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Comparative Glycomics of Fat Globule Membrane Glycoconjugates from Buffalo (*Bubalus bubalis*) Milk and Colostrum

Nagaraju Brijesha,[†] Shin-Ichiro Nishimura,[‡] and Huligerepura Sosalegowda Aparna*[,†]

[†]Department of Biotechnology, University of Mysore, Manasagangotri, Mysore 570 006, Karnataka, India

[‡]Faculty of Advanced Life Science, Hokkaido University, Sapporo 001-0021, Japan

Supporting Information

ABSTRACT: The health-promoting effects of milk fat globule membrane (MFGM) glycoconjugates has attracted curiosity especially with regard to the challenges encountered to unravel the glycan complexities of MFGM glycoproteins and glycosphingolipids. In this context, we characterized glycans present in buffalo milk and colostrum fat globule membranes by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS analysis by adopting chemoselective glycoblotting technique. Unlike human and bovine MFGM glycoproteins, the variations were obvious with respect to their number, size, heterogeneity, and abundance among the samples analyzed. Among N-linked glycans, mono-, di-, and trisialyl glycans were apparent in colostrum, while MFGM predominantly contained mono- and disialyl glycans, in addition to neutral and high-mannose glycoforms. The structural assignments of major glycans were confirmed by TOF/TOF analysis. Core 1 O-glycans were more common in both samples, and the major glycosphingolipids were GM3 and GD3 irrespective of the samples analyzed. The colostrum N-glycans, being effective antibacterials against human pathogens, established the structure−function relationship of oligosaccharides in early milk in providing innate protection to the newborn.

KEYWORDS: *fat globule membrane, colostrum, milk, glycomics, MALDI-TOF MS, antibacterials*

INTRODUCTION

Milk is a mammalian-specific biological fluid with potential health benefits. Colostrum, the early mammary secretion essential for the growth and development of newborn mammals, possesses additional constituents like growth factors, free oligosaccharides, glycoconjugates, and cellular components that offer protection to the newborn during the transition from the intrauterine to the extrauterine condition.[1] Although intensive research has been carried out to characterize free oligosaccharides from human, bovine, ovine, and many other mammalian species,[2,3] the glycoconjugates present in the membranes of milk fat globules are currently receiving a lot of interest.[4,5]

Milk fat globule membrane (MFGM) originating from the apical membrane of mammary epithelial cells is structurally complex with phospho- and sphingolipids and a wide variety of proteins and glycoproteins that represent 1−2 and 2−4% of total proteins in milk and colostrum, respectively.[6] Fat globule membrane with core proteins and lipids possesses a heterogeneous array of oligosaccharides that form glycocalyx on the surface.[7] Because of the differences in isolation, purification, and analytical techniques employed, the information on the composition of the MFGM proteins is highly variable.[8,9] The most prominent proteins include mucin 1 (MUC1), xanthine oxidoreductase/dehydrogenase (XO/XDH), periodic acid Schiff III (PASIII), cluster of differentiation (CD36), butyrophilin (BTN), periodic acid Schiff 6/7 (PAS 6/7; also called lactadherin), adipophilin (ADPH), and fatty acid binding protein (FABP). The majority of them are glycosylated, but the level, type of residues, and position of glycosylation differ among the identified glycoproteins.[10,11]

The glycoproteomics of human and bovine MFGM were analyzed by LC-MS MS, and recently, the structural and

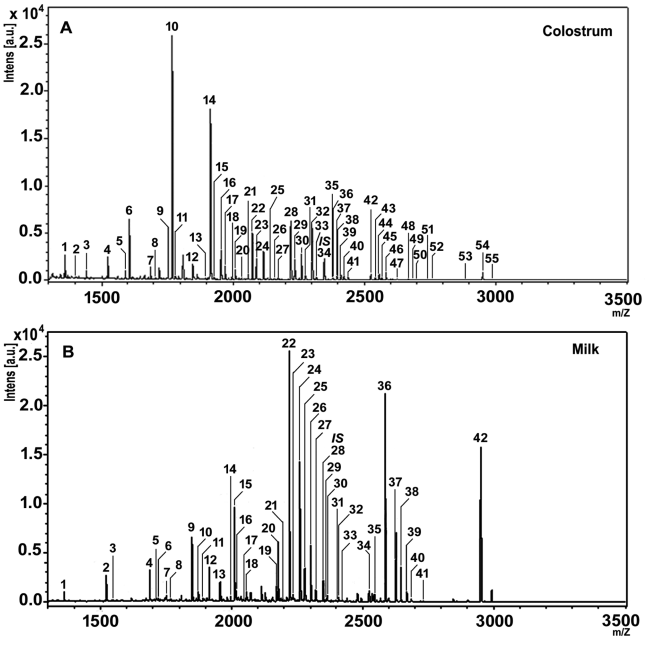

Figure 1. MALDI-TOF MS spectra of N-linked oligosaccharides from buffalo colostrum FGMP (A) and milk FGMP (B).

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Table 1. N-Linked Oligosaccharides of Buffalo Colostrum FGMP

