-12 years and healthy adults.

The assessment of effect on infant growth concluded that the addition of 2'-FL, alone or in combination with LNnT, to infant formula products has no effect on growth at the levels requested by the applicant. 2'-FL has been tested in formula in combination with short-chain fructooligosaccharide (scFOS) or galactooligosaccharide (GOS) or LNnT. The highest tested concentrations of 2'-FL and LNnT were 1.2 and 0.6 g/L, respectively. None of the studies examined by FSANZ found a difference in infant growth compared to a control formula. Based on a lack of adverse effects on growth in the clinical studies reviewed, and the limited gastrointestinal absorption of 2'-FL and LNnT, there is no evidence to indicate a nutritional concern at concentrations that are typically observed in human milk.

The concentration of 2'-FL in infant formula / follow-on formula / FSFYC considered in the dietary intake assessment was 2.4 g/L (rather than 1.2 g/L requested) as this level is similar to the mean concentration in mature human milk (2.4 – 3.0 g/L for 2'-FL secretors, which represents approximately 80% of women worldwide). This is approximately one fifth of the total concentration of oligosaccharides present in mature human milk (10 –15 g/L). The estimated dietary intake of 2'-FL based on 2.4 g/L in infant formula and follow-on formula is similar to 2'-FL intakes for 3 and 9 month old breastfed infants. Estimated mean intakes of 2'-FL from FSFYC, based on 2.4 g/L, for 12 month old infants and 2-3 year old children are similar to or less than those for younger formula-fed and breastfed infants (< 12 months).

The applicant's requested maximum of 0.6 g/L LNnT in infant formula products and FSFYC was considered in the dietary intake assessment. The mean concentration of LNnT in mature human milk is 0.28 – 0.31 g/L, noting all human milk contains LNnT. The estimated dietary intake of LNnT is therefore higher than that for 3 month and 9 month old breastfed infants due to the requested concentration of LNnT in infant formula and follow-on formula being higher than the mean concentration in human milk. However, the use level of 0.6 g/L is within the range of LNnT concentrations in mature human milk (0.09 – 1.08 g/L). Estimated mean intakes of LNnT from FSFYC for 12 month old infants and 2-3 year old children are similar to or lower than those for younger formula-fed infants (< 12 months).

The assessment of bifidogenic effect concluded that the ability of *Bifidobacterium* spp. to metabolise 2'-FL and LNnT is variable within and between species and that a bifidogenic effect is biologically and mechanistically plausible if the *Bifidobacterium* strains present in the infant and toddler colon are able to metabolise 2'-FL or LNnT. A single study, published as abstracts, demonstrated that infants fed formula supplemented with 2'-FL and LNnT at levels similar to those requested, had a gut microbiome at 3 months of age that more closely resembled that of breastfed infants and with a higher relative abundance of *Bifidobacterium* spp. compared to infants fed unsupplemented formula. As the reproducibility of this study has not been demonstrated in other populations the results are inconclusive. However, the biological plausibility of a bifidogenic effect occurring due to the requested addition of 2'-FL alone or with LNnT is further supported by a single clinical feeding trial for adults that showed a shift in the gut microflora to a higher relative abundance of bifidobacteria in a dose dependent manner following supplementation with either 2'-FL or LNnT alone or in combination at a 2:1 ratio of 2'-FL:LNnT.

The assessment of anti-infective effect concluded that the addition of 2'-FL to infant formula products and FSFYC may be detrimental to attachment and growth of invasive *Campylobacter jejuni* (*C. jejuni*) infection through binding inhibition. The biological and mechanistic plausibility of this health effect is supported by evidence from an *in vivo* murine model demonstrating decreased disease severity in animals fed 5 g/L 2'-FL, binding studies demonstrating a specific interaction between invasive *C. jejuni* strains and 2'-FL, and *in vitro* studies demonstrating *C. jejuni* binding inhibition in multiple cell lines. Evidence from a human study showing a decreased incidence of *Campylobacter* associated diarrhoea in infants of mothers with a higher proportion of 2'-FL in their milk provides additional supporting evidence. Based on the evidence assessed, FSANZ considers that this health effect could occur at the level of 2'-FL requested, although the extent of the effect in infants and toddlers at this level cannot be determined. The evidence for a health effect of 2'-FL and LNnT protecting against other pathogens and toxins is inconclusive and is primarily limited to *in vitro* inhibition studies with no specific mechanism of inhibition identified. A single human infant trial study provided limited evidence of a decreased rate of bronchitis and lower respiratory tract infection in infants fed formula supplemented with 2'-FL and LNnT. However, the reproducibility of this finding in multiple populations has not been demonstrated and is therefore inconclusive.

The assessment of immune modulation and improved barrier function concluded that there is insufficient evidence to support the assertion that infant formula supplemented with 2'-FL alone or with LNnT, will have an immune modulating effect or improve barrier function in infants and toddlers. The evidence to support these proposed health effects are largely based on *in vitro* studies and are not well supported by *in vivo* animal models or infant feeding studies. Of clinical significance in the assessment of food allergies, the available evidence demonstrates that 2'-FL does not prevent the production of allergen-specific IgE-immunoglobulins after sensitisation has occurred, and therefore 2'-FL does not protect against anaphylaxis.

FSANZ concludes that the bifidogenic effect and anti-infective effect against invasive *C. jejuni* are biologically plausible and the assessed evidence supports a mechanism for these effects, although direct and consistent evidence of association in infants and toddlers, as demonstrated by well-designed randomised control trials, are lacking. In reaching this conclusion, FSANZ has taken into consideration the complexity of definitively and reproducibly demonstrating a health effect for a substrate targeted at modulating gut microflora. Evidence from an *in vitro* laboratory study for anti-infective effect and an adult study for bifidogenic effect, indicates that these health effects may be enhanced as concentrations of 2'-FL (or LNnT in the case of the bifidogenic effect only) are increased. Evidence to support the health effects of improved barrier function, immune modulation and alleviation of allergic responses are inconclusive.

2'-FL and LNnT are naturally present in human milk in a range of concentrations and ratios, providing a history of safe human exposure to these substances for breastfed infants. FSANZ concludes there are no public health and safety concerns associated with the addition of 2'-FL alone or in combination with LNnT to infant formula products and FSFYC at the requested levels, or at higher estimated levels of dietary intakes based on 2.4 g/L 2'-FL.

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## Glossary of terms

|                        |   |
|------------------------|---|
| 2'-FL                  | 2'-O-fucosyllactose   |
| 2'-FL <sub>chem</sub>  | 2'-O-fucosyllactose produced by chemical synthesis                    |
| 2'-FL <sub>micro</sub> | 2'-O-fucosyllactose produced by microbial fermentation                |
| 2'-FL <sub>human</sub> | 2'-O-fucosyllactose naturally occurring in human milk                 |
| FSFYC                  | Formulated supplementary foods for young children (or 'toddler milk') |
| GOS                    | Galacto-oligosaccharide   |
| HMO                    | Human milk oligosaccharide  |
| HiMO                   | Human milk identical oligosaccharide                                  |
| LNT                    | lacto-N-tetraose  |
| LNnT                   | Lacto-N-neotetraose   |
| LNnT <sub>micro</sub>  | Lacto-N-neotetraose produced by microbial fermentation                |
| LNnT <sub>chem</sub>   | Lacto-N-neotetraose produced by chemical synthesis                    |
| LNnT <sub>human</sub>  | Lacto-N-neotetraose naturally occurring in human milk                 |
| scFOS                  | Short-chain fructo-oligosaccharide                                    |

# 1. Introduction

Glycom A/S has submitted an application to FSANZ to vary the *Australia New Zealand Food Standards Code* (the Code) to include two human milk identical oligosaccharides (HiMO), 2'-O-fucosyllactose (2'-FL) and lacto-N-neotetraose (LNnT) derived from genetically modified (GM) *Escherichia coli* strain K-12. 2'-FL is intended to be added alone or in combination with LNnT to infant formula products (includes infant formula, follow-on formula, and infant formula products for special dietary use) and formulated supplementary foods for young children (FSFYC or 'toddler milk').

The stated purpose for adding 2'-FL alone or in combination with LNnT to infant formula products or FSFYC is to better reflect the oligosaccharide composition of human milk. These HiMOs are stated by the applicant to confer functional benefits to infants and young children, consistent with the HMO fraction of human milk, with three specified health effects: a bifidogenic effect, an anti-infective effect against pathogens, and immune modulation, improved intestinal barrier function and alleviation of allergic responses.

The primary risk assessment problem to be addressed is whether addition of 2'-FL, alone or in combination with LNnT, would pose a public health and safety risk to the target population when added to infant formula products or FSFYC.

The applicant has requested a maximum of 1.2 g/L for 2'-FL alone or with an additional 0.6 g/L of LNnT, (i.e. totalling 1.8 g/L) for use in infant formula products and FSFYC.

## 2 Food Technology Assessment

The food technology assessment provides information on chemical identification, physicochemical properties, and specifications for the two oligosaccharides proposed to be added to infant formulas: 2'-FL and LNnT. The assessment primarily aimed to address whether the microbiologically-synthesised oligosaccharides proposed to be added to infant formula products and FSFYC are identical to those present in human milk. The assessment also considered the manufacturing process and the validity of analytical methods used to quantify and characterise 2'-FL and LNnT during production and as a component of infant formula and FSFYC.

[Section 3.1](#) provides information on the genetically modified organisms and the fermentation process for production of 2'-FL and LNnT.

### 2.1 Chemical properties

#### 2.1.1 Chemical names, properties, and structures

2'-FL and LNnT are naturally occurring oligosaccharides in human milk. Both have been isolated from human milk or produced through chemical synthesis. This application relates specifically to the production and isolation of 2'-FL and LNnT by a microbiological fermentation process. Characterisation of the substances from all three sources is important for their structural identification. Therefore, as required, the substances are distinguished according to the source using the relevant subscript (e.g. 2'-FL<sub>chem</sub> = chemically synthesised 2'-FL; 2'-FL<sub>micro</sub> = 2'-FL from microbiological fermentation; and 2'-FL<sub>human</sub> = 2'-FL in human milk). Chemical names and properties for 2'-FL and LNnT are listed in Table 2.1.

**Table 2.1: Chemical properties of 2'-FL and LNnT**

| Property                       | 2'-FL  | LNnT  |
|--------------------------------|--|---|
| Chemical name                  | $\alpha$ -L-Fucopyranosyl-(1→2)- $\beta$ -D-galactopyranosyl-(1→4)-D-glucopyranose | $\beta$ -D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy- $\beta$ -D-glucopyranosyl-(1→3)- $\beta$ -D-galactopyranosyl-(1→4)-D-glucopyranose |
| Common name                    | 2'-O-Fucosyllactose  | Lacto-N-neotetraose   |
| Alternative names <sup>1</sup> | Fucosyl- $\alpha$ -1,2-galactosyl- $\beta$ -1,4-glucose                            | Gal- $\beta$ -(1→4)-GlcNAc- $\beta$ -(1→3)-Gal- $\beta$ -(1→4)-Glc  |
|                                | Fuc- $\alpha$ -(1→2)-Gal- $\beta$ -(1→4)-Glc                                       | N-Acetyl-D-lactosamine- $\beta$ -(1→3)-D-lactose  |
|                                | 2'-O-L-Fucosyl-D-lactose   |   |
| CAS registry number            | 41263-94-9   | 13007-32-4  |
| Chemical formula               | C <sub>18</sub> H <sub>32</sub> O <sub>15</sub>                                    | C <sub>26</sub> H <sub>45</sub> NO <sub>21</sub>  |
| Molecular mass                 | 488.174 g/mol  | 707.63 g/mol  |
| Solubility <sup>2</sup>        | 240 g/L  | 214 g/L   |

<sup>1</sup> Fuc = fucose or fucosylpyranose; Gal = galactose or galactosylpyranose; Glc = glucose or glucosylpyranose

<sup>2</sup> For comparison, the solubility of lactose = 195 g/L.

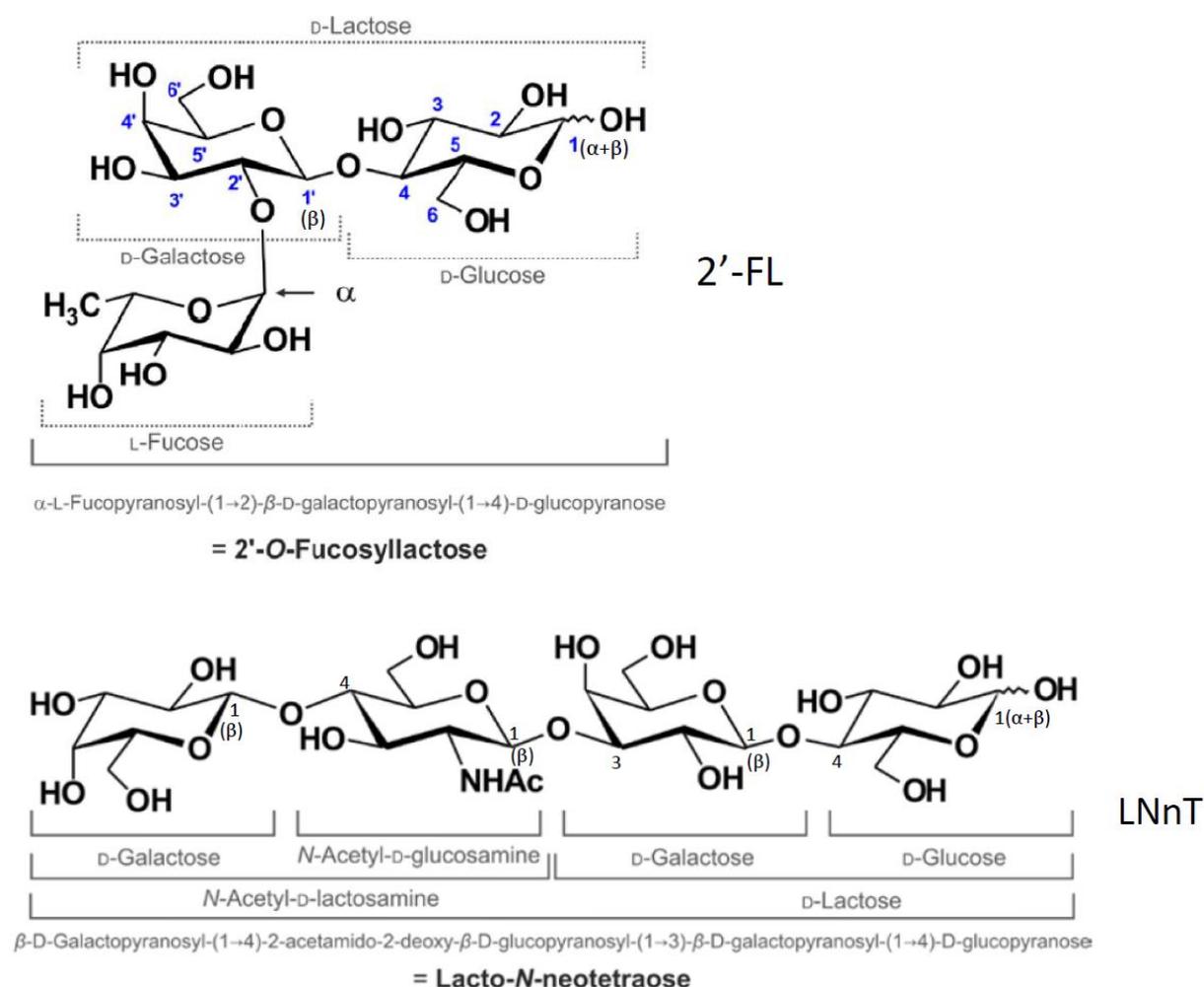
2'-FL is a trisaccharide consisting of the monosaccharides L-fucose, D-galactose, and D-glucose. It can also be described as the monosaccharide L-fucose and the disaccharide D-lactose, which are linked by an alpha (1→2) bond to form the trisaccharide. The structure includes a glucose moiety which is present as either the  $\alpha$  or  $\beta$  anomer (these are termed

conformational isomers).

LNnT is a linear tetrasaccharide consisting of D-galactose, *N*-acetyl-D-glucosamine, D-galactose and D-glucose. The structure also includes a glucose moiety which is present as either the  $\alpha$  or  $\beta$  anomer.

2'-FL and LNnT occur naturally in human milk as single specific constitutional isomers as shown in [Figure 2.1](#).

The substances 2'-FL and LNnT are white to off-white amorphous powders. Both are reducing sugars<sup>1</sup> and are therefore susceptible to Maillard reactions. They are not associated with any sweetening properties.



**Figure 2.1 Chemical Structures**

Structures show the conventional numbering of each monosaccharide moiety. The anomeric carbon (position 1) is a stereocenter where the OH group or glycosidic linkage is either in the  $\alpha$  or  $\beta$  position (as shown). The C-OH bond for a mixture of  $\alpha$  and  $\beta$  anomers is represented by a wavy line.

### 2.1.2 Structural identification

Several spectrometric methods were used to determine that 2'-FL and LNnT produced microbiologically are structurally and chemically equivalent as that derived from human milk

<sup>1</sup> Reducing sugars have a free hydroxyl group at the anomeric carbon (i.e. carbon position 1).

and chemically synthesised. Overall the approach for this determination involved:

- comparing spectral data for the microbiologically synthesised substances (2'-FL<sub>micro</sub> and LNnT<sub>micro</sub>) to the chemically synthesised substances 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub>;
- comparing the human milk derived substances (2'-FL<sub>human</sub> and LNnT<sub>human</sub>) to 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub>; and
- further spectral analyses to confirm the structure of 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub>.

The chemically synthesised forms of 2'-FL and LNnT were used in the studies submitted in the application to support the stated beneficial effects of the substances when added to infant formula products. Therefore, confirmation that the structural identification of 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub> was correct and showed the same characteristics as that sourced from human milk or microbial fermentation was an important consideration in this assessment.

### ***Chemically-derived versus microbiological substances***

<sup>1</sup>H Nuclear Magnetic Resonance (NMR) spectra of chemically- and microbiologically-synthesised 2'-FL and LNnT were submitted in Appendix IV of the application. For both 2'-FL and LNnT, spectra showed multiple peaks between 3.2-4.0 ppm which is typical for carbohydrate compounds.

For 2'-FL, four signals were present as doublets between 4.4-5.4 ppm representing the four anomeric carbons of 2'-FL: α H1 Fuc, β H1 Gal, and α/β H1 Glu (see [Figure 2.1](#)). For LNnT, five signals were present as doublets between 4.4-5.3 ppm representing the five anomeric carbons of LNnT: β-1Gal H1, β-3Gal H1, GlcNAc H1, and α/β-Glc H1. The chemical shifts were within 0.2 ppm of published NMR data which can be attributed to different concentrations of the solutions used for the measurement. For both 2'-FL and LNnT, the spectra for the microbiologically synthesised compounds were identical to the corresponding chemically synthesised compounds and showed no significant impurities.

Additionally, Nuclear Overhauser Effect Spectroscopy (NOESY<sup>2</sup>) was used to further characterise the structural properties of 2'-FL and LNnT. The NOESY spectra for 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> showed the same off-diagonal signals at similar intensities compared to 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub>. These NOESY data indicate that the stereochemical configuration and three dimensional structures of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> are comparable to the chemically-synthesised compounds.

### ***Chemically-derived versus human milk substances***

As above, spectral data for 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub> was compared to the human milk-derived 2'-FL and LNnT which were sourced commercially by the applicant. <sup>1</sup>H-NMR spectra for 2'-FL<sub>human</sub> and LNnT<sub>human</sub> were identical to the corresponding chemically synthesised substances, as summarised in the [previous section](#). NOESY spectra for 2'-FL<sub>human</sub> and LNnT<sub>human</sub> were also comparable to the chemically-synthesised substances.

### ***Additional structural data for 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub>***

Two analytical reports providing detailed spectrometric data for 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub> were provided.<sup>3</sup> This information was examined since chemically synthesised 2'-FL and LNnT were used in infant feeding trials ([Section 3.2.6](#)) and it is important to establish whether these substances are equivalent to the microbiologically-derived substances. Analyses using <sup>13</sup>C-

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<sup>2</sup> NOESY is an NMR method which runs spectra in 2 dimensions and obtains data on the spatial orientation of atoms.

<sup>3</sup> Analytical reports A-GH-2014-001 (2'-FL) and A-GH-2013-110 (LNnT) are detailed reports provided in confidence.

NMR, 2-dimensional NMR, and mass spectrometry confirmed the structural identification of 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub>.

The additional structural data<sup>3</sup> was not provided for microbiologically-synthesised 2'-FL and LNnT. The conclusion that 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> is structurally equivalent to the chemically synthesised substances is based on the analyses showing that spectral data were comparable for the substances derived from all three sources (see sections "[Chemically-derived versus microbiological substances](#)" and "[Chemically-derived versus human milk substances](#)") and on consistency with published data (Almond et al. 2004; Asres and Perreault 1996; Fura and Leary 1993; Ishizuka et al. 2008; Jenkins et al. 1984; Perreault and Costello 1999; Rundlöf et al. 2001; Strecker et al. 1989; Svensson et al. 2002; Urashima et al. 2002; Urashima et al. 2004; Urashima et al. 2005; Wada et al. 2008).

## 2.2 Analytical methods for detection

As there are no internationally recognised methods for the analysis of 2'-FL and LNnT, methods developed by the applicant were submitted to FSANZ in confidence. The information provided included:

- Description of methods using high performance liquid chromatography (HPLC) or high performance ion exchange chromatography (HPAEC)
- Details of methods including reagents, reference materials and standards, solution preparation, run parameters and procedures, evaluation, calculations, and typical chromatograms
- Analytical procedures to assay for carbohydrate impurities specified in the specification (sucrose, D-lactose, fucose, difucosyllactose, and 2-fucosyl-D-lactulose for 2'-FL; and D-lactose, lacto-*N*-triose II, *para*-lacto-*N*-neohexaose and LNnT fructose isomer for LNnT).
- Quantification of 2'-FL and LNnT in whey-predominate infant formula and data showing method validation (conducted by 3<sup>rd</sup> party laboratory)
- Validation reports on the development of quantitation methods by 3<sup>rd</sup> party laboratory.

The information demonstrated that the 2'-FL and LNnT can be manufactured and assayed to a purity that would be consistent with the specifications proposed by the applicant (see [Section 2.3.4](#)).

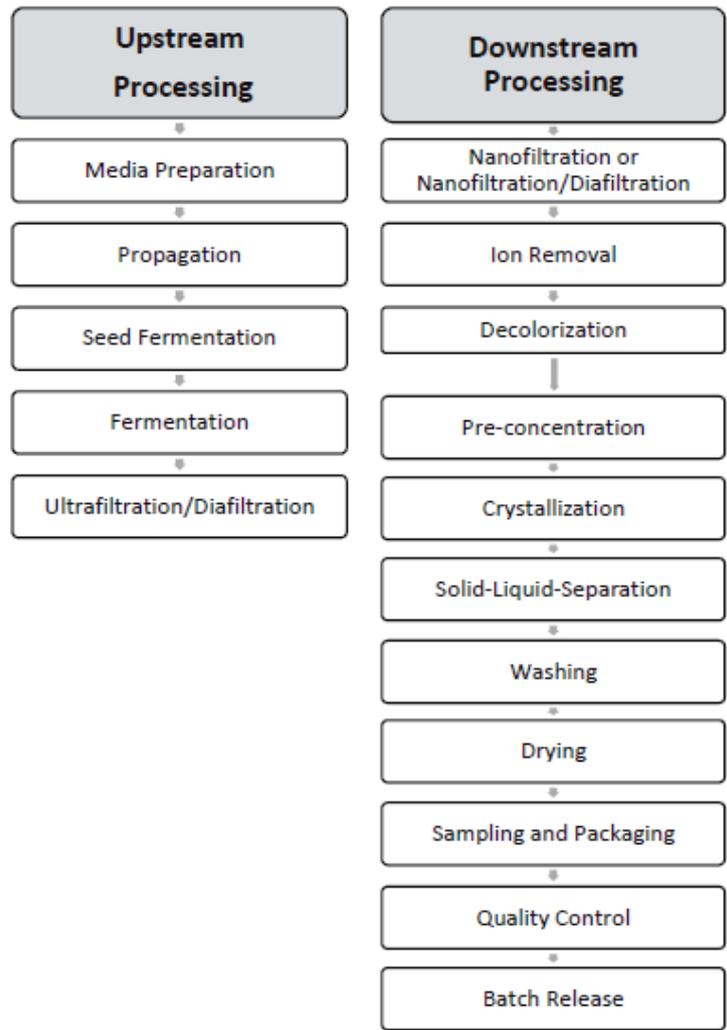
## 2.3 Manufacturing processes

### 2.3.1 Production of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub>

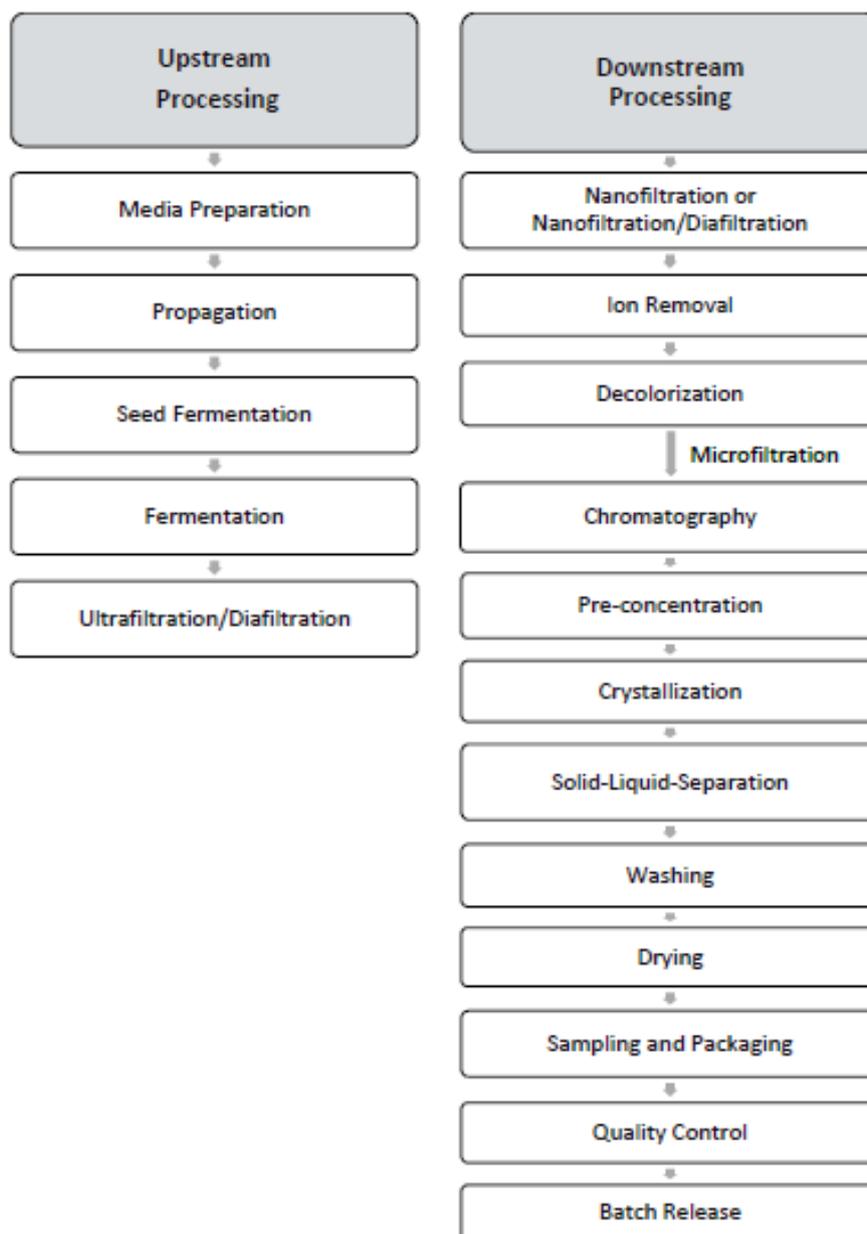
Production of 2'-FL is conducted by a two stage process: upstream and downstream processes are represented in [Figure 2.2](#). The upstream process involves conversion of D-lactose and D-sucrose to 2'-FL by a microbial fermentation process using *E. coli* K-12 (DH1) SCR6 (see [Section 3.1](#)). The downstream process involves the purification and isolation steps to produce high purity 2'-FL.

Similarly, production of LNnT is conducted through 2 stages: the upstream process where D-lactose is converted to LNnT by microbial fermentation using *E. coli* K-12 (DH1) MP572 (see [Section 3.1](#)) and the downstream process for the purification and isolation of high purity LNnT ([Figure 2.3](#)).

The applicant has provided details of the manufacturing processes for 2'-FL and LNnT including a description of the raw materials and processing aids, the production organism, purification and isolation, and quality controls. Manufacturing of 2'-FL and LNnT is conducted in accordance with Good Manufacturing Practices (GMP) and HACCP principles.



**Figure 2.2** Manufacturing process for 2'-FL<sub>micro</sub>



**Figure 2.3 Manufacturing process for LNnT<sub>micro</sub>**

### 2.3.2 Information on impurities

Carbohydrate impurities in multiple batches of the final isolated 2'-FL<sub>micro</sub> were assayed by HPLC using a Corona Charged Aerosol Detector (cCAD). It was noted that this method is characterised by high sensitivity and precision, a wide dynamic range, and reproducibility. The typical carbohydrate impurity profile showed the presence of small quantities of residual starting materials D-lactose and L-fucose, as well as the manufacturing by-products difucosyllactose and 2'-fucosyl-D-lactulose. In submitted batch analyses, amounts of these substances were within the specifications proposed by the applicant. Three of these substances (lactose, fucose and difucosyllactose) are natural components of human milk and exposures to these in 2'-FL preparations were considered to be insignificant compared to the exposure from each of these saccharides at the naturally occurring levels.

The by-product, 2'-fucosyl-D-lactulose, is formed from isomerisation of the terminal glucose

moiety to fructose in 2'-FL during heat treatment. The reaction is well-characterised for the analogous conversion of lactose to lactulose (Aider and Halleux 2007). Lactulose is formed through heat treatment of milk and levels in infant formula are considered to be an indicator of heat damage during processing (Pereyra González et al. 2003) (Segura et al. 2012) (Cattaneo et al. 2009). Consequently, lactulose levels in commercial infant formulas are low (Pereyra González et al. 2003). The 2'-FL → 2'-fucosyl-D-lactulose conversion occurs via the same mechanism as lactose → lactulose. Consequently, 2'-fucosyl-D-lactulose is likely to form at a proportion similar to lactulose during heat treatment. Low levels of 2'-fucosyl-D-lactulose would be expected to form during heat treatment of human milk although this has not been specifically evaluated. Therefore, it is concluded that the amounts of 2'-fucosyl-D-lactulose formed during heat treatment would be low and comparable to amounts that infants fed pasteurised human milk would receive.

Carbohydrate impurities in multiple batches of the final isolated LNnT<sub>micro</sub> were also assayed using HPLC-cCAD. The typical carbohydrate impurity profile showed the presence of small quantities of carbohydrate by-products formed during the fermentation process. These are lacto-*N*-triose II, *para*-lacto-*N*-neohexaose (both naturally present in human milk), and LNnT fructose isomer. In submitted batch analyses, these by-products were within the specifications proposed in the application. As described above for 2'-FL, the LNnT fructose isomer is formed through isomerisation of the glucose moiety to fructose during heat treatment. Isomerisation of LNnT to the LNnT fructose isomer would be expected to form during heat treatment to a degree similar to the lactose → lactulose conversion, although this has not been specifically evaluated in heat-treated human milk.

The application included a description of quality control measures and assay methods for analyses of amino acids and residual minerals from the fermentation medium, microbial endotoxins and residual proteins (from the fermentation organism), and residual amounts of the production organism itself. Batch analyses demonstrated that these contaminants and/or impurities were insignificant or absent in non-consecutive batches of isolated 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub>.

### 2.3.3 Stability

Results from stability studies for 2'-FL and LNnT as isolated substances were conducted under real-time and accelerated conditions ([Table 2.2](#)). These data demonstrated that 2'-FL and LNnT did not show changes in organoleptic properties, microbiological profile and/or impurity profiles or degradation of the ingredient over prolonged periods.

The stability of 2'-FL<sub>chem</sub> in combination with LNnT<sub>chem</sub> was assessed in infant formula powder. 2'-FL was added at 0.90g/100g (dry matter) and LNnT was added at 0.45 g/100 g (dry matter) to a whey-dominant, commercially available infant formula product. The amounts of 2'-FL and LNnT were representative of the amounts to be added under the intended conditions of use. The results indicated that the combined substances in this food matrix were stable for up to 900 days.

Study conditions and results from stability studies on 2'-FL and LNnT in liquid matrices (i.e. similar to a toddler milk product) were not included in the application. However, data were submitted for US FDA GRAS approval. The GRAS reports 546 and 547 (for 2'-FL and LNnT, respectively) indicated that the substances were stable under different processing conditions and throughout the shelf life for these select food products (US FDA 2015, 2015).

Stability studies specifically for the microbial-synthesised substances (2'-FL<sub>micro</sub> and LNnT<sub>micro</sub>) in a food matrix (either infant formula or other foods) were not submitted. However, it can be concluded that 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> would be as stable as 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub> based on (1) chemical and structural analyses showing that 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub> are

identical to 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub>, and (2) impurity studies showing that there are unlikely to be impurities present in preparations of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> that would accelerate their degradation.

**Table 2.2 Summary of results of stability tests for 2'-FL and LNnT**

| Stability study               | Source of substance | Conditions  | Stability of 2'-FL                                      | Stability of LNnT      |
|-------------------------------|---------------------|---|---|------------------------|
| Real-time                     | Chemical            | Isolated crystalline substance at 25°C and 60% relative humidity  | Stable up to 60 months                                  |                        |
| Real-time                     | Microbial           | Isolated crystalline substance at 25°C and 60% relative humidity  | Stable up to 18 months                                  | Stable up to 12 months |
| Accelerated                   | Chemical            | Isolated crystalline substance at 40°C and 75% relative humidity  | Stable up to 24 months                                  |                        |
| Accelerated                   | Microbial           | Isolated crystalline substance at 40°C and 75% relative humidity  | Stable up to 18 months                                  | Stable up to 12 months |
| In infant formula powder      | Chemical            | Both substances added to whey-based starter formula <sup>1</sup> stored in gassed (N <sub>2</sub> /CO <sub>2</sub> ) cans at 4-37°C | Stable up to 900 days                                   |                        |
| In general foods <sup>2</sup> | Chemical            | Substances added individually to selected food products; conditions and results not provided  | Stable over manufacturing and shelf life of the product |                        |

<sup>1</sup> Test used commercial infant formula for infants 0-6 months of age and considered to have typical nutrient composition.

<sup>2</sup> See US FDA GRAS notices 546 and 547 (2015). These included liquid matrices such as yoghurts, ready-to-drink flavoured milk, citrus fruit beverages.

### 2.3.4 Product specifications

Neither 2'-FL nor LNnT are covered under specifications listed in Schedule 3 (Identity and Purity) of the Code. As both substances have been approved for use in the EU and the US, the applicant has proposed specifications that have been defined in these jurisdictions ([Tables 2.3](#) and [2.4](#)). The specifications prescribe maximum amounts for carbohydrate by-products (see [Section 2.3.2](#)) and impurities related to the microorganisms used in the fermentation process. These specifications would not be applicable for 2'-FL and LNnT manufactured using chemical synthesis.

The applicant provided Certificates of Analysis for four non-consecutive batches of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> to demonstrate that the product meets the proposed specifications (see pages 41-42 of the application). Results were consistent across all batches tested for each substance. The Certificates of Analysis indicate that both substances meet S3-4 requirements for lead, arsenic, cadmium and mercury.

**Table 2.3 Product specifications proposed for 2'-FL (reproduced from application)**

| Parameter   | Specification   | Method  |
|---|---|---|
| Appearance  | Powder or agglomerates                                      | ISO 6658:2007   |
| Colour  | White to off white  | ISO 6658:2007   |
| Identification  | RT of main component corresponds to RT of standard $\pm$ 3% | Glycom method HPLC-202-2C4-002                                      |
| Assay (water free) for human-identical milk saccharides (HiMS) <sup>a</sup> | Not less than 96.0 w/w %                                    | Glycom methods HPLC-202-2C4-002, HPLC-206-2C4-001 and HPAEC-206-001 |
| Assay (water free) 2'-FL  | Not less than 94.0 w/w %                                    | Glycom method HPLC-202-2C4-002                                      |
| d-Lactose   | Not more than 3.0 w/w %                                     | Glycom method HPLC-206-2C4-001                                      |
| L-Fucose  | Not more than 1.0 w/w %                                     | Glycom method HPAEC-206-001   |
| Difucosyllactose  | Not more than 1.0 w/w %                                     | Glycom method HPAEC-206-001   |
| 2'-Fucosyl-d-lactulose  | Not more than 1.0 w/w %                                     | Glycom method HPLC-206-2C4-001                                      |
| pH (20°C, 5% solution)  | 3.2 to 5.0  | Ph. Eur. 2.2.3  |
| Water   | Not more than 5.0 w/w %                                     | Karl-Fischer (Ph. Eur. 2.5.32)                                      |
| Ash, sulphated  | Not more than 1.5 w/w %                                     | Ph. Eur. 6.7 04/2010:20414  |
| Acetic acid (as free acid and/or sodium acetate)                            | Not more than 1.0 w/w %                                     | Megazyme K-ACETRM 07/12   |
| Residual proteins   | Not more than 0.01 w/w %                                    | Bradford Assay; Glycom method UV-001                                |
| Heavy metals  |   |   |
| Lead  | Not more than 0.1 mg/kg                                     | ICP-MS by EPA 6020A:2007  |
| <b>Microbiological Parameters</b>   |   |   |
| <i>Salmonella</i>   | Absent in 25 g  | ISO 6579:2006   |
| Total plate count   | Not more than 500 CFU/g                                     | ISO 4833-1:2014   |
| Enterobacteriaceae  | Absent in 10 g  | ISO 21528-1:2004, ISO 21528-2:2007                                  |
| <i>Cronobacter (Enterobacter) sakazakii</i>                                 | Absent in 10 g  | ISO-TS 22964:2006   |
| <i>Listeria monocytogenes</i>   | Absent in 25 g  | ISO 11290-1:1996/A1:2005  |
| <i>Bacillus cereus</i>  | Not more than 50 CFU/g                                      | ISO 7932:2005   |
| Yeasts  | Not more than 10 CFU/g                                      | ISO 7954:1999   |
| Moulds  | Not more than 10 CFU/g                                      | ISO 7954:1999   |
| Residual endotoxins   | Not more than 10 EU/mg                                      | Eur. Ph. 2.6.14   |

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

**Table 2.4 Product specifications proposed for LNnT (reproduced from application)**

| Parameter   | Specification            | Method                               |
|---|--------------------------|--------------------------------------|
| Appearance  | Powder or agglomerates   | ISO 6658:2007                        |
| Colour  | White to off white       | ISO 6658:2007                        |
| Identification  | RT of standard $\pm$ 3%  | Glycom method HPLC-106-1C6-002       |
| Assay (water free) for human-identical milk saccharides (HiMS) <sup>a</sup> | Not less than 95.0 w/w % | Glycom method HPLC-106-1C6-002       |
| Assay (water free) Lacto-N-neotetraose                                      | Not less than 92.0 w/w % | Glycom method HPLC-106-1C6-002       |
| d-Lactose   | Not more than 3.0 w/w %  | Glycom method HPLC-106-1C6-002       |
| Lacto-N-triose II   | Not more than 3.0 w/w %  | Glycom method HPLC-106-1C6-002       |
| <i>para</i> -Lacto-N-neohexaose   | Not more than 3.0 w/w %  | Glycom method HPLC-106-1C6-002       |
| LNnT fructose isomer  | Not more than 1.0 w/w %  | Glycom method HPLC-106-1C6-002       |
| pH (20°C, 5 % solution)   | 4.0 – 7.0                | Eur. Ph. 2.2.3                       |
| Water   | Not more than 9.0 w/w %  | Karl-Fischer (Ph. Eur. 2.5.12)       |
| Ash, sulphated  | Not more than 1.5 w/w %  | Eur. Ph. 6.7 04/2010:20414           |
| Methanol  | Not more than 100 mg/kg  | Glycom method GC-109-1C6-001         |
| Residual proteins   | Not more than 0.01 w/w % | Bradford Assay; Glycom method UV-001 |
| <b>Heavy metals</b>   |                          |                                      |
| Lead  | Not more than 0.1 mg/kg  | ICP-MS by EPA 6020A:2007             |
| <b>Microbiological Parameters</b>   |                          |                                      |
| <i>Salmonella</i>   | Absent in 25 g           | ISO 6579:2006                        |
| Total plate count   | Not more than 500 CFU/g  | ISO 4833-1:2014                      |
| <i>Enterobacteriaceae</i>   | Absent in 10 g           | ISO 21528-1:2004, ISO 21528-2:2007   |
| <i>Cronobacter (Enterobacter) sakazakii</i>                                 | Absent in 10 g           | ISO-TS 22964:2006                    |
| <i>Listeria monocytogenes</i>   | Absent in 25 g           | ISO 11290-1:1996/A1:2005             |
| <i>Bacillus cereus</i>  | Not more than 50 CFU/g   | ISO 7932:2005                        |
| Yeasts  | Not more than 10 CFU/g   | ISO 7954:1999                        |
| Moulds  | Not more than 10 CFU/g   | ISO 7954:1999                        |
| Residual endotoxins   | Not more than 10 EU/mg   | Eur. Ph. 2.6.14                      |

CFU = colony forming units; Eur. Ph. = European Pharmacopeia; EU = endotoxin units; GC-HS = headspace gas chromatography; HPLC = high performance liquid chromatography; ISO = International Organization for Standardization; LNnT = lacto-N-neotetraose; RT = retention time. <sup>a</sup> Human-identical milk saccharides (HiMS) is defined as the sum of LNnT, lactose, lacto-N-triose II, and *para*-lacto-N-hexaose.

## 2.4 Key findings of the food technology assessment

FSANZ concludes that the data provided demonstrates that Glycom's 2'-FL and LNnT are chemically and structurally identical to the naturally occurring substances in human milk and to chemically synthesised substances. Spectral data submitted for 2'-FL and LNnT were consistent with published reports on these substances.

