

# Goat Milk Oligosaccharides: Their Diversity, Quantity, and Functional Properties in Comparison to Human Milk Oligosaccharides

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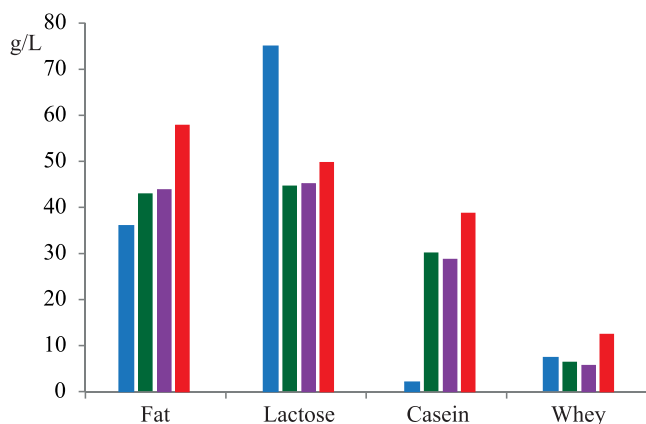
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**ABSTRACT:** Human milk is considered the golden standard in infant nutrition. Free oligosaccharides in human milk provide important health benefits. These oligosaccharides function as prebiotics, immune modulators, and pathogen inhibitors and were found to improve barrier function in the gut. Infant formulas nowadays often contain prebiotics but lack the specific functions of human milk oligosaccharides (hMOS). Milk from domesticated animals also contains milk oligosaccharides but at much lower levels and with less diversity. Goat milk contains significantly more oligosaccharides (gMOS) than bovine (bMOS) or sheep (sMOS) milk and also has a larger diversity of structures. This review summarizes structural studies, revealing a diversity of up to 77 annotated gMOS structures with almost 40 structures fully characterized. Quantitative studies of goat milk oligosaccharides range from 60 to 350 mg/L in mature milk and from 200 to 650 mg/L in colostrum. These levels are clearly lower than in human milk (5–20 g/L) but higher than in other domesticated dairy animals, e.g., bovine (30–60 mg/L) and sheep (20–40 mg/L). Finally, the review focuses on demonstrated and potential functionalities of gMOS. Some studies have shown anti-inflammatory effects of mixtures enriched in gMOS. Goat MOS also display prebiotic potential, particularly in stimulating growth of bifidobacteria preferentially. Although functional studies of gMOS are still limited, several structures are also found in human milk and have known functions as immune modulators and pathogen inhibitors. In conclusion, goat milk constitutes a promising alternative source for milk oligosaccharides, which can be used in infant formula.

**KEYWORDS:** goat milk oligosaccharides, prebiotics, health benefits, structures, quantitative analysis

## INTRODUCTION

The composition of human milk is very different from that of domesticated dairy animals (Figure 1).<sup>1–6</sup> One of the major components, human milk oligosaccharides (hMOS), constitutes a relatively large part of the carbohydrates present in mother's



**Figure 1.** Major milk components (g/L), fat, lactose, casein, and whey, in human (blue), goat (green), cow (purple) and sheep (red) milk, compiled from available literature sources.<sup>1,2,4–6</sup>

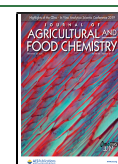
milk (5–20 g/L),<sup>3,7</sup> representing up to 20% of the total carbohydrate content. The diversity of hMOS is large, with 247 varieties observed and 162 hMOS structurally characterized.<sup>8</sup> The composition and abundance of hMOS vary between women with the genetics of the mothers and during lactation,<sup>9–12</sup> but also evidence emerges that gestational age, maternal health status, infant sex, and dietary habits influence hMOS composition.<sup>11,13,14</sup> These hMOS play a role in steering a healthy development of the infant gut microbiome, acting as prebiotics, stimulating the growth of beneficial bacteria. Beneficial bacteria on prebiotics secrete short-chain fatty acid (SCFA) and other metabolites (postbiotics), having a beneficial effect on host health.<sup>15</sup> Although other prebiotics are added to infant formula, such as galactooligosaccharides (GOS) and fructooligosaccharides (FOS), hMOS have well-established and specific functionalities. Oligosaccharides in human milk have been found to inhibit pathogen adhesion, stimulate the

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Scheme 1. Overview of All Neutral gMOS Structures Reported in Goat Colostrum and Milk, with Relative Abundances Reported in Neutral and Acidic Pools, Respectively<sup>a</sup>

RN	Structure	Name (Abbrev.)	Glycotope <sup>3</sup>	hMOS	Int <sup>31</sup> (%)	Refs
2 1 0 0 0		2'-fucosyllactose (2'-FL)	H1	Y	3	24,27,31,37
2 1 0 0 0		3-fucosyllactose (3-FL)	Lex	Y	2	24
3 0 0 0 0		alpha-3'-galactosyllactose (alpha3'-GL)	Galalpha3 Lac	N	10	24,31,37
3 0 0 0 0		3'-galactosyllactose (3'-GL)	Galbeta3 Lac	Y	27	24,31,37
3 0 0 0 0		6'-galactosyllactose (6'-GL)	Galbeta6 Lac	Y	15	24,231,37,44
1 1 1 0 0		3-fucosyllactosamine (3-FLN)	Lex	N	-	24,27,37
2 0 1 0 0		6'-N-acetylglucosaminyllactose (NAL)	GlcNAcbeta6 Lac	N	-	27,31
2 0 1 0 0		Lacto-N-triose II (LNT-II)	GlcNAcbeta3 Lac	Y	4	24,31,37
2 0 1 0 0		alpha-3'-N-acetylgalactosaminyllactose	GalNAcalpha3 Lac	N	<1	24,31,37
4 0 0 0 0	2x	DP4-GOS	Lac	N	<1	24,27,37
3 0 1 0 0		Lacto-N-neotetraose (LNnT)	LN2 Lac	Y	5	24,31,37
3 0 1 0 0		iso-Lacto-N-neotetraose (iLNT)	LN2 Lac	N	2	24,31,33,44
2 0 2 0 0	2x	di-N-acetyl-hexosaminyllactose	Lac	N	-	27,31
5 0 0 0 0	3x	DP5-GOS	Lac	N	-	27,37
3 1 1 0 0		iso-Lacto-N-fucopentaose II (iLNFP II)	Lex Lac	N	-	27
3 1 1 0 0		iso-Lacto-N-fucopentaose V (iLNFP V)	LN2 Lex	N	-	27
4 0 1 0 0		N-acetylglucosaminyldihexosyl-lactose (NADHL)	LNx Lac	N	-	27,31,37
4 0 1 0 0		novo-lacto-N-pentaose (nLNP)	LN2 Lac	N	2	24
3 0 2 0 0		Lacto-N-iso-pentaose (LNiP)	Galbeta3 LN2 GlcNAcbeta3 Lac	N	<1	24,27
6 0 0 0 0	4x	DP6-GOS	Lac	N	-	27
4 0 2 0 0		Lacto-N-neohexaose (LNnH)	LN2 LN2 Lac	Y	6	24,27,31
4 0 2 0 0		-	LN2 LN2 Lac	Y	<1	24
3 0 3 0 0		-	LN2 LN2 Lac	Y	2	24,27
2 0 4 0 0	2x	-	LN2 LN2	N	-	27
4 1 2 0 0	2x	-	Lac	N	-	27
3 1 3 0 0	1x	-	Lac	N	-	27
4 0 3 0 0	2x	-	Lac	N	-	31

● Glc      ▲ Fuc  
● Gal      ■ GalNAc  
■ GlcNAc    □ HexNAc

<sup>a</sup>The hMOS column (Y/N) indicates whether the structure has been observed in human milk. Hypothetical interactions with galectins are indicated, according to Urashima et al.<sup>8</sup> Possible interaction epitopes are marked green; blocked epitopes are marked red; and gray boxes indicate uncertain interactions where exact structures are not known, with relative peak intensities in the neutral and acidic pool, respectively (on the basis of the study by Albrecht et al.<sup>24</sup>).

**Scheme 2. Overview of All Acidic gMOS Structures Reported in Goat Colostrum and Milk, with Relative Abundances Reported in Neutral and Acidic Pools, Respectively<sup>a</sup>**