peak no	m/z	structure
1	1362.481	(Hex)2 + (Man)3 (GlcNac)2
2	1403.508	(Hex)1 (HexNAc)1 + (Man)3 (GlcNac)2
3	1444.534	(HexNAc)2 + (Man)3 (GlcNac)2
4	1524.534	(Hex)3 + (Man)3 (GlcNac)2
5	1590.592	(HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNac)2
6	1606.587	(Hex)1 (HexNAc)2 + (Man)3 (GlcNac)2
7	1686.587	(Hex)4 + (Man)3 (GlcNac)2
8	1708.61	(Hex)1 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNac)2
	1708.61	(HexNAc)1 (Deoxyhexose)1 (NeuGc)1 + (Man)3 (GlcNac)2
9	1752.645	(Hex)1 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNac)2
10	1768.640	(Hex)2 (HexNAc)2 + (Man)3 (GlcNac)2
11	1793.671	(HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNac)2
12	1848.640	(Hex)5 + (Man)3 (GlcNac)2
13	1895.703	(HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
14	1914.698	(Hex)2 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNac)2
15	1930.693	(Hex)3 (HexNAc)2 + (Man)3 (GlcNac)2
16	1955.724	(Hex)1 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNac)2
17	1971.719	(Hex)2 (HexNAc)3 + (Man)3 (GlcNac)2
18	1996.751	(HexNAc)4 (Deoxyhexose)1 + (Man)3 (GlcNac)2
19	2010.692	(Hex)6 + (Man)3 (GlcNac)2
20	2032.724	(Hex)3 (HexNAc)1 (NeuAc)1 + (Man)3 (GlcNac)2
	2032.724	(Hex)2 (HexNAc)1 (Deoxyhexose)1 (NeuGc)1 + (Man)3 (GlcNac)2
21	2057.756	(Hex)1 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
	2057.756	(HexNAc)2 (Deoxyhexose)2 (NeuGc)1 + (Man)3 (GlcNac)2
22	2073.750	(Hex)2 (HexNAc)2 (NeuAc)1 + (Man)3 (GlcNac)2
	2073.750	(Hex)1 (HexNAc)2 (Deoxyhexose)1 (NeuGc)1 + (Man)3 (GlcNac)2
23	2089.746	(Hex)2 (HexNAc)2 (NeuGc)1 + (Man)3 (GlcNac)2
24	2117.777	(Hex)2 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNac)2
25	2142.809	(HexNAc)4 (Deoxyhexose)2 + (Man)3 (GlcNac)2
26	2159.788	(Hex)1 (HexNAc)1 (Deoxyhexose)1 (NeuAc)2 + (Man)3 (GlcNac)2
27	2172.745	(Hex)7 + (Man)3 (GlcNac)2
28	2219.808	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
	2219.808	(Hex)1 (HexNAc)2 (Deoxyhexose)2 (NeuGc)1 + (Man)3 (GlcNac)2
29	2235.803	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuGc)1 + (Man)3 (GlcNac)2
	2235.803	(Hex)3 (HexNAc)2 (NeuAc)1 + (Man)3 (GlcNac)2
30	2260.835	(Hex)1 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
	2260.835	(HexNAc)3 (Deoxyhexose)2 (NeuGc)1 + (Man)3 (GlcNac)2
31	2276.830	(Hex)2 (HexNAc)3 (NeuAc)1 + (Man)3 (GlcNac)2
	2276.830	(Hex)1 (HexNAc)3 (Deoxyhexose)1 (NeuGc)1 + (Man)3 (GlcNac)2
32	2301.862	(HexNAc)4 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
33	2317.856	(Hex)1 (HexNAc)4 (NeuAc)1 + (Man)3 (GlcNac)2
	2317.856	(HexNAc)4 (Deoxyhexose)1 (NeuGc)1 + (Man)3 (GlcNac)2
34	2348.879	***internal standard***
35	2378.861	(Hex)2 (HexNAc)2 (NeuAc)2 + (Man)3 (GlcNac)2
	2378.861	(Hex)1 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 (NeuGc)1 + (Man)3(GlcNac)2
36	2381.861	(Hex)3 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
	2381.861	(Hex)2 (HexNAc)2 (Deoxyhexose)2 (NeuGc)1 + (Man)3 (GlcNac)2
37	2394.856	(Hex)2 (HexNAc)2 (NeuAc)1 (NeuGc)1 + (Man)3 (GlcNac)2
	2394.856	(Hex)1 (HexNAc)2 (Deoxyhexose)1 (NeuGc)2 + (Man)3 (GlcNac)2
38	2397.856	(Hex)3 (HexNAc)2 (Deoxyhexose)1 (NeuGc)1 + (Man)3 (GlcNac)2
	2397.856	(Hex)4 (HexNAc)2 (NeuAc)1 + (Man)3 (GlcNac)2
39	2410.852	(Hex)2 (HexNAc)2 (NeuGc)2 + (Man)3 (GlcNac)2
40	2422.888	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
	2422.888	(Hex)1 (HexNAc)3 (Deoxyhexose)2 (NeuGc)1 + (Man)3 (GlcNac)2
41	2438.883	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuGc)1 + (Man)3 (GlcNac)2
	2438.883	(Hex)3 (HexNAc)3 (NeuAc)1 + (Man)3 (GlcNac)2
42	2524.919	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)2 + (Man)3 (GlcNac)2
	2524.919	(Hex)1 (HexNAc)2 (Deoxyhexose)2 (NeuAc)1 (NeuGc)1 + (Man)3 (GlcNac)2
43	2540.914	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 (NeuGc)1 + (Man)3 (GlcNac)2
	2540.914	(Hex)3 (HexNAc)2 (NeuAc)2 + (Man)3 (GlcNac)2
	2540.914	(Hex)1 (HexNAc)2 (Deoxyhexose)2 (NeuGc)2 + (Man)3 (GlcNac)2
44	2556.909	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuGc)2 + (Man)3 (GlcNac)2
	2556.909	(Hex)3 (HexNAc)2 (NeuAc)1 (NeuGc)1 + (Man)3 (GlcNac)2

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Table 1. continued

peak no	m/z	structure
45	2565.946	(Hex)1 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 + (Man)3 (GlcNac)2
46	2584.941	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
	2584.941	(Hex)2 (HexNAc)3 (Deoxyhexose)2 (NeuGc)1 + (Man)3 (GlcNac)2
47	2625.967	(Hex)1 (HexNAc)4 (Deoxyhexose)2 (NeuGc)1 + (Man)3 (GlcNac)2
	2625.967	(Hex)2 (HexNAc)4 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
48	2666.994	(Hex)1 (HexNAc)5 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
	2666.994	(HexNAc)5 (Deoxyhexose)2 (NeuGc)1 + (Man)3 (GlcNac)2
49	2683.973	(Hex)2 (HexNAc)2 (NeuAc)3 + (Man)3 (GlcNac)2
50	2699.968	(Hex)2 (HexNAc)2 (NeuAc)2 (NeuGc)1 + (Man)3 (GlcNac)2
51	2746.993	(Hex)4 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
	2746.993	(Hex)3 (HexNAc)3 (Deoxyhexose)2 (NeuGc)1 + (Man)3 (GlcNac)2
52	2759.989	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuGc)2 + (Man)3 (GlcNac)2
	2759.989	(Hex)3 (HexNAc)3 (NeuAc)1 (NeuGc)1 + (Man)3 (GlcNac)2
53	2890.052	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)2 + (Man)3 (GlcNac)2
	2890.052	(Hex)1 (HexNAc)3 (Deoxyhexose)3 (NeuGc)2 + (Man)3 (GlcNac)2
	2890.052	(Hex)2 (HexNAc)3 (Deoxyhexose)2 (NeuAc)1 (NeuGc)1 + (Man)3 (GlcNac)2
54	2950.073	(Hex)4 (HexNAc)4 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
	2950.073	(Hex)3 (HexNAc)4 (Deoxyhexose)2 (NeuGc)1 + (Man)3 (GlcNac)2
55	2991.100	(Hex)3 (HexNAc)5 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
	2991.100	(Hex)2 (HexNAc)5 (Deoxyhexose)2 (NeuGc)1 + (Man)3 (GlcNac)2

functional characteristics of bovine milk glycoforms have been reviewed.[4,5] A detailed comparative N-glycomics of human and bovine milk reported by Nwosu et al.[12] revealed the abundance of fucosylation in human glycoproteins, while sialylation was prominent in bovine milk glycoproteins.[13] The presence of Neu5Gc was exclusive to bovine glycoproteins, with 1% of glycans containing both Neu5Ac and Neu5Gc residues. In addition, ∼31% of bovine milk N-glycans were found to be fucosylated, suggesting that protein-bound oligosaccharides were the major source of fucose in bovine milk. In addition to glycoproteins, glycosphingolipids (GSLs) formed an integral part of the fat globule membrane (FGM). Earlier methods utilized high-performance thin-layer chromatography (HPTLC) separation with a corresponding lipid-bound sialic acid quantitation for the isolation of glycosphingolipids from bovine milk.[14] More recently, high-performance liquid chromatography (HPLC)-MS/MS technique was employed to characterize human milk gangliosides.[15] Interestingly, GM3 and GD3 were abundant in both human and bovine milk.

The majority of the MFGM proteins play a vital role in various cellular processes and defense mechanisms in the newborn.[16] The proteins of MFGM reportedly have hypocholesterolemic, anticancer, and multiple sclerosis suppressive properties,[17] while the MFGM-associated glycoproteins encompass a range of antibacterial,[18] antiviral,[19] and antiadhesive properties.[20] The antiadhesive effect of MFGM glycoproteins against *Helicobacter pylori* (*H. pylori*), *Escherichia coli* (*E. coli*), and *Salmonella typhimurium* including colonic bacteria is well-documented.[21] Similarly, MUC1 and PAS6/7 were found to decoy the binding of pathogenic microbes to the gastrointestinal tract, while purified XDH/XO was shown to inhibit the growth of *Staphylococcus aureus* (*S. aureus*), *E. coli*, and *Salmonella enteritidis* either through hydrogen peroxide formation or stimulation of the lactoperoxidase system in milk.[22] The extremely diverse glycans found in FGM presumably mimic specific bacterial and viral ligands, and when ingested, they prevent the attachment of pathogenic organisms to the intestinal mucosa in the acidic environment of the stomach.[7] The GSLs in milk exhibit multiple benefits as they promote growth and development of the brain, the mucus layer of the gut, and its microflora; immunity; and inhibition of pathogens that bind to the gastrointestinal tract.[23] In addition, GSLs from bovine MFGM were found to be effective in binding to enterotoxigenic *E. coli*.[14]

Buffalo milk, being the second largest global source for milk, differs from its closely related ruminant species with higher proportions of proteins and fat. Although the differences in the carbohydrate content were studied during different stages of lactation in buffalo milk,[24] only the recent in situ investigations on the microstructure of buffalo milk fat globules indicated the presence of lipids, protein assemblies, glycoproteins, and glycolipids.[25] Hence, a more detailed analysis of buffalo FGM was envisaged as buffalo milk is an important component of human nutrition and a major source for many dairy products.