Infant formula milk powders generally have a shelf life of two years. The analysis showing

addition of 2'-FL in combination with LNnT is stable in infant milk powder for up to 900 days is therefore within the expected shelf life for this product. The application indicated that the substances are also stable over the shelf life of liquid matrices.

The applicant has proposed specifications to be included in Schedule 3 for the use of 2'-FL and LNnT in infant formula products and FSFYC. These are essentially identical to specifications that were approved for use in the EU and US at the time of FSANZ's assessment<sup>4</sup>. The specifications are publically available and provided in the application as per Tables 2.3 and 2.4 above. The CCI material provided in the application is not necessary for the inclusion of the specifications in the Code if the substances are approved. Additionally, the proposed specification in the application states a method for each parameter. This information assisted in FSANZ's assessment but the stated methods are not proposed to be included in Schedule 3.

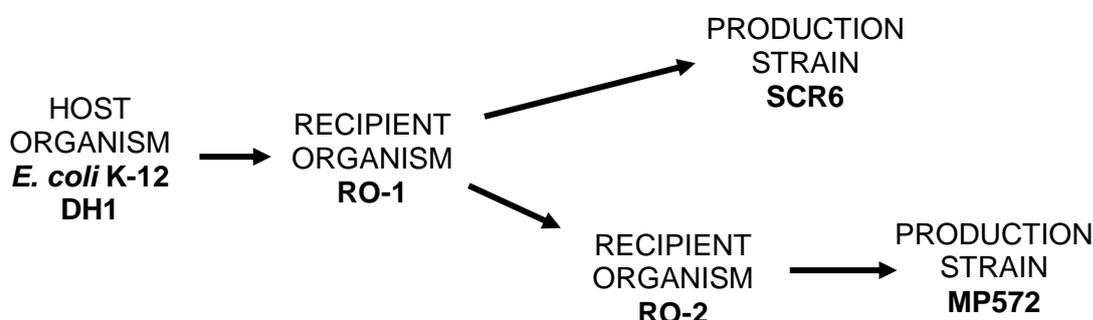
### 3. Safety assessment

#### 3.1 Genetically modified (GM) production strain assessment

The objectives of this safety assessment are to evaluate any potential public health and safety concerns that may arise from the use of the genetically modified (GM) production strains that have been generated for the large scale production of 2'-FL and LNnT by fermentation. Specifically by considering:

- history of use of the source organisms
- characterisation of the genetic modification(s)
- safety of the novel proteins being expressed.

The source organisms referred to in this assessment are outlined in [Figure 3.1](#). Individual production strains were created to produce the 2'-FL (strain SCR6) and LNnT (strain MP572) by fermentation. These production strains were derived from an *Escherichia coli* K-12 DH1 host. Several genetic modifications were made to the *E. coli* host organism prior to generation of the final production strains, in order to optimise production of oligosaccharides, and these have been referred to as recipient organisms RO-1 and RO-2. Different genetic modifications were performed on the host and recipient organisms and some of these modifications involved introduction of genetic material from organisms we have referred to as gene donors.



**Figure 3.1 Outline of the different source organisms assessed in this application**

<sup>4</sup> FSANZ notes the recent revision to the EU regulations to provide a generic specification for *E. coli* K-12 derived 2'-FL. See (EU) 2019/388 of 11 March 2019. This regulation is not listed as a primary source for specifications in S3—2 Identity and Purity.

### 3.1.1 History of use

#### **Host organism**

*Escherichia coli* K-12 is the most common bacterial laboratory strain in use globally. It was isolated from a human stool sample in 1922 (Bachmann 1996). Comparative genome sequencing and proteomic analysis of the K-12 strain and its derivatives, to well characterised pathogenic strains, have identified differences in the K-12 cell wall structure associated with reduced ability to colonise the human intestinal tract, and absence of adhesive proteins and virulence factors that meet requirements for pathogenicity (Bachmann 1996; EPA 1997; Sahl et al. 2013). These studies have also shown reduced toxin production in K-12 strains and absence of plasmids encoding antibiotic resistance. Under the U.S. National Institutes of Health (NIH) Research Involving Recombinant or Synthetic Nucleic Acid Molecules ([NIH Guidelines](https://osp.od.nih.gov/wp-content/uploads/NIH_Guidelines.pdf)<sup>5</sup>, 2016), *E. coli* K-12 is classified as a Risk Group 1 agent which is reserved for organisms that are not human or animal pathogens.

*E. coli* K-12 has a long history of use in the human biopharmaceutical industry, with ~30% of currently approved recombinant therapeutic proteins in the United States (US) being produced in *E. coli* K-12, starting with the US FDA approval of biosynthetic human insulin in 1983 (Huang et al. 2012; Jozala et al. 2016). The use of this bacterium as a source for the production of food enzymes began in the 1980s (JECFA 1991). *E. coli* K-12 is permitted as a source microorganism for the production of chymosin in the Food Standards Code. *E. coli* K-12 is considered a model organism and has been thoroughly characterised for use in research and industry, it is therefore considered a safe organism.

The strain (DH1) used to create the production organisms is a recombination deficient strain of K-12 (Hanahan 1983). Using DH1 (DSM No 4235) allows for expression of the introduced genes from plasmids rather than relying on integration of the introduced genes into the *E. coli* chromosome. Expression from a plasmid ensures that the regulatory elements associated with the genes remain fully functional, which guarantees optimal expression and also prevents disruption of chromosomal genes.

#### **Gene donor organisms**

The source of the main enzyme genes used to produce 2'-FL (futC) and LNnT (IgtA and GalT) were *Helicobacter pylori* and *Neisseria meningitides*. These organisms have been categorised in the NIH Guidelines as Risk Group 2 agents for organisms that are associated with human disease, where the disease is rarely serious and there is likely availability of preventative or therapeutic interventions (US FDA 2016a, 2016b).

Genetic material from *H. pylori* was initially isolated by targeted amplification then exposed to several rounds of standard DNA cloning, which would have eliminated the chance of extraneous genetic material or host pathogenic factors being carried across to the enzyme production organism. Genetic material from *N. meningitides* was chemically synthesised, based on published DNA sequences therefore no material from the source will be present in the production strain. Chemical synthesis was performed in order to optimise codon usage. These changes do not result in changes to the expressed protein sequence.

In some versions of the production strains, genes were expressed from plasmids. To ensure continual presence of the plasmids and to accommodate a modified metabolic profile of the recipient organism strains, sucrose operon genes were co-located on the plasmids. These operon genes were sourced from *Salmonella typhimurium* and *Klebsiella pneumonia*. These organisms have been categorised as Risk Group 2 agents. The genetic material that was

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<sup>5</sup> [https://osp.od.nih.gov/wp-content/uploads/NIH\\_Guidelines.pdf](https://osp.od.nih.gov/wp-content/uploads/NIH_Guidelines.pdf)

used was chemically synthesised, based on published DNA sequences. Chemical synthesis was used to introduce silent mutations, enabling modification of restriction sites to assist with DNA cloning. These silent mutations do not result in changes to the protein sequences from the two organisms.

Further genetic material was also sourced from the host. This included introduction of endogenous genes to increase expression of specific oligosaccharide synthesis pathway genes, thus increase yield. Other genes were introduced to allow selection of transformants (*NadC*). Finally, host DNA was used to provide regulatory elements (promoters and terminators).

### **3.1.2 Characterisation of the genetic modification(s)**

#### ***Optimisation of the recipient strain(s) RO-1 and RO-2***

In order to ensure efficient production of the target oligosaccharides 2'-FL and LNnT, an optimised recipient organism RO-1 was initially created through a series of genetic modifications to the DH1 host strain (US FDA 2016a, 2016b). These changes included 'knocking out' six genes by removing chromosomal DNA in order to:

1. suppress expression of unnecessary carbohydrates that could
  - a) impact culturing conditions
  - b) enhance purity of the desired oligosaccharides
2. modify the metabolism of the organism by
  - a) allowing utilisation of an alternate carbon source
  - b) allowing selection and maintenance of transformants

Another modification to RO-1 was the introduction of an *E. coli* promoter at a targeted location to enhance expression of an endogenous biosynthesis enzyme required for the production of 2'-FL.

Further modifications were then made to RO-1 specifically for the production of LNnT thus generating an RO-2 organism (US FDA 2016b). These changes included:

1. deletion of a crucial biosynthesis enzyme gene (*nadC*). This gene is later reintroduced via the plasmid used to generate MP572, as a selective marker for positive transformants
2. disruption of a repressor gene for the lac operon by insertion of DNA, in order to mediate gene expression
3. the introduction of multiple copies of the beta-1,3-N-acetylglucosaminyltransferase (*IgtA*) gene at loci involved in sugar metabolism, in order to increase yield and purity of the LNnT. The introduced DNA was chemically synthesised to allow codon optimisation and was based on the DNA sequence from a well-characterised source organism.

#### ***Description of the introduced DNA***

The production strains for 2'-FL (strain SCR6) and LNnT (strain MP572) were generated using standard bacterial transformation techniques on chemically competent cells, prepared from the optimised recipient strains RO-1 and RO-2, respectively.

For 2'-FL, the introduced DNA was cloned into two plasmids, designated plasmid A and plasmid B. Plasmid A is a derivative of pBBR1-MCS3 (Kovach et al. 1995) that encodes a tetracycline resistance gene and the backbone of plasmid B is a commercially available BlueScript plasmid that contains an ampicillin resistance gene. Although antibiotic resistance

genes are present, the use of antibiotics only occurred during the DNA cloning stage, for initial selection of transformants. No antibiotics were used at any stage during the production of 2'-FL.

Two groups of genes were introduced on the plasmids in the transformation step to create SCR6. One group introduced a sucrose operon, allowing SCR6 to be grown in the presence of sucrose as an energy and carbon source ([Table 3.1](#)). Growth on sucrose also improves the yield of 2'-FL and ensures minimal plasmid loss, as the sucrose operon genes were split between the two expression plasmids.

**Table 3.1 Outline of the sucrose operon genes and their products**

| Gene | Product  |
|------|--|
| scrA | Phosphotransferase (PTS) system sucrose-specific component |
| scrY | Sucrose porin  |
| scrR | Sucrose repressor  |
| scrB | Sucrose-6-phosphate hydrolase                              |

The second group of genes ([Table 3.2](#)) coded for enzymes to allow creation of the 2'-FL synthesis pathway in the *E. coli* ([Figure 3.2](#)). Before transformation, all gene sequences contained in the two plasmids were confirmed by DNA sequence analysis.

**Table 3.2 Outline of introduced genes and their products involved in 2'-FL synthesis**

| Gene | Product                           |
|------|-----------------------------------|
| manB | Phosphomannomutase                |
| manC | Mannose-1-P-guanosyltransferase   |
| gmd  | GDP-mannose-4,6- dehydratase      |
| wcaG | GDP-fucose synthase               |
| futC | $\alpha$ -1,2-fucosyl-transferase |

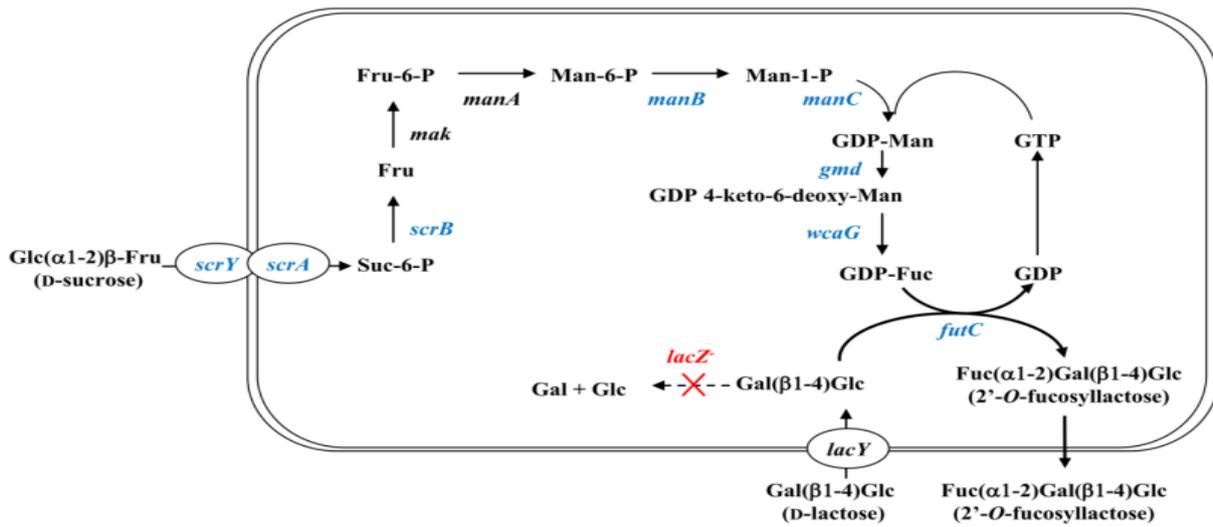


Figure 3.2 Outline of the biosynthesis pathway recreated in the *E. coli* 2'-FL production strain SCR6

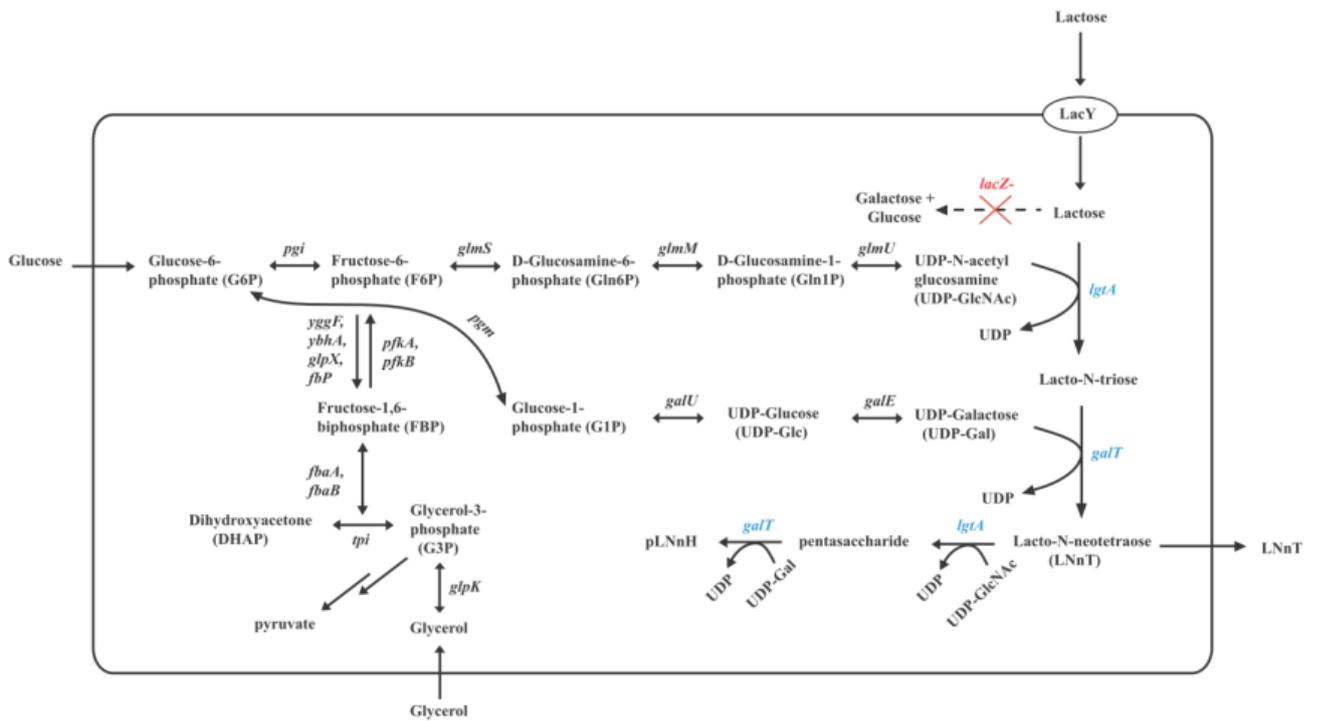


Figure 3.3 Outline of the biosynthesis pathway recreated in the *E. coli* LNT production strain MP572

Production of LNnT was achieved with the introduction of a single plasmid into RO-2 encoding two genes, beta-1,4-galactosyltransferase (*galT*) and *nadC*, on a BlueScript plasmid backbone. In this plasmid, the ampicillin gene has been replaced with the *nadC* gene to allow for selection of transformants without the need for antibiotics. Expression of *galT* combined with the multiple copies of *IgtA* introduced previously into RO-1 ([Section 3.1.2](#)) alongside endogenous enzymes already present in DH1, allows creation of the full LNnT synthesis pathway ([Figure 3.3](#)). The gene sequences were confirmed prior to transformation by DNA sequence analysis.

### ***Characterisation of the genetically modified organisms***

#### *Characterisation of the gene disruptions in the recipient strains RO-1 and RO-2*

Several gene disruptions were performed on the DH-1 host genomic DNA to generate RO-1 (as outlined in [Section 3.1.2](#)). These disruptions involved deletion of sequences from within the gene to disable the gene. To confirm these deletions, polymerase chain reaction (PCR) was performed on genomic DNA using primers that spanned each gene. Full length products were observed in DH1 indicating presence of intact genes whereas smaller products of expected sizes were observed in both RO-1 and RO-2, confirming disruption of the genes. The same smaller PCR products were detected in the production strains SCR6 and MP572, confirming that the gene disruptions were maintained after the final transformation used to generate the production strains.

Two further modifications were then performed on RO-1 in order to generate RO-2 and involved deletion of the *nadC* gene and disruption of a lac operon gene. PCR analysis of genomic DNA confirmed presence of the full length *nadC* gene and lac operon gene in DH1 and RO-1 and absence or disruption of these genes in MP572.

DNA sequence analysis was also performed and confirmed that during the modification of the recipient strains genome, there had been no rearrangement of the DNA.

#### *Characterisation of introduced DNA*

In the generation of RO-2, several copies of the *IgtA* gene were introduced into the genomic DNA of RO-1 at targeted locations. PCR performed on genomic DNA was used to confirm presence and orientation of *IgtA*, using primers spanning the junction between the genomic DNA and the introduced gene. The results confirmed that multiple copies of the *IgtA* gene were present in MP572 at the targeted loci and were in the correct orientation. No amplification was seen in DH1 or RO-1. DNA sequence analysis also confirmed the orientation of the *IgtA* sequences and showed there had been no rearrangement of the DNA.

PCR amplification of the plasmid-based *futC* gene confirmed presence in SCR6 but absence in DH1 and RO-1. Similarly, PCR amplification of the plasmid-based *galT* gene confirmed presence in MP572 but absence in DH1 and RO-2. The primers used in these PCR reactions spanned the introduced DNA and the plasmid backbone or regulatory elements, such as promoters and the introduced DNA.

#### *Genetic stability and inheritance of the introduced DNA*

Several approaches were used to demonstrate the genetic stability and inheritance of the introduced DNA in the production strains SCR6 and MP572. This was done using different cell generations collected during the cell bank preparation stage, as outlined in [Table 3.3](#).

**Table 3.3 Generation times for the cell samples analysed**

| Sample name and abbreviation |      | Generation Number |       |
|------------------------------|------|-------------------|-------|
|                              |      | SCR6              | MP572 |
| Initial cell clone           | ICC  | 0                 | 0     |
|                              | ICC+ | -                 | 61.8  |
| Master cell bank             | MCB  | 13.6              | 8.0   |
| Working cell bank            | WCB  | 26.2              | 18.8  |
|                              | WCB+ | 48.2              | 48.2  |

### **SCR6**

Cultures of cells taken from different generations (ICC, MCB and WCB+) were grown overnight in LB media containing ampicillin and tetracycline. After purification of plasmid DNA, gel electrophoresis was used to visualise the isolated plasmids. The results showed that the plasmids were present across all generations, confirming inheritance of the DNA. As a negative control, the absence of plasmid was confirmed in DH1 and RO-1.

PCR amplification of the plasmid-based *futC* gene from cultures established from different generations (ICC to WCB+) confirmed the presence and inheritance of the plasmid containing this gene over the generations analysed. The data also showed there was no rearrangement of the plasmid over time.

PCR targeting the disrupted genes in RO-1 outlined in [Section 3.1.2](#) was examined in the ICC, MCB and WCB+ cell populations and confirmed that these changes were consistent across all generations analysed.

Finally, data were obtained from a 120h fermentation production performance test to examine growth profiles, lactose utilisation and production levels of 2'-FL. Comparisons of cultures starting from cells at three different time points (MCB, WCB and WCB+), showed consistent growth, substrate utilisation and 2'-FL production levels.

Taken together, these data confirm the inheritance and stability of the DNA in SCR6.

### **MP572**

Cultures of cells taken from different generations (ICC, MCB, WCB and WCB+) were grown overnight in a defined minimal media. After extraction of plasmid DNA from each culture, gel electrophoresis was used to visualise the DNA. The results showed that the plasmid was present across all generations, confirming inheritance of the DNA. As a negative control, the absence of plasmid was confirmed in DH1 and RO-1.

PCR amplification of the plasmid-based *galT* gene from cultures established from different generations (ICC to WCB+) confirmed the presence and inheritance of this plasmid over the generations analysed. The data also showed there was no rearrangement of the plasmid over time.

PCR targeting the disrupted genes in RO-1 and RO-2 outlined in [Section 3.1.2](#) was also examined in the ICC through to WCB+ cell populations. The data confirmed these changes were consistent across the generations analysed.

PCR targeting the *lgtA* gene was also examined in the ICC through to WCB+ cell populations. The results confirmed presence across the generations analysed and that there was no rearrangement of the *lgtA* gene over time.

Finally, data were obtained from a 70h fermentation production performance test to examine growth profiles, lactose utilisation and production levels of LNnT. Comparisons of cultures starting from cells at four different time points spanning 50 generations, showed consistent growth, substrate utilisation and LNnT production levels.

Taken together, these data confirm the inheritance and stability of the DNA in MP572.

#### *Open reading frame (ORF) analysis*

The applicant performed an ORF analysis and identified two putative ORFs arising from the generation of RO-1. ORF1 could encode a protein of 136 amino acids and ORF2 could encode a protein of 60 amino acids. To be expressed, these ORFs would need to be linked to a functional promoter and other regulatory elements.

### **3.1.3 Safety of novel proteins**

In the consideration of whether the novel proteins being evaluated in this application are safe, it is important to take into account that the expressed proteins, along with the genetically modified bacteria themselves, will be removed during the purification of the oligosaccharides. Due to the degree of purification of the final food, it is highly unlikely that novel protein or DNA will be present.

A large and diverse range of proteins are ingested as part of the normal human diet, which do not cause adverse effects. Only a small number of dietary proteins have the potential to cause adverse health effects, either because they have anti-nutrient properties or they can trigger allergic reactions in some individuals (Delaney et al. 2008). Furthermore, proteins perform a wide range of functions in humans. To encompass the range of type and function, the safety assessment of any novel proteins must consider if there is a history of safe use, whether there are any potential toxic, anti-nutrient or allergenic effects and whether the protein is susceptible to digestion.

#### ***History of safe use***

The novel enzymes expressed in the SCR6 and MP572 strains are bacterial proteins that share a degree of homology to mammalian enzymes, as many mammals including humans produce 2'-FL and LNnT. Furthermore, there is no evidence from the scientific literature to indicate that presence of these enzymes in the final food would be a safety concern. Due to the similarity of these enzymes to endogenous human proteins, these enzymes could be considered to have a long history of safe use in humans.

In addition, the sucrose operon components and other bacterial specific genes expressed in SCR6 and MP572, are found in many bacterial species present in foods and the human gut microbiome, such as lactobacilli and bifidobacteria (Gänzle and Follador 2012; Pokusaeva et al. 2011). Although there will be some species-specific differences in the protein sequences, there is no evidence to indicate these proteins would be a safety concern in the unlikely event they were present in the final foods. With the presence of these proteins in commensal bacteria, it could be concluded there is a long history of safe use in humans.

### **Bioinformatic analysis for potential allergenicity**

The applicant provided results of an *in silico* analysis comparing the novel proteins and ORFs to known allergenic proteins in the Food Allergy Research and Resource Program dataset, which is available through [AllergenOnline](#)<sup>6</sup> (University of Nebraska). The database at the time of the search contained 2035 sequences (v.17).

Three types of comparisons were done:

- a) Full length sequence search – a FASTA alignment was performed comparing the whole sequence to the database entries. Significant homology was determined when there was more than 50% similarity between the query protein and database entry, with the E-value threshold set at  $1 \times 10^{-7}$  ( $<1e-7$ )
- b) 80-mer sliding window search – a FASTA alignment was performed comparing all contiguous 80 amino acids within the novel amino acid sequences to the database entries (Pearson and Lipman 1988). Matches were identified if there was greater than 35% homology.
- c) 8-mer exact match search – A FASTA alignment was performed comparing contiguous 8 amino acids within the novel amino acid sequences to the database entries (FAO/WHO 2001). Matches were identified if there was 100% homology.

Results from the full length and 8-mer search did not identify any similarity of the novel proteins expressed in the SCR6 and MP572 strains to known allergens. However, a match was identified with the 80-mer sliding window search, between one of the sucrose operon proteins introduced into SCR6 and a minor tomato allergen (*Solanum lycopersicum*). The similarity between the two proteins over a region of 80 amino acids was 46.3% (E-value of  $7.0 \times 10^{-16}$ ) while comparison of the full length protein expressed in SCR6 to the minor tomato allergen showed only 27% similarity (E-value of  $9.3 \times 10^{-19}$ ). Subsequent analysis of the peptide sequences showed that the sucrose operon protein expressed in SCR6 does not contain a region shown to be required for allergenicity of the tomato allergen. Combining this sequence information with the fact that the novel protein is unlikely to be present in the final food due to the production organism being removed by filtration and the oligosaccharides being highly purified, with undetectable levels of residual protein, it is unlikely this protein poses any risk.

### **Bioinformatic analysis for potential toxicity**

The applicant provided results from *in silico* analyses comparing the novel proteins and ORFs to known protein toxins identified in two custom UniProt databases: [animal venom proteins and toxins](#)<sup>7</sup> and [virulence factors](#)<sup>8</sup>. A BLASTP algorithm using the default BLOSUM62 scoring matrix was used and matches were identified if homology was  $\geq 35\%$ . The search identified similarity between the ORF2 potential protein to a neurotoxin found in some species of viper, with an E-value of 0.21. The two proteins share 80% homology at the C-terminus but did not share homology in the region where the active sites of the toxin are located. It is highly unlikely that this potential ORF will be present in the final food due to the production organism being removed by filtration and the oligosaccharides, 2'-FL and LNnT, being highly purified with undetectable levels of residual protein.

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<sup>6</sup> [AllergenOnline](#)

<sup>7</sup> <https://www.uniprot.org/uniprot/?query=taxonomy%3A%22Metazoa+%5b33208%5d%22+AND+%28keyword%3Atoxin++OR+annotation%3A%28type%3A%22tissue+specificity%22+AND+venom%29%29+AND+reviewed%3Ayes&sort=score>

<sup>8</sup> <https://www.uniprot.org/uniprot/?query=keyword:KW-0843>

### **Susceptibility of novel proteins to digestion**

To determine if the novel proteins and ORFs expressed in SCR6 and MP572 are likely to be digested, potential cleavage sites were investigated by FSANZ using the amino acid sequence of the novel proteins and the [PeptideCutter tool](http://web.expasy.org/peptide_cutter/)<sup>9</sup> in the ExPASy Proteomics Site. A summary of the data is presented in [Table 3.4](#). These data show that the novel proteins present in SCR6 and MP572 would likely be as susceptible to protein digestion as the vast majority of dietary proteins.

**Table 3.4 Number of potential cleavage sites found in the novel proteins expressed in the SCR6 and MP572 production strains**

| Gene Name | Pepsin |        | Trypsin | Chymotrypsin     |                 | Endopeptidases |
|-----------|--------|--------|---------|------------------|-----------------|----------------|
|           | pH 1.3 | pH > 2 |         | High specificity | Low specificity |                |
| scrA      | 121    | 142    | 27      | 43               | 117             | 35             |
| scrY      | 92     | 142    | 46      | 55               | 112             | 82             |
| scrR      | 74     | 85     | 30      | 22               | 81              | 56             |
| scrB      | 97     | 142    | 34      | 46               | 123             | 87             |
| manB      | 88     | 111    | 52      | 34               | 98              | 98             |
| manC      | 73     | 106    | 46      | 34               | 96              | 113            |
| gmd       | 69     | 92     | 38      | 35               | 89              | 83             |
| wcaG      | 49     | 69     | 31      | 25               | 74              | 56             |
| futC      | 69     | 97     | 34      | 38               | 82              | 60             |
| galT      | 61     | 82     | 31      | 32               | 77              | 53             |
| nadC      | 63     | 68     | 33      | 15               | 58              | 73             |
| lgtA      | 61     | 89     | 47      | 34               | 80              | 68             |
| ORF1      | 27     | 31     | 11      | 11               | 28              | 25             |
| ORF2      | 8      | 11     | 11      | 7                | 14              | 15             |

#### **3.1.4 Key findings of GM assessment**

The *E. coli* host organism has a long history of safe use for the production of human biopharmaceuticals and food enzymes. The gene donor organisms, except *E. coli*, have the potential to be pathogenic. However, DNA was either chemically synthesised or when obtained directly from the organisms, has undergone several stages of cloning. Carryover of infectious material or harmful compounds would be minimal and the gene donor organisms would therefore be unlikely to pose any risk.

<sup>9</sup>[http://web.expasy.org/peptide\\_cutter/](http://web.expasy.org/peptide_cutter/)

Characterisation of the genetic modifications made to SCR6 and MP572 showed that the changes were as expected, the DNA had not undergone rearrangement and were genetically stable.

The novel proteins introduced to create the 2'-FL and LNnT biosynthesis pathways were shown by bioinformatics analysis to have no biologically significant similarity with known toxins or allergens. Furthermore, bioinformatics analysis indicated that the novel proteins would likely be as susceptible to protein digestion as the vast majority of dietary proteins.

On the basis of the data provided in the present application, and other available information, no potential public health and safety concerns were identified in the assessment of SCR6 and MP572.

## 3.2 Toxicological assessment

### 3.2.1 Assessments by Other Agencies

In 2015 the European Food Safety Authority (EFSA) assessed 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub> produced by Glycom as novel food ingredients (EFSA 2015a, 2015b). It was concluded that 2'-FL and LNnT in combination are safe:

- for infants when added to infant and follow-on formulae at concentrations up to 1.2 g/L of 2'-FL and 0.6 g/L of LNnT, at a ratio of 2:1 in the reconstituted formulae
- when added to follow-on and young-child formulae at concentrations up to 1.2 g/L or 0.6 g/L respectively, either alone or in combination at a ratio of 2:1
- when added to other foods (e.g. cereal bars, dairy products and beverages) at the uses and use levels proposed.

EFSA did not establish Acceptable Daily Intake (ADI) values for these substances.

The Food Safety Authority of Ireland (FSAI) has issued opinions concluding that Glycom's 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> produced by fermentation are substantially equivalent to the previously approved chemically synthesised forms, and therefore raised no safety concerns (FSAI 2016, 2016).

The US Food and Drug Administration (FDA) has responded that it has 'no questions' to Glycom's self-assessment that 2'-FL and LNnT produced by both microbial fermentation and chemical synthesis are Generally Recognized as Safe (GRAS)<sup>10</sup>. The USFDA has also issued 'no questions' responses to other manufacturers' self-assessed GRAS notifications for 2'-FL produced by microbial fermentation (using different production strains) by Jennewein<sup>11</sup>, FrieslandCampina<sup>12</sup> and Dupont<sup>13</sup>.

### 3.2.2 Evaluation of Submitted Data

FSANZ has assessed the submitted data on the safety of 2'-FL and LNnT and information from published sources. The toxicological database includes:

- information on the absorption, distribution, metabolism and excretion of HMOs including 2'-FL and LNnT
- in vitro genotoxicity studies with 2'-FL or LNnT (including bacterial and mammalian mutagenicity studies and micronucleus assays in mammalian cells)
- An in vivo micronucleus assay with 2'-FL produced by Jennewein

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<sup>10</sup> GRAS GRN 650 (2'-FL<sub>micro</sub>); GRN 659 (LNnT<sub>micro</sub>); GRN 546 (2'-FL<sub>chem</sub>); GRN 547 (LNnT<sub>chem</sub>)

<sup>11</sup> GRAS GRN 571

<sup>12</sup> GRAS GRN 735

<sup>13</sup> GRAS GRN 749

- subchronic toxicity studies with 2'-FL in juvenile rodents and piglets, and with LNnT in juvenile rodents
- human clinical studies in infants and adults.

Subsequent to publication of the first CFS, the applicant provided an unpublished report on interim results of a clinical study of 2'-FL and LNnT in 5-12 year-old children.

The available data are considered suitable to assess the hazard of 2'-FL and LNnT.

The test article preparations used in the submitted toxicological studies were considered to be representative of Glycom's 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub>. Methods of manufacture and specifications of Glycom 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> and other preparations used in the submitted toxicological studies are compared in [Table 3.5](#) and [Table 3.6](#), respectively.

The applicant also provided analytical reports demonstrating that 2'-FL and LNnT produced by fermentation are structurally identical to those obtained by chemical synthesis, and that 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub> produced by chemical synthesis are identical to those found in human milk (refer to [Section 2.1.2](#) of this report).

**Table 3.5 Comparison of 2'-FL test articles used in toxicological studies**

| Parameter              | 2'-FL under the present application                              | 2'-FL produced by Glycom A/S | 2'-FL produced by Jennewein (Jennewein Biotechnologie GmbH 2015) | 2'-FL produced by FrieslandCampina (van Berlo et al. 2018) |
|------------------------|--|------------------------------|--|--|
| Manufacture Process    | Fermentation   | Chemical synthesis           | Fermentation   | Fermentation   |
| Parent Organism        | <i>E. coli</i> K-12  | N/A                          | <i>E. coli</i> BL21  | <i>E. coli</i> K12   |
| Test Article Purity    | 98.4% (as human identical milk saccharides);<br>97.6% (as 2'-FL) | 96.9%#                       | 92.4 – 97.9%   | 94%  |
| <b>Specification</b>   |  |                              |  |  |
| Assay by HPLC          | Min. 94.0%   | Min. 95.0%                   | ≥ 90%*   | Not reported   |
| D-Lactose              | Max. 3.0 % w/w   | Max. 3.0% w/w                | ≤ 5%*  | Not reported   |
| L-Fucose               | Max. 1.0% w/w  | Max 1.0% w/w                 | ≤ 3%*  | Not reported   |
| Difucosyllactose       | Max. 1.0% w/w  | Max. 1.0% w/w                | ≤ 5%*  | <1%**  |
| 2'-Fucosyl-D-lactulose | Max. 1.0% w/w  | Max. 0.6% w/w                | NS   | Not reported   |

| Parameter         | 2'-FL under the present application | 2'-FL produced by Glycom A/S | 2'-FL produced by Jennewein (Jennewein Biotechnologie GmbH 2015) | 2'-FL produced by FrieslandCampina (van Berlo et al. 2018) |
|-------------------|-------------------------------------|------------------------------|--|--|
| Fucosyl galactose | NS                                  | NS                           | ≤ 3%*  | Not reported   |
| Protein           | 0.01%                               | 0.1%                         | ≤ 100 µg/g   | 0.002%**   |
| Ash               | Max. 1.5%                           | 0.2%                         | ≤ 0.5%   | 0.06%**  |

HPLC: high-performance liquid chromatography; N/A: not applicable; NS: not specified; w/w: weight/weight.

# In the majority of toxicity studies with chemically synthesised 2'-FL the purity was reported to be 99.9%. However, subsequent re-analysis of the test material using an improved analytical method and a purer analytical reference standard indicated that the purity of the batch used in the toxicity studies (L06112K) was 96.9%.

\* Percent of total carbohydrates by HPLC (area under the curve)

\*\* Analytical values rather than specifications

**Table 3.6 Comparison of LNnT test articles used in toxicological studies**

| Parameter            | LNnT under the present application                             | LNnT produced by Glycom A/S | LNnT reported by Prieto (2005)   |
|----------------------|--|-----------------------------|--|
| Manufacture Process  | Fermentation   | Chemical synthesis          | Coupled fermentation with yeast/ <i>E. coli</i>  |
| Parent Organism      | <i>E. coli</i> K-12  | N/A                         | <i>Candida famata</i> ATCC 32550 and <i>E. coli</i> JM101 for overexpression of enzymes  |
| Test Article Purity  | 97.9 (as human identical milk saccharides);<br>94.4% (as LNnT) | 98.9%                       | Not reported   |
| Specification        |  |                             |  |
| Assay by HPLC        | Min. 95.0%   | Min. 95.0%                  | Specifications not reported. Authors reported that LNnT was 'practically devoid of other carbohydrates and organic contaminants with the exception of lactose' (present at less than 2%). The test material was also tested for metals, microbial toxins and microbial contamination and found to be virtually |
| D-Lactose            | Max 3.0%   | Max. 1.0%                   |  |
| Lacto-N-triose II    | Max 3.0%   | Max. 0.3%                   |  |
| LNnT fructose isomer | Max 1.0%   | Max. 0.6%                   |  |
| Water                | Max. 9.0%  | Max. 9.0%                   |  |
| Protein              | Max. 0.01%   | Max. 0.01%                  |  |
| Ash, sulfated        | Max. 1.5%  | Max. 0.4%                   |  |
| Aerobic mesophilic   | ≤ 500 CFU/g  | ≤ 500 CFU/g                 |  |

| Parameter         | LNnT under the present application | LNnT produced by Glycom A/S | LNnT reported by Prieto (2005) |
|-------------------|------------------------------------|-----------------------------|--------------------------------|
| total plate count |                                    |                             | free of known contaminants     |

HPLC: high-performance liquid chromatography; N/A: not applicable; CFU: colony-forming units.

### 3.2.3 Toxicological studies

#### ***Absorption, Distribution, Metabolism & Excretion studies***

Glycom's 2'-FL and LNnT are structurally and chemically identical to the forms of these substances naturally present in human milk. It is not anticipated that there will be any significant differences in pharmacokinetics between naturally occurring and manufactured forms of these HMOs.

#### *In vitro* studies

HMOs have been shown to be resistant to hydrolysis by digestive enzymes in *in vitro* studies using human or porcine enzyme preparations or intestinal brush border membranes (Engfer et al. 2000; Gnoth et al. 2000). *In vitro* studies with the human intestinal cell line Caco-2 showed that both neutral and acidic HMOs cross the epithelial barrier (Gnoth et al. 2001).

#### Animal studies

In laboratory rats, small amounts of 2'-FL, LNnT and/or other HMOs have been detected in serum or urine samples following oral administration (Jantscher-Krenn et al. 2013; Jennewein Biotechnologie GmbH 2015; Vazquez et al. 2017). Serum concentrations of 2'-FL in rats were < 60 µg/mL following oral administration of single doses in the range of 200 – 5000 mg/kg bw (Vazquez et al. 2017). In a 13-week repeated dose oral toxicity study involving dietary administration of 2'-FL at doses equivalent to 7700 and 8700 mg/kg bw/day in males and females, respectively, mean serum concentrations were below 11 µg/mL on test day 1 or 2, and approximately 2 µg/mL in test week 13 (Jennewein Biotechnologie GmbH 2015). LNnT concentrations in serum of rats were < 8 µg/mL after administration of single LNnT doses equivalent to 200 – 1000 mg/kg bw 2'-FL (Vazquez et al. 2017).

#### Studies in infants

Breath hydrogen tests in infants administered HMOs isolated from their mother's milk showed similar under the breath hydrogen curve values as those produced following consumption of the poorly absorbed sugar lactulose, indicating that HMOs reach the colon, where they undergo fermentation (Brand-Miller et al. 1995; Brand-Miller et al. 1998).

HMOs have been detected in the plasma (Goehring et al. 2014; Marriage et al. 2015; Ruhaak et al. 2014) and urine of breastfed infants (Chaturvedi et al. 2001; Dotz et al. 2014; Goehring et al. 2014; Jennewein Biotechnologie GmbH 2015; Marriage et al. 2015; Obermeier et al. 2006; Rudloff et al. 1996; Rudloff et al. 2012). Concentrations of intact HMOs in plasma and urine were generally low compared with concentrations in human milk (approximately 0.05 – 0.07% in plasma and 0.5 – 1.5% in urine compared with the concentration in milk or the amount ingested) (Chaturvedi et al. 2001; Marriage et al. 2015; Rudloff et al. 2012).

A recent study with a chemically synthesised form of 2'-FL (produced by Inalco SpA, Milan, Italy) compared the relative extent of absorption and excretion, defined as the concentration

detected in plasma or urine divided by the concentration in infant formula or human milk, in infants fed human milk or infant formula containing 2'-FL at 0.2 or 1.0 g/L (Marriage et al. 2015). The formula also contained 2.2 or 1.4 g/L galactooligosaccharides (GOS), respectively, to provide a total oligosaccharide concentration of 2.4 g/L. Comparisons on day of life 42 found similar relative absorption values in plasma for infants fed human milk or formula containing 2'-FL, ranging from 0.05-0.07%. Relative 2'-FL excretion in the urine was also similar, ranging from 1.26-1.50%. However the total amount of 2'-FL consumed, absorbed and eliminated in the urine was not reported. Concentrations of 2'-FL measured in plasma and urine samples from infants fed control formula, formula containing 2'-FL or human milk are shown in [Table 3.7](#).