RN	Structure	Name (Abbrev.)	Glycoform <sup>3</sup>	hMOS	Int <sup>21</sup> (%)	Refs	
2 0 0 1 0		3'-sialyllactose (3'-SL)	Neu5Ac	Lac	Y	13	24,27,31,33,37
2 0 0 1 0		6'-sialyllactose (6'-SL)	Neu5Ac	Lac	Y	18	24,27,31,33,37
2 0 0 0 1		3'-N-glycolyneuraminylactose (3'-NGL)	Neu5Gc	Lac	N	30	24,31,33,37
2 0 0 0 1		6'-N-glycolyneuraminylactose (6'-NGL)	Neu5Gc	Lac	N	27	24,31,33,37
1 0 1 1 0		3'-sialyllactosamine (3'-SLN)	Neu5Ac	LN2	N	<0.1	24,32
1 0 1 1 0		6'-sialyllactosamine (6'-SLN)	Neu5Ac	LN2	N	<1	24,27,33,37
1 0 1 0 1		3'-N-glycolyneuraminylactosamine (3'-NGLN)	Neu5Gc	LN2	N	<1	24,37
1 0 1 0 1		6'-N-glycolyneuraminylactosamine (6'-NGLN)	Neu5Gc	LN2	N	2	24,27,33,37
3 0 0 1 0		3'-Sialyl-6'-GL (3'-S6'-GL)	GlcNAcβ6 Neu5Ac	Lac	N	-	24,27,32,33,37
3 0 0 1 0		6'-Sialyl-3'-GL (6'-S3'-GL)	Neu5Ac Galβ3	Lac	N	-	24,27,32,33,37
3 0 0 1 0		3'-sialyl-3'-GL (3'-S-3'-GL)	Neu5Ac Galβ3	Lac	N	<1	24
3 0 0 0 1		3'-N-glycolyneuraminyl-3'-GL (3'-NG-3'-GL)	Neu5Gc Galβ3	Lac	N	<1	24,27,31,37
3 0 0 0 1		6'-N-glycolyneuraminyl-3'-GL (6'-S3'-GL)	Neu5Gc Galβ3	Lac	N	<1	24,27,31,37
2 0 1 1 0		3'-sialyl-Lacto-N-triose II (3'-SLN T II)	Neu5Ac GlcNAcβ3	Lac	N	<1	24,27,37
2 0 0 2 0		Di-N-acetyneuraminylactose (DSL)	Neu5Ac Neu5Ac	Lac	N	<1	24,27,31,37
2 0 0 1 1		N-glycolyl-N-acetyl-dineuraminylactose (NGSL)	Neu5xx Neu5xx	Lac	N	<1	24,37
2 0 0 0 2		di-N-glycolyneuraminylactose (DNGL)	Neu5Gc Neu5Gc	Lac	N	2	24,31,32
4 0 0 1 0		Sialyl-DP4-GOS		Lac	N	-	31
3 0 1 0 1		N-glycolyneuraminyl-LNnT (NGLNnT)	LN2	Lac	N	<1	24
3 0 0 2 0		di-sialyl-DP3-GOS		Lac	N	-	24,31
3 0 0 1 1		N-acetyl-N-glycolyl-di-neuraminyl-DP3-GOS		Lac	N	-	31
3 0 0 0 2		di-N-glycolyneuraminyl-3'-GL (DN-3'-GL)		Lac	N	<0.1	24,31
4 0 1 0 1		3'-N-glycolyneuraminyl-nLNP (3'-NGnLNP)	Neu5Gc LN2 Galβ3	Lac	N	<0.1	24
4 0 1 0 1		6'-N-glycolyneuraminyl-nLNP (6'-NGnLNP)	Neu5Gc LN2 Galβ3	Lac	N	<0.1	24
4 0 2 1 0		sialyl-Lacto-N-neohexaose (S-LNnH)	Neu5Ac LN2 LN2	Lac	Y	-	24,31
4 0 2 0 1		3'-N-glycolyneuraminyl-LNnH (3'-NG-LNnH)	Neu5Gc LN2 LN2	Lac	N	<0.1	24
4 0 2 0 1		6'-N-glycolyneuraminyl-LNnH (6'-NG-LNnH)	Neu5Gc LN2 LN2	Lac	N	<0.1	24

● Glc      ◆ Neu5Ac      ■ GlcNAc  
● Gal      ◇ Neu5Gc

<sup>a</sup>The hMOS column (Y/N) indicates whether the structure has been observed in human milk. Hypothetical interactions with galectins are indicated, according to Urashima et al.<sup>8</sup> Possible interaction epitopes are marked green; blocked epitopes are marked red; and gray boxes indicate uncertain interactions where exact structures are not known, with relative peak intensities in the neutral and acidic pool, respectively (on the basis of the study by Albrecht et al.<sup>24</sup>).

immune system of infants, modulate host receptors, and modify epithelial glycosylation as well as stimulating brain development.<sup>16,17</sup> Some of these functions have also been observed for GOS in a few studies, particularly stimulating gut barrier function, but have been less well-established.<sup>18–21</sup>

Bovine milk is traditionally the base for infant formula, as a substitute for mother's milk when breastfeeding is not possible or mothers choose not to breastfeed. There are fewer studies of bovine milk oligosaccharides (bMOS) than of hMOS, but the overall view is that bMOS concentrations are much lower (30–60 mg/L) compared to hMOS (5–20 g/L).<sup>3,22</sup> Moreover, the bMOS composition with 40–50 varieties observed and 37 structures identified is much less varied<sup>23,24</sup> than the hMOS composition (~247 varieties with 162 structures identified).<sup>8</sup> Goat milk is an approved protein source for infant formula and has attracted attention as a result of higher levels and diversity in milk oligosaccharides, with average concentrations reported of 60–350 mg/L in mature milk and up to 2.4 g/L in colostrum.<sup>25–30</sup> The oligosaccharide composition of goat milk (gMOS) has been studied<sup>24,27–29,31–35</sup> and compared to other domesticated animals.<sup>24,29</sup> A few studies have reported variations of oligosaccharides at the goat breed level,<sup>25,26</sup> over time during lactation,<sup>25,28,36</sup> in parity,<sup>26</sup> or at the individual goat level.<sup>37</sup> Such information is considered interesting, because gMOS are more abundant than bMOS and contain certain structures that are in common with hMOS (Schemes 1 and 2). Therefore, goat milk has potential to display special functional properties that may find application in a range of commercial products in various niches.

In this literature overview, we will evaluate the current knowledge of gMOS structures and quantities, also in relation to what is known about hMOS and bMOS. An overview will be presented on functional properties of MOS in general as well as specific structure–function relationships that have been discovered thus far for hMOS, bMOS, and gMOS.

## ■ GOAT MILK OLIGOSACCHARIDE (GMOS) STRUCTURES

The first gMOS were separated and purified from Japanese Saanen goat colostrum.<sup>31</sup> Detailed structural characterization identified three novel compounds, Gal( $\alpha$ 1–3)Gal( $\beta$ 1–4)Glc ( $\alpha$ 3'-GL), Gal( $\beta$ 1–3)Gal( $\beta$ 1–4)Glc (3'-GL), Gal( $\beta$ 1–6)Gal( $\beta$ 1–4)Glc (6'-GL), and Fuc( $\alpha$ 1–2)Gal( $\beta$ 1–4)Glc (2'-FL).<sup>31</sup> In 1997, the same research group reported the structures of the first four acidic gMOS from Japanese Saanen, namely, Neu5Ac( $\alpha$ 2–6)Gal( $\beta$ 1–4)Glc (6'-SL), Neu5Ac( $\alpha$ 2–3)Gal( $\beta$ 1–4)Glc (3'-SL), Neu5Gc( $\alpha$ 2–6)Gal( $\beta$ 1–4)Glc (6'-NGL), and Neu5Ac( $\alpha$ 2–6)Gal( $\beta$ 1–4)GlcNAc (6'-SLN).<sup>33</sup> This study showed non-human sialic acid *N*-glycolylneuraminic acid (Neu5Gc) occurring in gMOS.<sup>33</sup> Another group published the structures of two branched acidic gMOS from mature goat milk, i.e., Gal( $\beta$ 1–3)[Neu5Ac( $\alpha$ 2–6)]Gal( $\beta$ 1–4)Glc (6'-SHL) and Gal( $\beta$ 1–6)[Neu5Ac( $\alpha$ 2–3)]Gal( $\beta$ 1–4)Glc (3'-SHL).<sup>32</sup>

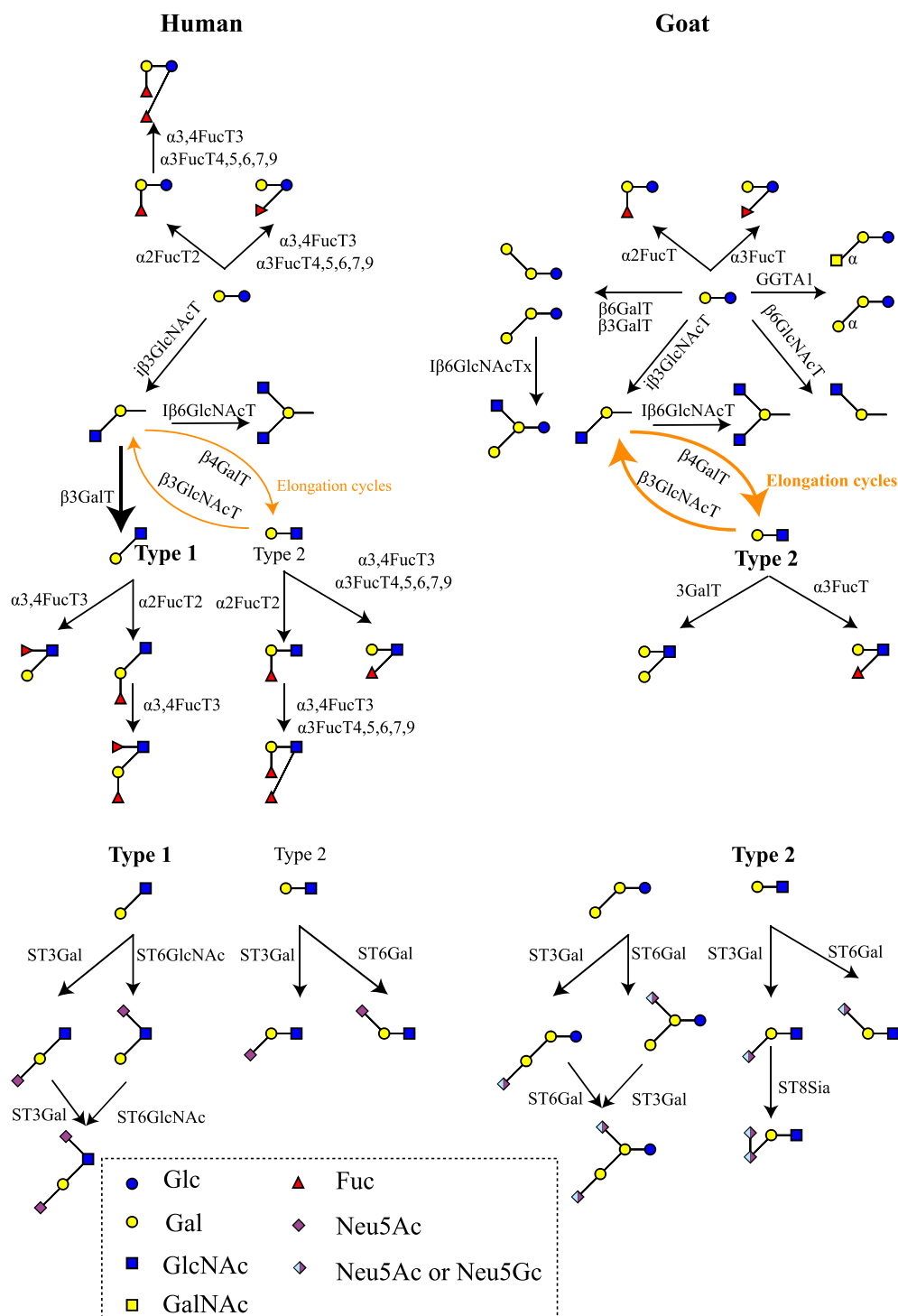
After these initial efforts to isolate specific oligosaccharides from goat milk and to identify their structures with detailed analytical methods, more comprehensive studies were performed, identifying structural compositions, i.e., the monosaccharide makeup of the structures, based on mass spectrometry (MS) data. When these data were combined with reference structures and previous literature, comparative studies could be made, following known structures and annotating newly found oligosaccharide structures. One of the first comparative studies

focused on the gMOS composition of fresh mature milk from Spanish goats from the Murciano-Granadina breed, from cows and sheep, compared to hMOS.<sup>29</sup>

