Structural Determination and Daily Variations of Porcine Milk Oligosaccharides

NANNAN TAO,[†] KAREN L. OCHONICKY,[§] J. BRUCE GERMAN,[#] SHARON M. DONOVAN,[§] AND CARLITO B. LEBRILLA*,[†]

[†]Department of Chemistry and Biochemistry (School of Medicine), University of California, Davis, California 95616, [§]Division of Nutritional Sciences, University of Illinois, Urbana, Illinois 61801, and [#]Department of Food Science and Technology, University of California, Davis, California 95616

Free milk oligosaccharides (OS) are major components of mammalian milk. Swine are important agricultural species and biomedical models. Despite their importance, little is known of the OS profile of porcine milk. Herein, the porcine milk glycome was elucidated and monitored over the entire lactation period by liquid chromatography profiling and structural determination with mass spectrometry. Milk was collected from second-parity sows (*n* = 3) at farrowing and on days 1, 4, 7, and 24 of lactation. Twenty-nine distinct porcine milk oligosaccharides (pMO) were identified. The pMO are highly sialylated, which is more similar to bovine milk than human milk OS. Six fucosylated pMO were detected at low levels in porcine milk, making it more similar to human milk than bovine milk. In general, the pMO content was highest in milk collected at farrowing and day 1 of lactation, decreased during early lactation, but then rose at day 24; however, the pMO displayed different patterns of variation across lactation. In summary, porcine milk contains both acidic (sialylated) and neutral OS, but sialic acid containing OS predominate throughout lactation.

KEYWORDS: Oligosaccharide; porcine milk; mass spectrometry; LC-MS; bovine milk

INTRODUCTION

Milk oligosaccharides (OS) are relatively resistant to digestion (*1*) and contribute to the anti-infective and prebiotic activities of milk (*2, 3*). Human milk is particularly rich in OS, containing 20−23 g/L in colostrum and 7−12 g/L in mature milk (*4*). In contrast, bovine milk, which forms the basis of most infant formulas, contains only ∼$^1/_{20}$th of the OS content (*5*). There are also marked differences in the structural diversity of human milk OS (hMO) (*6−8*) and bovine milk OS (bMO) (*5, 9*). Swine are important agricultural species and are considered to be an excellent model for nutrition studies because of their digestive system physiology and anatomical structure (*10, 11*). Additionally, the developing brain structure and function resemble those of human infants (*12*). These similarities have led to an increased interest in characterizing porcine milk composition; however, little is known of the OS profile of porcine milk.

Milk OS are complex carbohydrates that are typically composed of 3−10 monosaccharides, which are covalently linked through glycosidic bonds to yield free OS with aldehyde reducing ends. Because OS are produced by the same enzymes as O-linked glycans, they tend to have structural similarities to *O*-glycans. The monomers that make up milk OS include D-glucose (Glc),

D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc), *N*-acetylneuraminic acid (NeuAc), and *N*-glycolylneuraminic acid (NeuGc) (*4, 13*). Fucose and neuraminic acids (or sialic acids) are found on the nonreducing end and are generally associated with important biological functions (*2, 3*). Fucose and sialic acids are known to bind to bacterial walls, thereby preventing binding to epithelial cells (*14*). For example, higher levels of fucosyloligosaccharides in human milk have been reported to protect against infant diarrhea (*15*). Higher concentrations of sialic acids offer a higher level of protection from infection (*15*).

Milk OS are also thought to modulate the neonatal microbiota by serving as a prebiotic (*8, 16*). The microbiota of breast- versus formula-fed infants differ (*17*) and can be influenced by the addition of synthetic prebiotics (*18, 19*). Recent work from our laboratory demonstrated that the microbiota of the breast-fed infant ferments specific human milk OS (*19−21*) For example, when five strains of bifidobacteria were tested (*Bifidobacterium longum* biovar *infantis, Bifidobacterium bifidum, Bifidobacterium longum* biovar *longum, Bifidobacterium breve,* and *Bifidobacterium adolescentis*), differences in their ability to utilize hMO for growth or to generate free sialic acid, fucose, and *N*-acetylglucosamine were noted (*19*). *B. longum* bv. *infantis* demonstrated substantial degradation of hMO, whereas other strains demonstrated moderate (*B. bifidum*) or little degradation (*B longum* bv. *longum, B. breve,* and *B. adolescentis*). Thus, HMO may selectively promote the growth of certain bifidobacteria strains, and their catabolism may result in free monosaccharides in the colonic lumen.

