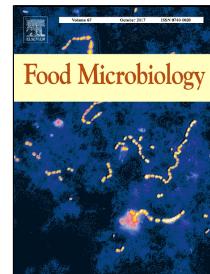


Accepted Manuscript

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PII: S0740-0020(17)30219-8

DOI: 10.1016/j.fm.2017.09.001

Reference: YFMIC 2857

To appear in: *Food Microbiology*

Received Date: 10 March 2017

Revised Date: 30 August 2017

Accepted Date: 02 September 2017

Please cite this article as: Pamela Thomson, Daniel A. Medina, Daniel Garrido, Human Milk Oligosaccharides and Infant Gut Bifidobacteria: Molecular Strategies for their Utilization, *Food Microbiology* (2017), doi: 10.1016/j.fm.2017.09.001

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Milk Oligosaccharides and Infant Gut Bifidobacteria: Molecular Strategies for Utilization

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Highlights

- This review provides recent updates regarding the beneficial role of human milk oligosaccharides on the infant gut microbiome, especially on *Bifidobacterium* species
- We first revised the different structures of HMO, to later discuss their influence on the gut microbiome.
- We provide an updated view on the molecular mechanisms devised by *Bifidobacterium* species, including recent genomic and transcriptomic studies.
- Finally, a current view regarding attempts to replicate the effect of HMO on the gut microbiome is provided, such as adding synthetic HMO or bovine milk oligosaccharides to infant formula.

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25 **Abstract**

26 Breast milk is the gold standard in infant nutrition. In addition to provide essential nutrients for the
27 newborn, it contains multiple bioactive molecules that provide protection and stimulate proper
28 development. Human milk oligosaccharides (HMO) are complex carbohydrates abundant in breast milk.
29 Intriguingly, these molecules do not provide energy to the infant. Instead, these oligosaccharides are key
30 to guide and support the assembly of a healthy gut microbiome in the infant, dominated by beneficial gut
31 microbes such as *Bifidobacterium*. New analytical methods for glycan analysis, and next-generation
32 sequencing of microbial communities, have been instrumental in advancing our understanding of the
33 positive role of breast milk oligosaccharides on the gut microbiome, and the genomics and molecular
34 strategies of *Bifidobacterium* to utilize these oligosaccharides. Moreover, novel approaches to simulate
35 the impact of HMO on the gut microbiome have been described and successfully validated, including the
36 incorporation of synthetic HMO and bovine milk oligosaccharides to infant formula. This review
37 discusses recent advances regarding the influence of HMO in promoting a healthy gut microbiome, with
38 emphasis in the molecular basis of the enrichment in beneficial *Bifidobacterium*, and novel approaches to
39 replicate the effect of HMO using synthetic or bovine oligosaccharides.

40

41 **Keywords:** Human milk oligosaccharides, infant gut microbiome, *Bifidobacterium*, glycosyl hydrolases.

42

43 **Abbreviations:** HMO: human milk oligosaccharides; Glc: glucose; Gal: galactose; GlcNAc: N-
44 acetylglucosamine; Fuc: fucose; NeuAc: *N*-acetylneuraminic acid; FL: fucosyllactose; SL: sialyllactose;
45 LNB: lacto-*N*-biose; LacNAc: *N*-acetyllactosamine; SBP: solute binding protein; GH: glycosyl hydrolase;
46 LNT: lacto-*N*-tetraose; LNnT: lacto-*N*-neotetraose; BMO: bovine milk oligosaccharides.

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51 **1. Introduction**

52 Breast milk is regarded as the natural and optimal way to nourish the newborn. Its composition
53 has been thought to be shaped through evolution exclusively for this purpose. Breast milk is a complex
54 food, rich in lactose, fatty acids and proteins, which directly provide energy to the infant.

55 According to the WHO, exclusive breastfeeding is recommended for six months, and certain
56 health benefits could be observed for infants breastfed up to a year (Gartner et al., 2005; Kramer and
57 Kakuma, 2012). Among other effects, breast-fed infants appear to be more protected against infections
58 and certain immune diseases such as asthma (Shamir, 2016). This protection could last beyond this
59 period, where evidence has suggested that breast-fed infants have a lower risk for developing obesity and
60 certain inflammatory diseases (Horta et al., 2015; Le Huerou-Luron et al., 2010).

61 In addition to provide key nutrients that are essential for proper growth and development, human
62 breast milk contains a wide array of bioactive molecules (Hennet and Borsig, 2016). They include
63 bioactive peptides, lactoferrin, glycolytic enzymes, antibodies and cytokines, nucleotides, fatty acids,
64 among others (Hennet and Borsig, 2016). These non-essential elements can for example modulate
65 inflammatory responses, promote proper development of the immune and gastrointestinal physiology, and
66 stimulate visual and brain development. These molecules are complex and their incorporation in infant
67 formula is difficult (Cicero et al., 2016; Hill and Newburg, 2015). Most of them are also absent in bovine
68 milk.

69 Evidence in the last years has supported a critical role for milk bioactives, especially human milk
70 oligosaccharides (HMO), in shaping the infant gut microbiome (Lemas et al., 2016). HMO are complex
71 carbohydrates present at large quantities in breast milk (10-15 g/L ; (Petherick, 2010; Smilowitz et al.,
72 2013)). They are not accessible for intestinal enzymes, which do not have the specificity to cleave
73 linkages in HMO. Therefore, these molecules reach the infant colon and serve as substrate for certain gut
74 microbes. In addition, HMO could play other physiological roles including pathogen deflection,
75 preventing their binding to the intestinal epithelium (Martín-Sosa et al., 2002; Morrow et al., 2010;
76 Weichert et al., 2016; Zhang et al., 2013). Breast-fed infants are characterized by a gut microbiome rich in

77 *Bifidobacterium* species, a genus well known for its safety, also regarded as beneficial. Indeed,
78 bifidobacteria are the target for several dietary interventions using prebiotics such as inulin, and fructo
79 and galactooligosaccharides, probably due to their ability to prevent pathogen colonization and regulate
80 host responses (Ruiz et al., 2016).

81 Advances in sequencing technologies and analytical analysis of milk glycans have expanded our
82 understanding of the genomic and molecular adaptations of beneficial *Bifidobacterium* to HMO.
83 Considering the interest of incorporating these molecules to infant formula, chemical synthesis and
84 recovery of bovine milk oligosaccharides from dairy streams are two successful strategies that have been
85 tested recently. These studies have shown that these HMO alternatives are safe, and modulate the infant
86 gut microbiota similar to HMO.

