A profile of sphingolipids and related compounds tentatively identified in yak milk

S. Qu,* G. Barrett-Wilt,† L. M. Fonseca,*‡§ and S. A. Rankin*¹

*Department of Food Science, and

†Biotechnology Center, Mass Spectrometry/Proteomics Facility, University of Wisconsin–Madison 53706

‡Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brasília BEX 18183-12-3, Brazil

§School of Veterinary Medicine, Universidade Federal de Minas Gerais, Belo Horizonte MG 31270-901, Brazil

ABSTRACT

This work characterized a fraction of constituents in yak milk within the realm of approximately 1,000 to 3,000 Da using matrix-assisted laser desorption/ionization (MALDI) time-of-flight mass spectrometry. Eleven samples of yak milk powder from the Sichuan province of China were received by the Department of Food Science, University of Wisconsin–Madison, and stored at room temperature until analysis. Sample preparation involved delipidation and deproteinization of yak milk samples and cold ethanol precipitation. Subsequently, MALDI time-of-flight mass spectrometry was performed in positive ion, reflector mode (AB Sciex TOF/TOF 4800 MALDI; AB Sciex, Foster City, CA). The instrument was first calibrated with the manufacturer's 6-peptide mixture, and each spectrum was internally calibrated using the accurate mass of ACTH Fragment 18–39 standard peptide (protonated mass at m/z 2464.199) present in each sample. Laser power was adjusted for the calibration standards and for each sample so that the signal obtained for the most-abundant ion in each spectrum could be maximized, or kept below $\sim 2 \times 10^4$ to preserve spectral quality. Structure and name based on mass were matched using the Metlin metabolite database (<https://metlin.scripps.edu/index.php>). Results of the current work for yak milk powder showed a large variety of sphingolipid structures with clusters around 1,200, 1,600, and 2,000 Da. The profiling matched several glycosphingolipids, such as gangliosides GA1, GD1a, GD1b, GD3, GM1, GM2, GM3, and GT2 and several other unique moieties, including deaminated neuraminic acid (KDN) oligosaccharides, and fucose containing gangliosides. Matrix preparation

and MALDI time-of-flight parameters were important factors established in this work to allow high resolution profiling of complex sphingolipids in yak powder milk.

Key words: yak milk, sphingolipid, ganglioside

INTRODUCTION

Milk contains a wide array of complex constituents important for the health of the mammalian offspring. Such constituents include the major components such as protein, milkfat, and lactose as well as some relatively low-molecular-weight, biologically important compounds including oligosaccharides, phospholipids, and sphingolipids (SL). Although the functions of these latter, minor compounds are not fully understood, they are thought to serve various important metabolic and functional roles to the imbiber beyond merely providing calories. The various roles of these compounds include functioning as prebiotics, prevention adhesion of pathogens to the human epithelial cells (Niñonuevo and Lebrilla, 2009), and functioning in cell membrane and neuron development as well as in various roles in the development of the immune system (O'Hara and Shanahan, 2006). For example, some of these compounds benefit human health as prebiotics through such pathways as the conversion of exogenously acquired L-fucose to guanosine diphosphate-L-fucose, which is then incorporated into multiple surface capsular polysaccharides and glycoproteins important for human symbionts (Coyne et al., 2005).

Structurally, these compounds routinely contain carbohydrate oligomers composed of various monosaccharide subunits bonded through covalent glycosidic linkages with sugar moieties or conjugated to other biomolecules, such as proteins and lipids. The glycan moieties of glycoproteins are a major class of oligosaccharides attached to proteins via amide or acyl linkages. Sphingolipids are considered a type of lipid material composed of sphingoid bases and aliphatic amino alcohols including sphingosine (2-amino-4-octadecene-1,3-

Received September 22, 2015.

Accepted February 20, 2016.

¹Corresponding author: sarankin@wisc.edu

diol). As a result of these complexities, complete identification and nomenclature of these compounds is notably complex and often may defy complete, positive molecular characterization.

In general, SL are thought to be concentrated in the milkfat globule bilayer membrane, primarily associated with the outer leaflet of the membrane. Although not thought to serve a direct role in human nutrition, the digestive products of SL are regarded as demonstrating substantial effects on various systems involving cell regulation including the development and prevention of disease (see Vesper et al., 1999). Although additional studies are in order, beneficial effects appear to be significant in the realm of gastrointestinal health (Rueda et al., 1998), cancer prevention (Parodi, 1997), and atherosclerosis and have prompted interests in the commercial preparation of milkfat globule membrane as a food ingredient (Spitsberg, 2005). We propose that an improved understanding of the molecular structure

of SL in diverse foods will aid the use of such a food fraction for human health.

Yak is a bovid species found throughout the Himalayan region of south central Asia, the Tibetan Plateau, and as far as Mongolia and Russia. Yaks are adapted to the extreme conditions of high altitudes and climate of these regions (Medhammar et al., 2012) and provide an almost singular, sustaining food source for the people who live in these harsh conditions. Yak milk and its derivatives, such as butter and fermented products, are consumed in these regions, providing a sustaining dietary source of health-imparting nutrients, calories, and compositional factors, such as protein and minerals. Given the unique environmental conditions wherein the yak has evolved and the ability of yak milk to act as a significant source of nutrition for many of the Tibetan peoples in this area, we suggest that yak milk may have some unique components or component profiles that influence human health, namely SL pro-

Figure 1. Representative mass spectrum of matrix-assisted laser desorption/ionization time-of-flight analysis of yak milk. m/z = mass-to-charge ratio. Color version available online.

files. With the proliferation of mass spectral databases accessible to the scientific community and the ability to generate high resolution separation and resolution of both molecular and ionic species, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a method of choice for initial profiling of such complex materials. Thus, the objective of this work was to characterize SL compounds found in yak milk using MALDI-TOF MS.

MATERIALS AND METHODS

Yak Milk Samples

Whole yak milk powder was manufactured in a dairy plant located in the Hongyuan country, Sichuan, China,

and packaged using a nitrogen gas flush with multilayer Mylar film packaging. Eleven samples were obtained during the milking season and sent to the Department of Food Science, University of Wisconsin–Madison, for analysis. Samples were stored at room temperature ($\sim 25^{\circ}\text{C}$) for no more than 3 mo until analyses were completed. No attempts were made to characterize the degree to which oxidation may or may not have influenced sample integrity; however, a 3-mo storage time at room temperature is very much in harmony with typical conditions for storage of dried dairy powders.