*Address correspondence to this author at the Department of Chemistry and Biochemistry (School of Medicine), University of California, One Shields Ave., Davis, CA 95616 [telephone (530) 752-0504; fax (530) 754-8995; e-mail cblebrilla@ucdavis.edu].

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In addition to their anti-infective and prebiotic activities within the gut, hMO can be absorbed and are excreted in the urine in breast-fed infants (*22*). As such, systemic effects have also been attributed to hMO. Due to their similarities to selectin ligands, the anti-inflammatory properties of hMOs have been tested in vitro. It has been shown that sialic acid containing OS reduce the adhesion of leukocytes to endothelial cells, an indication for an immune regulatory effect of certain hMO (*3*).

Lastly, sialic acids have important roles in the development of the infant brain; gangliosides make up 10% of the total lipid mass of brain tissue, with different numbers of negative sialic acid moieties (*23*, *24*). Neonates have a limited capacity for de novo endogenous synthesis of sialic acid (*25*). Data from neonatal models suggest that systemically administered or dietary sialic acid is readily incorporated into the developing brain (*26*) and may have functional outcomes (*27*). Approximately 80% of injected radiolabeled sialic acid was incorporated into the synaptosomal fraction of 12-day-old rat pups (*26*). A recent study conducted in piglets demonstrated that supplementary dietary sialic acid was dose-dependently associated with faster learning, higher concentrations of protein-bound sialic acid in the frontal cortex, and 2−3-fold higher mRNA levels of two learning-related genes, *GNE* and *ST8SIA4* (*27*).

The goals of the current study were to elucidate the structures of porcine milk OS (pMO) and to examine the changes in the content and composition during the complete lactation period using a HPLC-Chip/ToF system and nESI-FTICR mass spectrometry methods developed in our laboratory for human (*6*) and bovine (*5*, *9*) milks. Mass spectrometry with nanoflow liquid chromatography (nanoLC) provides a rapid quantitative method for glycan profiling. Each individual OS species was monitored to determine variation. Comparison of pMO against hMO and bMO was also performed.

MATERIALS AND METHODS

Materials. Nonporous graphitized carbon cartridges (150 mg of bed weight, 4 mL tube size) were obtained from Alltech (Deerfield, IL). Sodium borohydride (98%) was purchased from Sigma-Aldrich (St. Louis, MO). All reagents such as chloroform, acetonitrile, methanol, and alcohol were of analytical or HPLC grade.

Collection of Milk Samples. Colostrum and milk samples were collected from three second-parity sows at various intervals from within a few hours after delivery until late lactation (day 24). Milk samples were obtained by manual expression after intravenous injection of 10 units of oxytocin (Phoenix Scientific, St. Joseph, MO) and frozen at −80 °C immediately after collection.

Sample Preparation. Milk samples were totally thawed, and then 0.5 mL of deionized water was added into an equal amount of raw milk followed by mixing and centrifugation at 4000*g* for 30 min at 4 °C. After the top fat layer was removed, 4 volumes of chloroform/methanol (2v:1v) was added to the defatted milk samples. After centrifugation at 4000*g* for 30 min at 4 °C, the upper layer was carefully transferred. Two volumes of ethanol was added to the mixture, which was incubated overnight at 4 °C, followed by centrifugation at 4000*g* for 30 min at 4 °C to remove denatured protein precipitate. The supernatant (milk OS-rich fraction) was freeze-dried with a speed vacuum. Native milk OS were reduced to alditol forms by using 1.0 M sodium borohydride in water and incubating overnight at 42 °C. pMO were purified by solid-phase extraction using a nonporous graphitized carbon cartridge (GCC-SPE) and eluted with 20% acetonitrile in water (v/v) prior to mass spectrometry analysis.

Nanoelectrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (nESI-FTICR-MS) and Infrared Multiphoton Dissociation (IRMPD). Mass spectra were obtained using nESI-FTICR-MS (IonSpec Corp., Irvine, CA) equipped with a 9.4 T superconducting magnet in both positive and negative ion modes. Cone voltages were maintained at 2000−3000 V to obtain signals. Ions, which were transferred by the quadrupole ion guide prior to the ICR cell for detection, were accumulated in the hexapole. Tandem MS was
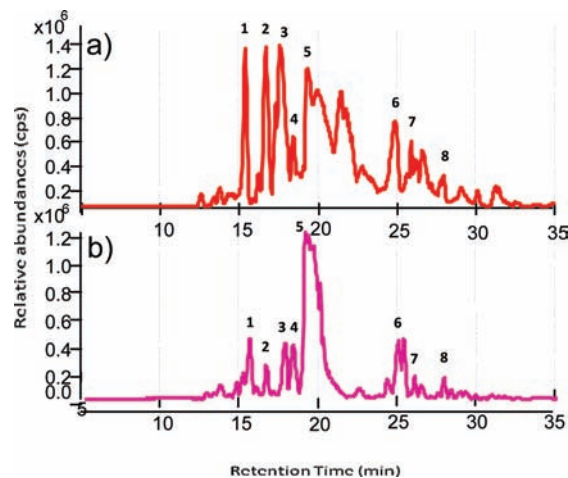
Figure 1. Comparison of high-performance liquid chromatography−chip/time-of-flight-MS chromatograms of porcine (**a**) and bovine (**b**) milk oligosaccharides.

performed with infrared multiphoton dissociation (IRMPD) with a 10.6 μm CO_2 laser (Parallax Laser Inc., Waltham, MA) by transmitting the laser beam into the ICR cell through a BaF_2 window to fragment isolated ions.

HPLC-Chip/TOF Mass Spectrometry. Milk OS fractions collected after solid-phase extraction with the graphitized carbon cartridge were analyzed using a microfluidic 6200 series HPLC-chip/TOF MS instrument (Agilent Technologies, Santa Clara, CA). The microfluidic HPLC-chip consists of an enrichment column, an LC separation column packed with porous graphitized carbon, and a nanoelectrospray tip. Separation was performed by a binary gradient: A, 3% acetonitrile in 0.1% formic acid solution; B, 90% acetonitrile in 0.1% formic acid solution. The column was initially equilibrated and eluted with the flow rate at 0.3 μL for nanopump and 4 μL for capillary pump. The 65 min gradient was programmed as follows: 2.5−20 min, 0−16% B; 20−30 min, 16−44% B; 30−35 min, increased to 100% B; 45 min, 100% B; and finally, 0% B for 20 min to equilibrate the chip column before the next sample injection. Each composition of milk OS was identified with an in-home program (Glycan Finder). Distinct compositions were identified on the basis of accurate mass, retention times, and relative abundances. The instrument performed automatic tuning using a dual nebulizer electrospray source with an automated internal calibrant consisting of an unknown fluorinated compounds delivery system, which introduced a constant flow (100 mL/min) of calibrating solution containing the internal reference masses (m/z 118.0863, 322.0481, 622.0290, 922.0098, 221.9906, 1521.9715, 1821.9523, 2121.9332, 2421.9140, and 2721.8948) in positive mode.

RESULTS AND DISCUSSION

Mass spectrometry (MS) provides a rapid, quantitative system for glycomics analysis. We have recently reported the characterization of hMO by using nanoLC and high-performance MS (*6*). The integration of nanoLC with MS in a HPLC-chip/TOF MS instrument allowed the rapid profiling of hMO and bMO in a precise and reproducible manner. The ultrahigh sensitivity of the instrumentation allows for a reduced sample requirement and the ability to detect lower abundance species. With this system, we were able to identify 40 bMO, most of which were in the sialylated forms (*9*), which doubled the number of total published bMO.