A detailed and comprehensive study was conducted by Albrecht et al., in which milk samples of different domesticated animals, including goats, were analyzed for their milk oligosaccharide composition.<sup>24</sup> Structural oligosaccharide libraries were created using a combination of analytical techniques. Purified MOS were fluorescently derivatized via reductive amination with 2-aminobenzamide (2-AB). This stoichiometric addition of one 2-AB label per oligosaccharide allowed for their relative quantification with fluorescence detection.<sup>38</sup> Some oligosaccharides were identified on the basis of their elution compared to standards. All other structures were identified by exoglycosidase assays in combination with tandem mass spectrometry (MS/MS) data. Sialic acid determination was performed to distinguish between Neu5Gc and Neu5Ac epitopes.<sup>31</sup>

Albrecht et al. found that milk from domesticated animals contained a much larger variety in complex MOS than previously known. More than 80% of the oligosaccharides found in pools of all domesticated animals were acidic oligosaccharides. In total, 77 structures were identified in all of the milk samples, of which 29 were neutral, 45 were sialylated, and 3 were phosphorylated.<sup>24</sup> A total of 40 structures were characterized in mature goat milk, of which 16 were neutral, 23 were acidic, and 1 was phosphorylated lactose (Schemes 1 and 2). Numerically, sheep and bovine milk are only slightly less diverse, with 37 and 32 distinct structures, respectively. However, in comparison of the total milk profile, bovine milk is dominated by one structure, i.e., 3'-SL, while sheep milk is dominated by 3'-*N*-glycolylneuraminylactose (3'-NGL) and 6'-*N*-glycolylneuraminylactose (6'-NGL) and goat milk shows a much more varied peak pattern in this comparative study.<sup>24</sup> From the sialylated pool, the proportion of Neu5Gc-containing oligosaccharides was highest in sheep colostrum (94%) and mature goat milk (64%). The acidic pool of cow colostrum contained 3% Neu5Gc oligosaccharides, whereas only trace amounts were found in the milk of other domesticated animals ( $\leq 1\%$ ).<sup>24</sup> Humans are unique in their inability to produce Neu5Gc but are able to incorporate dietary Neu5Gc in their glycosylation.<sup>39,40</sup> Incorporation of Neu5Gc has also been observed in hMOS.<sup>41</sup> Studies have associated Neu5Gc incorporation with several diseases, including cancer, type 2 diabetes, atherosclerosis, and autoimmune diseases.<sup>40,42</sup> Although there is no causality proven for Neu5Gc, potential health effects of the relatively high levels of Neu5Gc in goat milk should be taken into account. In the study by Albrecht et al., 19 structures were identified for the first time in goat milk along with 3-FL, one of the more abundant hMOS constituents.<sup>24</sup> A later study annotated 78 variations in goat colostrum but did not identify specific structures; only monosaccharide compositional data were obtained.<sup>37</sup>

Using more sensitive techniques, a comparative study between  $\alpha$ <sub>s1</sub>-casein-producing and non-producing goats was performed.<sup>27</sup> Previously reported structure compositions were observed and verified by MS/MS. This study annotated in total 37 oligosaccharide structures. A fucosyllactosamine structure with monosaccharide composition 1Hex, 1Fuc, 1HexNAc, 0Neu5Ac, and 0Neu5Gc was exclusively found in milk of goats unable to synthesize  $\alpha$ <sub>s1</sub>-casein. Another fucosylated gMOS composed of 3Hex, 1Fuc, 3HexNAc, 0Neu5Ac, and 0Neu5Gc was present in both types of milk but only in trace



**Figure 2.** Biosynthesis of neutral and acidic complex (left) human milk oligosaccharides (hMOS) and (right) goat milk oligosaccharides (gMOS). Enzymes probably involved are indicated in the human pathway. Required enzyme activities to achieve known structures are notated in the goat pathway. *I* $\beta$ 6GlcNAcTx refers to a potential separate enzyme as observed in marsupial lactation, capable of adding GlcNAc( $\beta$ 1–6) branching of 3'-GL.<sup>57</sup> Main pathways and structure types are depicted in bold with thicker arrows. The human hypothetical pathway is compiled from Urashima et al.,<sup>8</sup> Bode et al.,<sup>16</sup> Blank et al.,<sup>54</sup> and Van Leeuwen et al.<sup>53</sup> and combined with the Kyoto Encyclopedia of Genes and Genomes (KEGG) glycosphingolipid biosynthesis: lacto and neolacto series.<sup>56</sup>

amounts in milk of *as*<sub>1</sub>-casein-producing goats. For all other structures, no significant differences in abundance were observed.

Taken together, the structural diversity of human milk is much higher than that of any domesticated dairy animal, i.e., 247 compositional variations and 162 identified structures in human

milk, as compared to 78 compositions and 40 structures in goat milk, 50 compositions and 37 structures in bovine milk, and 32 structures in sheep milk; as a result of fewer studies, there is no extra data on composition variety in sheep milk at this time.<sup>8,23,24,37</sup> With focus on the major structures that are responsible for >95% of the quantitative composition on the

basis of relative abundance studies, human milk contains 22 major structures,<sup>3,43</sup> while goat milk is defined by 9 major structures, bovine milk is defined by 6 structures, and sheep milk is defined by only 4.<sup>24</sup> It is clear that goat milk presents a more diverse pallet of MOS structures than bovine or sheep milk.

There are some clear differences in the structural composition and number of oligosaccharides between human milk and milk from domesticated animals, e.g., goat, cow, and sheep.<sup>24</sup> In human milk, the lacto-*N*-biose type 1 structural epitope predominates over those containing the *N*-acetylglucosamine type 2 structure (Figure 2), whereas type 1 chains are rare in milk of domesticated dairy animals. Contrary to hMOS, domesticated animal milk oligosaccharides can also have *N*-acetylglucosamine [Gal( $\beta$ 1-4)GlcNAc] at the reducing end.<sup>44</sup> Around 70% of hMOS is neutral and often fucosylated,<sup>24</sup> whereas the fraction of fucosylated MOS in animal milk is relatively low and most of the oligosaccharides are acidic.<sup>44,45</sup> Domesticated animal milk can contain structural epitopes that are not present in human milk, such as GalNAc, Neu5Gc-containing oligosaccharides, and ( $\alpha$ 1-3)-linked Gal and GalNAc.<sup>24</sup> Moreover, in goat as well as bovine milk oligosaccharides, Gal( $\beta$ 1-4)GlcNAc branches on 3'-galactosylactose (lacto-*N*-novopentaose) have been observed as well as sialylation of both 3'-GL as well as 6'-GL (Schemes 1 and 2).

In bovine milk, *N*-linked glycans from glycoproteins were shown to contain *N,N'*-di-*N*-acetylglucosamine diamine [GalNAc( $\beta$ 1-4)GlcNAc (LacdiNAc)].<sup>46</sup> Although no comprehensive studies of goat milk protein glycosylation are known to us, a recombinant human erythropoietin expressed in transgenic goat milk was highly decorated with the LacdiNAc epitope.<sup>47</sup> In bovine milk, LacdiNAc disaccharide has been observed as well as other oligosaccharides where Gal has been substituted for GalNAc.<sup>38,48</sup> This activity has been ascribed to bovine  $\alpha$ -lactalbumin, which induces milk  $\beta$ -galactosyltransferase (B4GalT1) to accept uridine diphosphate (UDP)-GalNAc as a donor as well as UDP-Gal in the synthesis of lactose.<sup>49</sup> It is not known whether this is also responsible for the LacdiNAc glycotopes in protein glycosylation. Possibly  $\beta$ -*N*-acetylglucosaminyltransferases B4GalNT3 and B4GalNT4 are responsible for the synthesis of these glycotopes. It should be noted that, in humans, these enzymes are also encoded in the genome and are responsible for the presence of LacdiNAc epitopes in the gastric mucosa.<sup>50</sup> In view of the similarities between MOS and protein glycosylation patterns, such LacdiNAc glycotopes can also be expected to occur in bMOS and gMOS.

