Appendix III-a

Comprehensive Documentation of Case-of-Need for 2'-FL and LNnT

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1.1 Description

The 2'-FL and LNnT manufactured by Glycom A/S are identical in chemical structure to the natural 2'-FL and LNnT nutrients occurring at significant amounts in human breastmilk. Analytical data to support this is provided in Appendix IV. The 2'-FL and LNnT manufactured by Glycom are referred to as human-identical milk oligosaccharides (HiMOs)[1]. 2'-FL and LNnT are intended for use in specific foods at levels intended to reflect the naturally occurring concentrations of these compounds in human milk. The following sections detail the physiological role of human milk oligosaccharides and describe the technical need for these compounds in infant and follow-on formula.

1.2 Fundamentals. Infant Formula, Human Milk and its Oligosaccharides

Human milk is a complete source of nutrition that meets the needs of the fast growing and vulnerable infant from birth, and is widely recommended as the exclusive source of nutrients for the first 6 months of human life and should continue upon introduction to complementary foods up to 2 years (WHO, 2017). It is a highly complex emulsion containing many different biomolecules and has evolved under a trade-off optimization process between mother and child throughout evolutionary times (Trivers, 1974; German *et al.*, 2002; Petherick, 2010).

Infant formulas were developed and designed to be a substitute or complement for breastmilk. Infant formula composition has been refined over time to improve the formulas' suitability and to better reflect the composition of breastmilk, particularly given the beneficial health outcomes that have been observed in exclusively breastfed infants (Carver, 2003; Thompkinson and Kharb, 2007; Stevens *et al.*, 2009; EFSA, 2014; Green Corkins and Shurley, 2016).

If breastfeeding is not possible or mothers choose not to breastfeed, infant formulas are the sole source of nutrition for growing and developing infants until the introduction of complementary food and thereafter may serve as complement to the introduced weaning food. Due to its pivotal role in early nutrition and the vulnerability of its target consumers, infant formulas are highly regulated by the authorities in many countries, including Australia and New Zealand.

Yet, even today with much progress and control over the composition of infant formula, there are a large number of studies that show that breast-feeding provides a wide range of beneficial health effects to the infant as compared to formula feeding (Deoni *et al.*, 2013; Morrow and Chantry, 2013; Brion *et al.*, 2011; Iacovou and Sevilla-Sanz, 2010; Agostoni *et al.*, 2009; Horta *et al.*, 2007; Hanson, 2007; Gale and Martyn, 1996; Victora *et al.*, 2016; Quigley *et al.*, 2016).

Infant formulas are typically and traditionally based on cow milk and a comparison between the macronutrient content of cow milk, infant formula and human milk (Newburg, 2013; EFSA, 2014;

[1] Throughout this application, the term "human-identical milk oligosaccharides (HiMOs)" is used to refer to the manufactured forms of these substances (*e.g.*, 2'-FL and LNnT obtained by either chemical synthesis or microbial fermentation), while the term "human milk oligosaccharides (HMOs)" is used to refer to their naturally occuring counterparts in human breastmilk. Glycom has demonstrated that the HiMOs that are the subject of the current application (2'-FL and LNnT manufactured by fermentation) are identical to the 2'-FL and LNnT that are present naturally in human breastmilk.

Michaelsen *et al.*, 1994; Xu *et al.*, 2017; Bode, 2013; Newburg and Neubauer, 1995; Viverge *et al.*, 1990; Lönnerdal *et al.*, 2017; Hester *et al.*, 2012) reveals that the *biggest remaining compositional difference* between infant formulas and human milk today is the **oligosaccharide** fraction of human milk, as these are not present in mature cow milk to any significant degree.

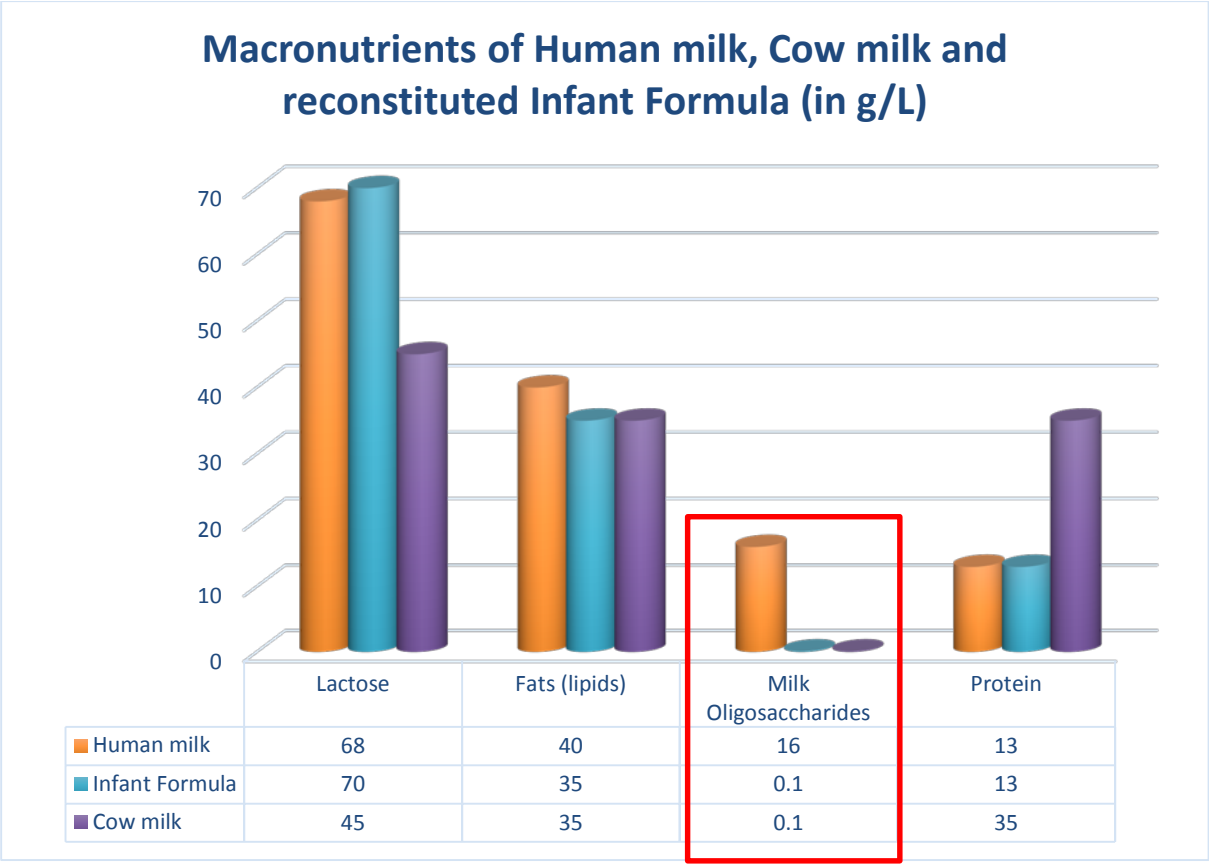


Figure 1.2-1: Macronutrients of human milk, cow milk and reconstitued infant formula. *As an estimate, in mature human milk (around 30 days postpartum) the milk sugar lactose is typically found at concentrations of 68 g/L (reported range 55-70 g/L) (Newburg, 2013; EFSA, 2014), fats (lipids) around 40 g/L (reported range 24 to 59 g/L) (Michaelsen et al., 1994; EFSA, 2014) oligosaccharides around 16 g/L (reported range 5-25 g/L) (Xu et al., 2017; Bode, 2013; Newburg and Neubauer, 1995; Viverge et al., 1990) and protein around 13 g (reported range 8-21 g/L) (Hester et al., 2012; Lönnerdal et al., 2017). Only trace amounts of milk oligosaccharides are present in mature cow milk and, as a consequence, in cow milk–based infant formula (Bode, 2006).*

It is estimated that oligosaccharide biosynthesis constitutes 10% of total energy expenditure of the mother for milk production (Yu *et al.*, 2013b). It is thus energetically costly for the mother to provide oligosaccharides to the infant and it would make little evolutionary sense to continue investing into their biosynthesis if they would not provide a beneficial function to the infant (Pike and Milligan, 2010).

It is not surprising that several health benefits of breastmilk are proposed to be mediated through the effects of the breastmilk oligosaccharides (generally dubbed human milk oligosaccharides, HMO) on the newly establishing intestinal microbiota. This is based on the high abundance of HMOs and the fact that they are not digestible, implying they do not serve as energy source for the growing infant and reach the large intestine, where the gut microbiota mainly establishes (Pacheco *et al.*, 2015; Newburg and Morelli, 2015; Sela and Mills, 2014). It has been established that HMOs are non-

digestible for the infant and reach the lower and large intestine intact (Rudloff and Kunz, 2012; Gnoth *et al.*, 2000; Engfer *et al.*, 2000; Brand-Miller *et al.*, 1995).

To date, commercially available infant formulas are not supplemented with oligosaccharides that are identical to the naturally occurring milk oligosaccharides and the accompanying dossier constitutes the very first application of human-identical milk oligosaccharides for infant formulas.

1.2.1 HMO Structures and Comparison to "Conventional" Non-Digestible Oligosaccharides

All HMOs are derived from the milk sugar lactose (i.e. galactosyl-β1-4-glucose) by possible extension with 4 principal monosaccharides: *N*-acetyl-D-glucosamine (GlcNAc), D-galactose (Gal), sialic acid (Neu5Ac or NANA[2]) and/or L-fucose (Fuc). GlcNAc and galactose are added in specific order and linkages to form the neutral core-structures, and the latter two monosaccharides (NANA and fucose) are always found on the terminal positions of either lactose or the core-structures, they are never glycosylated themselves.

The resulting composition of the human milk oligosaccharide fraction is complex and many aspects on the diversity of the milk oligosaccharide fraction have been summarized in excellent overview articles (Urashima *et al.*, 2011; Totten *et al.*, 2014; Chen, 2015). It is however important to emphasize that the collective body of analytical data on the quantities of individual structures (as reported in at least 39 scientific publications; see Appendix III-b) clearly shows that a subset of less than 20 individual structures makes up the large majority of the biomass and that 3 distinct structural classes can be defined, and representative HMOs identified for each class. This has been recognised by the authorities, for instance, the EFSA opinion on the essential composition of infant and follow-on formula states:

Approximately 20 oligosaccharides make up more than 90% of the total amount of oligosaccharides in human milk, with the principal oligosaccharides being fucosyllactoses, lacto-N-tetraose, lacto-N-neotetraose, sialyllactoses, lacto-N-fucopentaoses (I–V) and lacto-N-difucohexaoses (I–III). The neutral linear and branched-chain oligosaccharides are fucosylated to a varying degree and make up 80-85% of the total amount of oligosaccharides in human milk, whereas the acidic oligosaccharides contain sialic acid and make up 15-20% of the total amount.

Considering that the neutral fraction consists of *non-fucosylated* core structures and fucosylated HMOs, it is thus possible to categorize the complex oligosaccharide fraction into 3 principal classes of HMOs and to define key representative HMOs for each category:

- **Neutral core HMOs** (containing the aminosugar **GlcNAc**) – 15% of total biomass
- **Neutral fucosylated HMOs** (containing **fucose**) – 70% of total biomass
- **Acidic HMOs** (containing **sialic acid,"NANA"**) – 15% of total biomass

[2] *N*-Acetyl-D-neuraminic acid, abbreviated Neu5Ac or NANA, is colloqually and widely referred to as **sialic acid**

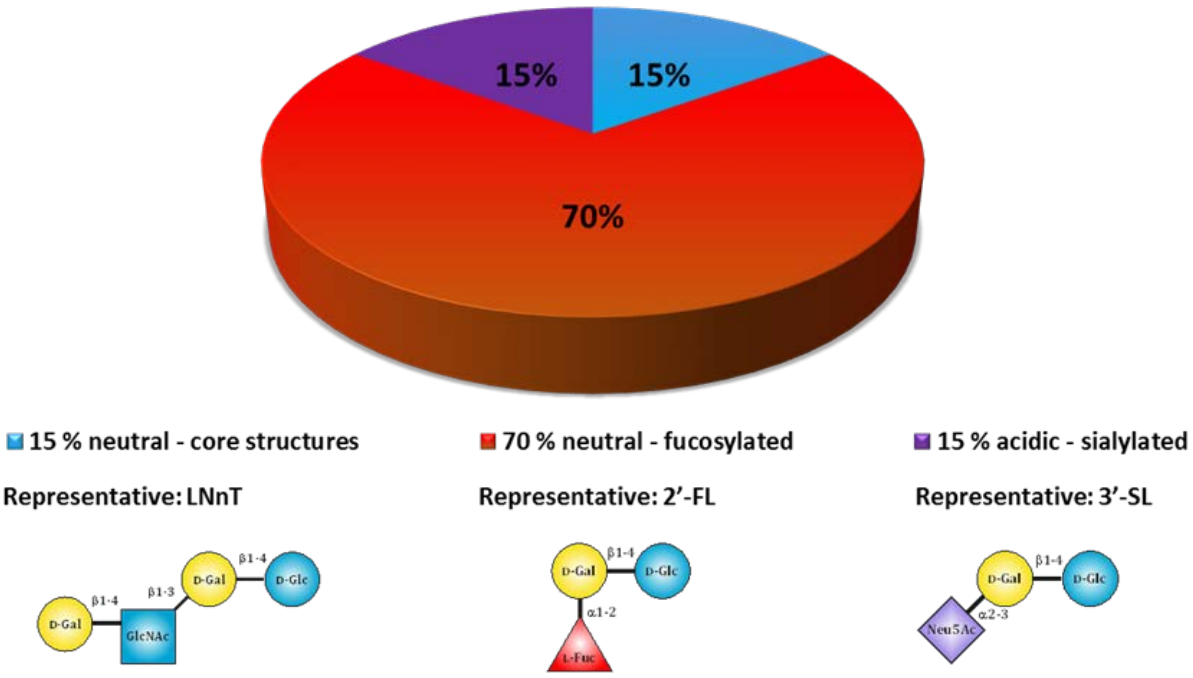


Figure 1.2.1-1 Principal Categories and representative HMOs

The structures of natural milk oligosaccharides are distinct from the non-digestible oligosaccharides that are added to infant formulas today (i.e. galacto-oligosaccharides, GOS, and fructo-oligosaccharides, FOS). The table below provides a comparative overview on the major carbohydrate components (building blocks), the general structure and the main components.