In the present investigation, we isolated FGM from buffalo milk and colostrum and analyzed their glycan diversity. The N- and O-linked oligosaccharides and GSLs were characterized by MALDI-TOF MS analysis by adopting chemoselective glycoblotting, and the abundant glycans were confirmed by TOF/TOF analysis. To establish the structure−function relationship, the potential role of glycans in imparting protection against bacterial pathogens was explored.

MATERIALS AND METHODS

The colostrum (first day of lactation) and milk samples were obtained locally. Sequence-grade trypsin, 1-propanesulfonic acid, 2-hydroxy-3-myristamido (PHM), ammonium bicarbonate (NH_4HCO_3), dithiothreitol (DTT), iodoacetamide (IAA), ammonium carbamate, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich, U.S.A. Peptide *N*-Glycosidase F was obtained from New England Biolabs. All other chemicals and reagents were of either spectroscopic or analytical grade.

Isolation of FGM. The colostrum fat globule membrane (CFGM) and milk fat globule membrane (MFGM) were extracted from cream as described by Basch et al.[26] with minor modifications. Briefly, the cream was washed twice (4500*g*, 10 min, 4 °C) with phosphate buffer saline (PBS 10 mM, pH 7.2), suspended in distilled water and allowed to crystallize at 4 °C for 20 h. The separated fat and serum fractions were warmed at 45 °C for 30 min to melt the fat and washed with distilled water to recover the residual serum. The total serum was centrifuged twice (5000*g*, 15 min, 4 °C) to remove the fat. The CFGM

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Table 2. N-Linked Oligosaccharides of Buffalo Milk FGMP

	m/z	structure
1	1362.481	(Hex)2 + (Man)3 (GlcNac)2
2	1524.534	(Hex)3 + (Man)3 (GlcNac)2
3	1549.566	(Hex)1 (HexNAc)1 (Deoxyhexose)1 + (Man)3 (GlcNac)2
4	1686.587	(Hex)4 + (Man)3 (GlcNac)2
5	1711.618	(Hex)2 (HexNAc)1 (Deoxyhexose)1 + (Man)3 (GlcNac)2
6	1727.613	(Hex)3 (HexNAc)1 + (Man)3 (GlcNac)2
7	1752.645	(Hex)1 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNac)2
8	1765.640	(HexNAc)2 (NeuGc)1 + (Man)3 (GlcNac)2
9	1848.640	(Hex)5 + (Man)3 (GlcNac)2
10	1873.671	(Hex)3 (HexNAc)1 (Deoxyhexose)1 + (Man)3 (GlcNac)2
11	1895.703	(HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
12	1914.698	(Hex)2 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNac)2
13	1955.724	(Hex)1 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNac)2
14	1996.751	(HexNAc)4 (Deoxyhexose)1 + (Man)3 (GlcNac)2
15	2010.692	(Hex)6 + (Man)3 (GlcNac)2
16	2016.729	(Hex)2 (HexNAc)1 (Deoxyhexose)1 (NeuAc)1 + (Man) 3 (GlcNac)2
	2016.729	(Hex)1 (HexNAc)1 (Deoxyhexose)2 (NeuGc)1 + (Man) 3 (GlcNac)2
17	2045.720	(Hex)1 (HexNAc)1 (NeuGc)2 + (Man)3 (GlcNac)2
18	2057.756	(Hex)1 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man) 3 (GlcNac)2
	2057.756	(HexNAc)2 (Deoxyhexose)2 (NeuGc)1 + (Man)3 (GlcNac)2
19	2172.745	(Hex)7 + (Man)3 (GlcNac)2
20	2178.782	(Hex)3 (HexNAc)1 (Deoxyhexose)1 (NeuAc)1 + (Man) 3 (GlcNac)2
	2178.782	(Hex)2 (HexNAc)1 (Deoxyhexose)2 (NeuGc)1 + (Man) 3 (GlcNac)2
21	2194.777	(Hex)3 (HexNAc)1 (Deoxyhexose)1 (NeuGc)1 + (Man) 3 (GlcNac)2
22	2219.808	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man) 3 (GlcNac)2
	2219.808	(Hex)1 (HexNAc)2 (Deoxyhexose)2 (NeuGc)1 + (Man) 3 (GlcNac)2
23	2238.803	(Hex)4 (HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNac)2
24	2260.835	(Hex)1 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man) 3 (GlcNac)2
	2260.835	(HexNAc)3 (Deoxyhexose)2 (NeuGc)1 + (Man)3 (GlcNac)2
25	2279.830	(Hex)3 (HexNAc)3 (Deoxyhexose)1 + (Man)3 (GlcNac)2
26	2301.862	(HexNAc)4 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2
27	2320.857	(Hex)2 (HexNAc)4 (Deoxyhexose)1 + (Man)3 (GlcNac)2
28	2348.879	*internal standard*
29	2361.883	(Hex)1 (HexNAc)5 (Deoxyhexose)1 + (Man)3 (GlcNac)2
30	2365.866	(Hex)2 (HexNAc)2 (Deoxyhexose)2 (NeuAc)1 + (Man) 3 (GlcNac)2
	2365.866	(Hex)1 (HexNAc)2 (Deoxyhexose)3 (NeuGc)1 + (Man) 3 (GlcNac)2
31	2406.893	(Hex)1 (HexNAc)3 (Deoxyhexose)2 (NeuAc)1 + (Man) 3 (GlcNac)2
	2406.893	(HexNAc)3 (Deoxyhexose)3 (NeuGc)1 + (Man)3 (GlcNac)2
32	2410.852	(Hex)2 (HexNAc)2 (NeuGc)2 + (Man)3 (GlcNac)2
33	2422.888	(Hex)2 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man) 3 (GlcNac)2
	2422.888	(Hex)1 (HexNAc)3 (Deoxyhexose)2 (NeuGc)1 + (Man) 3 (GlcNac)2
34	2524.919	(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)2 + (Man) 3 (GlcNac)2
	2524.919	(Hex)1 (HexNAc)2 (Deoxyhexose)2 (NeuAc)1 (NeuGc)1 + (Man)3 (GlcNac)2
35	2543.914	(Hex)3 (HexNAc)2 (Deoxyhexose)2 (NeuGc)1 + (Man) 3 (GlcNac)2
	2543.914	(Hex)4 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man) 3 (GlcNac)2
36	2584.941	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man) 3 (GlcNac)2
	2584.941	(Hex)2 (HexNAc)3 (Deoxyhexose)2 (NeuGc)1 + (Man) 3 (GlcNac)2
37	2625.967	(Hex)1 (HexNAc)4 (Deoxyhexose)2 (NeuGc)1 + (Man) 3 (GlcNac)2
	2625.967	(Hex)2 (HexNAc)4 (Deoxyhexose)1 (NeuAc)1 + (Man) 3 (GlcNac)2
38	2644.962	(Hex)4 (HexNAc)4 (Deoxyhexose)1 + (Man)3 (GlcNac)2
39	2666.994	(Hex)1 (HexNAc)5 (Deoxyhexose)1 (NeuAc)1 + (Man) 3 (GlcNac)2
	2666.994	(HexNAc)5 (Deoxyhexose)2 (NeuGc)1 + (Man)3 (GlcNac)2
40	2685.989	(Hex)3 (HexNAc)5 (Deoxyhexose)1 + (Man)3 (GlcNac)2
41	2730.998	(Hex)2 (HexNAc)3 (Deoxyhexose)3 (NeuGc)1 + (Man) 3 (GlcNac)2
	2730.998	(Hex)3 (HexNAc)3 (Deoxyhexose)2 (NeuAc)1 + (Man) 3 (GlcNac)2
42	2950.073	(Hex)4 (HexNAc)4 (Deoxyhexose)1 (NeuAc)1 + (Man) 3 (GlcNac)2
	2950.073	(Hex)3 (HexNAc)4 (Deoxyhexose)2 (NeuGc)1 + (Man) 3 (GlcNac)2

and MFGM samples thus separated were washed twice with acetone (1:4, v/v, 8000*g*, 20 min, 4 °C), dried, and stored at −20 °C until further analysis.

