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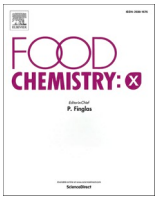

Comparative analysis of oligosaccharides in the milk of human and animals by using LC-QE-HF-MS

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ABSTRACT

The complex oligosaccharides (OS) in different milk are more difficult to detect and complicated to analyze as their enormous structural complexity. UPLC-QE-HF-MS was supposed to be a highly effective method for OS identification. In present study, 70 human milk oligosaccharides (HMOs), 14 bovine milk oligosaccharides (BMOs), 23 goat milk oligosaccharides (GMOs) and 24 rat milk oligosaccharides (RMOs) were detected by using UPLC-QE-HF-MS, respectively. There were highly differences in number and composition between the four milk OS. 14 neutral and 3 acidic OS were firstly found in rat milk. The composition and abundances of RMOs were might more similar to that of HMOs, comparing with BMOs and GMOs. The similarity between HMOs and RMOs might provide theoretical basis for better application of rats in biological/biomedical studies of HMOs as models. The BMOs and GMOs were expected to be suitable for applications in medical and functional foods as a promising bioactive molecular.

1. Introduction

Milk is a highly nutritional food containing various OS with complex structures. As a class of bioactive molecules, these OS are the third largest component after lactose and lipids in human milk (Lu, Zhang, Song, Zhang, Pang, Sari, et al., 2020). The milk OS play important roles in improving growth and development of newborns (Underwood, Gaerlan, De Leoz, Dimapasoc, Kalanetra, Lemay, et al., 2015). The OS are generally defined as carbohydrate polymers that contain 3 to 10 monosaccharide units covalently linked through glycosidic bonds (N Tao, DePeters, Freeman, German, Grimm, & Lebrilla, 2008), which are divided into (i) neutral OS, whose structures are mainly lactose linked with neutral monosaccharides such as glucose or galactose (Hex), *N*-acetylglucosamine or *N*-acetylgalactosamine (HexNAc) and fucose or deoxyhexose (Fuc) and (ii) acidic OS, containing acidic components such as *N*-acetylneuraminic (NeuAc) also known as sialic acid or *N*-glycolylneuraminic acid (NeuGc) (Martín-Ortiz, Salcedo, Barile, Bunyatratchata, Moreno, Martin-García, et al., 2016; Meyrand, Dallas, Caillat, Bouvier, Martin, & Barile, 2013).

There are large numbers of bioactive OS in human milk and animal milk, and the quite differences in contents and diversities of OS between human and animal milk are described according to an increasing number of reports (Li, Jiang, Zhou, Ding, Guo, Li, et al., 2021). The human milk contains more OS than non-human mammalian milk and there are more than 200 structures of HMOs have been identified so far (Albrecht, Lane, Marino, Al Busadah, Carrington, Hickey, et al., 2014; Ninonuevo, Park, Yin, Zhang, Ward, Clowers, et al., 2006). Currently, the number of GMOs have been found is up to 78 (Martín-Ortiz, et al., 2016), which is approximately 4 times higher than bovine milk, but the concentration is still much lower than that in human milk (Albrecht, et al., 2014; Sousa, Medeiros, Pintado, & Queiroga, 2019). There are 50 compositions and 37 structures of BMOs have been determined until now (Aldredge, Geronimo, Hua, Nwosu, Lebrilla, & Barile, 2013). Rats have been used as a common tooling model in various biomedical studies (Dvorak, Halpern, Holubec, Dvorakova, Dominguez, Williams, et al., 2004) and the anatomy, physiology, genetics, basic biology and biochemistry of rats and mice are well-understood (Gosling, 2001), but there are very limited studies on RMOs comparing with HMOs, BMOs and GMOs. Only

Abbreviations: OS, oligosaccharides; HMOs, human milk oligosaccharides; BMOs, bovine milk oligosaccharides; GMOs, goat milk oligosaccharides; RMOs, rat milk oligosaccharides; Hex, hexose; dHex, deoxyhexose; HexNAc, *N*-acetylhexosamine; Fuc, fucose; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycotylneuraminic acid; 3′-SL, 3′-sialyllactose; 6′-SL, 6′-sialyllactose; ESI, electrospray ionization; TIC, total ion chromatograms.