**Table 3.7 2'-FL concentrations (mg/L) in feeds, plasma and urine in infants fed control formula, formula containing 2'-FL<sub>chem</sub> or human milk (Marriage et al. 2015)**

| Day of life | Medium     | Control formula*   | Experimental formula 1* | Experimental formula 2* | Human milk        |
|-------------|------------|--------------------|-------------------------|-------------------------|-------------------|
| 42          | Feed (n)   | 0**                | 200                     | 1000                    | 1980 ± 170 (76)   |
| 42          | Plasma (n) | < 0.03 ± 0.01 (36) | 0.13 ± 0.02 (32)        | 0.52 ± 0.07 (33)        | 1.00 ± 0.17 (36)  |
| 42          | Urine (n)  | 0.08 ± 0.01 (59)   | 3.00 ± 0.33 (54)        | 12.60 ± 1.92 (61)       | 35.55 ± 6.89 (58) |
| 119         | Feed       | 0                  | 200                     | 1000                    | Not tested        |
| 119         | Plasma (n) | < 0.03 ± 0.00 (12) | 0.05 ± 0.01 (12)        | 0.29 ± 0.09 (14)        | 0.43 ± 0.17 (11)  |
| 119         | Urine (n)  | 0.09 ± 0.01 (53)   | 2.88 ± 0.71 (45)        | 11.18 ± 1.95 (45)       | 19.52 ± 4.51 (54) |

Data represent the mean ± the standard error of the mean (SEM)

\* Containing GOS at 2400 (Control formula), 2200 (Experimental Formula 1) or 1400 (Experimental Formula 2) g/L

\*\* No 2'-FL was added to the control formula

Several studies have shown the elimination of intact HMOs including 2'-FL and LNnT as well as degradation products in the faeces of breastfed infants (Albrecht et al. 2010; Albrecht et al. 2011a; Albrecht et al. 2011b; Chaturvedi et al. 2001; Coppa et al. 2001). In one study of a mother-infant pair, LNnT was not detected in the infant's faeces, although it was present in the mother's human milk (Davis et al. 2016).

Coppa et al. (2001) reported that the pattern of HMOs in the faeces of six breastfed infants was similar to that of their mother's milk, with the exception of an almost complete lack of lactose, present in mothers' milk, in the faeces of all infants. The amount of milk ingested over a 24 hour period was calculated by weighing the infant before and after each feeding, and faeces samples were collected during the same period. About 40-50% of the total ingested oligosaccharides was found in the faeces. For the majority of the ingested oligosaccharides, including 2'-FL and LNnT, the amount excreted in the faeces ranged from 35-65% of the amount ingested.

In a study of 16 breastfed infants aged 2-19 weeks, Chaturvedi et al. (2001b) estimated that approximately 97% of the HMOs consumed were excreted intact in the faeces, based on

assumptions of a mean milk consumption of 1050 mL/day and a mean faecal output of 120 g/day.

Analysis of the faeces from three infants found an average 75.3% decrease in the abundance of intact HMO from the first week of life to week 17 (Davis et al. 2016). This fall was accompanied by an increased abundance of digested HMOs. Digestion studies *in vitro* using a glycosidase from *Bifidobacterium longum* subsp. *longum* showed similar digestion products to those in faeces, suggesting that the digested oligosaccharides found in faeces corresponded to the action of glycosidases on HMOs. In a study of 10 breast feeding infants, a gradual fall in faecal HMOs over the first three to four months of life was observed, with an accompanying increase in oligosaccharide metabolic products (Albrecht et al. 2011a).

Although the available studies provide only limited quantitative information, the data indicate that absorption of HMOs is very limited. A large proportion of ingested HMOs including 2'-FL and LNnT pass to the large intestine, where they are fermented by the intestinal microbiota or excreted intact in the faeces.

### **3.2.4 Toxicological studies with 2'-FL**

#### ***Subchronic toxicity studies with 2'-FL***

*Studies conducted with the applicant's 2'-FL<sub>micro</sub>*

*13-week oral toxicity study in juvenile rats (Penard 2015)*

This study was conducted in compliance with OECD principles of GLP and OECD TG 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents) modified to use rat pups (starting postnatal day 7).

Juvenile Wistar (CrI:WI(Han)) rats (10/sex/group) were administered 2'-FL produced by fermentation (Batch number 00215, purity 97.6% [as 2'-FL]) by oral gavage from 7 days of age for 13 weeks. Doses of 0 (vehicle control (water)), 2000, 4000 and 5000 mg/kg bw/day were administered, and an additional group received 5000 mg/kg bw/day fructooligosaccharide (FOS), an oligosaccharide already permitted in infant formula, as a reference compound. Recovery groups of 5/sex/group were also included for all treatments to evaluate the possible regression of any signs of toxicity following a 4 week treatment-free period.

Morbidity/mortality checks were performed at least twice daily. Clinical observations were made daily, with a full clinical examination performed weekly. Ophthalmological examinations were performed in the controls, the high dose group and reference compound group during Week 13. Body weights were recorded at least twice weekly during the first eight weeks of treatment and then weekly up to the end of the treatment period or the end of the treatment-free period. Food consumption was measured for each cage of animals from the end of weaning at three weeks of age (study day 14). Physical development (pinna unfolding, eye opening, incisor eruption and left tibia length) and sexual maturation were evaluated. Reflex tests (surface righting reflex, gripping reflex, papillary reflex and auditory reflex) and neurotoxicity tests (water maze and open field) were performed. Blood and urine were collected for clinical laboratory determinations after 13 weeks of dosing (study day 91 for males or 92 for females) and from recovery animals during Week 13 (study day 119).

Animals that died during the study were necropsied and all surviving animals were killed and necropsied at the end of the treatment period or treatment-free period. Selected organs were weighed and organ/tissue samples fixed and preserved. Selected organs/tissues from the

control and high dose group animals killed at the end of the treatment period and from all animals found dead were examined histopathologically.

Two deaths occurred during the study. One male given 2'-FL at 4000 mg/kg bw/day that displayed abnormal behaviour on placement in the water maze on day 50 was removed from the water and found dead in its cage later that day. One female given FOS at 5000 mg/kg bw/day was found dead after treatment and the open field test on day 65. It had blood on the mouth and nose. Respiratory tract changes histologically correlated with moderate or marked multifocal alveolar haemorrhage/oedema were observed in both animals. Both deaths were considered to be accidental, most likely due to drowning following the water maze test for the male or related to the administration procedure (gavage) for the female. There were no treatment-related deaths in any group.

No treatment-related effects on body weight, body weight gain or food consumption were seen in any 2'-FL treated group. The majority of pups given 2'-FL at 4000 and 5000 mg/kg bw/day and those given FOS had frequent liquid faeces and/or soiled urogenital region before weaning. These clinical signs were not seen in control animals or those given 2'-FL at 2000 mg/kg bw/day. Hypersalivation, abnormal foraging and/or pedalling were observed in the reference compound group and in the 2'-FL groups at 4000 and 5000 mg/kg bw/day from day 35 but mainly on day 70 and later, the incidence being highest for the 2'-FL high dose group. Only one male and one female in the low dose group showed these signs on one occasion, and none in the control group. In the absence of other adverse changes these minor clinical signs were not considered to be adverse.

No treatment-related effects were observed on mean tibia length, reflex and physical development, sexual maturation or upon ophthalmological examination. There was no evidence of treatment-related effects on learning capacity, memory or motor activity in the water maze test, and no effects on exploratory behaviour and general movement were observed in the open field test.

There were no treatment-related effects on haematology, coagulation, serum clinical chemistry and urinary parameters. A number of changes that reached statistical significance compared with controls were observed, including slightly reduced red blood cell and white blood cell counts, haemoglobin and packed cell volume in females administered 4000 and 5000 mg/kg bw/day, slightly prolonged prothrombin time in both sexes at 4000 and 5000 mg/kg bw/day, as well as slightly lower triglyceride and cholesterol concentrations in males at all doses and lower triglyceride concentrations in females given 4000 and 5000 mg/kg bw/day. These variations and other statistical differences between treated and control groups were not considered to be of toxicological relevance because they were of low magnitude and mostly within the laboratory's historical control data range.

No changes in organ weights and no histopathological alterations associated with administration of the test substance were observed.

The no observable adverse effect level (NOAEL) for 2'-FL in this study was 5000 mg/kg bw/day, the highest dose tested.

#### *Studies conducted with other 2'-FL preparations*

*13 week oral toxicity study with Glycom's 2'-FL<sub>chem</sub> in juvenile rats (Coulet et al. 2014; Manciaux 2012)*

The oral toxicity of 2'-FL<sub>chem</sub> (Batch no. L06112K) was assessed in a 90 day study in Wistar (CrI: WI(Han)) rats. The study was performed under GLP conditions in accordance with OECD TG 408, with a modification to include the use of neonatal rats. The purity of the test

substance was reported as 99.9%, however subsequent re-analysis indicated that the purity was 96.9%.

Rat pups (10/sex/group) were administered 2'-FL at doses of 0 (water vehicle control), 2000, 5000 or 6000 mg/kg bw/day by oral gavage from postnatal day (PND) 7 for 13 weeks. A comparative control group was administered FOS at 6000 mg/kg bw/day. Additional groups of recovery animals (5/sex/group) were included that received the vehicle, 2'-FL or FOS (both at 6000 mg/kg bw/day) for 13 weeks and were then assessed following a 4 week treatment-free period.

Morbidity/mortality checks and clinical observations were performed at least twice daily. A full clinical examination was performed weekly. Ophthalmological examinations were performed on animals in the control, high dose 2'-FL and FOS groups during week 13. Individual body weights were recorded twice weekly during the first 8 weeks of treatment, and once weekly thereafter. Food consumption was measured twice weekly for each cage of animals starting from weaning at three weeks of age (i.e. from study day 14) and until nine weeks of age (i.e. eighth week of treatment) and then once weekly. Clinical laboratory determinations were performed during week 14. All pups that died during the study were necropsied. All surviving pups were killed at the end of the treatment period or after the 4 week treatment-free period and necropsied. Selected organs/tissue from the control, high dose and reference control groups were examined histopathologically.

One male and one female given 6000 mg/kg bw/day of 2'-FL were found dead on day 2 of the study and two males given FOS were found dead on day 12 or day 13. One female given FOS was found dead during the treatment-free period. The cause of death could not be determined after gross or histopathological investigations, and a relationship to treatment could not be excluded.

Coloured/liquid faeces were noted for a few animals given 2000 mg/kg bw/day of 2'-FL and for all animals given 5000 and 6000 mg/kg bw/day, and those given FOS, from day 0 up to day 12/13. These were associated with erythema in the urogenital area for most animals given 6000 mg/kg bw/day 2'-FL and FOS. Marked hypersalivation was noted mainly on days 57 and 71, 1 hour after dosing, for half of the animals given 2'-FL at 5000 mg/kg bw/day and for most animals given 6000 mg/kg bw/day of 2'-FL or FOS.

A transient lower mean body weight gain compared with controls was observed between days 0 and 3 in animals given 5000 or 6000 mg/kg bw/day of 2'-FL (reductions of 21% and 41%, respectively in males and 31% and 37% in females). A slight difference in mean body weight (ranging from -11% on day 10 to -2% on day 84) persisted throughout the treatment period in males given 2'-FL at 6000 mg/kg bw/day and resulted in a minimally lower (-2%) mean body weight on day 90 compared with vehicle controls. Females given 2'-FL had a mean body weight similar to or higher than that of vehicles controls at the end of the dosing period.

No treatment-related changes in food consumption, ophthalmology, haematology, serum clinical chemistry, coagulation and urine parameters were observed in treated groups. Statistically significant reductions in red blood cells, haemoglobin and packed cell volume were observed in female rats given 5000 or 6000 mg/kg bw/day of 2'-FL compared to vehicle controls, however these changes were small in magnitude (< 6%) and within the range of the test facility's historical negative control data. A statistically significant reduction in serum bilirubin was observed in males given 6000 mg/kg bw/day 2'-FL, however this was within the range of historical control data and not considered to be adverse. Statistically significant reductions in serum protein and globulin were observed in males given 5000 and 6000 mg/kg bw/day 2'-FL, however these changes were small in magnitude (< 10%) and not considered adverse.

Minimal cortical tubular epithelial cytoplasmic vacuolation in the kidney was seen with a higher incidence in females given 5000 or 6000 mg/kg bw/day of 2-FL or FOS at the end of the dosing period. As this was also seen in controls after the treatment-free period and was not associated with any relevant clinical pathology changes or histological evidence of degeneration, it was of unclear origin but not considered to be adverse. Relative kidney weight in females was lower than controls at 6000 mg/kg bw/day of 2'-FL, but this was not considered likely to be correlated with the vacuolation, as the vacuolation was also seen in animals given FOS without any changes in kidney weight.

The NOAEL of 2'-FL in this study was 5000 mg/kg bw/day, based on the unexplained deaths that occurred at 6000 mg/kg bw/day.

*90-day oral toxicity study with 2'-FL<sub>micro</sub> (produced by FrieslandCampina) in rats (van Berlo et al. 2018)*

A 90-day dietary toxicity study was conducted with FrieslandCampina's 2'-FL<sub>micro</sub> in juvenile rats. The study was performed under GLP conditions in accordance with OECD TG 408. The test item had a purity of 94%. Wistar Han IGS rats (CrI:WI(Han)), 10/sex/group, were administered 2'-FL via the diet from PND 25 for 90 days at concentrations of 0, 3, 6 and 10% (w/w). Mean intake of 2'-FL was calculated to be 2170, 4270 and 7250 mg/kg bw/day for males and 2450, 5220 and 7760 mg/kg bw/day for females in the low, mid and high dose groups, respectively.

Animal condition and behaviour were monitored twice daily with more detailed clinical observations conducted weekly. A functional observation battery and motor activity assessment was performed in all animals 12 weeks after initiation of treatment. Ophthalmological examinations were conducted on the control and high dose animals in the last week of the exposure period. Body weight was recorded weekly and food consumption was measured twice weekly. Blood samples were collected at necropsy for haematology and clinical chemistry evaluations, and urine was collected from all animals in the final week of the study. Macroscopic examination was performed at necropsy and organ weights were determined. Histopathological examination was performed on organs and tissues from animals in the control and high dose group, and from animals that died during the study.

No treatment-related mortality or clinical signs were observed. One female animal in the mid dose group died in the fourth week of the study. No cause of death could be established following histopathological examination of organs and tissues from this animal. As no further deaths occurred in any dose group the mortality was not considered to be due to 2'-FL exposure. Detailed clinical observations, the functional observational battery and the motor activity assessment did not reveal any neurotoxic potential of 2'-FL. No exposure-related changes were found in the ophthalmological evaluation. There were no significant differences in body weights between the control and test groups throughout the course of the study. Food consumption was significantly decreased in females in the high dose group, but the reduction was slight (< 10%) and body weights were not affected. No treatment-related adverse effects on haematology, clinical chemistry and urinalysis parameters were observed. Relative liver weights were significantly increased (+8%) in high dose males, but in the absence of accompanying histopathological changes or alterations in clinical chemistry this was not considered to be an adverse effect. Caecal enlargement was noted in all groups of treated males and in females given the mid and high dose of 2'-FL. This finding was considered to be a physiological response to consumption of the test substance, which is a non-digestible carbohydrate. No exposure-related macroscopic or microscopic histopathological changes were reported.

The NOAEL of 2'-FL in this study was the highest concentration tested, corresponding to 7250 mg/kg bw/day in males and 7760 mg/kg bw/day in females.

*7 day pilot oral toxicity study with 2'-FL<sub>micro</sub> (produced by Jennewein) in rats (Jennewein Biotechnologie GmbH 2015)*

A pilot study was conducted with Jennewein's 2'-FL<sub>micro</sub> in preparation for a 90 day toxicity study. The test item had a purity of 96.0% (Batch no. 2FL-2013-36-22). Two groups of five female rats (CD®/CrI:CD(SD)) were administered 0 or 10% 2'-FL in the diet for seven days.

None of the rats given 2'-FL died or showed clinical signs of toxicity during the treatment period. Body weight and feed consumption were similar in control and treated animals.

*90 day oral toxicity study with 2'-FL<sub>micro</sub> (produced by Jennewein) in rats (Jennewein Biotechnologie GmbH 2015)*

The test item in this study was Jennewein's 2'-FL<sub>micro</sub> and had a purity of 94.1% (Batch no. 2FL-2013-43-2632). The study was conducted in accordance with GLP and following OECD TG 408, although only one dose of the test item was used in the study. Four-week old CD® CrI:CD rats (10/sex/group) were administered 2'-FL in the diet at concentrations of 0 or 10% for 90 days. Additional groups of satellite animals of 3 and 9 animals per sex were included in the control and treatment groups, respectively, and were used for blood sampling for evaluation of toxicokinetics. Checks for mortality/morbidity and clinical signs were made daily and a detailed clinical examination was performed once per week. Neurological screening, including assessment of sensory reactivity, grip strength and locomotor activity, was conducted in the final week of the study. Urine and blood samples were collected from the main study animals prior to necropsy at the end of the study. Blood samples for toxicokinetics were taken from control and treated satellite animals on at least two time points on study day 1/2 and from treated satellite animals in test week 13.

The mean dietary intake of 2'-FL over the 13 weeks of the study was 7660 mg/kg bw/day in males and 8720 mg/kg bw/day in females. Absorption of 2'-FL was demonstrated in this study, as discussed in the [section on absorption, distribution, metabolism and excretion earlier in this document](#).

All animals survived until the end of the study, and no treatment-related clinical signs were observed. No effects of 2'-FL on body weight, body weight gain, food and drinking water consumption, haematological and biochemical parameters, urinary status, the eyes, organ weights and the myeloid:erythroid ratio were observed. The neurological screening did not identify any test item-related changes, and there were no macroscopic and/or microscopic changes in organs and tissues.

The NOAEL in this study was a 10% concentration of 2'-FL in the diet, equivalent to average doses of 7660 mg/kg bw/day in males and 8720 mg/kg bw/day in females.

*3 week oral toxicity study with 2'-FL<sub>micro</sub> (produced by Jennewein) in neonatal piglets (Hanlon and Thorsrud 2014)*

The safety of Jennewein's 2'-FL<sub>micro</sub> has also been evaluated in a neonatal piglet model, conducted in accordance with GLP. The 2'-FL used in this study had a purity of 97.9% 2'-FL (Lot no. 2013-50-3436).

A total of 27 male and 21 female two-day old Domestic Yorkshire Crossbred Swine were available for inclusion in the study. The piglets had been allowed to nurse from the sow during the first two days after birth, and the piglets received injections of iron supplement and antibiotics according to standard practices. Due to the imbalance in the numbers of male and female piglets available at the start of the study, animals were assigned to treatment groups so as to ensure that the control and high dose groups had an equal distribution of males and

females. As a result, 6, 8, 7 and 6 male piglets and 6, 4, 5 and 6 female piglets were assigned to receive liquid diets containing 0, 200, 500 and 2000 mg/L of 2'-FL, corresponding to 0, 29, 72 and 292 mg/kg bw/day, respectively, in males and 0, 29, 74 and 299 mg/kg bw/day, respectively, in females. The test vehicle and control was a commercially available milk replacer. Piglets were administered the liquid diet via a feeding bowl from age two days for three weeks.

All animals were monitored for morbidity, mortality, injury and availability of food twice daily, and a detailed clinical examination was performed twice weekly. Food consumption was measured daily, and body weights were recorded daily for the first week and every other day thereafter. Blood samples were collected from all animals prior to dosing on study days 7 and 21. Gross necropsies were performed on all animals following termination on study day 22, and microscopic evaluations of selected organs and tissues were conducted.

All animals survived to scheduled necropsy and no definitive test article-related changes in clinical observations were recorded during the course of the study. There were no differences in body weight gain or feed consumption between the control and treatment groups. Watery faeces were noted in 3/6 males and 2/6 females given 2000 mg/L 2'-FL, in 1/7 males and 2/5 females in the 500 mg/L group and in 2/8 males and 2/4 females in the 200 mg/L group. This was not considered to be test article-related based on the lack of dose-response and the absence of any associated pathological findings.

One male and two females in the 2000 mg/L group had a lack of appetite on one day of the study, and one female in the 200 mg/L dose showed a lack of appetite for two days during the study. The specific days were not reported. As there was no dose response and no impact on growth or feed intake, this observation was not considered to be adverse.

There were no test article-related effects on haematology, coagulation, urinalysis and clinical chemistry parameters. Male piglets in the 2000 mg/L dose group had statistically significant increased alanine aminotransferase (ALT) concentrations on day 7 and day 21 compared with controls. This was not considered to be treatment related or toxicologically relevant as there were no significant differences in other clinical chemistry markers of toxicity, no effects on liver weight and no histopathological changes in the liver.

No adverse macroscopic or histopathological changes were observed. Animals in all treatment groups had variable, minimal to mild, focal, acute inflammation within the keratinised portion of the nonglandular stomach and variable thickness of the keratinised portion. As some degree of this finding was observed in animals in all groups, there was no clear dose-response and similar findings were not observed in the stomach, this finding was not considered to be clearly treatment related. As noted by the study authors, similar findings have been observed in control piglets of the same age in previous studies at the same test facility (Mahadevan et al. 2014).

It was concluded that dietary exposure to 2'-FL at concentrations up to 2000 mg/L was well tolerated by neonatal farm piglets and did not result in adverse health effects or impact piglet growth. The NOAEL for 2'-FL in this study was 2000 mg/L, equivalent to 292 mg/kg bw/day in males and 299 mg/kg bw/day in females.

### ***Chronic and carcinogenicity studies with 2'-FL***

No chronic or carcinogenicity studies of 2'-FL were submitted in the application or located from other sources. Such studies are not considered to be necessary because the results of genotoxicity assays are negative and there is no evidence from subchronic studies of lesions that could lead to neoplasia through nongenotoxic mechanisms.

## **Genotoxicity Studies with 2'-FL**

*Studies conducted with the applicant's 2'-FL<sub>micro</sub>*

*Bacterial reverse mutation assay (Verspeek-Rip 2015)*

The study was conducted in compliance with Good Laboratory Practice (GLP) and following OECD Test Guideline (TG) 471.

The test material for this experiment had a purity of 97.6% (as 2'-FL; Batch no. 00215), and the vehicle and negative control was water. The bacterial strains tested were *Salmonella typhimurium* TA1537, TA98, TA1535 and TA100, and *Escherichia coli* strain WP2uvrA. The test was initially conducted by the plate incorporation method and then repeated using the pre-incubation method, and both assays were performed in the presence and absence of metabolic activation (S9 mix). Based on the findings of a dose range finding study all assays were conducted with concentrations up to 5000 µg 2'-FL /plate. The positive controls in the assays without S9 mix were sodium azide for strain TA1535, 2-nitrofluorene (NF) for TA98, methylmethanesulfonate (MMS) for TA100 and 4-nitroquinoline N-oxide (4-NQO) for WP2uvrA. For TA1537, the positive control was ICR-91 in the plate incorporation assay and NF in the pre-incubation assay. The positive control in the assays conducted in the presence of S9 was 2-aminoanthracene (2AA) for all bacterial strains.

No increase in the number of revertant colonies was observed at any of the concentrations of 2'-FL tested in any of the tester strains, in either of the two experiments. Negative control values were within the laboratory historical control ranges, while the positive controls produced the expected increases in revertant colonies, confirming the validity of the test system. It was concluded that 2'-FL was not mutagenic under the conditions of this study.

*In vitro micronucleus assay with human lymphocytes (Verbaan 2015)*

This study was conducted in compliance with GLP and following OECD TG 487.

The test material for this experiment had a purity of 97.6% (as 2'-FL; Batch no. 00215), and the vehicle and negative control was water. The assay was conducted using cultured peripheral human lymphocytes obtained from healthy, non-smoking volunteers (one volunteer per test). Concentrations of 0, 512, 1600 and 2000 µg 2'-FL/mL were used, based on the results of a dose range finding test. Two cytogenetic tests were conducted. In the first assay, cells were treated with 2'-FL for 3 hours in the presence or absence of S9 fraction, then cultured in fresh medium and harvested 27 hours after the beginning of treatment. In the second assay, cells were exposed to 2'-FL for 24 hours in the absence of S9 and then harvested. In both of these assays cytochalasin B was added to the cultures to prevent cell division. Positive control clastogens were mitomycin C or cyclophosphamide in the absence or presence of metabolic activation, respectively. Colchicine was used as a positive control for aneugenicity in the absence of metabolic activation. Assays were conducted in duplicate with 1000 mononucleated and 1000 binucleated cells scored for micronuclei in each replicate (2000 of each in total).

2'-FL did not induce a statistically significant increase in the number of mono- or binucleated cells with micronuclei in the absence or presence of metabolic activation in either of the two tests. The numbers of 2'-FL-treated cells with micronuclei were also within the laboratory historical negative control data range. The positive controls induced the expected increases in the number of cells with micronuclei. The test conditions were therefore considered to be acceptable.

It was concluded that 2'-FL was not clastogenic or aneugenic in human lymphocytes under the conditions of this study.

#### Studies conducted with other 2'-FL preparations

Genotoxicity studies have also been conducted with Glycom's 2'-FL<sub>chem</sub>, as well as 2'-FL<sub>micro</sub> produced by Jennewein and FrieslandCampina. These studies were GLP compliant and conducted according to appropriate test guidelines.

As summarised in [Table 3.8](#), 2'-FL showed no evidence of mutagenic, clastogenic or aneugenic activity in these assays.

**Table 3.8 Summary of genotoxicity studies with other 2'-FL preparations**

| Test  | Test system   | Test article  | Concentration or dose range  | Result                               | Reference                             |
|---|---|---|--|--------------------------------------|---------------------------------------|
| <b>2'-FL<sub>chem</sub></b>   |   |   |  |                                      |                                       |
| Bacterial reverse mutation (Ames Test; OECD TG 471)   | <i>S. typhimurium</i> strains TA98, TA100, TA1535, TA1537 & TA102 | 2'-FL (96.9% purity <sup>#</sup> ; Batch No. L06112K)<br><br>Vehicle: water | Plate incorporation test: 52 – 5000 µg/plate<br><br>Pre-incubation test: 492 – 5000 µg/plate   | Negative ± S9<br><br>No cytotoxicity | (Chalendard 2011; Coulet et al. 2014) |
| <i>In vitro</i> mammalian cell gene mutation (mouse lymphoma assay; OECD TG 476 [now TG 490]) | L5178Y TK <sup>+/-</sup> mouse lymphoma cells                     | 2'-FL (96.9% purity <sup>#</sup> ; Batch No. L06112K)<br><br>Vehicle: water | Short term exposure (4 h): 492 – 5000 µg/mL<br><br>Long term exposure (24 h): 1.7 – 5000 µg/mL | Negative ± S9<br><br>No cytotoxicity | (Chalendard 2011; Coulet et al. 2014) |
| <i>In vitro</i> mammalian cell micronucleus assay (OECD TG 487)                               | Cultured human lymphocytes  | 2'-FL (96.9% purity; Batch No. L06112K)<br><br>Vehicle: RPMI 1640 medium    | 512 – 2000 µg/mL   | Negative ± S9<br><br>No cytotoxicity | (Verbaan 2015)                        |
| <b>2'-FL<sub>micro</sub> produced by Jennewein</b>  |   |   |  |                                      |                                       |
| Bacterial reverse mutation (Ames Test; OECD TG 471)   | <i>S. typhimurium</i> strains TA98, TA100, TA1535, TA1537 & TA102 | 2'-FL (92.4% purity; Batch No. 2FL-2013-43-                                 | 31.6 – 5000 µg/plate   | Negative ± S9<br><br>No cytotoxicity | (Jennewein Biotechnologie GmbH 2015)  |

| Test   | Test system  | Test article   | Concentration or dose range   | Result  | Reference                            |
|--|--|--|---|---|--------------------------------------|
|  |  | 2632R)   |   |   |                                      |
|  |  | Vehicle:<br>DMSO   |   |   |                                      |
| <i>In vivo</i> mammalian erythrocyte micronucleus test (OECD TG 474) | Rats (CrI:CD (SD))<br><br>PO, gavage<br><br>24 and 48 h sampling times | 2'-FL (92.4% purity; Batch No. 2FL-2013-43-2632R)<br><br>Vehicle: 0.8% hydroxymethyl-cellulose | Preliminary toxicity test: 500, 1000 & 2000 mg/kg bw<br><br>Main study: 500, 1000 & 2000 mg/kg bw | Negative<br><br>No clinical signs of toxicity | (Jennewein Biotechnologie GmbH 2015) |

#### 2'-FL<sub>micro</sub> produced by FrieslandCampina

|   |   |  |                    |                                      |                         |
|---|---|--|--------------------|--------------------------------------|-------------------------|
| Bacterial reverse mutation (Ames Test; OECD TG 471)             | <i>S. typhimurium</i> strains TA98, TA100, TA1535, TA1537'<br><br><i>E. coli</i> strain WP2 <i>uvrA</i> | 2'-FL (94% purity; Batch No. not reported)<br><br>Vehicle: Phosphate Buffered Saline | 62 – 5000 µg/plate | Negative ± S9<br><br>No cytotoxicity | (van Berlo et al. 2018) |
| <i>In vitro</i> mammalian cell micronucleus assay (OECD TG 487) | Cultured human lymphocytes  | 2'-FL (94% purity; Batch No. not reported)<br><br>Vehicle: RPMI1640                  | 500 – 2000 µg/mL   | Negative ± S9<br><br>No cytotoxicity | (van Berlo et al. 2018) |

# In the majority of toxicity studies with chemically synthesised 2'-FL the purity was reported to be 99.9%. However, subsequent re-analysis of the test material using an improved analytical method and a purer analytical reference standard indicated that the purity of the batch used in the toxicity studies (L06112K) was 96.9%.

### Allergenicity

No detectable proteins are found in the 2'-FL<sub>micro</sub> ingredient. 2'-FL<sub>micro</sub> is specified to contain <0.01% protein, and batch analyses have found protein levels to be below the limit of quantification (0.0017%). Therefore 2'-FL is unlikely to pose an allergenicity concern.

### 3.2.5 Toxicological studies with LNNt

#### ***Subchronic toxicity studies with LNNt***

*Studies conducted with the applicant's LNNt<sub>micro</sub>*

*13-week oral toxicity study in juvenile rats (Penard 2016)*

The oral toxicity of LNNt<sub>micro</sub> (Batch no. 2547750901; 97.9% purity as human-identical milk saccharides; 94.4% as LNNt) was assessed in a 13-week study in Wistar (CrI: WI(Han)) rats. The study was performed under GLP conditions in accordance with OECD TG 408, with an adaptation to include the use of neonatal rats, starting from PND 7.

Juvenile rats (10/sex/group) were administered LNNt by oral gavage from 7 days of age for 13 weeks. Doses of 0, 1000, 2500 and 5000 mg/kg bw/day were administered, and an additional group received 5000 mg/kg bw/day FOS as a reference compound. The vehicle control was water. Additional recovery groups of 5/sex/group were included for all treatments to evaluate the possible regression of any signs of toxicity following a 4 week treatment-free period.

Animals were observed daily for morbidity/mortality and clinical signs, with a full clinical examination conducted weekly. Body weights were recorded at least twice weekly during the first eight weeks of treatment and weekly for the remainder of the study. Food consumption was recorded twice weekly for the first six weeks of treatment, starting after weaning (study day 15) and then weekly. Ophthalmological examinations were performed on the control animals and those given 5000 mg/kg bw/day LNNt or FOS once during week 13. Physical development (pinna unfolding, eye opening, incisor eruption and left tibia length) and sexual maturation (vaginal opening or preputial cleavage) were evaluated. Reflex tests (surface righting reflex, gripping reflex, pupillary reflex and auditory reflex) and neurotoxicity tests (water maze and open field) were performed. Blood and urine samples were collected from all main study animals in week 13. All animals were necropsied at the end of the study, and microscopic examinations were conducted on selected organs/tissues from animals given the vehicle control or the high dose of LNNt, as well as animals that died before the end of study.

There were no treatment-related deaths during the study. One control female was killed on study day 20 due to an enlarged and protruding left eye, which correlated histologically with minimal retrobulbar fibroplasia. One female in the group administered FOS was found dead on day 28. The cause of death was considered to be accidental, as the animal was trapped between the cage and the cage rack. Microscopic examination revealed minimal, bilateral tubular degeneration in the kidneys which may have been secondary to the trauma.

No treatment-related clinical signs were observed during the study. Hypersalivation was seen for one male (one occasion) and five females (one occasion for four females and on two occasions for the other) given 5000 mg/kg bw/day LNNt. As these were isolated occurrences they were considered to be unrelated to LNNt administration.

LNNt did not have an effect on body weight, food consumption, ophthalmology, haematology, serum chemistry or urinalysis parameters. While some statistically significant differences were observed in haematology and clinical chemistry parameters they were small in magnitude, did not show a dose-response relationship and were inconsistent between the sexes. Therefore they were considered to be unrelated to LNNt administration. Statistically significant increases in urine volume and decreases in specific gravity were observed for males and females given 5000 mg/kg bw/day LNNt compared with controls. In the absence of any microscopic renal changes these differences were considered to be incidental.

There was no effect of LNNt treatment on reflex and physical development, sexual maturation or performance in neurotoxicity tests.

At necropsy there were no test-item related effects on organ weights and no macroscopic or microscopic findings considered to be related to LNNt administration.

The NOAEL for LNNt in this study was 5000 mg/kg bw/day, the highest dose tested.

#### *Studies conducted with other LNNt preparations*

##### *14-day dose range finding study with LNNt<sub>chem</sub> in juvenile rats (Coulet et al. 2013)*

LNNt<sub>chem</sub> was investigated in a series of toxicity studies with juvenile rats. Initially, a 14 day non-GLP dose range finding study was performed, in which Wistar IGS:CrI:WI Han rats (5/sex/group) were administered 0, 1000, 2500 or 5000 mg/kg bw/day of LNNt (98.9% purity, Batch no. L01032K) by gavage from PND 7 until weaning on PND 20. The vehicle control was water. An additional reference control group was administered FOS at 5000 mg/kg bw/day, on the basis that it is already used in infant formula.

Animals were observed twice daily for mortality and morbidity, and clinical observations were performed daily. Detailed clinical examinations were made weekly and body weights were measured at intervals over the course of the study. All animals were euthanised on PND 21 and macroscopic examinations were performed.

No mortality and no abnormalities in clinical signs, body weights and necropsy were observed in rats administered LNNt. The NOAEL for LNNt in this study was 5000 mg/kg bw/day, the highest dose tested.

##### *28-day oral toxicity study with LNNt<sub>chem</sub> in juvenile rats (Coulet et al. 2013; Séret 2012)*

Juvenile Wistar IGS:CrI:WI Han rats (10/sex/group) were administered Glycom's LNNt<sub>chem</sub> (98.9% purity, Batch no. L01032K) by gavage at doses of 0, 1000, 2500 and 5000 mg/kg bw/day for 28 (males) or 29 (females) days, starting from PND 7. The study was performed under GLP conditions and following OECD TG 407 modified to use rat pups. The vehicle control was water, and an additional group of rats were administered FOS (5000 mg/kg bw/day) as a reference item. Additional groups of five males and five females were administered 0 or 5000 mg/kg bw/day of LNNt or FOS for 28/29 days, followed by a 14 day treatment-free period.

Rats were observed twice daily for morbidity and mortality during the treatment period, and clinical signs were recorded daily before and at least once after dosing. During the treatment-free period clinical signs were observed once daily. A full clinical examination was performed weekly. Body weights were assessed prior to dosing, every 2 days until weaning on PND 21 and twice weekly thereafter. Food and water intake were also recorded twice weekly after weaning. Ophthalmology was performed on all animals in the control, high dose LNNt and FOS groups on study day 24, blood and urine samples were collected at the end of the administration or treatment-free periods, and animals were killed and necropsied. Histopathological examinations were conducted on all organs and tissues from animals in the control, high dose LNNt and FOS groups killed at the end of the treatment period, and for the animal that died during the study.

There were no LNNt-related deaths during the study. One female treated with FOS died on day 4. There were no remarkable macroscopic or microscopic findings and the cause of death was not determined. In addition, two control males and two males given 1000 mg/kg

bw/day LNnT died just after blood sampling on the day of necropsy. This was considered to be due to the high volume of blood (3.5 mL) taken from each animal.

The majority of animals given FOS had erythema on the urogenital area during the first week of treatment and all animals in the FOS group had soft/liquid/coloured (yellow) faeces on some occasions during the first two weeks. One male treated with 5000 mg/kg bw/day LNnT had decreased activity and piloerection on day 14, and some males and females in the high dose group had soft/coloured faeces on some occasions during the first two weeks of the study. These findings were isolated and not considered to be adverse. No treatment-related clinical signs were observed during the treatment-free period.

There were no treatment-related changes in food or water consumption, and body weight and body weight gain were similar in control and LNnT-treated animals. There were no treatment-related ophthalmological findings.

At the end of the treatment period total white blood cell, absolute lymphocyte and absolute monocyte counts were statistically significantly higher in females administered LNnT at 5000 mg/kg bw/day compared with controls. Percentages of lymphocytes and monocytes were not altered. At the end of the treatment-free period white blood cell and absolute lymphocyte counts were statistically significantly lower in females given 5000 mg/kg bw/day LNnT compared with controls. These changes were not observed in males and were considered by the study authors to be incidental and not adverse since they were slight in nature. Values in treated animals were also within the historical negative control ranges for the test facility.

In males given 2500 or 5000 mg/kg bw/day LNnT, statistically significant decreases in mean corpuscular volume and percentage reticulocyte counts were observed compared with controls. A statistically significant increase in mean corpuscular haemoglobin concentration was seen in males given 5000 mg/kg bw/day LNnT compared with controls. These changes were small in magnitude and not associated with any clinical or histopathological alterations, and so were not considered to be of toxicological relevance.

Mean total protein, albumin and globulin serum concentrations were slightly lower for females treated with LNnT at 5000 mg/kg bw/day, and in males and females administered FOS. However, the albumin/globulin ratio was comparable between control, LNnT and FOS groups and there were no associated changes at the macroscopic or microscopic evaluation.

There were no treatment-related changes in coagulation or urine parameters in any group, and no treatment-related changes in organ weight, macroscopic or microscopic evaluations were observed. Lobular degeneration/atrophy of acinar cells was reported in 1/10 males treated with 5000 mg/kg bw/day of LNnT. This change was considered to be unrelated to the test item and part of the background seen in Wistar rats. A minimal increase in zymogen content of acinar cells was noted focally in 2/10 females given the high dose of LNnT. Due to the focal distribution of the finding and its low incidence and severity, it was considered to be incidental and related to individual variation.

The NOAEL for LNnT in this study was considered to be 5000 mg/kg bw/day, the highest dose tested.

#### *13-week oral toxicity study with LNnT<sub>chem</sub> in juvenile rats (Coulet et al. 2013; Leroy 2011)*

Juvenile Wistar Crl:WI(Han) rat pups (15/sex/group) were administered Glycom's LNnT<sub>chem</sub> (98.9% purity, Batch no. L01032K) by oral gavage at doses of 0, 1000, 2500 or 5000 mg/kg bw/day from PND 7 for 13 weeks. An additional reference group of 15 male and 15 female rats was administered FOS at 5000 mg/kg bw/day. Five males and five females in each group were assigned to undergo a 4-week treatment-free period at the end of the

administration period. The study was conducted in compliance with OECD principles of GLP and in accordance with OECD TG 408 modified to use rat pups (starting PND 7).

Morbidity and mortality checks were performed at least twice daily and clinical signs were recorded daily. A detailed clinical examination was performed weekly. Body weights were recorded twice weekly and food consumption was determined weekly for each cage of animals after weaning on PND 21. An ophthalmological examination was performed during the last week of treatment on all animals in the control, high dose LNnT and FOS groups that were scheduled to be killed at the end of the treatment period. Blood and urine samples were scheduled to be collected at the end of the treatment and treatment-free periods prior to termination. However, samples were not collected from rats in the 1000 and 2500 mg/kg bw/day LNnT treatment groups due to an oversight in the original study plan. Therefore urine and blood samples were also collected from recovery animals in all groups nine days after the last day of dosing (study day 100). All animals found dead were necropsied, and all surviving animals were killed at the end of the treatment or treatment free period and necropsied. Selected organs/tissues from the control, high dose LNnT and FOS groups, as well as all animals found dead, were examined histopathologically. The spleen, thymus and pancreas from all animals in the low and mid dose groups were also microscopically examined.

One male and one female given 2500 mg/kg bw/day of LNnT were found dead on day 14 of treatment, and one female given FOS was found dead on day 13. Although the cause of death in these animals could not be clearly established, they were not considered clearly related to treatment given the lack of a dose-response. The study authors considered that possible intubation error or trauma during dosing was likely to have contributed to the deaths of these animals.

The majority of pups given FOS had yellow liquid faeces, often accompanied by erythema of the urogenital region, in the period before weaning. These signs were generally no longer observed after weaning. Yellow liquid faeces, soft faeces or erythema of the urogenital region were also occasionally seen in animals given 5000 mg/kg bw/day LNnT before weaning. The incidence of these signs in the control animals and those given 1000 or 2500 mg/kg bw/day LNnT were very low. No other treatment-related clinical signs were observed.

No significant effects of LNnT on body weight, body weight gain or food consumption were observed. Male and female pups given 5000 mg/kg bw/day of FOS showed a transient marked reduction in body weight gain over the first three days of dosing compared with controls, but this recovered and no significant differences were observed by the end of the treatment or treatment-free period.

A persistent, but slight, reduction in total white blood cell count was noted at all sampling times in all groups of males given either FOS or LNnT. The effect was generally more marked with LNnT than with FOS regardless of dose level. The low WBC counts were mainly due to a reduction in neutrophils, though minor reductions in lymphocytes and monocytes were noted in the LNnT-treated groups. In females, effects on white blood cells were restricted to statistically significantly lower white blood cell and lymphocyte counts at the two highest dose levels after nine days of recovery, but not in the high dose group at the end of the treatment period or in any group after the full treatment-free period. These differences did not show a clear dose response, were generally within historical control ranges and were not correlated with any histopathological changes. Therefore the effects were not considered to be adverse.

Other changes in haematology parameters at the end of the treatment period were statistically significant reductions in mean haemoglobin concentration and packed cell volume in females given 5000 mg/kg bw/day LNnT, compared with controls. However, the

individual values for both parameters generally remained within historical control values, with the exception of haemoglobin values for two females given 5000 mg/kg bw/day LNNt. In males, statistically significantly lower reticulocytes and platelet counts were observed in the high dose LNNt and FOS groups compared with controls at day 90. No significant changes in reticulocytes were observed in any group on day 100 or at the end of the treatment-free period, and the only statistically significant effect on platelet count was a decrease in males given 1000 mg/kg bw/day, compared with controls, on day 100. The haematological changes in males were also close to or within the historical control range, and the individual data did not suggest a treatment-related effect.