Sample Preparation

Powdered milk samples were dissolved in microfiltered water (resistance of 18 M Ω) at 10% (wt/vol).

a) $\text{C}_{51}\text{H}_{78}\text{O}_{15}$

Glycerolipids, glycosyldiradylglycerols, glycosyldiacylglycerols

b) $\text{C}_{54}\text{H}_{105}\text{NO}_{26}\text{P}_2$

Sphingolipids, phosphosphingolipids, ceramide phosphoinositols

c) $\text{C}_{69}\text{H}_{123}\text{N}_3\text{O}_{31}$

Sphingolipids, acidic glycosphingolipids, gangliosides

d) $\text{C}_{84}\text{H}_{150}\text{N}_4\text{O}_{38}$

Sphingolipids, neutral glycosphingolipids, GalNAc β 1-3Gal α 1-3Gal β 1-4Glc- (Isoglobo series)

Figure 2. Examples of some lipid- and oligosaccharide-containing compounds tentatively found in yak milk using matrix-assisted laser desorption/ionization time-of-flight analysis.

Table 1. Glycans tentatively identified in yak milk as analyzed by matrix-assisted laser desorption/ionization time-of-flight analysis¹

<i>m/z</i> ²	Systematic name	Molecular formula	Relative mass error (ppm)	Class of compound, subclasses classes
953.5233 [M + Na] ⁺	1-(3Z,6Z,9Z,12Z,15Z-octadecapentaenoyl)-2-(6Z,9Z,12Z,15Z-octadecetraenoyl)-3-O-(6'-O-α-D-galactosyl-β-D-galactosyl)-sn-glycerol	C ₅₁ H ₇₈ O ₁₅	4	Glycerolipids, glycosyl-diacylglycerols, glycosyl-diacylglycerols
1,246.6473 [M + H] ⁺	N-(2-hydroxyhexadecanoyl)-4-R-hydroxyicosasphinganine-1-O-[myo-inositol-1-phosphoryl-6-D-mannopyranosyl-α1-2-myoinositol-1-phosphate]	C ₅₄ H ₁₀₅ NO ₂₆ P ₂	2	Sphingolipids, phosphosphingolipids, ceramide phosphoinositols
1,252.6133 [M + K] ⁺	N-(octadecanoyl)-sphinganine-1-O-[myo-inositol-1-phosphoryl-6-D-mannopyranosyl-α1-2-myoinositol-1-phosphate]	C ₅₄ H ₁₀₅ NO ₂₄ P ₂	17	Sphingolipids, phosphosphingolipids, ceramide phosphoinositols
1,264.7542 [M + K] ⁺	Galα1-3(Fuco1-2)Galβ1-4Glcβ-Cer(d18:1/20:0)	C ₆₂ H ₁₁₅ NO ₂₂	9	Sphingolipids, neutral glycosphingolipids, Galβ1-4GlcNAcβ1-3Galβ1-4Glc- (neolacto series)
1,377.8383 [M + K] ⁺	Galα1-3(GalNAcβ1-4)Galβ1-4Glcβ-Cer(d18:1/24:0)	C ₆₈ H ₁₂₆ N ₂ O ₂₃	40	Sphingolipids, neutral glycosphingolipids, Galβ1-4GlcNAcβ1-3Galβ1-4Glc- (neolacto series)
1,382.8018 [M + Na] ⁺	Fuco1-2Galα1-3Galα1-4Galβ1-4Glcβ-Cer(d18:1/18:0)	C ₆₆ H ₁₂₁ NO ₂₇	48	Sphingolipids, neutral glycosphingolipids, GalNAcβ1-3Galα1-4Galβ1-4Glc- (globoseries)
1,474.8628 [M + H] ⁺	Ganglioside GD3 (d18:0/18:0) ³	C ₇₀ H ₁₂₇ N ₃ O ₂₉	7	Sphingolipids, acidic glycosphingolipids, gangliosides
1,490.8213 [M + H] ⁺	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ-Cer(d18:1/14:0) ⁴	C ₆₉ H ₁₂₃ N ₃ O ₃₁	22	Sphingolipids, acidic glycosphingolipids, gangliosides
1,490.9069 [M + Na] ⁺	Ganglioside GM2 (d18:1/24:0) [GalNAc-β1- > 4(Neu5Ac-α2- > 3)Gal-β1- > 4Glc-β1- > 1'Cer]	C ₇₃ H ₁₃₃ N ₃ O ₂₆	6	Sphingolipids, acidic glycosphingolipids, gangliosides
1,512.8033 [M + Na] ⁺	Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ-Cer(d18:1/14:0)	C ₆₉ H ₁₂₃ N ₃ O ₃₁	15	Sphingolipids, acidic glycosphingolipids, gangliosides
1,522.8604 [M + Na] ⁺	Ganglioside GD3 (d18:1/20:0) ⁵ [α-N-acetylneuraminyl-2,8-α-N-acetylneuraminyl-2,3-β-D-galactosyl-1,4-β-glucosyl-1,1'-ceramide]	C ₇₂ H ₁₂₉ N ₃ O ₂₉	22	Sphingolipids, acidic glycosphingolipids, gangliosides
1,537.8755 [M + K] ⁺	Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Cer(d18:1/15Z))	C ₇₄ H ₁₃₄ N ₂ O ₂₈	25	Sphingolipids, neutral glycosphingolipids, Galβ1-4GlcNAcβ1-3Galβ1-4Glc- (neolacto series)
1,589.9513 [M + H] ⁺	KDNα2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Cer(d18:1/24:0)	C ₇₇ H ₁₄₀ N ₂ O ₃₁	12	Sphingolipids, acidic glycosphingolipids, gangliosides
1,601.9513 [M + H] ⁺	Ganglioside GM1 (d18:0/22:1(13Z)) ⁶	C ₇₈ H ₁₄₀ N ₂ O ₃₁	13	Sphingolipids, acidic glycosphingolipids, gangliosides
1,606.9333 [M + K] ⁺	GalNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Cer(d18:1/26:1(17Z))	C ₇₈ H ₁₄₁ N ₃ O ₂₈	46	Sphingolipids, neutral glycosphingolipids, Galβ1-4GlcNAcβ1-3Galβ1-4Glc- (neolacto series)
1,617.9826 [M + H] ⁺	KDNα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Cer(d18:1/26:0)	C ₇₉ H ₁₄₄ N ₂ O ₃₁	36	Sphingolipids, acidic glycosphingolipids, gangliosides
1,631.9982 [M + H] ⁺	Fuco1-2Galβ1-4(Fuco1-3)GlcNAcβ1-3Galβ1-4Glcβ-Cer(d18:1/24:0)	C ₈₀ H ₁₄₆ N ₂ O ₃₁	29	Sphingolipids, neutral glycosphingolipids, Galβ1-4GlcNAcβ1-3Galβ1-4Glc- (neolacto series)
1,669.8772 [M + Na] ⁺	Ganglioside GD2 (d18:1/16:0) ⁷ [GalNAc-β1- > 4(NeuAc-α2- > 8NeuAc2- > 3)Gal-β1- > 4Glc-β1- > 1'Cer; GalNAc-β1- > 4(NeuAc-α2- > 8NeuAc2- > 3)LacCer]	C ₇₆ H ₁₃₄ N ₄ O ₃₄	4	Sphingolipids, acidic glycosphingolipids, gangliosides