Isolation of FGM GSLs. The cream from colostrum and milk samples was washed several times in 5 volumes of Tris HCl (10 mM, pH 7.2) and kept at cold temperature (4 °C) for 1 h. The suspension was then shaken on a laboratory shaker until butter formed. The process of membrane release was completed by incubating the mixture at 4 °C for 30 min. The membranes were recovered as pellet after centrifugation (3500*g*, 30 min) and washed twice with water prior to freeze-drying.[27] The GSLs from CFGM and MFGM pellets were isolated as described by Puente et al.[28] The samples were homogenized in 10 volumes of chloroform/methanol mixture (C/M, 1:1, v/v) overnight at 4 °C followed by centrifugation (1000*g*, 10 min, 4 °C). The pellets were suspended in 10 volumes of C/M (1:2, v/v), stirred, and centrifuged (1000*g*, 10 min, 4 °C). The pellets thus obtained were rehomogenized in 10 volumes of C/M (1:2, v/v), stirred, and centrifuged as described above. The volume was then reduced to one-quarter by rotary evaporation, and GSLs were isolated by modified Folch partition method.[29]

Release of N- and O-Glycans. The release of N-glycans from the samples was carried out as described by Kita et al.[30] Briefly, the samples (250 μg) were mixed with 0.4% PHM (15 μL), 0.33 M NH_4HCO_3 (30 μL), and 120 mM DTT (10 μL) and incubated at 60 °C for 30 min. The alkylation was carried out by adding 123 mM IAA (20 μL) followed by incubation in the dark at room temperature. Trypsin (400 U) was added to the reaction mixture and incubated at 37 °C for 3 h. The reaction was terminated by heat inactivation at 90 °C for 10 min. The sample was finally incubated with PNGase F (2U) at 37 °C overnight and vacuum-dried.

The O-glycans were released from the samples as per the method of Miura et al.[31] Briefly, the samples (1500 μg) were mixed with

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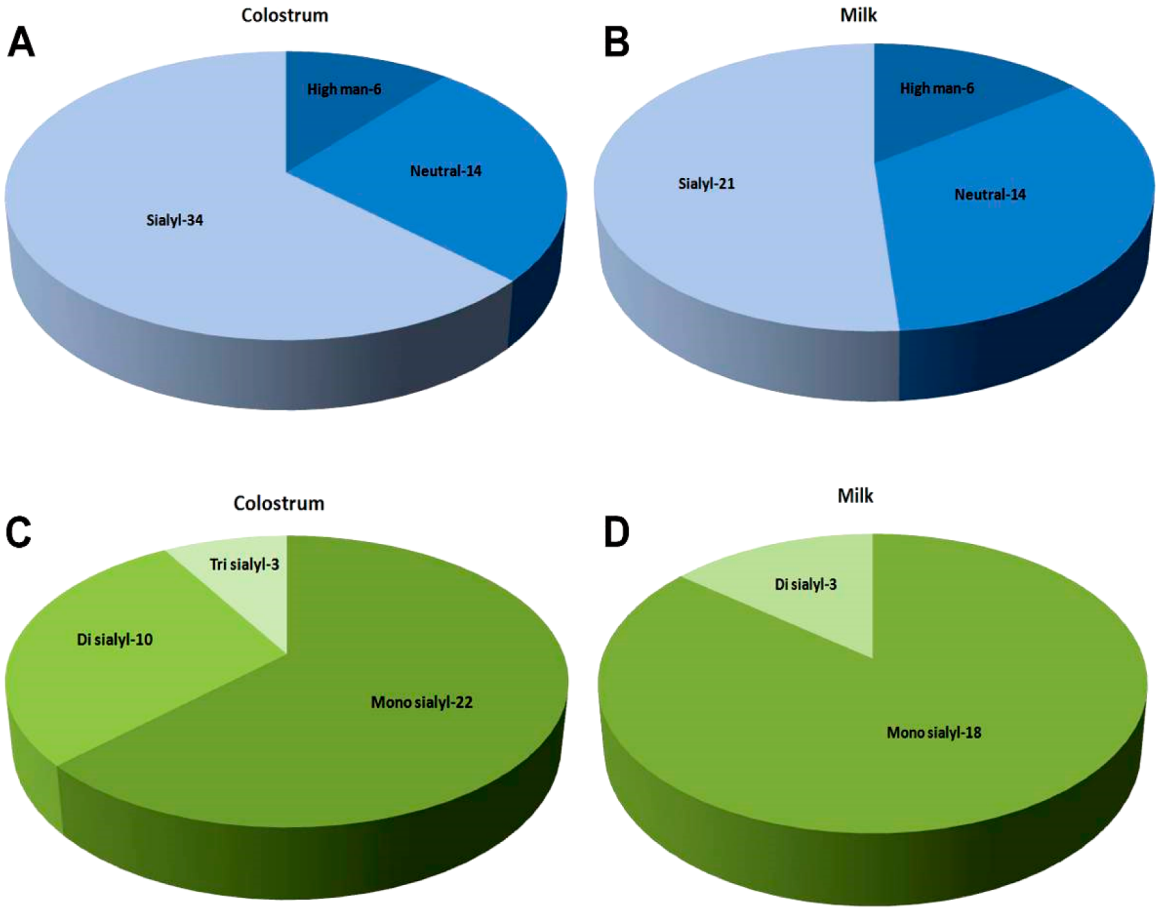

Figure 2. Pie chart showing the relative abundance of N-glycans in buffalo colostrum and milk FGMP: total N-glycans in CFGM (A), total N-glycans in MFGM (B), sialylated N-glycans in CFGM (C), and sialylated N-glycans in MFGM (D).

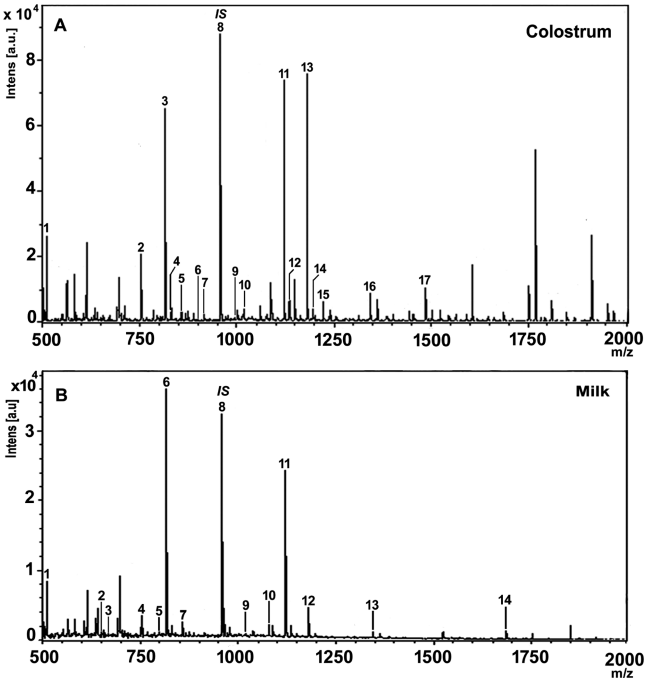

Figure 3. MALDI-TOF MS spectra of O-linked oligosaccharides from buffalo colostrum FGMP (A) and buffalo milk FGMP (B).

ammonium carbamate (1 mg/ μL) and incubated at 60 °C for 20−40 h. The reaction was stopped by adding water (500 μL) and vacuum-dried at 60 °C. To the dried sample, 150 mM acetic acid (500 μL) was added, the pH was adjusted to 3−4, and the sample was vacuum-dried.