87 This review presents recent advances regarding the influence of human milk oligosaccharides on
88 the infant gut microbiome and strategies devised for replicating this effect, and molecular and genomic
89 adaptations of bifidobacteria to utilize HMO.

90

91 **2. Structural diversity of HMO**

92 Human milk provides in a single food all the nutrients necessary for the growth of the infant and
93 its composition reflects all the nutritional and physiological demands of the newborn. It contains more
94 than 100 different compounds; which tend to vary between mothers, and also during the breastfeeding
95 period in term and preterm infants (Smilowitz et al., 2013, Sundekilde et al., 2016). Lactose, fatty acids
96 and proteins are the most abundant macronutrients of human milk, which are absorbed and metabolized
97 mostly in the small intestine (Ballard and Morrow, 2013).

98 Human milk contains a complex mixture of HMO, which are delivered to the infant at high
99 concentrations (10-15 g/L). This value is even higher than milk proteins (7-10 g/L; (Petherick, 2010;
100 Smilowitz et al., 2013)). Since HMO are resistant to intestinal enzymes (Engfer et al., 2000), the energy
101 invested in synthetizing these molecules is not directly assimilated by the newborn, suggesting their
102 bioactive role is very important for the infant.

103 HMO chain length varies from 3 to 15 carbohydrate units, being composed of five
104 monosaccharides: glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), and *N*-
105 acetylneuraminic acid (NeuAc) or sialic acid. HMO are synthesized from a lactose core (Gal β 1-4Glc) by
106 glycosyl transferases in the lactocyte. Among several, less than 50 HMO have a representative abundance
107 in breast milk (Kunz et al., 2000; Wu et al., 2011; Wu et al., 2010). Several representative HMO
108 structures abundant in breast milk are shown in Figure 1.

109 Some HMO are decorated with a fucose or sialic acid residue attached to the lactose core,
110 rendering molecules such as 2' or 3'-fucosyllactose (FL), or 3' or 6'-sialyllactose (SL) (Figure 1). In other
111 HMO, the lactose core is coupled to repeats of lacto-*N*-biose (Gal β 1-3GlcNAc; LNB), also defined as
112 type 1 chains. This renders lacto-*N*-tetraose (LNT), the most abundant HMO in milk (Urashima et al.,
113 2012). Type 2 chains in HMO are formed when an *N*-acetyllactosamine unit (LacNAc; Gal β 1-4GlcNAc)
114 is attached to lactose, rendering lacto-*N*-neotetraose (LNnT). Type 2 chain HMO are less abundant than
115 type 1 (Urashima et al., 2012). These two types of building blocks could be further elongated forming
116 hexaooses, octaooses and larger HMO, and decorated by fucosyl and sialyl residues in α -linkages. These
117 modifications significantly increase the number of HMO structures (Wu et al., 2011; Wu et al., 2010).

118 HMO are defined as acidic if they contain NeuAc in their structures. Neutral HMO could be
119 either fucosylated or non-fucosylated. 50% of HMO structures are fucosylated, and up to 14% could be
120 sialylated (Totten et al., 2012a).

121 It has been reported that the total HMO concentration tends to decrease across lactation (Andreas
122 et al., 2015). One factor that varies between mothers and that has been shown to influence the gut
123 microbiome is the secretor status (Lewis et al., 2015). Secretors are individuals who secrete ABH antigens
124 in their bodily fluids, and are characterized by glycoconjugates containing the Fuc α 1-2Gal determinant
125 (Varki, 2009). The α 1-2 fucosyltransferase responsible for this linkage (FUT2) is encoded by the secretor
126 locus; this genotype can be observed phenotypically by analyzing the HMO composition of milk (Lewis
127 et al., 2015). Another fucosyl transferase (FUT3) is associated to the Lewis (Le) gene and

128 glycoconjugates in these individuals containing the Fucα1-3Gal determinant. Allelic variation of these
 129 genes results in milks with both types of fucosylated HMO (Se+Le+), with no fucosyl residues in HMO
 130 or glycoconjugates (Se-Le-), or its combinations (Totten et al., 2012b). These differences in fucose
 131 content of HMO in breast milk have been associated to certain health outcomes (Lewis et al., 2015).

132 A recent study observed that secretor milk contains higher concentrations of total and fucosylated
 133 HMO than non-secretor milk (Xu et al., 2017), including 2'-fucosyllactose (2FL), lacto-*N*-fucopentaose I,
 134 lactodifucotetraose, and difucosyllacto-*N*-hexaose. In contrast, secretor milk contains lower relative
 135 concentrations of undecorated and sialylated HMO (Totten et al., 2012a).

136

137 **3. HMO as prebiotics and modulators of the infant gut microbiome**

138 The first year of the baby's life is critical for the establishment of the intestinal microbiome. The
 139 gut microbiome represents a complex community composed of a large number of microorganisms, similar
 140 to the number of cells of the human body, also present at high cell densities (Sender et al., 2016). Not
 141 unexpectedly, the microbiome plays roles in human health and disease (Sekirov et al., 2010). The
 142 influence of the gut microbiome is well exemplified on our metabolism (Lewis et al., 2017). Gut microbes
 143 synthesize important vitamins and other molecules that serve nutritional roles for the host. Saccharolytic
 144 microbial fermentation in the gut results in the production of short-chain fatty acids (acetate, propionate
 145 and butyrate), which reduce luminal pH therefore helping in preventing colonization of acid-sensitive
 146 enteropathogens (Fukuda et al., 2011b). Acetate production also contributes, through bacterial cross-
 147 feeding, to the production of butyrate. This SCFA is a primary substrate for colonocytes thus contributing
 148 to epithelial integrity (Roberfroid et al., 2010). These acids are used as energy source by the intestinal
 149 epithelium and modulate physiological responses in the host (Morrison and Preston, 2016).

150 The infant diet is one of the most important factors that shape the gut microbiome in the first year
 151 of life (Tamburini et al., 2016). Breast milk supports a healthy infant gut microbiome often dominated by
 152 *Bifidobacterium* species. These bacteria can represent up to the 90% of the total microbiome (Yatsunenko
 153 et al., 2012). This effect has been explained in part by the high amounts of HMO in breast milk. These

154 molecules arrive intact in the infant colon and stimulate the growth of beneficial microorganisms such as
155 *Bifidobacterium*. (Garrido, Dallas, et al., 2013).