Continued

Table 1 (Continued). Glycans tentatively identified in yak milk as analyzed by matrix-assisted laser desorption/ionization time-of-flight analysis¹

m/z^2	Systematic name	Molecular formula	Relative mass error (ppm)	Class of compound, subclasses classes
1,683.9292 [M + Na] ⁺	GalNAco1-3GalNAcβ1-3(GalNAcβ1-4)Galα1-4Galβ1-4Glcβ-Cer(d18:1/18:0)	C ₇₈ H ₁₄₀ N ₄ O ₃₃	11	Sphingolipids, neutral glycosphingolipids, GalNAcβ1-3Galα1-4Galβ1-4Glc- (globo series)
1,684.9286 [M + K] ⁺	NeuGco2-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Cer(d18:1/24:0)	C ₇₉ H ₁₄₃ N ₃ O ₃₂	22	Sphingolipids, acidic glycosphingolipids, gangliosides
1,700.9204 [M + H] ⁺	Galα1-3Galα1-3Galα1-3Galα1-3Galβ1-4Glcβ-Cer(d18:1/18:0)	C ₇₈ H ₁₄₁ NO ₃₈	46	Sphingolipids, neutral glycosphingolipids, GalNAcβ1-3Galα1-4Galβ1-4Glc- (globo series)
1,718.0099 [M + H] ⁺	GalNAco1-3GalNAcβ1-3Galα1-3(GlcNAcβ1-6)Galβ1-4Glcβ-Cer(d18:1/22:0)	C ₈₂ H ₁₄₈ N ₄ O ₃₃	8	Sphingolipids > neutral glycosphingolipids, GalNAcβ1-3Galα1-3Galβ1-4Glc- (isogloblo series)
1,738.9473 [M + H] ⁺	Galβ1-4(Fuco1-3)GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Cer(d18:1/16:0)	C ₈₀ H ₁₄₃ N ₃ O ₃₇	3	Sphingolipids, neutral glycosphingolipids, Galβ1-4GlcNAcβ1-3Galβ1-4Glc- (neolacto series)
1,744.0255 [M + H] ⁺	GalNAco1-3GalNAcβ1-3Galα1-3(GlcNAcβ1-6)Galβ1-4Glcβ-Cer(d18:1/24:1(15Z))	C ₈₄ H ₁₅₀ N ₄ O ₃₃	2	Sphingolipids, neutral glycosphingolipids, GalNAcβ1-3Galα1-3Galβ1-4Glc- (isogloblo series)
1,755.9755 [M + Na] ⁺	KDNα2-3Galβ1-4(Fuco1-3)GlcNAcβ1-3Galβ1-4Glcβ-Cer(d18:1/24:1(15Z))	C ₈₃ H ₁₄₈ N ₂ O ₃₅	2	Sphingolipids, acidic glycosphingolipids, gangliosides
1,797.9763 [M + K] ⁺	GalNAc-β1- > 4(NeuAc-α2- > 8NeuAc2- > 3)Gal-β1- > 4Glc-β1- > 1'Cer [ganglioside GD2 (d18:1/24:0)]	C ₈₄ H ₁₅₀ N ₄ O ₃₄	24	Sphingolipids, acidic glycosphingolipids, gangliosides
1,798.9967 [M + K] ⁺	Ganglioside GD2 (d18:0/24:0) ⁸	C ₈₅ H ₁₅₃ N ₃ O ₃₄	13	Sphingolipids, acidic glycosphingolipids, gangliosides
1,799.9765 [M + Na] ⁺	GalNAcβ1-4(NeuAcα2-3)Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ-Cer(d18:1/20:0)	C ₈₃ H ₁₄₈ N ₄ O ₃₆	20	Sphingolipids, acidic glycosphingolipids, gangliosides
1,824.0001 [M + H] ⁺	GalNAco1-3GalNAcβ1-3Galα1-3(Galβ1-4GlcNAcβ1-6)Galβ1-4Glcβ-Cer(d18:1/18:0)	C ₈₄ H ₁₅₀ N ₄ O ₃₈	16	Sphingolipids, neutral glycosphingolipids, GalNAcβ1-3Galα1-3Galβ1-4Glc- (isogloblo series)
1,853.0154 [M + H] ⁺	KDNα2-3Galβ1-3(NeuAcα2-6)GalNAcβ1-4Galβ1-4Glcβ-Cer(d18:1/22:0)	C ₈₆ H ₁₅₃ N ₃ O ₃₉	40	Sphingolipids, acidic glycosphingolipids, gangliosides
1,881.0467 [M + H] ⁺	KDNα2-3Galβ1-3(NeuAcα2-6)GalNAcβ1-4Galβ1-4Glcβ-Cer(d18:1/24:0)	C ₈₈ H ₁₅₇ N ₃ O ₃₉	24	Sphingolipids, acidic glycosphingolipids, gangliosides
1,907.0623 [M + H] ⁺	Ganglioside GD1b (d18:1/23:0) ⁹	C ₉₀ H ₁₅₉ N ₃ O ₃₉	22	Sphingolipids, acidic glycosphingolipids, gangliosides
1,946.0345 [M + Na] ⁺	NeuAcα2-3Galβ1-3(Fuco1-4)GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4Glcβ-Cer(d18:1/20:0)	C ₈₉ H ₁₅₈ N ₄ O ₄₀	17	Sphingolipids, acidic glycosphingolipids, gangliosides
1,961.0705 [M + Na] ⁺	NeuAcα2-3Galβ1-4(Fuco1-3)GlcNAcβ1-3Galβ1-4Glcβ-Cer(d18:1/24:0)	C ₉₁ H ₁₆₃ N ₃ O ₄₀	20	Sphingolipids, acidic glycosphingolipids, gangliosides
1,973.0828 [M + H] ⁺	Galα1-3Galα1-3Galα1-3Galα1-3Galα1-4Galβ1-4Glcβ-Cer(d18:1/26:1(17Z))	C ₉₂ H ₁₆₅ NO ₄₃	3	Sphingolipids, neutral glycosphingolipids, GalNAcβ1-3Galα1-4Galβ1-4Glc- (globo series)
1,975.0246 [M + Na] ⁺	GalNAcβ1-4(NeuAcα2-3)Galβ1-3GlcNAcβ1-3(GalNAcβ1-4)Galβ1-4Glcβ-Cer(d18:1/18:0)	C ₈₉ H ₁₅₇ N ₃ O ₄₁	7	Sphingolipids, acidic glycosphingolipids, gangliosides
2,020.0736 [M + H] ⁺	Ganglioside GT2 (d18:1/22:1(13Z)) ¹⁰	C ₉₄ H ₁₆₂ N ₄ O ₄₂	32	Sphingolipids, acidic glycosphingolipids, gangliosides
2,036.1049 [M + H] ⁺	Ganglioside GT2 (d18:1/23:0) ¹¹	C ₉₅ H ₁₆₆ N ₄ O ₄₂	15	Sphingolipids, acidic glycosphingolipids, gangliosides
2,042.1155 [M + H] ⁺	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Cer(d18:1/22:0)	C ₉₄ H ₁₆₈ N ₄ O ₄₃	9	Sphingolipids, neutral glycosphingolipids, Galβ1-4GlcNAcβ1-3Galβ1-4Glc- (neolacto series)