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three acidic OS, including 3′-sialyllactose (3′-SL), 6′-sulphated lactose (6′-Su-L) and 6′-sulphate-3′-sialyllactose (6′-Su-3′-SL), have been identified in rat milk a few decades ago (Carubelli, Ryan, Trucco, & Caputto, 1961; Choi & Carubelli, 1968; Naccarato, Ray, & Wells, 1975). In a recent study, 15 acidic RMOs that containing 9 monosialylated, 2 disialylated, 1 monosulphated, and 3 both monosulphated and monosialylated OS have been detected by a high-sensitivity online solid-phase extraction and hydrophilic interaction chromatography coupled with electrospray tandem mass spectrometry (HPLC-ESI-CID-MS/MS)(Li, et al., 2021). However, the neutral RMOs are still unclear so far. The well-understanding of completely basic information of bioactive RMOs are of great interest to biological/biomedical researches using rats as model.

To date, a large number of studies using different analytical methods to detect or quantify the OS in human and animal milk, such as high performance liquid chromatography (HPLC)(Tadasu Urashima, Asakuma, Leo, Fukuda, Messer, & Oftedal, 2012), microfluidic chips and mass spectrometry (Leo, Asakuma, Fukuda, Senda, & Urashima, 2010), HPLC-mass spectrometry (HPLC-MS)(Austin & Bénet, 2018), high performance anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD)(Wang, Zhou, Gong, Chen, Feng, Liu, et al., 2020), nuclear magnetic resonance (NMR)(Urakami, Saeki, Watanabe, Kawamura, Nishizawa, Suzuki, et al., 2018), capillary electrophoresis (Monti, Cattaneo, Orlandi, & Curadi, 2015), ultra performance liquid chromatography coupled with Q-Exactive Focus mass spectrometry (UPLC-Q-Exactive Focus–MS/MS)(Lu, et al., 2020) and HPLC-ESI-CID-MS/MS (Li, et al., 2021). In recent years, the advanced liquid chromatography coupled with a triple quadrupole mass spectrometer (LC-QQQ-MS/MS) was used to detect and absolute quantify twelve OS in human milk (Zhang, et al., 2022). UPLC-QE-HF-MS was successfully adopted to identify the profiles of HMOs in breast milk of four mothers and fecal OS in the feces of their breast-fed infant in our previous study (Li, Zhou, & Xu, 2023). Although several methods have been reported to successfully identify and analyze OS in human and animal milk, the complex OS in different milk are more difficult to detect and more complicated to analyze due to their enormous structural complexity. The ultra-performance liquid chromatography-Q Exactive-HF hybrid quadrupole-Orbitrap-mass spectrometry (UPLC-QE-HF-MS) is an advance technique with higher resolutional, higher precise, and higher sensitive, compared with other existing methods in some previous reports stated above. The usage of UPLC-QE-HF-MS for analyzing OS in animal milk has not been reported yet. The more accurate information of newly OS were speculated to be found using this technique. There aren't much OS data on animal milk from other species, especially for the rat milk, that show how many different oligosaccharide structures are typically present compared to human milk. More information is required, nevertheless, regarding the structural and analytical properties of oligosaccharides found in the milk of various animals. Meanwhile, the feasibility of the new method UPLC-QE-HF-MS for HMOs identification has been verified in our previous research (Li, Zhou, & Xu, 2023). We supposed that UPLC-QE-HF-MS could also be a highly effective method for animal OS identification, especially for the unknown neutral RMOs, since UPLC-QE-HF-MS has higher resolution than regular triple/four-stage quad or orbitrap system, and the results of identification will be more accurate. In general, the aim of this study was to investigate the composition of OS present in rats, goats, bovine and human milk and compare their differences using UPLC-QE-HF-MS. In this work, we have firstly identified and analyzed the neutral and acidic OS in various milk by using LC-QE-HF-MS, and then comparative analyzed the differences in composition between human milk and animals milk, such as goat milk, bovine milk and rat milk. These complete knowledge of RMOs was regarded as basic information and should be helpful for further biological and biomedical researches using rats as models, especially for fundamental studies on OS that could not be performed in human beings. Furthermore, it was necessary for comprehensively comparing the distinctions of OS among human milk and animal milk so as to provide the theoretical basic for better understanding the potential values of animal milk as commercially nutritional substitutes for mother's milk in food industry. Meanwhile, it was of great significance to clarify the composition of RMOs and their similarities or differences with HMOs as for further exploring the mechanism of healthy effects of OS.