156 Bifidobacteria are Gram positive, heterofermentative, strict anaerobes rods; with a saccharolytic
157 metabolism (Sela, 2010). *Bifidobacterium* species are among the first bacteria to colonize the human
158 gastrointestinal tract (Bertelsen et al., 2016; Fukuda et al., 2011a). Certain species appear to be more
159 commonly found in the infant gut, such as *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) and *B.*
160 *breve*, among others (Lewis et al., 2015; Matsuki et al., 2016; Yatsunenko et al., 2012). Other species are
161 present both in the infant and adult gut microbiome, such as *B. bifidum*, *B. pseudocatenulatum* and
162 *Bifidobacterium longum* subsp. *longum* (*B. longum*) (Avershina et al., 2013; Turroni et al., 2012).

163 *Bifidobacterium* genomes are known to contain a large set of genes for carbohydrate utilization
164 (Khoroshkin et al., 2016; Milani et al., 2016), indicating a preference for complex carbohydrates.
165 Moreover, some of these genomes display a clear adaptation to the nursing period dominated by HMO
166 (Sela et al., 2008). Several strains of *Bifidobacterium* have been shown to be able to utilize HMOs
167 (Garrido et al., 2016), especially of infant origin (Table 1).

168 Compared to breast-fed infants, exclusively formula-fed infants are characterized by higher gut
169 microbiome diversity, with a significant proportion of bifidobacteria (Bäckhed et al., 2014) and signatures
170 such as increased abundance of *Firmicutes* and *Bacteroides*, *C. difficile*, *B. adolescentis* and certain
171 species of *Proteobacteria* (Azad et al., 2013).

172 Two recent studies have addressed the impact on genetics and HMO on the infant gut
173 microbiome using next-generation sequencing. Secretor status allows the production and delivery of
174 HMO containing 2`fucosyl linkages, such as 2FL and LNFP I (Smilowitz et al., 2013). Conversely,
175 mutations in the corresponding FUT2 gene prevent their synthesis. It has been shown that secretor status
176 correlates with a higher abundance of *Bifidobacterium* species in the gut microbiome of infants receiving
177 these milks (Lewis et al., 2015). In contrast, infants fed non-secretor milk showed a delay in the
178 colonization by these beneficial microorganisms, and more *Clostridium* and *Enterobacteria* in their feces.
179 *Bifidobacterium* species in these two groups were catalogued as *B. breve*, *B. infantis* and *B. longum*, and a

180 higher percentage of bifidobacteria isolated from secretor milk-fed infants was able to grow using 2FL as
181 the sole carbon source (Lewis et al., 2015). This suggested that secretor breast milk is able to select gut
182 microbes with specific glycan foraging capabilities for fucosylated HMO. Moreover, this bacterial
183 selection correlated with lower amounts of residual HMO and larger concentrations of lactate in their
184 feces, indicating a more pronounced bifidobacterial metabolic activity targeting fucosylated HMO. SCFA
185 production by bifidobacteria has been shown to be beneficial for the host (Fukuda et al., 2011a).

186 Another clinical study focused on the fecal microbiome of Japanese breast-fed infants in their
187 first month of life (Matsuki et al., 2016). Their microbiome gradually shifted from a predominance of
188 facultative anaerobes to a *Bifidobacterium*-dominated microbiome. This dominance also correlated with a
189 lower fecal pH and higher production of short-chain fatty acids, similar to other studies (Davis et al.,
190 2016). Predominance of *Bifidobacterium* in secretor milk-fed infants was also observed. From this cohort,
191 one set of children who received secretor milk did not show a predominance of bifidobacteria nor a
192 significant fecal HMO consumption. The genotype of the bacteria isolated from these infants resulted to
193 be important to explain the low fecal HMO consumption, lower pH and acid production (Matsuki et al.,
194 2016). In this study, the ability to consume FL across several bifidobacterial isolates was explained by
195 discrete genes encoding ABC transporters, and GH29 and GH95 fucosidases, providing a genetic basis
196 for the FL utilization and predominance of these microorganisms in the infant gut. These molecular
197 strategies will be discussed in the next section.

198

199 **4. Molecular strategies for HMO utilization**

200 The enrichment in *Bifidobacterium* species in the infant gut microbiome has been associated to
201 the wide presence of HMO in breast milk. HMO consumption is mainly associated to *Bifidobacterium*
202 genus, being only found in a few *Bacteroides* and *Lactobacillus* species (Bidart et al., 2014; Marcabal et
203 al., 2010). Advances in genomics, transcriptomics and glycobiology have been useful to study the
204 molecular basis of bifidobacterial enrichment by HMO, contributing to our understanding of the
205 beneficial effects of breast milk on the gut microbiome establishment.

206 While HMO consumption is spread among bifidobacteria, this ability is not characteristic of all
 207 bifidobacterial isolates, and there are certain HMO more utilized by bifidobacteria than others. Table 1
 208 shows a summary of the ability of several infant gut-associated *Bifidobacterium* strains to grow on HMO
 209 purified from breast milk, or individual HMO (Garrido et al., 2015; Garrido, 2016; Ruiz-Moyano et al.,
 210 2013). This table shows that the consumption of HMO is well conserved among *B. infantis* strains,
 211 consuming all types of HMO including fucosylated and sialylated molecules. This phenotype is
 212 somewhat variable in *B. bifidum* strains (Table 1). Among these four species, LNT is well utilized by all
 213 strains tested, similarly to LNNT with the exception of *B. longum* strains. Recent studies have isolated
 214 unique strains of *B. longum* and *B. breve* with a more competitive HMO consumption phenotype targeting
 215 complex fucosylated HMO (Garrido, 2016).

216

217 **4.1 *B. infantis* strategies for HMO consumption**

218 *B. longum* subsp. *infantis* (*B. infantis*) ATCC 15697 is the archetypical HMO-utilizing bacteria
 219 (Ward et al., 2007), vigorously consuming several classes of HMO. This ability is largely conserved
 220 across several isolates of the subspecies (Table 1). In contrast, this phenotype diverges from others *B.*
 221 *longum*, where most *B. longum* strains appear to be better adapted to the adult gut microbiome, dominated
 222 by fiber-derived oligosaccharides (Schell et al., 2002).