Continued

Table 1 (Continued). Glycans tentatively identified in yak milk as analyzed by matrix-assisted laser desorption/ionization time-of-flight analysis¹

m/z^2	Systematic name	Molecular formula	Relative mass error (ppm)	Class of compound, subclasses classes
2,101.0435 [M + H] ⁺	NeuAco2-8NeuAco2-3Gal31-3(NeuAco2-6)GalNAc31-4Gal31-4Glc3-Cer(d18:1/16:0)	C ₉₃ H ₁₆₁ N ₅ O ₄₇	34	Sphingolipids, acidic glycosphingolipids, gangliosides
2,133.1553 [M + Na] ⁺	GalNAco1-3GalNAco31-3Galo1-3(GalNAco31-4Gal31-4GlcNAco31-6)Gal31-4Glc3-Cer(d18:1/24:0)	C ₉₈ H ₁₇₅ N ₅ O ₄₃	30	Sphingolipids, neutral glycosphingolipids, GalNAc31-3Galo1-3Gal31-4Glc- (isoglobosin series)
2,172.1785 [M + H] ⁺	GalNAco1-3(Fuco1-2)Gal31-3GalNAco1-3(Fuco1-2)Gal31-4GlcNAco31-3Gal31-4Glc3-Cer(d18:1/22:0)	C ₁₀₀ H ₁₇₅ N ₄ O ₄₆	9	Sphingolipids, neutral glycosphingolipids, Gal31-4GlcNAco31-3Gal31-4Glc- (neolactosin series)
2,187.1530 [M + H] ⁺	NeuGco2-3Gal31-4GlcNAco31-3(GlcNAco31-6)Gal31-4GlcNAco31-3Gal31-4Glc3-Cer(d18:1/22:0)	C ₉₉ H ₁₇₅ N ₅ O ₄₇	19	Sphingolipids, acidic glycosphingolipids, gangliosides
2,205.1191 [M + K] ⁺	GalNAco31-4(NeuGco2-3)Gal31-3GalNAco31-4(NeuAco2-3)Gal31-4Glc3-Cer(d18:1/17:0)	C ₁₀₀ H ₁₇₅ N ₅ O ₄₅	25	Sphingolipids, acidic glycosphingolipids, gangliosides
2,254.1816 [M + Na] ⁺	Galo1-3Gal31-4GlcNAco31-3Gal31-4GlcNAco31-3Gal31-4GlcNAco31-3Gal31-4Glc3-Cer(d18:1/24:0)	C ₁₀₂ H ₁₈₂ N ₄ O ₄₈	39	Sphingolipids, neutral glycosphingolipids, Gal31-4GlcNAco31-3Gal31-4Glc- (neolactosin series)
2,255.2156 [M + H] ⁺	GalNAco1-3(Fuco1-2)Gal31-3GlcNAco31-3(GlcNAco31-6)Gal31-3GlcNAco31-3Gal31-4Glc3-Cer(d18:1/15:0)	C ₁₀₄ H ₁₈₃ N ₅ O ₄₇	24	Sphingolipids, neutral glycosphingolipids, Gal31-3GlcNAco31-3Gal31-4Glc- (lactosin series)
2,347.2266 [M + H] ⁺	NeuGco2-3Gal31-4GlcNAco31-3Gal31-3GalNAco31-4(NeuGco2-3)Gal31-4Glc3-Cer(d18:1/26:0)	C ₁₀₆ H ₁₈₇ N ₅ O ₅₁	24	Sphingolipids, acidic glycosphingolipids, gangliosides
2,356.1308 [M + K] ⁺	GalNAco1-3GalNAco31-3Galo1-3(NeuAco2-3)GalNAco31-4Gal31-4GlcNAco31-6)Gal31-4Glc3-Cer(d18:1/18:0)	C ₁₀₃ H ₁₈₀ N ₆ O ₅₁	32	Sphingolipids, acidic glycosphingolipids, gangliosides
2,461.2947 [M + H] ⁺	Gal31-4GlcNAco31-3Gal31-4GlcNAco31-3Gal31-4GlcNAco31-3Gal31-4Glc3-Cer(d18:1/26:1(17Z))	C ₁₁₂ H ₁₉₇ N ₅ O ₅₃	18	Sphingolipids, neutral glycosphingolipids, Gal31-4GlcNAco31-3Gal31-4Glc- (neolactosin series)
2,609.3682 [M + H] ⁺	Fuco1-2Gal31-4GlcNAco31-3Gal31-4GlcNAco31-3Gal31-4GlcNAco31-6)Gal31-4GlcNAco31-3Gal31-4Glc3-Cer(d18:1/26:0)	C ₁₁₈ H ₂₀₉ N ₅ O ₅₇	37	Sphingolipids, neutral glycosphingolipids, Gal31-4GlcNAco31-3Gal31-4Glc- (neolactosin series)
2,648.2675 [M + Na] ⁺	NeuAco2-3Gal31-4GlcNAco31-3(GlcNAco1-3(Fuco1-2)Gal31-4GlcNAco31-6)Gal31-4GlcNAco31-3Gal31-4Glc3-Cer(d18:1/18:0)	C ₁₁₅ H ₂₀₀ N ₆ O ₆₀	2	Sphingolipids, acidic glycosphingolipids, gangliosides
2,838.3517 [M + Na] ⁺	NeuAco2-3Gal31-4GlcNAco31-3Gal31-4(Fuco1-3)GlcNAco31-3Gal31-4GlcNAco31-3Gal31-4Glc3-Cer(d18:1/20:0)	C ₁₂₃ H ₂₁₄ N ₆ O ₆₅	17	Sphingolipids, acidic glycosphingolipids, gangliosides
2,870.4167 [M + H] ⁺	NeuAco2-3Gal31-4GlcNAco31-3Gal31-4(Fuco1-3)GlcNAco31-3Gal31-4GlcNAco31-3Gal31-4Glc3-Cer(d18:1/15:0)	C ₁₂₇ H ₂₂₀ N ₆ O ₆₅	47	Sphingolipids, acidic glycosphingolipids, gangliosides
2,908.3726 [M + K] ⁺	NeuAco2-3Gal31-4GlcNAco31-3Gal31-4(Fuco1-3)GlcNAco31-3Gal31-4GlcNAco31-3Gal31-4Glc3-Cer(d18:1/24:1(15Z))	C ₁₂₇ H ₂₂₀ N ₆ O ₆₅	23	Sphingolipids, acidic glycosphingolipids, gangliosides
2,998.4043 [M + K] ⁺	Cer(d18:1/24:1(15Z))	C ₁₃₀ H ₂₂₀ N ₆ O ₆₈	11	Sphingolipids, neutral glycosphingolipids, Gal31-4GlcNAco31-3Gal31-4Glc- (neolactosin series)
3,061.4250 [M + K] ⁺	Galo1-3Gal31-4GlcNAco31-3(GlcNAco31-6)Gal31-4Glc3-Cer(d18:1/24:1(15Z))	C ₁₃₂ H ₂₃₁ N ₅ O ₇₁	39	Sphingolipids, neutral glycosphingolipids, Gal31-4GlcNAco31-3Gal31-4Glc- (neolactosin series)
3,122.5012 [M + H] ⁺	Fuco1-2(Galo1-3)Gal31-4GlcNAco31-6(Galo1-3(Fuco1-2)Gal31-4GlcNAco31-3Gal31-4GlcNAco31-3Gal31-4Glc3-Cer(d18:1/22:0))	C ₁₃₆ H ₂₃₀ N ₆ O ₇₃	28	Sphingolipids, neutral glycosphingolipids, Gal31-4GlcNAco31-3Gal31-4Glc- (neolactosin series)