Comparison of Bovine and Porcine Milk OS. Our previous experience elucidating the OS structures in bMO (*9*) and hMO (*9*) facilitated the structural identification of pMO. **Figure 1** shows a direct comparison of the HPLC chromatograms performed on the nanoLC-chip/TOF MS of pMO (**Figure 1a**) and bMO (**Figure 1b**). On the basis of the accurate masses and retention times, we find strong similarities between bMO and pMO; the eight major OS peaks shown in the two chromatograms have

Table 1. Common Porcine Milk Oligosaccharides (OS) with Compositions and Relative Abundances[a]

	m/z (expt)[1]	m/z (cal)	Hex	Fuc	HexNAc	NeuAc	NeuGc	Retention Time (min)	Rel. Abund.[2]	Existence in BMO[4]	Proposed Structures[5]
1	490.190	490.190	2	1				11.471	○	☆	
2	506.184	506.185	3					10.429	○	★	
3	506.185	506.185	3					9.216	○	★	
4	506.186	506.185	3					15.065	○	★	
5	635.227	635.227	2			1		17.348	●	★	
6	635.227	635.227	2			1		11.67	○	★	
7	636.249	636.248	2	2				14.572	○	☆	
8	676.252	676.254	1		1	1		20.68	○	★	
9	692.238	692.249	1		1		1	12.574	○[3]	★	
10	709.264	709.264	3		1			15.304	◎	★	
11	750.289	750.291	2		2			14.598	○	★	
12	797.279	797.280	3			1		22.182	○	★	
13	797.280	797.280	3			1		19.915	○	★	
14	871.315	871.317	4		1			16.459	○	★	
15	912.347	912.343	3		2			19.692	○	★	
16	967.348	967.349	1		1	2		18.47	○	★	
17	1000.357	1000.359	3		1	1		26.043	○	★	
18	1000.358	1000.359	3		1	1		22.346	○	★	
19	1017.372	1017.375	4	1	1			14.033	○	☆	
20	1041.390	1041.386	2		2			20.637	○	★	
21	1074.400	1074.396	4		2			19.693	◎	★	
22	1162.416	1162.412	4		1	1		26.272	○	★	
23	1162.429	1162.412	4		1	1		25.784	○	★	
24	1187.439	1187.444	2	1	2			24.319	○	☆	
25	1308.475	1308.470	4	1	1	1		25.585	○	☆	
26	1365.492	1365.492	4		2	1		26.398	○	★	
27	1365.493	1365.492	4		2	1		25.185	◎	★	
28	1511.552	1511.550	4	1	2	1		24.565	○	☆	
29	1656.584	1656.587	4		2	2		28.852	○	☆	

[a] [1] expt and cal, experimental and calculated *m/z* values; Hex, hexose; Fuc, Fucose; HexNAc, *N*-acetylhexosamine; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid. [2] Relative abundances (%): black circles, >50%; concentric circles, 5−50%; open circles, <5%. [3] Trace amount or not detectable in most samples. [4] OS exist (black stars) or do not exist (white stars) in bovine milk. Proposed bovine structures are available (*5, 9*). [5] Red circles and yellow circles indicate glucose (Glc) and galactose (Gal), respectively; orange squares represent *N*-acetylglucosamine (GlcNAc); purple and yellow diamonds represent *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc), respectively; blue triangles represent fucose (Fuc).