2. Materials and methods

2.1. Reagents and materials

The rat milk sample was collected from mature breast rats in Health Science Center of Peking University. The bovine milk and goat milk were obtained from Lvneng farms (Shanxi, China). The puerperal women who underwent routine postpartum examination at postnatal 42 days were recruited in Maternal and Child Health Care Hospital of Changping District, Beijing, from January 1st, 2021 to June 30th, 2021. The inclusions were as follows: being with no diabetes, hypertension, abnormal thyroid function, abnormal liver function, abnormal kidney function and other diseases, no mastitis and other breast related diseases, no serious genetic defects and mental diseases; no history of long-term use of antibiotics; agreeing to participate in this study and signing the informed consent. 10 mL breast milk was collected with a sterile electric breast pump, and the nipple and areola were disinfected before collection. After collection, all milk samples were mixed together ($n \geq 5$ in each group) and stored at −80℃ for further use. Methanol, methanoic acid, acetonitrile and ammonium formate were purchased from Beijing Chemical Reagent Co. (China). All reagents were of analytical grade or chromatographic grade.

2.2. Sample preparation

The methods of sample preparation were performed according to a previously published method (Lu, et al., 2020) and with some modifications. Samples were centrifuged at 10,000 g for 30 min at 10 °C to remove the upper fat. Two volumes of methanol were subsequently added and incubated at 4 °C for 2 h for removing the whey protein. The mixture solution was centrifuged at 4000 g for 30 min at 4 °C for complete phase separation. The supernatant contains OS fraction was carefully transferred to a new tube for later analysis.

2.3. OS detection by UPLC-QE-HF-MS

In current research, an ACQUITY UPLC BEH Amide Column (2.1 mm × 100 mm, 1.7 μm) was used to separate OS on Waters system. The basic parameters were listed as follows: The column temperature: 35 ℃; Flow rate: 0.3 mL/min; Mobile phase A: acetonitrile; Mobile phase B: 10 mM ammonium formate; Gradient elution condition: 0–20 min 95 %-78 % A; 20–35 min, 78 %-73 % A; 35–38 min, 73 %-62 % A; 38–45 min, 62 %-50 % A; The injection volume: 10 μL.

The OS was determined by UPLC-QE-HF-MS with an electrospray ionization (ESI) source. The mass spectrum conditions included the heated capillary of 320 ℃, the spray voltage was 3.8 kV in positive mode and 3.4 kV in negative mode, the curtain gas was at 35 psi, collision-activated dissociation was at medium. Each ion is scanned based on optimized declustering voltage and collision energy during detection. The analysis was operated with full scan (m/z 300–2000) in positive and negative mode for all OS. The matching and analysis of all possible OS was performed according to JCGG database (https://jcggdb.jp/idb/indexList.do?id=inchikey) and OS database (This database is a self-built database and the data was obtained from the literatures). The matching degree between the actual molecular weight and the theoretical molecular weight is controlled within 5 ppm. The mass range for MS was set to m/z 400–2000. The difference of retention time (RT) less than 0.5 min was used as a repeating substance.