The  $\alpha$ -Gal glycotope is found abundantly in mammalian glycosylation, except for humans, apes, and Old World monkeys.<sup>51</sup> The responsible enzyme  $\alpha$ -galactosyltransferase (GGTA1) is functionally encoded in the genomes of most mammals. In the human genome, two non-functional variants have been identified, GGTA1 and GGTAIP.<sup>52</sup>

From the structures known thus far (Schemes 1 and 2), it is possible to construct a hypothetical biosynthetic scheme for gMOS, as presented previously for hMOS (Figure 2).<sup>53,54</sup> Although for gMOS the biosynthetic enzymes are less established, many of the enzymes postulated to be involved in hMOS biosynthesis have homologues in the goat genome that are also expressed in the mammary gland during lactation.<sup>55,56</sup> There are some interesting differences, particularly in the presence of major levels of structures consisting of lactose elongated with ( $\beta$ 1-3) galactose units. Also, the presence of the 6' isotope of LNT (iLNT) in goat milk indicates that, contrary to the human biosynthetic pathway, the GlcNAc( $\beta$ 1-3)Gal

epitope is not required for the attachment of a 6'-GlcNAc residue. Also, the structure Gal( $\beta$ 1-3)[GlcNAc( $\beta$ 1-6)]Gal( $\beta$ 1-4)Glc may be formed by transfer of Gal( $\beta$ 1-3) to iLNT or alternatively by transfer of GlcNAc by a separate I $\beta$ 6GlcNAcT enzyme, as observed in lactating tamar wallaby.<sup>57</sup> The human biosynthetic pathway does not include enzymes for Gal( $\beta$ 1-3), with Gal( $\beta$ 1-4) disubstitution on a GlcNAc residue, which has been tentatively observed in goat milk.<sup>24</sup> It should be noted that the first elucidation of this structure is tentative, because the papers identifying this structure initially contained mistakes in other structures. The goat structures indicate activity of ST8Sia, to generate Sia( $\alpha$ 2-8)Sia epitopes, with either Neu5Ac or Neu5Gc residues. Activity of such ST8Sia transferases in the mammary gland of lactating goats was observed in a comprehensive transcriptomics study.<sup>55</sup> Finally, the goat milk oligosaccharides contain some Gal( $\alpha$ 1-3)Gal and GalNAc( $\alpha$ 1-3)Gal glycotopes that are absent in human milk.

## ■ QUANTITATIVE ASPECTS OF GMOS

Milk is a complex fluid, containing many classes of biomolecules (Figure 1). A major class is carbohydrates, of which the majority is usually lactose. The second class of carbohydrates in human milk is that of hMOS, while in bovine milk, this is monosaccharides, with bMOS constituting the third carbohydrate class.<sup>58,59</sup> For goat milk, the concentrations of monosaccharides have not been extensively studied, but one study puts them at a comparable level with gMOS.<sup>34</sup> Analysis of milk oligosaccharides often requires extraction of MOS prior to analysis. The extraction procedure and required level of purity depend upon the analytical methods, which are, in turn, determined by the research question. For identification of specific, unknown structures, the compounds need to be isolated in pure form for further analysis. A recent review outlines the challenges and pitfalls in analysis of hMOS.<sup>53</sup> The most common methods for hMOS analysis have been described in detail here, and the same challenges hold for gMOS analysis. In short, many analytical methods have been employed, and particularly, comprehensive quantitative analysis is difficult. The risk of loss of MOS in the extraction phase is one major issue that has been understudied thus far. Also, the choice of analytical method determines to a large part the precision of quantitation. While for human milk many studies have been performed, a wide variation in quantitative results has been observed.<sup>3</sup> Some of the variation can be explained by population-based differences<sup>60</sup> or genetic variation between individuals.<sup>53</sup> For goats, as well, the breed and geographical factors may influence the results. It is, however, also possible that differences in analytical approaches yield deviating results.

Several studies have been performed quantifying gMOS or comparing MOS quantities between domesticated animal species. In a comparative study between goat, sheep, and cow milk, quantification was based on standards using high-pressure/pH anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD).<sup>29</sup> They found that the mature Murciano-Granadina goat milk contained 250–300 mg/L gMOS, representing about 5 times the amount of bMOS measured in cow milk (30–60 mg/L) and about 10 times more than sMOS in sheep milk (20–40 mg/L) in the same study.<sup>29</sup> Another study on this breed investigated the levels of gMOS in colostrum and mature milk of four individual goats as well as a pooled milk sample from 12 goats.<sup>37</sup> A large variation in total gMOS levels was observed in colostrum of the four individual Murciano-Granadina goats and the pooled colostrum, ranging

**Table 1. Overview of Average Levels of gMOS Determined in Quantitative Studies from Different Breeds, Murciano-Granadina (MG), New Zealand Saanen (NZS), Alpine (A), Garganica (G), Maltese (M), and Saanen (S)**

breed	gMOS (mg/L)	method <sup>a</sup>	comments	reference
MG	250–300	HPAEC–PAD		29
MG	251–572	HILIC–MS	acidic, 83–251 mg/L; neutral, 140–315 mg/L	37
NZS	260–320	LC–MS	colostrum, ~320 mg/L; mature milk, ~260 mg/L	34
A	1110–1250	GC–MS	monosaccharide-analysis-based method; high levels of Glc and Gal suggest significant levels of lactose	27
G	240–700	HPAEC–PAD	only 3'-SL, 6'-SL, and DSL; colostrum, 700 g/L; mature milk, 240 g/L	25
M	190–625	HPAEC–PAD	only 3'-SL, 6'-SL, and DSL; colostrum, 625 mg/L; mature milk, 190 g/L	25
S	125–365	HPAEC–PAD	only 3'-SL, 6'-SL, and DSL; colostrum, 365 g/L; mature milk, 125 g/L	26
MG	112–488	HILIC–MS	colostrum, 488 mg/L; mature milk, 112–178 mg/L	28
MG	703–2398	LC–MS	first 4 days of lactation; day 1, 2400 mg/L; day 4, 700 mg/L	30

<sup>a</sup>HPAEC–PAD, high-performance/pH anion-exchange chromatography–pulsed amperometric detection; GC–MS, gas chromatography–mass spectrometry; LC–MS, liquid chromatography–mass spectrometry; and HILIC–MS, hydrophilic interaction chromatography–mass spectrometry.

from 251 to 572 mg/L (Table 1).<sup>37</sup> Total neutral gMOS levels ranged from 140 to 315 mg/L, and total acidic gMOS levels ranged from 83 to 251 mg/L. The most abundant gMOS were galactosylactoses, with values ranging from 128.7 to 274.3 mg/L. Higher 2'-FL concentrations (2.2–31.6 mg/L) were measured in comparison to fucosylactosamine (3.1–6.15 mg/L). Furthermore, the authors noted that, similar to human milk, all five colostrum samples contained higher 6'-SL concentrations (29–124 mg/L) than 3'-SL (3–12 mg/L).<sup>37</sup> It should be noted that several other gMOS studies showed opposite findings, identifying predominant levels of 3'-SL instead.<sup>25,26,61</sup> Also, in one study on human milk in Vietnam, specific individuals were identified with predominantly 3'-SL or near equal levels of 3'-SL and 6'-SL rather than a clear predominance of 6'-SL.<sup>62</sup>

Other studies on gMOS concentrations in New Zealand Saanen goats showed ~320 mg/L in colostrum and ~260 mg/L in mature milk (Table 1).<sup>34</sup> This study applied liquid chromatography with mass spectrometric detection (LC–MS), separating structures on a porous graphitized carbon (PGC) column. Quantitation was achieved in relation to a calibration curve of commercial standards (4'-GL, 3'-SL, 6'-SL, 3-FL, 2'-FL, LNH, DSL, lactose-1P, and GOS from Yakult). Only structures fitting *m/z* of these compounds were taken into account for the quantitation. This might result in some underestimation of gMOS, because other structural compositions were not taken into account.

Quantitation was also performed in a comparative study between Alpine goats that were either capable or incapable of producing  $\alpha_{s1}$ -casein.<sup>27</sup> In the study, the milk of eight A/A goats (capable of producing  $\alpha_{s1}$ -casein) was compared to the milk from eight O/O goats (null mutant). The study observed a broad spread in concentrations for each group with  $1110 \pm 290$  mg/L for A/A goats and  $1250 \pm 320$  mg/L for O/O goats (Table 1) but no statistically significant difference between groups. These values are significantly higher than the values observed in the aforementioned studies,<sup>25,26,28,29,37</sup> and the applied method relies on monosaccharide quantitation by gas chromatography coupled to MS, showing almost equivalent amounts (44.2–46.9 mol %) of Gal and Glc in all samples, suggesting a significant portion of remaining lactose as part of the gMOS fraction.<sup>27</sup>

A series of studies by Claps et al. analyzed the concentrations of 3'-SL, 6'-SL, and DSL in the colostrum and mature milk of Garganica ( $n = 15$ ), Maltese ( $n = 20$ ), and Saanen ( $n = 5$ ) goats, using a HPAEC–PAD method with external standard calibration.<sup>25,26</sup> Significant differences in the levels of the three

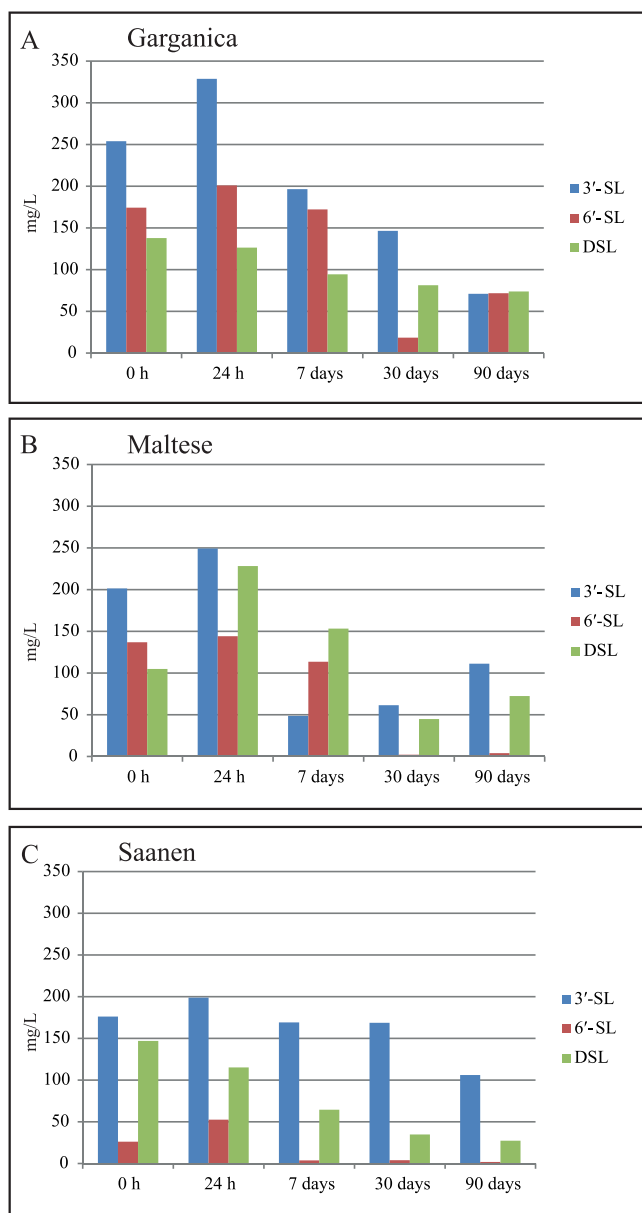
sialyloligosaccharides were found between the breeds (Figure 3 and Table 1).