Continued

m/z	Systematic name	Molecular formula	Relative mass error (ppm)	Class of compound, subclasses
3; 306.5360 [M + Na] ⁺	Galα1-3Galβ1-4GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Cer(d18:1/24:1(15Z))	C ₁₁₂ H ₂₁₆ N ₆ O ₇₈	2	Sphingolipids, neutral glycosphingolipids, Galβ1-4GlcNAcβ1-3Galβ1-4Glc- (neolacto series)

m/z	Systematic name	Molecular formula	Relative mass error (ppm)	Class of compound, subclasses
3; 306.5360 [M + Na] ⁺	Galα1-3Galβ1-4GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ-Cer(d18:1/24:1(15Z))	C ₁₁₂ H ₂₁₆ N ₆ O ₇₈	2	Sphingolipids, neutral glycosphingolipids, Galβ1-4GlcNAcβ1-3Galβ1-4Glc- (neolacto series)

 $m/z = \text{mass-to-charge ratio.}$

IIUPAC: GalNAc- β 1- > 4 (Neu5Ac- α 2- > 3)Gal- β 1- > 4Glc- β 1- > 1Cer; Ganglioside GM2; Ii3NeuAcGlcOsc3Cer; N-acetyl-D-galactosaminyl-(N-acetylneuraminy)-D-galactosyl-
N-acetylneuraminy-(N-acetylneuraminy)-D-galactosyl-D-glucosylceramide; N-acetyl-ganglioside GM2; N-acetyl-ganglioside GM2;
N-acetyl-D-glucosyl-N-acylsphingosine; N-acetyl-D-galactosaminyl-(N-acetylneuraminy)-D-galactosyl-D-glucosylceramide; N-acetyl-D-galactosyl-1,4- β -glucosyl-
N-acetylneuraminy-(N-acetylneuraminy)-delta-galactosyl-1,4- β -glucosyl-N-acylsphingosine; N-acetyl-D-galactosaminyl-(N-acetylneuraminy)-delta-galactosyl-
N-acetylneuraminy-(N-acetylneuraminy)-delta-galactosyl-D-glucosylceramide.

IUUPAC: (2*S*,4*S*,5*R*)-2-[[[(2*S*,3*R*,4*R*,5*S*,6*R*)-6-[[[(2*S*,3*R*)-2-[(1*S*,2*S*)-docos-13-enamido]-3-hydroxyoctadecyl]oxy]-4,5-dihydroxy-2-(hydroxymethyl)oxan-3-yl]oxy]-3-hydroxy-5-[[[(2*S*,3*R*,4*R*,5*R*,6*R*)-5-hydroxy-6-(hydroxymethyl)-3-(2-oxopropyl)-4-[[[(2*R*,3*R*,4*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy]oxan-2-yl]oxy]-6-(hydroxymethyl)oxan-4-yl]oxy]-5-acetamido-4-hydroxy-6-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]oxane-2-carboxylic acid.

UIUPAC: (2*S*,4*S*,5*R*)-6-[(1*S*,2*R*)-2-[[[(2*S*,4*S*,5*R*)-2-carboxy-5-acetamido-4-hydroxy-6-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]oxan-2-yl]oxy]-1,3-dihydroxypropyl]-2-[[[(2*R*,3*S*,4*R*,5*R*,6*S*)-3-oxopropyl]-3-(2-oxopropyl)oxan-2-yl]oxy]-6-[[[(2*R*,3*S*,4*R*,5*R*,6*R*)-4,5-dihydroxy-6-[(2*S*,3*R*)-3-hydroxy-2-tetracosanamidooctadecyl]oxy]-2-(hydroxymethyl)oxan-3-yl]oxy]-5-hydroxy-2-(hydroxymethyl)oxan-4-yl]oxy]-5-acetamido-4-hydroxyoxane-2-carboxylic acid.