3. Results and discussion

There were approximately 40 HMOs standards commercially available at the different suppliers available for determination of HMOs, but these standards were still insufficient for quantifying hundreds of founded HMOs. Therefore, the advanced UPLC-QE-HF-MS technique was applied for identifying the large number of OS in human, bovine, goat and rat milk. The total ion chromatogram (TIC) of different samples were shown in Fig. 1. The neutral OS and acidic OS at each peak of Fig. 1 were analyzed by two databases as seen in Table 1 and Table 2, each MS/MS spectra of LC fractions of OS in Table 1 and 2 can be found in Supplementary Fig. S1 and S2, respectively.

3.1. Identification and analysis of neutral OS in various milk

As seen in Table 1, Hex2HexNAc1Fuc1, Hex3HexNAc1dHex1, Hex4HexNAc2dHex1, Hex3HexNAc3dHex1, Hex5HexNAc3, Hex4HexNAc2dHex3, Hex5HexNAc2dHex2, Hex5HexNAc3dHex1, Hex5HexNAc3dHex2 and Hex6HexNAc4 were exclusively detected in human milk. Except bovine milk, the Hex2Fuc1 at *m/z* 488.1741 and its isomers

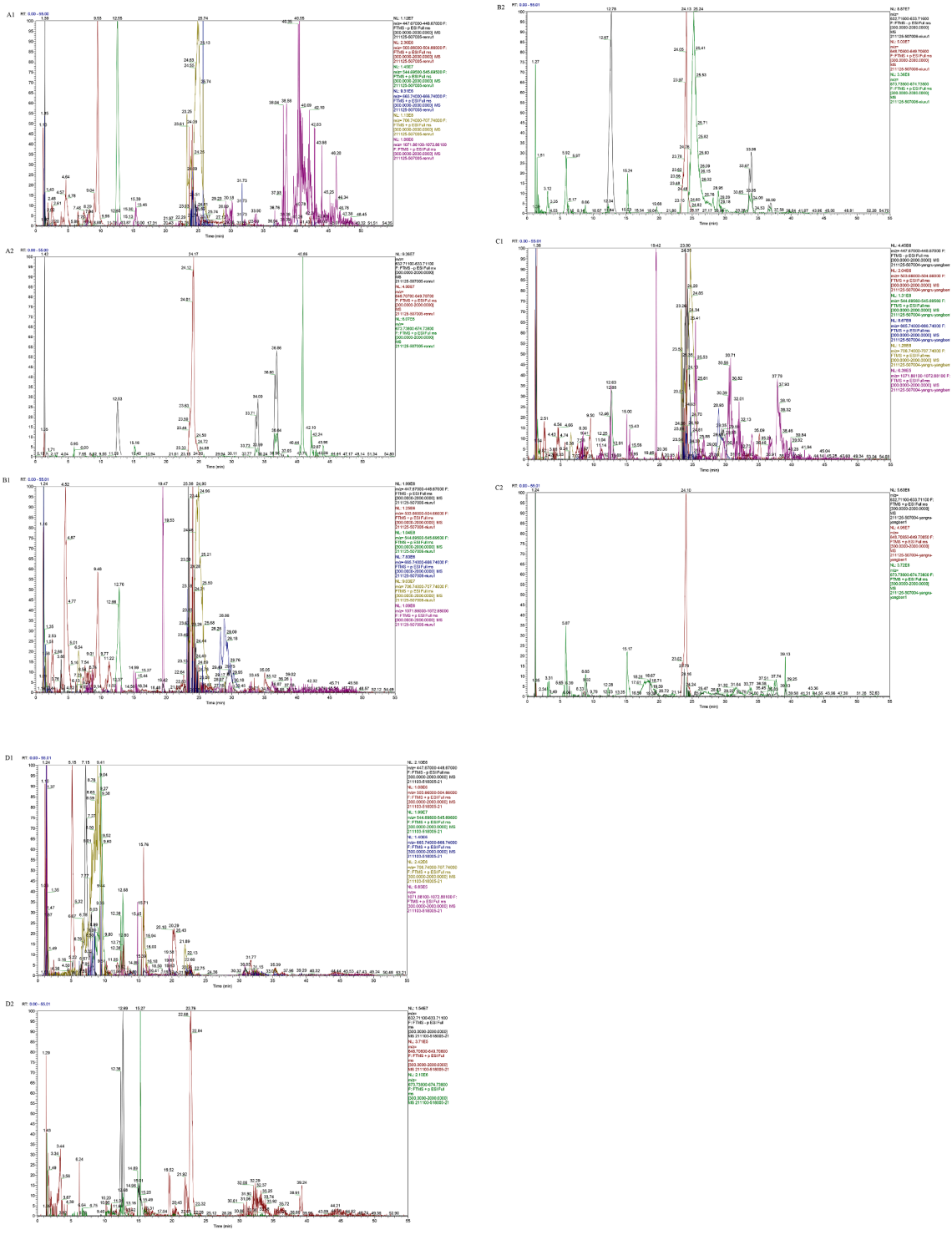
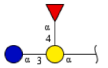
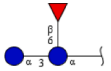
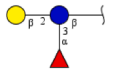
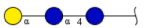