Garganica colostrum and milk samples contained higher concentrations of 3'-SL and 6'-SL than Maltese and Saanen goat colostrum, whereas the DSL content was highest in colostrum from Saanen goats. In the first 24 h after kidding, a significant increase in the 3'-SL concentration in the milk of all three breeds was observed, followed by a decline toward mature milk.<sup>25,26</sup> The same pattern was observed for 6'-SL concentrations for the three goat breeds. The DSL content in Garganica (137.9–126.4 mg/L) and Saanen (150.0–113.9 mg/L) goat colostrum dropped slightly in the first 24 h, from 137.9 to 126.4 mg/L and from 150.0 to 113.9 mg/L, respectively, but significantly increased in colostrum of Maltese goats, from 104 to 228 mg/L (Table 1). Toward mature milk, the DSL levels gradually decreased for all breeds (Figure 3). The general trend of decrease in MOS concentrations from colostrum toward mature milk is found in common also with hMOS and bMOS.<sup>22,63</sup> A recent study comparing Saanen goats to local Guanzhong goats at 40–50 days of lactation showed similar levels of 3'-SL, 17.17 mg/L in Guanzhong and 18.51 mg/L in Saanen, while 6'-SL and DSL are significantly increased in Guanzhong goats, i.e., 33.41 mg/L 6'-SL versus 9.98 mg/L in Saanen goats and 1.16 mg/L DSL in Guanzhong versus 0.39 mg/L in Saanen.<sup>64</sup> The Claps studies on Saanen goats showed much higher concentrations at 90 days postpartum.<sup>26</sup>

Saanen goats have a high frequency of defective alleles at the  $\alpha_{s1}$ -casein locus, and this is associated with a low  $\alpha_{s1}$ -casein content in milk (450 mg/L compared to up to 8.4 g/L).<sup>26,65</sup> On the basis of these results, Claps et al. speculated that the defective  $\alpha_{s1}$ -casein production negatively affected the whole secretion process of milk constituents, including the synthesis of sialyltransferases and, thus, the production of sialylated gMOS. This is in contrast, however, with the findings from Meyrand et al., where no significant differences in acidic gMOS concentrations were observed between Alpine goats with and without  $\alpha_{s1}$ -casein deficiency.<sup>27</sup>

Claps and co-workers also investigated the influence of parity on the sialyl gMOS content in colostrum and milk.<sup>26</sup> Goat milk samples from five goats in second parity and five goats in third parity were analyzed, showing only a significant difference in 3'-SL concentrations between second and third parity, with values of 203 and 125 mg/L, respectively.<sup>26</sup> A recent study has shown that, in protein glycosylation of goat milk glycoproteins, fucosylation and sialylation increase with parity.<sup>66</sup>

Martin-Ortiz et al. examined gMOS levels in milk of Murciano-Granadina goats at different lactation stages.<sup>28</sup> Eight



**Figure 3.** Average levels (mg/L) of 3'-SL, 6'-SL, and DSL in milk of (A) Garganica, (B) Maltese, and (C) Saanen goats over time and adapted from Claps et al.<sup>25,26</sup>

major gMOS (3'-SL, 6'-SL, sialyllactosamine, three glycolylneuraminylactose isomers, and two galactosyllactose isomers) were quantified with hydrophilic interaction chromatography–quadrupole–mass spectrometry (HILIC–Q–MS), and 49 gMOS structures were monitored.

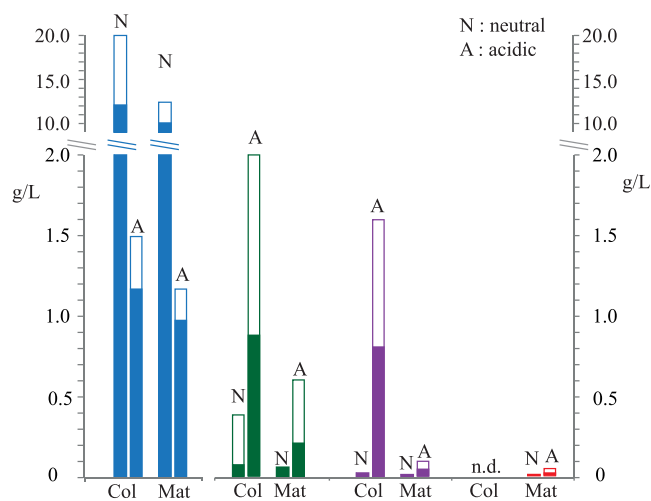
A comparison between milk of a single goat and a pooled milk sample from eight goats showed a considerable difference in total gMOS between the individual goat (GM) and the pooled milk (GP) samples of 58.9 and 178.1 mg/L, respectively (Table 1).<sup>28</sup> This large difference in results between the individual goat and the pooled milk shows the large variability in gMOS composition between individual goats of the same species. Similar to results from Claps et al., over time, concentrations of the most abundant gMOS were highest at the beginning of lactation and decreased with lactation time.<sup>25,26</sup> In Murciano-Granadina goats, 6'-SL was by far the most abundant acidic gMOS in the pooled samples, whereas in the individual goat

samples, 3'-SL levels almost reached those of 6'-SL. Also notably, 3'-SL in pooled milk of Saanen, Garganica, and Maltese goats was the most abundant gMOS.<sup>25,26</sup>

The concentration of neutral gMOS (265.2 mg/L) was higher than that of acidic gMOS (222.8 mg/L) in the beginning of lactation but rapidly dropped with lactation time, mainly as a result of a significant decrease in galactosyllactose isomers, the most abundant neutral gMOS. The acidic gMOS content also decreased with lactation time but not as much as that of the neutral gMOS, resulting in higher acidic (149.3 mg/L) than neutral (28.8 mg/L) gMOS concentration at 30 days of lactation. Notably, the concentrations of 3'-SL are much lower than those observed in the three other goat species, while the 6'-SL levels at day 1 are comparable to that of the Maltese goats. The levels of 6'-SL remain higher in the Murciano-Granadina goats. In this study, also three Neu5Gc–lactose isomers were monitored, without giving specific structures. These oligosaccharides were only observed at relatively low levels (together ~25–50 mg/L) and do not seem to follow the same concentration trend over time as analogous compounds containing Neu5Ac. The concentrations of two neutral galactosyllactose structures, most likely 3'-GL and 6'-GL, were also measured, ranging from ~30 to 270 mg/L; approximately half of the gMOS determined in this study were neutral. In the study by Albrecht et al., the analysis of acidic versus neutral structures showed 95% acidic structures in goat milk.<sup>24</sup> Other reports also state that acidic oligosaccharides are the major component in goat milk.<sup>25,45</sup> It is possible that the levels of acidic versus neutral gMOS are strongly affected by goat breed, suggesting the potential for a comprehensive and comparative study of goat milk from different breeds.

A recent study on goat colostrum composition analyzed lipid and oligosaccharide composition and concentration.<sup>30</sup> This paper showed levels decreasing from 2398.4 mg/L at day 1 to 702.7 mg/L at day 4 of lactation, markedly higher than in other studies thus far (Table 1).

When all quantitative studies on gMOS are taken into account (Table 1 and Figure 4), a very broad range of concentrations has



**Figure 4.** Comparison of acidic and neutral MOS concentration levels between human (blue), goat (green), cow (purple), and sheep (red) colostrum (Col) and mature milk (Mat). For sheep colostrum, there are no data (n.d.) available. The open part of the bars shows the highest concentration level reported, and the filled part of the bars represents the lowest reported concentration.<sup>3,25,26,29,30,37,60,63</sup>

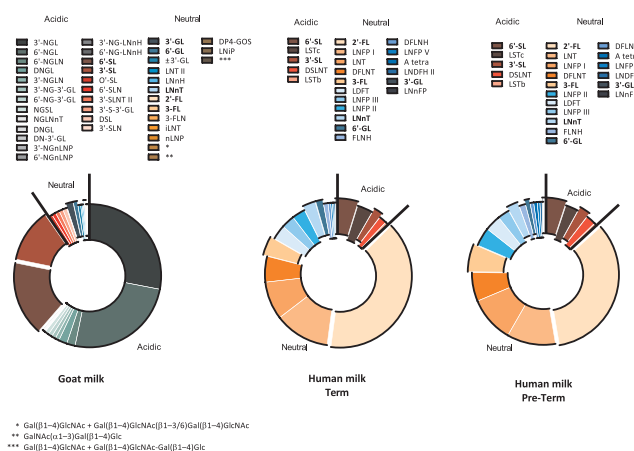


been reported. The lowest value measured in a single goat was 58.9 mg/L in mature milk and ~200 mg/L in early colostrum.<sup>28</sup> The highest values in mature milk were observed in a single study on Saanen goats at 1110–1250 mg/L.<sup>27</sup> The methodology of this study, however, seems to have incorporated a significant amount of lactose into the quantitation and should therefore be discounted as an assessment of gMOS quantity. The highest colostrum values were ~2.4 g/L in Murciano-Granadina goats.<sup>30</sup> On average, the values reported range from ~100 to 250 mg/L in mature milk and from ~300 to 2400 mg/L in colostrum across different breeds. Interestingly, one study on bovine milk showed that the major oligosaccharides, i.e., 3'-SL, 6'-SL, and 6'-SLN, start at high concentrations in colostrum, i.e., 855 mg/L for 3'-SL, 117 mg/L for 6'-SL, and 141 mg/L for 6'-SLN, followed by a rapid decrease.<sup>67</sup> An ultrafiltrate of whey permeate was shown to contain particularly high levels of bMOS, indicating that processing streams may be a promising source of dairy-derived MOS.<sup>68</sup> A comparison of MOS composition of goat milk with human, bovine, and sheep milk shows that, in both the colostrum phase as well as mature milk phase, the quantities of gMOS are higher than those for bovine and sheep milk but still far lower than those in human milk (Figure 4). The physiological and molecular basis for these variations in gMOS concentrations remains to be elucidated. It should be noted that quantitative studies on hMOS also vary greatly, and recent recommendations were to evaluate different methods in a cross-laboratory study to determine how much of the variation is caused by experimental bias.<sup>3,53</sup> A similar recommendation could be made for analysis of goat milk oligosaccharides.