complementary retention times and masses. For example, with bMO, peak 1 was determined to be lacto-*N*-neotetraose (LNnT) with *m/z* 710.275 [3Hex + 1HexNAc + H]⁺ (*5, 9*). The corresponding peak in the pMO chromatogram can be assigned to the same structure, LNnT, because they share the same retention times and accurate masses. Structure and composition of LNnT are listed as oligosaccharide-10 (**OS-10**) in **Table 1**. The broad

peak assigned as peak 5 with a retention time at around 19−21 min is another example. This OS is found in both bMO and pMO and corresponds to sialyllactose (NeuAc α(2−3)Galβ(1−4)Glc) (SL) (*5*), designated **OS-5** (**Table 1**). Both bovine and porcine milks have SL (molecular mass 635.227) as the most abundant MO, which comprised > 50% in total pMO followed by three common OS with *m/z* 709.264 (**OS-10**), 1074.400 (**OS-21**), and
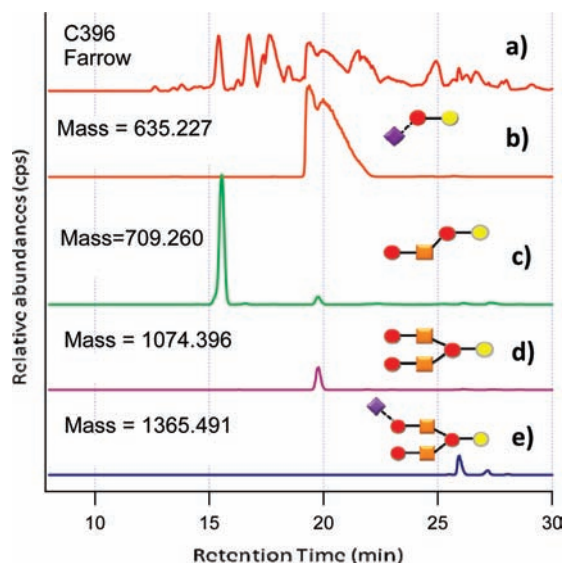
Figure 2. Base peak chromatogram (**a**) and extracted ion chromatograms (XIC) of selected ions (**b**−**e**) from a milk sample collected at farrowing from a single sow.

1365.493 (**OS-27**) with intensities of 5−50% (**Table 1**). Tandem MS of the compounds was used to provide further confirmation that the compounds are identical (data not shown).

Characterization of Porcine Milk Oligosaccharides. Extracted ion chromatograms of the most abundant OS in porcine colostrum are shown in **Figure 2**. In general, there was significantly less structural diversity in the pMO compared to hMO as most masses yielded only one or two isomers. A similar lack of OS structural diversity is present in bovine milk as well (*5*). In contrast, up to eight isomers can be observed for some hMO (*7*).

A summary of the pMO carbohydrate composition and retention times is listed in **Table 1**. Approximately 29 different OS were identified in porcine milk, compared to 40 in bovine milk (*9*) and > 130 in human milk (*3*). Under the conditions used in these experiments, acidic OS elute after approximately 20 min; SL has a retention time around 20 min. Another major anionic species has the retention time of around 26 min and corresponds to a bMO structure, monosialyllacto-*N*-neohexaose (SLNnH) (*5*). Similar to bMO, pMO is composed primarily of anionic OS. Over 70% of the OS are composed of sialylated OS in both bMO and pMO, whereas for humans this value is between 5 and 20% (*7*). Six fucosylated OS species were observed in pMO, albeit at very low abundance. Total fucosylation is around 1% of the OS abundance in porcine colostrum and even less in mature porcine milk. Compared with up to 70% fucosylation of hMO, there is little or no fucosylated OS in the milk of domestic animals, including bovine (*5*). Only one NeuGc-linked OS, $\alpha(2-6)$NeuGc-lactosamine, was present in porcine colostrum. It was undetectable in mature porcine milk. Thus, compared to bovine milk, there are many fewer NeuGc-linked OS present in porcine milk.