Release of Glycans from GSLs. The GSL glycans were released as described by Nagahori et al.[32] Briefly, into the samples suspended in C/M (1:1,v/v), O_3 and N_2 bubbling was done for 5 min and 20 s, respectively, and the samples were vacuum-dried. Both samples were then incubated at room temperature for 30 min with the addition of 500 mM NaOMe in methanol (100 μL). The samples were neutralized by adding AcOH (6 μL) and vacuum-dried.

Chemoselective Glycoblotting. The glycoblotting was performed as previously described by Furukawa et al.[33] In brief, the vacuum-dried N-, O-linked and GSL glycans constituted in distilled water (18 μL) were mixed with internal standards (20−50 pmol; N-glycans, m/z 2348.879 (Hex)2 (HexNAc)2 (NeuAcAm)2 + (Man)3 (GlcNac)2; and O- and GSLs, m/z 958.376 (HexNAc)4) and aliquoted on to a MultiScreen Solvinert filter plate containing Blotglyco H beads (250 μL, 10 mg/mL) followed by addition of 2% AcOH/acetonitrile (ACN) (180 μL).The plate was incubated at 80 °C for 60 min, and the beads were sequentially washed with 200 μL of 2 M guanidine HCl, distilled water, and 1% triethylamine/CH_3OH. The plate was then incubated with 100 μL of 10% Ac_2O/CH_3OH at room temperature for 30 min and washed stepwise in 200 μL of 10 mM HCl, CH_3OH, and dioxane. The esterification of sialic acids was performed in the presence of 100 μL of 100 mM MTT/dioxane with incubation at 60 °C for 60 min with subsequent washings in 200 μL of dioxane, CH_3OH, and distilled water. The N- and O-glycans were labeled as benzyloxiamine (BOA) derivatives by adding BOA (20 μL, 20−50 mM) and 2% AcOH/ACN (180 μL) and were incubated at 80 °C for 60 min. The BOA-labeled glycans were recovered by washing the beads with distilled water (100 μL) and

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Table 3. O-Linked Oligosaccharides of Buffalo Colostrum FGMP

peak no.	m/z	structure
1	511.190	(Hex)1 (HexNAc)1
2	755.296	(HexNAc)3
3	816.301	(Hex)1 (HexNAc)1 (NeuAc)1
	816.301	(HexNAc)1 (Deoxyhexose)1 (NeuGc)1
4	832.296	(Hex)1 (HexNAc)1 (NeuGc)1
5	857.328	(HexNAc)2 (NeuAc)1
6	901.354	(HexNAc)3 (Deoxyhexose)1
7	917.349	(Hex)1 (HexNAc)3
8	958.376	*internal standard*
9	994.349	(Hex)2 (HexNAc)1 (NeuGc)1
10	1019.380	(Hex)1 (HexNAc)2 (NeuAc)1
	1019.380	(HexNAc)2 (Deoxyhexose)1 (NeuGc)1
11	1121.412	(Hex)1 (HexNAc)1 (NeuAc)2
	1121.412	(HexNAc)1 (Deoxyhexose)1 (NeuAc)1 (NeuGc)1
12	1137.407	(Hex)1 (HexNAc)1 (NeuAc)1 (NeuGc)1
		(HexNAc)1 (Deoxyhexose)1 (NeuGc)2
13	1181.433	(Hex)2 (HexNAc)2 (NeuAc)1
		(Hex)1 (HexNAc)2 (Deoxyhexose)1 (NeuGc)1
14	1197.429	(Hex)2 (HexNAc)2 (NeuGc)1
15	1222.460	(Hex)1 (HexNAc)3 (NeuAc)1
		(HexNAc)3 (Deoxyhexose)1 (NeuGc)1
16	1343.486	(Hex)3 (HexNAc)2 (NeuAc)1
		(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuGc)1
17	1486.544	(Hex)2 (HexNAc)2 (NeuAc)2
		(Hex)1 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 (NeuGc)1
		(HexNAc)2 (Deoxyhexose)2 (NeuGc)2

Table 4. O-Linked Oligosaccharides of Buffalo Milk FGMP

peak no.	m/z	structure
1	511.190	(Hex)1 (HexNAc)1
2	654.249	(HexNAc)1 (NeuAc)1
3	670.244	(HexNAc)1 (NeuGc)1
4	755.296	(HexNAc)3
5	803.306	(Hex)1 (HexNAc)1 (Deoxyhexose)2
6	816.301	(Hex)1 (HexNAc)1 (NeuAc)1
		(HexNAc)1 (Deoxyhexose)1 (NeuGc)1
7	857.328	(HexNAc)2 (NeuAc)1
8	958.376	*internal standard*
9	1019.380	(Hex)1 (HexNAc)2 (NeuAc)1
		(HexNAc)2 (Deoxyhexose)1 (NeuGc)1
10	1079.402	(Hex)2 (HexNAc)3
11	1121.412	(Hex)1 (HexNAc)1 (NeuAc)2
		(HexNAc)1 (Deoxyhexose)1 (NeuAc)1 (NeuGc)1
12	1181.433	(Hex)2 (HexNAc)2 (NeuAc)1
		(Hex)1 (HexNAc)2 (Deoxyhexose)1 (NeuGc)1
13	1343.486	(Hex)3 (HexNAc)2 (NeuAc)1
		(Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuGc)1
14	1692.623	(Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1
		(Hex)2 (HexNAc)3 (Deoxyhexose)2 (NeuGc)1

vacuum-dried. The total labeled glycans (1 μL) mixed with 2,3-dihydroxybenzoic acid (DHB)/DHB Na (9:1, 1 μL) were spotted on the MALDI plate and subjected for MALDI-TOF MS.

Analysis of N-, O-, and GSL Glycans. The BOA labeled N-, O-, and GSL glycans were analyzed by MALDI-TOF mass spectrometer in positive ion reflector mode using Auto flex III TOF/TOF (Bruker Daltonics, Bremen, Germany). The spectra were recorded in a linear mode using FLEX CONTROL 3.0 software.

The MALDI-TOF MS data was analyzed using FLEX ANALYSIS 3.0 software. The N-glycans, O-glycans, and GSL glycans were identified by database search using http://www.expasy.ch/tools/glycomod, http://glycosuitedb.expasy.org, and http://jcggdb.jp. The TOF/TOF analysis was performed with the abundant glycan peaks.

Antimicrobial Analysis. The bacterial strains *E. coli*, *S. aureus*, *Klebsiella pneumoniae* (*K. pneumoniae*), and *Pseudomonas aeruginosa* (*P. aeruginosa*) were subcultured from frozen glycerol stocks on to the appropriate agar medium and were incubated at 37 ± 2 °C in aerobic atmosphere in nutrient broth. After 18−24 h of incubation, the optical density (OD) was adjusted spectrophotometrically at 600 nm and was standardized at 0.1 ± 0.02 by diluting with sterile broth. A bacterial suspension adjusted in this way contains ∼1.5 × 10^8 CFU/mL. The inoculums on further dilution (1:10) had resulted in a final inoculum of ∼1.5 × 10^5 CFU/mL. This was inoculated onto the nutrient broth containing varied concentrations of test samples (500 ng−2 mg/mL) along with reference standard chloramphenicol (0.0625−32 μg/mL) in a microtiter plate and incubated for 18−24 h at 37 ± 2 °C in aerobic condition. The antimicrobial effect (MIC) was read manually as the lowest concentration of sample, which completely inhibited the visible growth. Experiments were carried out in triplicate and repeated thrice.