## FUNCTIONAL ANALYSIS OF MILK OLIGOSACCHARIDES

Currently, increasing evidence is generated for specific functions of specific hMOS, being antiadhesive, immune-modulating, or a targeted prebiotic for specific desirable bacterial/microbiota strains in early infant development.<sup>16</sup> At the moment, there is a lack of studies into the required concentrations of hMOS to achieve optimal functionality. Also missing in the current understanding is the level of diversity required for optimal health effects in the infant development. The addition of a single hMOS structure as a supplement to infant formula clearly has benefits, as observed for the additions of 2'-FL.<sup>69,70</sup> When gMOS and bMOS composition is observed, it is clear that goat milk shows more similarities with hMOS composition than bMOS.<sup>24,27,29,37</sup> It should be noted, however, that there is still a significant difference between gMOS and hMOS. One particular difference is the dominance of acidic MOS in goat milk compared to human milk (Figure 4). Moreover, the neutral fraction of hMOS is dominated with fucosylated structures, while gMOS contain only minor levels of fucosylated neutral structures (Figure 5). Figure 5 shows a comparison of structures and relative quantities based on the study by Albrecht et al. for goat milk<sup>24</sup> to term and preterm human milk on the basis of the study by Austin et al.<sup>63</sup> Although the composition of hMOS is significantly different from gMOS, there are sialylated, neutral non-fucosylated, and neutral fucosylated structures in common between the two. Therefore, in comparison to bMOS and sMOS, gMOS have more potential as a functional substitute for hMOS. Moreover, the levels of gMOS are significantly higher than those of bMOS and sMOS, suggesting that goat milk has more potential as a source for such functional oligosaccharides.

**Prebiotic Potential.** Thum and co-workers used a previously obtained gMOS-enriched fraction (labeled CMOF



**Figure 5.** Overview of relative intensities of gMOS derived from Albrecht et al.<sup>24</sup> compared to hMOS in term and preterm milk derived from Austin et al.<sup>63</sup> Clockwise from the top, first, acidic oligosaccharides are grouped together, presented according to relative abundance, followed by neutral oligosaccharides in order of abundance. Structures similar between human and goat milk are presented in bold, and corresponding segments are offset from the ring chart.

in their work)<sup>34</sup> in a study to investigate the growth of bifidobacteria that were isolated from the feces from breast-fed infants.<sup>71</sup> It should be noted that processing conditions and use of  $\beta$ -galactosidase in the enrichment of gMOS has some influence on the composition of the oligosaccharide mixture.<sup>72</sup> A total of 17 strains of bifidobacteria were isolated from infant feces, and their growth on gMOS was analyzed in comparison to GOS, FOS, inulin, and 3'-SL or 6'-SL.<sup>71</sup> All bifidobacteria grew faster and reached higher optical densities on the gMOS compared to the other substrates. No growth was observed on medium supplemented with inulin. Interestingly, one *Bifidobacterium bifidum* strain was able to use 3'-SL or 6'-SL as the sole carbon source, whereas the other *B. bifidum* strains were able to ferment the gMOS-enriched fraction while partially degrading 3'-SL and 6'-SL in the gMOS. Carbohydrate depletion after 36 h of incubation was evaluated by LC-MS and HPLC analysis. Two *B. bifidum* strains (AGR2166 and AGR2168) used more gMOS than *Bifidobacterium breve* or *Bifidobacterium longum* subspecies *longum* isolates, and this was related to an enhanced sialidase activity. Furthermore, for gMOS, the *in vitro* production of lactate and SCFAs, such as acetate, compared better to other fermentable substrates.<sup>71</sup> These SCFAs are important substrates for gut health and gut microbiota.<sup>73,74</sup> The production of SCFAs by beneficial bacteria has been linked to protection against several pathologies on the long term, including cardiovascular disease, inflammatory bowel disease (IBD), metabolic syndrome, and even some types cancer.<sup>75–77</sup> Butyrate is an important SCFA and considered the preferred energy source for colonocytes.<sup>78</sup> Incubation of *B. longum* subsp. *infantis* with gMOS has also shown increased adhesion to HT-29 cells of more than 8-fold compared to controls and more than 2.5-fold better than immunoglobulin G (IgG)-enriched bovine whey.<sup>61</sup> The authors of that study used the same gMOS mixture, rich in 3'-SL, in a study showing enhanced SCFA production when *B. longum* subsp. *infantis* is incubated with gMOS. Moreover, the pre-incubation with gMOS enhanced the ability of *B. longum* subsp. *infantis* to inhibit *Campylobacter jejuni* infection of HT-29 cells.<sup>79</sup>

The prebiotic potential of gMOS was confirmed in another study.<sup>80</sup> Here, bifidobacteria *Bifidobacterium animalis* and *B.*

*longum* strains were tested as well as *Lactobacillus casei* and *Lactobacillus acidophilus* strains. The growth rate of both bifidobacteria strains as well as *L. casei* was higher with gMOS isolated from goat-milk-derived infant formula than with the established GOS prebiotic ( $p < 0.05$ ).<sup>80</sup> It should be noted that both prebiotic studies have only tested probiotic species in monoculture and have not assessed growth of pathogens and/or commensal species nor assessed interspecies competition in mixed culture experiments. A shift toward more probiotic species in a mixed culture or *in vivo* is required to fully substantiate a prebiotic function.

An *in vivo* study by Thum et al. determined the effect of prenatal consumption of gMOS (CMOF) on the colon microbiota and milk composition of pregnant mice.<sup>81</sup> The development of the mice offspring and whether these effects in offspring persisted after 30 days of consuming the control diet were also determined. The diet did not affect the food intake, body weight, gastrointestinal tract (GIT) length, small intestine length, or weight of stomach, colon, spleen, kidneys, brain, femur, and visceral fat of the dam. However, in comparison to negative-control-diet- and GOS-diet-fed dams, the gMOS-diet-fed dams had an increased colon length and lower liver weight at 30 days after delivery. The pups of the gMOS-fed dams had increased body weight and length at weaning, similar to GOS control pups. However, only pups of gMOS-fed dams had an increased microbial diversity and relative abundance of bifidobacteria at weaning as well as higher levels of the SCFA butyric acid in the colon. Bifidobacteria produce acetate and lactate, which can be converted into butyrate by other bacteria.<sup>82</sup> It was concluded that consumption of gMOS by the dams during gestation and lactation improved the development of the offspring and the relative abundance of bifidobacteria in the colon at weaning. Although there is currently no data on human interventions with gMOS, studying the potential prebiotic effects, the studies thus far suggest prebiotic potential more closely resembling hMOS than GOS. This may be explained by the structural overlap between gMOS and hMOS (Figure 4). There is currently no intervention study with gMOS; a study with bMOS has been performed, using a bMOS enriched whey permeate, obtained through ultrafiltration and anion-exchange chromatography. The bMOS product is well-tolerated by healthy adults in a single-blind placebo-controlled study.<sup>83,84</sup> The bMOS were fully digested by the intestinal microbiota but did not illicit a significant change in the microbial transcriptome nor on the exfoliated gut epithelia.<sup>84</sup> There was only a short-term effect on bifidobacterial levels, showing an increase after 4 days of intervention but no longer after 11 days.<sup>83</sup> The product contained mostly 3'-SL and to a lesser extent 6'-SL,<sup>85</sup> fitting the composition observed for bovine milk.<sup>24</sup> It should be noted that recent advances in hMOS synthesis technology have allowed for sufficient levels of 2'-FL and LNnT to be produced, leading to the first European Food Safety Authority (EFSA)-approved infant formulas supplemented with these hMOS for functionality. Tests thus far have shown that the addition of 2'-FL and LNnT shifts the microbiota more toward breast-fed compositions than formulas with GOS and FOS.<sup>86,87</sup> Moreover, analysis of goat milk has shown the presence of both 2'-FL and LNnT, underscoring the potential of goat milk as an infant formula ingredient.