Changes in Porcine Milk Oligosaccharides across Lactation. HPLC chromatograms of pMO from colostrum and milk collected at farrowing and days 1, 4, 7, and 24 of lactation from the same sow are shown in **Figure 3**. The pMO peak at 15.5 min (see also peak 1, **Figure 1**; **OS-10**, **Table 1**) corresponds to LNnT, which varies across lactation. LNnT is also a predominant OS in both bMO (*5*) and hMO (*7*). To illustrate the relative abundances of the major OS species in porcine milk, three milking points were chosen to represent early (farrow), mid (day 4), and late lactation (day 24) (**Figure 4**). SL, with *m/z* at 635.2 (**OS-5**, **Table 1**), was
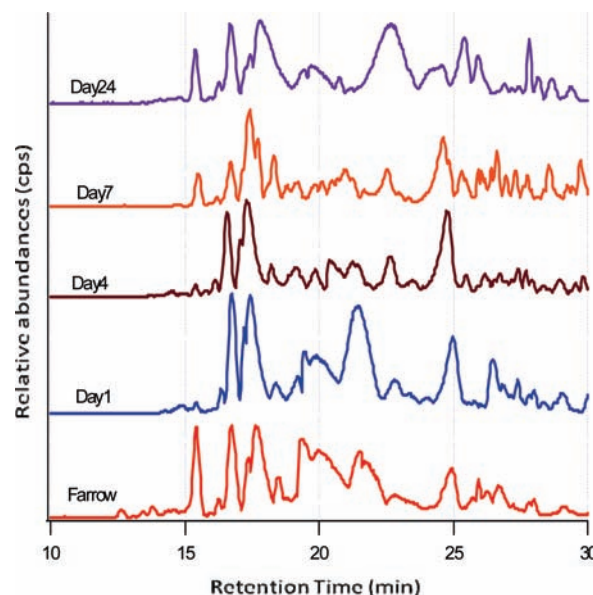

Figure 3. High-performance liquid chromatography−chip/time-of-flight-MS chromatograms of porcine milk oligosaccharides in samples collected at farrowing and days 1, 4, 7, and 24 of lactation from a single sow.
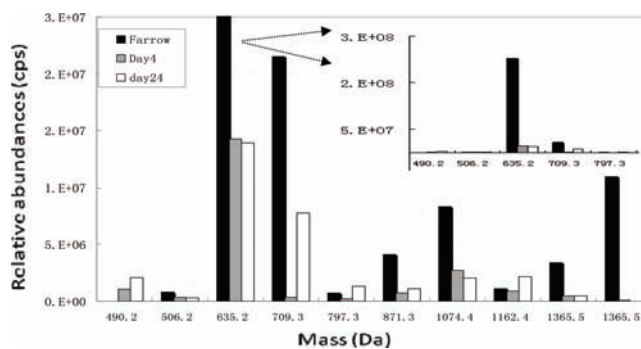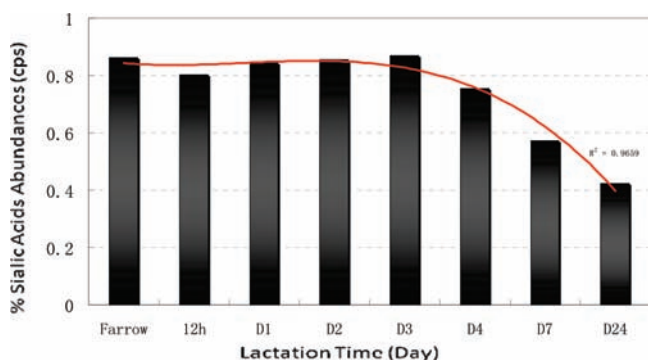

Figure 4. Changes in oligosaccharide abundances for several oligosaccharide masses in samples collected at farrowing and days 4 and 24 of lactation from a single sow.

clearly the most dominant species (**Figure 4**, inset), with relative abundances 10 times higher than the next largest OS peak. The figure also shows that all major peaks decrease significantly from farrowing to midlactation and some increase at late lactation.