Fig. 1. Total ion chromatogram of OS from (A1&A2) Human milk, (B1&B2) Bovine milk, (C1&C2) Goat milk and (D1&D2) Rat milk.

Table 1
Neutral OS identified and their peak area in various milk.

Composition	RT(min)	Mass		Ion	Peak area ($\times 10^6$)				Database	Proposed Structure
		Found	Cal		Human	Bovine	Goat	Rat		
Hex2Fuc1	30.218	488.1741	488.17399	H-	16860.9498	-	-	-	OS	
Hex2Fuc1	29.257	488.1741	488.17397	H-	14384.7856	-	-	-	OS	
Hex2Fuc1	28.291	488.1741	488.17443	H-	-	-	36.6928	-	OS	
Hex2Fuc1	28.253	488.1741	488.17374	H-	-	-	-	475.3737	OS	
Hex3	32.89	504.169	504.16836	H+	666.6315	-	-	-	OS	
Hex3	34.281	504.169	504.16839	H+	60.5216	1414.7185	650.2258	-	OS	
Hex3	24.147	504.169	504.16722	H+	-	1188.6947	379.5678	-	OS	

(*continued on next page*)

Table 1 (*continued*)

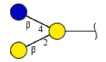
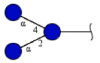
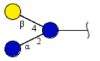
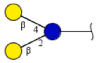
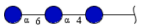
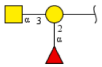
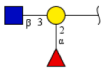
Hex3	32.239	504.169	504.16808	H+	582.9735	-	1180.2832	-	OS	
Hex3	33.326	504.169	504.16882	H-	-	1137.8513	418.0317	-	OS	
Hex3	23.541	504.169	504.16748	H-	-	-	-	60.6201	OS	
Hex3	32.099	504.169	504.16763	H-	-	-	-	2114.7499	OS	
Hex3	32.751	504.169	504.16732	H+	-	-	-	3275.2829	OS	
Hex3	33.935	504.169	504.1708	H-	-	-	-	23.0783	OS	
Hex3	37.996	504.169	504.16865	H-	-	-	-	22.3400	OS	
Hex1HexNAc1Fuc1	26.567	529.2007	529.19997	H+	192.3755	-	422.3607	-	OS	
Hex1HexNAc1Fuc1	28.163	529.2007	529.20025	H+	-	-	43.7740	-	OS	

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Table 1 (*continued*)