In serum clinical chemistry, slight but statistically significant reductions in cholesterol were seen at all sampling times in all groups of males given LNNt or FOS. Females were not affected. Urea concentrations were reduced at the end of the dosing period in males given FOS or LNNt, but the difference did not persist in the recovery group. Females in all groups given LNNt had an increased urea concentration on day 100, but not at the other sampling times. These changes were generally within historical control values and were not of sufficient magnitude to be considered adverse.

Urine analysis did not indicate any adverse effects of treatment with LNNt or FOS.

No treatment related lesions were found at necropsy, and organ weights were similar in all treated and control groups. At the end of the treatment period a decreased zymogen content was observed in pancreatic acinar cells at a minimal or slight severity in two males and one female receiving 5000 mg/kg bw/day of LNNt. At the end of the treatment-free period, this change was found at minimal severity in one male and one female previously treated with LNNt at 5000 mg/kg bw/day, and one male and one female previously treated with FOS. Due to the low incidence and severity of this finding, and its occurrence in FOS-treated animals only in the recovery phase, this finding was considered to be incidental.

The NOAEL of LNNt in this study was 5000 mg/kg bw/day, the highest dose tested. The study authors noted that LNNt was clinically better tolerated than FOS at the same dose level, based on the reduced level of gastrointestinal signs during the first two weeks of treatment with LNNt, and the lack of a transient reduction in body weight gain.

*28-day oral toxicity study with LNNt<sub>micro</sub> produced by coupled fermentation with yeast/E. coli in rat pups (Prieto 2005)*

LNNt<sub>micro</sub> produced by a coupled yeast/*E. coli* system was assessed in a 28-day study in immature rats. The journal article reporting this study does not indicate whether the study was conducted under GLP conditions, or whether any specific test guideline was followed.

Twelve litters (5/sex/litter) of CrICD®BR rat pups, approximately 15 days old, were assigned to receive 0, 10, 200 or 400 mg/kg bw/day LNNt (purity not reported) by oral gavage for 28 days. Urinalysis, haematology and faecal analysis were performed throughout the study, and anatomical pathology of all pups was performed at the end of the study. There were no significant differences in any of the parameters measured.

*Dietary toxicity study with LNNt<sub>micro</sub> produced by coupled fermentation with yeast/E. coli in rats (Prieto 2005)*

LNNt<sub>micro</sub> produced by a coupled yeast/*E. coli* system was also tested in a dietary toxicity study in which 31 – 37 day old rats (sex, strain and number not reported) were fed diets containing 1.0 or 5.0% LNNt (purity not reported). Food consumption and body weights were not reported but using default dose conversion factors (WHO 2015) these would be equivalent to doses of 1000 and 5000 mg/kg bw/day, respectively. The duration of this study

is unclear, as the methods section of the journal article reporting this study states that LNnT-containing diets were administered for four weeks, whereas the results section refers to the study as a four month study. It is not stated whether a control group was included in this study, and there is no indication as to whether the study was GLP or in accordance with any test guidelines.

No adverse effects were reported in rats fed either concentration of LNnT. There were no macroscopic or microscopic changes that appeared to be related to treatment with LNnT.

*Reviewers comment:* This study was not considered suitable for regulatory purposes due to limitations in reporting.

### **Chronic and carcinogenicity studies with LNnT**

No chronic or carcinogenicity studies of LNnT were submitted in the application or located from other sources. Such studies are not considered to be necessary because the results of genotoxicity assays are negative and there is no evidence from subchronic studies of lesions that could lead to neoplasia through nongenotoxic mechanisms.

### **Genotoxicity studies with LNnT**

*Studies conducted with the applicant's LNnT<sub>micro</sub>*

*Bacterial reverse mutation assay (Verspeek-Rip 2016)*

The test material for this assay had a purity of 94.4% (as LNnT, Batch No. 2547750901) and the vehicle and negative control was water. The study was conducted in compliance with Good Laboratory Practice (GLP) and following OECD Test Guideline (TG) 471.

The bacterial strains tested were *S. typhimurium* TA1535, TA1537, TA100 and TA98, and *E. coli* strain WP2uvrA. The test was initially conducted by the plate incorporation method and then repeated using the pre-incubation method, and both assays were performed in the presence and absence of metabolic activation (S9 mix). Based on the findings of a dose range finding study all assays were conducted with concentrations up to 5000 µg LNnT/plate. Positive controls in the assays without S9 mix were sodium azide for strain TA1535, NF for TA98, MMS for TA100 and 4-NQO for WP2uvrA. For TA1537, the positive control was ICR-91 in the plate incorporation assay and NF in the pre-incubation assay. In the presence of S9 the positive control was 2AA for all strains.

LNnT did not induce an increase in the number of revertant colonies in any strain in either the plate incorporation or pre-incubation assay, with and without metabolic activation. Acceptable responses were obtained with the negative and positive controls, demonstrating the validity of the test system. It was concluded that LNnT was not mutagenic under the conditions of this study.

*In vitro micronucleus assay with human lymphocytes (Verbaan 2016)*

The test material for this experiment had a purity of 94.4% (as LNnT, Batch no. 2547750901), and the vehicle and negative control was water. The study was conducted in compliance with GLP and following OECD TG 487.

The assay was conducted using cultured peripheral human lymphocytes obtained from healthy, non-smoking volunteers (one volunteer per test). Concentrations of 0, 512, 1600 and 2000 µg LNnT/mL were used, based on the results of a dose range finding test. Two cytogenetic assays were conducted. In the first test, cells were treated with LNnT for 3 hours

in the presence or absence of S9 fraction, then cultured in fresh medium and harvested 27 hours after the beginning of treatment. In the second assay, cells were exposed to LNNt for 24 hours in the absence of S9 and then harvested. In both of these assays cytochalasin B was added to the cultures to prevent cell division. Positive control clastogens were mitomycin C or cyclophosphamide in the absence or presence of metabolic activation, respectively. Colchicine was used as a positive control for aneugenicity in the absence of metabolic activation. Assays were conducted in duplicate with 1000 mononucleated and 1000 binucleated cells scored for micronuclei in each replicate (2000 of each in total).

LNNt did not induce a statistically significant increase in the number of mono- or binucleated cells with micronuclei in the absence or presence of metabolic activation in either of the two experiments. The numbers of LNNt-treated cells with micronuclei were also within the laboratory historical negative control data range. The positive controls induced the expected increases in the number of cells with micronuclei, and the test conditions were therefore considered to be acceptable.

It was concluded that LNNt was not clastogenic or aneugenic in human lymphocytes under the conditions of this study.

#### *Studies conducted with other LNNt preparations*

A series of *in vitro* genotoxicity studies with Glycom's LNNt<sub>chem</sub> have also been conducted (summarised in [Table 3.9](#)). These studies were GLP compliant and conducted according to appropriate test guidelines. LNNt showed no evidence of mutagenic, clastogenic or aneugenic activity in these assays.

**Table 3.9 Summary of genotoxicity studies with other LNNt preparations**

| Test  | Test system   | Test article   | Concentration or dose range  | Result                               | Reference                             |
|---|---|--|--|--------------------------------------|---------------------------------------|
| <b>LNNt<sub>chem</sub></b>  |   |  |  |                                      |                                       |
| Bacterial reverse mutation (Ames test; OECD TG 471)   | S. <i>typhimurium</i> strains TA98, TA100, TA1535, TA1537 & TA102 | LNNt (98.9% purity; Batch No. L01032K)<br><br>Vehicle: water | Plate incorporation test: 52 – 5000 µg/plate<br><br>Pre-incubation test: 492 – 5000 µg/plate   | Negative ± S9<br><br>No cytotoxicity | (Chalendard 2010; Coulet et al. 2013) |
| <i>In vitro</i> mammalian cell gene mutation (mouse lymphoma assay; OECD TG 476 [now TG 490]) | L5178Y TK <sup>+</sup> mouse lymphoma cells                       | LNNt (98.9% purity; Batch No. L01032K)<br><br>Vehicle: water | Short term exposure (4 h): 418 – 4250 µg/mL<br><br>Long term exposure (24 h): 1.4 – 4250 µg/mL | Negative ± S9<br><br>No cytotoxicity | (Chalendard 2011; Coulet et al. 2013) |
| <i>In vitro</i> mammalian cell  | Cultured human lymphocytes  | LNNt (98.9% purity;  | 512 – 2000 µg/mL   | Negative ± S9                        | (Verbaan 2015)                        |

| Test                             | Test system | Test article       | Concentration or dose range | Result          | Reference |
|----------------------------------|-------------|--------------------|-----------------------------|-----------------|-----------|
| micronucleus assay (OECD TG 487) |             | Batch No. L01032K) |                             | No cytotoxicity |           |
|                                  |             | Vehicle: DMSO      |                             |                 |           |

### Allergenicity

No detectable proteins are found in the LNnT<sub>micro</sub> ingredient. The specification for protein content of LNnT<sub>micro</sub> is < 0.01% protein, and batch analyses have found protein levels to be below the limit of quantification (0.0017%). Therefore LNnT is unlikely to pose an allergenicity concern.

### 3.2.6 Human studies with 2'-FL and/or LNnT

#### Clinical studies in infants

*Infant study with 2'-FL<sub>chem</sub> in combination with LNnT<sub>chem</sub> (Puccio et al. 2017)*

Glycom's 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub> were evaluated in a multi-centre, randomised, double-blind placebo-controlled trial of two parallel groups of formula-fed infants. Healthy infants of ≤ 14 days of age were randomly assigned to consume a standard cow's milk-based infant formula containing long-chain polyunsaturated fatty acids without added oligosaccharides, with or without supplementation with 2'-FL and LNnT at concentrations of 1.0-1.2 g/L and 0.5-0.6 g/L of reconstituted formula, respectively<sup>14</sup>. Randomisation was stratified to ensure a balance of infant sex and delivery method (vaginal or caesarean) between groups. Infants were scheduled to receive the test (n = 88) or control (n = 87) formula up to six months of age, when infants in both groups were switched to an intact protein, cow's milk-based follow-up formula without the two HiMOs for feedings up to 12 months of age. The study was powered to detect a difference of 3 g/day in bodyweight gain as statistically significant with a one-sided test. The infants were exclusively fed the test or control formula for the first 4 months of age, at which point complementary foods were allowed to be introduced. The study had two populations: an intention-to-treat (ITT) population which included all infants randomised to study formula, and a per-protocol (PP) population which included infants with none of the following major protocol violations before age four months: being hospitalized for >3 consecutive days one week before age four months, being off study formula for ≥3 consecutive days, and taking ≥4 teaspoons of complementary food per day. The primary outcome was body weight gain through to age four months. Secondary outcomes included other anthropometric measures (weight, length, body mass index (BMI), head circumference and corresponding z scores), digestive tolerance (flatulence, spitting-up and vomiting), stool characteristics, behaviour patterns and morbidity, which were monitored up to 12 months of age.

The six-month intervention was completed by 64 infants in the control group and 58 in the test group, and 64 and 57 infants, respectively, completed the 12-month study. The drop-out rate was similar in both groups. Mean daily formula intake was similar between groups at all study visits. As noted in [Section 3.3](#), mean body weight gain at age four months in the test group was not significantly different to those in the control group in both the ITT and PP

<sup>14</sup> Analytical results indicated concentration ranges in test formula of 1.04-1.143 g/L (SD 0.073 – 0.08 g/L) for 2'-FL and 0.52-0.61 g/L (SD 0.028-0.033 g/L) for LNnT.

study populations. No differences in the mean weight, length, head circumference and BMI or in weight-for-age, length-for-age, head circumference-for age or BMI-for-age z-scores were noted at any visit during the study. The least square mean (SE) weight gain from enrolment through to age 4 months was 29.39 (0.538) g/day for infants receiving the test formula and 29.53 (0.541) g/day for infants receiving the control formula. These growth data also tracked closely with WHO growth standards.

Stool consistency scores were similar in both the test and control formula-fed infants at most time points, although the test group had a tendency towards softer stools at one month and significantly softer stools at two months (graded on the Bristol scale score as 6.1 in the control group versus 5.7 in the test group,  $p = 0.021$ ). No significant differences in stool frequency were reported at any visit.

The overall incidence of adverse events was not significantly different between the control and test formula-fed groups. However, when adverse events were organised by the Preferred Term, bronchitis was reported more frequently in the control group than in the test group at 4 months (12.6% vs 2.3%; OR 0.16,  $p = 0.01$ ), 6 months (21.8% vs 6.8%, OR 0.26, 95% CI, 0.08-0.74  $p = 0.005$ ), and 12 months (27.6% vs 10.2%, OR 0.30, 95% CI 0.11-0.73,  $p=0.004$ ) and in the subgroup of Caesarean-born infants at 12 months (6.3% in Test vs. 31.3% in Control, OR 0.15, 95% CI 0.01-0.81,  $p=0.022$ ). An analysis of the adverse events by adverse event cluster and delivery method also revealed a significantly higher incidence of lower respiratory tract infections among infants delivered by Caesarean section assigned to the control formula at 6 months (28.1% vs 6.3%, OR 0.17; 95% CI, 0.02-0.96,  $p = 0.043$ ) compared to those receiving test formula. At 12 months, this difference persisted among infants delivered by Caesarean section (40.6% vs 12.5%, OR 0.21; 95% CI 0.04-0.83,  $p = 0.022$ ) and was also observed in the overall population (24.5% vs 19.3%, OR 0.45; 95% CI 0.21-0.95,  $p = 0.027$ ). Infants receiving the test formula had significantly fewer reports of the use of antipyretics during the four month exclusive formula-feeding period, although no statistically significant differences were seen at 6 or 12 months. Infants receiving the test formula had significantly fewer reports of antibiotic use at 6 months or 12 months, but not at 4 months.

It was concluded that infant formula supplemented with 2'-FL and LNnT was safe and well tolerated under the conditions of this study (refer to [Section 4.3.5](#) for further details).

#### *Infant study with 2'-FL<sub>chem</sub> in combination with galactooligosaccharides (Marriage et al. 2015)*

The safety of 2'-FL<sub>chem</sub> produced by Inalco SpA, Milan, Italy was assessed in a prospective, randomised, controlled, growth and tolerance study in healthy, full-term singleton infants. Infants were enrolled by day of life (DOL) 5 and randomised to one of three formulas: a control formula containing 2.4 g/L GOS, one containing 0.2 g/L 2'-FL and 2.2 g/L GOS (Experimental formula 1), or one with 1.0 g/L 2'-FL and 1.4 g/L GOS (Experimental formula 2). All formulas had a caloric density of 64.3 kcal/dL and their composition was similar to a milk-based commercially available formula. An additional group of infants were fed human milk. Infants were exclusively fed formula ( $n=189$ ) or human milk ( $n=65$ ) from enrolment to DOL 119. The primary endpoint was body weight gain from DOL 14-119, with secondary endpoints including measures of other anthropometric parameters, tolerance and adverse events. Levels of 2'-FL in blood and urine were assessed in a subset of infants at DOL 42 and 119, and in human milk collected from breast feeding mothers at DOL 42.

There were no significant differences in mean weight, length or head circumference between any of the groups, and no significant differences in mean gains in these measures from DOL 14 to 119. From enrolment to DOL 28 the percentage of feedings with spitting up or vomit within one hour of feeding was significantly higher in all three of the formula-fed groups compared with the group given human milk ([Table 3.10](#)). There were no differences between

any of the groups in feedings with spitting up or vomit after DOL 28. Repeated measure analysis during the DOL 42, 84 and 119 visits found that the mean rank stool consistency was significantly greater (i.e. harder stools) for the formula-fed groups compared with the human milk-fed group (Control formula > human milk,  $p = 0.004$ ; Experimental formula 1 > human milk,  $p = 0.001$ ; Experimental formula 2 > human milk,  $p = 0.009$ ), but there was no significant difference among the three formula-fed groups (specific scores not reported). There were no significant differences in the overall percentage of infants with adverse events or serious adverse events in the control formula group compared with those given formula containing 2'-FL. A comparison with the incidence of adverse events in infants fed human milk was not reported.

**Table 3.10 Percent of feedings with spitting up or vomit within 1 hour of feeding (Marriage et al. 2015)**

| Control formula<br>(2.4 g/L GOS) | Experimental<br>formula 1<br>(0.2 g/L 2'-FL; 2.2<br>g/L GOS) | Experimental<br>formula 2<br>(1.0 g/L 2'-FL; 1.4<br>g/L GOS) | Human milk |
|----------------------------------|--|--|------------|
| 17.5 ± 2.6 %*                    | 21.5 ± 2.9*  | 18.0 ± 2.5*  | 10.5 ± 1.6 |

Values represent mean ± SEM

\* Formula-fed versus human milk-fed infants:  $p \leq 0.05$

2'-FL was detected in the urine and plasma of infants fed 2'-FL containing formula and in infants fed human milk. Further details can be found in the [section on absorption, distribution, metabolism and excretion studies](#).

The authors concluded that all of the formulas were well tolerated in this study, and no safety concerns were identified.

The effects of 2'-FL supplementation on biomarkers of immune function in a self-selected (i.e. maternally selected) subset of the infants included in this study have been reported in a paper by Goehring et al. (2016). This study is reviewed in [Section 4.4.1](#).

*Infant study with 2'-FL in combination with short-chain fructo-oligosaccharides (Kajzer et al. 2016)*

A summary of results of a prospective, randomised, multi-centre, double-blind controlled tolerance study with an infant formula containing 2'-FL (source unspecified) in combination with short-chain fructo-oligosaccharides (scFOS) is available in a conference abstract. A total of 121 infants enrolled at age 0-8 days were allocated to receive a milk-based infant formula that did (n=46) or did not contain 2 g/L scFOS and 0.2 g/L 2'-FL (n=42). A third group of infants were fed with human milk (n=43) as a comparison. Infants were exclusively fed formula or human milk from enrolment until 35 days of age. Data relating to intake, stool patterns, anthropometrics and parental questionnaires were collected. Further experimental details, including the source of the 2'-FL used in the study, are not reported in the abstract.

There were no significant differences in the mean rank stool consistency or the predominant stool consistency between the three groups. The average number of stools per day was significantly higher in the human milk-fed group than in those given either form of infant formula ( $p < 0.0001$ ; stool numbers not reported). No significant differences in formula intake, number of formula feedings per day, anthropometric data or percent feedings with spit-up or vomit were observed. The study authors concluded that the formula containing 2'-FL and scFOS was well tolerated in young infants.

### *Infant study with LNnT<sub>micro</sub> from coupled fermentation with yeast/E.coli (Prieto 2005)*

A randomised, double-blind placebo controlled study in human infants was conducted with LNnT<sub>micro</sub> produced by a coupled yeast/*E. coli* fermentation process. In this study 228 children (111 females and 117 males) aged 6-24 months were assigned to receive infant formula containing 220 mg LNnT/L or a control formula without LNnT *ad libitum* for 112 days. Consumption was recorded by parents and day care centre workers and verified by formula monitors. Oropharyngeal swabs were carried out every two weeks to determine the effects of LNnT on oropharyngeal colonisation with *Streptococcus pneumoniae*. Children were also monitored for an exploratory variable, 'ear status'. For this, children were assessed for otitis media or other signs of inflammation or disruption of the ear canal, and classified as 'abnormal ear', 'normal ear' or 'indeterminable'. 'Indeterminable' was assigned when the child was either unwilling or unavailable to undergo ear examination.

There were no statistically significant differences in formula ingestion volumes between the control and the LNnT group. No statistically significant differences in body weight or body length were observed, and there was no significant difference in the colonisation rate of *S. pneumoniae*. The statistical power of the study was not reported. Children in the LNnT group had a statistically significant lower rate of abnormal ears compared with children who received the control formula, but this was not significant once antibiotic administration was considered as a covariate in the analysis. The author concluded that LNnT was well tolerated by children at a concentration of 220 mg/L.

### **Clinical studies in children**

#### *Child study with 2'-FL<sub>chem</sub> alone and in combination with LNnT<sub>chem</sub> (Holm 2018)*

An interim report of a randomised, placebo-controlled, double-blind parallel study with Glycom's 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub> involving 75 children admitted to a hospital childhood obesity programme was submitted by the applicant. Children (25 per group) were randomised to consume an oral bolus dose of 4.5 g placebo (glucose), 2'-FL or a 4:1 mixture of 2'-FL and LNnT once daily for 8 weeks. Bowel habits were monitored by daily completion of the Bristol Stool Form Scale (BSFS) and faecal samples were collected from the children at the start of the intervention and after 4 and 8 weeks of treatment. Gastrointestinal symptoms were measured using the Gastrointestinal Symptom Rating Scale (GSRS) at the beginning of the study and at weeks 4 and 8, and blood samples for clinical chemistry and haematology parameters were collected at the start of the study and at end of the 8-week intervention period. Effects on body weight and body composition (% fat mass and fat free mass) were also assessed. This study is ongoing and results of further analyses of the parameters outlined above at selected time periods following the intervention, as well as additional analyses of exploratory blood markers potentially relevant to pathogenesis of obesity, are not yet available.

The number of children that completed the study intervention were 18, 21 and 24 in the placebo, 2'-FL and 2'-FL plus LNnT groups, respectively. GSRS measures indicated that both 2'-FL and 2'-FL plus LNnT were well tolerated, and the proportion of abnormal bowel movements was similar in all three groups. No adverse changes in haematology or clinical chemistry parameters were observed in any treatment group. Levels of faecal calprotectin, a measure of neutrophil migration into intestinal mucosa, were generally well below the upper limit for normal values, and the few observations outside the normal range were equally distributed between groups. Adverse events occurred with a similar frequency in all three groups, and none were considered to be severe or probably related to the investigational product. The group receiving 2'-FL plus LNnT had a higher frequency of adverse events possibly related to the investigational product, mainly driven by two participants who each reported several gastrointestinal symptoms at a single incidence. One of these participant's

symptoms were classified as mild, while the other experienced four moderate adverse events (abdominal pain, diarrhoea, reflux and flatulence). As expected due to the weight loss programme, small changes in anthropometric measures and body composition were observed from baseline to the end of the intervention period. The only statistically significant differences between groups were greater weight loss in the placebo group than in the 2'-FL group (-2.2 kg versus -0.6 kg;  $p = 0.047$ ), and a greater reduction in body mass index Z-score in the placebo compared to the 2'-FL and 2'-FL plus LNnT groups.

FSANZ notes that the report of this study is preliminary, and the applicant has advised that the anthropometry and body composition measures such as body weight and BMI are still undergoing analysis to account for dropout rates and confounding variables such as baseline differences, age and sex.

### ***Clinical studies in adults***

#### *Adult study with 2'-FL<sub>chem</sub> and/or LNnT<sub>chem</sub> (Elison et al. 2016)*

Glycom's 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub> were evaluated in a parallel, double-blind, randomised, placebo-controlled study in 100 healthy adult volunteers. Participants were randomly assigned to consume either 2'-FL or LNnT at doses of 5, 10 or 20 g/day, a 2:1 mix of 2'-FL and LNnT at 5, 10 or 20 g mixture/day, or glucose (2 g/day) as a placebo control. The duration of the intervention was 14 days. All interventions were provided as a daily bolus dose dissolved in water, which participants were instructed to consume at breakfast. All subjects were examined physically at entry to the study and again at the end of the intervention. Blood samples for clinical chemistry and haematology analyses, as well as faecal samples for biomarker and microbiota composition analyses, were collected at baseline and at the end of the intervention period. Adverse events were monitored throughout the study, and participants completed a self-administered Gastrointestinal Symptom Rating Scale (GSRS) form, recorded bowel movement frequency and evaluated stool consistency using the Bristol Stool Form Scale (BSFS).

All participants completed the study according to the protocol without any dropouts. No changes of clinical significance in any physical parameter including pulse rate and blood pressure were found during the 2-week intervention. Clinical chemistry and haematological analyses found no alterations considered to be due to the intake of study products. One participant taking 20 g/day 2'-FL and one taking 5 g/day 2'-FL had a normal glomerular filtration rate (GFR  $\geq 60$ ) at the start of the study but an impaired GFR ( $< 60$ ) at the end. GFR values for each individual were not reported, limiting the ability to interpret this finding which was not discussed by the study authors. All other parameters were within normal ranges for all subjects and any minor changes at the end of the study compared with baseline values were not considered clinically relevant.

A total of 56 adverse events were reported by 44 participants. All were judged as 'mild', with many of the symptoms being common gastrointestinal symptoms including flatulence, bloating and constipation. The authors considered it was difficult to determine whether these were actually related to the study product, part of normal day-to-day variation or a result of increased awareness of gastrointestinal symptoms during the study period.

GSRS scores were low at baseline and remained low at the end of the intervention. Compared with baseline, changes in GSRS within an intervention group were mostly not significant, with a few exceptions. Volunteers taking 20 g of 2'-FL and LNnT combined reported increased bloating and passing of gas. Individuals receiving 20 g of 2'-FL reported increased rumbling while those taking 20 g LNnT reported harder stools. Those receiving 10 g LNnT reported increases in passing gas. Compared with placebo, statistically significant changes in GSRS scores were increased nausea, rumbling, bloating, passing of gas,

diarrhoea, loose stools and urgency to pass stools in individuals receiving 20 g/day 2'-FL, and increased passing of gas in the groups receiving 10 or 20 g/day LNnT. Although these changes were statistically significant, the mean scores remained low (mean score <3; mild discomfort or below). No statistically significant changes in GSRS compared with placebo were found in individuals taking any of the doses of the 2'-FL/LNnT mixture.

Statistically significant changes in stool frequency and consistency at the end of the study compared with baseline were an increased average number of bowel movements in groups taking 20 g/day of 2'-FL, 20 g/day of LNnT and 5 g/day of LNnT, and significantly higher BSFS scores (indicating softer stools) in participants taking 20 g/day of 2'-FL or LNnT. However, these changes were small, were similar to scores in the placebo group and not considered clinically relevant. No significant difference in the average number of bowel movements were observed between the intervention groups and the placebo group. Effects of 2'-FL and/or LNnT on microbiota composition are discussed in [Section 4.2](#).

Overall the results indicate that the consumption of 2'-FL and/or LNnT at the doses tested was safe and generally well tolerated in healthy adults.

### 3.2.7 Discussion

The available data were considered suitable to assess the hazard of 2'-FL and LNnT. This included information on the absorption, distribution, metabolism and excretion of HMOs, *in vitro* and *in vivo* genotoxicity studies, subchronic toxicity studies in juvenile animals and human clinical studies in infants and adults.

2'-FL and LNnT are also naturally present in human milk, providing a history of human exposure to these substances for breastfed infants. The requested maximum concentrations of 2'-FL<sub>micro</sub> (1.2 g/L) and LNnT<sub>micro</sub> (0.6 g/L) in infant formula products, and the 2'-FL concentration used in the dietary intake assessment (based on 2.4 g/L), are within the range of concentrations reported in mature human milk (1.0 – 7.8 g/L for 2'-FL secretors and 0.09 – 1.08 g/L for LNnT; see [Tables 3.13](#) and [3.14](#)).

#### ***Absorption, distribution, metabolism and excretion***

Glycom's 2'-FL and LNnT are structurally and chemically identical to the forms of these substances present in human milk. Therefore no differences in pharmacokinetics between naturally occurring and synthetic forms of these HMOs are expected.

HMOs have been shown to be resistant to hydrolysis by digestive enzymes in *in vitro* studies using human or porcine enzyme preparations or intestinal brush border membranes (Engfer et al. 2000; Gnoth et al. 2000). A large proportion of ingested HMOs including 2'-FL and LNnT pass to the large intestine, where they are fermented by the intestinal microbiota or excreted intact in the faeces.

Small amounts of HMOs have been detected in serum and urine of laboratory animals following oral administration of these substances. In breastfed humans, levels of HMOs in plasma and urine are generally very low in comparison with their concentration in human milk.

A recent study comparing breastfed infants with those consuming infant formula supplemented with a chemically-synthesised form of 2'-FL found no evidence to suggest that absorption or urinary elimination of 2'-FL<sub>chem</sub> is significantly different to that of 2'-FL in human milk.

## **In vitro and animal studies with 2'-FL**

2'-FL<sub>micro</sub> produced by Glycom was not genotoxic in an *in vitro* bacterial mutagenicity assay or an *in vitro* micronucleus assay in human lymphocytes. Other forms of 2'-FL were not mutagenic, clastogenic or aneugenic in a number of *in vitro* and *in vivo* studies.

In subchronic oral toxicity studies in juvenile rats, no adverse effects were seen with Glycom's 2'-FL<sub>micro</sub> or Glycom's 2'-FL<sub>chem</sub> at doses up to 5000 mg/kg bw/day. Unexplained deaths were observed for two animals (one female, one male) aged 9 days with Glycom's 2'-FL<sub>chem</sub> at a dose of 6000 mg/kg bw/day. The cause of death could not be determined after gross or histopathological investigations, and a relationship to treatment could not be excluded. However the deaths were not consistent with the absence of any signs of toxicity in other treated animals, or other studies in the database.

No adverse effects were observed in a 90 day oral toxicity study in which Jennewein's 2'-FL<sub>micro</sub> was administered via the diet to rats, starting from four weeks of age, at doses up to 7660 and 8720 mg/kg bw/day in males and females, respectively.

In a 3 week toxicity study in neonatal piglets given Jennewein's 2'-FL<sub>micro</sub> via a liquid diet, no adverse effects were observed at doses up to 292 mg/kg bw/day in males and 299 mg/kg bw/day in females.

No adverse effects were observed in a 90 day oral toxicity study in juvenile rats with FrieslandCampina's 2'-FL<sub>micro</sub> at doses up to 7250 mg/kg bw/day in males and 7760 mg/kg bw/day in females.

No chronic toxicity/carcinogenicity studies are available, but because 2'-FL is not genotoxic and no lesions that might progress to neoplasia by non-genotoxic mechanisms were observed in subchronic studies, such studies are not considered to be necessary.

2'-FL<sub>micro</sub> does not contain detectable proteins, and is considered unlikely to pose an allergenicity concern.

Based on the available data, the overall NOAEL for 2'-FL is 5000 mg/kg bw/day.

## **In vitro and animal studies with LNnT**

LNnT<sub>micro</sub> and LNnT<sub>chem</sub> produced by Glycom were not genotoxic in an *in vitro* bacterial mutagenicity assay or an *in vitro* micronucleus assay in human lymphocytes. LNnT<sub>chem</sub> was also tested in an *in vitro* mammalian cell gene mutation assay, with negative results.

No adverse effects were observed in subchronic oral toxicity studies in juvenile rats with Glycom's LNnT<sub>micro</sub> or Glycom's LNnT<sub>chem</sub> in juvenile rats at doses up to 5000 mg/kg bw/day.

LNnT is not genotoxic and no lesions that might progress to neoplasia by non-genotoxic mechanisms were observed in subchronic studies. As such carcinogenicity studies are not considered to be necessary.

LNnT<sub>micro</sub> does not contain detectable proteins, and is considered unlikely to pose an allergenicity concern.

Based on the available data, the NOAEL for LNnT is identified as 5000 mg/kg bw/day.

## Human studies

In human studies, infants receiving formula supplemented with both 2'-FL and LNnT at concentrations of 1.0-1.2 g/L and 0.5-0.6 g/L, respectively, for the first six months of life showed age-appropriate increases in body weight and other growth measures (additional analysis and discussion of growth is provided in [Section 3.3](#)). The formula was also well tolerated.

No indications of adverse effects were reported in infant studies with formula supplemented with 0.2 or 1.0 g/L 2'-FL in combination with 2.2 or 1.4 g/L GOS, respectively, or with formula containing 0.2 g/L 2'-FL in combination with scFOS (2 g/L).

No study in infants investigated supplementation of infant formula with 2'-FL in combination with both GOS and scFOS. As such, the tolerance of infants to this combination of added oligosaccharides could not be assessed.

Interim results from a study in obese children aged 5-12 years indicated that a daily dose of 4.5 g 2'-FL or a 4:1 mixture of 2'-FL and LNnT was well tolerated with no adverse effects on haematology or clinical chemistry parameters, or on faecal markers of inflammation.

In a study in adults, consumption of a daily dose of up to 20 g 2'-FL or LNnT alone for two weeks, or a combination of both at a 2:1 ratio up to a total of 20 g/d was well tolerated. Some increases in gastrointestinal symptoms compared with placebo controls were observed in individuals taking 20 g 2'-FL alone, or 10 or 20 g LNnT alone, but the scores for these symptoms were rated as minor discomfort and were not considered to be clinically relevant.

While no clinical studies have been conducted in young children, the available data indicating that 2'-FL and LNnT were well tolerated without adverse effects in infants, children aged 5-12 years and adults provide supporting evidence that the inclusion of 2'-FL alone or in combination with LNnT in supplementary foods for young children is unlikely to result in adverse effects.

Estimated levels of intake are lower for young children than those of 3 month old infants (highest estimated 90<sup>th</sup> percentile (P90) intakes of 660 and 160 mg/kg bw/day for 2'-FL and LNnT, respectively, for infants versus 310 and 77 mg/kg bw/day, respectively, for 2-3 year olds; [Table 3.22](#)). Young children are also expected to be a less sensitive subgroup than young infants, for whom infant formula would be the sole source of nutrition and whose metabolic capacities are not yet fully developed (EFSA Scientific Committee et al. 2017, 2017; IPCS 2009).

There are no clinical studies in infants with 2'-FL alone. However, based on the lack of adverse effects in toxicological studies with 2'-FL at doses up to 5000 mg/kg bw/day, as well as in clinical studies of 2'-FL in combination with LNnT, GOS or scFOS or of 2'-FL alone in children aged 5-12 years, there is no evidence to indicate a toxicological concern for the proposed uses of 2'-FL alone in infant formula, follow-on formula and formulated supplementary foods for young children.

For 2'-FL, the NOAEL of 5000 mg/kg bw/day is more than 7-fold greater than the highest estimated P90 dietary intake for 3 month old infants (660 mg/kg bw/day), 12-fold higher than the highest estimated P90 intake for 12 month old infants (400 mg/kg bw/day), and 16-fold greater than the highest estimated P90 intake for children aged 2-3 years (310 mg/kg bw/day; [Table 3.22](#)). These estimated dietary intakes are based on a 2'-FL level of 2.4 g/L (i.e. 96 mg/100 kJ for infant formula and follow-on formula; 0.56 g/serving for FSFYC).

For LNnT, the NOAEL of 5000 mg/kg bw/day is more than 30-fold greater than the highest estimated P90 dietary intake for 3 month old infants (160 mg/kg bw/day), 50-fold greater than the highest estimated P90 intake for 12 month old infants (100 mg/kg bw/day), and more than 60-fold greater than the highest estimated P90 intake for children aged 2-3 years (77 mg/kg bw/day ; [Table 3.22](#)). These estimated dietary intakes are based on a LNnT level of 0.6 g/L (i.e. 24 mg/100 kJ for infant formula and follow-on formula; 0.14 g/serving for FSFYC).

### 3.2.8 Key findings of toxicological assessment

Based on an assessment of the available toxicological and clinical evidence for 2'-FL and LNnT, it was concluded that there were no public health and safety concerns associated with the addition of 2'-FL, alone or in combination with LNnT, to infant formula products and formulated supplementary foods for young children, at the levels requested by the applicant and at the estimated levels of dietary intake based on 2.4 g/L of 2'-FL and 0.6 g/L of LNnT (see [Section 3.4](#)).

## 3.3 Effect on infant and toddler growth

As children grow relatively slowly in relation to the day-to-day variation in weight and measurement error of weight and height, a study investigating anthropometric outcomes needs to typically run for several months if it is to detect a difference that might be present. Most of the studies reviewed elsewhere in this report have lasted days or weeks. Only five studies conducted in infants and young children which reported anthropometric results were found – one cohort study in breastfed infants and four trials of infant formula containing either or both of the HMOs of interest. Four of these studies had observation periods longer than one month. FSANZ did not find any studies which tested the administration of HMOs via solid food.

### 3.3.1 Cohort studies

Because the manufacture of HMOs is recent, there are no cohort studies comparing the growth of infants who have received these formulas to date. FSANZ found one study that compared the growth of breastfed infants classified in relation to the concentration of 2'-FL in maternal human milk.

Sprenger et al. (2017b) measured the concentrations of 2'-FL and LNnT, and three other HMOs, in human milk samples collected at 30, 60, and 120 days postpartum from 50 Singaporean women who gave birth to singleton infants (25 girls and 25 boys) and intended to breastfeed until the infants were at least four months old. The authors stated that this was an exploratory study and so they had not calculated a sample size in relation to any particular outcome. Anthropometric measures of the breastfeeding infants were recorded at birth and at 30, 60 and 120 days of age. Based on the 2'-FL concentrations in the 30 day milk sample, 16 women were classed as low 2'-FL secretors (mean 0.026 g/L, 95% CI 0.012–0.042 g/L) and 34 as high 2'-FL secretors (mean 2.17 g/L, 95% CI 1.88–2.46 g/L). Among the low secretors, mean 2'-FL concentration declined from 0.026 to 0.011 g/L by 120 days postpartum and LNnT declined from 0.19 to 0.066 g/L. Among the high 2'-FL secretors, mean concentration of 2'-FL declined from 2.17 to 1.38 g/L and the concentration of LNnT declined from 0.26 to 0.11 g/L. At 30 days and 120 days, the ratio of the mean 2'-FL to LNnT concentration was approximately 1:7.3 and 1:6 in the low secretors, and 8.3:1 and 12.7:1 in the high secretors.

Results for mean length, weight, head circumference and body mass index by maternal secretor status were plotted on the sex-specific WHO growth charts and were not significantly different from the chart median at birth and any of the three subsequent ages.

No numerical data were presented for the anthropometric parameters. The authors do not comment on whether any of the infants had commenced eating solids at 4 months. This small study shows no difference in infant growth by maternal 2'-FL secretor status.

### 3.3.2 Clinical trials

Of the four clinical trials identified by FSANZ ([Table 3.11](#)), one was reported only as an abstract (Kajzer et al. 2016). The formulas in this study contained either no added oligosaccharides or 2'-FL (0.2 g/L) in combination with FOS (2 g/L). The authors' stated that there were no significant differences in anthropometric measurements; however, as follow-up finished when the infants were aged 35 days, this study was not of adequate duration to examine whether weight or other anthropometric parameters varied between the two groups.

The other three trials used intervention oligosaccharides added to formula as shown in [Table 3.11](#). Two trials tested the oligosaccharides in infants recruited within 14 days of birth, while the third study recruited children aged 6-24 months in day care centres. Further details of the methods used in these studies are summarised in [Section 3.2.6](#). As noted above, there were no statistically significant differences in anthropometric data in any of the studies.

Marriage et al. (2015) compared formulas containing a total of 2.4 g/L oligosaccharides (0.2 g/L 2'-FL and 2.2 g/L GOS) to a control formula containing 2.4 g/L GOS. The study was powered to detect a 3 g/day or larger difference in body weight change using a 2-sided test. Anthropometric data were presented for only 60% of those randomised and also for the non-randomised breastfed comparison group.

Puccio et al. (2017) compared a formula containing 2'-FL (1.0-1.2 g/L) and LnNT (0.5-0.6 g/L) to a control formula containing no added oligosaccharides in Belgian and Italian babies. The study was powered to detect a difference in bodyweight gain of 3 g or greater per day in the intervention as statistically significantly different from the control group using a one-sided test. They did not justify their decision to use a one-sided rather than a two-sided test. Given that formula-fed infants are typically heavier than breastfed infants (Dewey 1998), it would seem reasonable to have a hypothesis that would allow for an undesirable increase in weight resulting from a new formula. Although there was a 25% loss to follow-up, missing data was imputed to allow an intention-to-treat analysis to be conducted.

Therefore both Marriage et al. (2015) and Puccio et al. (2017) were adequately powered to detect small differences in weight gain. Consequently the finding of no statistically significant difference does not reflect inadequate powering. The exact differences are shown in [Table 3.11](#) and it is evident that clinically relevant differences in growth between the groups do not exist in these two studies. In particular, Puccio et al. (2017) has conducted an intention-to-treat analysis and so more confidence can be placed in their results than the per protocol analysis of Marriage et al. (2015). Puccio et al. (2017) is the only study to use both the oligosaccharides of interest together in infant formula.

In both Marriage et al. (2015) and Puccio et al. (2017) mean z-scores travelled along the WHO growth chart median, which is unexpected as formula fed infants are typically heavier than breastfed infants by 12 months (Dewey 1998). Marriage et al. (2015) and Kajzer et al. (2016), who are from the same research group, used a formula containing 64.3 kcal/dL (269 kJ/100 mL) which Marriage et al. (2015) describe as a "lower calorie formula". The formula used by Puccio et al. (2017) contained 67 kcal/dL (280 kJ per 100 mL). Standard 2.9.1 of the Australia New Zealand Food Standards Code requires infant and follow-on formula to contain "an energy content of no less than 2500 kJ/L and no more than 3550 kJ/L". In 2015, FSANZ conducted a survey of 35 infant formulas for sale in Australia and New Zealand and their energy content ranged between 270 and 294 kJ per 100 mL. Therefore the energy content of the test formulas used by these authors lie within the range

of infant formula used in Australia and New Zealand, albeit in the lower half of the range. This may explain the similarity in the growth of formula fed infants in these studies compared to the WHO growth chart.

The study in infants and toddlers aged 6-24 months described by Prieto (2005) is difficult to interpret because it was conducted by a separate investigator (M. O’Ryan, in Chile in 1998) and it was stated that the study’s methods have been published before but they do not reference this statement. Prieto (2005) states that they are presenting previously unreported results from this trial. The investigator has confirmed that the study was conducted, had negative results, and has not been published (M. O’Ryan, personal communication, 2018).

There are no clinical studies with 2'-FL alone. However, based on the lack of adverse effects on growth in clinical studies of 2'-FL in combination with LNnT, GOS or scFOS, there is no evidence to indicate a nutritional concern for the requested addition of 2'-FL alone to infant formula products and FSFYC. Further, due to the limited oral absorption of 2'-FL and LNnT (as discussed in [Section 3.2.7](#)), no adverse effects on growth are expected at concentrations corresponding to those typically observed in human milk.

### **3.3.3 Key findings of effect on growth**

In summary, trials in infants have tested 2'-FL in combination with either scFOS, GOS, or LNnT. The highest tested concentrations of 2'-FL and LNnT in infant formula were 1.2 and 0.6 g/L, respectively. None of these studies found a difference in growth compared to a control formula. Based on this, and the limited oral absorption of 2'-FL and LNnT, FSANZ concludes that no adverse effects on growth are expected at concentrations corresponding to those typically observed in human milk.