To illustrate more clearly the behavior of the most abundant species, **Figure 5** shows selected OS ion abundances of six of the predominant pMO across lactation. The left panels (**a**−**c**) represent neutral OS, whereas the right panels (**d**−**f**) represent sialylated OS. In general, there is a significant decline in OS during the first few days of lactation. LNnT decreases significantly (**Figure 5a**) as well as LNnH *m/z* 1074.398 (**Figure 5c**), SL (**Figure 5d**) and a large (as yet unidentified) sialylated OS (**Figure 5f**). However, there are significant variations in the behavior of individual OS. The patterns of LNnT (**Figure 5a**), LNH (**Figure 5c**), and SL (**Figure 5d**) and the unidentified sialylated OS (**Figure 5f**) are very similar, with a significant drop during the first few days and an increase at day 24. The temporal change of these OS components are in agreement with Nakamura et al. (*28*), who reported that the milk OS concentrations increase during late lactation. However, there are other pMO, such as that shown in **Figure 5b**, that do not follow the same behavior, whereas another (**Figure 5e**) increased to a greater degree at day 24 than LNnT, LNH, and SL. To determine whether variations among individuals were present, the milk samples of the two other sows
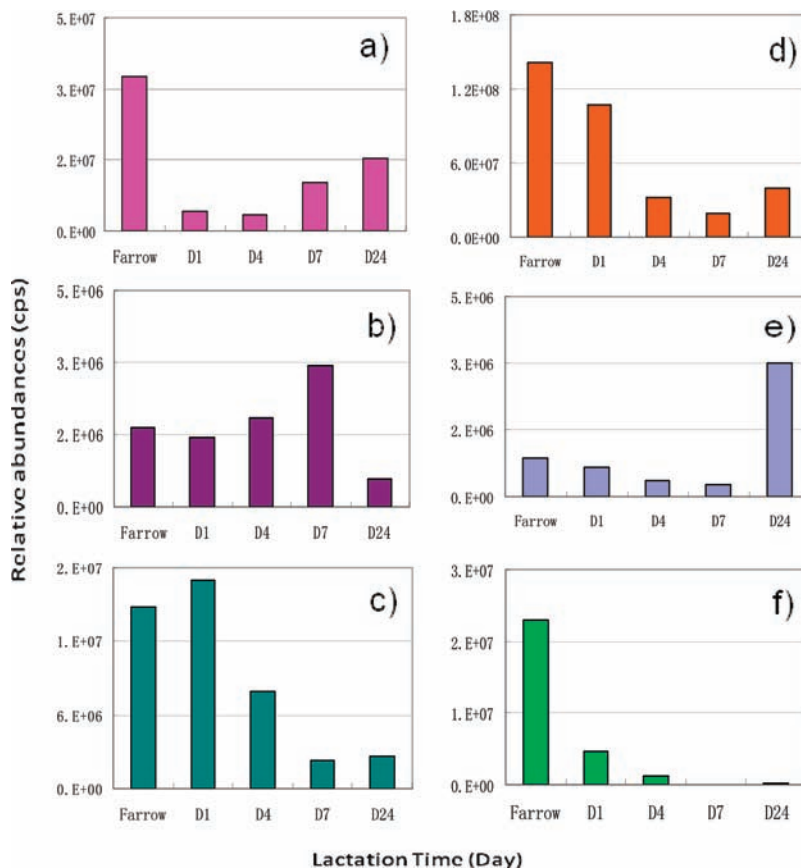
Figure 5. Variations in oligosaccharide abundances across lactation in samples collected from three sows: (**a**) 732 mass; (**b**) 871 mass; (**c**) 1074 mass; (**d**) 635 mass; (**e**) 797 mass; (**f**)1365 mass. Panels **a**−**c** present neutral OS, and panels **d**−**f** are sialylated OS.

Figure 6. Percent changes in abundances of sialylated oligosaccharides in colostrum and milk samples from days 4 and 24.

were studied. Similar patterns of individual OS species from early to late lactation were observed (data not shown).