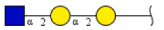
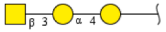
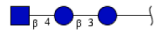
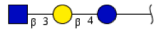
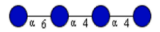
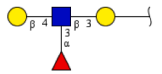
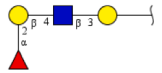
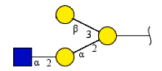
Hex2HexNAc1	29.715	545.1956	545.19476	H+	-	2790.5137	-	-	OS	
Hex2HexNAc1	38.393	545.1956	545.19447	H+	271.8341	-	-	-	OS	
Hex2HexNAc1	40.715	545.1956	545.19601	H-	97.2819	-	-	-	OS	
Hex2HexNAc1	28.915	545.1956	545.19442	H+	-	-	39.5737	-	OS	
Hex2HexNAc1	30.59	545.1956	545.19408	H+	-	-	-	5792.382727	OS	
Hex2HexNAc1	35.669	545.1956	545.19478	H+	-	-	-	1234.7152	OS	
Hex4	24.133	666.2219	666.2196	H+	-	4292.5669	5178.4416	5620.8959	OS	
Hex4	29.241	666.2219	666.22034	H-	96.2865	-	-	-	JCGG	
Hex2HexNAc1Fuc1	42.289	691.2535	691.25406	H-	315.0260	-	-	-	OS	
Hex2HexNAc1Fuc1	40.953	691.2535	691.25234	H+	310.5983	-	-	-	OS	
Hex3HexNAc1	38.155	707.2484	707.24614	H+/-	2752.8191	-	-	-	OS	

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Table 1 (*continued*)

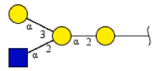
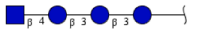
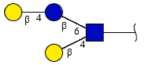
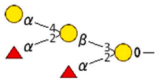
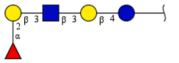
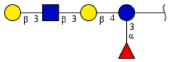
Hex3HexNAc1	40.897	707.2484	707.24614	H+	152.7245	-	-	-	OS	
Hex3HexNAc1	45.407	707.2484	707.24614	H+	48.6068	-	-	-	OS	
Hex3HexNAc1	44.441	707.2484	707.24614	H+	29.2453	-	-	-	OS	
Hex3HexNAc1	42.01	707.2484	707.24614	H+	14.4868	-	-	-	OS	
Hex3HexNAc1	42.961	707.2484	707.24614	H+	14.3968	-	-	-	OS	
Hex3HexNAc1	38.681	707.2484	707.24625	H+	1533.0851	-	-	-	OS	
Hex3HexNAc1	38.595	707.2484	707.24828	H+	-	-	-	36.8902	OS	
Hex3dHex2	2.702	814.2953	814.29242	H-	-	-	-	19.5947	JCGG	
Hex3HexNAc1dHex1	40.516	853.3062	853.30419	H+/-	192.7747	-	-	-	JCGG	
Hex3HexNAc1dHex1	45.381	853.3062	853.30419	H+	22.2075	-	-	-	JCGG	

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Table 1 (*continued*)

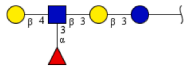
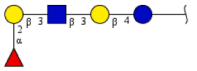
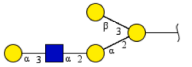
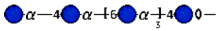
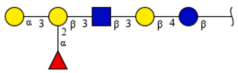
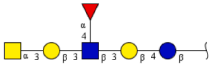
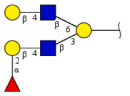
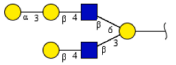
Hex3HexNAc1dHex1	46.281	853.3062	853.30419	H+	17.7162	-	-	-	JCGG	
Hex3HexNAc1dHex1	44.437	853.3062	853.30419	H+	12.8089	-	-	-	JCGG	
Hex4HexNAc1	41.226	869.3012	869.30156	H-	68.3350	-	-	-	OS	
Hex3HexNAc2	40.542	910.3278	910.3272	H+	30.5081	-	-	-	OS	
Hex6	32.776	1008.3379	1008.33652	H+	-	-	-	53.4843	JCGG	
Hex4HexNAc1dHex1	42.414	1015.359	1015.35837	H+	260.1292	-	-	-	JCGG	
Hex3HexNAc2dHex1	43.238	1056.3856	1056.38371	H+	9.8275	-	-	-	JCGG	
Hex3HexNAc2dHex1	41.765	1056.3856	1056.38556	H+	17.2520	-	-	-	JCGG	
Hex4HexNAc2	41.991	1072.381	1072.38049	H+	1009.0949	-	-	-	OS	