**Table 3.11 Clinical trials in infants and toddlers using formulas containing one or both of 2'-FL and LNnT**

| Reference                             | Age at recruitment;<br>duration of study      | N randomised<br>(control,<br>intervention)   | Added oligo-<br>saccharide<br>content of<br>control<br>formula (g/L) | Concentration in<br>tested infant formula        |               | Power/<br>sample<br>size   | Difference<br>in weight<br>vs control  |
|---------------------------------------|---|--|--|--|---------------|--|--|
|                                       |   |  |  | 2'-FL<br>(g/L)                                   | LNnT<br>(g/L) |  |  |
| Kajzer et al. 2016<br>(Abstract only) | 0-8 days;<br>Followed until 35 days<br>of age | 42/46<br><br>N followed:<br>(36/41) Also 43<br>(42 followed-up)<br>non-randomised<br>breastfed<br>comparison group | 0  | 0.2 plus<br>2 g/L<br>scFOS                       | 0             | Not<br>reported  | “there were no<br>differences<br>between<br>groups for<br>anthropometric<br>data”                  |
| US                                    | 5 days;<br>Followed until 119<br>days of age  | 314 (189 followed-<br>up)<br><br>Also 106 non-<br>randomised<br>breastfed<br>comparison<br>infants                 | 2.4 g/L GOS  | Test<br>formula 1:<br>0.2 plus<br>2.2 g/L<br>GOS | 0             | To detect a<br>3 g/day<br>difference<br>or greater<br>using a 2-<br>sided test | 0.2 g/day less<br>in boys;<br>1 g/day less in<br>girls (not an<br>intention-to-<br>treat analysis) |
|                                       |   |  |  | Test<br>formula 2:<br>1.0 plus<br>1.4 g/L<br>GOS | 0             |  | 1 g/day more<br>in boys,<br>0.3 g/day less<br>in girls (not an<br>intention-to-<br>treat analysis) |

| Reference<br><br>Country where study conducted | Age at recruitment;<br>duration of study            | N randomised<br>(control,<br>intervention)<br><br>N followed-up                            | Added oligo-<br>saccharide<br>content of<br>control<br>formula (g/L) | Concentration in<br>tested infant formula |              | Power/<br>sample<br>size   | Difference<br>in weight<br>vs control   |
|--|---|--|--|---|--------------|--|---|
|  |   |  |  | 2'-FL<br>(g/L)                            | LNT<br>(g/L) |  |   |
| Puccio et al. 2017<br><br>Belgium and Italy    | 0-14 days;<br><br>Followed until 4<br>months of age | 87/88<br><br>N followed-up<br>58/64 although an<br>intention-to-treat<br>analysis was done | 0  | 1.0-1.2                                   | 0.5-0.6      | To detect -<br>3 g/day<br>difference<br>or greater<br>using a 1-<br>sided test | Intervention<br>group gained<br>0.13 g/day less<br>weight (95%<br>CI: -1.63 to<br>1.37) i.e. 13 g<br>over 100 days<br>(intention-to-<br>treat analysis) |
| Prieto 2005<br><br>Chile                       | 6-24 months;<br><br>Duration 16 weeks               | Total 228  | 0  | 0   | 0.2          | Not<br>reported  | LNT group<br>gained 0.07 g<br>less in weight<br>and 0.37 cm<br>less in length<br>than control<br>group over the<br>16 weeks                             |

## 3.4 Dietary intake assessment

### 3.4.1 Approach to estimating dietary intakes of 2'-FL and LNnT

Dietary intake assessments require data on the concentrations of the chemical of interest in the foods requested, including any naturally-occurring sources and any current permissions for additions to food; and consumption data for the foods that have been collected through a national nutrition survey. The dietary intakes of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> were estimated using: (1) a higher use level of 2'-FL than requested in the application based on a similar concentration to human milk, and the requested use level of LNnT, in infant formula, follow-on formula and FSFYC (or 'toddler milks'); (2) model diets for infants aged 3 months, 9 months and 12 months; and (3) food consumption data from the most recent Australian national nutrition survey for children 2-3 years. The dietary intake assessment for children aged 2-3 years was undertaken using FSANZ's dietary modelling computer program, Harvest<sup>15</sup>.

Dietary intakes were also estimated from naturally occurring sources in human milk for comparative purposes.

A summary of the general FSANZ approach to conducting the dietary intake assessment for this application is in [Appendix 1](#). A detailed discussion of the FSANZ methodology and approach to conducting dietary intake assessments is set out in *Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes* (Food Standards Australia New Zealand 2009).

#### **Consumption data used**

The food consumption data used for the dietary intake assessments were:

- 2011-12 Australian National Nutrition and Physical Activity Survey (2011-12 NNPAS), one 24 hour food recall survey of 12,153 Australians aged 2 years and above, with a second 24-hour recall undertaken for 64% of respondents (Australian Bureau of Statistics 2014). Only those respondents who had two days of food consumption data (n=7,735) were used in the assessment of dietary intakes of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub>. This data set was used for the assessment for 2-3 year old children.

The design of this nutrition survey and the key attributes, including survey limitations, are set out in [Appendix 1](#).

The hazard identification and characterisation did not identify any population sub-groups for which there were specific safety considerations in relation to intake of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub>. The population groups that are used for the dietary intake assessment are:

- Infants aged 3 months – representing exclusively formula-fed / breastfed infants
- Infants aged 9 months – representing infants who consume food as well as follow-on formula or human milk
- Infants aged 12 months – representing infants who consume food as well as FSFYC
- Children aged 2-3 years – representing children who consume food as well as FSFYC.

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<sup>15</sup> Harvest is FSANZ's custom-built dietary modelling program that replaced the previous program, DIAMOND.

Model diets were used for the population groups 3 months, 9 months and 12 months, to represent the consumption of infant formula/follow-on formula/FSFYC and solid foods (where appropriate) for these groups. How the model diets were constructed is outlined further below. There are no national consumption data for New Zealand children aged 2-3 years as the 2002 National Children's nutrition survey included respondents from 5 years of age and above. Therefore it is assumed that the model diets for infants, and the assessments for 2-3 year old Australian children are representative of New Zealand infants and young children.

### **Concentrations of 2'-FL and LNnT**

*Proposed concentrations of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> in infant formula, follow-on formula and FSFYC, from the application*

The food categories requested in the application to contain 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> and the proposed maximum use levels are listed in [Table 3.12](#).

**Table 3.12 Proposed maximum use levels of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> in foods, from the application**

| Food   | Maximum use level      |        |                       |        |
|--|------------------------|--------|-----------------------|--------|
|  | 2'-FL <sub>micro</sub> |        | LNnT <sub>micro</sub> |        |
|  | (g/L)                  | (g/kg) | (g/L)                 | (g/kg) |
| Infant formula<br>(as prepared or ready-to-feed)                                       | 1.2                    | 1.15   | 0.6                   | 0.58   |
| Follow-on formula<br>(as prepared or ready-to-feed)                                    | 1.2                    | 1.15   | 0.6                   | 0.58   |
| Infant formula products for special<br>dietary use<br>(as prepared or ready-to-feed)   | 1.2                    | 1.15   | 0.6                   | 0.58   |
| Formulated supplementary foods for<br>young children<br>(as prepared or ready-to feed) | 1.2                    | 1.15   | 0.6                   | 0.58   |

**Note:** 1 litre of infant formula, follow-on formula and FSFYC is equal to 1,050 grams.

### *Concentrations of 2'-FL and LNnT in mature human milk*

The applicant reported the mean and the range of concentrations of 2'-FL<sub>human</sub> and LNnT<sub>human</sub> in samples of mature human milk (10 days post-partum and above). The maximum use levels of 2'-FL<sub>micro</sub> proposed by the applicant are approximately less than or equal to half of the mean concentration of 2'-FL<sub>human</sub> in mature human milk for women who secrete the enzyme α-1,2-fucosyltransferase in their mammary glands ('secretors'): 1.2 g/L ([Table 3.12](#)) and 2.4 – 3.0 g/L ([Table 3.13](#)), respectively. The proposed maximum use levels of 2'-FL<sub>micro</sub> (1.2 g/L) are at the lower end of the range of 2'-FL concentrations in mature human milk (for secretors) (1.0 – 7.8 g/L).

The maximum use levels of LNnT<sub>micro</sub> proposed by the applicant are approximately double the mean concentrations of LNnT<sub>human</sub> in mature human milk: 0.6 g/L ([Table 3.12](#)) and 0.28 – 0.31 g/L ([Table 3.14](#)), respectively. The proposed maximum use levels of LNnT<sub>micro</sub> (0.6 g/L) are within the range of LNnT<sub>human</sub> concentrations in mature human milk (0.04 – 1.08 g/L).

**Table 3.13 Range of 2'-FL<sub>human</sub> concentrations in mature human milk, from 'secretor' milk samples, as provided by the applicant**

| Days post-partum | 2'-FL <sub>human</sub> concentration (g/L) |      | References   |
|------------------|--|------|--|
|                  | Range                                      | Mean |  |
| 10-60            | 1.0 – 7.8                                  | 3.0  | (Bao et al. 2013; Coppa et al. 1999; Coppa et al. 2011; Galeotti et al. 2012, 2014; Hong et al. 2014; Kunz et al. 2017; Leo et al. 2009; McGuire et al. 2017; Olivares et al. 2015; Sprenger et al. 2017b) |
| 60+              | 1.0 – 3.6                                  | 2.4  | (Coppa et al. 1999; Kunz et al. 2017; McGuire et al. 2017; Sprenger et al. 2017b; Thurl et al. 1996)   |

Note: 1 litre of human milk is equivalent to 1,040 grams

**Table 3.14 Range of LNnT<sub>human</sub> concentrations in mature human milk, from pooled milk samples, as provided by the applicant**

| Days post-partum | LNnT <sub>human</sub> concentrations (g/L) |      | References   |
|------------------|--|------|--|
|                  | Range                                      | Mean |  |
| 10-60            | 0.09 – 1.08                                | 0.31 | (Austin et al. 2016; Bao et al. 2013; Chaturvedi et al. 1997; Coppa et al. 1999; Erney et al. 2000; Galeotti et al. 2014; Hong et al. 2014; Kunz et al. 2017; Leo et al. 2009; McGuire et al. 2017; Spevacek et al. 2015; Sprenger et al. 2017b; Sumiyoshi et al. 2003; Thurl et al. 2010) |
| 60+              | 0.04 – 1.08                                | 0.28 | (Asakuma et al. 2011; Austin et al. 2016; Chaturvedi et al. 2001; Coppa et al. 1999; Engfer et al. 2000; Kunz et al. 2017; Leo et al. 2010; McGuire et al. 2017; Nakhla et al. 1999; Smilowitz et al. 2013; Sprenger et al. 2017b; Sumiyoshi et al. 2003; Thurl et al. 1996)               |

**Concentrations of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> in infant formula, follow-on formula and FSFYC used in the dietary intake assessment**

The mean 2'-FL<sub>human</sub> concentration in mature human milk (for secretors at 60+ days) is 2.4 g/L. In this assessment, the maximum concentration of 2'-FL<sub>micro</sub> in infant formula / follow-on formula / FSFYC that has been considered is 2.4 g/L on the basis of considering a similar concentration of 2'-FL in infant formula / follow-on formula / FSFYC as is naturally-occurring in human milk.

The permissions for addition of substances to infant formula and follow-on formula in the Code (Standard 2.9.1) are based on mg/100 kJ units. For FSFYC, existing permissions (Standard 2.9.3) are on a g/serving units basis. Consequently, the proposed maximum levels of 2'-FL<sub>micro</sub> considered in this assessment are 96 mg/100 kJ for infant formula and follow-on formula and 0.56 g/serving for FSFYC (Table 3.15). The concentrations of LNnT<sub>micro</sub> are 24 mg/100 kJ for infant formula and follow-on formula and 0.14 g/serving for FSFYC (Table 3.15).

**Table 3.15 Concentrations of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> in infant formula, follow-on formula and FSFYC used in the dietary intake assessment**

| Product           | Units     | Concentration of 2'-FL <sub>micro</sub> | Concentration of LNnT <sub>micro</sub> |
|-------------------|-----------|---|--|
| Infant formula    | mg/100 kJ | 96                                      | 24                                     |
| Follow-on formula | mg/100 kJ | 96                                      | 24                                     |
| FSFYC             | g/serve   | 0.56                                    | 0.14                                   |

#### *Concentrations of 2'-FL and LNnT in domestic mammalian milk*

Infant formula, follow-on formula and FSFYC are made with domestic mammalian milk bases, particularly cow milk and goat milk. The milk itself and other foods made from cow, sheep and goat milk could be consumed by Australian and New Zealand infants and children aged 1-3 years. Consequently, the sources of naturally occurring 2'-FL and LNnT from domestic mammalian milks were investigated. These are discussed below.

#### **Cows' milk**

Several authors reported that 2'-FL and LNnT are absent in domestic cows' milk (Castanys-Muñoz et al. 2013; Coulet et al. 2013; Tao et al. 2009). However, Aldredge et al. (2013) reported that 0.3% of bovine milk oligosaccharides are 2'-FL and 3.78% are LNnT. This author did not present data on the bovine milk oligosaccharide content of cows' milk. Other literature sources reported that cows' milk contains 0.03 – 0.06 g oligosaccharides per litre (Albrecht et al. 2014; Martín-Ortiz et al. 2016). If the Aldredge et al. (2013) proportions are applied to the total oligosaccharide from Albrecht et al. (2014) and Martín-Ortiz et al. (2016), the concentrations of naturally occurring 2'-FL and LNnT in cows' milk are 0.00009 – 0.00018 g/L and 0.0011 – 0.0023 g/L, respectively.

The highest derived concentration of 2'-FL in cows' milk is approximately 13,330 times lower than the concentration used in the dietary intake assessment permission for 2'-FL<sub>micro</sub> in infant formula / follow-on formula / FSFYC and 13,330 – 16,670 times lower than the mean concentration of 2'-FL<sub>human</sub> in human milk for secretors (Table 3.17).

The derived concentration of naturally occurring LNnT in cows' milk is approximately 265 times lower than the requested permission for LNnT<sub>micro</sub> in infant formula / follow-on formula / FSFYC and 123 – 137 times lower than the mean concentration of LNnT<sub>human</sub> in human milk (Table 3.17).

Due to the low concentrations of 2'-FL and LNnT in cows' milk, there is likely to be minimal contribution of cows' milk, its products (e.g. cheese, yoghurt, cream etc.) and foods made from these foods to naturally occurring 2'-FL and LNnT dietary intakes. The contribution of these foods is discussed in further detail below for 2-3 year old children. The mean and P90 consumption of cows' milk and all of its products, expressed as milk equivalents, for 2-3 year

old Australian children is listed in [Table 3.16](#). All 2-3 year old children are consumers of cows' milk in some form (e.g. as a drink, yoghurt, cheese, as an ingredient in recipes etc.) across the two days of the nutrition survey.

**Table 3.16 Mean and P90 consumption of cows' milk and all of its products, expressed in milk equivalence, for 2-3 year old Australian children**

| Consumption of cows' milk foods,<br>expressed as milk equivalents<br>(grams per day) |       |
|--|-------|
| Mean   | 707   |
| P90  | 1,156 |

### Goat's milk

2'-FL is present in domestic goat milk but not LNnT (Castanys-Muñoz et al. 2013; Urashima et al. 2001; Urashima et al. 2013). Thum et al. (2015) reported the concentration of 2'-FL in goat's milk as  $0.002 \pm 0.001$  g/L. Martín-Ortiz et al. (2016) reported the range of 2'-FL concentrations in goat colostrum samples as 2.2 – 31.6 mg/L (0.0022 – 0.0316 g/L). The average of the concentrations provided is 0.012 g/L.

The Thum et al. (2015) concentration of 2'-FL in goats' milk is 1,200 times lower than the concentration of 2'-FL<sub>micro</sub> proposed in infant formula / follow-on formula / FSFYC and 1,200 – 1,500 times lower than the mean concentration of 2'-FL<sub>human</sub> in human milk. The Martín-Ortiz et al. (2016) average concentration of naturally occurring 2'-FL in goat's milk is 200 times lower than the proposed permission for 2'-FL<sub>micro</sub> in infant formula / follow-on formula / FSFYC and 200 – 250 times lower than the mean concentration of 2'-FL<sub>human</sub> in human milk.

No child aged 2-3 years specifically reported eating goats' cheese or goats' milk either on its own or as an ingredient in mixed foods (e.g. salad) in the 2011-12 NNPAS. The contribution of goats' milk foods to naturally occurring 2'-FL dietary intakes is likely to be minimal.

**Table 3.17 Comparison between mean 2'-FL and LNnT contents of cows' milk, goats' milk and human milk and the proposed permissions in infant formula, follow-on formula and FSFYC**

|  | Oligosaccharide concentration (mg/L) |           |
|--|--------------------------------------|-----------|
|  | 2'-FL                                | LNnT      |
| Cows' Milk                                 | 0.18                                 | 2.27      |
| Goats' Milk                                | 12.1                                 | 0         |
| Infant formula / follow on formula / FSFYC | 2,400                                | 600       |
| Human milk <sup>♦</sup>                    | 2,400 – 3,000                        | 280 – 310 |

♦ Mean concentration, based on data from [Table 3.13](#) and [Table 3.14](#)

### Sheep's milk

2'-FL and LNnT have not been detected in sheep (ovine) milk (Urashima et al. 2001).

### 3.4.2 How were the estimated dietary intakes calculated?

#### *Children aged below 2 years*

As there are no data available from the 2011-12 NNPAS or the New Zealand National Children's Nutrition Survey (2002 NZNNS) for children aged less than 2 years, model diets were constructed to estimate dietary 2'-FL and LNnT intakes for the target groups of children aged 3 months, 9 months and 12 months. The same model diets were used for Australia and New Zealand.

As the 3 month, 9 month and 12 month old infant model diets are based on mean food consumption amounts only, a distribution of food consumption was not available and hence, a distribution of 2'-FL and LNnT dietary intakes was not able to be produced. Therefore, the 90<sup>th</sup> percentile dietary intakes were estimated using the calculation shown in Equation 1.

#### **Equation 1: 90<sup>th</sup> percentile dietary exposure calculation for the 3 month, 9 month and 12 month old infant model diets**

$$90^{\text{th}} \text{ percentile exposure} = \text{mean exposure} \times 2^*$$

\* (World Health Organization et al. 1985)

The energy contents of infant formula, follow-on formula, FSFYC and human milk are required for the calculation of the amount of infant formula / follow-on formula / FSFYC / human milk in the model diets for 3 month, 9 month and 12 month old infants. AUSNUT is the latest nutrient data set published for Australian foods. In this dataset, the energy content of *Infant formula, 6-12 months, prepared with water* is 264 kJ/100 g and *Milk, human/breast, mature, fluid* is 286 kJ/100 g (Food Standards Australia New Zealand 2016). A set of model diets were developed using the AUSNUT energy contents for infant formula / follow-on formula / human milk in the calculation of infant formula, follow-on formula and human milk consumption for 3 month and 9 month old infants ([Table 3.18](#) – [Table 3.21](#)).

A set of model diets was not established for infants consuming infant formula products for special dietary uses as the energy and/or fluid requirements can vary depending on the medical conditions of the infant. Additionally, the energy content of the various infant formula products for special dietary uses can be variable. From an examination of a range of products currently on the market, including formulas for premature infants, formulas for use by infants with inborn errors of metabolism and formulas for use by infants with severe food allergies, the range of energy contents is 269 – 415 kJ/100 g. If an infant consuming infant formula products for special dietary uses has similar energy requirements to those used in the model infant diets and their specific formula has a similar energy content to that used in the model diets, then their intake of 2'-FL and LNnT is anticipated to be similar to that outlined in the assessment below. If an infant consuming infant formula products for special dietary uses has similar energy requirements to those used in the model infant diets and their specific formula has a higher energy content to that used in the model diets, then their intake of 2'-FL and LNnT is anticipated to be similar to or lower than that outlined in the assessment below.

#### *Infants aged 3 months*

The recommended energy intake for a three-month-old boy (343 kJ/kg bw/day) (United Nations University et al. 2004) and the 50<sup>th</sup> percentile weight (6.4 kg) (World Health Organization 2006) for the same age and sex were used as the basis for the model diet. Boys' weights were used because boys tend to be heavier than girls at the same age and

therefore have higher overall energy and food requirements. The entire energy requirement in the 3 month old infant diet is derived from infant formula or human milk, depending on the assessment. The body weight of 6.4 kg was used to estimate dietary intakes for 3 month old infants on a body weight basis.

#### *Infants aged 9 months*

By the age of 9 months, infants are consuming a mixed diet of solids and follow-on formula / human milk. The model diet was constructed based on recommended energy intakes, mean body weight and the proportion of milk and solid foods in the diet for a 9 month old infant. The recommended energy intake for a 9 month old boy (330 kJ/kg bw/day) (United Nations University et al. 2004) and the 50<sup>th</sup> percentile weight (8.9 kg) (World Health Organization 2006) for the same age and sex was used as the basis for the model diet. It was assumed that 50% of energy intake was derived from follow-on formula / human milk and 50% from solids and other fluids (Butte et al. 2004; Hitchcock 1986; Pan American Health Organization 2003). The body weight of 8.9 kg was used to estimate dietary intakes for 9 month old infants on a body weight basis.

#### *Infants aged 12 months*

Infants aged under 12 months should be breastfed or fed a commercial infant/follow-on formula as their main drink, not cow's milk (National Health and Medical Research Council (Australia) 2013). At 12 months of age, infants can drink cows' milk (or other appropriate substitute) as their main drink. 'Toddler milk' / FSFYC is not a requirement for healthy children (National Health and Medical Research Council (Australia) 2013). Infants aged 12 months are consuming a mixed diet. The model diet was constructed based on the recommended energy intake for a 12 month old boy (335 kJ/kg bw/day) (United Nations University et al. 2004) at the 50<sup>th</sup> percentile weight (9.6 kg) (World Health Organization 2006) for the same age and sex. It was assumed that 35% of energy intake was derived from FSFYC and 65% from solids and other fluids (Hitchcock 1986). It was also assumed that all 12 month old infants are consuming FSFYC as their source of milk. The body weight of 9.6 kg was used to estimate dietary intakes for 12 month old infants on a body weight basis.

#### ***Children aged 2-3 years***

2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> dietary intakes from FSFYC were calculated for each individual child aged 2-3 years in the 2011-12 NNPAS using their individual food consumption records from the dietary survey (n=200). The Harvest program multiplied the specified concentrations of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> for FSFYC by the amount of the food that an individual consumed in order to estimate the intake of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub>. Once this had been completed, the total amount of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> consumed from all FSFYC was summed for each individual. Mean intakes were then derived from the individuals' ranked intakes. Where intakes are estimated on a body weight basis, each individual's body weight was used for the calculation before summary statistics for the group were derived. Of the 200 children aged 2-3 years, only 8 (4%) were consumers of FSFYC. Consequently, there are insufficient consumers to derive P90 dietary intakes of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub>. Like the model diets, P90 dietary intakes for 2-3 year old children were derived using Equation 1.

### ***Assumptions and limitations of the dietary intake assessment***

The aim of the dietary intake assessment was to make the most realistic estimation of dietary intakes of 2'-FL and LNnT as possible. However, where significant uncertainties in the data existed, conservative assumptions were generally used to ensure that the estimated dietary intake was not an underestimate of intake.

Assumptions made in the dietary intake assessment included:

- Unless otherwise specified, all foods within a category contain 2'-FL and LNnT at the concentrations specified in [Table 3.15](#) for infant formula, follow-on formula and FSFYC and [Table 3.13](#) and [Table 3.14](#) for human milk and [Table 3.17](#) for domestic mammalian milks
- 1 litre of infant formula, follow-on formula or FSFYC equals 1,050 grams
- 1 litre of human milk equals 1,040 grams
- The smallest serving size for FSFYC is 115 ml (121 g)
- The largest serving size for FSFYC is 230 ml (242 g)
- The amount of FSFYC consumed by children aged 2-3 years is as per the 2011-12 NNPAS, irrespective of the serve size for the FSFYC product
- where a food was not included in the intake assessment, it was assumed to contain a zero concentration of 2'-FL and LNnT
- there is 100% market penetration of the use of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> into the infant formula, follow-on formula and FSFYC markets
- infants aged 3 months are exclusively infant formula fed
- infants aged 9 months consume follow-on formula
- infants aged 12 months consume FSFYC
- consumption of foods as outlined in the model diets represent current food consumption amounts for Australian and New Zealand children aged 3 months, 9 months and 12 months
- consumption of FSFYC by Australian children 2-3 years is as per the 2011-12 NNPAS
- consumption of FSFYC by Australian children 2-3 years is the same for New Zealand children aged 2-3 years
- children aged 12 months and above are no longer consuming breast milk
- there is no contribution to 2'-FL and LNnT intakes through foods and beverages other than from infant formula, follow-on formula and FSFYC and cows' and goat's milks
- there is no contribution to 2'-FL and LNnT intakes through the use of complementary or other medicines.

In addition to the specific assumptions made in relation to this dietary intake assessment, there are a number of limitations associated with the nutrition surveys from which the food consumption data used for the assessment are based. A discussion of these limitations is included in Section 6 of the *Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes* (Food Standards Australia New Zealand 2009).

### 3.4.3 Estimated dietary intakes of 2'-FL

#### *Infants aged <2 years*

##### *2'-FL<sub>human</sub> from human milk*

When it is assumed that infants aged <12 months are consuming human milk (and no infant formula or follow-on formula), the estimated mean and P90 dietary intakes of 2'-FL<sub>human</sub> from human milk are 1.8 – 2.2 g/day and 3.5 – 4.4 g/day for 3 month old infants and 1.2 g/day and 2.4 g/day for 9 month old infants.

On a grams per kilogram body weight per day basis, the estimated mean and P90 dietary intakes of 2'-FL<sub>human</sub> from human milk are 0.28 – 0.34 g/kg bw/day and 0.55 – 0.69 g/kg bw/day for 3 month old infants and 0.13 g/kg bw/day and 0.27 g/kg bw/day for 9 month old infants.

Further details can be found in [Table 3.18](#), [Table 3.22](#), [Figure 3.4](#) and [Figure 3.5](#).

**Table 3.18 Calculation of estimated dietary intakes of 2'-FL<sub>human</sub> from human milk for infants aged 3 months and 9 months**

|   | Units           | 10-60 day<br>secretor<br>human<br>milk | 60+ days secretor human<br>milk |          |
|---|-----------------|--|---------------------------------|----------|
|   |                 | 3 months                               | 3 months                        | 9 months |
| Recommended energy intake <sup>1</sup>  | kJ/kg<br>bw/day | 343                                    | 343                             | 330      |
| P50 Body Weight <sup>2</sup>  | kg              | 6.4                                    | 6.4                             | 8.9      |
| Recommended energy intake   | kJ/day          | 2,195                                  | 2,195                           | 2,937    |
| Amount of human milk required to meet energy requirements <sup>3</sup>        | g/day           | 765                                    | 765                             | n/a      |
| Amount of human milk required to meet 50% of energy requirements <sup>3</sup> | g/day           | n/a                                    | n/a                             | 515      |
| Mean dietary intake of 2'-FL <sup>4</sup>                                     | g/day           | 2.2                                    | 1.8                             | 1.2      |
|   | g/kg<br>bw/day  | 0.34                                   | 0.28                            | 0.13     |
| P90 dietary intake of 2'-FL <sup>4</sup>                                      | g/day           | 4.4                                    | 3.5                             | 2.4      |
|   | g/kg<br>bw/day  | 0.69                                   | 0.55                            | 0.27     |

1. (United Nations University et al. 2004)

2. (World Health Organization 2006)

3. Energy content of human milk is 286 kJ/100 g (Food Standards Australia New Zealand 2016)

4. Mean concentration of 2'-FL in human milk used in calculation ([Table 3.13](#))

### *Dietary intakes of 2'-FL<sub>micro</sub>*

The estimated mean and P90 dietary intakes of 2'-FL<sub>micro</sub> are 2.1 g/day and 4.2 g/day for 3 month old infants, 1.4 g/day and 2.8 g/day for 9 month old infants, and 0.97 – 1.9 g/day and 1.9 – 3.9 g/day for 12 month old infants. The estimated dietary intakes of 2'-FL<sub>micro</sub> for 3 month old and 9 month old infants are similar to those for 2'-FL<sub>human</sub> from the mean concentration of 2'-FL in 'secretor' human milk ([Table 3.19](#), [Table 3.22](#) and [Figure 3.4](#)).

On a grams per kilogram body weight per day basis, the estimated mean and P90 dietary intakes of 2'-FL<sub>micro</sub> are 0.33 g/kg bw/day and 0.66 g/kg bw/day for 3 month old infants, 0.16 g/kg bw/day and 0.32 g/kg bw/day for 9 month old infants, and 0.10 – 0.20 g/kg bw/day and 0.20 – 0.40 g/kg bw/day for 12 month old infants ([Table 3.19](#), [Table 3.22](#) and [Figure 3.5](#)).

### **Children aged 2-3 years**

On a grams per day basis, the estimated mean and P90 consumer dietary intakes of 2'-FL<sub>micro</sub> for Australian children aged 2-3 years from FSFYC are 1.1 – 2.2 g/day and 2.2 – 4.4 g/day ([Figure 3.4](#) and [Table 3.22](#)). The estimated dietary intake of naturally occurring 2'-FL from all cows' milk foods is low at the P90 consumption level: 0.00020 grams 2'-FL per day (<0.05% of the estimated mean intake of 2'-FL<sub>micro</sub> from FSFYC), confirming that cows' milk makes a minor contribution to 2'-FL dietary intakes in comparison to FSFYC with 2'-FL<sub>micro</sub> added.

On a gram per kilogram body weight per day basis, the estimated mean and P90 dietary intakes are 0.077 – 0.15 g/kg bw/day and 0.15 – 0.31 g/kg bw/day ([Figure 3.5](#) and [Table 3.22](#)).

**Table 3.19 Estimated dietary intakes of 2'-FL<sub>micro</sub> for infants aged 3 months, 9 months and 12 months from infant formula / follow on formula / FSFYC**

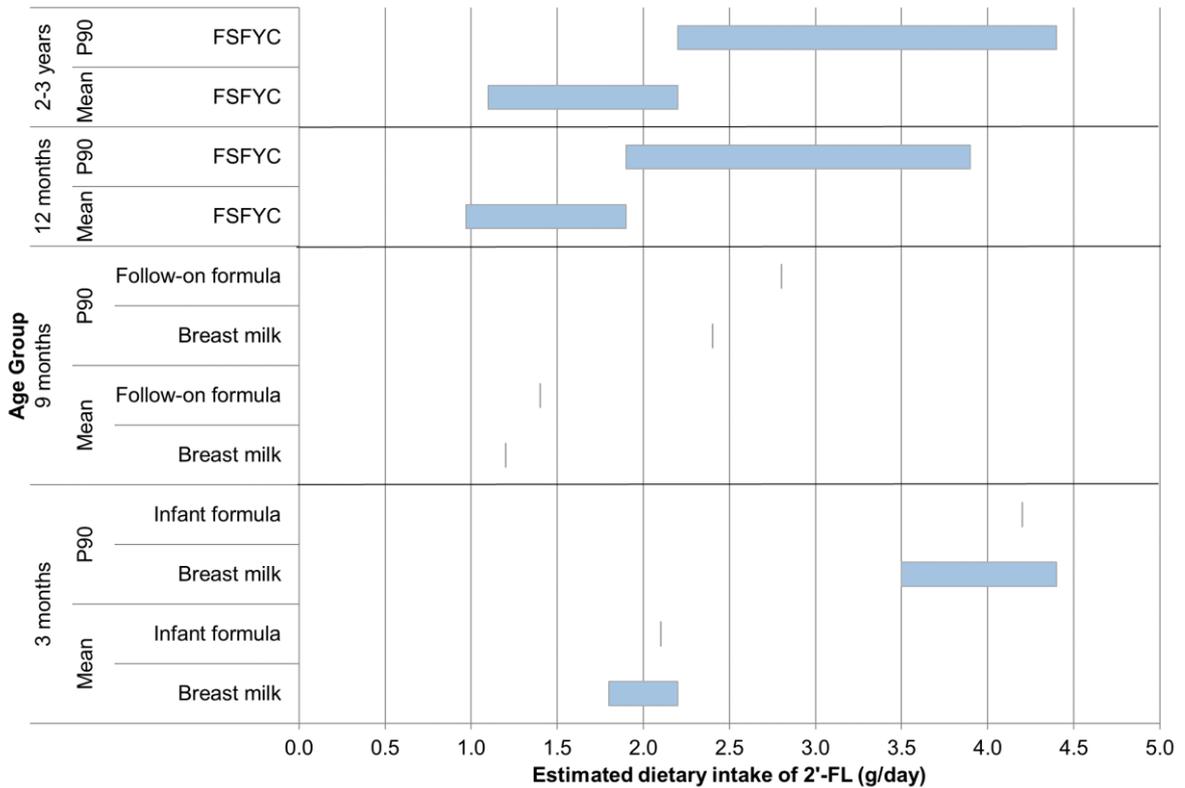
|   | Units        | 3 months <sup>3</sup> | 9 months <sup>3</sup> | 12 months                       |                                 |
|---|--------------|-----------------------|-----------------------|---------------------------------|---------------------------------|
|   |              |                       |                       | Minimum serve size <sup>4</sup> | Maximum serve size <sup>4</sup> |
| Recommended energy intake <sup>1</sup>                      | kJ/kg bw/day | 343                   | 330                   | 335                             | 335                             |
| P50 Body Weight <sup>2</sup>                                | kg           | 6.4                   | 8.9                   | 9.6                             | 9.6                             |
| Recommended energy intake                                   | kJ/day       | 2,195                 | 2,937                 | 3,216                           | 3,216                           |
| 100% of energy requirements <sup>3</sup>                    | kJ/day       | 2,195                 | n/a                   | n/a                             | n/a                             |
| 50% of energy requirements <sup>3</sup>                     | kJ/day       | n/a                   | 1,469                 | n/a                             | n/a                             |
| Amount of FSFYC required to meet 35% of energy requirements | g/day        | n/a                   | n/a                   | 418<br>(3.5 serves)             | 418<br>(1.7 serves)             |
| Mean dietary intake of 2'-FL <sub>micro</sub>               | g/day        | 2.1                   | 1.4                   | 1.9                             | 0.97                            |
|   | g/kg bw/day  | 0.33                  | 0.16                  | 0.20                            | 0.10                            |
| P90 dietary intake of 2'-FL <sub>micro</sub>                | g/day        | 4.2                   | 2.8                   | 3.9                             | 1.9                             |
|   | g/kg bw/day  | 0.66                  | 0.32                  | 0.40                            | 0.20                            |

1. (United Nations University et al. 2004)

2. (World Health Organization 2006)

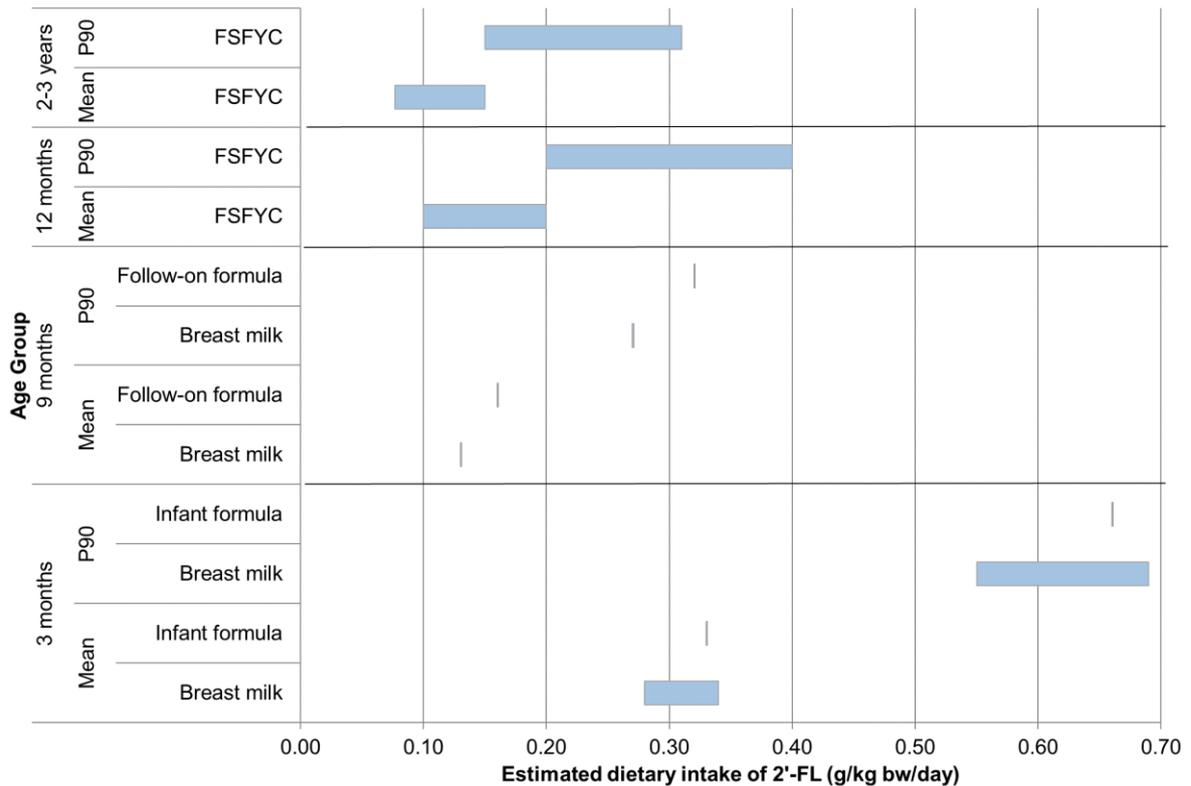
3. Energy content of infant formula and follow on formula is 264 kJ/100 g formula (Food Standards Australia New Zealand 2016), with the proposed concentration of 2'-FL<sub>micro</sub> in infant formula and follow-on formula being 96 mg/100 kJ.

4. Energy content of FSFYC (toddler milk) is 269 kJ/100 g formula (Food Standards Australia New Zealand 2016), with the proposed concentration of 2'-FL<sub>micro</sub> in FSFYC being 0.56 g/serving. The minimum serve size is 115 ml (121 g) and the maximum serve size is 230 ml (242 g).



**Notes** 1) for FSFYC, the lower bound is for the largest serving size (230ml) and the upper bound represents the smallest serving size (115 ml); 2) for breast milk for 3 month old infants, the lower bound represents secretor milk for 10-60 days post-partum and the upper bound represents secretor milk for 60+ days post-partum

**Figure 3.4 Estimated dietary intakes of 2'-FL for infants aged 3 months, 9 months and 12 months and children aged 2-3 years from human, natural and microbial sources, in grams per day**



Notes 1) for FSFYC, the lower bound is for the largest serving size (230ml) and the upper bound represents the smallest serving size (115 ml); 2) for breast milk for 3 month old infants, the lower bound represents secretor milk for 10-60 days post-partum and the upper bound represents secretor milk for 60+ days post-partum

**Figure 3.5 Estimated dietary intakes of 2'-FL for infants aged 3 months, 9 months and 12 months and children aged 2-3 years from human, natural and microbial sources, in grams per kilogram body weight per day**

### 3.4.4 Estimated dietary intakes of LNnT

#### Children aged <2 years

##### *LNnT<sub>human</sub> from human milk*

When it is assumed that infants aged <12 months are consuming human milk (and no infant formula or follow-on formula), the estimated mean and P90 dietary intakes of LNnT<sub>human</sub> from human milk are 0.21 – 0.23 g/day and 0.41 – 0.46 g/day for 3 month old infants and 0.14 g/day and 0.28 g/day for 9 month old infants. On a grams per kilogram body weight per day basis, the estimated mean and P90 dietary intakes of LNnT<sub>human</sub> from human milk are 0.032 – 0.036 g/kg bw/day and 0.065 – 0.072 g/kg bw/day for 3 month old infants and 0.016 g/kg bw/day and 0.031 g/kg bw/day for 9 month old infants ([Table 3.20](#), [Table 3.22](#), [Figure 3.6](#) and [Figure 3.7](#)).

**Table 3.20 Estimated dietary intakes of LNnT<sub>human</sub> from human milk for infants aged 3 months and 9 months**

|   | Units        | 10-60 day human milk | 60+ days secretor human milk |          |
|---|--------------|----------------------|------------------------------|----------|
|   |              | 3 months             | 3 months                     | 9 months |
| Recommended energy intake <sup>1</sup>  | kJ/kg bw/day | 343                  | 343                          | 330      |
| P50 Body Weight <sup>2</sup>  | kg           | 6.4                  | 6.4                          | 8.9      |
| Recommended energy intake   | kJ/day       | 2,195                | 2,195                        | 2,937    |
| Amount of human milk required to meet energy requirements <sup>3</sup>        | g/day        | 765                  | 765                          | n/a      |
| Amount of human milk required to meet 50% of energy requirements <sup>3</sup> | g/day        | n/a                  | n/a                          | 515      |
| Mean dietary intake of LNnT <sup>4</sup>                                      | g/day        | 0.23                 | 0.21                         | 0.14     |
|   | g/kg bw/day  | 0.036                | 0.032                        | 0.016    |
| P90 dietary intake of LNnT <sup>4</sup>                                       | g/day        | 0.46                 | 0.41                         | 0.28     |
|   | g/kg bw/day  | 0.072                | 0.065                        | 0.031    |

1. (United Nations University et al. 2004)
2. (World Health Organization 2006)
3. Energy content of human milk is 286 kJ/100 g formula (Food Standards Australia New Zealand 2016)
4. Mean concentration of LNnT in human milk used in calculation ([Table 3.14](#))

#### *LNnT<sub>micro</sub> from infant formula / follow-on formula / FSFYC*

The estimated mean and P90 dietary intakes of LNnT<sub>micro</sub> are 0.53 g/day and 1.1 g/day for 3 month old infants, 0.35 g/day and 0.71 g/day for 9 month old infants, and 0.24 – 0.48 g/day and 0.480 – 0.97 g/day for 12 month old infants. The estimated dietary intakes of LNnT<sub>micro</sub> for 3 month old and 9 month old infants are higher than those for LNnT<sub>human</sub> from human milk ([Table 3.21](#), [Table 3.22](#) and [Figure 3.6](#)).