⁰TUPAC: (2*S*,4*S*,5*R*)-6-[(1*S*,2*R*)-2-[(2*S*,4*S*,5*R*)-2-carboxy-5-acetamido-4-hydroxy-6-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]oxan-2-yl]oxy]-1,3-dihydroxypropyl]-5-acetamido-4-hydroxyoxan-2-yl]oxy]-1,3-dihydroxypropyl]-2-[(2*R*,3*S*,4*R*,5*R*,6*S*)-3-[(2*S*,3*R*,4*R*,5*R*,6*R*)-4,5-dihydroxy-6-(hydroxymethyl)-3-(2-oxopropyl)oxan-2-yl]oxy]-6-[(2*R*,3*S*,4*R*,5*R*,6*R*)-6-[(13*Z*)-docos-13-enamido]-3-hydroxyoctadec-4-en-1-yl]oxy]-4,5-dihydroxy-2-(hydroxymethyl)oxan-3-yl]oxy]-5-hydroxy-2-(hydroxymethyl)oxan-4-yl]oxy]-5-acetamido-4-hydroxyoxan-2-carboxylic acid.

¹H NMR (CDCl₃, 25 °C): δ 7.35 (d, 2H, *trans*-H₂C=CH-), 6.85 (d, 2H, *trans*-H₂C=CH-), 6.45 (d, 2H, *trans*-H₂C=CH-), 6.35 (d, 2H, *trans*-H₂C=CH-), 6.25 (d, 2H, *trans*-H₂C=CH-), 6.15 (d, 2H, *trans*-H₂C=CH-), 6.05 (d, 2H, *trans*-H₂C=CH-), 5.95 (d, 2H, *trans*-H₂C=CH-), 5.85 (d, 2H, *trans*-H₂C=CH-), 5.75 (d, 2H, *trans*-H₂C=CH-), 5.65 (d, 2H, *trans*-H₂C=CH-), 5.55 (d, 2H, *trans*-H₂C=CH-), 5.45 (d, 2H, *trans*-H₂C=CH-), 5.35 (d, 2H, *trans*-H₂C=CH-), 5.25 (d, 2H, *trans*-H₂C=CH-), 5.15 (d, 2H, *trans*-H₂C=CH-), 5.05 (d, 2H, *trans*-H₂C=CH-), 4.95 (d, 2H, *trans*-H₂C=CH-), 4.85 (d, 2H, *trans*-H₂C=CH-), 4.75 (d, 2H, *trans*-H₂C=CH-), 4.65 (d, 2H, *trans*-H₂C=CH-), 4.55 (d, 2H, *trans*-H₂C=CH-), 4.45 (d, 2H, *trans*-H₂C=CH-), 4.35 (d, 2H, *trans*-H₂C=CH-), 4.25 (d, 2H, *trans*-H₂C=CH-), 4.15 (d, 2H, *trans*-H₂C=CH-), 4.05 (d, 2H, *trans*-H₂C=CH-), 3.95 (d, 2H, *trans*-H₂C=CH-), 3.85 (d, 2H, *trans*-H₂C=CH-), 3.75 (d, 2H, *trans*-H₂C=CH-), 3.65 (d, 2H, *trans*-H₂C=CH-), 3.55 (d, 2H, *trans*-H₂C=CH-), 3.45 (d, 2H, *trans*-H₂C=CH-), 3.35 (d, 2H, *trans*-H₂C=CH-), 3.25 (d, 2H, *trans*-H₂C=CH-), 3.15 (d, 2H, *trans*-H₂C=CH-), 3.05 (d, 2H, *trans*-H₂C=CH-), 2.95 (d, 2H, *trans*-H₂C=CH-), 2.85 (d, 2H, *trans*-H₂C=CH-), 2.75 (d, 2H, *trans*-H₂C=CH-), 2.65 (d, 2H, *trans*-H₂C=CH-), 2.55 (d, 2H, *trans*-H₂C=CH-), 2.45 (d, 2H, *trans*-H₂C=CH-), 2.35 (d, 2H, *trans*-H₂C=CH-), 2.25 (d, 2H, *trans*-H₂C=CH-), 2.15 (d, 2H, *trans*-H₂C=CH-), 2.05 (d, 2H, *trans*-H₂C=CH-), 1.95 (d, 2H, *trans*-H₂C=CH-), 1.85 (d, 2H, *trans*-H₂C=CH-), 1.75 (d, 2H, *trans*-H₂C=CH-), 1.65 (d, 2H, *trans*-H₂C=CH-), 1.55 (d, 2H, *trans*-H₂C=CH-), 1.45 (d, 2H, *trans*-H₂C=CH-), 1.35 (d, 2H, *trans*-H₂C=CH-), 1.25 (d, 2H, *trans*-H₂C=CH-), 1.15 (d, 2H, *trans*-H₂C=CH-), 1.05 (d, 2H, *trans*-H₂C=CH-), 0.95 (d, 2H, *trans*-H₂C=CH-), 0.85 (d, 2H, *trans*-H₂C=CH-), 0.75 (d, 2H, *trans*-H₂C=CH-), 0.65 (d, 2H, *trans*-H₂C=CH-), 0.55 (d, 2H, *trans*-H₂C=CH-), 0.45 (d, 2H, *trans*-H₂C=CH-), 0.35 (d, 2H, *trans*-H₂C=CH-), 0.25 (d, 2H, *trans*-H₂C=CH-), 0.15 (d, 2H, *trans*-H₂C=CH-), 0.05 (d, 2H, *trans*-H₂C=CH-).

Solubilized powdered milk samples were heated to 70°C for 30 min, cooled to 4°C, and centrifuged at $16,000 \times g$ for 10 min. The lipid-rich upper layer was removed and discarded. Ethanol at -20°C was added at 1:1 with the sample volume, vortexed, and incubated at 4°C overnight to precipitate proteins. The sample was then centrifuged again at $16,000 \times g$ for 10 min at 25°C. The supernatant was transferred to another tube, and ethanol was removed by a centrifugal vacuum concentrator (SpeedVac, Savant, Thermo Scientific, Waltham, MA). The volumes were adjusted to the original volume with purified water. The samples were centrifuged a final time at $16,000 \times g$ at room temperature for 5 min immediately before spotting for MALDI-TOF analysis.