GOS and FOS are linear oligomers of galactose and fructose, respectively, elongated from lactose or a sucrose core, respectively. FOS can also be derived from the enzymatic hydrolysis of inulin. These compounds do not possess any aminosugar (*i.e.*, N-acetylglucosamine, GlcNAc), sialic acid (NANA) or fucose as building blocks and they do not possess any branching and sidechains like many HMOs do (Figure 1.5.1-2).

Section 3.5 will provide information about the impact of the structural difference on biological effects and the data that is available to demonstrate that the structural diversity of HMOs is a requirement to achieve a broader range of benefits as compared to the structurally simple non-digestible oligosaccharides (GOS and FOS).

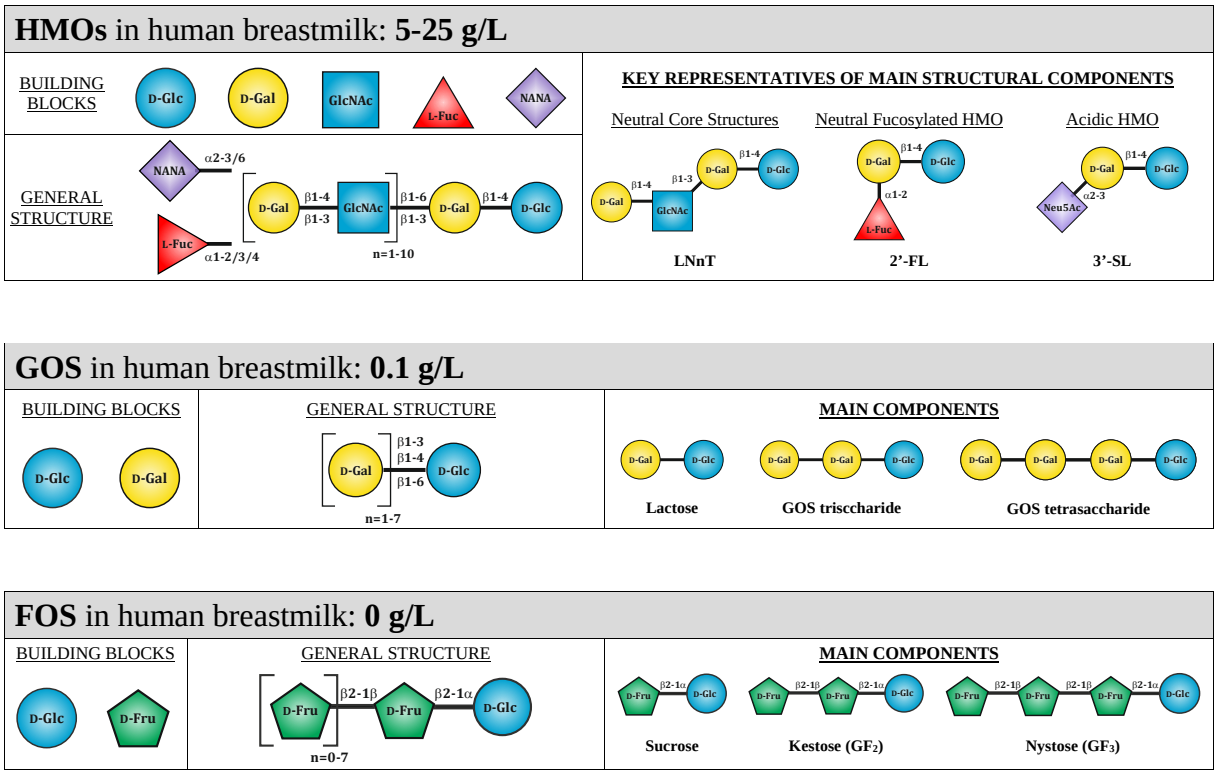


Figure 1.5.1-2: Principle building blocks, structures and main components of HMOs, GOS and FOS

1.2.2 Evolutionary Rationale for Infant Formula Fortification with Milk Oligosaccharides

All cellular surfaces are covered by a dense carbohydrate layer, the glycocalix (Varki, 2011; Varki, 2017), and presentation of select carbohydrate epitopes differs between species characteristically (Bishop and Gagneux, 2007). Human epithelial surfaces are rich in carbohydrate blood group and Lewis antigen epitopes (Ravn and Dabelsteen, 2000; Hod *et al.*, 2009; Oriol *et al.*, 1986). These human blood group and Lewis antigen epitopes are common binding receptors for pathogens (viral, bacterial, fungal, protozoan and parasitical), and selective recognition is needed for infection (Le Pendu *et al.*, 2006; Marionneau *et al.*, 2005; Zopf and Roth, 1996; Sharon and Ofek, 2000).

Human milk has evolved under a trade-off optimization process between mother and child during mammalian evolution (Petherick, 2010; German *et al.*, 2002; Trivers, 1974; Oftedal, 2012). HMOs are freely occurring oligosaccharides, but their structures present identical epitopes to human cell surface-linked carbohydrate epitopes (i.e. human blood group and Lewis antigens) (Bode and Jantscher-Krenn, 2012; Newburg and Grave, 2014). The latter are part of the defensive barrier lining the epithelial surfaces (in the form of the glycocalyx and mucin glycans), and were subject to strong selection (evolutionary pressure) due to a „molecular arms race" between infectious agents and humans (Bishop and Gagneux, 2007; Urashima *et al.*, 2012; Messer and Urashima, 2002; Springer and Gagneux, 2013). The fact that identical carbohydrate epitopes are expressed as free oligosaccharides in the breast and excreted into milk thus proposes that these free oligosaccharides serve as an intriguing defense mechanism of mammals in general and humans in particular. Human milk contains the largest number and amounts of oligosaccharides of all mammalian species investigated (Newburg, 2000; Newburg *et al.*, 1999; Tao *et al.*, 2011).

This hypothesis is supported by many preclinical studies that demonstrated that HMOs bind efficiently to a diverse range of pathogens [reviewed by (Hickey, 2012; Bode, 2015; Newburg *et al.*, 2005; Kunz and Rudloff, 2006; Sharon and Ofek, 2000; Zopf and Roth, 1996)] and their toxins [reviewed by (El-Hawiet *et al.*, 2015; Newburg, 2009)]. In addition, the human microbiome evolved concurrently with the evolution of human epithelial surface glycans and milk oligosaccharides (Schluter and Foster, 2012; Moeller *et al.*, 2016). Milk oligosaccharides are not found as freely occurring oligosaccharides anywhere else in nature but almost exclusively in milk (with exception of tiny quantities being found in urine from lactating mothers) (Newburg, 2000; Bode, 2006).

1.2.3 Four HMO Phenotypes Including Secretor and Non-Secretors

For the data discussed in the coming sections it is relevant to understand a key feature of human milk biology, which is the fact that 4 structurally-distinct milk groups can be differentiated in all human populations.

Already in the 1960s ground-breaking work performed by Victor Ginsburg (Kobata *et al.*, 2004) and Akira Kobata (Endo, 2010) has revealed the enzymatic basis for the human blood groups, which are *carbohydrate-based* cell-surface antigens, and their close structural and biosynthetic relationship to the freely occurring milk oligosaccharides (Grollman *et al.*, 1969; Shen *et al.*, 1968; Kobata *et al.*, 1968). In this context it had been initially recognized in 1967 that not all mothers excrete 2'-FL into their milk, because they don't express a specific enzyme that is needed for 2'-FL biosynthesis (Grollman and Ginsburg, 1967). This observation is not limited to the freely occurring 2'-FL in milk, but includes other cell-surface bound "2'-FL" epitopes that are typically secreted into other bodily fluids like blood and saliva. Therefore, the term "non-secretor" was coined for this phenotype. Based on this initial finding it was established that human milk can be categorized into four different "milk groups" (*i.e.*, phenotypes) based on the presence (or absence) of distinct structural features in their oligosaccharide fraction and that these different phenotypes contain a significantly different amount of fucosylated HMOs in general and 2'-FL in particular.

The categorization of milk types is indeed related and comparable to "blood groups", but with the important difference that all mothers are "universal donors" of milk, as can be concluded from the long and safe tradition of wet nursing that was commonplace before the invention of infant formulas (Stevens *et al.*, 2009; Mason *et al.*, 2013), as well as the availability of human breastmilk through "collection banks" in present day. It means that all milk groups are fundamentally safe for an infant; however, there are indeed differences in the nutritional effects of each milk group for the infant which can be investigated in observational clinical trials where mother-child pairs are stratified according to milk phenotypes (and possibly also infant genotypes). The biological effects caused by the difference of Secretor and non-Secretor milk and how they related to 2'-FL are discussed in Section 1.4. Here in this section we shortly summarize the scientific basis and structural consequences of the 4 different milk groups.

The milk groups are categorized according to the presence (or absence) of the specific oligosaccharide products of 2 distinct fucosyltransferase enzymes, namely α1,2- and

α1,4-fucosyltransferase, encoded by the genes *FUT2* and *FUT3*, respectively (see **Table 1.2.3-1** below).

Table 1.2.3-1 Milk Groups by Maternal Secretor and Lewis Phenotypes

Milk Group:	**1**	**2**	**3**	**4**
Milk Phenotype:	Se+ / Le$^{(a-\ b+)}$	Se– / Le$^{(a+\ b-)}$	Se+ / Le$^{(a-\ b-)}$	Se– / Le$^{(a-\ b-)}$
α-1,2-fucosylated HMOs: α-1,2-FT enzyme (FUT2)	+	–	+	–
α-1,3-fucosylated HMOs: α-1,3-FT enzymes (FUT3, FUT5, FUT6)[(a)]	+	+	+	+
α-1,4-fucosylated HMOs: α-1,4-FT enzyme (FUT3)[(b)]	+	+	–	–
Typically observed frequency:[(c)]	~ 70%	~ 20%	~ 9%	~ 1%

FUT = fucosyltransferase.
[(a)] α-1,3-fucosylated structures are synthesized by different FUT enzymes.
[(b)] The FUT3 enzyme possesses α-1,3 and α-1,4 FT activity.
[(c)] As reported in the literature (Thurl *et al.*, 2010; Castanys-Muñoz *et al.*, 2013; Austin *et al.*, 2016).

Expressed in words:

- **Group 1**. LEWIS-positive **Secretor** mothers with LEWIS blood group (Le$^{a-\ b+}$) express both *FUT2* and *FUT3* and thus can synthesize both α-1,2-fucosylated HMO and α-1,4-fucosylated HMOs.
- **Group 2**. LEWIS-positive non-Secretor mothers with LEWIS blood group (Le$^{a+\ b-}$) express *FUT3* but not *FUT2* and thus produce α-1,4-fucosylated HMOs but not α-1,2-fucosylated HMOs.
- **Group 3**. LEWIS-negative **Secretor** mothers with LEWIS blood group (Le$^{a-\ b-}$) express *FUT2* but not *FUT3* and therefore synthesize α-1,2-fucosylated HMOs but not α-1,4-fucosylated HMOs.
- **Group 4.** LEWIS-negative non-Secretor mothers with LEWIS blood group (Le$^{a-\ b-}$) express neither *FUT2* nor *FUT3* and cannot produce either α-1,2-fucosylated HMOs nor α-1,4-fucosylated HMOs.

The concentration of fucosylated HMO in mothers' milk, and particularly that of 2'-FL goes in ascending order from milk group 4 < 2 < 3 < 1 as is shown in the Figure below.

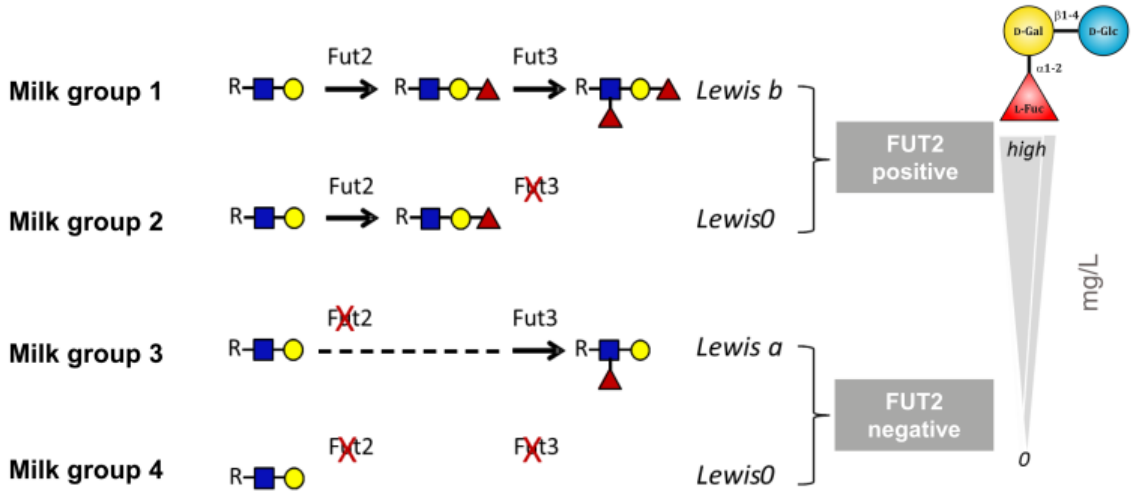


Figure 1.2.3-1 Concentration of 2'-FL increases from milk group 4 < 2 < 3 < 1 1.