On a grams per kilogram body weight per day basis, the estimated mean and P90 dietary intakes of LNnT<sub>micro</sub> are 0.082 g/kg bw/day and 0.16 g/kg bw/day for 3 month old infants, 0.040 g/kg bw/day and 0.079 g/kg bw/day for 9 month old infants, and 0.025 – 0.050 g/kg bw/day and 0.050 – 0.10 g/kg bw/day for 12 month old infants ([Table 3.21](#), [Table 3.22](#) and [Figure 3.7](#)).

**Table 3.21 Estimated dietary intakes of LNnT<sub>micro</sub> for infants aged 3 months, 9 months and 12 months from infant formula / follow on formula / FSFYC**

|  | Units        | 3 months | 9 months | 12 month                        |                                 |
|--|--------------|----------|----------|---------------------------------|---------------------------------|
|  |              |          |          | Minimum serve size <sup>4</sup> | Maximum serve size <sup>4</sup> |
| Recommended energy intake <sup>1</sup>                                   | kJ/kg bw/day | 343      | 330      | 335                             | 335                             |
| P50 Body Weight <sup>2</sup>   | Kg           | 6.4      | 8.9      | 9.6                             | 9.6                             |
| Recommended energy intake  | kJ/day       | 2,195    | 2,937    | 3,216                           | 3,216                           |
| 100% of energy requirements <sup>3</sup>                                 | kJ/day       | 2,195    | n/a      | n/a                             | n/a                             |
| 50% of energy requirements <sup>3</sup>                                  | kJ/day       | n/a      | 1,469    | n/a                             | n/a                             |
| Amount of FSFYC required to meet 35% of energy requirements <sup>4</sup> | g/day        | n/a      | n/a      | 418<br>(3.5 serves)             | 418<br>(1.7 serves)             |
| Mean dietary intake of LNnT <sub>micro</sub>                             | g/day        | 0.53     | 0.35     | 0.48                            | 0.24                            |
|  | g/kg bw/day  | 0.082    | 0.040    | 0.050                           | 0.025                           |
| P90 dietary intake of LNnT <sub>micro</sub>                              | g/day        | 1.1      | 0.71     | 0.97                            | 0.48                            |
|  | g/kg bw/day  | 0.16     | 0.079    | 0.10                            | 0.050                           |

1. (United Nations University et al. 2004)

2. (World Health Organization 2006)

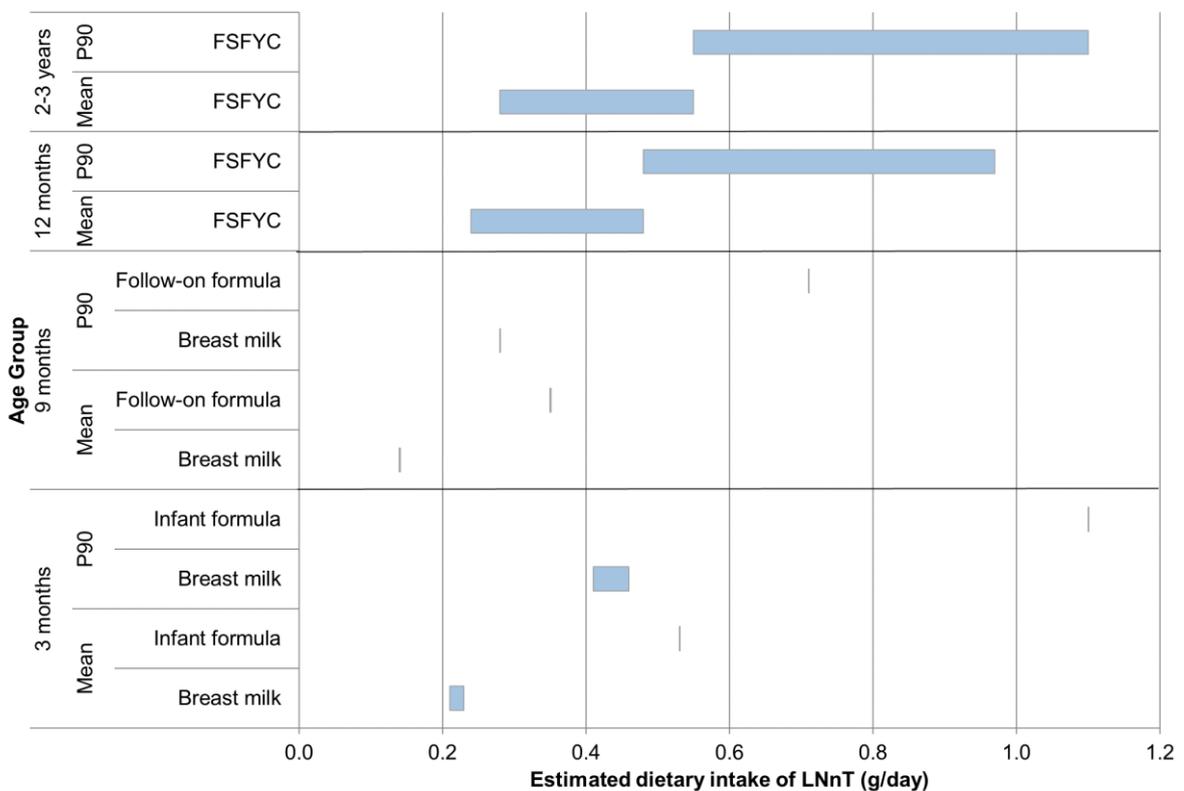
3. Energy content of infant formula and follow-on formula is 264 kJ/100 g formula (Food Standards Australia New Zealand 2016)

4. Energy content of 'toddler milk' (FSFYC) is 269 kJ/100 g formula (Food Standards Australia New Zealand 2016) , with the proposed concentration of 2'-FL<sub>micro</sub> in FSFYC being 0.56 g/serving. The minimum serve size is 115 ml (121 g) and the maximum serve size is 230 ml (242 g).

Children aged 2-3 years

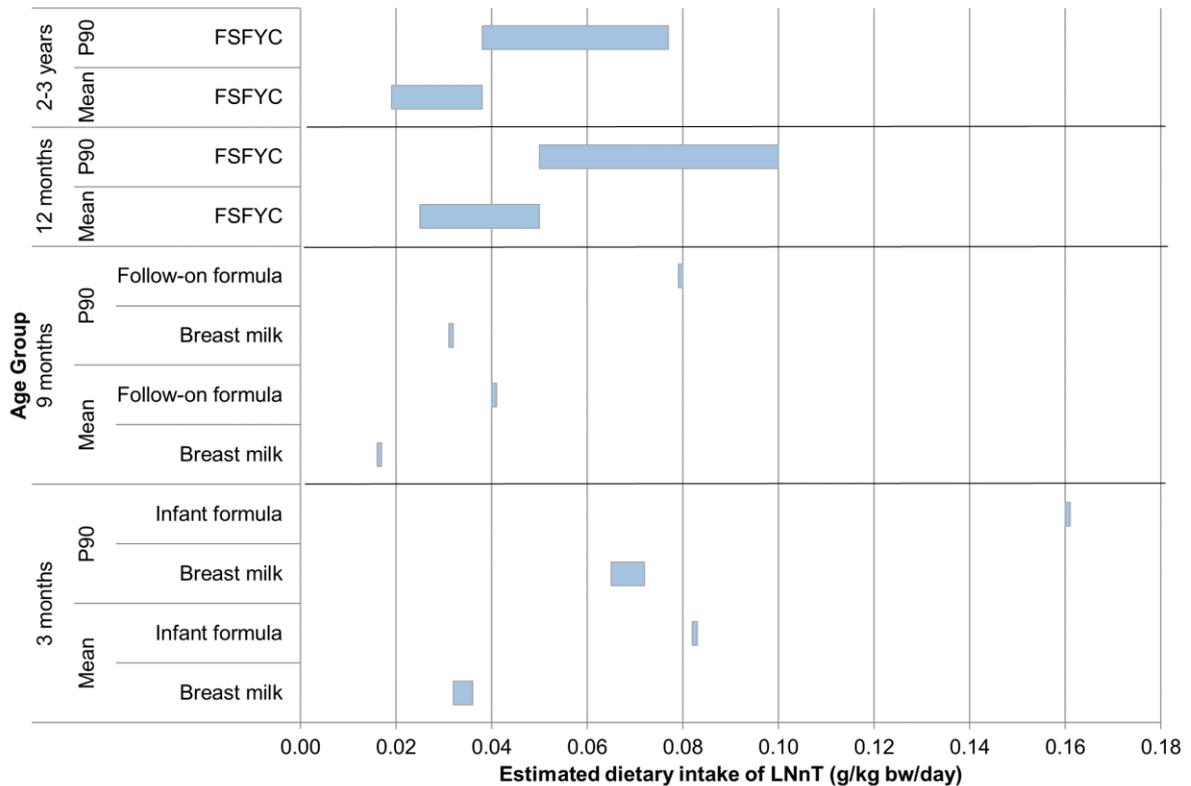
On a grams per day basis, the estimated mean and P90 consumer dietary intakes of L<sub>NnT</sub><sub>micro</sub> for Australian children aged 2-3 years are 0.28 – 0.55 g/day and 0.55 – 1.1 g/day (Table 3.22 and Figure 3.6). The estimated dietary intake of naturally occurring L<sub>NnT</sub> from other cows' milk foods is low at the P90 consumption of cows' milk: 0.0025 grams L<sub>NnT</sub> per day (<1% of the estimated mean intake of L<sub>NnT</sub><sub>micro</sub> from FSFYC), confirming that cows' milk makes a minor contribution to L<sub>NnT</sub> dietary intakes in comparison to FSFYC with L<sub>NnT</sub><sub>micro</sub> added.

On a gram per kilogram body weight per day basis, the estimated mean and P90 dietary intakes are 0.019 – 0.038 g/kg bw/day and 0.038 – 0.077 g/kg bw/day (Table 3.22 and Figure 3.7).



**Notes** 1) for FSFYC, the lower bound is for the largest serving size (230ml) and the upper bound represents the smallest serving size (115 ml); 2) for breast milk for 3 month old infants, the lower bound represents secretor milk for 10-60 days post-partum and the upper bound represents secretor milk for 60+ days post-partum

**Figure 3.6 Estimated dietary intakes of L<sub>NnT</sub> for infants aged 3 months, 9 months and 12 months and children aged 2-3 years from human, natural and microbial sources, in grams per day**



**Notes** 1) for FSFYC, the lower bound is for the largest serving size (230ml) and the upper bound represents the smallest serving size (115 ml); 2) for breast milk for 3 month old infants, the lower bound represents secretor milk for 10-60 days post-partum and the upper bound represents secretor milk for 60+ days post-partum

**Figure 3.7 Estimated dietary intakes of LNnT for infants aged 3 months, 9 months and 12 months and children aged 2-3 years from human, natural and microbial sources, in grams per kilogram body weight per day**

### 3.4.5 Comparison of estimated dietary intakes of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> to dietary intakes from human milk

The estimated dietary intakes of 2'-FL<sub>micro</sub> are similar to intakes of 2'-FL<sub>human</sub> for 3 month and 9 month old infants. This is due to the maximum use level of 2'-FL<sub>micro</sub> in infant formula and follow-on formula (2.4 g/L / 96 mg/100 kJ) being similar to the mean concentration of 2'-FL<sub>human</sub> for human milk (for secretors).

The dietary intakes of LNnT<sub>micro</sub> are higher than for LNnT<sub>human</sub> for 3 month and 9 month old infants. This is due to the proposed LNnT<sub>micro</sub> concentration in infant formula and follow-on formula (0.6 g/L) being higher than the mean concentration in human milk (0.28 – 0.31 g/L). The proposed maximum use level of LNnT<sub>micro</sub> (0.6 g/L) is within the range of LNnT concentrations in mature human milk (0.09 – 1.08 g/L).

The dietary intake assessment results for all age groups and all sources of 2'-FL and LNnT are summarised in [Table 3.22](#).

**Table 3.22 Summary of estimated dietary intakes of 2'-FL and LNnT (from all sources assessed) for infants aged 3 months, 9 months and 12 months and children aged 2-3 years**

| Substance | Unit        | Age group | Mean dietary intake         |                              | P90 dietary intake          |                              |
|-----------|-------------|-----------|-----------------------------|------------------------------|-----------------------------|------------------------------|
|           |             |           | From microbial fermentation | From human milk <sup>◆</sup> | From microbial fermentation | From human milk <sup>◆</sup> |
| 2'-FL     | g/day       | 3 months  | 2.1                         | 1.8 – 2.2                    | 4.2                         | 3.5 – 4.4                    |
|           |             | 9 months  | 1.4                         | 1.2                          | 2.8                         | 2.4                          |
|           |             | 12 months | 0.97 – 1.9 <sup>Ω</sup>     | n/a                          | 1.9 – 3.9 <sup>Ω</sup>      | n/a                          |
|           |             | 2-3 years | 1.1 – 2.2 <sup>Ω</sup>      | n/a                          | 2.2 – 4.4 <sup>Ω</sup>      | n/a                          |
|           | g/kg bw/day | 3 months  | 0.33                        | 0.28 – 0.34                  | 0.66                        | 0.55 – 0.69                  |
|           |             | 9 months  | 0.16                        | 0.13                         | 0.32                        | 0.27                         |
|           |             | 12 months | 0.10 – 0.20 <sup>Ω</sup>    | n/a                          | 0.20 – 0.40 <sup>Ω</sup>    | n/a                          |
|           |             | 2-3 years | 0.077 – 0.15 <sup>Ω</sup>   | n/a                          | 0.15 – 0.31 <sup>Ω</sup>    | n/a                          |
| LNnT      | g/day       | 3 months  | 0.53                        | 0.21 – 0.23                  | 1.1                         | 0.41 – 0.46                  |
|           |             | 9 months  | 0.35                        | 0.14                         | 0.71                        | 0.28                         |
|           |             | 12 months | 0.24 – 0.48 <sup>Ω</sup>    | n/a                          | 0.48 – 0.97 <sup>Ω</sup>    | n/a                          |
|           |             | 2-3 years | 0.28 – 0.55 <sup>Ω</sup>    | n/a                          | 0.55 – 1.1 <sup>Ω</sup>     | n/a                          |
|           | g/kg bw/day | 3 months  | 0.082                       | 0.032 – 0.036                | 0.16                        | 0.065 – 0.072                |
|           |             | 9 months  | 0.040                       | 0.016                        | 0.079                       | 0.031                        |
|           |             | 12 months | 0.025 – 0.050 <sup>Ω</sup>  | n/a                          | 0.050 – 0.10 <sup>Ω</sup>   | n/a                          |
|           |             | 2-3 years | 0.019 – 0.038 <sup>Ω</sup>  | n/a                          | 0.038 – 0.077 <sup>Ω</sup>  | n/a                          |

Ω lower bound of the range is for the FSFYC serve size of 230 ml; the upper bound of the range is for the FSFYC serve size of 115 ml

◆ lower bound of the range is for breast milk 60+ days post-partum; upper bound of the range if for 10-60 days post-partum

## 4. Health effects assessment

The stated purpose for adding 2'-FL, alone or in combination with LNnT, to infant formula products or FSFYC is to better reflect the oligosaccharide composition of human milk. The addition of these HiMOs to infant formula products and FSFYC are stated by the applicant to confer functional benefits to infants and young children, consistent with the human milk oligosaccharide (HMO) fraction of human milk, with three specified health effects: (1) a bifidogenic effect; (2) an anti-infective effect against pathogens; and (3) immune modulation, improved barrier function and alleviation of allergic responses. The health effects stated by the applicant relate either directly or indirectly to the selective microbial metabolism of 2'-FL and/or LNnT in the gut of infants and young children to modulate the intestinal microflora. This creates conditions that are purported to favour growth of bifidobacteria (bifidogenic effect), be detrimental to growth and attachment of pathogens (anti-infective effect), and mediate immune system functioning (immune modulation, improved barrier function and alleviation of allergic responses).

The effects of substrates that are targeted at modulating gut microflora have the potential to vary widely from individual to individual (Gibson et al. 2017). This is due to the unique microbial ecology of individuals and a variety of host and environmental factors that may influence the effect of a dietary intervention. Moreover, microbial utilisation of 2-FL and /or LNnT can only occur if the appropriate bacteria are a component of the infant or young child's gut microflora. As such, it is difficult to definitively and reproducibly demonstrate causality of an effect associated with the addition of 2'-FL and LNnT to infant formula products and FSFYC.

FSANZ assessed data submitted by the applicant and information from published sources, including an additional child study with 2'-FL<sub>chem</sub> alone and in combination with LNnT<sub>chem</sub> that was received after FSANZ published the 1<sup>st</sup> CFS report. Both direct (clinical trial data) and indirect evidence (animal and *in vitro* studies) was assessed to determine the biological and mechanistic plausibility of the stated health effects.

FSANZ's assessment of the stated health effects is for the purpose of the requested voluntary compositional permission. The term 'health effect(s)' is therefore used in the context of our assessment for compositional permission. Although suggested by some submitters to the 1<sup>st</sup> CFS, FSANZ has not applied the health claims substantiation framework (Standard 1.2.7) for assessing evidence for the purpose of compositional permission. The applicant has not applied for a permitted health claim for FSFYC in the Code and infant formula products are prohibited from making claims.

### 4.1 Concentrations of 2'-FL and LNnT in human milk

The concentrations of 2'-FL and LNnT reported for human milk, cow milk and goat milk are summarised in [Table 3.17](#). One of the major differences between human milk and most infant and follow-on formula products, regardless of whether they are derived from animal milk or plant sources, is in their oligosaccharide content (Austin et al. 2016; Castanys-Muñoz et al. 2013). For example, bovine milk contains low total concentrations of oligosaccharides (~0.05 g/L) compared to breastmilk (20–25 g/L in colostrum and 10–15 g/L in mature milk) (Bode 2012; Gidrewicz and Fenton 2014; Kunz et al. 1999; Newburg 2013). As such, oligosaccharides are the third most abundant component of human milk solids after lactose and fat.

A limiting factor in comparing data on HMO concentrations is that a range of methods of analysis have been used in the available studies (Galeotti et al. 2012; Kunz et al. 2017).

Genetic variations and differences between mothers in their diet and health, in addition to differences in sampling time, also contribute to the reported variability in HMO concentrations (Asakuma et al. 2008; Olivares et al. 2015; Thurl et al. 2010). In the context of applications to amend the Code regarding infant formula composition, FSANZ has previously identified variations in human milk composition for the same reasons (FSANZ 2013, 2016).

In addition, an estimated 80% of women world-wide produce human milk that contains 2'-FL (secretors). These women express the  $\alpha$ -1,2-fucosyltransferase enzyme responsible for fucosylating oligosaccharides and lactose at the 2'-O-position (Austin et al. 2016; Castanys-Muñoz et al. 2013; McGuire et al. 2017). Unlike 2'-FL, LNnT is present in the milk of all women and accounts for ~5% of total oligosaccharide content (Thurl et al. 2017). In women that secrete 2'-FL, the ratio of 2'-FL to LNnT concentrations has been shown to vary widely, from approximately 1:1 (McGuire et al. 2017) to greater than 10:1 (Spevacek et al. 2015; Austin et al. 2016; Kunz et al. 2017).

## 4.2 Bifidogenic effect

### 4.2.1 Intestinal microflora development in breast fed babies and infants

For the purpose of this assessment, a bifidogenic effect is defined as a proliferation and increase in the relative abundance of bifidobacteria in the intestinal microbiota.

The development of the microbiota of the babies and infants is complex and dynamic. The types of bacteria present in the gut are highly individual and progressively matures and develops a more adult-like microbiota by one year of age (Milani et al. 2017; Palmer et al. 2007). The composition of the microbiota is dependent on a range of factors including delivery method (vaginal vs caesarean), diet (breast vs infant formula), geographical origin and the use of antibiotics (Milani et al. 2017). The introduction of solids (around 6 months) and weaning leads to a reduction on the abundance of bifidobacteria as other bacteria become more dominant (Davis et al. 2017).

Studies of infant faecal samples have found that the microflora of breastfed infants is more homogeneous than those on infant formula diets and is dominated by bifidobacteria (Bezirtzoglou et al.; Bezirtzoglou et al. 2011). The genus *Bifidobacterium* contains 32 species which are generally recognised as being non-pathogenic. *Bifidobacterium* species grow in anaerobic conditions with an optimum growth temperature of 37-41°C. HMOs are metabolised by *Bifidobacterium* species in the colon of babies and infants (Brand-Miller et al. 1998; Gnoth et al. 2000). End products of metabolism include acetic and lactic acid, although organic acids such as butyric and propionic acid are not produced (Biavati and Mattarelli 2012).

A range of *Bifidobacterium* species have been identified in infant and adult faecal samples ([Table 4.1](#)). The major species identified in infant faeces include *B. longum* subsp. *infantis*, *B. breve* and *B. bifidum*. In adults, *B. adolescentis* is the major species found in faeces.

Specific adaptations for carbohydrate metabolism of HMOs have been suggested to explain the differences in the *Bifidobacterium* species present in infant and adult faeces (Bunesova et al. 2016). Species such as *B. longum* subsp. *infantis* and *B. bifidum* are hypothesised to have adapted to consume HMOs, while the adult associated species *B. adolescentis* and *B. longum* subsp. *longum* are capable of metabolising plant derived polysaccharides. Other bacterial genera, such as *Bacteroides* can metabolise a variety of polysaccharides from human milk and plant sources.

Examples of studies describing the development of the microflora of breastfed and unsupplemented infant formula fed infants are described below.

A study by Smith-Brown et al. (2016) investigated the effect of secretor status (determined using blood and saliva samples; refer to [Section 4.1](#)) and breast-feeding on the microflora composition at 2 to 3 years of age. Mothers with inactive secretor genes have different HMO profiles and concentrations. The study included 37 children and 17 eligible mothers. The microflora composition of faecal samples was determined using 16S rRNA gene sequencing. The majority of the mothers (11/17, 64.7%) were found to be secretors of HMOs. For children exclusively breastfed for 4-months (11/37) the abundance of Bifidobacteria was significantly higher for those with secretor mothers (n=8) compared to non-secretor mothers (n=3), although the relative abundance data for the two groups were not reported.

**Table 4.1 Species of *Bifidobacterium* found in infant and adult faecal samples (adapted from Biavati and Mattarelli (2012))**

| Species                                 | Infant | Adult |
|---|--------|-------|
| <i>B. bifidum</i>                       | ✓      | ✓     |
| <i>B. adolescentis</i>                  |        | ✓     |
| <i>B. angulatum</i>                     |        | ✓     |
| <i>B. breve</i>                         | ✓      |       |
| <i>B. catenulatum</i>                   | ✓      | ✓     |
| <i>B. dentum</i>                        |        | ✓     |
| <i>B. gallicum</i>                      | ✓      | ✓     |
| <i>B. longum</i> subsp. <i>longum</i>   | ✓      | ✓     |
| <i>B. longum</i> subsp. <i>infantis</i> | ✓      |       |
| <i>B. pseudocatenulatum</i>             | ✓      |       |

Lewis et al. (2015) investigated the influence of secretor status of the mother on the development of infant gut microflora using 16S rRNA gene amplicon sequencing. A total of 44 infant/mothers were selected for the study. The infants gut microflora was measured at four time periods ranging from 7 to 120 days. The majority of mothers (32/44, 67%) were found to be secretors. The most common bacteria found in the infant guts were Bifidobacteriales, Lactobacillales (mostly *Streptococcus*), Bacteroides, Enterobacteriaceae, and Clostridiaceae. A comparison of the relative abundance of gut microflora for secretor and non-secretor breastfed infants found statistically significant differences for *Bifidobacterium* (higher in the secretor group) and *Streptococcus* (lower in the secretor group). No differences were found between the two groups for Bacteroides, *Clostridium*, Enterobacteriaceae (Other) and *Escherichia/Shigella*. The types of bifidobacteria were found in the different groups. *B. longum* subsp. *infantis* dominated the microflora in the secretor-fed group. The faeces of these infants were found to have lower percentages of fucosylated oligosaccharides. An explanation is that *B. longum* subsp. *infantis* possesses two classes of 2'-fucosyllactose enzymes capable of metabolising this group of oligosaccharides. *B. breve* was found in the faeces of both groups suggesting that it may be an oligosaccharide generalist. The study concluded that breastfed infants from non-secretor mothers had a

lower relative abundance and delayed establishment of a bifidobacteria dominant gut microflora.

Bezirtzoglou et al. (2011) investigated the development of the gut microflora for exclusively breastfed and infant-formula newborn infants in Greece. All infants were delivered by vaginal delivery. Faecal samples were collected between 11-22 days of life for breastfed infants and 14-36 days for formula-fed infants. The formula-fed infants consumed the same infant formula product. The number of infants in this study was small, with only six infants in each group. Single cells within the infant microflora were identified using fluorescence in situ hybridization with specific 16S rRNA-based oligonucleotide probes. *Bifidobacterium* genus dominated the gut microflora of the breastfed infants (average 69%) compared with formula-fed infants (average 32%) and at lower numbers. The faecal microflora of the formula-fed infants were found to be more diverse than the breastfed infants.

Studies into the composition of the gut microflora of Australian and New Zealand infants are few.

Lahtinen et al. (2009) investigated the prenatal administration of *Lactobacillus rhamnosus* by Victorian mothers to influence the development of the infant's Bifidobacteria microflora. A placebo group did not receive the *Lactobacillus* supplement. For this group, infants delivered by caesarean section had a lower prevalence of the *B. longum* group (consisting of *B. longum* subsp. *infantis*, *B. longum* subsp. *longum* and *B. bifidum*) and a higher prevalence of *B. adolescentis*.

At 7 days after birth, any *Bifidobacterium* species were found in 91.7% of infants in the placebo group. The majority of infants in the placebo group were colonised with species belonging to the *B. longum* group (54.2%) followed by *B. breve* (25.0%) and the *B. adolescentis* group (consisting of *B. adolescentis* and *B. dentium*; 25.0%). At age 90 days, the proportion of infants with the *B. longum* group had increased to 60.7% with similar increases in both the *B. breve* (33.9%) and the *B. adolescentis* group (33.9%). Other *Bifidobacterium* species found colonising infants in the placebo group included *B. angulatum* (21.4%) and the *B. catenulatum* group (consisting of *B. catenulatum* and *B. pseudocatenulatum*; 17.9%) (Lahtinen et al. 2009).

Only 46% of the infants in the Lahtinen et al. (2009) study were exclusively breastfed for greater than three months. The infants in the study will have included a mix of breast fed, formula fed and mixed fed infants.

The use of groups of similar species such as the *B. longum* group which consists of *B. longum* subsp. *infantis*, *B. longum* subsp. *longum* and *B. bifidum* limits the usefulness of the study to inform the development of the infant gut microflora.

Tannock et al. (2013) investigated the gut microflora of infants in South Australia fed goat or cow milk based formulas and human milk. The composition of the stool samples was determined using pyrosequencing of 16S rRNA gene sequences. The study found that Bifidobacteriaceae were abundant in the microflora of the two formula and human milk fed groups. The proportion of Bifidobacteriaceae was greatest in the human milk group (about 80%) compared to the goat and cow milk groups, both around 50%.

For the human milk group, *B. longum* (26.1%) was the most abundant bifidobacterial species followed by *B. breve* (19.6%), *B. bifidum* (6.9%), *B. dentium* (4.8%) and *B. pseudocatenulatum* (2.9%).

The PCR primers used in this study made it possible to differentiate *B. longum* subsp. *longum* and *B. longum* subsp. *infantis*. It was found that when *B. longum* was present it was

usually *B. longum* subsp. *longum* and not *B. longum* subsp. *infantis*. This subspecies (*B. longum* subsp. *infantis*) was rarely detected in each of the three groups (n=30 infants in each group): two detections were made in the breast fed group and only one in the goat milk infant formula group. There were no detections made in the cow milk formula group.

#### 4.2.2 *In vitro* studies of bacterial growth/utilisation on 2'-FL and LNnT

##### *Bifidobacterium* strains

Asakuma et al. (2011) investigated the ability of *B. bifidum*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum* and *B. breve* to grow and metabolise mixtures of HMOs extracted from human milk. Milk was collected from mothers (age  $32.7 \pm 4.3$  years old) who had given birth to babies at term (36-41 weeks). The mothers and babies had no health issues. The mothers had not taken oral antibiotics prior to being recruited for the study. Human milk samples were collected at  $74.9 \pm 27.2$  days, with a range of 30–120 days. The HMO mixtures, including both 2'-FL and LNnT were extracted from human milk by chromatography as neutral oligosaccharides, with between 3 and 8 monomeric units.

Strong growth in the media containing the HMO mixture was observed for *B. longum* subsp. *infantis* and *B. bifidum*. The concentrations of 2'-FL and LNnT decreased rapidly during the early stages of growth. Weak growth was observed for *B. longum* subsp. *longum* and *B. breve*. The concentrations of 2'-FL and LNnT were constant throughout the cultivation period, indicating that these HMO's were not utilized for growth. Lactose and lacto-N-tetraose (LNT) were the only HMOs which were utilised for growth. All four strains grew well when glucose was added as a carbon source.

Differences in the ability of *B. bifidum*, *B. longum* subsp. *infantis* and *B. longum* subsp. *longum* to grow on 2'-FL and LNnT was reported in Garrido et al. (2015) and Garrido et al. (2016). *B. bifidum* and *B. longum* subsp. *infantis* strains grew strongly in media supplemented with HMO mixtures, 2'-FL and LNnT. By contrast, only a single *B. longum* subsp. *longum* strain grew in 2'-FL supplemented media from the 17 strains tested. Growth was more common for LNnT with 11/17 (65%) strains growing, most with weak to moderate growth. Strong growth of *B. longum* subsp. *longum* was only observed in media supplemented with LNT.

Ruiz-Moyano et al. (2013) investigated the growth response of 24 *B. breve* strains using total HMO isolated from human milk or individually on 2'-FL and LNnT as the sole carbon source. Growth on HMO mixtures was generally moderate with only three strains exhibiting extensive growth. High growth was observed for all strains grown on LNnT. The majority of strains (20/24, 83%) did not grow on 2'-FL. Only two strains grew to high levels.

Yu et al. (2013a) and Yu et al. (2013b) investigated the growth and utilization of 2'-FL by *B. longum* subsp. *infantis* (seven strains) and *B. longum* subsp. *longum* (three strains). All strains exhibited strong growth and utilisation of 2'-FL with the exception of a single strain of *B. longum* subsp. *longum* strain.

**Table 4.2 Summary of in vitro monoculture growth response of *Bifidobacterium* spp. grown on 2'-FL, LNnT or HMO mixtures**

| HMO   | Growth/utilisation   | No growth/utilisation   | Reference                              |
|-------|--|---|--|
| 2'-FL | <i>B. bifidum</i><br><i>B. longum</i> subsp. <i>infantis</i>   | <i>B. breve</i><br><i>B. longum</i> subsp. <i>longum</i>  | Asakuma et al. (2011)                  |
| 2'-FL | <i>B. bifidum</i> (8 strains)<br><i>B. kashiwanohense</i> (4 strains)<br><i>B. longum</i> subsp. <i>infantis</i> (5 strains)<br><i>B. longum</i> subsp. <i>suis</i> (1 strain) | <i>B. breve</i> (3 strains)<br><i>B. longum</i> subsp. <i>longum</i> (1 strain)<br><i>B. pseudolongum</i> subsp. <i>globosum</i> (1 strain)                           | Bunesova et al. (2016)                 |
| 2'-FL | <i>B. bifidum</i> (12 strains)<br><i>B. infantis</i> (21 strains)  |   | Garrido et al. (2015)                  |
| 2'-FL | <i>B. longum</i> subsp. <i>longum</i> (1 strain)   | <i>B. longum</i> subsp. <i>longum</i> (16 strains)  | Garrido et al. (2016)                  |
| 2'-FL | <i>B. breve</i> (4 strains)  | <i>B. breve</i> (20 strains)  | Ruiz-Moyano et al. (2013)              |
| 2'-FL | <i>B. longum</i> subsp. <i>infantis</i> (7 strains)<br><i>B. longum</i> subsp. <i>longum</i> (2 strains)   | <i>B. longum</i> subsp. <i>longum</i> (1 strain)  | Yu et al. (2013a)<br>Yu et al. (2013b) |
| LNnT  | <i>B. bifidum</i> (2 strains)<br><i>B. longum</i> subsp. <i>infantis</i>   | <i>B. breve</i><br><i>B. longum</i> subsp. <i>longum</i>  | Asakuma et al. (2011)                  |
| LNnT  | <i>B. bifidum</i> (8 strains)<br><i>B. breve</i> (2 strains)<br><i>B. longum</i> subsp. <i>infantis</i> (5 strains)  | <i>B. kashiwanohense</i> (4 strains)<br><i>B. longum</i> subsp. <i>longum</i><br><i>B. longum</i> subsp. <i>suis</i><br><i>B. pseudolongum</i> subsp. <i>globosum</i> | Bunesova et al. (2016)                 |
| LNnT  | <i>B. bifidum</i> (12 strains)<br><i>B. infantis</i> (21 strains)  |   | Garrido et al. (2015)                  |
| LNnT  | <i>B. longum</i> subsp. <i>longum</i> (11 strains)   | <i>B. longum</i> subsp. <i>longum</i> (6 strains)   | Garrido et al. (2016)                  |
| LNnT  | <i>B. breve</i> (24 strains)   |   | Ruiz-Moyano et al. (2013)              |

### **Non-Bifidobacterium species**

Yu et al. (2013a) found that *Clostridium perfringens* and *Escherichia coli* did not grow or utilise 2'-FL after 48 hours of incubation.

In a related *in vitro* study, Yu et al. (2013b) tested the ability of a wider range of human gut bacteria to grow on 2'-FL as the sole sugar, including seven strains of *B. longum* subsp. *infantis*, three strains of *B. longum* subsp. *longum*, *Bacteroides vulgatus*, *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Clostridium perfringens*, *Clostridium leptum*, *Enterobacter aerogenes*, *Enterobacter cloacae* subsp. *cloacae*, *Enterococcus faecalis*, *Escherichia coli*, *Lactobacillus delbrueckii* subsp. *lactis*, *Lactobacillus rhamnosus*, *Staphylococcus epidermidis* and *Streptococcus thermophilus*. With the exception of one strain of *B. longum* subsp. *longum*, all other bifidobacteria and all three *Bacteroides* spp. consumed 40% or more of 2'-FL in 48 hours, and between 10 and 40% for one strain of *B. longum* subsp. *longum*, *Lactobacillus delbrueckii* subsp. *lactis*, *Streptococcus thermophilus* and one of the *Enterococcus faecalis* strains. This result suggests that the ability to utilise 2'-FL as a sole sugar source is not limited to *Bifidobacterium* spp. and supports the suggestion that strains of *Bacteroides* spp. can metabolise 2'-FL from human milk.

#### **4.2.3 Human studies with 2'-FL and/or LNnT**

Details of the clinical studies can be found in [Section 3.2.6](#).

##### **Clinical studies in infants**

*Infant study with 2'-FL in combination with LNnT Puccio et al. (2017)*

Observations of changes in the microflora from the Puccio et al. (2017) study were briefly described in two abstracts by Alliet et al. (2016) and Steenhout et al. (2016). Supporting CCI information for the infant study was provided by the applicant but cannot be provided in this report.

Healthy infants of  $\leq 14$  days of age were randomly assigned to consume a standard cow's milk-based infant formula containing long-chain polyunsaturated fatty acids without added oligosaccharides, with or without supplementation with 2'-FL and LNnT at concentrations of 1.0-1.2 g/L and 0.5-0.6 g/L of reconstituted formula, respectively. Randomisation was stratified to ensure a balance of infant sex and delivery method (vaginal or caesarean) between groups. Infants were scheduled to receive the test (n = 88) or control (n=87) formula up to six months of age, when infants in both groups were switched to an intact protein, cow's milk-based follow-up formula without the two HMOs for feedings up to 12 months of age.

Stool samples were collected from the infants at 3 months of age. The microbial composition was determined using 16S rRNA gene sequencing and metagenomics. The metabolite profiles of the stools included measurement of amino acids (phenylalanine, isoleucine and tyrosine), faecal organic acids and fucosylated compounds using proton NMR. Stool pH was not measured.

The global average microbial composition profile of the Control and Test groups were similar at the genus level. The microbial composition of the Test group's stools was observed to be closer to the breastfed group than the Control group consuming unsupplemented infant formula. The observed difference was statistically assessed using random permutations of the redundancy analysis using the microbial alpha diversity. The statistical test supports the observation that the Test group was different from Control group at the genus level ( $p < 0.001$ ) and closer to the breastfed group.

Further statistical analysis was performed to identify differences in the abundance of taxa between the Control and Test groups. Six groups were identified: *Bifidobacterium* ( $p=0.01$ ), *Escherichia* ( $p=0.008$ ), unclassified Coprobacillaceae ( $p=0.01$ ), unclassified Peptostreptococcaceae ( $p=0.026$ ), Dorea ( $p=0.033$ ), and Megamonas ( $p=0.035$ ). Correction for False Discovery Rate confirmed the first three taxa. Main discriminants between Test and Control by random forest analysis were *Bifidobacterium*, *Escherichia* and Peptostreptococcaceae. The direction of the differences of abundance were not reported.

A multivariate analysis of the stool metabolites identified phenylalanine, isoleucine, tyrosine, fecal organic acids and fucosylated compounds as influential to discriminate between the Test, Control and breastfed groups. The Test and breastfed groups were more similar in composition compared to the Control. The observed profiles were claimed to indicate reduced protein fermentation in the Test group compared to the Control group.

The authors concluded that infant formula supplemented with 2'-FL and LNnT shifted the stool microflora and metabolic profile towards that observed in breastfed infants.

*Infant study with 2'-FL in combination with galactooligosaccharides (Marriage et al. 2015)*

This study was excluded from the assessment of bifidogenic effect due to the inclusion of GOS.

*Infant study with 2'-FL in combination with short-chain fructo-oligosaccharides (Kajzer et al. 2016)*

This study was excluded from the assessment of bifidogenic effect due to the inclusion of scFOS.

*Infant study with LNnT (Prieto 2005)*

A randomised, double-blind placebo controlled study in human infants was conducted with LNnT produced by a coupled yeast/*E. coli* fermentation process. In this study 228 children (111 females and 117 males) aged 6-24 months were assigned to receive infant formula containing 220 mg LNnT or a control formula without LNnT *ad libitum* for 112 days. Stool pH was not measured.

Results of the analysis of the microflora of infant stools were only presented for *Lactobacillus* spp. from the FOS component of the study.

### **Clinical studies in children and adults**

*Child study with 2'-FL<sub>chem</sub> alone and in combination with LNnT<sub>chem</sub> (Holm 2018)*

An interim report of a randomised, placebo-controlled, double-blind parallel study with Glycom's 2'-FL<sub>chem</sub> and LNnT<sub>chem</sub> involving 75 children aged 5-12 years admitted to a hospital childhood obesity programme was submitted by the applicant after the 1<sup>st</sup> CFS was published. Children (25 per group) were randomised to consume an oral bolus dose of 4.5 g placebo (glucose), 2'-FL or a 4:1 mixture of 2'-FL and LNnT once daily for 8 weeks. Faecal samples were collected from the children at the start of the intervention and after 4 and 8 weeks of treatment for microbiota composition analyses using 16S RNA sequencing. Due to the ongoing nature and short duration of the study, FSANZ have not used the preliminary microbiota composition results in its assessment of bifidogenic effect.

### *Adult study with 2'-FL and/or LNnT (Elison et al. 2016)*

The applicant's 2'-FL and LNnT were evaluated in a parallel, double-blind, randomised, placebo-controlled study in 100 healthy adult volunteers. Participants were randomly assigned to consume either 2'-FL or LNnT at doses of 5, 10 or 20 g/day, a 2:1 mix of 2'-FL and LNnT at 5, 10 or 20 g mixture/day, or glucose (2 g/day) as a placebo control. The duration of the intervention was 14 days. All interventions were provided as a daily bolus dose dissolved in water, which participants were instructed to consume at breakfast. Clinical checks were made on the study participants at entry to the study and again at the end of the intervention. Blood samples for clinical chemistry and haematology analyses, as well as faecal samples for biomarker and microbiota composition analyses, were collected at baseline and at the end of the intervention period. Stool pH was not measured.

The composition of the gut microflora was determined using 16S RNA sequencing analysis. The primary impact of HMO supplementation on the microflora was an increase in the relative abundance of *Actinobacter* and *Bifidobacterium* and reduction in the relative abundance of Firmicutes and Proteobacteria. In total, 77% of all study participants responded to the HMO interventions. A dose response was observed whereby intervention groups given higher HMO concentrations had a greater abundance of *Actinobacter* and *Bifidobacterium*. No statistically significant differences were observed in the SCFA acetate, butyrate or propionate concentrations despite the shifts in the gut microflora.

## **4.3 Anti-infective effect**

### **4.3.1 Anti-infective effect of human milk**

In full-term infants, breastfeeding exclusively from 3-6 months of age and partially thereafter, has been associated with a significant reduction in infections of the gastrointestinal tract of infants (Kramer et al. 2001; Kramer et al. 2003; Tarrant et al. 2010)(Duijts et al. 2010). The protective effects of breastfeeding on infections can be attributed to several components in human milk (Lawrence and Pane 2007). The components in human milk that interact with innate immunity of infants can be classified as chemical, cellular, pathogen recognition and anti-inflammatory (Cacho and Lawrence 2017). The chemical component consists of human milk oligosaccharides and glycoproteins, such as lactoferrin, mucins and lactadherin, and have been proposed to directly bind to pathogens and/or regulating immune cell activation (Legrand 2016).

Antibodies, specifically immunoglobins (Ig), are secreted in human milk which provides pathogen recognition and subsequent immune functions to the infant (Cacho and Lawrence 2017). The majority of the Ig fraction in human milk is secretory IgA which can bind to pathogens in the intestinal mucosa and interfere with the infection processes without stimulating a major inflammatory response (Mantis et al. 2011). Other fractions of Ig present in human milk include IgM and IgG which contribute to the innate and adaptive immune responses from pathogen exposure in infants (Cacho and Lawrence 2017).