Mass Spectrometry

Fresh MALDI matrix was prepared before collection of data by dissolving solid α -cyano-4-hydroxycinnamic acid [Sigma-Aldrich (Fluka), St. Louis, MO >99.0%] in 75% acetonitrile, 0.1% trifluoroacetic acid in water at a concentration of 6 mg/mL. For each sample, 0.4 μ L of yak milk sample was pipetted to the MALDI target and overlaid, first with 0.4 μ L of the ProteoMass ACTH Fragment 18–39 MALDI-MS standard peptide (Sigma-Aldrich) dissolved in 0.1% formic acid in water at 200 fmol/ μ L, and then with 0.4 μ L of α -cyano-4-hydroxycinnamic acid MALDI matrix. The samples were mixed on plate by aspiration and air dried. The MALDI-TOF MS was performed in positive ion, reflector mode on an AB Sciex TOF/TOF 4800 MALDI instrument (AB Sciex, Foster City, CA). The instrument was first calibrated using the manufacturer's 6-peptide mixture, and then each spectrum was internally calibrated using the accurate mass of ACTH Fragment 18–39 standard peptide (protonated mass at m/z 2,464.199; Sigma-Aldrich) present in each sample. Laser power was adjusted for the calibration standards and for each sample so that the signal obtained for the most-abundant ion in each spectrum could be maximized, or kept below $\sim 2 \times 10^4$ to preserve spectral quality. Structure and name identification based on mass were matched using the Metlin metabolite database (<https://metlin.scripps.edu/index.php>) by Scripps Center for Metabolomics (La Jolla, CA).

RESULTS AND DISCUSSION

In this work, a technique using MALDI-TOF was developed and applied to a fraction of molecules in the range of 1,000 to 3,000 Da extracted from yak milk. Because such compounds are formed by the combination of a relatively limited set of sugar residues and the majority of the glycans have published structures, ten-

tative structures with unknown samples can be conditionally annotated from the molecular mass of the parent molecules. Sugar unit components of carbohydrate side chains of mammalian proteins and lipids include D-mannose, D-galactose, L-fucose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid (Dass, 2006). Lack of accuracy of this approach may result from differences in isotope ratios as well as the inability of MALDI-TOF analysis to differentiate isomers of similar molecular weight differentiated by such features as the positional sites of glycosylation (Stahl et al., 1994).

Initial preparation technique trials for this work involved delipidated and deproteinized yak milk samples using membrane deproteinization (results not shown); however, improvement of the technique was reached with cold ethanol precipitation (Kobata, 1972; Thurl et al., 1991; Sen et al., 2011) with lactose and protein removal. The MALDI-TOF MS has been used as one of the most advanced soft ionization methods, which facilitates the fast ionization of large molecules, such as proteins, polysaccharides, compound lipids, and nucleic acids. The MALDI-TOF MS has also been successfully used as a fast screening method of gangliosides accumulation in the central nervous system of dogs (Satoh et al., 2011).

Results of the current work for yak milk powder show a large variety of complex oligosaccharide-containing structures with main molecular clusters around 1,200, 1,600, and 2,000 Da (Figure 1). These structures included complex sphingolipids, such as glycosphingolipids, and inositol-containing ceramides (see Figure 2). The profile of yak milk gangliosides included GA1, GD1a, GD1b, GD3, GM1, GM2, GM3, and GT2 (Table 1). One common structural feature of these gangliosides is the ceramide core. Gangliosides (Christie, 2014) are oligoglycosylceramides containing a sialic acid residue such as *N*-acetylneuraminic acid. Less often, the sialic acid component is *N*-glycolylneuraminic acid or an *N*-acetylneuraminic acid analog. Gangliosides can constitute up to 6% of the lipids in brain tissue and are present in all animal tissues (Christie, 2014). Martin et al. (2003) described GM3, 9-*O*-acetyl-GD3, GD3, and GT3 as gangliosides found in native bovine milk (see also Hanagata et al., 1991).

Some authors have reported that GD3 dominates mature bovine milk (and human colostrum), and GM3 dominates mature human milk (Bode et al., 2004). Others have reported more GM3 than GD3 in bovine milk (Sanchez-Juanes et al., 2009). One aspect that occurs in human milk gangliosides is the selective enrichment with certain fatty acids with potential physiological effects (Bode et al., 2004). The gangliosides GM3 and GD3 were observed to bind enterotoxigenic *Escherichia*

coli strains (Martin et al., 2003). Although our work did not investigate fatty acid composition in the yak milk gangliosides, one can speculate that the unique composition of the gangliosides may be an interesting aspect for further study.

In a report by Bode et al. (2004), MALDI-TOF MS and GLC were used to analyze the fatty acid composition of the predominant gangliosides in pooled human and bovine milk (i.e., monosialoganglioside GM3 and the disialoganglioside GD3). Human milk resulted in mass peaks in the ranges of 1,176 to 1,326 Da (GM3) and 1,523 to 1,624 Da (GD3). These mass peaks were thought to result from MALDI-induced generation of ganglioside-alkali adduct ions ($[M + Na]^+$ and $[M + K]^+$). Peaks inside the cluster had 28-Da intervals possibly due to chain-length variations in the sphingoid base or fatty acid with an even number of methylene groups. In bovine milk, MALDI-TOF MS of the predominant ganglioside fractions were similar to those isolated from human milk; however, 14-Da intervals in the peak clusters were identified, possibly the result of chain length variations in the sphingoid base or fatty acid with singular methylene groups (Bode et al., 2004). Compared with human milk, bovine milk is generally lower in fucose (<5%) but high in sialic acid (>60%; Ruhaak and Lebrilla, 2012). These variations in gangliosides structure in different species suggest alternate or diverse biological functions (Martin et al., 2003; Bode et al., 2004).

Profiling matched gangliosides containing several hexoses, including fucose, and deaminated neuraminic acid (KDN). One common aspect of the majority of the profiled compounds is the presence of sialic acid. Sialic acid derivatives include 9-carbon sugar acids and are found in large amounts in human milk oligosaccharides. Nutritionally, sialic acids are important components of gangliosides and sialylated glycoproteins (Wang, 2012). Deaminated neuraminic acid was another relatively abundant compound with mass around 1,590 Da $[M + H]^+$. The metabolomics database assigned this molecular weight to sphingolipid compounds KDN (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid), the KDN- α -2-3Gal- β -1-4Glc-NAc- β -1-3Gal- β -1-4Glc- β -Cer (d18:1/24:0), and an acidic glycosphingolipid with the molecular formula of $C_{77}H_{140}N_2O_{31}$. Another study also identified gangliosides and neutral glycosphingolipids in cow milkfat globule membranes with similar qualitative composition (Sanchez-Juanes et al., 2009). A notable characteristic of these compounds is the ability to bind enterotoxigenic *Escherichia coli* and to inhibit bacterial hemagglutination (Sanchez-Juanes et al., 2009). In human milk, oligosaccharides containing α -1,2 linked fucose are related to *Escherichia coli* growth inhibition (Newburg, 1999; Chaturvedi et al., 2001; Newburg et

al., 2004). Fucosylated structures were found in yak milk. Both, fucosylated and nonfucosylated structures are of potential beneficial importance. For instance, bifidobacteria, important in human intestinal health, have been shown to grow better in the presence of fucosylated oligosaccharides (Ruhaak and Lebrilla, 2012). Milk oligosaccharides, together with host cells, target glycoconjugate variations, which may be related to a coordinated type of defense against pathogens (Chaturvedi et al., 2001).