The prevalence of these four milk groups varies to some extend globally according to ethnicity or geographical location; but is typically and in most ethnicities around 70% Group 1 (express *FUT2* and *FUT3*), 19% Group 2 (express FUT3 but not FUT2), 10% Group 3 (express FUT2 but not FUT3) and 1% Group 4 (no expression of either *FUT2* or *FUT3*) (Thurl *et al.*, 2010; Castanys-Muñoz *et al.*, 2013; Austin *et al.*, 2016), so that it can be roughly stated that ~ 80% of the population are Secretors and express 2'-FL into their milk (compare also to **Table 1.2.4-2**).

These phenotypes (i.e. Secretor and LEWIS) can be best understood in the context of general human polymorphisms for the carbohydrate histo-blood group and LEWIS antigens and we refer to excellent overview articles for more detail (Oriol *et al.*, 1986; Ferrer-Admetlla *et al.*, 2009; Cooling, 2016).

1.2.4 Natural Concentration of 2'-FL In Human Milk

The concentration of 2'-FL in human milk has been measured and reported to date in at least 28 independent publications (see Appendix III-b). The data show that 2'-FL is, on average, by far the most abundant HMO of pooled human milk, even though approximately 20% of the female population (i.e. non-secretors) don't express it into milk at any relevant levels (Austin *et al.*, 2016; Castanys-Muñoz *et al.*, 2013). To note, the biological significance of secretor vs non-secretor milk, and how it affects the mother and the infant, is summarised in Section 1.4.2.

The following table summarizes the levels of 2'-FL that have been reported in breast milk across these various studies.

Table 1.2.4-1 2'-FL Concentration in Human Milk After Full-Term Birth

Lactation time	Key findings	References
Pooled milk		
Days 1-4 ("colostrum")	**Reported Range: 1.0 to 8.4 g/L** Average: 3.2 g/L	(Spevacek *et al.*, 2015; Asakuma *et al.*, 2008; Morrow *et al.*, 2004a; Erney *et al.*, 2000)
Days 5-14 ("transitional milk")	**Reported Range: 2.1 to 2.8 g/L** Average: 2.5 g/L	(Austin *et al.*, 2016; Spevacek *et al.*, 2015; Erney *et al.*, 2000)
Days 10-60 ("mature milk")	**Reported Range: 0.7 to 3.9 g/L** Average: 2.2 g/L	(Austin *et al.*, 2016; Spevacek *et al.*, 2015; Musumeci *et al.*, 2006; Chaturvedi *et al.*, 2001; Erney *et al.*, 2001; Erney *et al.*, 2000; Chaturvedi *et al.*, 1997; McGuire *et al*., 2017)
After 2 months ("mature milk")	**Reported Range: 0.7 to 3.4 g/L** Average: 1.9 g/L	(Austin *et al.*, 2016; Smilowitz *et al.*, 2013; Asakuma *et al.*, 2011; Erney *et al.*, 2000; McGuire *et al*., 2017)
Secretor milk		
Days 1-4 ("colostrum")	**Reported Range: 3.9 to 4.1 g/L** Average: 4.0 g/L	(Kunz *et al.*, 2017; Leo *et al.*, 2009; Coppa *et al.*, 1999)
Days 5-14 ("transitional milk")	**Reported Range: 3.0 to 3.6 g/L** Average: 3.3 g/L	(Kunz *et al.*, 2017; Coppa *et al.*, 1999)
Days 10-60 ("mature milk")	**Reported Range: 1.0 to 7.8 g/L** Average: 3.0 g/L	(Sprenger *et al.*, 2017a; Kunz *et al.*, 2017; Olivares *et al.*, 2015; Hong *et al.*, 2014; Galeotti *et al.*, 2014; Bao *et al.*, 2013; Galeotti *et al.*, 2012; Coppa *et al.*, 2011; Leo *et al.*, 2009; Coppa *et al.*, 1999; McGuire *et al*., 2017)
After 2 months ("mature milk")	**Reported Range: 1.0 to 3.6 g/L** Average: 2.4 g/L	(Sprenger *et al.*, 2017a; Kunz *et al.*, 2017; Coppa *et al.*, 1999; Thurl *et al.*, 1996; McGuire *et al*., 2017)

The **average** levels in pooled milk are highest in colostrum (3.2 g/L), followed by transitional milk (2.5 g/L) and continue to decline slowly in mature milk (2.2 g/L) and mature milk from a lactation stage later than 2 months (1.9 g/L). In the context of relative abundance, 2'-FL ranks first with approximately 15-20 w/w% (corresponding to 24-30 mol%) of the total HMO biomass (Castanys-Muñoz *et al.*, 2013).

In milk from Secretor mothers the corresponding average levels are significantly higher, 4.0 g/L in colostrum, 3.3 g/L in transitional milk, 3.0 g/L in mature milk and 2.4 g/L in mature milk from a lactation stage later than 2 months. Non-Secretor milk is reported to contain less than 0.08 mg/L independent of lactation stage (Sprenger *et al.*, 2017a; Kunz *et al.*, 2017; Olivares *et al.*, 2015; Galeotti *et al.*, 2014; Galeotti *et al.*, 2012; Coppa *et al.*, 2011; Leo *et al.*, 2009; McGuire *et al.*, 2017). Of note is the high variability of 2'-FL concentrations between different mothers, as can be seen from the reported ranges which oftentimes reach levels beyond 5 g/L.

Several studies have also investigated the regional (ethnic) dependency of the 2'-FL concentration of milk and reveal that the correlation to the Secretor frequency is predictive. There are negligible differences of average 2'-FL concentrations (2.2 to 2.4 g/L) between Asia, China, Europe and the U.S., regions which all possess Secretor frequencies between 70 and 80% (see Table below). In Mexico, Peru, and the Hispanic populations of the U.S., where the Secretor frequency is reported to reach nearly 100%, the average concentration of 2'-FL is highest with 3.2 to 3.4 g/L, which is in surprisingly good agreement with the levels found in the Secretor subpopulation of other regions (see Table above).

Table 1.2.4-2 2'-FL Concentration in Mature Human Milk (lactation days 10-60) by Global Regions

Region	Reported Secretor frequency	Key findings	References
U.S. (Hispanic)	~ 95%	**Average: 3.4 g/L**	McGuire *et al.*, 2017
Latin America (Mexico, Peru)	~ 98 to 100%	**Average: 3.2 g/L**	(Morrow *et al.*, 2004a; Erney *et al.*, 2000; McGuire *et al.*, 2017)
Asia	~ 80%	**Average: 2.3 g/L**	(Erney *et al.*, 2000)
China	~ 80%	**Average: 2.3 g/L**	(Austin *et al.*, 2016)
Europe	~ 76 to 80%	**Average: 2.4 g/L**	(Erney *et al.*, 2000; McGuire *et al.*, 2017)
U.S.	~ 68 to 75%	**Average: 2.2 g/L**	(Spevacek *et al.*, 2015; Chaturvedi *et al.*, 2001; Erney *et al.*, 2000; McGuire *et al.*, 2017)
Africa	~ 65 to 85%	**Average: 1.3 g/L**	McGuire *et al.*, 2017
World		**Reported Range: 0 to 7.8 g/L**	

2'-FL = 2'-*O*-fucosyllactose; U.S. = United States.

1.2.5 Natural Concentration of LNnT In Human Milk

The concentration of LNnT in human milk has been measured and reported to date in at least 26 independent publications (see Appendix III-b). A detailed overview on all data can be provided upon request. The first important aspect that the data shows is that LNnT is present in the milk of all mothers.

Table 1.2.5-1 gives an overview on the most significant findings of these investigations in regards to LNnT in dependence of lactation time and Secretor status.

Table 1.2.5-1 LNnT concentration in human milk after full-term birth

Lactation time	Key findings	References
Pooled milk		
Days 1-4 ("colostrum")	**Reported Range: 0.21 to 0.49 g/L** Average: 0.34 g/L Outlier: 2.04 g/L (Coppa)	(Coppa *et al.*, 1999; Erney *et al.*, 2000; Sumiyoshi *et al.*, 2003; Asakuma *et al.*, 2008; Thurl *et al.*, 2010; Bao *et al.*, 2013; Spevacek *et al.*, 2015; Kunz *et al.*, 2017)
Days 5-14 ("transitional milk")	**Reported Range: 0.15 to 0.55 g/L** Average: 0.32 g/L Outlier: 1.83 g/L (Coppa)	(Coppa *et al.*, 1999; Erney *et al.*, 2000; Sumiyoshi *et al.*, 2003; Leo *et al.*, 2009; Leo *et al.*, 2010; Spevacek *et al.*, 2015; Austin *et al.*, 2016; Kunz *et al.*, 2017)
Days 10-60 ("mature milk")	**Reported Range: 0.09 to 1.08 g/L** Average: 0.31 g/L Outliers: 0.95 to 4.1 g/L (Coppa, Galeotti)	(Chaturvedi *et al.*, 1997; Coppa *et al.*, 1999; Erney *et al.*, 2000; Sumiyoshi *et al.*, 2003; Leo *et al.*, 2009; Thurl *et al.*, 2010; Bao *et al.*, 2013; Galeotti *et al.*, 2014; Hong *et al.*, 2014; Spevacek *et al.*, 2015; Austin *et al.*, 2016; Kunz *et al.*, 2017; Sprenger *et al.*, 2017a; McGuire *et al.*, 2017)
After 2 months ("mature milk")	**Reported Range: 0.04 to 1.08 g/L** Average: 0.28 g/L Outliers: 1.37 g/L (Coppa)	(Thurl *et al.*, 1996; Coppa *et al.*, 1999; Nakhla *et al.*, 1999; Erney *et al.*, 2000; Chaturvedi *et al.*, 2001; Sumiyoshi *et al.*, 2003; Leo *et al.*, 2010; Asakuma *et al.*, 2011; Smilowitz *et al.*, 2013; Austin *et al.*, 2016; Kunz *et al.*, 2017; Sprenger *et al.*, 2017a; McGuire *et al.*, 2017)
Secretor milk		
Days 1-30	**Reported Range: 0.24 to 0.36 g/L** Average: 0.30 g/L Outliers: 2.57 g/L (Galeotti)	(Thurl *et al.*, 2010; Galeotti *et al.*, 2012; Hong *et al.*, 2014; Kunz *et al.*, 2017; Sprenger *et al.*, 2017a; McGuire *et al.*, 2017)
Non-secretor milk		
Days 1-30	**Reported Range: 0.11 to 0.25 g/L** Average: 0.19 g/L Outliers: 3.53 g/L (Galeotti)	(Thurl *et al.*, 2010; Galeotti *et al.*, 2012; Hong *et al.*, 2014; Kunz *et al.*, 2017; Sprenger *et al.*, 2017a)

LNnT = lacto-*N*-neotetraose.

The levels of LNnT is generally consistent across the lactation stages. The average levels in pooled milk are highest in colostrum (0.34 g/L), followed by transitional milk (0.32 g/L) and continue to decline slowly in mature milk (0.31 g/L) and mature milk from a lactation stage later than 2 months (0.28 g/L). The reported ranges are between 0.04 and 1.08 g/L. Some studies find significantly higher levels of LNnT in breast-milk, but as these studies are all connected to the same group of investigators and are inconsistent with numerous reports by a several independent researchers, applying a set of different analytical methods, they have been considered as outliers for this analysis (Coppa *et al.*, 1999; Gabrielli *et al.*, 2011; Galeotti *et al.*, 2012; Galeotti *et al.*, 2014).

In milk from Secretor mothers the corresponding LNnT levels (0.30 g/L) are consistently higher than in the milk of non-Secretor mothers (0.19 g/L), as confirmed by four independent studies (Thurl *et al.*, 2010; Hong *et al.*, 2014; Kunz *et al.*, 2017; Sprenger *et al.*, 2017a) and suggesting a possible co-regulation of Secretor function and LNnT expression.

Several studies have also investigated the regional (ethnic) dependency of the LNnT concentration of milk. There are insignificant differences of average LNnT concentrations (0.25 to 0.33 g/L) between Latin America, Asia, Europe, and USA. The reported levels for China appear to be lower (0.15 g/L), but are based on a single study to date. Although the African populations appear to possess higher levels of LNnT in their milk (0.7 g/L), this data is also based on a single study. Therefore, it is possible that the reported low and high extremes may be study-biased, rather than real differences. The most parsimonious conclusion is that there is a wide variation between individual mothers that covers ranges up to more than 1 g/L of LNnT.

Table 1.2.5-2 LNnT Concentration in Mature Human Milk (lactation days 1-60) by Global Regions

Region	Key findings	References
Latin America (Mexico, Peru)	**Average: 0.33 g/L**	Chaturvedi *et al.*, 1997; Erney *et al.*, 2000; McGuire *et al.*, 2017
Asia	**Average: 0.25 g/L**	Erney *et al.*, 2000; Sumiyoshi *et al.*, 2003; Asakuma *et al.*, 2008, 2011; Sprenger *et al.*, 2017a
China	**Average: 0.15 g/L**	Austin *et al.*, 2016
Europe	**Average: 0.32 g/L**	Erney *et al.*, 2000; Thurl *et al.*, 2010; Kunz *et al.*, 2017; McGuire *et al.*, 2017
U.S.	**Average: 0.33 g/L**	Erney *et al.*, 2000; Bao *et al.*, 2013; Hong *et al.*, 2014; Spevacek *et al.*, 2015; McGuire *et al.*, 2017
Africa	**Average: 0.70 g/L**	McGuire *et al.*, 2017
World	**Reported Ranges: 0.04 to 1.08 g/L**	

LNnT = lacto-*N*-neotetraose; U.S. = United States.

1.2.6 Co-Occurrence of 2'-FL/LNnT and Secretor Milk

A study by Sprenger *et al.* (Sprenger *et al.*, 2017a) observed that the concentration of LNnT is positively correlated to the concentration of 2'-FL, while the concentation of LNT is negatively correlated. The effect is statistically significant and suggests that a mixture of 2'-FL and LNnT is a practical proxy for the oligosaccharide composition of Secretor milk (see Figure 1.2.6-1).

This effect has been also seen in other studies (compare to Section 3.2.5 below) (Thurl *et al.*, 2010; Hong *et al.*, 2014; Kunz *et al.*, 2017; Sprenger *et al.*, 2017a).