Living cells, including stem cells, macrophages and T cells, are also found in human milk, particularly during early lactation (Ballard and Morrow 2013). Maternal leukocytes in human milk provide immunity to the infant by phagocytosis of pathogens and producing bioactive components (Witkowska-Zimny and Kaminska-El-Hassan 2017). Pathogen recognition components, such as toll-like receptors and cluster of differentiation 14 (CD-14), are soluble in human milk that regulate toll-like receptor signalling in intestinal epithelial cells of the infant (He et al. 2016a). Furthermore, anti-inflammatory cytokines, immune/growth factors and antioxidants are also co-secreted in human milk that modulate the immune and inflammatory responses of the infant gastrointestinal tract (Garofalo 2010). The overall effect of the

maternal transfer of living cells and pathogen recognition molecules has been proposed to modulate the immune system and suppress inflammation in the infant.

With specific regard to the anti-infective effects of 2'-FL in human milk, Morrow et al. (2004) investigated the protective effects of HMOs against diarrhoeal illness in breastfed infants in San Pedro Martir, Mexico City. Ninety three breast-feeding mother-infant pairs were prospectively studied from birth to 2 years from 1988 to 1991 to determine if one or more major 2-linked fucosylated oligosaccharides of human milk are inversely associated with the incidence of diarrhoea caused by *Campylobacter* and calicivirus (norovirus). A single milk sample was analysed by high performance liquid chromatography for each mother; samples were collected between 1 and 5 weeks postpartum. The representativeness of this sample for the course of lactation was analysed using longitudinal data from 11 Mexican secretor mothers. Infant stool samples were collected weekly with additional samples obtained whenever diarrhoea occurred. Diarrhoea samples were routinely tested for *Campylobacter jejuni*, pathogenic *Escherichia coli*, *Shigella*, *Salmonella*, *Aeromonas*, and rotavirus. The incidence of *Campylobacter* diarrhoea in infants whose mother's milk contains low levels of 2'-FL (<29% of total HMO) was approximately 8.7 cases per 100 child months, verses an incidence of approximately 1.5 and 1.6 cases per 100 child months for mothers with intermediate (29-36% of total HMO) and high levels of 2'-FL ( $\geq 37\%$  of total HMO), respectively. Protective effects associated with the level of 2'-FL in human milk were not reported for other pathogens or moderate-to-severe diarrhoea of any cause. High levels of lacto-N-difucohexaose (>12% of total HMO) in mothers human milk was associated with a decreased incidence of calicivirus-related diarrhoea in infants compared to low levels (<7% of total HMO). High (>77% of total HMO) and intermediate (72-77% of total HMO) levels of all 2-linked fucosyl oligosaccharides were associated with a decreased incidence of moderate-to-severe diarrhoea of any cause, compared to low levels in mother's milk (<72% of total HMO). The concentration of 2'-FL and the total concentration of HMO in human milk was not reported by Morrow et al. (2004) and so it is not possible to infer a protective concentration of 2'-FL in milk. Furthermore, all mothers in the study were 2'-FL secretors and the incidence of *Campylobacter* associated diarrhoea in non-secretors was not determined, as such, the potential protective effects of 2'-FL in the low secretor cohort could not be determined.

#### 4.3.2 *In vitro* studies of bacterial toxin inhibition

FSANZ has assessed the submitted data on the inhibitory effects of 2'-FL and LNnT on bacterial toxins ([Appendix 2, Table A2.1](#)). Two studies (Crane et al. 1994; Newburg et al. 1990) were excluded from the assessment due to the use of mixed fucosylated oligosaccharide fractions from human donor milk, rather than 2'-FL in a purified form.

Two submitted studies (El-Hawiet et al. 2011; El-Hawiet et al. 2015) demonstrated weak binding affinity of purified 2'-FL to *Clostridium difficile* toxin A (TcdA) and toxin B (TcdB), *Vibrio cholerae* toxin (cholera toxin), *Escherichia coli* heat labile enterotoxin and *E. coli* Shiga toxin type 1 and Shiga toxin type 2 by direct electrospray ionization mass spectrometry. No inference can be drawn on the competitive inhibitory binding of 2'-FL to these toxins in the infant gut.

A single study on the binding of LNnT to streptolysin O was submitted. Shewell et al. (2014) used a red blood cell binding inhibition assay to demonstrate that binding to red blood cell glycans is required for the haemolytic activity of streptolysin O, which is a cholesterol-dependent cytolysin produced by *Streptococcus pyogenes*. Hemolytic activity of streptolysin O against red blood cells was blocked by the LNnT by inhibiting binding to the cell surface.

While the submitted studies provide evidence of a potential mechanism for a health effect of HMOs by competitively binding to bacterial toxins, no conclusions can be drawn on whether 2'-FL or LNnT binding will effectively inhibit toxin interactions with their cellular receptors in

the infant gut. The health effect of protecting infants and toddlers against bacterial toxins released in the gastrointestinal tract is not demonstrated.

#### 4.3.3 *In vitro* studies of anti-infective effect on microbial pathogens

FSANZ has assessed the submitted data on the *in vitro* anti-infective effects of 2'-FL on microbial pathogens ([Appendix 2, Table A2.2](#)). Eight studies were excluded from the assessment due to inclusion of mixed oligosaccharide fractions from human donors or sialylated HMOs (Andersson et al. 1983; Brassart et al. 1991; Coppa et al. 2006; Cravioto et al. 1991; Li et al. 2014; Ramphal et al. 1991) or the study did not address an anti-infective mechanism (Hoeflinger et al. 2015; Mezzoff et al. 2016). Two additional studies were identified by FSANZ and included in the assessment (Duska-McEwen et al. 2014; Hester et al. 2013).

A range of *in vitro* studies have demonstrated that 2'-FL and LNnT are able to inhibit, to varying degrees, binding or invasion of bacterial and viral pathogens to intestinal and respiratory epithelial cells ([Appendix 2, Table A2.2](#)).

Cellular and antigen binding studies have demonstrated that invasive *C. jejuni* strains bind specifically to  $\alpha$ 1,2-fucosyl moieties on the H2 blood group antigens of intestinal epithelial cells, but not for non-invasive strains, and that binding of invasive strains can be inhibited by 2'-FL (Ruiz-Palacios et al. 2003). Cell binding assays using biosensors also demonstrate a strong binding affinity of 2'-FL with invasive *C. jejuni*, but not for other bacteria tested including *Pseudomonas aeruginosa*, *Cronobacter sakazakii*, *Salmonella enterica* serotype Typhimurium, *Staphylococcus aureus*, *Listeria monocytogenes*, *Streptococcus dysgalactiae*, *Streptococcus mutans* (Lane et al. 2011). *In vitro* studies in various human cell lines, including HEp-2 (cervical epithelial cells) and HT-29 (intestinal epithelial cells), and Caco-2 (intestinal epithelial cells), and Chinese hamster ovarian cells transfected with the FUT1 gene (CHO-FUT1) have demonstrated evidence of 2'-FL inhibiting *C. jejuni* adhesion (Lane et al. 2012; Ruiz-Palacios et al. 2003; Weichert et al. 2013; Yu et al. 2016) in an apparent dose dependent manner (Yu et al. 2016).

Evidence from *in vitro* cell lines demonstrating an inhibitory effect of 2'-FL and LNnT for bacteria and viruses other than *C. jejuni* are variable and do not demonstrate specific binding inhibition ([Appendix 2, Table A2.2](#)). These studies present a possible but non-specific mechanism for a health effect, however a specific homologous relationship between the pathogen binding receptor and 2'-FL or LNnT has not been demonstrated. As such, conclusions cannot be drawn on whether formula supplemented with 2'-FL and/or LNnT will be effective in inhibiting pathogen interactions with their cellular receptors in the infant gut.

Overall, the evidence indicates that a health effect is possible in circumstances where there is homology between 2'-FL or LNnT and the cellular binding receptor required for adhesion and pathogenesis, such as for 2'-FL and the H2 blood group antigen binding receptor for invasive *Campylobacter jejuni*.

#### 4.3.4 *In vivo* studies of anti-infective effect on microbial pathogens

FSANZ has assessed the submitted data on the *in vivo* anti-infective effects of 2'-FL on microbial pathogens ([Appendix 2, Table A2.2](#)). Two studies were excluded from the assessment due to the use of mixed fucosylated oligosaccharide fractions from human donor milk in the murine challenge model (Ruiz-Palacios et al. 2003) or the inclusion of 6' and 3'-sialyllactose in oligosaccharide test mix (Li et al. 2014).

*Murine challenge study with 2'-FL (Glycosyn) and C. jejuni (Yu et al. 2016)*

The experimental murine model for *C. jejuni* strain 81-176 infection was optimised at 7 days of antibiotic treatment followed by 3 daily inoculation of  $10^8$  cfu/mouse in 100  $\mu$ L saline gavage. Four week-old C57BL/6 male mice were separated in to five treatment arms and received 7 days of an antibiotic treatment to disrupt the microflora and enhance the infection model. Group 1 received no treatment and were uninfected negative controls, Group 2 were uninfected mice administered 5 g/L of 2'-FL in their water for 3 days, Group 3 mice were infected with *C. jejuni* with no further intervention (positive controls), Group 4 mice were infected with an inoculum of *C. jejuni* that also contained 2'-FL, and Group 5 mice were administered 5 g/L of 2'-FL in their drinking water for 3 days before and concurrent with the inoculum containing both *C. jejuni* and 2'-FL. A disease activity index (DAI) schema was applied to infected and control mice which was a composite of weight loss, bleeding, and diarrhoea symptoms. *C. jejuni* was also quantified in faeces and tissues by real-time PCR.

The addition of 5 g/L of 2'-FL concurrently with *C. jejuni* challenge (Group 4), was protective against *C. jejuni* infection and faecal shedding and infection of the intestine, spleen and mesenteric lymph nodes were reduced by 90%, 80%, 96% and 93%, respectively, and DAI was reduced by 57% compared to infected positive controls. The addition of 5 mg/ml for 3 days before and concurrent with *C. jejuni* challenge (Group 5), was protective against *C. jejuni* infection and infection of the intestine, spleen and mesenteric lymph nodes were reduced by 99%, 97%, 97% and 98%, respectively, and DAI was reduced by 77% compared to infected positive controls.

Overall, these results indicate that 2'-FL added at relatively high concentrations to the diet of mice, is protective against invasive *C. jejuni* strains that require binding to fucosylated receptors on the surface of intestinal epithelial cells to initiate infection.

*Murine challenge study with 2'-FL (Glycosyn) and adherent-invasive E. coli (He et al. 2016b)*

Eight-week-old female C57BL/6 mice received 0.25% dextran sodium sulphate (DSS) in their drinking water for 3 days, and were given 20 mg of streptomycin by gavage on day 4; half also received 100 mg of 2'-FL in 200  $\mu$ L by gavage for each of the 4 days. On day 5, the two groups of experimental mice were inoculated with  $10^9$  colony forming units of adherent-invasive *E. coli* via 200  $\mu$ L gavage and sacrificed after an additional 4 days; a control group received DSS and antibiotic, but only a sham PBS inoculation. Body weight was monitored daily and adherent-invasive *E. coli* in faeces and colonic tissue were quantified.

Pre-treatment of mice with 100 mg of 2'-FL daily for 4 days prior to challenge with adherent-invasive *E. coli* prevented weight loss by approximately 10% by day four post infection. The bacterial count in colon tissue of infected mice was significantly less in the 2'-FL supplemented group compared to the positive control group, approximately 100 cfu/g of tissue verses approximately 750 cfu/g.

Overall, these results indicate that a high dose of 2'-FL is moderately protective against invasive adherent-invasive *E. coli* in a murine model. The very high dose of 2'-FL used in this study makes it difficult to infer an anti-infective effect of 2'-FL against adherent-invasive *E. coli* if administered at concentrations consistent with the proposed use in infant formula products.

*Pig challenge study with 2'-FL (Du Pont) and enterotoxigenic E. coli (Cillieborg et al. 2017)*

Twenty-five pigs were delivered at term by caesarean section from 2 FUT-1 homozygous sows. The pigs were prepared with umbilical and orogastric tubes, infused with parenteral nutrition and sow's plasma, inoculated with maternal faeces, and from day 2 transferred to

full enteral feeding with boluses of milk replacer. Pigs were allocated into 3 groups. One group was inoculated daily with  $7.5 \times 10^{10}$  enterotoxigenic *E. coli* F18 (n=9), 1 group was given the same dose of *E. coli* F18 plus 10 g/L 2'-FL in the milk replacer (n=8) and 1 control group received only milk replacer (n=8).

All of the 9 pigs challenged with *E. coli* F18 without 2-FL were euthanized before completion of the protocol because of extensive diarrhoea and poor clinical condition. Also, 6 out of 8 of the 2'-FL supplemented group and 2 of 8 control pigs were similarly euthanized. Administration of 2'-FL to *E. coli* F18 challenged pigs failed to prevent diarrhoea, although the relative weight loss tended to be reduced (-19 vs -124 g/kg,  $P=0.12$ ) and higher villi were observed in the distal small intestine (351  $\mu\text{m}$  vs 303  $\mu\text{m}$ ,  $P<0.05$ ).

Overall, these results indicate that a high dose of 2'-FL is weakly protective against enterotoxigenic *E. coli* F18 in a pig model and did not improve intestinal function or prevent diarrhoea in new born pigs. The unrealistically high dose of 2'-FL and the very high challenge dose of *E. coli* F18 used in this study makes it difficult to infer an anti-infective effect of 2'-FL against enterotoxigenic *E. coli* if administered at concentrations consistent with the proposed use in infant formula products.

#### 4.3.5 Infant feeding trials

*Infant study with 2'-FL in combination with LNnT (Puccio et al. 2017)*

Secondary outcomes measured in this study (refer to [Section 3.2.6](#)) included parent-reported adverse events that were classified according to the Medical Dictionary for Regulatory Activities (MedDRA) System, Organ, and Class (SOC) categories. For adverse events classified as 'Infections and Infestations', further categories were identified *a priori*, including upper respiratory tract infection, pyrexia, rhinitis, bronchitis, bronchiolitis, otitis media, pharyngitis and gastroenteritis. In addition, three adverse event clusters were identified: upper respiratory tract infection, lower respiratory tract infection, and otitis/ear infection. Morbidity outcomes were analysed in the ITT population using the Fisher exact test and reported as odds ratio (OR) and 95% confidence interval (CI).

Infants receiving test formula had significantly fewer reports of bronchitis through 4 months (2.3% vs 12.6%, OR=0.16, 95% CI 0.02-0.78,  $P\leq 0.01$ ), 6 months (6.8% vs 21.8%, OR=0.26, 95% CI 0.08-0.74,  $P\leq 0.01$ ) and 12 months (10.2% vs 27.6%, OR=0.30, 95% CI 0.11-0.73,  $P\leq 0.01$ ), and the adverse event cluster of lower respiratory tract infection through 12 months (19.3% vs 34.5%, OR=0.45, 95% CI 0.21-0.95,  $P<0.05$ ). Similar statistically significant differences between the Test and Control groups were observed in the subgroup of infants born by caesarean section for bronchitis through 12 months (3.1% vs 34.4%, OR=0.06, 95% CI 0.00-0.50,  $P=0.003$ ) and lower respiratory tract infection through 6 months (6.3% vs 28.1%, OR=0.17, 95% CI 0.02-0.96,  $P=0.043$ ) and 12 months (12.5% vs 40.6%, OR=0.21, 95% CI 0.04-0.83,  $P=0.022$ ). Infants receiving test formula had significantly fewer reports of antibiotic use through 6 months (34.1% vs 49.4%, OR=0.53, 95% CI 0.27-1.02,  $P<0.05$ ) and 12 months (42.0% vs 60.9%, OR=0.47, 95% CI 0.24-0.89,  $P<0.05$ ). No effect of 2'-FL and LNnT on gastrointestinal-related morbidities were observed.

Overall these results provide limited evidence that infant formula supplemented with 1.0-1.2 mg/ml 2'-FL and 0.5-0.6 mg/ml LNnT are associated with reduced rates of parent-reported morbidity compared to unsupplemented formula. No associations with reduced rates of gastrointestinal illness were reported. These results have not been reproduced elsewhere and are therefore limited in the context of inferring an anti-infective health effect for formula supplemented with 2'-FL and LNnT.

## 4.4 Intestinal barrier function, immune modulation and alleviation of allergic responses

### *Intestinal barrier function*

Maintenance of intestinal barrier function is essential for life and involves a dynamic process, mediated by intestinal epithelial cells (IECs). IECs play two major roles in this process. The first role involves uptake of nutrients and water. The second role is to provide a physical barrier against exogenous substances in the lumen. These substances include degraded food products, digestive enzymes, bacteria and bacterial-derived products, and antigenic components of the degraded food and bacteria. The uptake of nutrients and barrier function of the intestinal epithelial cells (IECs) are supported by junctional complexes, that provide adhesive contacts between neighbouring epithelial cells. The junctional complexes involve a range of different proteins and include tight junctions, adherens junctions and desmosomes (Lechuga and Ivanov 2017).

IECs in conjunction with immune cells regulate the expression and function of the junctional proteins. When barrier function is disrupted, there are associated changes in junctional proteins that lead to increased permeability. This can occur when immune cells need to cross the intestinal barrier, when pathogens target the barrier or there is trauma or surgical intervention to the intestinal barrier. When permeability is not restored, mucosal injury and inflammation can occur, as has been observed in inflammatory bowel diseases, like ulcerative colitis and Crohn's disease and in necrotising enterocolitis (NEC). Increased intestinal permeability is also prevalent in preterm infants, due to immaturity of the preterm gastrointestinal tract (Taylor et al. 2009; van Elburg et al. 2003). The immaturity of the preterm gut is associated with increased risk of developing gut-associated sepsis and NEC.

One of the consistent factors providing a positive impact in gastrointestinal health in neonates is human milk. Studies examining the health effects of human milk in full-term neonates have identified that infants exclusively fed human milk for 3-6 months experience a significant reduction in the incidence of gastrointestinal infections (Kramer et al. 2001; Kramer et al. 2003; Tarrant et al. 2010). In pre-term neonates, inclusion of human milk in enteral feeds has been shown to decrease intestinal permeability and reduce incidence of NEC and gut-associated sepsis (Patel et al. 2014; Taylor et al. 2009). Which components of human milk that mediates this protective effect has not yet been identified.

### *Immune modulation*

The immune system plays a crucial role in maintaining homeostasis by responding to various stimuli. In the classical "danger-sensing" paradigm (Matzinger 1994), injury to an epithelial barrier such as skin or the intestinal lining can lead to exposure to foreign or (i.e. non-self) material. This exposure can result in an inflammatory response, which aims to eliminate that foreign material and restore homeostasis. The immune system also senses and interacts with other body systems, in a collaborative process that most often benefits both systems and this is particularly pertinent in the interaction with the microbiota (Brodin and Davis 2017; Rook et al. 2015; Tanaka and Nakayama 2017). When the interaction between the immune system and other body systems is not functioning optimally, this can lead to the development of autoimmune disease and allergies.

The components of the immune system that leads to an inflammatory response can be divided into innate and adaptive systems. The innate system is typically a rapid and non-specific response involving myeloid cells, natural killer cells and innate lymphoid cells that express recognition and effector molecules, such as pattern recognition receptors and cytokines. The adaptive system is a slower and targeted response, often triggered by the

innate immune system. The adaptive response is mediated by T- and B-lymphocytes and can involve both cell-mediated and humoral responses.

Immune modulation involves changing the way the immune system operates. The most common method used to modulate the immune response is through vaccination. In the neonate and infant population, immune modulation is also impacted by a multitude of factors that include maternal-foetal interactions, method of delivery, nutrition and the development and composition of microbiota (Marques et al. 2013; Rautava 2016; Tanaka and Nakayama 2017). Nutrition source is important, as evidenced by studies showing a favourable decrease in incidence of infections in different cohorts where infants exclusively fed with human milk are compared to those fed formula (Kramer et al. 2003; Tanaka and Nakayama 2017; Tarrant et al. 2010). What is still unknown is whether an individual component of human milk can modulate the immune system in isolation.

#### *Exclusion of references*

Only primary sources of data were included in FSANZ's assessment of intestinal barrier function, immune modulation and allergy.

#### **4.4.1 Intestinal barrier function effects related to 2'-FL and LNnT**

A series of *in vitro* studies were presented that examined whether 2'-FL or LNnT could directly influence barrier function. A study by Holscher et al. (2014) examined the impact of 2'-FL and LNnT on epithelial maturation and differentiation *in vitro*. Treatment with 2g/L 2'-FL for 72h significantly increased cell differentiation and maturation of undifferentiated human epithelial cells (HT-29 and Caco-2Bbe) and increased digestive capacity in fully differentiated Caco-2 cells. These results were not observed for lower doses of 2'-FL (0.02 and 0.2 g/L). Treatment with 2g/L LNnT for 72h modestly enhanced cell differentiation and maturation and treatment with 0.2g/L LNnT for 72h also modestly decreased barrier permeability. There are several limitations with this study. While 72h might have been appropriate for measuring changes in cell proliferation and apoptosis in the undifferentiated cells, this time point may have been too late for identifying changes in HMO-induced biochemical pathways and gene transcriptional events in the differentiated cells. Furthermore, the LNnT-induced changes in barrier permeability may not be biologically significant considering the affect was not dose-responsive and was only minimally increased.

The cells examined by Holscher et al. (2014) were IECs obtained from adult colorectal cancer lines. Another *in vitro* study was provided that examined whether HMOs could impact differentiation of foetal IECs (Hester and Donovan 2012). This study used a mixture of HMOs consisting of 85% 2'-FL and 15% 3'-FL and was therefore excluded from FSANZ's assessment due to the inclusion of 3'-FL.

Only one study was presented where changes in junctional complex proteins was examined *in vitro* (Chichlowski et al. 2012). In this study, two bifidobacteria strains (*B. infantis* and *B. bifidum*) were cultured in the presence of either lactose or 2'-FL and then binding studies between the bifidobacteria and IECs (Caco-2 and HT-28) cells was examined. This study was excluded from assessment because there was no direct application of 2'-FL to the IECs and there was no characterisation of HMO-induced differences between the bifidobacterial strains to identify factors that could be impacting junctional complex protein expression and function.

While *in vitro* studies provide information on the impact of HMOs on a single cell type, *in vivo* studies allow examination of a broader range of effects. In experimental models of NEC induced by hypoxia, mice pups fed formula supplemented with 2'-FL (5g/L) were rated in disease severity more similar to dam-fed than unsupplemented formula-fed pups (Good et al.

2016). Disease severity was determined by the degree of gross and microscopic changes in intestinal mucosal injury, body weight and expression of pro-inflammatory mediators. Similar results were reported for rat pups fed formula supplemented with 2g/L 2'-FL although the results were not significantly different to formula-fed controls (Autran et al. 2016). Another result presented in this study showed that supplementation with 10g/L human milk derived-HMOs could not replicate the results for dam-fed rat pups, indicating that the concentration of HMOs was not optimal or there are other critical factors not present in the mix.

In a dextran sulphate sodium-induced colitis model of NEC, the overall results showed that 2'-FL supplementation of dam-fed animals did not impact loss of body weight or minimise the presence of a faecal biomarker for inflammation, calprotectin (Weiss et al. 2014). However, the authors note the possibility of gender-specific effects. This included diet-related differences in body weight and the inflammatory marker in male mice that was not present in females, in a feeding group where supplementation occurred from birth to day 45. The author concluded that 2'-FL may be protective in males. However, in the data presented, the control males in this feeding group showed increased susceptibility to the chemically-induced colitis. This was demonstrated by a greater loss of body weight and increased levels of the inflammatory marker compared to the control animals in all the other groups. While changes were apparent in the unsupplemented controls, the body weight changes from the supplemented males in this group was no different to the supplemented and unsupplemented mice in all the other groups. Furthermore, a comparison of the inflammatory marker between the genders could not be performed because the data for the females in the feeding group was not provided. The conclusion that 2'-FL supplementation was protective in males in this model is not supported by the data.

Only one of the *in vivo* studies examined the impact of HMOs on intestinal permeability in the induced-NEC model (Good et al. 2016) and the data showed that supplementation with 2'-FL resulted in restoration of barrier function. This effect on permeability was associated with 2'-FL regulation of endothelial nitric oxide synthase (eNOS) expression. A reduction in eNOS expression has previously been reported in experimental NEC and has been correlated with impaired intestinal perfusion (Yazji et al. 2013).

Changes in barrier function can also occur after surgical intervention in neonates and infants, leading to the development of short bowel syndrome. Surgical intervention is often a requirement in children who develop NEC, have birth defects associated with gastroschisis, volvulus or intestinal atresia or have congenital conditions like Hirschsprung's disease (Amin et al. 2013). A study was provided that examined the impact of 2'-FL on intestinal recovery in an *in vivo* model of short bowel syndrome (Mezoff et al. 2016). The surgery involved resection of the ileum to cecum of adolescent mice. During recovery, the mice were placed on a liquid diet. The control group received the liquid diet without supplementation and the intervention group received a supplemented liquid diet containing 2.5 g/L 2'-FL. At 21 days post-surgery, there was no difference in villus height, crypt depth or bowel circumference in animals supplemented with 2'-FL compared to non-supplemented animals. However, at 56 days post-surgery, there was a significant increase in crypt depth in animals supplemented with 2'-FL. Associated with this was a non-significant trend showing increased villus height and bowel circumference. Furthermore, there was a favourable weight divergence in 2'-FL supplemented animals from day 27 post-surgery. The data indicated that in an experimental model of short bowel syndrome that long term supplementation with 2'-FL improved intestinal recovery after surgery.

In the studies provided, there is plausible biochemical evidence that 2'-FL and LNnT may have an effect in promoting epithelial cell differentiation and maturation. 2'-FL may also be protective in experimental models of NEC or post intestinal surgery but the observed effects reported in the animal models were only seen at considerably high doses of 2'-FL to body

weight. None of the studies assessed by FSANZ provided clinically relevant data to support the protective effect of 2'-FL or LNnT in infants in restoring barrier function.

#### 4.4.2 Immune modulating effects related to 2'-FL

A series of *in vitro* and *ex vivo* studies have provided biochemical evidence that 2'-FL can directly influence immune cell signalling and trafficking. Early studies examining angiogenesis in autoimmune conditions discovered that 2'-FL, being used as a glucose analog of a blood group antigen, could induce expression of the leukocyte adhesion receptor ICAM-1 on human endothelial cell lines and promoted leukocyte rolling on epithelial surfaces (Zhu et al. 2003). Further analysis showed that these events were mediated by the activation of the cell signalling pathways JAK2-STAT3 and NFκβ in the endothelial cells (Zhu et al. 2003; Zhu et al. 2005). JAK2-STAT3 and NFκβ are key cell signalling pathways in immune cells, involved in regulating gene expression required for cell activation and cytokine expression. Subsequent studies showed these cell signalling pathways were activated by 2'-FL in monocytes (Amin et al. 2008; Rabquer et al. 2012) and mediated monocyte recruitment to sites of inflammation and expression of the cytokine IL-8.

Characterisation of 2'-FL in an infection model (Sotgiu et al. 2006), showed this HMO was not mitogenic for human monocytes but was effective at reducing the mitogenic effect of bacterial lipopolysaccharide (LPS). Even though 2'-FL reduced the LPS-stimulated proliferation of cells it had no impact on the LPS-induced cytokine response. A similar result was reported for swine monocytes, where 2'-FL was shown to be non-mitogenic and did not change the cytokine response induced by LPS or the plant-based mitogen phytohaemagglutinin (Comstock et al. 2014).

In a study examining epithelial cell responses to infection (in T84 and HCT-8 cell lines), 2'-FL was shown to reduce internalisation of pathogenic *E. coli* and the associated LPS-induction of IL-8 expression (He et al. 2016b). Further analysis demonstrated that 2'-FL was suppressing the transcription of an LPS cell surface receptor, CD14, while simultaneously triggering internalisation of the receptor. This reduction in CD14 on the surface of the cell correlated well with the decreased transcription of IL-8. The reduction in IL-8 expression could also have been mediated by a demonstrated downregulation of NFκβ by 2'-FL. Although 2'-FL was shown to have an effect in an *in vitro* infection model, this was not the case in a piglet study of enterotoxigenic *E. coli* infection (Cilieborg et al. 2017) (Refer to [Section 4.3.4](#)).

The immune response is also activated after injury. To investigate whether 2'-FL provided a benefit after injury, an animal surgical model has been examined (Mezoff et al. 2016). The surgery involved resection of the ileum to cecum of adolescent mice. During recovery, the mice were placed on a liquid diet. The control group received the liquid diet without supplementation and the intervention group received the liquid diet supplemented with 2.5 g/L 2'-FL. Subsequent analysis of the transcriptome at 8-weeks post-surgery identified 2030 significant changes in gene expression between the control and intervention groups. In particular, there was upregulation of mucosal immune response genes and downregulation of pathways related to IL-17 signalling. Although physiological changes were fully characterised (see [Section 4.4.1](#)), there was no further characterisation of the gene expression changes or determination of any associated changes to protein expression or function.

To determine if 2'-FL could mediate immune modulating effects in infants, a feeding study was performed comparing breastfed (BF) babies to formula-fed babies (Marriage et al. 2015). In the formula-fed cohort, there were three formula groups, supplemented with a total of 2.4 g/L oligosaccharides. The control formula (CF) contained 2.4 g/L GOS, experimental formula 1 (EF1) contained 2.2 g/L GOS plus 0.2 g/L 2'-FL and experimental formula 2 (EF2)

contained 1.4 g/L GOS and 1.0 g/L 2'-FL. Reported incidence of general adverse effects was no different between the formula-fed groups, however there were significantly more reported incidences of infections and infestations in the CF and EF2 groups. No data was provided for adverse effects in breastfed babies. A follow-on study provided an immunological characterisation of the infants on day 42 (Goehring et al. 2016). The CF group were reported to have less circulating T cells than the other feeding groups, associated with a decreased number of CD8+ cells. Levels of circulating proinflammatory cytokines (TNF $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$  and IL-6) and IL-1RA were significantly different across groups, with the rank from highest to lowest being: CF > EF2 > EF1 > BF. There was no feeding group effect seen in a phytohemagglutinin (PHA)-stimulation of T cell proliferation and differentiation assay, indicating that T cell maturation was not impacted by 2'-FL. The results from a respiratory syncytial virus (RSV)-stimulation assay were similar to the PHA-stimulation assay, where there was no feeding group effect seen in an immune response profile to RSV-infection. The CF group did however show generally higher levels of proinflammatory cytokines compared to the other feeding groups.

There are limitations to how the data from Goehring et al. (2016) can be interpreted. Although the authors reported a lower number of circulating T cells in the CF group, the reference range of T cells in infants is broad (Kotylo et al. 1993; Tosato et al. 2015) and a change in 5-10% as reported in the CF group is unlikely to be clinically significant. There is also no information provided on the current health status of the infants at the time of blood collection therefore the differences in T cell levels could be related to health status and not presence or absence of HMOs. Another limitation is the lack of a dose-response, which the author claims has been reported in other infant formula feeding studies. The previously summarised *in vitro* studies that examined different concentrations of 2'-FL all report concentration-response effects of 2'-FL (He et al. 2016b; Sotgiu et al. 2006; Zhu et al. 2003; Zhu et al. 2005). As the reported plasma levels of 2'-FL in these infants ranked from highest to lowest is BF > EF2 > EF1 > CF (Marriage et al. 2015), it may be expected that the BF and CF groups show the largest difference in T cell counts and this is what is observed. The fact that there is variance between the EF1 and EF2 groups suggest there are other factors coming into play that have not yet been elucidated. Furthermore, the potential role GOS may be playing in the variable results further complicates the interpretation of these results.

In the studies provided, there is biochemical evidence that 2'-FL can mediate changes in immune signalling and trafficking. However, the clinical significance of this data is inconclusive and an immune modulating effect for the proposed use of 2'-FL in infant formula products is therefore also inconclusive.

#### **4.4.3 Immune modulating effects related to LNnT**

Two *in vitro* studies were presented examining the role of LNnT in immune modulation. In the first study, LNnT (125 mg/L) was shown to be non-mitogenic to swine monocytes, did not induce cytokine production nor had any impact on LPS-stimulation of these monocytes (Comstock et al. 2014). However, LNnT tended to act synergistically with PHA, slightly enhancing proliferation and expression of the cytokines TNF $\alpha$  and IL-10.

In the second study, an analysis of the mitogenic and B cell enrichment potential of LNnT in splenocytes was investigated in an infection mouse model (Velupillai and Harn 1994). In uninfected control mice, LNnT conjugated to horse serum albumin (HSA) was only mildly mitogenic however it did not significantly enrich the B cell population or increase expression of IL-10 and IL-4. To show that this result was related to LNnT and not the HSA, competitive binding was demonstrated with non-conjugated LNnT, which inhibited the slight increase in lymphocyte proliferation. In the infected mice (schistosome), the HSA-LNnT conjugate showed significant mitogenic potential and enrichment of B cells yet only minimally induced IL-10 expression.

Several studies were provided demonstrating that LNnT can bind to a range of galectins, which are carbohydrate binding proteins produced by many cell types, including immune cells (Bohari et al. 2016; Collins et al. 2014; Halimi et al. 2014; Noll et al. 2016; Shams-Ud-Doha et al. 2017). As the focus of these studies was just to identify binding interaction and did not investigate the role of this binding interaction in immune modulation, these studies were excluded from assessment.

In the biochemical studies provided, there has been minimal demonstration that LNnT plays a significant role in immune modulation. Furthermore, no evidence has been provided to demonstrate an immune modulating effect of LNnT in infants.

#### **4.4.4 Immune modulating effects related to mixtures of HMOs**

Several *in vitro* and *in vivo* animal studies were provided that presented data on HMO modulation of the immune response. These studies included partially characterised HMO mixtures from pooled human colostrum samples (He et al. 2014) and pooled HMOs from mature milk (He et al. 2016b) or from chemically synthesised mixtures of HiMOs (Comstock et al. 2017; Li et al. 2014). These studies were excluded from the assessment as the mixtures contained HMOs other than just 2'-FL and LNnT.

#### **4.4.5 Allergic responses effects related to 2'-FL**

Food allergies are an adverse effect mediated by the immune response, resulting from exposure to an allergenic food. The initial event in food allergy development is sensitisation, where exposure triggers an immunoglobulin response mediated by B cells, producing IgE antibodies. Subsequent exposure or a challenge event results in both an IgE-mediated and non-IgE-mediated allergic response. The IgE-mediated response may involve gastrointestinal hypersensitivity, oesophageal inflammation and cutaneous urticaria and is associated with activation of mast cells and basophils. The most extreme IgE-mediated response is anaphylaxis. The non-IgE-mediated response is immune cell mediated and can be associated with activation of eosinophils and CD4+ T cells and release of histamine from mast cells (degranulation).

Evidence that human milk offers protection for development of food allergies is inconclusive but some studies have shown increased incidence of food allergies in formula fed infants or in infants that only receive human milk for less than 4 months (Schoetzau et al, 2002; Luccioli et al, 2014). The identification of the component of human milk that provides this protective effect was not reported in these studies.

Since food allergies are mediated by the immune response, where HMOs have been shown to play a potential immune modulating role ([Sections 4.4.2-4.4.3](#)), one study investigated if 2'-FL could impact food allergies using the ovalbumin-sensitised mouse model (Castillo-Courtade et al. 2015). Daily intake of 5g/L 2'-FL post-sensitisation was able to reduce the challenge-induced mast cells response to ovalbumin compared to unsupplemented controls. This attenuated response was associated with a reduction in mast cell degranulation. No changes in immune signalling were observed between the groups but there were increased numbers of IL-10 producing T cells present in the intestinal lymphatic tissue in the mice treated with 2'-FL. Although the mast cell response was attenuated in the 2'-FL group, the development of allergen-specific IgE antibodies was not modified, thereby the risk of anaphylaxis remains. The impact of 2'-FL was also examined in a passive cutaneous anaphylaxis model, and was shown to be effective at reducing inflammation as determined by decreased extravasation. The major limitations of this study was the absence of examination of a dose response, especially as the dose examined (5g/L 2'-FL) would be considered a high dose per body weight when comparing mice and infants.

Food allergies are one example of allergic responses that can develop in childhood. Other allergic responses, often associated with food allergies, include eczema, asthma and allergic rhinitis. The impact of breastmilk on incidence of these allergies was examined in a prospective study of children, considered at high-risk for developing allergies (Sprenger et al. 2017a). In particular, the 2'-FL secretor status of the mothers was examined. The study showed that the secretor status of the mothers had no impact on the prevalence of allergies at 2 and 5 years of age, in the cohort of children delivered vaginally. In those delivered by C-section, there was a trend showing higher rates of allergies when the mothers were non-secretors. There are several limitations with this study. Firstly, the data presented was not statistically significant indicating that this data was not biologically important and the cohort where the trend was evident contained only seven children. Furthermore, the secretor status of the children was not examined and this could have impacted the results.

The two studies examining the impact of HMOs on allergies (Castillo-Courtade et al. 2015; Sprenger et al. 2017a) did not provide strong evidence to support the protective effect of HMOs, such as 2'-FL, in preventing development of allergies in infants and children. Although 2'-FL was shown to have the potential to alleviate allergic responses post-sensitisation, the data was only observed in an animal model, using a considerably high dose of 2'-FL to body weight, thus the relevance to infants is inconclusive.

## 4.5 Key findings

FSANZ undertook assessments to determine if the proposed health effects of adding 2'-FL and/or LNnT to infant formula products and FSFYC are supported by evidence. As a part of the assessment, FSANZ did not identify evidence that would indicate the proposed effects would be limited to a particular age group of infants or toddlers. The key findings for each proposed health effect are therefore applicable to all the infant formula products and FSFYC to which this application applies.

### 4.5.1 Bifidogenic effect

FSANZ's assessment sought to determine if evidence supports the assertion that the addition of 2'-FL and LNnT to infant formula products and FSFYC could have a bifidogenic effect. FSANZ has previously recognised that the presence of *Bifidobacterium* and *Lactobacillus* in the intestinal microflora largely benefit the host<sup>16,17</sup>.

The microflora of the infant gut is complex and develops with time. Early in the infants life the microflora is dominated by *Bifidobacterium* spp., although the abundance and type of species present is dependent on many factors including delivery method, feeding method, environmental exposures and the use of antibiotics.

The ability of *Bifidobacterium* spp. to metabolise HMOs, specifically 2'-FL and LNnT, is variable within and between species. A review of the scientific literature highlights *B. longum* subsp. *infantis* as the most important component of the infant gut microflora for metabolising HMOs. *In vitro* studies of strains of this subspecies demonstrates a well-developed capacity to metabolise a wide range of HMOs including 2'-FL and LNnT. Subspecies associated with adults, such as *B. longum* subsp. *longum* have a reduced ability to metabolise HMOs. The available evidence therefore supports the biological and mechanistic plausibility that the addition of 2'-FL and/or LNnT to infant formula products will have a bifidogenic effect if the *Bifidobacterium* strains present are able to metabolise these HMOs. No bifidogenic effect would be expected if the *Bifidobacterium* strains present in infants gut are unable to metabolise 2'-FL and LNnT.

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<sup>16</sup> <http://www.foodstandards.gov.au/code/proposals/Pages/proposalp306addition3639.aspx>

<sup>17</sup> <http://www.foodstandards.gov.au/code/applications/Pages/applicationa1055shor4991.aspx>

A clinical feeding trial for infants supplied by the applicant provided some limited evidence that the addition of 2'-FL and LNnT to infant formula products will influence the gut microbiome to more closely resemble the microbiome of breastfed infants and with a higher relative abundance of *Bifidobacterium* spp. compared to infants fed unsupplemented formula. However, the reproducibility of this finding in multiple populations has not been demonstrated and the evidence is inconclusive.

No evidence was provided by the applicant to assess the bifidogenic effect for toddlers consuming FSFYC. Evidence from the development and maturation of the gut microflora suggests that toddlers older than one year of age will be more similar to that of adults than infants. A single clinical feeding trial for adults supplemented with either 2'-FL or LNnT alone or in combination at a 2:1 ratio of 2'-FL:LNnT demonstrated a shift in the gut microflora to higher relative abundance of bifidobacteria in a dose dependent manner. However, the bifidogenic effect remains dependent on the species present being able to metabolise 2'-FL or LNnT.

#### **4.5.2 Anti-infective effect**

The evidence assessed by FSANZ supports the plausibility of a health effect of 2'-FL being detrimental to invasive *C. jejuni* infection. The biological plausibility of the health effect has been demonstrated by evidence from animal studies and the plausible mechanism by which binding inhibition occurs is demonstrated by *in vitro* studies. These studies include evidence from an *in vivo* murine model demonstrating decreased disease severity in animals fed 5 g/L 2'-FL and binding studies demonstrating a specific interaction between invasive *C. jejuni* strains and 2'-FL and *in vitro* studies demonstrating *C. jejuni* binding inhibition in multiple cell lines. Evidence from a human study showing a decreased incidence of *Campylobacter* associated diarrhoea in infants of mothers with a higher proportion of 2'-FL in their milk provides additional supporting evidence.

The extent of the health effect at the levels requested in infants and young children cannot be quantified. FSANZ's assessment did not establish an anti-infective dose response effect for 2'-FL and invasive *C. jejuni* infection *in vivo*, although it is possible that higher concentrations of 2'-FL could enhance the anti-infective effect as was observed in an *in vitro* inhibition assay ([Appendix 2](#), [Table A2.2](#)).

The evidence for a health effect of 2'-FL and LNnT protecting against other pathogens and toxins is inconclusive and is primarily limited to *in vitro* inhibition studies. The human infant trial data provided by the applicant provides some limited evidence of a decreased rate of bronchitis and lower respiratory tract infection but no decreased rate of gastrointestinal illness in infants fed formula supplemented with 2'-FL and LNnT. However, the reproducibility of this finding in multiple populations has not been demonstrated and the evidence is inconclusive.

#### **4.5.3 Intestinal barrier function**

There is plausible biochemical evidence that 2'-FL and LNnT may have an effect in promoting epithelial cell differentiation and maturation in *in vitro* systems, which may be important in pre-term infants where the intestinal barrier is too immature to function properly or in full term infants that have trauma or surgical intervention. 2'-FL may also reduce mucosal permeability and inflammation in experimental models of NEC or after intestinal surgery but the observed effects reported in the animal models were only seen at considerably high doses of 2'-FL to body weight. None of the studies assessed by FSANZ provided clinically relevant data to support the protective effect of 2'-FL or LNnT in infants in restoring barrier function. It is therefore not possible to draw conclusions on the health effect of 2'-FL and LNnT in restoring barrier function in infants and toddlers.