CONCLUSIONS

A unique sphingolipid composition in yak milk, with an abundance of complex and diverse glycosphingolipids structures, are reported. Further work to refine and quantitate these compounds will shed further light on the potential of yak milk to influence human health. Applications of MALDI-TOF MS play an important role to enhance our knowledge of the identity and diversity of biologically active compounds in milk. Such knowledge can be significantly advanced because of the access to high-resolution MS technologies and comprehensive databases of these complex molecules. Matrix preparation and the parameters established in this work allowed the profiling of complex sphingolipids in yak powder with high resolution.

ACKNOWLEDGMENTS

The authors express appreciation to Hongyuan Tianyu Yak Dairy Ltd. Co. (Qiongx, China) for financial support.

REFERENCES

- Bode, L., C. Beermann, M. Mank, G. Kohn, and G. Boehm. 2004. Human and bovine milk gangliosides differ in their fatty acid composition. *J. Nutr.* 134:3016–3020.
- Chaturvedi, P., C. D. Warren, M. Altaye, A. L. Morrow, G. Ruiz-Palacios, L. K. Pickering, and D. S. Newburg. 2001. Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. *Glycobiology* 11:365–372.
- Christie, W. W. 2014. Gangliosides: Structure, Occurrence, Biology and Analysis. Lipid Library. Am. Oil Chem. Soc., Urbana, IL. Accessed Dec. 27, 2015. <http://aocs.files.cms-plus.com/FTPReceiving/lipidimporthtml/lipidlibrary/Lipids/gang/index.htm>.
- Coyne, M. J., B. Reinap, M. M. Lee, and L. E. Comstock. 2005. Human symbionts use a host-like pathway for surface fucosylation. *Science* 307:1778–1781.
- Dass, C. 2006. Fundamentals of Contemporary Mass Spectrometry. John Wiley Sons Inc., Hoboken, NJ.
- Hanagata, G., S. Yanahira, K. T. Yanahira, T. Yakabe, and E. Deya, inventors. 1991. Nouveau ganglioside provenant du lait et son procede d'obtention [New ganglioside of milk origin and process for obtaining it]. Snow Brand Milk Products Co Ltd., assignee. France Pat. No. FR2662697 A1.
- Kobata, A. 1972. [24] Isolation of oligosaccharides from human milk. Pages 262–271 in *Methods in Enzymology: Complex Carbohydrates Part B*. Vol. 28. Acad. Press, London, UK.

- Martin, M. J., S. Martin-Sosa, J. M. Alonso, and P. Hueso. 2003. Enterotoxigenic *Escherichia coli* strains bind bovine milk gangliosides in a ceramide-dependent process. *Lipids* 38:761–768.
- Medhammar, E., R. Wijesinha-Bettoni, B. Stadlmayr, E. Nilsson, U. R. Charrondiere, and B. Burlingame. 2012. Composition of milk from minor dairy animals and buffalo breeds: A biodiversity perspective. *J. Sci. Food Agric.* 92:445–474.
- Newburg, D. S. 1999. Human milk glycoconjugates that inhibit pathogens. *Curr. Med. Chem.* 6:117–127.
- Newburg, D. S., G. M. Ruiz-Palacios, M. Altaye, P. Chaturvedi, J. Meinzen-Derr, M. L. Guerrero, and A. L. Morrow. 2004. Innate protection conferred by fucosylated oligosaccharides of human milk against diarrhea in breastfed infants. *Glycobiology* 14:253–263.
- Niñonuevo, M. R., and C. B. Lebrilla. 2009. Mass spectrometric methods for analysis of oligosaccharides in human milk. *Nutr. Rev.* 67:S216–S226.
- O'Hara, A. M., and F. Shanahan. 2006. The gut flora as a forgotten organ. *EMBO Rep.* 7:688–693.
- Parodi, P. W. 1997. Cows' milk fat components as potential anticarcinogenic agents. *J. Nutr.* 127:1055–1060.
- Rueda, R., J. L. Sabatel, J. Maldonado, J. A. Molina-Font, and A. Gil. 1998. Addition of gangliosides to an adapted milk formula modifies levels of fecal *Escherichia coli* in preterm newborn infants. *J. Pediatr.* 133:90–94.
- Ruhaak, L. R., and C. B. Lebrilla. 2012. Analysis and role of oligosaccharides in milk. *BMB Rep.* 45:442–451.
- Sanchez-Juanes, F., J. M. Alonso, L. Zancada, and P. Hueso. 2009. Glycosphingolipids from bovine milk and milk fat globule membranes: A comparative study. Adhesion to enterotoxigenic *Escherichia coli* strains. *Biol. Chem.* 390:31–40.
- Satoh, H., T. Yamauchi, M. Yamasaki, Y. Maede, A. Yabuki, H. Chang, T. Asanuma, and O. Yamato. 2011. Rapid detection of GM1 ganglioside in cerebrospinal fluid in dogs with GM1 gangliosidosis using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Vet. Diagn. Invest.* 23:1202–1207.
- Sen, D., A. Gosling, G. W. Stevens, P. K. Bhattacharya, A. R. Barber, S. E. Kentish, C. Bhattacharjee, and S. L. Gras. 2011. Galactosyl oligosaccharide purification by ethanol precipitation. *Food Chem.* 128:773–777.
- Spitsberg, V. L. 2005. Invited review: Bovine milk fat globule membrane as a potential nutraceutical. *J. Dairy Sci.* 88:2289–2294.
- Stahl, B., S. Thurl, J. Zeng, M. Karas, F. Hillenkamp, M. Steup, and G. Sawatzki. 1994. Oligosaccharides from human milk as revealed by matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Biochem.* 223:218–226.
- Thurl, S., J. Offermanns, B. Muller-Werner, and G. Sawatzki. 1991. Determination of neutral oligosaccharide fractions from human milk by gel permeation chromatography. *J. Chromatogr.* 568:291–300.
- Vesper, H., E. M. Schmelz, M. N. Nikolova-Karakashian, D. L. Dillehay, D. V. Lynch, and A. H. Merrill Jr. 1999. Sphingolipids in food and the emerging importance of sphingolipids to nutrition. *J. Nutr.* 129:1239–1250.
- Wang, B. 2012. Molecular mechanism underlying sialic acid as an essential nutrient for brain development and cognition. *Adv. Nutr.* 3:465S–472S.