223 The genome sequence of *B. infantis* ATCC 15697 shows a singular specialization for HMO
 224 utilization. This was evident from the overabundance of Family 1 Solute Binding Proteins (SBPs), part of
 225 ABC transporters for oligosaccharides (Garrido et al., 2011), and glycosyl hydrolases that appeared to
 226 target host-derived carbohydrates (such as mucin glycans and ABO group oligosaccharides) containing
 227 fucose and sialic acid (Sela et al., 2012). Many of these genes are located in a specific segment of the
 228 genome, the HMO cluster I (Garrido, Dallas, et al., 2013).

229 A mechanistic model for HMO consumption has been described in *B. infantis*, which is based on
 230 the bacterial import of intact HMO inside the cytoplasm, mediated by several SBPs (Figure 2A). Their

231 respective genes are induced several fold in the presence of HMO (Garrido et al., 2015), and the
 232 transporters display affinity for different subsets of HMO (Garrido et al., 2011).

233 The genome sequence of *B. infantis* ATCC 15697 also revealed a large number of glycosyl
 234 hydrolases (GHs) genes, which participate in the enzymatic processing of HMO (Table 2). These
 235 enzymes are located intracellularly, suggesting a consumption strategy that does not release
 236 monosaccharides outside the cell (Figure 2). Table 2 presents a summary of the GHs in *B. infantis* that
 237 participate in HMO utilization. The affinities of these enzymes cover the whole spectrum of HMO
 238 linkages and molecules in breast milk.

239 Activities of these GHs release monosaccharides in the cytoplasm (Figure 2). Proteomic analysis
 240 of HMO consumption based on the *B. infantis* genome indicated that these carbohydrates are assimilated
 241 in central metabolic pathways (termed as “bifid shunt”) (Kim et al., 2013), releasing large quantities of
 242 acetic and lactic acid. These acids are key players modulating intestinal physiology and preventing
 243 pathogen colonization (Fukuda et al., 2011a).

244 Global transcriptomics showed that gene expression of *B. infantis* during growth on HMO
 245 resembles neutral LNT and LNnT, and that the global response mounted to HMO is steady during
 246 bacterial growth (Garrido et al., 2015). RNA-seq transcriptomic analysis shows that HMO-utilization
 247 genes appear to be induced not only during growth in presence of abundant LNT and LNnT, but also on
 248 6SL. In contrast, 2FL and 3FL induced the expression of alternate gene clusters for fucose metabolism
 249 and utilization, different from those in the HMO cluster I (Garrido et al., 2015). These analyses are
 250 important to understand the regulatory networks behind HMO utilization, and to design novel HMO
 251 analogues that trigger similar responses in select beneficial bifidobacteria.

252

253 ***4.2 B. bifidum* strategy**

254 Several isolates of *B. bifidum* are capable to use fucosylated or sialylated HMO (Table 1). A
 255 different HMO consumption mechanistic model has been described in *B. bifidum* (Figure 2B). In contrast
 256 to the import of intact HMO and intracellular degradation showed in *B. infantis*, *B. bifidum* relies on a set

257 of diverse membrane-associated extracellular GHs, with similar enzymatic affinities for HMO compared
258 to *B. infantis* intracellular enzymes (Kitaoka, 2012).

259 Two unique extracellular GHs presents in *B. bifidum* are lacto-*N*-biosidase and endo-*N*-
260 acetylgalactosaminidase. Lacto-*N*-biosidase is an endoglycosidase, able to cleave the tetrasaccharide LNT
261 producing lacto-*N*-biose (LNB) and lactose (Wada et al., 2008). In this model LNB is internalized inside
262 the *B. bifidum* cell by an oligosaccharide binding SBP (Figure 2B). The corresponding gene is located in
263 another important cluster found in most all bifidobacteria, the LNB/GNB cluster (Kitaoka et al., 2005;
264 Nishimoto and Kitaoka, 2007). This cluster also participates in LNT processing, and it is induced several
265 fold in the presence of HMO or LNT in these species (Garrido et al., 2015). Nevertheless, the lacto-*N*-
266 biosidase enzymatic activity is absent in *B. infantis*, which displays a sequential and intracellular
267 degradation of HMO.

268 The global transcriptome response of *B. bifidum* SC555 on pooled or individual HMO has been
269 recently described (Garrido et al., 2015). RNA-seq analysis showed that these responses are similar to
270 mucin, or neutral HMO such as LNT and LNnT. These glycans contain Gal and GlcNAc. Moreover,
271 global transcriptomes of SC555 during growth on 2FL, 3FL or 6SL were mostly identical to lactose.
272 While this strain is endowed with α -fucosidases and α -sialidases, the results suggest that neither fucose or
273 sialic acid are used as a carbon source (Figure 2), and that this strain appears to prefer the lactose moiety
274 (Garrido et al., 2015). This idea is supported by the lack of feeder metabolic pathways for utilization of
275 fucose and sialic acid. It is possible that *B. bifidum* releases fucose and sialic acid during growth to the
276 extracellular media, making these monosaccharides available for other gut microbes and potentially to gut
277 pathogens (Egan, O'Connell Motherway, et al., 2014). This is in contrast to the *B. infantis* model, which
278 appears to be more "selfish". These two different strategies also displayed a high contrast at the global
279 transcriptome level during growth on HMO. When taking into account only orthologous genes present in
280 both species, their gene expression was markedly different in both genomes, remarking their unique
281 responses to HMO.

282

283 **4.3 *B. breve* strategy**

284 Recently, new strategies for HMO utilization have been described for other infant gut microbes.
 285 *B. breve* is a dominant species in the infant gut and it appears to be exclusively found in this environment
 286 (Matsuki et al., 2016). A more accurate picture of the adaptations of this species has been recently
 287 provided. Among 24 fecal infant isolates of *B. breve*, consumption of neutral HMO LNT and LNnT was
 288 well conserved (Table 1). It was found that they all contained a GH95 α -fucosidase, and only a few
 289 isolates a GH29 α -fucosidase (Ruiz-Moyano et al., 2013). Moreover, a GH33 α -sialidase was also found
 290 in these strains. Interestingly, only those who have the GH29 α -fucosidase displayed growth on 2FL, and
 291 larger fucosylated HMOs LNFP I and LNFP III. The gene encoding this α -fucosidase was induced several
 292 fold times in the presence of 2FL, in contrast to the GH95 enzyme (Ruiz-Moyano et al., 2013).