RESULTS

Isolation of FGM Proteins from Colostrum and Milk Samples. The FGM proteins were isolated from first day buffalo colostrum and milk samples. The average yield of FGM proteins was found to be higher in colostrum (700 μg) compared to milk sample (500 μg) per mg of dry weight of cream. The samples resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed the presence of major FGM proteins (Supporting Information) and their glycoprotein nature (data not shown). Both samples were analyzed to unravel their glycan complexities to get better insight into their functionalities.

Glycome Characterization of Colostrum and Milk FGM. The structural analysis of N- and O-linked and GSL glycans was performed by chemoselective glycoblotting technique. Quantitation of individual glycans was carried out using internal standards prior to glycoblotting.

The N-linked oligosaccharides released from the protein backbone after PNGase F treatment were derivatized as reported earlier.[30] The combined mass spectra of MALDI-TOF-MS separation of colostrum and milk fat globule membrane proteins were shown in Figure 1A and B. A sum total of 54 and 41 N-linked oligosaccharides were identified in colostrum (Table 1) and milk (Table 2) samples, respectively. On the basis of their structural complexity, N-linked glycans were classified as sialyl, neutral, and high-mannose type of oligosaccharides. Both colostrum and milk samples were found to contain bi-, tri-, and tetra-antennary sialylated complex N-linked glycoforms. There were higher numbers of sialyl glycans in colostrum (34) compared to milk (21) FGM, while neutral (14) and high-mannose (6) glycans were found to be the same in both samples. Among sialyl oligosaccharides, mono-, di-, and trisialyl glycans were present in CFGM, while MFGM was found to contain only mono- and disialyl glycans (Figure 2). In fact, monosialyl glycans were predominant in both CFGM and MFGM proteins. Many of the glycan structures showed increasing complexity either by the addition of fucose on the reducing end GlcNAc or by the addition of fucose and/or sialic acid on the exposed nonreducing end. Thus, we unravelled significant structural differences between CFGM and MFGM oligosaccharides.

O-linked oligosaccharides are the major glycan structures from the high molecular weight glycoproteins from colostrum

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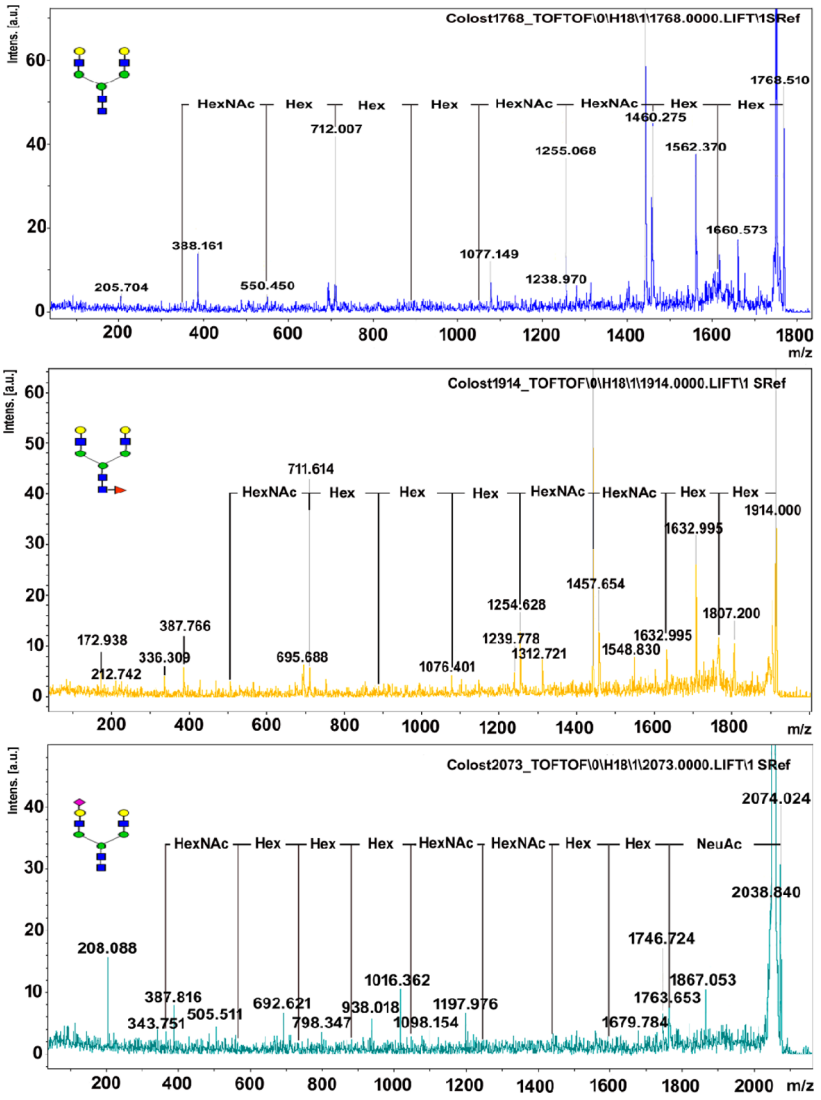

Figure 4. MALDI-TOF/TOF of major buffalo colostrum FGMP N-linked oligosccharides with m/z 1768, 1914, and 2073.

and milk FGM. It was observed that the O-linked glycan profile of CFGM was different from that of the MFGM sample. The MALDI-TOF MS of O-linked glycans of both colostrum and milk FGM was as shown in parts A and B of Figure 3. Buffalo MFGM was typically found to contain only 13 oligosaccharides, while in CFGM there were 16 oligosaccharides (Tables 3 and 4). In both samples predominantly core 1 oligosaccharides were present, and possible structures of core 2, core 3, and core 4 glycans were noticeable, which needs further confirmation. The overall observation explicates the differences in their number and type of sialyl glycans between colostrum and milk samples, wherein CFGM was found to be rich in sialylated oligosaccharides that decreased during the course of its maturation to milk.

To further confirm the glycoforms of abundant glycans, TOF/TOF analysis was performed. The m/z 1768, 1914, and 2073 of CFGM and 2219, 2260, and 2584 of MFGM were chosen for analysis. As represented in Figures 4 and 5 based on the spectral signatures, the structures (Hex)2(HexNAc)2 + (Man)3(GlcNac)2, (Hex)2(HexNAc)2 (Deoxyhexose)1 + (Man)3 (GlcNac)2, and (Hex)2 (HexNAc)2 (NeuAc)1 + (Man)3 (GlcNac)2 for m/z 1768, 1914, and 2073 of CFGM, respectively, and (Hex)2 (HexNAc)2 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2, (Hex)1 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2, and (Hex)3 (HexNAc)3 (Deoxyhexose)1 (NeuAc)1 + (Man)3 (GlcNac)2 for m/z 2219, 2260, and 2584 of MFGM, respectively, were deduced.

To characterize the sugar moieties of fat globule membrane GSL, glycomic analysis was performed. The glycan analysis from colostrum and milk fat globule membrane GSL revealed the variations between the samples as shown in Figure 6A and B. The major gangliosides were found to be GM3 and GD3 irrespective of the samples, but their proportions were almost 3-fold higher in colostrum than in milk. The other glycans identified were as shown in Table 5 for colostrum and milk samples, respectively. Thus, characterization of total glycome of fat globule membrane was achieved by adopting chemoselective glycoblotting strategy.