#### 4.5.4 Immune modulation and alleviation of allergic responses

There is biochemical evidence that 2'-FL can mediate changes in immune signalling, cytokine expression and trafficking in *in vitro* systems, thereby demonstrating that 2'-FL has the potential to modulate the immune response. However the data obtained from animal studies and the one infant feeding study are ambiguous in directly linking 2'-FL to an immune modulating effect in whole body systems and are therefore inconclusive. In summary, there has been no demonstration to date that 2'-FL has an immune modulating effect in infant populations.

There is limited biochemical evidence of any immune modulating effect of LNnT and no data has been provided to demonstrate health effects in infant populations.

A single study showed that 2'-FL has the potential to alleviate allergic responses post-sensitisation in an animal model. This required a considerably high dose of 2'-FL relative to body weight. Of clinical relevance was that 2'-FL did not prevent production of allergen-specific IgE-immunoglobulins, therefore the risk of anaphylaxis remains.

A prospective study examining the impact of 2'-FL in human milk on development of allergies in high-risk children showed minimal effects.

The evidence that 2'-FL or LNnT is immune modulating in infants is inconclusive, due to the reliance on *in vitro* studies and lack of clinically relevant studies

## 5. Conclusion

The application is requesting a variation to the *Australia New Zealand Food Standards Code* (the Code) to permit two human milk identical oligosaccharides (HiMO), 2'-O-fucosyllactose (2'-FL) and lacto-N-neotetraose (LNnT) derived by microbial fermentation from genetically modified (GM) *Escherichia coli* K-12. These two substances are found naturally in human milk. The applicant (Glycom) has requested permission for the addition of 1.2 g/L of 2'-FL alone or with an additional 0.6 g/L of LNnT (i.e. totalling 1.8 g/L) in infant formula products (includes infant formula, follow-on formula, and infant formula products for special dietary use) and formulated supplementary foods for young children (FSFYC or 'toddler milks').

The stated purpose for adding 2'-FL, alone or in combination with LNnT, to infant formula products or FSFYC is to better reflect the oligosaccharide composition of human milk. These HiMOs are stated by the applicant to confer functional benefits to infants and young children (also referred to as 'toddlers' in this report), consistent with the human milk oligosaccharide (HMO) fraction of human milk, with three specified health effects: (1) a bifidogenic effect, (2) an anti-infective effect against pathogens, and (3) immune modulation, improved intestinal barrier function and alleviation of allergic responses.

The safety, technical and health effects assessment includes: (i) a food technology assessment of 2'-FL and LNnT; (ii) a safety assessment to identify potential adverse effects associated with 2'-FL and LNnT; (iii) a dietary intake assessment to estimate the total dietary intake of 2'-FL and LNnT for breastfed infants and intake resulting from the addition of 2'-FL and LNnT to infant formula products and FSFYC; and (iv) an assessment of the stated health effects.

The food technology assessment concluded that the applicant's 2'-FL and LNnT are chemically and structurally identical to the naturally occurring oligosaccharides in human milk and to chemically synthesised oligosaccharides, using appropriate methods of analysis. The shelf-life and specifications are appropriate for addition to infant formula products and FSFYC.

The GM safety assessment concluded that no public health and safety concerns have been identified for 2'-FL and LNnT derived from genetically modified *E. coli* K-12, production strains SCR6 and MP572, respectively.

Based on an assessment of the available toxicological and clinical evidence for 2'-FL and LNnT, it was concluded that there were no public health and safety concerns associated with the addition of 2'-FL, alone or in combination with LNnT, to infant formula products and formulated supplementary foods for young children, at the levels requested by the applicant and at the estimated levels of dietary intake based on 2.4 g/L of 2'-FL and 0.6 g/L of LNnT. Since the applicant's 2'-FL and LNnT are structurally and chemically identical to the forms of these substances present in human milk, no differences in pharmacokinetics between naturally occurring and manufactured forms of 2'-FL and LNnT are expected. Overall, the available data indicated that intestinal absorption is limited, and a significant proportion of HMOs including 2'-FL and LNnT reach the large intestine where they are fermented by the microbiota or excreted unchanged in the faeces.

Both 2'-FL and LNnT produced by microbial fermentation were not genotoxic in *in vitro* bacterial mutagenicity assays or in *in vitro* micronucleus assays in human lymphocytes. No adverse effects were observed in subchronic oral toxicity studies with 2'-FL or LNnT in juvenile rats at doses up to 5000 mg/kg bw/day. In human studies, infant formula supplemented with 2'-FL and LNnT was well tolerated with age-appropriate increases in body weight and other growth measures, and no significant increases in adverse events. 2'-FL and

LNnT were also well tolerated in studies with obese children aged 5-12 years and healthy adults.

The assessment of effect on infant growth concluded that the addition of 2'-FL, alone or in combination with LNnT, to infant formula products has no effect on growth at the levels requested by the applicant. 2'-FL has been tested in formula in combination with short-chain fructooligosaccharide (scFOS) or galactooligosaccharide (GOS) or LNnT. The highest tested concentrations of 2'-FL and LNnT were 1.2 and 0.6 g/L, respectively. None of the studies examined by FSANZ found a difference in infant growth compared to a control formula. Based on a lack of adverse effects on growth in the clinical studies reviewed, and the limited gastrointestinal absorption of 2'-FL and LNnT, there is no evidence to indicate a nutritional concern at concentrations that are typically observed in human milk.

The concentration of 2'-FL in infant formula / follow-on formula / FSFYC considered in the dietary intake assessment was 2.4 g/L (rather than 1.2 g/L requested) as this level is similar to the mean concentration in mature human milk (2.4 – 3.0 g/L for 2'-FL secretors, which represents approximately 80% of women worldwide). This is approximately one fifth the total concentration of oligosaccharides present in mature human milk (10-15 g/L). The estimated dietary intake of 2'-FL based on 2.4 g/L in infant formula and follow-on formula is similar to 2'-FL intakes for 3 and 9 month old breastfed infants. Estimated mean intakes of 2'-FL from FSFYC, based on 2.4 g/L, for 12 month old infants and 2-3 year old children are similar to or less than those for younger formula-fed and breastfed infants (< 12 months).

The applicant requested a maximum of 0.6 g/L LNnT in infant formula products and FSFYC. This concentration was considered in the dietary intake assessment. The mean concentration of LNnT in mature human milk is 0.28 – 0.31 g/L, noting all human milk contains LNnT. The estimated dietary intake of LNnT is therefore higher than that for 3 month and 9 month old breastfed infants due to the requested concentration of LNnT in infant formula and follow-on formula being higher than the mean concentration in human milk. However, the use level of 0.6 g/L is within the range of LNnT concentrations in mature human milk (0.09 – 1.08 g/L). Estimated mean intakes of LNnT from FSFYC for 12 month old infants and 2-3 year old children are similar to or lower than those for younger formula-fed infants (< 12 months).

The assessment of bifidogenic effect concluded that the ability of *Bifidobacterium* spp. to metabolise 2'-FL and LNnT is variable within and between species and that a bifidogenic effect is biologically and mechanistically plausible if the *Bifidobacterium* strains present in the infant and toddler colon are able to metabolise 2'-FL or LNnT. A single study, published as abstracts, demonstrated that infants fed formula supplemented with 2'-FL and LNnT at levels similar to those requested, had a gut microbiome at 3 months of age that more closely resembled that of breastfed infants and with a higher relative abundance of *Bifidobacterium* spp. compared to infants fed unsupplemented formula. As the reproducibility of this study has not been demonstrated in other populations the results are inconclusive. However, the biological plausibility of a bifidogenic effect occurring due to the requested addition of 2'-FL alone or with LNnT is further supported by a single clinical feeding trial for adults that showed a shift in the gut microflora to a higher relative abundance of bifidobacteria in a dose dependent manner following supplementation with either 2'-FL or LNnT alone or in combination at a 2:1 ratio of 2'-FL:LNnT.

The assessment of anti-infective effect concluded that the addition of 2'-FL to infant formula products and FSFYC may be detrimental to attachment and growth of invasive *C. jejuni* infection through binding inhibition. The biological and mechanistic plausibility of this health effect is supported by evidence from an *in vivo* murine model demonstrating decreased disease severity in animals fed 5 g/L 2'-FL, binding studies demonstrating a specific interaction between invasive *C. jejuni* strains and 2'-FL, and *in vitro* studies demonstrating *C.*

*jejuni* binding inhibition in multiple cell lines. Evidence from a human study showing a decreased incidence of *Campylobacter* associated diarrhoea in infants of mothers with a higher proportion of 2'-FL in their milk provides additional supporting evidence. Based on the evidence assessed, FSANZ considers that this health effect could occur at the level of 2'-FL requested, although the extent of the effect in infants and young children at this level cannot be determined. The evidence for a health effect of 2'-FL and LNnT protecting against other pathogens and toxins is inconclusive and is primarily limited to *in vitro* inhibition studies with no specific mechanism of inhibition identified. A single human infant trial study provided limited evidence of a decreased rate of bronchitis and lower respiratory tract infection in infants fed formula supplemented with 2'-FL and LNnT. However, the reproducibility of this finding in multiple populations has not been demonstrated and is therefore inconclusive.

The assessment of immune modulation and improved barrier function concluded that there is insufficient evidence to support the assertion that infant formula supplemented with 2'-FL alone or with LNnT, will have an immune modulating effect or improve barrier function in infants and toddlers. The evidence to support these proposed health effects are largely based on *in vitro* studies and are not well supported by *in vivo* animal models or infant feeding studies. Of clinical significance in the assessment of food allergies, the available evidence demonstrates that 2'-FL does not prevent the production of allergen-specific IgE-immunoglobulins after sensitisation has occurred, and therefore 2'-FL does not protect against anaphylaxis.

FSANZ concludes that the bifidogenic effect and anti-infective effect against invasive *C. jejuni* are biologically plausible and the assessed evidence supports a plausible mechanism for these effects, although direct and consistent evidence of association in infants and toddlers, as demonstrated by well-designed randomised control trials, are lacking. In reaching this conclusion, FSANZ has taken into consideration the complexity of definitively and reproducibly demonstrating a health effect for a substrate targeted at modulating gut microflora. Evidence from an *in vitro* laboratory study for anti-infective effect and an adult study for bifidogenic effect, indicates that these health effects may be enhanced as concentrations of 2'-FL (or LNnT in the case of the bifidogenic effect only) are increased. Evidence to support the health effects of improved barrier function, immune modulation and alleviation of allergic responses are inconclusive.

2'-FL and LNnT are naturally present in human milk in a range of concentrations and ratios, providing a history of safe human exposure to these substances for breastfed infants. FSANZ concludes there are no public health and safety concerns associated with the addition of 2'-FL alone or in combination with LNnT to infant formula products and FSFYC at the requested levels, or at higher estimated levels of dietary intakes based on 2.4 g/L 2'-FL.

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# Appendix 1: Dietary Intake Assessments at FSANZ

A dietary intake assessment is the process of estimating how much of a food chemical a population, or population sub group, consumes. Dietary intake of food chemicals is estimated by combining food consumption data with food chemical concentration data. The process of doing this is called 'dietary modelling'.

*Dietary intake = food chemical concentration x food consumption*

FSANZ's approach to dietary modelling is based on internationally accepted procedures for estimating dietary intake of food chemicals. Different dietary modelling approaches may be used depending on the assessment, the type of food chemical, the data available and the risk assessment questions to be answered. In the majority of assessments FSANZ uses the food consumption data from each person in the national nutrition surveys to estimate their individual dietary intake. Population summary statistics such as the mean intake or a high percentile intake are derived from the ranked individual person's intakes from the nutrition survey.

An overview of how dietary intake assessments are conducted and their place in the FSANZ Risk Analysis Process is provided on the FSANZ website at:

<http://www.foodstandards.gov.au/science/riskanalysis/Pages/default.aspx>

FSANZ has developed a custom-built computer program 'Harvest' to calculate dietary intakes. Harvest replaces the program 'DIAMOND' that was been used by FSANZ for many years. Harvest has been designed to replicate the calculations that occurred within DIAMOND using a different software package.

Further detailed information on conducting dietary intake assessments at FSANZ is provided in *Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes* (Food Standards Australia New Zealand 2009), available at:

[http://www.foodstandards.gov.au/science/exposure/documents/Principles%20 %20practices %20exposure%20assessment%202009.pdf](http://www.foodstandards.gov.au/science/exposure/documents/Principles%20%20practices%20exposure%20assessment%202009.pdf)

## A1.1 Food consumption data used

The most recent food consumption data available were used to estimate intakes of 2'-FL<sub>micro</sub> and LNnT<sub>micro</sub> for the Australian and New Zealand populations. The national nutrition survey (NNS) data used for these assessments were:

- The 2011-12 Australian National Nutrition and Physical Activity Survey (2011-12 NNPAS).

The design of this survey and the key attributes are set out below. Further information on the National Nutrition Surveys used to conduct dietary intake assessments is available on the FSANZ website at:

<http://www.foodstandards.gov.au/science/exposure/Pages/dietaryexposureandin4438.aspx>

Where data from national surveys do not exist for a population subgroup, model diets can be constructed to approximate a typical diet for that group. This was the case in this assessment for children under 2 years of age.

### **A1.1.1 2011–12 Australian National Nutrition and Physical Activity Survey (2011-12 NNPAS)**

The 2011–12 Australian National Nutrition and Physical Activity Survey (2011-12 NNPAS), undertaken by the Australian Bureau of Statistics, is the most recent food consumption data for Australia. This survey includes dietary patterns of a sample of 12,153 Australians aged from 2 years and above. The survey used a 24-hour recall method for all respondents, with 64% of respondents also completing a second 24-hour recall on a second, non-consecutive day. The data were collected from May 2011 to June 2012 (with no enumeration between August and September 2011 due to the Census). Only those respondents who had two days of food consumption data were used to estimate 2'-FL and LNnT dietary intakes for this assessment. The Day 1 2 average provides the best estimates of 2'-FL and LNnT dietary intakes for Australians and New Zealanders aged 2-3 years (noting that the New Zealand national nutrition surveys do not contain data for children <5 years of age). Consumption and respondent data from the survey were incorporated into the Harvest program from the Confidentialised Unit Record Files (CURF) data set (Australian Bureau of Statistics 2014).

### **A1.3 Limitations of dietary intake assessments**

Dietary intake assessments based on 2011-12 NNPAS food consumption data provide the best estimation of actual consumption of a food and the resulting estimated dietary intake assessment for the Australian population aged 2 years and above. However, it should be noted that NNS data do have limitations. Further details of the limitations relating to dietary intake assessments undertaken by FSANZ are set out in the FSANZ document, *Principles and Practices of Dietary Exposure Assessment for Food Regulatory Purposes* (Food Standards Australia New Zealand 2009).

As there were no data available from the 2011–12 Australian National Nutrition and Physical Activity Survey (2011-12 NNPAS) and the 2002 NZNNS for children aged less than 2 years, model diets were constructed to estimate dietary 2'-FL and LNnT intakes for the target groups of children aged 3 months (representing exclusively formula-fed infants), 9 months (representing infants who consume food as well as follow-on formula) and 12 months (for children who consume FSFYC).

## Appendix 2: Summary of evidence considered in health effects assessment

**Table A2.1 Summary of evidence for HMO inhibition of bacterial toxins**

| Reference               | Experimental model   | HMO assessed  | Pathogen (toxin)  | Main findings  |
|-------------------------|--|---|---|--|
| El-Hawiet et al. (2011) | Direct electrospray ionization mass spectrometry assay.<br><br>Verocytotoxicity neutralisation assays on HMO extracts from donor human milk. | 2'-FL<br><br>Oligosaccharide fraction from donor milk | 1. <i>Clostridium difficile</i> (toxin A (TcdA))<br>2. <i>Clostridium difficile</i> (toxin B (TcdB))  | The binding of the toxin fragments to the panel of 21 HMOs was uniformly weak. Specifically, 2'-FL had low binding affinity for both toxin fragments, with the highest affinity for the A2 fragment of TcdA. Study authors suggest that HMO concentrations need to be very high to successfully compete with the vero cells for binding to TcdA and TcdB.<br><br>Verocytotoxicity neutralization assays on HMO extracted from donor human milk indicate that the HMOs do not significantly inhibit the cytotoxic effects of TcdA or TcdB |
| El-Hawiet et al. (2015) | Direct electrospray ionization<br><br>mass spectrometry assay  | 2'-FL   | 1. <i>Vibrio cholerae</i> (cholera toxin)<br>2. <i>Escherichia coli</i> (heat labile enterotoxin)<br>3. <i>Escherichia coli</i> (Shiga toxin type 1)<br>4. <i>Escherichia coli</i> (Shiga toxin type 2) | The binding of the toxins to the panel of 20 HMOs was uniformly weak. 2'-FL had weak binding affinity for all four toxins studied with the highest affinity for the <i>E. coli</i> heat labile enterotoxin. The authors hypothesise that if consumed in sufficiently high quantity then HMOs could competitively inhibit toxin from interacting with cellular binding sites.   |
| Crane et al. (1994)     | T84 intestinal cell binding inhibition assay   | Fucosylated oligosaccharide fraction from donor milk  | 1. <i>Escherichia coli</i> (heat-stable enterotoxin)  | The fucosylated oligosaccharide fraction of human milk inhibited toxin binding to T84 cells by approximately 25% compared to control. No specific conclusions can be drawn on 2'-FL.<br><br>Study excluded from assessment due to use of mixed fucosylated oligosaccharide fraction.   |
| Newburg et al. (1990)   | Suckling mouse challenge   | Fucosylated oligosaccharide fraction from donor milk  | 1. <i>Escherichia coli</i> (heat-stable enterotoxin)  | The neutral fucosylated oligosaccharide fraction of human milk protected suckling mice from lethal fluid losses. No conclusions can be drawn on 2'-FL.<br><br>Commercially acquired 2'-FL did not protect suckling mice  |

| Reference             | Experimental model                            | HMO assessed | Pathogen (toxin)                                  | Main findings  |
|-----------------------|---|--------------|---|--|
|                       |   |              |   | against heat stable enterotoxin.<br>Study excluded from assessment due to use of mixed fucosylated oligosaccharide fraction.   |
| Shewell et al. (2014) | Red blood cell (RBC) binding inhibition assay | LNT          | 1. <i>Streptococcus pyogenes</i> (streptolysin O) | LNT inhibits streptolysin O (SLO) binding to glycan receptor on RBC. The inclusion of LNT in this study was to demonstrate that the SLO binding site on RBC was a glycan rather than cholesterol. The study was not designed to demonstrate that LNT in infant formula would prevent haemolysis by SLO in infants infected with <i>S. pyogenes</i> . |

**Table A2.2 Summary of evidence for HMO anti-infective effect for bacterial and viral pathogens**

| Reference                   | Experimental model  | HMO assessed   | Pathogen                    | Main findings and comments  |
|-----------------------------|---|--|-----------------------------|---|
| Brassart et al. (1991)      | Human buccal epithelial cell binding inhibition assay   | 2'-FL and 3'FL fraction obtained by chromatography from pooled human donor milk  | <i>Candida albicans</i>     | <p>2'-FL and 3'FL fraction inhibited <i>C. albicans</i> binding to buccal cells by approximately 32%.</p> <p>No specific conclusions can be drawn on the anti-infective or anti-adhesion effect of 2'-FL from this study due to the HMO fraction containing both 2'-FL and 3'FL.</p> <p>Study excluded from assessment due to use of mixed fucosylated oligosaccharide fraction.</p>  |
| Ruiz-Palacios et al. (2003) | <ol style="list-style-type: none"> <li>H-1 and H-2 blood group antigen binding inhibition assay</li> <li>Human intestinal mucosa binding inhibition assay</li> <li>CHO-FUT1 transfection binding assay</li> <li>HEp-2 cell binding inhibition assay</li> <li>BALB/c mice challenge</li> </ol> | <p>Fucosylated oligosaccharide fraction from donor milk used in Hep-2 cell binding inhibition assay and neutral milk oligosaccharides fraction used in mouse challenge experiment.</p> <p>2'-FL used in H1 and H2 blood group antigen binding experiment, CHO-FUT1 transfection experiment and human intestinal mucosa binding inhibition assay.</p> | <i>Campylobacter jejuni</i> | <p>Ten out of twelve invasive <i>C. jejuni</i> strains tested bind specifically to <math>\alpha</math>1,2-fucosylated containing H2 blood group antigens (which are expressed in the intestinal mucosa) and this binding can be inhibited by 2'-FL. Invasive <i>C. jejuni</i> binding to H1 antigen is non-specific.</p> <p>Chinese hamster ovary (CHO) cells were transfected with either FUT1, FUT3 or FUT4 genes that encode for the <math>\alpha</math>1,2-fucosyltransferase (CHO-FUT1), <math>\alpha</math>1,3/4-fucosyltransferase (CHO-FUT3), and <math>\alpha</math>1,3-fucosyltransferase (CHO-FUT4). Substantially more invasive <i>C. jejuni</i> bound to the FUT1 transfected cells than to other cell types demonstrating specific binding affinity for the <math>\alpha</math>1,2-fucosyl moiety.</p> <p>2'-FL incubated with <i>C. jejuni</i> culture at a concentration of 2 mg/ml was able to inhibit invasive <i>C. jejuni</i> binding to ileum specimens by 69%, whereas 2'-FL was able to inhibit non-invasive <i>C. jejuni</i> binding by only 8%. The study authors conclude that the epithelial cell surface H2 blood group (fucosyl <math>\alpha</math>1,2) epitopes are the preferred binding site of invasive strains of <i>C. jejuni</i> but not for non-invasive strains.</p> <p>No specific conclusions can be drawn on the anti-infective or anti-adhesion effect of 2'-FL from the Hep-2 cell and mice challenge studies due to the HMO fraction containing all fucosylated or neutral oligosaccharides in the pooled donor milk.</p> |

| Reference          | Experimental model   | HMO assessed          | Pathogen  | Main findings and comments   |
|--------------------|--|-----------------------|---|--|
|                    |  |                       |   | Hep-2 in vitro and murine in vivo studies excluded from assessment due to use of mixed oligosaccharide fraction.   |
| Lane et al. (2011) | Biosensor binding assay  | Biotin labelled 2'-FL | <ol style="list-style-type: none"> <li>1. <i>Campylobacter jejuni</i></li> <li>2. <i>Pseudomonas aeruginosa</i></li> <li>3. <i>Cronobacter sakazakii</i></li> <li>4. <i>Salmonella enterica</i> serotype Typhimurium</li> <li>5. <i>Staphylococcus aureus</i></li> <li>6. <i>Listeria monocytogenes</i></li> <li>7. <i>Streptococcus dysalactiae</i></li> <li>8. <i>Streptococcus mutans</i></li> </ol> | <p>Biotin labelled 2'-FL captured on streptavidin coated biosensor surface and <i>C. jejuni</i> passed over sensor surface. Sensor signal indicates binding to 2'-FL. Binding demonstrated for <i>C. jejuni</i> and weak binding signal for <i>S. aureus</i>. No binding signal detected for other bacteria screened.</p> <p>Use of biotin labelled 2'-FL provides evidence to demonstrate specific binding affinity of invasive <i>C. jejuni</i>.</p>   |
| Lane et al. (2012) | HT-29 cell adhesion and invasion inhibition assay  | 2'-FL                 | <i>Campylobacter jejuni</i>   | <p>Incubation of 2'-FL with invasive <i>C. jejuni</i> prior to addition to HT-29 cells resulted in a reduction in adhesion to cells by 55% compared to control (<i>C. jejuni</i> only). 2'-FL did not prevent <i>C. jejuni</i> invasion into HT-29 cells.</p> <p>The source and concentration of 2'-FL was not reported but possibly added at 1 mg/ml consistent with addition of other human milk oligosaccharides.</p>   |
| Yu et al. (2016)   | <ol style="list-style-type: none"> <li>1. HEp-2 and HT-29 cell invasion inhibition assay</li> <li>2. C57BL/6 murine challenge study</li> </ol> | 2'-FL                 | <i>Campylobacter jejuni</i>   | <p>Incubation of 2'-FL with invasive <i>C. jejuni</i> prior to addition to HEp-2 and HT-29 cells line inhibited cell invasion in a dose-dependent manner and 1 mg/ml resulted in approximately 50% inhibition in both cell lines.</p> <p><i>In vivo</i> challenge with invasive <i>C. jejuni</i> in a murine infection model demonstrated a protective effect of 2'-FL. When administered at 5 mg/ml concurrently with <i>C. jejuni</i> challenge, faecal shedding and infection of the intestine, spleen and mesenteric lymph nodes were reduced by 90%, 80%, 96% and</p> |

| Reference              | Experimental model   | HMO assessed | Pathogen   | Main findings and comments   |
|------------------------|--|--------------|--|--|
|                        |  |              |  | 93%, respectively, compared to uninfected controls. When 2'-FL was administered at 5 mg/ml for 3 days before and concurrent with <i>C. jejuni</i> challenge, faecal shedding and infection of the intestine, spleen and mesenteric lymph nodes were reduced by 99%, 97%, 97% and 98%, respectively, compared to uninfected controls.   |
| Weichert et al. (2013) | <ol style="list-style-type: none"> <li>1. Caco-2 cell adhesion inhibition assay</li> <li>2. Lung carcinoma cell line A549 adhesion inhibition assay</li> </ol>                 | 2'-FL        | <ol style="list-style-type: none"> <li>1. <i>Salmonella enterica</i> serovar fryis</li> <li>2. Enteropathogenic <i>Escherichia coli</i> (EPEC)</li> <li>3. <i>Pseudomonas aeruginosa</i></li> <li>4. <i>Campylobacter jejuni</i></li> </ol>              | <p>Infection of Caco-2 cell line in the presence of 10 mg/ml 2'-FL inhibited adhesion of <i>S. enterica</i> fryis, EPEC, <i>P. aeruginosa</i> and <i>C. jejuni</i> by 12%, 18%, and 17% and 26%, respectively.</p> <p>Infection of cell line A549 in the presence of 10 mg/ml 2'-FL inhibited adhesion of <i>P. aeruginosa</i> by 24%.</p> <p>Concentration of 2'-FL used in these inhibition assays were at least 8-fold more concentrated than the proposed use in infant formula of up to 1.2 mg/ml with only moderate inhibition of adhesion.</p>  |
| He et al. (2016b)      | <ol style="list-style-type: none"> <li>1. Intestinal epithelium T84 and HCT8 cell adhesion and invasion inhibition assay</li> <li>2. In vivo murine challenge study</li> </ol> | 2'-FL        | <ol style="list-style-type: none"> <li>1. Enterotoxigenic <i>Escherichia coli</i>,</li> <li>2. Uropathogenic <i>Escherichia coli</i></li> <li>3. Adherent-invasive <i>Escherichia coli</i></li> <li>4. <i>Salmonella enterica</i> Typhimurium</li> </ol> | <p>Pre-treatment of intestinal cells lines T84 or HCT8 with 2 mg/ml 2'-FL for 48 hours prior to inoculation resulted in inhibition of intracellular invasion of enterotoxigenic <i>E. coli</i> by approximately 60% and greater than 80% in T84 and HCT8 cell lines, respectively. Intracellular invasion by uropathogenic <i>E. coli</i> and adherent-invasive <i>E. coli</i> was also inhibited by pre-treatment of cells with 2'-FL for 48 hours. Intracellular invasion by <i>S. enterica</i> was not inhibited by 2'-FL.</p> <p>In a murine model of adherent-invasive <i>E. coli</i> infection, mice received 0.25% dextran sodium sulphate (DSS) in their drinking water for 3 days, and were given 20 mg of streptomycin by gavage on day 4; half also received 100 mg of 2'-FL in 200 µl by gavage for each of the 4 days. On day 5, mice were challenged with 10<sup>9</sup> cfu via 200 µl gavage and sacrificed after 4 days. Feeding mice DSS followed by streptomycin disrupted mouse microbiota and led to overt infection leading to 10% weight loss by day 3 and 4 post challenge. Feeding mice with 2'-FL prior to challenge prevented weight loss and resulted in a reduction in bacterial counts in faeces and colonic tissue.</p> |

| Reference                 | Experimental model  | HMO assessed   | Pathogen   | Main findings and comments   |
|---------------------------|---|--|--|--|
| Coppa et al. (2006)       | Caco-2 cell adhesion inhibition assay                     | Neutral oligosaccharide fraction containing 2'-FL and other HMOs obtained from pooled donor milk | <ol style="list-style-type: none"> <li>1. Enteropathogenic <i>Escherichia coli</i></li> <li>2. <i>Salmonella enterica</i> serovar fyris</li> <li>3. <i>Vibrio cholerae</i></li> </ol>  | <p>Pre-incubation of cell line with neutral low-molecular-weight oligosaccharides fraction prior to inoculation with pathogens inhibited adhesion by 33%, 25% and 1% for <i>E. coli</i>, <i>S. enterica</i> and <i>V. cholera</i>, respectively. Incubation of cell lines with purified 2'-FL prior to pathogen inoculation inhibited adhesion by 2% for all pathogens.</p> <p>Excluded from assessment due to use of neutral fraction and presence of other HMOs.</p>   |
| Thomas and Brooks (2004)  | Lung carcinoma cell line A549 adhesion inhibition assay   | 2'-FL  | <ol style="list-style-type: none"> <li>1. <i>Pseudomonas aeruginosa</i></li> <li>2. <i>Legionella pneumophila</i></li> <li>3. <i>Burkholderia cenocepacia</i></li> <li>4. <i>Burkholderia pseudomallei</i></li> <li>5. <i>Yersinia pestis</i></li> <li>6. <i>Bacillus anthracis</i></li> </ol> | Co-inoculation of cell line with bacteria in combination with 2'-FL at a concentration of 50mM inhibited adhesion of <i>B. cenocepacia</i> by 57%. Adhesion was not inhibited for the other pathogens tested.  |
| Koromyslova et al. (2017) | Histo-blood group antigen (HBGA) binding inhibition assay | 2'-FL  | Norovirus virus-like particle (VLP)  | <p>Pre-incubation of 2'-FL with 0.5 µg/ml of norovirus GI.1 VLP and 5 µg/ml of norovirus GII.17 VLP prior to addition to HGBA from human A-type saliva and porcine gastric mucin type III (PGM) inhibited adhesion of VLP to HBGA in a mostly dose dependent manner. For GI.1 VLPs, the half-maximal inhibitory concentrations for 2'-FL were 50 mM and 38 mM with PGM and A-type saliva, respectively. For GII.17 VLPs, the half-maximal inhibitory concentrations for 2'-FL were 20 mM and 13 mM with PGM and A-type saliva, respectively.</p> <p>The half-maximal inhibitory concentrations of 2'-FL substantially exceed the proposed concentration in infant formula. The binding assays while demonstrating a mechanism of VLP binding inhibition, the binding and inhibition of norovirus in vivo cannot be elucidated.</p> |
| Laucirica et al.          | Green monkey kidney                                       | 2'-FL  | Rotavirus  | Infectivity of two clinical strains of rotavirus were inhibited by 2'-   |

| Reference               | Experimental model                                | HMO assessed  | Pathogen                                 | Main findings and comments  |
|-------------------------|---|---|--|---|
| (2017)                  | epithelial cells (MA104) binding inhibition assay |   |  | FL at 2.5 or 5 mg/ml ranging from 15% to 62% inhibition depending on incubation conditions. When 2'-FL was added at 5 mg/ml after virus absorption onto MA104 cell line, infectivity was inhibited by 62% in one strain and 45% in the other.   |
| Andersson et al. (1983) | Epithelial cell binding inhibition assay          | LNT (purified from human milk fraction)                   | <i>Streptococcus pneumoniae</i>          | <p>Pre-incubation of LNT with <i>S. pneumoniae</i> prior to inoculation on to epithelial cells inhibited adhesion in a dose dependent manner. The effective dose inhibiting cell adhesion by 75% was 1.5 mg/ml. The method of purification of LNT and the purity of the final product was not reported in the article.</p> <p>Excluded from assessment due to possible presence of other lactotetraose oligosaccharides.</p>  |
| Andersson et al. (1985) | Epithelial cell binding inhibition assay          | LNT   | <i>Streptococcus pneumoniae</i>          | Commercial LNT product inhibited adhesion of <i>S. pneumoniae</i> to epithelial cells in a dose dependent manner, from 99% inhibition for 5 and 10 mg/ml and by approximately 90% at a concentration of 1 mg/ml. The experimental methods were poorly described and cell lines and incubation conditions not reported for LNT.  |
| Andersson et al. (1986) | Epithelial cell binding inhibition assay          | LNT (purified from human milk fraction and synthetic LNT) | <i>Streptococcus pneumoniae</i>          | Preincubation of LNT with <i>S. pneumoniae</i> prior to inoculation on to epithelial cells inhibited adhesion in a dose dependent manner. The effective dose inhibiting cell adhesion by 50% was 0.2 mg/ml LNT purified from human milk and 0.6 mg/ml for synthetic LNT.  |
| Cravioto et al. (1991)  | HEp-2 cells inhibition assay                      | Lacto and neolactotetraose fraction of human milk         | Enteropathogenic <i>Escherichia coli</i> | <p>Preincubation of the lacto- and neolactotetraose fraction at 3 mg/ml inhibited adhesion of <i>E. coli</i> to HE2 cells by 55-68% for three different <i>E. coli</i> strains.</p> <p>Unable to draw specific conclusions on the anti-infective or anti-adhesion effect of LNT from the Hep-2 cell inhibition assay as the study used a the lacto- and neolactotetraose fraction at 3 mg/ml which is inconsistent with the proposed use of purified LNT at a concentration of 0.6 mg/ml.</p> <p>Excluded from assessment due to HMO fraction other lactotetraose oligosaccharides.</p> |

| Reference                | Experimental model   | HMO assessed   | Pathogen  | Main findings and comments  |
|--------------------------|--|--|---|---|
| Ramphal et al. (1991)    | Adhesion assays  | LNnT purified from human milk and conjugated to 4-hexadecylaniline | <i>Pseudomonas aeruginosa</i>   | <p>Experiments designed to investigate binding receptors for <i>P. aeruginosa</i> and the conjugation 4-hexadecylaniline is not consistent with the proposed use of LNnT. No inference on mechanisms of binding and anti-infective potential <i>in vivo</i> can be drawn.</p> <p>Excluded from assessment due to conjugation of human milk derived LNnT to 4-hexadecylaniline.</p>  |
| Tong et al. (1999)       | Binding inhibition assay in chinchilla (rodent) trachea organ cultures | LNnT   | <i>Streptococcus pneumoniae</i>   | Three strains of <i>S. pneumoniae</i> pretreated for 30 min with LNnT (1–140 mM) demonstrated a 20–65% dose dependent decrease in adherence to trachea epithelial cells.  |
| Li et al. (2014)         | In vivo pig challenge study  | 40% 2'-FL, 35% LNnT, 10% 6'SL (sialyllactose) and 5% 3'SL          | Rotavirus   | <p>The data showed that piglets fed formula containing 4 mg/ml of the HMO mix ten days prior to rotaviral infection were as susceptible to infection as those fed control formula or formula containing FOS/GOS. However, there was an indication that presence of HMOs reduced the ability of the virus to replicate or to re-infect cells in a second-wave of infection. The effect of 2'-FL and LNnT in comparison to the 3'SL and 6'SL cannot be determined and the challenge study is inconsistent with the proposed use of 2'-FL and LNnT.</p> <p>Excluded from assessment due to use of 6'SL and 3'SL.</p> |
| Hoeflinger et al. (2015) | Carbohydrate utilisation study   | 2'-FL, LNnT and 6'SL   | Nine pathogenic and two nonpathogenic <i>Enterobacteriaceae</i> strains                                   | <p>All 11 strains tested were unable to utilise the HMO as a sole carbon source supplemented at a concentration of 1% (w/v). This study demonstrates that these <i>Enterobacteriaceae</i> will not be able to utilise 2'-FL and LNnT for growth but it does not demonstrate an inhibitory or anti-infective mechanism.</p> <p>Excluded from assessment of anti-infective effect as the study does not address an inhibitory mechanism.</p>  |
| Mezoff et al. (2016)     | Murine resected small bowel adaptation model                           | 2'-FL  | Measuring alpha diversity and relative abundance of intestinal bacterial families pre- and post-operation | This study demonstrated that mice supplemented with 2'-FL have a lower relative abundance of <i>Enterobacteriaceae</i> in resected bowel compared to control. This study does not demonstrate an inhibitory or anti-infective effect, rather that bacterial families including <i>Clostridiaceae</i> and <i>Lactobacillaceae</i>  |

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|--------------------------|---|--------------|--|---|
|                          |   |              |  | <p>had a growth advantage in the presence of 2'-FL as their relative abundance increased relative to control.</p> <p>Excluded from assessment of anti-infective effect as the study does not address an inhibitory mechanism.</p>   |
| Cillieborg et al. (2017) | <ol style="list-style-type: none"> <li>1. Binding inhibition assay in pig jejunal epithelium cell line PS1c1</li> <li>2. In vivo pig challenge study</li> </ol> | 2'-FL        | Enterotoxigenic <i>Escherichia coli</i> F18 and non-pathogenic <i>E. coli</i> ATCC 25922 | <ol style="list-style-type: none"> <li>1. Incubation of 2'-FL at concentrations of 1 mg/ml or 5 mg/ml with enterotoxigenic <i>E. coli</i> immediately prior to inoculation onto PS1c1 cell line resulted in less than 20% and greater than 70% inhibition of cell adhesion, respectively, in comparison to saline control. Adhesion of non-pathogenic <i>E. coli</i> strain to PS1c1 cell line was not inhibited by 2'-FL at either 1 or 5 mg/ml.</li> <li>2. New born, caesarean-derived piglets fed milk formula supplemented with 10 mg/ml of 2'-FL were not protected from developing diarrhoea when challenged with <i>E. coli</i> F18 was inoculated at a dose of <math>7.5 \times 10^{10}</math> cfu/day. Pigs challenged with <i>E. coli</i> F18 and supplemented with 2'-FL in milk formula tended to show less relative weight loss compared to pigs challenged with <i>E. coli</i> F18 without 2'-FL (-19 g/kg verses -124 g/kg).</li> </ol> <p>The dose of 2'-FL used in the experimental challenge model exceeds the proposed use in infant formula by approximately 8 fold.</p> |
| Weichert et al. (2016)   | Histo-blood group antigen (HBGA) binding inhibition assay   | 2'-FL        | Norovirus virus-like particle (VLP)  | <p>Pre-incubation of 2'-FL with 2.5 µg/ml of norovirus GII.10 VLP prior to addition to HBGA from human saliva (A and B types) and porcine gastric mucin type III (PGM) inhibited adhesion of VLP to HBGA in a mostly dose dependent manner. The half-maximal inhibitory concentration for 2'-FL was 5.5 mM, 11.2 mM and 26.9 mM for PGM, A-type saliva and B-type saliva assays, respectively.</p> <p>The half-maximal inhibitory concentrations of 2'-FL exceed the proposed concentration in infant formula from 2-fold to 10-fold higher concentration. The binding assays while demonstrating a mechanism of VLP binding inhibition, the binding and inhibition of norovirus infection <i>in vivo</i> cannot be elucidated.</p>   |

| Reference                  | Experimental model  | HMO assessed             | Pathogen   | Main findings and comments  |
|----------------------------|---|--------------------------|--|---|
| Duska-McEwen et al. (2014) | <ol style="list-style-type: none"> <li>16HBE human upper respiratory epithelial cell line</li> <li>Calu-3 human sub-bronchial gland cell line</li> </ol>                  | <p>2'-FL</p> <p>LNnT</p> | <ol style="list-style-type: none"> <li>Respiratory Syncytial Virus (RSV)-A strain</li> <li>Influenza A Virus (IAV)-H1N1</li> </ol> | <p>Respiratory epithelial cells were pre-incubated with HMOs at various concentrations for 24 hours, followed by removal of HMOs, rinsing of cells and adsorption of RSV or IAV viruses for 1 hour. Virus was removed and cells were incubated in media containing HMOs for a further 48 hours. Viral gene copy number was measured as a surrogate for viral load in cell lysates from the 48 hours post-infection samples. 2'-FL significantly reduced viral copy number of RSV in 16HBE cells by &gt;90% at concentrations from 50 to 1000 µg/ml whereas LNnT had no effect. In contrast, LNnT significantly reduced IAV copy number in 16HBE cells in a dose-dependent manner from 1 to 1000 µg/ml, with &gt;80% reduction observed for 100 and 1000 µg/ml of LNnT. 2'-FL had no effect on of IAV viral copy number. In Calu-3 cells, 2'-FL significantly decreased viral gene copy number at or above 1 µg/mL but to a lesser extent than observed in 16HBE cells. LNnT results were not reported for Calu-3 cells.</p> |
| Hester et al. (2013)       | <ol style="list-style-type: none"> <li>MA-104 cell line focus-forming unit (FFU) inhibition assay</li> <li><i>In situ</i> rotavirus infection model in piglets</li> </ol> | <p>2'-FL</p> <p>LNnT</p> | <ol style="list-style-type: none"> <li>Porcine rotavirus OSU strain</li> <li>Human rotavirus Wa strain</li> </ol>                  | <p>LNnT or 2'-FL were pre-incubated with rotavirus OSU strain or Wa strain (10<sup>3</sup> infectious units) at either 1 or 10 mg/ml for 30 min before the application to MA-104 cells. The LNnT and 2'-FL treatments at either 1 or 10 mg/ml did not reduce infectivity of either rotavirus OSU strain or Wa strain.</p> <p>A midline laparotomy was performed on sedated 21 day old piglets and 10 cm loops of ileum were isolated. 2 ml porcine rotavirus OSU strain at 1x10<sup>7</sup> FFU with or without addition of 2mg/ml LNnT was injected in to ileum loop. After 6 hours of treatment, piglets were euthanised and the ileum section was washed and frozen. mRNA expression of non-structural protein 4 (NSP4) was measured as a proxy of virus replication. Rotavirus infected ileal loops co-incubated with 2mg/ml LNnT had significantly less NSP4 mRNA expression compared to rotavirus alone, an approximately 3-fold decrease was observed..</p>  |