293 Several *B. breve* isolates display significant levels of consumption of sialylated HMO such as
 294 sialyl-LNT, at levels comparable to *B. infantis*. Moreover, mass-spectrometry based analysis revealed that
 295 *B. breve* SC95 has a preference for sialylated over neutral HMO (LNT, LNH and 2FL). A gene cluster for
 296 utilization of sialic acid has been characterized in *B. breve* UCC2003, encoding enzymes that convert this
 297 monosaccharide into fructose-6-P, a putative sialic acid-ABC transporter, and an intracellular α -sialidase
 298 (Egan, O'Connell Motherway, et al., 2014). Using transcriptomics and mutagenesis, it has been described
 299 the ability of *B. breve* UCC2003 to use LNT and LNnT through two different mechanisms (James et al.
 300 2016), which resemble previously described mechanistic evidence on *B. infantis* and *B. bifidum*.

301 Interestingly, *B. breve* UCC2003 is able to utilize extracellular sialic acid released by the activity
 302 of another gut bacterium, *B. bifidum* PRL2010. During growth on sialyl-lactose (SL) or mucin glycans, *B.*
 303 *bifidum* releases to the extracellular environment fucose and sialic acid, which in turn are utilized by *B.*
 304 *breve* UCC2003, a strain that does not use directly mucin glycans or SL (Egan, O'Connell Motherway, et
 305 al., 2014; Egan, O'Connell Motherway, et al., 2014).

306

307 **4.4 *B. longum* strategy**

308 *B. longum* subsp. *longum* is dominant in the infant gut microbiome (Lewis et al., 2015).
 309 Nevertheless, only a few strains have been studied regarding HMO utilization, especially strains obtained
 310 from adult origin (Ward et al., 2007). Previous studies indicated a preference of *B. longum* for plant-
 311 derived oligosaccharides, but these data did not explain the high abundance of this species in the infant
 312 gut. Lately, 17 *B. longum* isolates from infant origin were characterized with respect to their HMO
 313 consumption phenotype (Garrido, 2016). All these strains consumed LNT, but only a few isolates utilized
 314 LNnT and 2FL (Table 1).

315 Total HMO consumption determined by mass spectrometry, allowed the identification of one
 316 isolate, strain *B. longum* SC596, which displayed a novel phenotype not previously reported for this
 317 subspecies. SC596 displayed a clear preference for fucosylated HMO, over other more abundant
 318 molecules such as LNT, which were consumed later during exponential growth (Garrido, 2016). The
 319 genome sequence of *B. longum* SC596 provided clues to this remarkable adaptation to HMOs. A novel
 320 gene cluster devoted to the utilization of fucosylated HMO was found and described in SC596 (Garrido,
 321 2016). This cluster was expressed as a sole transcriptional unit, in a coordinated fashion only in the
 322 presence of 2FL, 3FL, and during early growth on HMO. It contained two α -fucosidases with
 323 complementary enzymatic activities, in addition to fucose metabolism enzymes, an ABC transporter, and
 324 a LacI-type regulator. This gene cluster appeared to be unique to this strain, and it is homologous to
 325 certain segments of the *B. infantis* HMO cluster I.

326 Transcriptomic analysis during exponential growth showed that the global response of *B. longum*
 327 SC596 to HMO changed from the expression of genes for fucosylated HMO metabolism, to expression of
 328 gene clusters for neutral HMO consumption. The molecular strategy of SC596 to utilize HMO resembles
 329 the *B. infantis* model of consumption (Figure 2A). SC596 was endowed with several ABC transporters
 330 and intracellular glycosyl hydrolases. The SBPs associated to these transporters bind LNT and
 331 fucosylated HMO, and the GHs cleaved all linkages found in neutral and fucosylated HMO.

332

333 **5. Novel strategies to replicate the bifidogenic effect of breast milk**

334 Under certain circumstances breastfeeding is not possible, and food alternatives for infant feeding
335 are required. Considering the complexity of HMO, it is not currently possible to supplement formula milk
336 with these molecules. We will discuss three recent approaches that have been studied to replicate the
337 beneficial effects of HMO on the gut microbiome.

338

339 **5.1. Fructo and galactooligosaccharides**

340 Certain non-digestible food ingredients such as fructo-oligosaccharides (FOS) and galacto-
341 oligosaccharides (GOS) have been included in infant formula as prebiotics for decades (Gibson et al.,
342 2004; Torres et al., 2010). They are usually added in a ratio GOS/FOS 9:1, with the goal of endowing
343 formula with bioactives aimed to increase lactobacilli and bifidobacteria. FOS and GOS are linear
344 polymers, much simpler structures compared to HMO. FOS are a mixture of polymers of fructose in β -2-
345 1 linkage with a terminal glucose and a degree of polymerization (DP) of 3 to 6 (Roberfroid et al., 2010).
346 Commercially available FOS are obtained by partial enzymatic hydrolysis of inulin extracted from
347 chicory roots using an endo-inulinase, or by synthesis from sucrose using fructosyl-transferase (Franck
348 2002). GOS are produced enzymatically using transglycosylation reactions, and have a DP of 3-15
349 (Barboza et al., 2009).

350 Several reports indicate that FOS and GOS promote a healthy gut microbiome dominated by
351 *Bifidobacterium* in the infant, resulting in lower fecal pH and larger SCFA production (Davis et al., 2011;
352 Oozeer et al., 2013). In that sense, a potentially useful approach to improve lactose digestion and
353 tolerance is by direct modulation of the colonic bacteria to metabolize lactose effectively. A recent study
354 conducted in human subjects demonstrated that administration of a highly purified GOS, significantly
355 improved clinical outcomes for lactose digestion and tolerance (Azcarate-Peril et al. 2017). Changes in
356 the fecal microbiome demonstrate a bifidogenic response in 90% GOS-consumer subjects. Furthermore,
357 relative abundance of lactose-fermenting *Bifidobacterium*, *Faecalibacterium*, and *Lactobacillus* were
358 significantly increased in response to GOS, suggesting that the microbiome is responsive to GOS
359 utilization (Azcarate-Peril et al. 2017).

360 On the other side, the molecular clues of GOS utilization by *B. thetaiotaomicron*, a prominent
 361 member of the human microbiota, have been described. Several active pathways for targeting O-mucin
 362 glycans as well as the galactan utilization system were induced by GOS (Lammerts van Bueren et al.
 363 2017). The authors identified two distinct mechanisms directed at GOS metabolism by *B.*
 364 *thetaiotaomicron*: firstly, extended linear β -(1 → 4)-linked GOS molecules were degraded via the action
 365 of an extracellular GH53 endo- β -(1 → 4)-galactanase. Secondly, a subset of host glycan metabolism
 366 factors was activated, showing that specific GOS molecules mimic HMOs by expressing enzymes and
 367 transport components implicated in mucin-O-glycan degradation (Lammerts van Bueren et al. 2017).