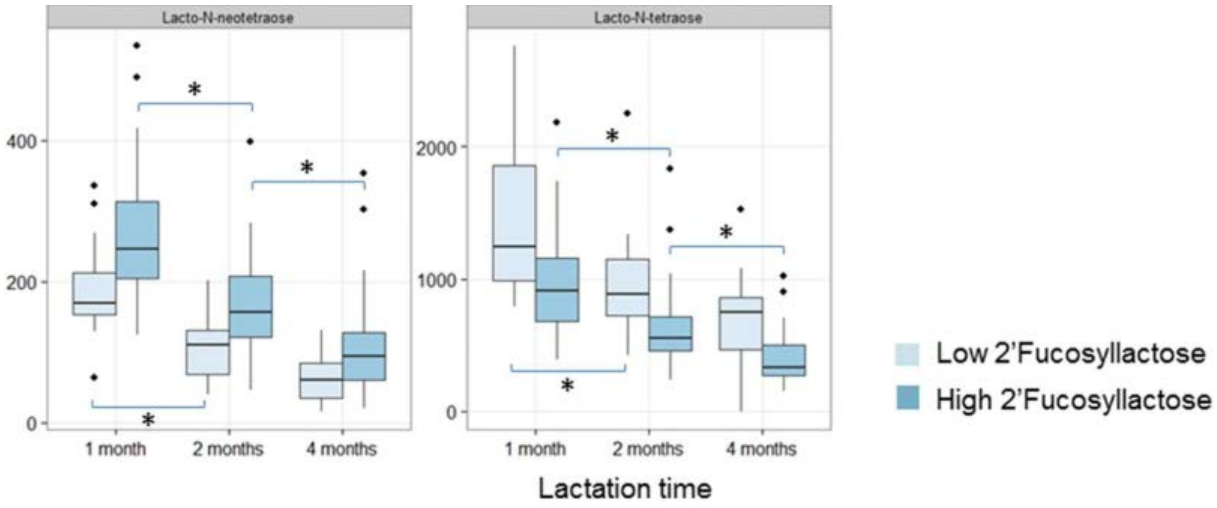


Figure 1.2.6-1 Box plot of LNnT and LNT concentrations over first 4 months of lactation separated by group with Low- and High 2'-O-Fucosyllactose in milk. (* indicates difference at a $p<0.05$; n = 50 for samples 1 and 2 months postpartum, n = 48 for samples 4 months postpartum).

1.3 Function Mechanisms of 2'-FL and LNnT

The biological roles of HMOs were investigated from the moment of their discovery in the 1950's [reviewed in (Kunz, 2012)]. The discovery itself was directly connected to their bifidogenic effect, since they were identified as the predominant "*bifidus factor*" of human milk (György and Rose, 1955; György *et al.*, 1954a,b; Gauhe *et al.*, 1954; György, 1953). However, owing to the complex composition of the total HMO fraction (and intricate molecular structure of individual components) it was for a very long time impossible to isolate − or produce − significant amounts of HMOs allowing for larger scale study of their explicit nutritional effects. This circumstance has even been highlighted in the latest European Food Safety Authority (EFSA) opinion on the essential composition of infant- and follow-on formula (EFSA, 2014), where it states in Chapter 5.4.5.3 on page 35:

"*Because of the variety, variability, complexity and polymorphism of human milk oligosaccharides, the addition to IF and FOF of a mixture of oligosaccharides mimicking those found in breast milk is not feasible and oligosaccharides which are currently added to IF and FOF are not comparable to human milk oligosaccharides. Instead, oligofructosyl-saccharose (oligofructose; FOS) and oligogalactosyl-lactose (oligogalactose; GOS) have been used in IF and FOF. FOS is not found in human milk and GOS is found only in trace amounts.*"

However, despite the principal verity of the above EFSA comment there already exists a huge body of basic research studies (including receptor binding studies, single cell and cell culture studies and smaller animal studies) that were performed using smaller amounts of either the whole HMO fraction isolated from human milk, or individually isolated and purified HMOs like 2'-FL and LNnT. In fact, research and technological capabilities in manufacturing is accelerating exponentially in this area, thus building the body of evidence. The mechanisms behind the main physiological roles of HMOs, and 2'-FL and LNnT in particular, are described in further detail in the subsections below.

1.3.1 Bifidogenic Function and Microbiota Shift in Infant Gut

The bifidogenic effects found in breast-fed infants include proliferation of specific bacterial strains such as *Bifidobacterium infantis*, *B. breve* and *B. bifidum* (Bezirtzoglou *et al.*, 2011). Bifidobacteria are considered beneficial for human health due to their ability to maintain intestinal health (Picard *et al.*, 2005; Hidalgo-Cantabrana *et al.*, 2017). Since *Bifidobacterium* is abundant in the microbiota of breast-fed infants, their acquisition and HMO metabolism has drawn a lot of attention in recent years.

Mono-, mixed or fecal culturing has clearly confirmed the decisive role of HMOs in promoting the growth of bifidobacteria, and these techniques have helped unravel the mechanistic understanding of HMO utilization by bifidobacteria (Vester Boler *et al.*, 2013). Many bifidobacterial genomes encode a large proportion of oligosaccharide processing and transporting genes clustered within conserved loci (Garrido *et al.*, 2016; De Leoz *et al.*, 2015; Pacheco *et al.*, 2015; Sela and Mills, 2014; LoCascio *et al.*, 2010; Sela *et al.*, 2008). These loci typically contain regulatory elements, ABC transporters, carbohydrate-binding proteins and highly specific glycoside hydrolases (rarely found across the bacterial kingdom). The genome sequence from *B. infantis* reveals the existence of five HMO-related loci. The largest of these clusters (43 kb) contains several glycoside hydrolases, ABC transporters and extracellular solute binding proteins. This unique set of regulatory elements, transporters, carbohydrate-binding proteins and highly specific glycoside hydrolases makes *B. infantis* exceptionally well adapted to the utilization of HMOs.

Analysis of other bifidobacterial genomes suggests a broad range of strategies for consuming milk glycans. Although strains of *B. breve* lack an HMO cluster similar to *B. infantis,* initial examination has observed a comparable intracellular degradation of HMO components. Strains belonging to *B. bifidum* express extracellular enzymes for degradation of HMOs outside the cells (Marcobal and Sonnenburg, 2012; Pacheco *et al.*, 2015).

More specifically, 2'-FL is hydrolysed to fucose and lactose by α-1,2-fucosidases. These enzymes are rare in the bacterial domain of life but have been identified in common infant gut commensals like *B. infantis* (Kim *et al.*, 2013) and *B. bifidum* (Katayama *et al.*, 2004; Nagae *et al.*, 2007; Katayama *et al.*, 2008).

LNnT in turn is hydrolysed to galactose and lacto-*N*-triose II by specific β-1,4-galactosidases. These enzymes are equally rare in the bacterial domain but have been identified in *B. infantis* (Yoshida *et al.*, 2012; Garrido *et al.*, 2012) and *B. bifidum* (Miwa *et al.*, 2010). Lacto-*N*-triose II is then hydrolyzed to *N*-acetyl-glucosamine (GlcNAc) and lactose by N-acetyl-β-D-hexosaminidases also identified in *B. infantis* (Garrido *et al.*, 2012) and *B. bifidum* (Miwa *et al.*, 2010).

Hence, due to their specific genes that coevolved with humans to utilize HMOs (Moeller *et al.*, 2016; Segre and Salafsky, 2016), *Bifidobacterium* species in the infant's intestine have the growth advantage compared to other members of the intestinal microbial community (Sela *et al.*, 2008; Yu *et al.*, 2013b; Hoeflinger *et al.*, 2015). For a summary of studies elucidating the molecular mechanisms of 2'-FL and LNnT utilization see Table 1.3.1-1.

Besides being able to grow on HMOs, studies have shown that 2'-FL and LNnT utilized by *B. infantis*, *B. breve* and *B. bifidum* result in the production of metabolites such as short-chain fatty acids (SCFA) and through this mechanism, a lowering of the pH is achieved (Garrido *et al.*, 2015; Vester Boler *et al.*, 2013). The generation of SCFAs as an end product of fermentation is also important for metabolic cross-feeding which in turn generates butyrate, all of which helps to maintain gastrointestinal health (*e.g.*, Goverse *et al.*, 2017; den Besten *et al.*, 2013). A recent study has shown that the *Eubacterium hallii*, one of the first butyrate producers in the infant gut, consume acetate released by *Bifidobacterium longum* subsp. *infantis* after 2'-FL utilization, and produces butyrate (Schwab *et al*., 2017). Butyrate is the primary energy source for colonocytes and is important for gut microbiota/host homeostasis as it interact with the infant epithelium and impact the immune system (Wong *et al.*, 2006; Plöger *et al.*, 2012). Additionally, infant-related bifidobacteria produce bacteriocins, proteinaceous toxins which inhibit the growth of unfavourable bacteria (Collado *et al.*, 2005; Yildirim and Johnson, 1998).

In conclusion, the selective growth of bifidobacteria on 2'-FL and LNnT creates a beneficial microbiota composition that

i) inhibits the growth of harmful bacteria due to an unfavourable environment mediated by the production of metabolites such as SCFA and bacteriocins.
ii) inhibits their colonization due to competition for nutrients and adhesion sites in the intestine.

Hence, 2'-FL and LNnT support creation of an ecological niche that may be more resistant against the colonization of pathogens.For a summary of studies showing bifidogenic effects of 2'-FL and LNnT see Table 1.3.1-2.

Table 1.3.1-1 summarises the key studies that reveal the molecular mechanisms underlying the utilisation biochemistry of the bifidogenic effects of 2'-FL and LNnT.

Table 1.3.1-1 Mechanisms of 2'-FL and LNnT Utilization by Bifidobacteria

HMO	Main findings	Origin	Method	References
2'-FL	*B. bifidum* JCM1254 produces 1,2-α-L-fucosidase (afcA) to utilize 2'FL	Type strain	Enzyme assay and crystal structure	(Katayama *et al.*, 2008; Nagae *et al.*, 2007; Katayama *et al.*, 2005; Katayama *et al.*, 2004)
2'-FL	*B. infantis* (7 strains), *B. longum* (3 strains), *B. vulgatus* (1 strain), *B. fragilis* (1 strain) and *B. thetaiotaomicron* (1 strain) induce α-L-fucosidase activity	Type strains	*In vitro* monoculturing and enzyme assay	(Yu *et al.*, 2013b)
2'-FL	Identification of novel gene cluster in *B. longum* SC596 for utilization of fucosylated HMO, including genes for import of fucosylated molecules, fucose metabolism and two α-fucosidases.	Strains isolated from breast-fed infants	*In vitro* monoculturing and genome sequencing	(Garrido *et al.*, 2016)
2'-FL and LNnT	*B. infantis* expresses an assortment of transporters to internalize HMO substrates while *B. bifidum* expresses enzymes to hydrolyse HMO extracellularly	Type strains	*In vitro* monoculturing and genome sequencing	(Garrido *et al.*, 2015)
LNnT	*B. infantis* ATCC 15697 has a gene (Bga2A) encoding a β-1,4-galactosidase specific to degrade LNnT	Type strains	Enzyme assay	(Yoshida *et al.*, 2012)
LNnT	β-hexosaminidases from *B. infantis* ATCC 15697 are induced during early growth *in vitro* on LNnT.	Type strains	*In vitro* monoculturing and Molecular cloning	(Garrido *et al.*, 2012)
LNnT	BbgIII (β-galactosidase) and BbhI (β-N-acetylhexosaminidases) from *B. bifidum* JCM1254 are highly specific for degradation of LNnT.	Type strain	*In vitro* monoculturing and Molecular cloning	(Miwa *et al.*, 2010)
LNnT	*B. breve* UCC2003 metabolizes LNnT through overlapping yet distinct pathway from LNT. Homologs of key genetic loci involved identified in *B. breve*, *B. bifidum*, *B. longum* subsp. *infantis* and *B. longum* subsp. *longum*	Type strain	*In vitro* monoculturing and transcriptome analysis	(James *et al.*, 2016)

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

Table 1.3.1-2 summarises the key studies that show the specific bifidogenic effects of 2'-FL and LNnT. This is further supported by data from observational studies, as well as clinical studies that have been conducted with 2'-FL and LNnT (described in Section 1.4).