**Anti-inflammatory Effects.** Goat milk oligosaccharides have shown to be anti-inflammatory in a rat model of hapten-induced colitis. Daddaoua et al. investigated the role of gMOS in a model of experimental colon inflammation induced by the

hapten, trinitrobenzenesulfonic acid (TNBS), in rats.<sup>88</sup> TNBS-induced colon inflammation is characterized by anorexia with body weight loss, epithelial necrosis, bowel wall thickening, and colon shortening. For the treatment of rats, an enriched gMOS permeate of Martinez-Ferez et al. was used.<sup>89</sup> This permeate contains ~200–240 mg/L gMOS but also ~2.25 g/L lactose. Rats that were fed with 500 mg of the lyophilized gMOS permeate/kg of body weight per day showed significantly less symptoms compared to control rats that did not receive the gMOS. Downregulation of colonic expression of inflammatory factors interleukin 1 $\beta$  (IL-1 $\beta$ ) and inducible nitric oxide synthase (iNOS) was also observed as well as reduced cyclooxygenase 2 (COX2), which is not directly involved in inflammation but can be used as a marker for inflammation.<sup>88</sup> Because the gMOS sample still contained a lot of lactose, it is unclear whether all of the observed effects are only caused by gMOS or if lactose also had an effect.<sup>88</sup>

Lara-Villoslada et al. also studied the effect of gMOS on colon inflammation in rats induced by dextran sodium sulfate (DSS).<sup>90</sup> They also used the enriched gMOS permeate produced by Martinez-Ferez et al. but first removed the residual lactose from the gMOS fraction. The body weight gain and food intake of rats fed with gMOS (PF-GMO) were similar to the control rats that did not receive gMOS (PF-control). Interestingly, the colonic content of probiotic lactobacilli and bifidobacteria was significantly higher in the rats receiving gMOS but not of the potential pathogens. After DSS treatment, histological analysis showed that the gMOS rats (DSS-GMO) had no ulceration and recovered from inflammation, while the DSS control rats had significant ulceration and inflammation. Also, blood granulocyte levels were reduced in gMOS-fed rats compared to control rats. In gMOS-fed rats, the levels of myeloperoxidase activity, a proxy for neutrophil infiltration, do not increase upon DSS treatment, while in control rats, a 5-fold activity increase is observed upon DSS treatment.<sup>90</sup>

Both studies indicated that gMOS reduce intestinal inflammation and contributed to the recovery of damaged colonic mucosa and suggested gMOS as a suitable treatment of inflammatory bowel disease. Previous studies showed that GOS, which are commonly added to infant formula to substitute hMOS, were unable to reduce inflammation in a rat model for colitis.<sup>91</sup> Similar studies have not been performed with hMOS thus far; however, anti-inflammatory effects have been well-established for hMOS.<sup>16,92</sup>

A recent mini-review showed an overview of hMOS with the potential for galectin binding and, thus, galectin-directed signaling.<sup>8</sup> Many hMOS have potential galectin recognition epitopes, i.e., all structures with a non-reducing terminal galactose linked to a Glc, GlcNAc, or GalNAc residue, with free OH on Gal OH4 and OH6 and a free OH3 on the preceding Glc, GlcNAc, or GalNAc. Other glycotopes were shown to not fit galectin-binding sites.<sup>93</sup> This interaction may be one of the mechanisms of hMOS immune modulation.<sup>8</sup> Many of the structures shown in Schemes 1 and 2 have clear potential for galectin recognition, marked in green. Galectins play a pivotal role in immune modulation and inflammatory control.<sup>94</sup> Therefore, many of the relatively abundant gMOS, such as 3'-GL, LNnT, LNnH, 3'-SL, and 3'-NGL (Schemes 1 and 2), have potential immune-modulating and anti-inflammatory effects through galectin interactions. Detailed studies on galectin specificity have shown, moreover, that some galectins have enhanced affinity when the Gal residue is sialylated on the 3 position, e.g., 3'-SL, while affinity for galectin 8-N is even further

enhanced for ganglioside GD3, containing a Neu5Ac( $\alpha$ 2–8)Neu5Ac( $\alpha$ 2–3)Gal epitope.<sup>93</sup> This epitope is also present on the gMOS structure DSL (Scheme 2). Specifically, a strong interaction of galectin 3-C for the Gal( $\alpha$ 1–3)Gal and GalNAc( $\alpha$ 1–3)Gal glycotopes has also been documented.<sup>93,95</sup> Elevated serum levels of galectin 3 have been associated with long-term risks in cardiovascular disease.<sup>96</sup> While human milk does not contain oligosaccharides with this glycotope, goat milk does contain a low amount of 3'- $\alpha$ -galactosyllactose (Scheme 1). Galectin 9-N and 9-C binding domains both interact strongly with poly- $\beta$ 1–3-linked Gal residues, which do not occur in human milk but are present in goat milk (Scheme 1). Where it was previously argued that hMOS might be a powerful tool to study galectin-binding kinetics and functionality,<sup>8</sup> gMOS might fill some gaps in glycotopes that are absent in hMOS, further enhancing the understanding of galectin interactions. Similarly, gMOS might be applied to study the binding of other lectins, including siglecs, selectins, and DC-SIGN, but also bacterial and viral glycan adhesion molecules. In the gut, intestinal epithelial cell-derived galectin 9 has been implicated in GOS/FOS-associated immunomodulation.<sup>97,98</sup> Galectins are involved in neutrophil regulation, and triggering of neutrophil response is implicated in necrosis in colitic inflammation.<sup>99</sup> Stimulation of galectins by hMOS or gMOS in the gut might play a role in immune modulation and reducing necrotizing effects of inflammation.

Besides galectins, interactions between oligosaccharides and toll-like receptors (TLRs) in the gut have also been shown. In some studies, oligosaccharides, including GOS, hMOS, and gMOS, have been shown to mediate TLR-mediated response.<sup>100–102</sup> In 2013, a mouse study showed that 3'-SL, which is also abundant in gMOS, interacts with TLR4, which is normally associated with LPS-mediated inflammatory response, leading to inflammation.<sup>103</sup> Later, a study using human TLR4 was unable to reproduce the effect, concluding that differences in specificity of mouse and human TLR4 might be responsible.<sup>104</sup>

**Barrier Function.** Barnett et al. studied the effect of a semi-purified gMOS-enriched fraction of goat milk<sup>34</sup> on the barrier integrity and mucin production of co-cultures of small and large intestine epithelial cells.<sup>105</sup> They showed that the gMOS-enriched fraction enhanced the intestinal epithelial barrier function. The *trans*-epithelial electrical resistance (TEER) increased significantly and in a dose-dependent manner. Although the researchers did not separately investigate the effect of other components in the gMOS-enriched fraction, it is known that lactose, glucose, and galactose have no effect on TEER as a readout of barrier integrity.<sup>18,19</sup> The gMOS did not significantly affect the metabolic flux of the epithelial cells. The expressions of the mucin-synthesizing genes for Muc2, Muc4, and Muc5AC were only slightly affected by the presence of gMOS, and total mucin production compared to controls was not different when cells were incubated with gMOS.<sup>105</sup> The conclusion was that gMOS did increase the barrier function of intestinal cells but not through adaptation of metabolic flux or increased mucin production.

In a recent publication, different GOS formulations were investigated for their effect on mucin production and expression of mucin-related genes.<sup>100</sup> This study observed that GOS with lactose had a more pronounced effect on the gene expression in cell lines than GOS that was purified of lactose. A further study using hMOS observed upregulation of mucin-production-related genes, MUC2, TFF3, and RETNLB, when LS174T

cells were incubated with 3-FL, while MUC2 and TFF3 were upregulated by lacto-*N*-triose (LNT2).<sup>106</sup> Furthermore, under challenge with tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin 13 (IL-13), and tunicamycin, differential effects of 2'-FL, 3-FL, and LNT2 were observed, supporting a direct effect of these structures on barrier-function-related production of mucin.<sup>106</sup> It should be noted that 3-FL, the most effective modulator, is not observed in goat milk, while LNT2 and 2'-FL have been found (Schemes 1 and 2); therefore, a certain level of induction of mucin production by gMOS may be expected.

A recent study using a murine NEC model observed that hMOS increased barrier function, by stimulating Muc2 production in the intestine.<sup>107</sup> This was further verified in a human gut epithelial cell line. Furthermore, it was observed that the protective effect was mediated through chaperones, particularly protein–disulfide isomerase; suppression of protein–disulfide isomerase abolished the effect of hMOS.<sup>107</sup> Lactose controls also had a noticeable effect on mucin-related gene expression. It seems that the effect of lowly digestible oligosaccharides on the mucin production is not straightforward.

**Pathogen Inhibition.** The pathogen inhibition activity of hMOS is presumed to be caused by similarity in structures between hMOS and epithelium-associated glycans.<sup>16</sup> Pathogens present lectin-like receptors that recognize a specific glycan epitope to adhere to the epithelial layer as a first step in infection. In the presence of significant levels of hMOS with similar structural elements, hMOS will bind to these receptors and, thereby, prevent adhesion.<sup>108</sup> In some cases, the hMOS block the epithelial receptor to which pathogen glycans adhere. For example, the HIV gp120 anchor protein presents high-mannose-type glycans that adhere to DC-SIGN as an adhesion mechanism for the pathogen.<sup>109</sup> DC-SIGN has affinity for high-mannose as well as fucose-containing glycans, particularly glycans containing a Le<sup>x</sup> glycotope; i.e., Fuc( $\alpha$ 1–3)[Gal $\beta$ (1–4)]GlcNAc.<sup>110</sup> Human milk contains LNFP III, with a Le<sup>x</sup> glycotope, as well as Muc1, containing glycans with Le<sup>x</sup> glycotopes that bind to DC-SIGN, thereby preventing viral adhesion.<sup>111–113</sup> There is also evidence that 3'-SL directly interacts with epithelial cells, modulating gene expression of sialyltransferases, thereby modifying epithelial glycosylation.<sup>114</sup> It has been hypothesized that the altered glycosylation might result in reduced binding of sialic-acid-specific pathogens.<sup>16</sup> The large diversity of hMOS is thought to exist because of the large diversity in pathogens.<sup>16,115</sup> It has been shown for *N*-glycans as well as hMOS that not only is a specific glycotope required for action, but also that the presentation of the epitope matters.<sup>116,117</sup> The levels of hMOS are also relevant, because some interactions require multivalency.<sup>118</sup> This can be achieved by not only highly complex, branched carbohydrates but also high levels of smaller oligosaccharides, resulting in indirect multivalency. This theory was further supported by research showing that the same functional epitope on different scaffold hMOS has different affinities for pathogen receptors.<sup>119</sup> Well-established structures related to decoy effects against pathogens are collected in Table 2. Although it has not been established as a pathogen inhibition effect, a microbial imbalance or pathogenic species is most likely involved in the development of necrotizing enterocolitis. It is worth noting that the most profound effects have been observed with disialyl-LNT (DSLNT), which has not been observed in goat milk. However, protective effects against necrotizing enterocolitis have also been shown for 2'-FL and sialylated GOS.<sup>120</sup> While human milk contains only minor levels of the GOS trisaccharides 3'-GL and 6'-GL<sup>43,121,122</sup> and no sialylated