Because sialylated OS appear to predominate in pig milk, the relative amount of sialylation of OS was determined across lactation. Shown in **Figure 6** is the total abundance of sialylated OS relative to total OS (sialylated plus neutral). The total proportion of sialylated OS content declines from 80% at farrowing to 60% in early lactation (days 4−7) to approximately 40% at late lactation (day 24) (**AS**). This pattern resembles the pattern reported in bMO (5); however, in that publication only milks from early and midlactation were studied due to the longer lactation duration in the cow. This OS pattern differs from that of hMO, in which sialylation is less abundant (~10−25% of total OS) and remains relatively constant throughout lactation (7). Both bMO and pMO have SL as its most abundant component,

whereas SL is a minor component in human milk. hMO are highly fucosylated, accounting for up to 70% of the OS species (7). Whereas only 1% of pMO was fucosylated, this represents a significant amount. In contrast, we previously detected no fucosylated OS in bovine milk (5, 9).

DISCUSSION

Milk OS have at least two important potential functions: prevention of pathogen binding to the intestinal epithelial and stimulation of growth of beneficial bacterial (29). Milk OS have been reported to differ among species (30) and within human populations (31). The ratio of neutral-to-anionic (acidic) hMO is ~80:20, whereas the opposite ratio is observed in bMO. hMO are highly fucosylated, whereas ~70% of bMO are sialylated and no fucosylated bMO are present (5). pMO resemble bMO in being predominately sialylated, but unlike bMO, six fucose-containing pMO were detected, suggesting a closer relationship between pMO and hMO than between bMO and hMO. However, the abundance and complexity of fucosylated OS in porcine milk is low relative to human milk.

There are OS that are common to hMO, bMO, and pMO. LNnT is the major component of hMO and is also abundant in bMO and pMO. LNnT has been shown to be a prebiotic that stimulates the growth of the beneficial *Bifidobacterium* (19−21) and is one of a few OS that increases during the late lactation period.

The mucosal surfaces of enterocytes within the mammalian intestine contain glycoconjugates that participate in cellular interactions, communication with the cell's surroundings, and may be exploited as receptor analogues for pathogens (32). Oligosaccharides found within colostrum and milk potentially may serve as receptors for pathogens, thus inhibiting their

attachment to mucosal surfaces (*29*). Therefore, a relationship has been proposed between the structure and function of oligosaccharides and their ability to decrease enteric infection (*29*).

Many viruses are dependent upon sialic acids for binding to cells. For example, rotavirus (RV) cell attachment and entry are considered as either dependent on or independent of the presence of sialic acids, for example, **N**-acetylneuraminic acid (Neu5Ac), in cell-surface glycoconjugates such as gangliosides (*33*). This relationship between virus and sialic acids has provided the basis for the now widely accepted RV classification into sialidase-sensitive and sialidase-insensitive strains. Human rotaviruses have been typically classified as sialidase-insensitive, whereas the infectivity of RV that commonly infects calves and piglets is sialic acid-dependent. Their infectivity can be blocked by neuraminidase treatment (*33*). Thus, the high concentrations of sialylated-OS in bovine and porcine milks may confer protection for the neonates of these species.

However, data obtained from a recent study suggest that the commonly used RV classification into sialidase-sensitive and sialidase-insensitive strains does not encapsulate the nature of human RVWa interactions with its host cell receptors (*34*). Neuraminidase treatment of Wa generated novel cellular receptors leading to increased infectivity, suggesting that Wa virus is not sialic acid independent, contrary to the widely accepted paradigm (*33, 34*). The data of Haselhorst and colleagues (*34*) suggest that a RV classification system based on ganglioside specificity rather than sialidase sensitivity may be required.

In addition, the type of fucosylated hMO may affect the ability to avoid infection (*29, 35*). There are reports, for example, that fucosylated OS inhibit diarrhea caused by the action of the heat-stable toxins of *Escherichia coli*, *Campylobacter jejune*, *Calicivirus*, and others (*15, 35−37*).

In summary, the HPLC-chip/TOF MS system allowed for the identification of nearly 30 different OS species in porcine milk. The predominant OS in porcine milk as in bovine milk was SL, possibly conferring protection of neonates against infection. An understanding of the anti-infective properties of porcine milk OS (pMO) is important given the reported increase in antibiotic-resistant microbes in healthy pigs (*38*) and recommendations against the use of low-dose antibiotics as growth promoters in the Europe Union (*39*).

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