Table 1.3.1-2 Bifidogenic Effects of 2'-FL and LNnT

HMO	Main findings	Origin	Method	Reference
2'-FL	*B. infantis* and *B. longum* grow on 2'-FL *E. coli* or *C. perfringens* does not utilize 2-'FL	Infant	*In vitro* monoculturing	(Yu *et al.*, 2013a)
2'-FL	Growth on 2'-FL: *B. infantis, B. longum*, *B. vulgatus, B. fragilis* and *B. thetaiotaomicron* No growth on 2'-FL: *Lactobacillus rhamnosus, Lactobacillus delbrueckii subsp. Lactis, Clostridium perfringens, Clostridium leptum, Staphylococcus epidermidis, Streptococcus thermophiles, Enterococcus faecalis, Enterobacter cloacae subsp. Cloacae, Enterobacter aerogenes, Escherichia coli*	Human	*In vitro* monoculturing	(Yu *et al.*, 2013b)
2'-FL and LNnT	Growth on 2'-FL and LNnT: *B. infantis* (21 strains) Moderate growth on 2'-FL and LNnT: *B. bifidum* (12 strains) No growth on: *B. animalis* subsp. *lactis*	Infant	*In vitro* monoculturing	(Garrido *et al.*, 2015)
2'-FL and LNnT	Growth on 2'-FL and LNnT: *B. bifidum* (2 strains), *B. longum subsp. infantis* No growth on 2'-FL or LNnT: *B. longum subsp. longum, B. breve*	Infant	*In vitro* monoculturing	(Asakuma *et al.*, 2011)
2'-FL and LNnT	Growth on 2'-FL: *B. bifidum* (8 strains), *B. kashiwanohense* (4 strains), *B. longum subsp. infantis* (5 strains), *B. longum subsp. suis* (1 strain) No growth on 2'-FL: *B. pseudolongum subsp. pseudolongum* (1 strain), *B. pseudolongum subsp. globosum* (1 strain), *B. breve* (3 strains), *B. longum subsb. longum* (1 strain) Growth on LNnT: *B. bifidum* (8 strains), *B. breve* (2 strains) *B. longum subsp. infantis* (5 strains) No growth on LNnT: *B. kashiwanohense* strains, *B. longum subsb. longum, B. longum subsb. suis, B. pseudolongum subsp. pseudolongum* or *B. pseudolongum subsp. globosum*	Infant	*In vitro* monoculturing	(Bunesova *et al.*, 2016)
2'-FL and LNnT	Growth on LNnT: *B. breve* (26 strains) Few strains grew on 2'-FL	Infant	*In vitro* monoculturing	(Ruiz-Moyano *et al.*, 2013)
2'-FL and LNnT	Growth on LNnT: *B. longum* (11 strains) Few strains grew on 2'-FL	Infant	*In vitro* monoculturing	(Garrido *et al.*, 2016) Scientific report
2'-FL and LNnT	Increase of bifidobacteria on 2' -FL and LNnT. Decrease of pH. Increased production of SCFA	Infant	*In vitro* fecal fermentation	(Vester Boler *et al.*, 2013)
2'-FL	Significant increase of bifidobacteria compared to control. Significant decrease of *Escherichia* and *Clostridium perfringens* compared to control. Significant lower pH than control	Infant	*In vitro* fecal fermentation	(Yu *et al.*, 2013a)
LNnT	Significant growth advantage for *B. infantis* over the *Bacteroides* strain.	Type strains	Co-inoculation of *Bacteroides thetaiotaomicron* and *B. infantis* in germ free-mice	(Marcobal *et al.*, 2011)
LNnT	Significant increase in bifidogenic activity, as indicated by the increase in galactosidase acivity (β-galactosidase utilization index) and esterase activity (Acetate Utilization Index)	Not applicable	Assessment of metabolic activity of cultured *B. infantis* ATCC 15697	Prieto, 2005

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

1.3.2 Anti-Infective Effect against Pathogens

The anti-infective effects against a range of pathogens of HMOs in general and 2'-FL and LNnT in particular are based on three principle mechanisms:

1) Inhibiting the growth and colonization of pathogens by providing a competitive advantage to non-pathogenic commensals such as bifidobacteria (through the mechanisms as described in the section above);

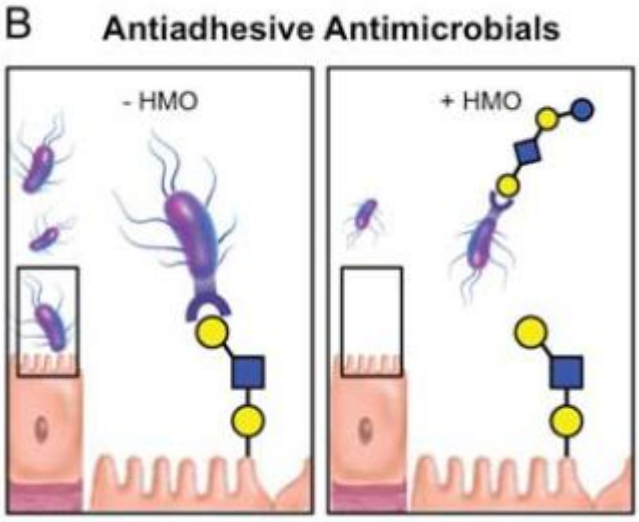
2) Competitive binding to the carbohydrate recognition domains of pathogen-generated proteins, e.g. surface proteins and/or toxins [reviewed by (Hickey, 2012; Bode, 2015; Newburg *et al.*, 2005; Kunz and Rudloff, 2006; Sharon and Ofek, 2000; El-Hawiet *et al.*, 2015; Newburg, 2009; Zopf and Roth, 1996)];

3) Modulation of the immune response either locally in the intestine or systemically (Abrahamsson and Sherman, 2014; He *et al.*, 2014; Yu *et al.*, 2016; Goehring *et al.*, 2016).

The competitive binding of pathogen-derived toxins to HMOs has been demonstrated in several *in vitro* studies using a set of different binding assays (see Table 1.3.2-1). Interestingly, an *in vivo* study by Newburg and colleagues found that fucosylated HMOs like 2'-FL were protective against a heat-stabile enterotoxin from *E. coli* in a suckling mouse lethality test indicating that the toxin binds to the fucosylated HMOs and because of this, the lethal impact of the enterotoxin is reduced (Newburg *et al.*, 1990).

Similarly, a range of *in vitro* studies have proven the anti-infective activity of 2'-FL and LNnT through competitive binding and inhibiting adhesion of pathogens (these studies are not discussed here but summarized in Table 1.3.2-2). It is notable that many viral, bacterial pathogens or toxins need to adhere to mucosal surfaces to colonize or invade the host and cause disease (Bode 2012, 2015). Most of pathogens express binding proteins (lectins) that bind to glycans on the host's epithelial cell surface. This initial attachment to epithelial cell surface sugars is also known as the glycocalyx (Bode, 2015). Some HMOs are structurally similar to the intestinal epithelial cell surface glycans and serve as decoy receptors to prevent pathogen binding and enhance pathogen clearance (Simon *et al.,* 1997; Gustafsson *et al*., 2006) (Fig. 1.3.2-1). This beneficial effect of HMOs is highly dependent on their structure.

Figure 1.3.2-1: HMOs are antiadhesive antimicrobials that serve as soluble glycan receptor decoys and prevent pathogen attachment.



Reference: Bode, L. Human milk oligosaccharides: Every baby needs a sugar mama. Glycobiology 2012a; 22(9): 1147-1162.

Furthermore, animal studies have proven anti-infective effects of 2'-FL and LNnT by reduction of pathogens and immune modulation. Yu *et al*., 2016 found that 2'-FL reduced *C. jejuni* colonization in mice, but also improved histologic features of intestinal inflammation and induced inflammatory signaling molecules of acute-phase mucosal immune response (Yu *et al.*, 2016).

Li *et al.,* 2014 found that a mixture of HMOs containing 40% 2'-FL and 35% LNnT (plus 10% 6'-SL, 5% 3'-SL and 10% free NANA) could reduce duration of rotavirus induced diarrhoea in piglets, and that the protective effect was the ability of HMOs to influence the host immunity by stimulating a balanced Th1 and Th2 cytokine response (Li *et al.*, 2014). A follow-up study by the same researchers provided insight into the possible mechanism of action that underlies partial protection against rotavirus-infection by the HMO mixture and showed that the same effect was not elicited by other non-digestible oligosaccharides (*i.e*., short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides) (Comstock *et al.*, 2017; Donovan, 2017). The protective effect of HMOs is not only relevant to enteric infections, but HMOs can also reduce upper respiratory track diseases (see Table 1.3.2-2). As an example, data from cell binding-assay studies demonstrate that LNnT inhibits adhesion of *Streptococcus pneumoniae* to epithelial cells (Andersson *et al.*, 1986; Andersson *et al.*, 1983) and intratracheal administration of LNnT concurrently with *Streptococcus pneumoniae* drastically decreased pneumococcal load in the lungs of rabbits and conferred protection from bacteremia (Idänpään-Heikkilä *et al.*, 1997).

Studies addressing toxin binding of HMOs are summarized in Table 1.3.2-1.

Table 1.3.2-1 Toxin Inhibition

HMO	Main findings	Pathogen	Method	Reference
2'-FL	Binds toxin A and toxin B	*C. difficile*	Binding assay	(El-Hawiet *et al.*, 2011)
2'-FL	Binds extensively to four bacterial exotoxins	*Vibrio cholera* and *E. coli*	Binding assay	(El-Hawiet *et al.*, 2015)
2'-FL	Inhibition of binding and activity of heat-stable enterotoxin	*E. coli*	T84 intestinal cells	(Crane *et al.*, 1994)
2'-FL	Protective against heat-stable enterotoxin	*E. coli*	Suckling mouse lethality test	(Newburg *et al.*, 1990)
LNnT	Blocks binding of streptolysin O	*Streptococcus pyogenes*	Red blood cells from humans	(Shewell *et al.*, 2014)

2'-FL = 2'-*O*-fucosyllactose; HMO = human milk oligosaccharide; LNnT = lacto-*N*-neotetraose.

Studies addressing anti-adhesive and anti-infective activities of HMOs against whole pathogens are summarised in Table 1.3.2-2.

Table 1.3.2-2 Anti-adhesion and Anti-infective

HMO	Main findings	Pathogen	Method	Reference
2'-FL	Inhibits adhesion	*Candida albicans*	Buccal epithelial cell assay	(Brassart *et al.*, 1991)
2'-FL	Inhibits adhesion *in vitro* Inhibits colonization *ex vivo* and *in vivo*	*Campylobacter jejuni*	HEp-2 cell assay, human intestinal mucosa assay, BAlb/c mice	(Ruiz-Palacios *et al.*, 2003)
2'-FL	Binds to pathogen	*Campylobacter jejuni*	Gold chip surface	(Lane *et al.*, 2011)
2'-FL	Inhibits adhesion	*Campylobacter jejuni*	HT-29 cell assay	(Lane *et al.*, 2012)
2'-FL	Attenuates infection and suppresses IL-8, IL-1beta and MIP-2 *in vitro*. Reduces pathogen colonization *in vivo*	*Campylobacter jejuni*	HEp-2, HT-29 cell assays, C57BL/6 mice	(Yu *et al.*, 2016)
2'-FL	Inhibits adhesion	*Campylobacter jejuni, Enteropathogenic E. coli, Salmonella fyris*	CaCo2 and Alveolar epithelial cell assays	(Weichert *et al.*, 2013)
2'-FL	Directly inhibits LPS-mediated inflammation and suppresses CD14 transcription and translation	*Enterotoxigenic E. coli, uropathogenic E. coli and adherent-invasive E. coli*	T84 and H4 intestinal cell assays	(He *et al.*, 2016)
2'-FL	Inhibits adhesion	*Pseudomonas aeruginosa*	Alveolar epithelial cell assay	(Weichert *et al.*, 2013)
2'-FL	Inhibits adhesion	*Enteropathogenic E. coli*	CaCo2 cell assay	(Coppa *et al.*, 2006)
2'-FL	Inhibits adhesion	*Burkholderia cenocepacia*	Alveolar epithelial cell assay	(Thomas and Brooks, 2004)
2'-FL	Binds to pathogen	Norovirus	Binding assay	(Koromyslova *et al.*, 2017)
2'-FL	Inhibits adhesion	Rotavirus	African green monkey kidney epithelial cells (MA104 cells)	Laucirica *et al.*, 2017
LNnT	Inhibits adhesion	*Streptococcus pneumoniae*	Nasopharyngeal epithelial cells	(Andersson *et al.*, 1983)
LNnT	Inhibits adhesion	*Streptococcus pneumoniae and Haemophilus influenzae*	Buccal & oropharyngeal epithelial cells	(Andersson *et al.*, 1985)

Table 1.3.2-2 Anti-adhesion and Anti-infective				
HMO	**Main findings**	**Pathogen**	**Method**	**Reference**
LNnT	Inhibits adhesion	*Streptococcus pneumoniae and Haemophilus influenzae*	Buccal & oropharyngeal epithelial cells	(Andersson *et al.*, 1986)
LNnT	Inhibits adhesion	*Enteropathogenic E coli*	HEp-2 cell assay	(Cravioto *et al.*, 1991)
LNnT	Binds to pathogen	*Pseudomonas aeruginosa*	Binding assay	(Ramphal *et al.*, 1991)
LNnT	Inhibits adhesion	*Streptococcus pneumoniae*	Ciliated chinchilla respiratory epithelium	(Tong *et al.*, 1999)
2'-FL, LNnT	Reduce duration of rotavirus-induced diarrhea	*Rotavirus*	Piglets	(Li *et al.*, 2014)
2'-FL, LNnT	*Enterobacteriaceae* strains don't grow on 2'-FL and/or LNnT	*Klebsiella pneumoniae, K. oxytoca, Salmonella enterica* serovar *Typhimurium*, *E. coli*, *Cronobacter sakazakii*	*In vitro* monoculturing	(Hoeflinger *et al.*, 2015)
2'-FL	Reduced the growth of *Enterobacteriaceae*	*Enterobacteriaceae*	Resected mice (model for small bowel syndrome)	(Mezoff *et al.*, 2016)
2'-FL	Decreased adhesion	*Escherichia coli* F18 (enterotoxigenic)	*In vitro* binding assay	Cilieborg *et al.*, 2017
2'-FL	Did not reduce the diarrhea induced by inoculation of piglets with *E.coli* F18	*Escherichia coli* F18 (enterotoxigenic)	Piglets	
2'-FL	Inhibits pathogen adhesion	Norovirus	Binding assay	Weichert *et al.*, 2016

2'-FL = 2'-*O*-fucosyllactose; HMO = human milk oligosaccharide; LNnT = lacto-*N*-neotetraose.

1.3.3 Intestinal Barrier Function, Immune Modulation and Allergy

The intestinal barrier function plays an important role in maintaining host health. Intestinal barrier dysfunction plays a role in infant diseases such as inflammatory bowel disease, infectious enteritis, and necrotizing enterocolitis (NEC). Several studies have shown that human breast milk decreases the intestinal permeability and therefore enhances the physical barrier of an infant intestine (Halpern and Denning, 2015).

The protective effect of HMOs on gut barrier function and immune modulation has been examined using both *in vitro* and animal models. A study by Good *et al.* (Good *et al.*, 2016) found that administration of 2'-FL to NEC induced mice reduced the severity of NEC, decreased pro-inflammatory markers and preserved the small intestinal mucosal architecture. The ability of 2'-FL and LNnT to support the intestinal barrier function and modulate the immune system can be explained through both indirect and direct mechanisms (for a recent review, please see Donovan and Comstock, 2016). Indirectly, it has been shown that stimulating the growth of infant-dominate bifidobacteria by HMOs can enhance tight junction protein expression and immunomodulatory IL-10 in CaCo2 cells and intestinal epithelial cells. However, this effect could not be observed, when the bacteria grew on lactose (Chichlowski *et al.*, 2012).