**Table 2. Structural MOS Epitopes with Shown Pathogen Inhibition Functionalities<sup>a</sup>**

glycotope <sup>b</sup>	pathogen	possible gMOS	reference
Fuc( $\alpha$ 1–2)Gal glycotopes	<i>Campylobacter jejuni</i>	maybe	125
	<i>Candida albicans</i>	maybe	126
	norovirus	maybe	127
3-FL	<i>Escherichia coli</i>	yes	128
	<i>Salmonella fyris</i>	yes	128
Le <sup>x</sup> (LNFP III) <sup>b</sup>	HIV	maybe	111
LNT	<i>Entamoeba histolytica</i>	no	129
	Group B <i>Streptococcus</i>	no	130–132
	<i>Streptococcus pneumoniae</i>	no	133
LNFP I	<i>Pseudomonas aeruginosa</i>	no	130
	Group B <i>Streptococcus</i>	no	131,132
	<i>Streptococcus pneumoniae</i>	yes	133,134
LNnT	<i>Pseudomonas aeruginosa</i>	yes	130
	<i>Escherichia coli</i>	yes	128
	influenza B	yes	130
3'-SL	<i>Helicobacter pylori</i>	yes	135
	<i>Pseudomonas aeruginosa</i>	yes	136
	influenza A	yes	130
	<i>Salmonella fyris</i>	yes	128
6'-SL	<i>Escherichia coli</i>	yes	128
	<i>Pseudomonas aeruginosa</i>	yes	136
	<i>Pseudomonas aeruginosa</i>	yes	136
general hMOS	<i>Vibrio cholera</i>	maybe	128

<sup>a</sup>The presence of a certain glycotope in gMOS is provided. <sup>b</sup>3-FL, 3-fucosyllactose; LNT, lacto-N-tetraose; LNFP I, lacto-N-fucopentaose I; LNnT, lacto-N-neotetraose; 3'-SL, 3'-sialyllactose; and 6'-SL, 6'-sialyllactose.

GOS, goat milk does contain a wider array of GOS structures and several sialylated GOS structures (Scheme 2). These may potentially have similar effects, although the structural overlap with the sialylated GOS used in the necrotizing enterocolitis study is limited.<sup>123</sup>

Recently, it was shown that gMOS can trigger bifidobacteria to inhibit *C. jejuni* infection in an *in vitro* study, showing a form of indirect pathogen inhibition.<sup>79</sup> Two recent studies have investigated the direct antiadhesive capacity of isolated gMOS. One study observed reduced adhesion of *Escherichia coli* and *Salmonella typhimurium* to CACO-2 cells when pre-incubated with gMOS.<sup>80</sup> This was observed independent of beneficial microbiota. The second study showed the same results with *Salmonella* by green fluorescent antibodies against the *Salmonella* strain used.<sup>124</sup> Here, the effect of sialylated gMOS was shown to be stronger than that of neutral gMOS. Although not all structures in Table 2 are present in similar quantities in goat milk as in human milk, hypothetically, some functionalities may also occur with other gMOS if they present the suitable glycotopes as well as with hMOS. Further research into functionality of hMOS as well as non-human MOS would be beneficial for the development of the field.

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## ABBREVIATIONS USED

2-AA, anthranilic acid; 2-AB, 2-aminobenzamide; 2'-FL, 2'-fucosyllactose, Fuc( $\alpha$ 1–2)Gal( $\beta$ 1–4)Glc; 3-FL, 3-fucosyllactose, Gal( $\beta$ 1–4)[Fuc( $\alpha$ 1–3)]Glc; 3'-GL, 3'-galactosyllactose, Gal( $\beta$ 1–3)Gal( $\beta$ 1–4)Glc; 3'-NGL, 3'-N-glycolylneuraminylactose, Neu5Gc( $\alpha$ 2–3)Gal( $\beta$ 1–4)Glc; 3'-SHL, 3'-sialylhexosyllactose, Gal( $\beta$ 1–6)[Neu5Ac( $\alpha$ 2–3)]Gal( $\beta$ 1–4)Glc; 3'-SL, 3'-sialyllactose, Neu5Ac( $\alpha$ 2–3)Gal( $\beta$ 1–4)Glc; 4'-GL, 4'-galactosyllactose, Gal( $\beta$ 1–4)Gal( $\beta$ 1–4)Glc; 6'-GL, 6'-galactosyllactose, Gal( $\beta$ 1–6)Gal( $\beta$ 1–4)Glc; 6'-NGL, 6'-N-glycolylneuraminylactose, Neu5Gc( $\alpha$ 2–6)Gal( $\beta$ 1–4)Glc; 6'-SHL, 6'-sialylhexosyllactose, Gal( $\beta$ 1–3)[Neu5Ac( $\alpha$ 2–6)]Gal( $\beta$ 1–4)Glc; 6'-SL, 6'-sialyllactose, Neu5Ac( $\alpha$ 2–6)Gal( $\beta$ 1–4)Glc; 6'-SLN, 6'-sialyllactosamine, Neu5Ac( $\alpha$ 2–6)Gal( $\beta$ 1–4)-GlcNAc; B4GalNT,  $\beta$ -1,4-N-acetylgalactosaminyltransferase; B4GalT,  $\beta$ -galactosyltransferase; bMOS, bovine milk oligosaccharide(s); COX2, cyclooxygenase 2; DC-SIGN, dendritic cell-specific intercellular adhesion molecule; DSL, disialyllactose, Neu5Ac( $\alpha$ 2–8)Neu5Ac( $\alpha$ 2–3)Gal( $\beta$ 1–4)Glc; DSLNT, disialyllacto-N-tetraose, Neu5Ac( $\alpha$ 2–3)Gal( $\beta$ 1–3)[Neu5Ac( $\alpha$ 2–6)]GlcNAc( $\beta$ 1–3)Gal( $\beta$ 1–4)Glc; DSS, dextran sodium sulfate; FOS, fructooligosaccharide(s); Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GGTA1,  $\alpha$ -galactosyltransferase; GIT, gastrointestinal tract; Glc, glucose; GlcNAc, N-acetylglucosamine; gMOS, goat milk oligosaccharide(s); GOS, galactooligosaccharide(s); Hex, hexose; HexNAc, N-acetylhexosamine; HILIC–Q–MS, hydrophilic interaction chromatography–quadrupole–mass spectrometry; HIV,

human immunodeficiency virus; hMOS, human milk oligosaccharide(s); HPAEC–PAD, high-pressure/pH anion-exchange chromatography–pulsed amperometric detection; HPLC, high-pressure liquid chromatography; IBD, inflammatory bowel disease; IL-13, interleukin 13; IL-1 $\beta$ , interleukin 1 $\beta$ ; iLNT, isolacto-*N*-tetraose, Gal( $\beta$ 1–3)GlcNAc( $\beta$ 1–6)Gal( $\beta$ 1–4)Glc; iNOS, inducible nitric oxide synthase;  $\beta$ 1,6-*N*-acetylglucosaminyltransferase; LacdiNAC, *N,N'*-di-*N*-acetylglucosamine diamine, GalNAC( $\beta$ 1–4)GlcNAc; LC–MS, liquid chromatography–mass spectrometry; Le<sup>x</sup>, Lewis x blood group determinant; LNFP III, lacto-*N*-fucopentaose III, Gal( $\beta$ 1–4)[Fuc( $\alpha$ 1–3)]GlcNAc( $\beta$ 1–3)Gal( $\beta$ 1–4)Glc; LNH, lacto-*N*-hexaose, Gal( $\beta$ 1–4)GlcNAc( $\beta$ 1–6)[Gal( $\beta$ 1–3)GlcNAc( $\beta$ 1–3)]Gal( $\beta$ 1–4)Glc; LNnH, lacto-*N*-neohexaose, Gal( $\beta$ 1–4)GlcNAc( $\beta$ 1–6)[Gal( $\beta$ 1–4)GlcNAc( $\beta$ 1–3)]Gal( $\beta$ 1–4)Glc; LNnT, lacto-*N*-neotetraose, Gal( $\beta$ 1–4)GlcNAc( $\beta$ 1–3)Gal( $\beta$ 1–4)Glc; LNT, lacto-*N*-tetraose, Gal( $\beta$ 1–3)GlcNAc( $\beta$ 1–3)Gal( $\beta$ 1–4)Glc; LNT2, lacto-*N*-triose; LPS, lipopolysaccharide; MOS, milk oligosaccharide(s); MS, mass spectrometry; Muc, mucin; MUC2, mucin 2; NEC, necrotizing enterocolitis; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; PGC, porous graphitized carbon; SCFA, short-chain fatty acid; sMOS, sheep milk oligosaccharide(s); ST8Sia,  $\alpha$ -2,8-sialyltransferase; TEER, *trans*-epithelial electrical resistance; TFF3, trefoil factor 3; TLR, toll-like receptor; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; TNSB, trinitrobenzenesulfonic acid; UDP, uridine diphosphate;  $\alpha$ 3'-GL, 3' $\alpha$ -galactosyllactose, Gal( $\alpha$ 1–3)Gal( $\beta$ 1–4)Glc

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