368

369 **5.2. Synthetic HMO**

370 Large-scale synthesis of HMO is difficult, and it has been attempted by several approaches
 371 including metabolic engineering and chemical synthesis (Petschacher and Nidetzky, 2016). The clinical
 372 safety of synthetic HMO produced by chemical methods, such as LNnT and 2FL, has been tested in
 373 preclinical animal models (Coulet et al., 2014; Coulet et al., 2013), paving their way for the first human
 374 studies with HMO.

375 A recent clinical study looked at the immune response of 200 babies divided into three feeding
 376 groups: exclusively breastfed, formula-fed without synthetic 2FL, and formula-fed with 2FL (Goehring et
 377 al. 2016). After six weeks, results showed that when comparing the breastfed group to the group fed
 378 formula with 2FL, the levels of five immune markers were statistically similar. Conversely, in the group
 379 fed formula without 2FL, the levels of immune markers were different, suggesting that 2FL-formula
 380 intake results in an immune response similar to breast-fed infants (Goehring et al. 2016).

381 Another recent study showed that oral supplementation in healthy adults with 2FL and/or LNnT
 382 modified their gut microbiota. In this study, the primary effect was a substantial increase in relative
 383 abundance of *Actinobacteria* (especially *Bifidobacterium*) and a reduction in relative abundance of
 384 *Firmicutes* and *Proteobacteria*. This study represents a clear indication that supplementing the diet with
 385 HMO is a valuable strategy to shape the human gut microbiota and specifically promote the growth of

386 beneficial bifidobacteria (Elison et al. 2016). Another clinical study evaluated the effect of infant formula
387 supplemented with 2FL or LNnT on growth and morbidity of newborns. This work indicated that the
388 administration of infant formula with 2FL and LNnT was safe, well tolerated and supports age-
389 appropriate growth compared to those fed the control formula (Puccio et al. 2017).

390

391 **5.3 Bovine Milk Oligosaccharides**

392 The content of oligosaccharides in other mammal milks is much smaller compared to human
393 milk. However, these small amounts could still be attractive if obtained from industrial byproducts.
394 Bovine milk could contain 20 times less oligosaccharides compared to human milk, and they are majorly
395 sialylated (Albrecht et al., 2014; Sundekilde et al., 2012; Tao et al., 2008). Dairy streams from cheese
396 production have been used to recover bovine milk oligosaccharides (BMO) in sufficient quantities (Mehra
397 et al., 2014).

398 Certain beneficial effects of BMO in health through the gut microbiome have been recently
399 demonstrated. In some countries, malnutrition puts millions of children at risk of stunting, a severe
400 disease characterized by impaired growth, defects on cognitive capacity and weakened immune system
401 (Blanton et al., 2016). A contribution of the gut microbiome on this disease has been demonstrated, where
402 the composition of the microbiome of stunted children has been referred as “immature”. Gut microbes
403 influence the body growth of these infants, and replicating these observations in animal models indicate
404 that specific key species, such as *Ruminococcus gnavus* and *Clostridium symbiosum*, might correct and
405 restore body growth of stunted children (Blanton et al., 2016). Therefore, a connection between breast
406 milk composition and the gut microbiome of stunted children has been established (Charbonneau et al.,
407 2016). Sialic acid-containing HMO were shown to be less abundant in the breast milk fed to stunted
408 infants, and this was hypothesized to contribute in part to this disease. Charbonneau and collaborators
409 used a preclinical model in mice and sialylated BMO (sBMO) isolated from whey streams, hypothesizing
410 that these oligosaccharides might correct some of the defects in the microbiome of these infants and
411 restore health. Germ-free mice were colonized with the microbiome of a healthy or a severely stunted

412 infant and these mice replicated the stunting phenotype of the children, displaying several characteristics
413 of malnutrition. When supplementing their diet with sBMO, the animals displayed a dramatic systemic
414 recovery in their bones and muscles, and in their liver and brain activity (Charbonneau et al., 2016).

415 Among several gut species from the microbiome of these infants, it was found that only a small
416 number of these bacteria were responsive to sBMO. These commensal species, *Bacteroides fragilis* and
417 *Escherichia coli*, consumed these substrates via cross-feeding of sialic acid. Unexpectedly, when germ-
418 free mice were inoculated with these two species and fed sBMO, growth enhancement was not
419 appreciated. This indicates that other gut microbes or host factors are required to replicate this effect, and
420 highlights the complexity of gut microbiome assembly and interactions.

421

422 **5.4 Other Bovine Milk Glycoconjugates**

423 Certain members of the gut microbiome possess the ability to hydrolyze glycolipids (Larson and
424 Midtvedt, 1989), and bovine milk is rich in these glycoconjugates. Recently, the ability of representative
425 bifidobacteria to utilize gangliosides purified from breast milk was determined *in vitro*, using high-
426 resolution mass spectrometry (Lee et al., 2014). Major milk glycolipids, GM3 and GD3, were largely
427 consumed during growth of *B. infantis* and *B. bifidum*, supporting the growth of these microorganisms.
428 Interestingly, in both cases the major end product was lactosylceramide, indicating that the sialidase
429 activity of these microorganisms targets milk gangliosides, and that the release of sialic acid allows
430 bacterial growth (Lee et al., 2014).

431 Proteins in breast milk are at a concentration of 10 g/L (Hennet and Borsig, 2016), and they could
432 be classified in insoluble caseins and soluble whey proteins. Oligosaccharides in milk proteins could
433 either be *N*-linked (attached to Asn) or *O*-linked (attached to Ser/Thr). Breast milk contains several
434 glycosylated proteins, including lactoferrin and immunoglobulins (*N*-glycosylated), and mucins κ-casein
435 (*O*-glycosylated) (Froehlich et al., 2010). *N*-linked glycans in breast milk proteins are largely fucosylated,
436 compared to bovine milk glycans which are mostly sialylated. Bovine milk glycans also contain *N*-
437 glycolylneuraminic acid (NeuGc) instead of *N*-acetylneuraminic acid (Nwosu et al., 2012).