Antimicrobial Activity of CFGM and MFGM Proteins on Human Pathogens. To verify the antimicrobial potency of both colostrum- and milk-derived fat globule membrane proteins (FGMPs), the minimum inhibitory concentration on human pathogens *E. coli*, *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* was tested. FGMPs from colostrum and milk were tested from concentrations ranging from 0.05 to 2 mg/mL. Both samples in their native form did not show any effect on

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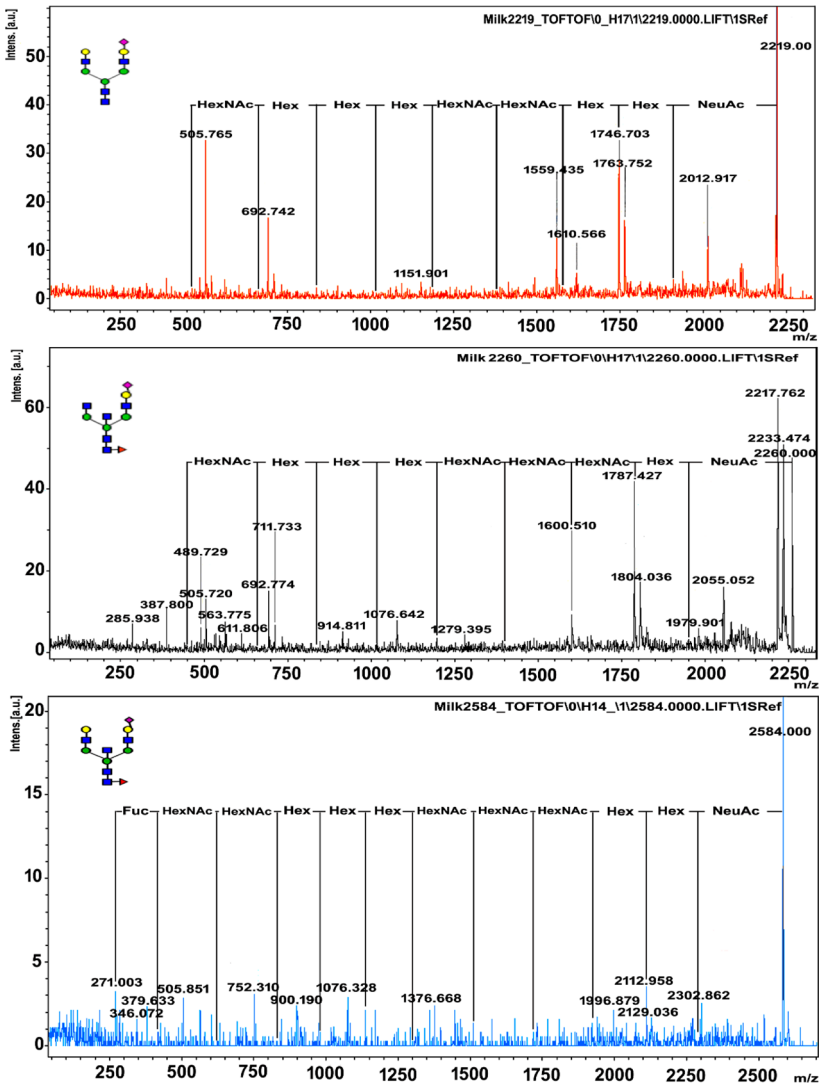

Figure 5. MALDI-TOF/TOF of major buffalo milk FGMP N-linked oligosaccharides with m/z 2219, 2260, and 2584.

any test organisms. Interestingly, CFGM N-glycan-enriched sample after trypsin digestion followed by PNGase treatment exhibited growth inhibition with MIC as low as 128 μg/mL for *P. aeruginosa* and 256 μg/mL for *E. coli*, *S. aureus*, and *K. pneumoniae* (Table 6). However, MFGM sample after PNGase treatment showed MIC of 256 and 512 μg/mL for *P. aeruginosa* and *S. aureus*, respectively, with no effect on *E. coli* and *K. pneumoniae*. Chloramphenicol was used as a standard positive control for all organisms tested (4−38 μg/mL).

DISCUSSION

Milk fat globule membrane is unique, comprising phospholipids, sphingolipids, and several proteins and glycoproteins. Although fat globule membrane proteins from human and bovine milk have been already characterized with well-assigned functional attributes, no or little information is available on the glycoconjugates present in buffalo milk or colostrum.[4,16,25] In this context, the present study undertaken unravels structural complexity of glycans bound to glycoproteins and glycosphingolipids of fat globule membranes from buffalo colostrum and milk. Protein-bound oligosaccharides were predominantly higher with a greater degree of heterogeneity in colostrum compared to milk fat globule membrane proteins. The major glycosphingolipids were common in both samples, but their relative proportions differed significantly. In addition, the colostrum-based, glycan-enriched fraction was found to be more effective in eliciting antibacterial effect against human pathogens compared to milk fat globule membrane proteins.

The FGMs, being complex with various health benefits, have created growing interest to characterize their structural complexities in relation to biological effects among lactating mammals. The major (glyco)proteins of FGM include mucin 1, xanthine dehydrogenase/oxidase, mucin 15, cluster of differentiation 36, PAS, butyrophilin, adipophilin, lactadherin, and fatty acid binding protein.[19] A recent review on the isolation of FGM clearly indicated that the subtle variations in the methods of isolation reflect on their yield and composition and suggested quantitating proteins in the FGM samples with the particular method of their isolation.[9] Accordingly, in the present study the protein quantification indicated the presence of more proteins in the FGM of early milk than in that of the mature milk. Earlier studies have focused on milk FGM proteome from various mammals with limited focus on buffalo milk.[34−36] The proteomic characterization of buffalo milk FGMP has been reported by D'Ambrosio et al.[36] and Nguyen et al.,[25] but few studies reflect on the glycomic characterization of FGMP

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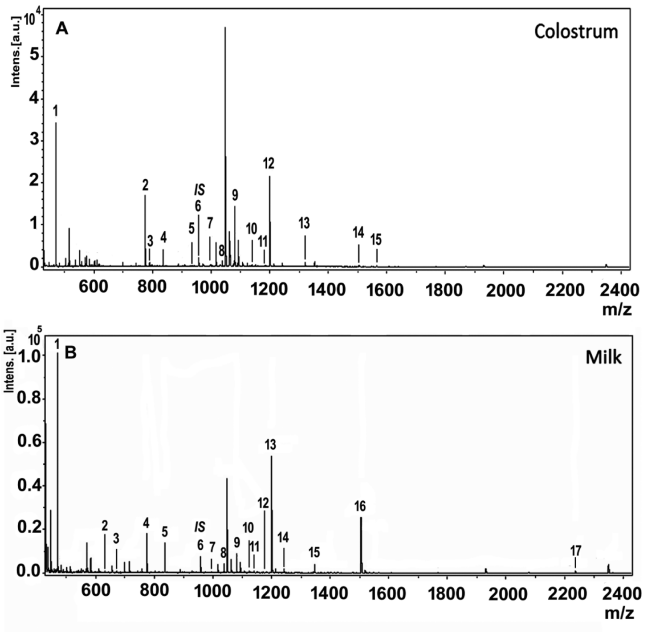

Figure 6. MALDI-TOF MS spectra of glycosphingolipids from buffalo colostrum FGM (A) and milk FGM (B).

and GSLs.[3,4,36] Structural studies on the colostrum and milk fat globule membrane proteins showed colostrum samples to possess a greater diversity with respect to both their number as well as their complexity in comparison to milk fat globule membrane proteins. However, interestingly, structural glycoforms were found to be significantly higher compared to bovine milk fat globule membrane proteins, and many glycans were similar to those present in human milk fat globule membrane proteins.[3,4] In fact, we made similar observations during the characterization of N-linked oligosaccharides from buffalo colostrum IgG.[37] The buffalo colostrum IgG oligosaccharides were more similar to human IgG than bovine IgG with a higher degree of fucosylation, a feature common to human IgG. However, further investigation on glycotransferases may explicitly show the complexity in glycosylation among ruminants as well as between colostrum and milk samples.