In a mouse model for small bowel syndrome Mezoff *et al.* reported that 2'-FL changed the intestinal microbial community and affected characteristic histological changes such as increase in crypt depth

and villus height. In addition, the study showed that administration of 2'-FL indirectly or directly initiated an upregulation in genes related to mucosal immune response (Mezoff *et al.*, 2016). A direct effect of HMOs on gut barrier function has been examined in a study by Holscher *et al.*, where LNnT was found to increase transepithelial resistance in CaCo2 cells (Holscher *et al.*, 2014). Additionally, Hester *et al*. found that LNnT could reduce intestinal crypt cell proliferation and apoptosis (Hester and Donovan, 2012).

As discussed previously (Section 3.2.2), HMOs are highly similar in structure to other human glycans, presenting many identical epitopes. Lectins are carbohydrate-binding proteins on the surfaces of mammalian cells that translate recognition of specific carbohydrate motifs and the spatial presentation of those motifs into action. The intestinal dendritic cells (DC) present in the organized lymphoid structures of the intestinal immune system are implicated both in the maintenance of tolerance, and in the generation of protective immune responses against pathogens. This flexibility in function is due to the ability of accurately sense their local environment and use these signals to shape the nature of the ensuing immune response. One class of lectins, called C-type lectins, is found amongst others on the surface of intestinal DC can use fucosylated glycans as ligands, hence the interaction between C-type lectins on intestinal DC and fucosylated HMOs, such as 2'-FL, can contribute to immunity and immune tolerance (Donovan and Comstock, 2016). This interaction can be assumed to be the mechanism behind the results found by Castillo-Courtade *et al*., where dietary 2'-FL was shown to attenuate food allergy symptoms and help establish oral tolerance by inducing IL-10^{+} regulatory cells and stabilizing mast cells in an animal model (Castillo-Courtade *et al.*, 2015).

Galectins are a group of soluble carbohydrate-binding proteins with immunomodulatory properties that act either intracellularly or extracellularly. Galectins bind predominantly glycosphingolipids of the lacto- and neolacto-series, including poly-*N*-acetyl-lactosamine (poly-Galβ1,4GlcNAc), but have also been explicitly shown to use LNnT as efficient ligand (Collins *et al.*, 2014; Halimi *et al.*, 2014; Noll *et al.*, 2016; Bohari *et al.*, 2016; Shams-Ud-Doha *et al.,* 2017). Recently, efficient and selective binding of 2'-FL to 4 human galectins was also determined (Shams-Ud-Doha *et al.,* 2017). Galectins plays a distinct role in the control of immune cell homeostasis by affecting the differentiation of regulatory DCs, which promotes T cell tolerance through mechanisms involving IL-27 and IL-10, hence the interaction between galectins and HMOs, such as LNnT, contributes to immunity and immune tolerance (Davicino *et al.*, 2011; Rabinovich *et al.*, 2012).

The absorption rate of HMOs from the gastrointestinal (GI) tract has been estimated to be approximately 1% of the total HMO intake, thus their systemic levels are in the range of 10-100 mg/mL (Bode *et al.*, 2004). This concentration is sufficient to directly affect and activate immune cells circulating in the blood. Several *in vitro* and *ex vivo* experiments have shown that both 2'-FL and LNnT can interact with immune cells affecting immune cell activation and cytokine production (Velupillai and Harn, 1994; Zhu *et al.*, 2003; Zhu *et al.*, 2005; Amin *et al.*, 2008; Rabquer *et al.*, 2012; Comstock *et al.*, 2014). As an example, Velupillai and Harn found that LNnT could impact splenic B cell proliferation and induction of IL-10 production *ex vivo* (Velupillai and Harn, 1994).

For an overview of these and further studies see Table 1.3.2-3.

Table 1.3.3-1 Gut Barrier Function and Immune Modulation			
HMO	**Main findings**	**Method**	**Reference**
2'-FL	Gut maturation by inhibiting cell proliferation	HT-29 and Cao2Bbe cells	(Holscher *et al.*, 2014)
2'-FL	Reduces cell proliferation	Peripheral blood mononuclear cell from piglets	(Comstock *et al.*, 2014)
2'-FL	Inhibits cell proliferation and modulates cytokine production of mononuclear cells (MNC)	MNC isolated from healthy controls and multiple sclerosis patients	(Sotgiu *et al.*, 2006)
2'-FL	Decreases severity of colitis	DSS-induced colitis mice	(Weiss *et al.*, 2014)
2'-FL	Improves mucosal architecture and upregulates genes related to mucosal immune responses	Resected mice (model for small bowel syndrome)	(Mezoff *et al.*, 2016)
2'-FL	Reduces pathology scores in necrotizing enterocolitis (NEC)	Rats with NEC	(Autran *et al.*, 2016)
2'-FL	Protects against NEC. Decreases pro-inflammatory markers. Preserves the small intestinal mucosal architecture	Neonatal wild-type mice with NEC	(Good *et al.*, 2016)
2'-FL	Affect monocyte activation and migration	In vitro monocyte assay	(Rabquer *et al.*, 2012; Amin *et al.*, 2008)
2'-FL	Impact on cell signaling by affecting angiogenesis	Rat aortic ring model	(Zhu *et al.*, 2005)
2'-FL	Impact on cell expression	Human microvascular endothelial cell lne-1	(Zhu *et al.*, 2003)
2'-FL	Induces IL-10+ regulatory cells and stabilizes mast cells	Food allergy treatment model (mice model)	(Castillo-Courtade *et al.*, 2015)
2'-FL	2'-FL attenuated lipopolysaccharide-induced inflammation in Intestinal epithelial cells stimulated with enterotoxigenic *Escherichia coli*	*In vitro* model of inflammation (T84 and H4 intestinal epithelial cells)	He *et al.*, 2016
LNnT	Affect splenic B-cell proliferation and IL-10 production	B cells isolated from infected animals	(Velupillai and Harn, 1994)
LNnT	Gut maturation by inhibiting cell proliferation Increases transepithelial resistance	HT-29 and Caco2Bbe cells	(Holscher *et al.*, 2014)
LNnT	Gut maturation by inhibiting cell proliferation	Human fetal intestinal cell lines	(Hester and Donovan, 2012)

2'-FL = 2'-*O*-fucosyllactose; DSS = dextran sulfate sodium; HMO = human milk oligosaccharide; LNnT = lacto-*N*-neotetraose; MNC = mononuclear cells; NEC = necrotizing enterocolitis.

1.3.4 Other Potential Functions

Positive impact on cognitive development

In breastfed children, higher intelligence quotient and better performance in intelligence tests later in life have been consistently observed compared to those who were formula fed (Horta *et al.*, 2015; Deoni *et al.*, 2013; Brion *et al.*, 2011; Iacovou and Sevilla-Sanz, 2010; Kramer *et al.*, 2008; Horta *et al.*, 2007; Der *et al.*, 2006; Mortensen *et al.*, 2002). Although the most prominent milk ingredients that are discussed in context of cognitive development are long-chain polyunsaturated fatty acids (LC-PUFA), such as docosa-hexaenoic acid (DHA) and arachidonic acid (AA or ARA), a large body of data also supports an important role of fucose and fucose-containing HMOs like 2'-FL for cognitive development and memory formation.

An enhanced fucosylation of brain glycoproteins, which are involved in memory formation, has been observed upon dietary administration of 2'-FL (Santos-Benito *et al.*, 1992; Krug *et al.*, 1994; Matthies *et al.*, 1996; Vázquez *et al.*, 2015; Vazquez *et al.*, 2016).

Interestingly, recent studies have also shown that dietary supplementation of 2'-FL can affect cognitive outcomes by stimulating central nerve system (CNS) function such as hippocampal LTP and learning and memory capabilities in rodents (Vázquez *et al.*, 2015; Vazquez *et al.*, 2016). The mechanism behind this has been suggested to be mediated through the vagus nerve. In 2016, Vazquez and colleagues showed that dietary administration of 2'-FL, but not fucose, enhanced LTP, but vagotomy inhibited the effects both on LTP and associative learning related paradigms. Since ingested fucose did not exert any effect on synapsis regulation, the molecular integrity of 2'-FL in the intestine seems necessary to induce beneficial effects on CNS function (Vazquez *et al.*, 2016). Hence, one mechanism for the effect of dietary 2'-FL on cognitive benefits has been postulated to be dependent on the vagus nerve, implicating the role of the gut-brain axis. Another study by Bienenstock *et al.* (2013) has also proven the impact of 2'-FL on the gut-brain axis in respect to intestinal contraction and motility (Bienenstock *et al.*, 2013). The study found that 2'-FL could affect colonic neuronally dependent smooth muscle contractions in mice by diminishing colon motor contractions. The mechanism was attributed to the ability of 2'-FL to regulate gut motility. Additionally, the study found that the modulation of neuronally dependent migrating motor complexes affected by fucosylated molecules demonstrated anti-nociceptive activity. Large amplitude motor complexes are essential for the perception of visceral pain, hence a reduction in amplitude as demonstrated by 2'-FL moderated the nociceptive stimulus (Bienenstock *et al.*, 2013).

For a summary of studies reporting cognitive effects of 2'-FL please see Table 1.3.4-1.

Table 1.3.4-1 Neural Development and Gut-Brain Axis

HMO	Main findings	Model	Administration	References
2'-FL	Inhibits proliferation of normal and transformed neural cells	Rat astrocytes	Cell culture	(Santos-Benito *et al.*, 1992)
2'-FL	Enhances hippocampal long-term potentiation	Rat	Intrahippocampal	(Krug *et al.*, 1994)
2'-FL	Enhances hippocampal long-term potentiation	Rat hippocampus	Cell culture	(Matthies *et al.*, 1996)
2'-FL	Regulates gut motility *ex vivo*	Mice	Dietary	(Bienenstock *et al.*, 2013)
2'-FL	Enhances synaptic plasticity Increases expression of brain functional markers Affects cognitive domains Improve learning and memory	Mice and rats	Dietary	(Vázquez *et al.*, 2015)
2'-FL	Affects cognitive domains Improves learning and memory Effect depends on the vagus nerve	Rats	Dietary	(Vazquez *et al.*, 2016)
2'-FL	Enhances cognitive abilities	Rats	Dietary	(Oliveros *et al.*, 2016)

2'-FL = 2'-*O*-fucosyllactose; HMO = human milk oligosaccharide; LNnT = lacto-*N*-neotetraose.

1.4 Comparison of HMO Effects by Observational Correlation Trials

A number of observational clinical trials have analysed mother-infant pairs in regards to properties of the mother's milk in correlation to infant outcomes. This included trials that reported a correlation between the content of individual HMOs in mother's milk, or more generally the Secretor status of the mother (and her milk), with infant outcomes.

These trials are summarised in Table 1.4-1 and discussed in below sections, followed by a rationale why adding the combination of 2'-FL and LNnT to infant formula provides a feasible proxy for Secretor milk.

The prominent role of 2'-FL and/or LNnT is illustrated by the proportion of observational studies in which these compounds are specifically identified by investigators as among the HMO compounds having a correlative association with positive infant endpoints.

Table 1.4-1 Overview on Observational Clinical Trials Correlating Milk Composition and Infant Outcomes

Reference	Study Group	Relevant study parameter	Relevant Correlations reported
(Sprenger *et al.*, 2017a) NCT01805011	50 Healthy mother-infant pairs	Individual HMO concentrations	• 2'-FL concentration negatively co-regulated with LNT and positively co/regulated with LNnT, suggesting that LNnT is Secretor-characteristic HMO. • 2'-FL and LNnT concentrations drop over lactation. • Up to 4 months of age, no significant differences were observed in body weight, body length, body mass index and head circumference of the infants who consumed breast milk with low or high FUT2 associatedHMO concentra-tions and composition
(Davis *et al.*, 2017) ISRCTN49285450	33 Healthy mother-infant pairs	Individual HMO concentrations	• During wet season, when food is scarce and stressors are high, breast milk contained 20% less HMOs • LNnT was positively correlated with *B. infantis* • More α(1-2)-fucosylated LNT (i.e. LNFP I) less sick days and higher height-for-age scores at 20 weeks postpartum
(Andreas *et al.*, 2016)	183 Healthy mother-infant pairs	Individual HMO concentrations	• LNDFH I and 3FL correlated to reduced Group B Streptococcus colonization
(Bender *et al.*, 2016)	50 HIV Mother-healthy infant pairs	Individual HMO concentrations	• Non-infected Infants from HIV-positive mothers show increased incidence of dysbiotic microbiota • HMO composition differs between HIV-positive and negative mothers • HIV-positive mothers show increased levels of 2'-FL and decreased levels of LNnT in their milk
(Charbonneau *et al.*, 2016)	78 Mother-infant pairs (29 healthy and 59 stunted infants)	Individual HMO concentrations	• Mothers of healthy infants had significantly higher concentrations of total, sialylated, and fucosylated HMOs than mothers of severely stunted infants • Most growth discriminatory sialylated HMO for entire cohort was sialyllacto-N-tetraose b (LSTb), while most discriminatory fucosylated HMOs were 2'-FL and LNFP I
(Davis *et al.*, 2016) NCT01817127	1 mother/infant pair and six infant fecal samples	Individual HMO concentrations in milk and infant feces	• LNT and LNnT, which were the two most abundant HMOs in this study, where completely lacking from the feces showing preferential consumption of these structures • The 2'-FL peaks were considerably reduced in feces compared to breastmilk • Bacterial profiling revealed that bifidobacteria comprised on average 68.0% of all bacteria in the infant gut at week 17
(Sprenger *et al.*, 2017b) NCT00298337	266 Healthy mother-infant pairs	Mother Secretor status	• Reduced risk for onset of certain allergies in infants from Secretor mothers born *via* c-section
(Smith-Brown *et al.*, 2016) ERP016646	17 Healthy mother-infant pairs	Mother Secretor status	• Persistence of *Bifidobacteria*-dominated microbiota demonstrated in offspring from Secretor mothers until 2-3 years of age
(Lewis *et al.*, 2015) NCT01817127	44 Healthy mother-infant pairs	Mother Secretor status	• Accelerated establishment of *Bifidobacteria*-dominated microbiota in offspring from Secretor mothers
(Kuhn *et al.*, 2015)	958 HIV-infected mothers, 103 HIV-infected infants, 143 uninfected infants	Mother Secretor status	• Higher breast milk 2'-fucosylated HMOs, 2'-FL and LNFP I, as well as non-2'-fucosylated HMOs were associated with lower infant mortality in HIV-exposed infants during lactation, but not thereafter
(Alderete *et al.*, 2015)	25 Healthy mother-infant pairs	Mother Secretor status	• Inverse relation between FUT2-dependent LNFP I, but not 2'-FL, in breast milk at 6 months and body weight, lean and fat mass of infants at 6 months. • LNnT inversely related to body fat mass at 6 months of age
(Flanders Stepans *et al.*, 2006)	49 Healthy mother-infant pairs	Total HMO content	• LNFP II levels used as proxy for total HMO content: levels in milk at 2 weeks postpartum associated with fewer infant respiratory problems by 6 weeks (P 0.01), as were levels in infant feces (P 0.003). Levels in milk at 2 weeks associated with fewer respiratory problems by 12 weeks (P 0.038), and fewer enteric problems by 6 weeks (P 0.004) and 12 weeks (O 0.045).