438 Early evidence showed the utilization of milk glycoproteins by gut microbes (Hoskins et al.,
439 1985). Several strains of bifidobacteria are endowed with endo- β -N-acetylglucosaminidases (GH18, EC
440 3.2.1.96), enzymes that catalyze the hydrolysis of the N-N'-diacetyl-chitobiose core found in all N-
441 glycans (Garrido, Nwosu, et al., 2012). EndoBI-1 in *B. infantis* releases complex and high-mannose N-
442 glycans from milk glycoproteins such as bovine and human lactoferrin, in addition to IgA and IgG
443 (Garrido, Nwosu, et al., 2012). This dual activity is not common among these enzymes. In addition,
444 EndoBI-1 is thermostable, and deploys full activity in breast milk.

445 This enzyme has been shown to be an attractive tool to release and recover prebiotic N-glycans
446 from dairy glycoproteins from bovine colostrum whey (Karav et al., 2015). This glycan source contains
447 mostly sialylated and fucosylated complex N-glycans, molecules that resemble the structure of HMO.
448 Interestingly, *B. infantis* is able to vigorously consume these extracts as the sole carbon source, but not
449 the deglycosylated milk proteins (Karav et al., 2016). This growth promotion was only observed on *B.*
450 *infantis*, and not for *B. animalis*, a species not adapted to the infant gut.

451

452 **6. Conclusions and future directions**

453 Human milk is the best source of nutrients for the newborn. Exclusive breastfeeding not only
454 promotes proper growth, it also provides a myriad of bioactive mechanisms that protect and stimulates
455 infant development. One of the most remarkable bioactive effects of breast milk is the selection of
456 beneficial microorganisms and a healthy microbiome, which is mostly carried out by HMO.

457 Breastfeeding has been associated with a variety of long-term health impacts. Considering that
458 alterations of the initial assembly of the gut microbiome have also been associated to long-term negative
459 effects, mechanistic studies regarding the influence of breastfeeding, milk glycans and the gut microbiome
460 on infant health are largely necessary.

461 Finally, it is an exciting time where the biological effect of HMO could be replicated, using either
462 synthetic approaches, or recovering similar molecules from dairy byproducts. Foods containing these
463 HMO analogues will certainly revolutionize the market, and most importantly will give the chance to

464 improve infant health worldwide. Current research on the molecular mechanisms of HMO utilization is
465 important in validating the claims on HMO on the microbiome, also being useful in identifying novel
466 isolates highly adapted to the infant gut that could be paired with HMO in symbiotic preparations.
467 Mechanistic research on the beneficial effects of these microbes, in the context of a complex developing
468 gut microbiome, will also expand our knowledge of the benefits of HMO and their role in health.

469

470 **Acknowledgments**

471 We acknowledge all the researchers in the UC Davis Foods for Health Institute, especially Dr.
472 David Mills and his group. We also thank UC Davis Chile Life Innovation Center (Pablo Zamora and
473 Allan Bennet) for their wonderful work and the opportunity to show this research, and the OECD Co-
474 operative Research Programme on Biological Resource Management for Sustainable Agricultural
475 Systems. We appreciate the support of grants Fondecyt de Iniciación [11130518], Fondecyt de
476 Postdoctorado grant [3160525], FONDEF [ID16I10045], SeedFund UC [COL 0316], and Beca
477 Postdoctorado Escuela de Ingenieria UC 2016.

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691 **Table 1:** In-vitro HMO utilization of a panel of infant-gut isolates of *Bifidobacterium*. Individual isolates
 692 were grown on each HMO (pooled or individual) as the sole carbon source. Values indicate the number of
 693 isolates able to grow moderately or vigorously on each substrate. Data were obtained from (Garrido et al.,
 694 2015; Garrido, 2016; Ruiz-Moyano et al., 2013).

Growth (OD600>0.5)	Structure	<i>B.</i> <i>infantis</i>	<i>B.</i> <i>bifidum</i>	<i>B.</i> <i>longum</i>	<i>B. breve</i>
HMO	-	22/22	12/14	8/17	10/23
Lacto- <i>N</i> -tetraose	Gal β 1-3GlcNAc β 1-4Gal β 1-4Glc	22/22	14/14	17/17	23/23
Lacto- <i>N</i> -neotetraose	Gal β 1-4GlcNAc β 1-4Gal β 1-4Glc	22/22	13/14	2/17	23/23
2-fucosyllactose	Fuc α 1-2Gal β 1-4Glc	22/22	13/14	1/17	2/23
3-fucosyllactose	Gal β 1-4Glc α 1-3Fuc	22/22	13/14	1/17	0/23
6-sialyllactose	NeuAc α 2-6Gal β 1-4Glc	21/22	11/14	0/17	0/23
Mucin	-	0/22	10/14	2/17	0/23

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705 **Table 2:** Major glycosyl hydrolases in *B. infantis* targeting HMO.

Enzyme	HMO affinity	Induction of gene expression during growth on HMO
α-sialidase		
Blon_2348	3'SL, 6'SL and sialyl-LNT (Sela et al., 2011).	Yes (Sela et al., 2011)
α-fucosidase		
Blon_2335	2FL, 3FL, lacto- <i>N</i> -fucopentaose I, H-disaccharide	Yes (Sela et al., 2012)
Blon_2336	3FL, lacto- <i>N</i> -fucopentaose III Lewis ^a (Gal β 1-3(Fuc α 1-4)GlcNAc), Lewis ^x (Gal β 1-4(Fuc α 1-3)GlcNAc)	Yes (Sela et al., 2012)
β-galactosidases		
Blon_2016	Type 1 HMO: LNB, LNT, LNH	Constitutive (Garrido, Ruiz-Moyano, et al., 2013; Yoshida et al., 2012)
Blon_2334	Type 2 HMO: LacNAc, LNnT; lactose; galactooligosaccharides	Yes (Garrido, Ruiz-Moyano, et al., 2013; Yoshida et al., 2012)
β-<i>N</i>-acetylhexosaminidases		
Blon_0459	GlcNAc β 1-3Gal (linear); GlcNAc β 1-6Gal (branched).	Yes (Garrido, Ruiz-Moyano, et al., 2012)
Blon_0732	GlcNAc β 1-3Gal	Yes (Garrido, Ruiz-Moyano, et al., 2012)
Blon_2355	GlcNAc β 1-3Gal (linear); GlcNAc β 1-6Gal (branched).	Yes (Garrido, Ruiz-Moyano, et al., 2012)