Earlier studies on fat globule membrane proteins revealed that O-linked glycans in bovine milk are predominantly of core 1 structure.[5] In accordance, even in buffalo colostrum and milk samples, the core 1 glycans were more common, but the presence of other core structures including core 2 O-glycans was also indicated. Thus, the occurrence of such variations in epitopes needs detailed investigations. A previous report on MFGM glycoproteins indicates significant differences between CFGM and MFGM samples during lactation.[38] Ujita et al.[39] observed a decrease in MFGM sialylation in the first 5 days post-parturition, with a decrease in acidic oligosaccharide structures from 74% at day 1 to 59% at day 5. A similar pattern was observed by Wilson et al.,[4] who characterized the decline of monosialylated core-2 O-glycans after the third day of lactation. Thus, the present study also supports the fact that variations are prevalent in glycan composition between colostrum and milk samples in buffalo fat globule membrane proteins too. Further, the divergence observed between human and buffalo glycoprofiles reflects different biological needs of neonates and calves during their growth and development.

Fat globule membranes isolated from mature milk and first-day colostrum samples exhibited several individual GSL species ranging from neutral to acidic glycoforms. The major gangliosides were GD3 and GM3 in both samples in addition to minor ones. The pattern was, however, in analogy to bovine milk fat globule membrane.[14,40] Interestingly, similar results were also observed in human colostrum fat globule membrane gangliosides with slight variation in minor gangliosides.[15] The beneficial effects of human milk gangliosides in infant formula improved cognitive disorders, and supplements enriched with a MFGM product in preschool children decreased febrile episodes and improved behavioral regulation.[41,42] Hence, the current data set showing the presence of similar GSL molecular species may also have implications in designing biomimetic lipid droplets in infant milk formulas mimicking human milk, as reported by Gallier et al.[43] Thus, ruminant glycans with remarkable degrees of structural, functional, and nutritional similarity/diversity may have possible interventions in the preparation of fortified complementary foods that support normal growth and development as well as enhance immunity and cognitive performances in formula-fed infants.

Table 5. Glycosphingolipids of Buffalo Colostrum FGM and Milk FGM

colostrum		
peak no.	*m/z*	structure
1	470.164	(Hex)2
2	775.275	(Hex)2 (NeuAc)1
3	791.270	(Hex)2 (NeuGc)1
4	835.296	(Hex)3 (HexNAc)1
5	934.328	(Hex)1 (NeuAc)1 (NeuGc)1
6	958.376	***internal standard***
7	997.349	(Hex)4 (HexNAc)1
8	1038.375	(Hex)3 (HexNAc)2
9	1080.386	(Hex)2 (NeuAc)2
10	1140.407	(Hex)3 (HexNAc)1 (NeuAc)1
		(Hex)2 (HexNAc)1 (Deoxyhexose)1 (NeuGc)1
11	1184.433	(Hex)3 (HexNAc)2 (Deoxyhexose)1
12	1200.428	(Hex)4 (HexNAc)2
13	1321.455	(Hex)6 (HexNAc)1
14	1505.539	(Hex)4 (HexNAc)2 (NeuAc)1
		(Hex)3 (HexNAc)2 (Deoxyhexose)1 (NeuGc)1
15	1565.560	(Hex)5 (HexNAc)3
milk		
peak no.	*m/z*	structure
1	470.164	(Hex)2
2	632.217	(Hex)3
3	673.243	(Hex)2 (HexNAc)1
4	775.275	(Hex)2 (NeuAc)1
5	835.296	(Hex)3 (HexNAc)1
6	958.376	***internal standard***
7	994.349	(Hex)2 (HexNAc)1 (NeuGc)1
8	1038.375	(Hex)3 (HexNAc)2
9	1080.386	(Hex)2 (NeuAc)2
10	1127.412	(Hex)3 (HexNAc)1 (Deoxyhexose)2
11	1140.407	(Hex)3 (HexNAc)1 (NeuAc)1
		(Hex)2 (HexNAc)1 (Deoxyhexose)1 (NeuGc)1
12	1184.433	(Hex)3 (HexNAc)2 (Deoxyhexose)1
13	1200.428	(Hex)4 (HexNAc)2
14	1239.439	(Hex)1 (NeuAc)2 (NeuGc)1
15	1346.486	(Hex)4 (HexNAc)2 (Deoxyhexose)1
16	1505.539	(Hex)4 (HexNAc)2 (NeuAc)1
		(Hex)3 (HexNAc)2 (Deoxyhexose)1 (NeuGc)1
17	2235.804	(Hex)5 (HexNAc)4 (Deoxyhexose)1 (NeuGc)1

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Table 6. Antimicrobial Activity of PNGase-F Treated Buffalo Colostrum FGMP and Milk FGMP[a]

		bacterial strains (μg/mL)			
test sample	solvent	*Escherichia coli* MTCC7410	*Staphylococcus aureus* MTCC7443	*Klebsiella pneumoniae* MTCC109	*Pseudomonas aeruginosa* ATCC9027
colostrum	DMSO	256	256	256	128
milk	DMSO	NI[b]	512	NI[b]	256
chloramphenicol	DMSO	4	4	8	38

[a]Values are mean of triplicates. [b]NI, no inhibition.

FGMP possess antibacterial,[18,22] immunomodulatory,[44] antiviral,[19] and antiadhesive[20,45] properties. The antibacterial activity of buffalo CFGM and MFGM on the selected human pathogens tested in the present study indicated the inhibitory role of N-glycans on the growth of *E. coli*, *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* unlike the native CFGM or MFGM samples. This differential effect may be attributed to the fact that in the native FGMP sample the glycans may not be exposed to elicit their actions, which upon enzymatic treatment act as effective antibacterials. This is in analogy to our recent report showing growth inhibitory potential of N-glycans from colostrum IgG on *K. pneumoniae* that reflected the anti-infective property of colostrum-derived oligosaccharides against undesirable human pathogens.[37] The role of the glycan chains as decoy receptors to function as antimicrobial agents for the prevention of infection is becoming more apparent with several studies.[2,6,46,47] In fact, high-mannose glycans from bovine lactoferrin, in particular, are attractive potential ingredients in the functional food industry.[48] Further, FGM mucins have been shown to inhibit *H. pylori* colonization with Neu5Ac playing a key role in *H. pylori* inhibition.[47,49] Similarly, human milk mucin glycoproteins are also attributed to bind to rotavirus and inhibit HIV-1 transmission.[19] In summary, the present study unravelled the structure−function relationship of oligosaccharides of early and mature milk. The analysis led to the identification of glycan heterogeneity among the samples. The N- and O-glycans were higher and more complex in CFGM than in MFGM, while major GSLs were common in both samples but their proportions were significantly different, suggesting their functional role in early neonatal growth and development. In addition, CFGM was found to be a more efficient antibacterial than MFGM. The structural similarities of buffalo CFGM and MFGM to human and bovine MFGM glycoconjugates shed light for the future developments of buffalo colostrum-/milk-based infant food formulations from neonatal health perspective.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b03330.

SDS-PAGE analysis of fat globule membrane proteins (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel. (O): 91-821-2419482. Fax (O): 91-821-2419363. E-mail: hsa.uom@gmail.com.

ORCID

Huligerepura Sosalegowda Aparna: 0000-0001-5890-8407

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