Table 1.4-1	Overview on Observational Clinical Trials Correlating Milk Composition and Infant Outcomes		
Reference	**Study Group**	**Relevant study parameter**	**Relevant Correlations reported**
(Newburg *et al.*, 2004b; Morrow *et al.*, 2004a; Newburg *et al.*, 2004a; Morrow *et al.*, 2004b)	93 Healthy mother-infant pairs	Mother Secretor status	• Moderate-to-severe diarrhea of all causes (77 cases) occurred less often (P = 0.001) in infants whose milk contained high levels of total 2-linked fucosyl-OS • Campylobacter diarrhea (31 cases) occurred less often (P = .004) in infants whose milk contained high levels of 2'-FL • Calicivirus diarrhea (16 cases) occurred less often (P = .012) in infants whose milk contained high levels of lacto-N-difucohexaose (LNDFH-I) • *E. coli* stable toxin diarrhea (4 cases) occurred in infants consuming lower ratio of 2-linked fucosyl-OS

2'-FL = 2'-*O*-fucosyllactose; 3FL = 3-fucosyllactose; HMO = human milk oligosaccharide; LNDFH = lacto-*N*-difucohexaose; LNnT = lacto-*N*-neotetraose; LNT = lacto-*N*-tetraose; LST b = sialyllacto-N-tetraose b; OS = oligosaccharide

1.4.1 Observation Trials Correlating HMO Content of Breastmilk with Infant Outcomes

In a pilot study by Davis *et al.,* 2017 (Davis *et al.*, 2017), the HMO composition in the breast milk and the infant gut microbiota of 33 rural Gambian mother/infant pairs were profiled at 4, 16, and 20 weeks postpartum and correlated to growth and morbidity in the infants. Among the findings of this study was that *B. infantis* was the only microbe that was positively correlated with LNnT abundance, and that infants from mothers with more α(1-2)-fucosylated LNT (i.e. LNFP I) had less sick days and achieved higher height-for-age scores at 20 weeks postpartum (Davis *et al.*, 2017). In this study cohort LNT, LNFP I and LNnT were the most abundant HMOs with relative abundances of 23, 10 and 10% respectively, while 2'-FL with approximately 9% relative abundance was below its typically abundance of 15-20% found in other studies and populations (see Section 3.2.4). Whether the positive effect of LNFP I was directly connected to this specific HMO or the general upregulation of the FUT2 gene could not be determined in this study.

1.4.2 Comparison of Effects of Secretor and Non-Secretor Milk

Secretors are individuals who secrete ABH antigens in their bodily fluids and can link Fucose in an α(1–2)-position on a terminal Galactose residue. The α(1-2)-fucosyltransferase FUT2 is responsible for this linkage and is encoded for by the secretor locus (Ferrer-Admetlla *et al.*, 2009); this genotype corresponds in phenotype with HMO composition of milk, with Secretor mothers having higher relative concentrations of fucosylated HMOs [particularly 2'-FL, but further including lacto-N-fucopentaose I (LNFP I), lactodifucotetraose (LDFT), and difucosyllacto-N-hexaose I (DFLNH I)], but lower relative concentrations of undecorated and sialylated HMO (Blank *et al.*, 2011; Totten *et al.*, 2012).

It is of high interest to note that the diversity ("polymorphism") of the female population in regards to the Secretor genotype appears to have been maintained over evolutionary times due to a "parent-offspring conflict" (Springer and Gagneux, 2013): while the non-secretor phenotype appears to provide a net benefit to the mother to escape infectious agents (Marionneau *et al.*, 2005; Lindén *et al.,* 2008; Carlsson *et al.*, 2009), there is a growing body of evidence that the breastfed infant actually benefits from the inverse situation, the secretor phenotype as expressed into milk, as is described in the following paragraphs.

Newburg and Morrow (Newburg *et al.*, 2004b; Morrow *et al.*, 2004a) have investigated the association between maternal milk levels of 2-linked fucosylated oligosaccharides and prevention of diarrhea as a result of *E. coli* stable toxin, campylobacter, calciviruses or diarrhea of all causes in 93 breastfeeding mother-infant pairs in Mexico. They reported that moderate-to-severe diarrhea of all causes (77 cases) occurred less often (P = 0.001) in infants whose milk contained high levels of total 2-linked fucosyl-OS. Campylobacter diarrhea (31 cases) occurred less often (P = 0.004) in infants whose milk contained high levels of 2'-FL. Calicivirus diarrhea (16 cases) occurred less often (P = 0.012) in infants whose milk contained high levels of lacto-N-difucohexaose (LNDFH-I) and *E. coli* stable toxin diarrhea (4 cases) occurred in infants consuming lower ratio of 2-linked fucosyl-OS.

The results of this observational trial and the fact that almost 100% of the Mexican population are Secretors (Morrow *et al.*, 2004a; Erney *et al.*, 2000) suggests that the mother's Secretor status was under indirect positive selection pressure due to the higher content of 2'-fucosylated oligosaccharides exercising a protective effect on her offspring. Thus, an overall statistical effect on the frequency of the Secretor gene is maintained.

Lewis *et al.* (Lewis *et al.*, 2015) observed in a cohort of 44 mother-infant pairs that on average, bifidobacteria were established earlier and more often in infants fed by Secretor mothers than in infants fed by non-Secretor mothers. In Secretor-fed infants, the relative abundance of the *Bifidobacterium longum* group was most strongly correlated with high percentages of the order Bifidobacteriales (as determined by quantitative PCR). Conversely, in non-Secretor-fed infants, *Bifidobacterium breve* was positively correlated with Bifidobacteriales, while the *B. longum* group was negatively correlated. Infant feces with high levels of bifidobacteria had lower milk oligosaccharide levels in the feces (i.e. 2'-FL was being consumed by these bifidobacteria) and higher amounts of lactate. Furthermore, feces containing different bifidobacterial species possessed differing amounts of oligosaccharides, suggesting differential consumption *in situ*. Lewis *et al.* concluded that infants fed by non-Secretor mothers are delayed in the establishment of a bifidobacteria-laden microbiota. This delay may be due to difficulties in the infant acquiring a species of bifidobacteria able to consume the specific milk oligosaccharides delivered by the mother an accelerated establishment of Bifidobacteria-dominated microbiota in offspring from Secretor mothers.

Smith-Brown *et al.* (Smith-Brown *et al.*, 2016) reported that in 2- to 3-year-old Australian children, who were exclusively breast-fed for at least 4 months of life, the abundance of the known HMO consumers Bifidobacterium were increased in the children of Secretor mothers compared to non-Secretor mothers while the relative abundance of *Bacteroides plebeius*, a bacterium noted for its capacity to utilise sulphated polysaccharides for growth, was decreased in these children. It is remarkable that the effect remains observable for several years and highlights that HMOs may have an important role in early nutritional programming of a child for diverse health outcomes (Symonds *et al.*, 2013).

1.5 Comparison of Effects with and without Addition in Food Category

1.5.1 Overview on Clinical Trials with 2'-FL, LNnT and combination

We provide here an overview on clinical trials that have been performed with 2'-FL and/or LNnT in human study populations. A summary of the key study characteristics of these studies are summarized in Table 1.6.1-1, and their main findings in relation to the beneficial health effects are described in brief below. A specific focus on each of the main endpoints assessed in these studies are further described in Sections 1.6.2 to Section 1.6.5 that follow. Of note, 2'-FL and LNnT were reported to be well tolerated in all of these studies, as described in the main application.

A randomised, blinded, controlled, multi-centre, parallel-design study has been conducted where infants were administered formula containing up to 1.2 g 2'-FL together with up to 0.6 g LNnT for the first 4 months of life. Another group of infants received a control formula without these HiMOS. The infants were exclusively fed the test or control formulas for the first 4 months of age. Complementary foods were allowed to be introduced beginning at 4 months of age. At 6 months of age, the infants in both study groups (test and control formula) were switched to an intact protein, cow's milk–based, follow-up formula without HiMOs for feedings through to 12 months of age. Both the 2'-FL and LNnT ingredients used in this study were produced by Glycom. Additional details of this study, including the outcomes of the safety-related parameters assessed, have been provided in Section C.4.1. Although the primary objective of this study was to investigate the safety and tolerability of 2'-FL and LNnT (Puccio *et al.*, 2017), the secondary endpoints collected provides evidence that supplementation with 2'-FL and LNnT may have beneficial health outcomes. Infants receiving the formula supplemented with HiMOs had significantly fewer parental reports of bronchitis, lower respiratory tract infections, antipyretic use, and antibiotic use, when compared to infants receiving the control formula (Puccio *et al.,* 2017).

In the same study, stool samples were collected at 3 months of age for assessment of microbiota using 16S rRNA gene sequencing and metagenomics; metabolic signature was also examined using proton NMR-based metabolite profiling (Alliet *et al.*, 2016; Steenhout *et al*., 2016). Stool samples that were collected from a sample of breast-fed infants in the same study served as a reference control. Infants fed formula containing the 2 HiMOs experienced a shift in their faecal microbiota profile towards one that more closely resemble those of breast-fed infants. The global average microbial composition for the sub-group of infants with stool samples that followed the study protocol showed similar pattern between control (n=65) and test (n=58) at the genus level, although samples obtained from infants receiving the test formula were closer to breastfed (n=34) than control samples. Calculations of microbial alpha diversity and comparison of the global microbiota composition confirmed that test was different from control at the genus level ($p<0.001$) and closer to the breastfed reference. Statistical analysis (corrected for false discovery rate) identified several taxa differentially present in control and test including *Bifidobacterium* (p=0.01), *Escherichia* (p=0.008) and unclassified *Coprobacillaceae* (p=0.01). Multivariate analysis identified several influential metabolites that discriminated between test, control and breastfed groups including phenylalanine, isoleucine, tyrosine, faecal organic acids and fucosylated compounds. The values observed for the

test formula group were more similar to those observed in the breast-fed group compared with control, a finding that suggests reduced protein fermentation. The study authors concluded that: "*Together, the stool microbiota and metabolic signature show that the addition of 2'FL and LNnT to a starter infant formula shift the stool microbiota towards that observed in breastfed infants, both in composition and function.*"

Another randomised, controlled study has been conducted to investigate the safety of 2'-FL, in which infants were administered one of the following 3 formulas for 4 months: i) a standard, milk-based, commercially available infant formula containing 2.4 g GOS/L (control formula); ii) the standard formula supplemented with 0.2 g 2'-FL/L and 2.2 g GOS/L; or iii) the standard infant formula supplemented with 1.0 g 2'-FL/L and 1.4 g GOS/L (Marriage *et al.*, 2015; Goehring *et al.*, 2016). A comparator (reference) group comprised of infants consuming human milk (by breast and/or bottle) was also included. Additional details of this study, including the outcomes of the safety-related parameters assessed, have been provided in Section C.4.1. The outcomes related to biomarkers of immune function are described in a publication by Goehring *et al.* (2016). At 6 weeks of age, 2 to 3 mL of non-fasting venous blood was drawn and analysed for immunophenotypic markers (by flow cytometry) for the following cell surface markers: CD4, CD8, CD20, and CD56. Plasma samples were analyzed for cytokines: IFN-α2, IFN-δ, IL-10, IL-1 receptor antagonist (IL-1ra), IL-1α, IL-1β, IL-6, IFN-δ–induced protein 10, RANTES (regulated upon activation, normal T cell expressed and secreted), and TNF-α. RNA from peripheral blood mononuclear cells were quantified and used to detect a respiratory syncytial virus (RSV)-specific gene product, NS1, to quantify viral load.

The control formula group exhibited lower percentages of circulating T lymphocytes and CD8+ lymphocytes compared to the breastfed group; however, no significant differences in any cell type were observed between the 2'-FL supplemented groups and the control and breastfed groups with the exception of a lower CD8+ population in infants receiving 1.0 g 2'-FL/L compared to the breastfed group. The inflammatory cytokine profiles revealed a statistically significant higher concentration of circulating inflammatory cytokines IFN-α2, IL-1β, IL-6, TNF-α and IL1ra in the control formula group when compared to be breastfed group. However, no statistically significant differences were observed in the groups receiving the experimental formulas containing the 2 different doses of 2'-FL, when compared to the breastfed group. No significant differences in the other plasma cytokines or RANTES were observed between any of the groups. Furthermore, no significant differences in RSV NS1 viral load were observed between any groups. In *ex vivo* RSV-induced PMBC culture, cytokine production in the breastfed group did not significantly differ from the groups receiving the formulas containing 2'-FL; however, TNF-α and IFN-γ were significantly lower, and a non-significant trend towards reduced IL-1ra and IL-6 was observed, in the breastfed group compared to the control formula. The study authors concluded that infants provided 2'-FL fortified formulas exhibited lower plasma and *ex vivo* inflammatory cytokine profiles, similar to those of a breastfed reference group. In contrast, such effects were not observed among infants administered the control formula containing GOS only.