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707 **LEGEND TO FIGURES**

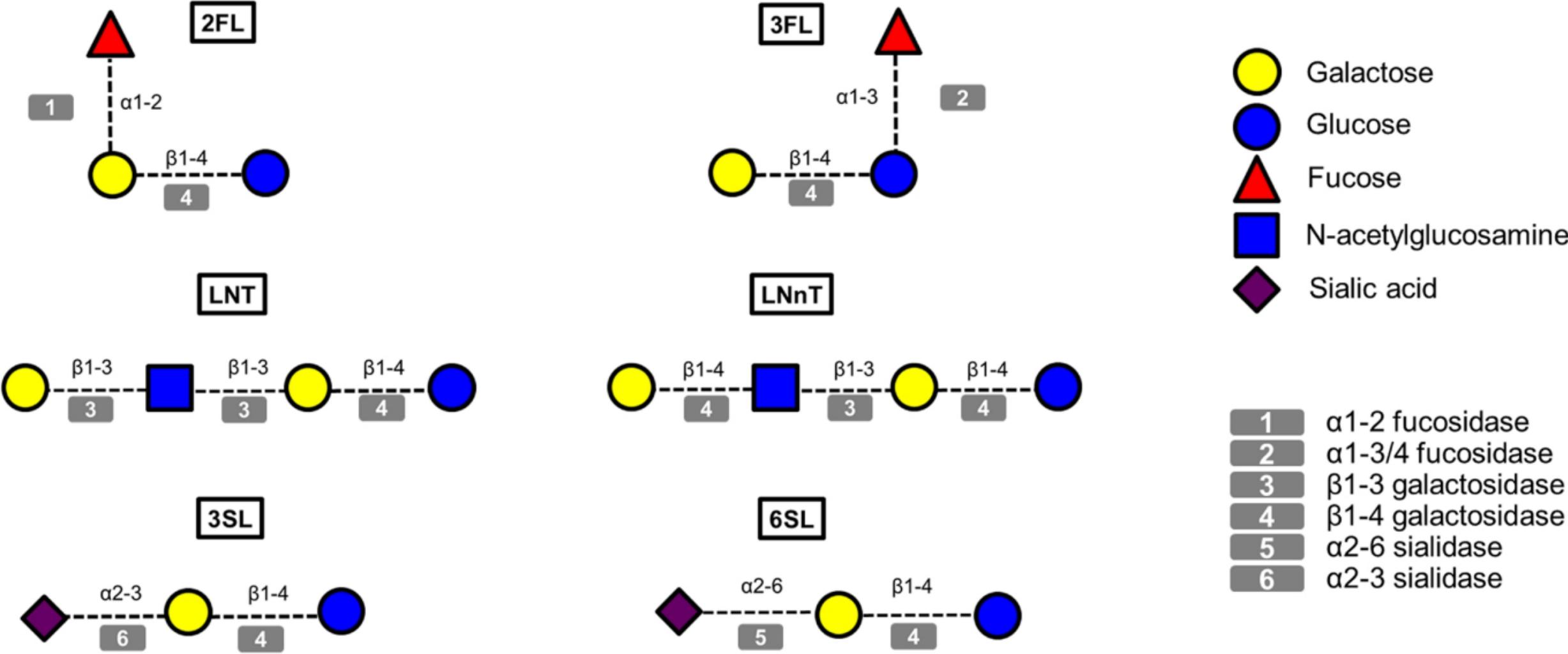
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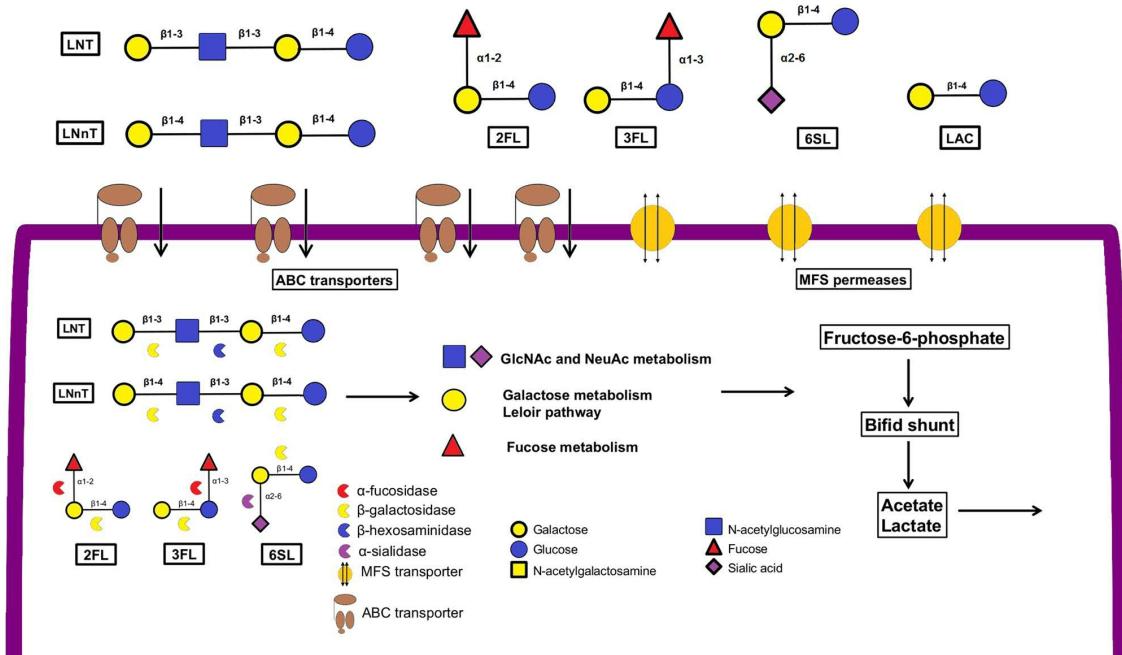
709 **Figure 1:** Representative HMO found in breast milk, and the corresponding bifidobacterial glycosyl
710 hydrolases acting on them. Legend for monosaccharides is in the bottom left, and the respective glycosyl
711 hydrolase activity in the bottom right. 2FL: 2-fucosyllactose; 3FL: 3-fucosyllactose; LNT: lacto-N-
712 tetraose; LNnT: lacto-N-neotetraose; 3SL: 3-sialyllactose; 6SL: 6-sialyllactose.

713 **Figure 2:** Representation of two major molecular strategies in infant-associated *Bifidobacterium* for
714 HMO utilization. A: *B. infantis*, *B. breve* and *B. longum*; B: *B. bifidum*.

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