Table 1.6.1-1 Overview of Clinical Trials Conducted with 2'-FL and LNnT

References	Study Groups / Test Article / Dose	Study Population, Duration of intervention and Study Completion
References (Puccio *et al.*, 2017) (Alliet *et al.*, 2016) ***Clinical trial number*** NCT01715246	***Control Formula*** Intact protein whey based infant formula (670 kcal/L) with LC-PUFA ***Test Formula*** Same as control, plus **2'-FL** (1.0-1.2 g/L) and **LNnT** (0.5-0.6 g/L) ***Reference group*** Breast fed infants enrolled at 3 months of age	***Study population*** 175 healthy full-term singleton **infants** (87-88 per group), 0-14 days of age at enrolment ***Duration of intervention*** 6 months, primary outcome assessment at 4 months **Study Completion at 4 months** 77% Control Formula, 73% Test Formula (no statistical difference)
References (Marriage *et al.*, 2015) (Goehring *et al.*, 2016) ***Clinical trial number*** NCT01808105	***Control Formula*** Lower Calorie Formula (643 kcal/L) with 2.4 g/L GOS ***Test Formula 1*** Same as control, plus 2.2 g/L **GOS** and 0.2 g/L **2'-FL** ***Test Formula 2*** Same as control, plus 1.4 g/L **GOS**, plus 1.0 g/L **2'-FL** ***Reference group*** Breast fed infants	***Study population*** 338 healthy full-term singleton **infants** (101-109 per group), day of life 5 at enrolment ***Duration of intervention*** 5 to 119 days of age **Study Completion at 4 months** 78% Control Formula, 67% Test Formula 1, 66% Test Formula 2, 78% Breast-fed
References (Kajzer *et al.*, 2016) ***Clinical trial number*** NCT01808105	***Control Formula*** Milk-based infant formula (643 kcal/L), no oligosaccharides ***Test Formula*** Same as control, plus 2 g/L **scFOS** and 0.2 g/L **2'-FL** ***Reference group*** Breast fed infants	***Study population*** 131 healthy full-term singleton **infants** (42-46 per group), 0-8 days of age at enrolment ***Duration of intervention*** Until 35 days of age **Study Completion until day 35** 86% Control Formula, 89% Test Formula, 98% Breast-fed
References (Prieto, 2005) ***Clinical trial number*** n/a	***Control Formula*** Intact protein formula (733 kcal/L) without LNnT ***Test Formula*** Same as control, plus 0.2 g/L **LNnT**	***Study population*** 228 infants and young **children** (113-115 per group), aged 6-24 months ***Duration of intervention*** 16 weeks **Study Completion at 4 months** 100% in both groups
References (Elison *et al.*, 2016) ***Clinical trial number*** NCT01927900	***Control*** Glucose ***Test Articles*** (1) 20 g 2'-FL, (2) 10 g 2'-FL, (3) 5 g 2'-FL, (4) 20 g LNnT, (5) 10 g LNnT, (6) 5 g LNnT, (7) 20 g 2'-FL/LNnT 2:1, (8) 10 g 2'-FL/LNnT 2:1, (9) 5 g 2'-FL/LNnT 2:1	***Study population*** 100 healthy **adults** ***Duration of intervention*** 14 days **Study Completion** 100% in all groups

2'-FL = 2'-*O*-fucosyllactose; LC-PUFA = long-chain polyunsaturated fatty acid; LNnT = lacto-*N*-neotetraose.

1.5.2 Selective Shift of Microbiota Composition and Bifidogenic Effects

HMOs including 2'-FL and LNnT contain glycosidic linkages that are resistant to hydrolysis by human digestive enzymes (Brand-Miller *et al.*, 1995, 1998; Engfer *et al*., 2000; Gnoth *et al*., 2000). Therefore, they are expected to largely escape digestion in the upper gastrointestinal tract and reach the colon intact, where they may serve as growth substrates for the commensal microflora present. It is well recognized that HMOs can facilitate the selective proliferation of beneficial gut bacteria; accordingly, they are often referred to in the literature as having "prebiotic" properties. It is generally understood by the industry and the scientific community to mean: "*a substrate that is selectively utilized by host microorganisms conferring a health benefit*" (Gibson *et al.*, 2017). Although the term "prebiotic" is not currently defined in the Australia New Zealand Food Standards Code, FSANZ has recognized that HMOs exhibit prebiotic properties. For example, in FSANZ's Proposal to amend the Code, which included provisions to allow for the addition of inulin-derived substances and GOS to infant formula products, infant foods, and formulated supplementary foods for young children 306 (Proposal P306; FSANZ, 2008), it is stated that: "Human breast milk oligosaccharides are recognised prebiotics, because of their resistance to digestion by host enzymes

and their ability to selectively promote the growth of Bifidobacterium and Lactobacillus in the colon (Coppa et al., 2004a)".

Recently, the effects of 2'-FL and LNnT supplementation on the microbiota composition of formula-fed infants compared to a breastmilk fed control were described by Alliet *et al.* (Alliet *et al.*, 2016). The study authors reported:

- Calculation of microbial alpha diversity and comparison of the global microbiota composition using random permutations of the redundancy analysis (RDA) confirmed that Test (2'-FL and LNnT) was different from Control at genus level ($p<0.001$) and closer to the breastfed group.
- By statistical analyses Alliet *et al.* identified several taxa differentially present in Control and Test. These were 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. Clinically relevant pathogens were very rarely found; however, Clostridium difficile toxin A/B was detected in 14% of Test and 26% Control (OR 0.47, CI 0.17–1.27, $p=0.15$).

The stool microbiota shows that the addition of 2'-FL and LNnT to a starter infant formula shift the stool microbiota towards that observed in breastfed infants, both in composition and function.

1.5.3 Effects on Metabolic Profiles

Further to the bifidogenic effects reported above, Alliet *et al.* (Alliet *et al.*, 2016) also observed that:

- Multivariate stool metabolite analysis identified influential metabolites that discriminate between Test (2'-FL and LNnT), Control and breastfed group. These were the amino acids phenylalanine, isoleucine, tyrosine, fecal organic acids and fucosylated compounds.

- Globally, the metabolic signatures observed in Test (2'-FL and LNnT) were more similar to those observed in stool of breastfed infants than Control. The observed profiles may indicate reduced protein fermentation in Test compared to Control.

Together the stool microbiota and metabolic signature show that the addition of 2'-FL and LNnT to a starter infant formula shift the stool microbiota towards that observed in breastfed infants, both in composition and function.

Goehring *et al*. (Goehring *et al.*, 2016) report:

- Breastfed infants and infants fed either formula with 2'-FL were similar and had lower plasma inflammatory cytokines than infants fed the control formula.

- Cytokine secretion by PBMC from breastfed infants and infants fed either 2'-FL-containing formula that were stimulated *ex vivo* with respiratory syncytial virus was similar and secreted less tumour necrosis factor-□ and interferon-□ and tended to have lower IL-1Ra, IL-6, and IL-1□ than cells from infants fed the control formula.

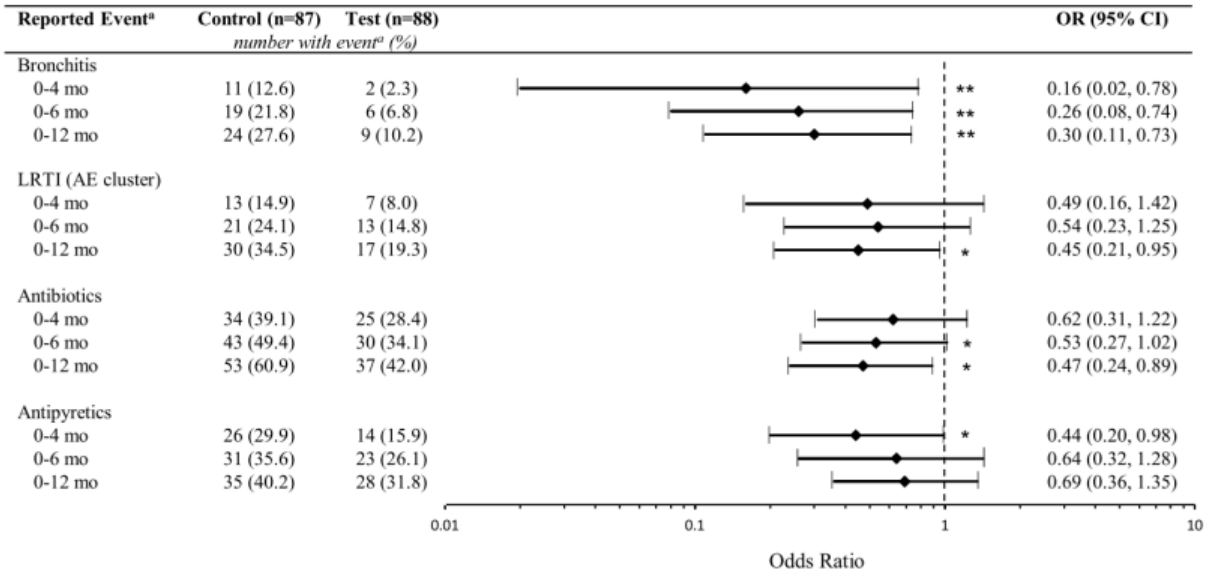
1.5.4 Anti-infective effects

Puccio *et al*. (Puccio *et al.*, 2017) report:

- The overall percentage of infants who had at least one reported adverse event (AE) was similar in both groups during the 4-month exclusive feeding period (Test: 84.1%; Control 90.8%). At 4 months, at least one serious AE was reported in 6 infants (6.8%) in the Test group and 10 infants (11.5%) in the Control group. The incidence of AEs are presented in Figure 1.6.4-1 below.
- the percentage of infants who reported at least one AE in the SOC category of 'Infections and Infestations' from 0-12 months was numerically lower in the Test group, a difference that approached statistical significance (69.3% vs. 82.8%, OR 0.47, 95% CI 0.21-1.02, P=0.051)
- Regarding the a priori-identified AEs of interest within the "Infections and Infestations" SOC category, as shown in Figure 1.6.5-1, infants receiving Test had significantly fewer reports of bronchitis through 4, 6 and 12 months, and the AE cluster of lower respiratory tract infection through 12 months
- 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 (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). Well-documented aberrations in the early stages of gut colonization in caesarean-born infants may make this group of infants particularly sensitive to nutritional interventions (e.g., HMOs) that are aimed at supporting the development of a healthy gut microbiota and a healthy immune system in early infancy.
- Fewer reports of the AE cluster of otitis/ear infection were observed in the Test group through 12 months, but the difference between groups did not reach statistical significance (6.8% vs. 12.6%, P=0.213).
- Infants receiving Test had significantly fewer reports of the use of antipyretics through 4 months, however, this difference was not statistically significant at 6 or 12 months.
- Infants receiving Test also had significantly fewer reports of antibiotic use through 6 and 12 months.
- It is noteworthy that most of the morbidity-related differences between the Test and Control groups persisted through 12 months, which was beyond the first 6 months of age when HMOs were consumed. The potential immune-modulation of HMOs may be long-lasting;

early exposure to HMOs may program the immune system in a way that lowers the later risk of infections within the respiratory tract.

Figure 1.6.4-1 Incidence (and Odds ratio, 95% Confidence Interval) of Parent-Reported Adverse Events in Control and Test Groups Receiving 1.0 to 1.2 g/L 2'-FL and 0.5 to 0.6 g/L LNnT



1.5.5 Other Reported Beneficial Effects

Puccio *et al*. (Puccio *et al.*, 2017) report:

- comparison of mean ± SE stool consistency ratings showed that the Test group had a tendency towards softer stools at 1 month (5.9 ± 0.2 vs. 5.5 ± 0.2, P=0.064) and had significantly softer stools at 2 months (6.1 ± 0.1 vs. 5.7 ± 0.2, P=0.021). Although these differences did not persist at later time points, the findings highlight the beneficial effect of HMOs on stool characteristics during the first few months of life when the neonatal GI tract undergoes profound growth and functional maturation.
- among the subgroup of infants delivered by caesarean section, colic at 4 months was reported less frequently in the Test group (P=0.035). Specifically, among caesarean-born infants, colic was reported by caregivers as "never" and "sometimes" in 96% and 4%, respectively, in the Test group compared to 74% and 26% in the Control group.
- at 2 months, night-time awakenings were reported less frequently in the Test group (P=0.036), with parents reporting "never," "sometimes" and "often" in 27%, 69% and 4% of infants in the Test group, compared to 25%, 57% and 18% in the Control group

1.5.6 Dose Justification for Combination Of 2'-FL And LNnT

2'-FL and LNnT are proposed for addition to infant formula at levels of up to 1.2 g/L for 2'-FL and up to 0.6 g/L for LNnT. These levels are clearly within the natural *ranges* of 2'-FL and LNnT as reported

for mature human breast milk (see Section 3.2.4 and 3.2.5), resulting in a formula that is representative of the nutritive composition of human breast milk.

At the same time, while clearly within the natural *range* of LNnT, the proposed level of LNnT has been set slightly above the *average* found in pooled breast milk. This renders the mixture of 2'-FL and LNnT to be closer in composition to Secretor milk with the advantages described in Section 3.4 and as tested in the clinical trial described in Section 3.6. Furthermore, its use level is supported by the results of the infant study in which LNnT is administered at concentrations of 0.5 to 0.6 g/L in infant formula (Puccio *et al.*, 2017).

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