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Table 1 (*continued*)

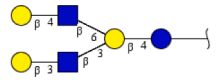
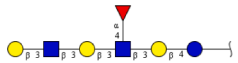
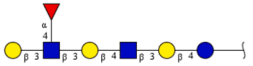
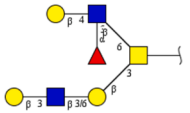
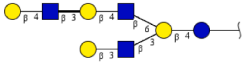
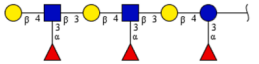
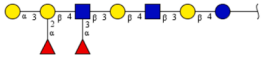
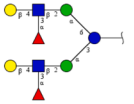
Hex4HexNAc2	30.978	1072.381	1072.37694	H+	-	-	-	15.8513	OS	
Hex4HexNAc2dHex1	42.95	1218.4384	1218.43683	H-	4438.8777	-	-	-	JCGG	
Hex4HexNAc2dHex1	45.383	1218.4384	1218.43499	H+	38.9259	-	-	-	JCGG	
Hex3HexNAc3dHex1	42.518	1259.465	1259.46147	H+	5.0217	-	-	-	JCGG	
Hex5HexNAc3	45.785	1437.5127	1437.50908	H+	14.7947	-	-	-	JCGG	
Hex5HexNAc3	43.68	1437.5127	1437.51413	H-	39.2527	-	-	-	JCGG	
Hex4HexNAc2dHex3	45.025	1510.5542	1510.55752	H-	27.6768	-	-	-	JCGG	
Hex5HexNAc2dHex2	45.214	1526.5491	1526.55337	H-	10.7729	-	-	-	JCGG	
Hex5HexNAc2dHex2	44.092	1526.5491	1526.55442	H+	7.4627	-	-	-	JCGG	

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Table 1 (*continued*)

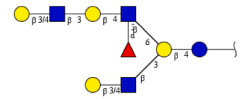
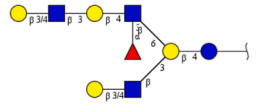
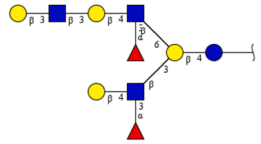
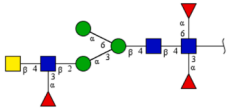
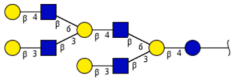
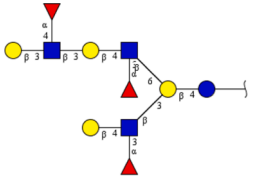
Hex5HexNAc3dHex1	44.657	1583.5706	1583.57241	H-	25.2506	-	-	-	JCGG	
Hex5HexNAc3dHex1	45.385	1583.5706	1583.56784	H+	10.1890	-	-	-	JCGG	
Hex5HexNAc3dHex2	45.384	1729.6285	1729.6299	H-	399.4512	-	-	-	JCGG	
Hex3HexNAc4dHex3	15.287	1754.6602	1754.66715	H+	-	-	-	10.4639	JCGG	
Hex6HexNAc4	44.919	1802.6449	1802.64219	H+	63.1626	-	-	-	JCGG	
Hex5HexNAc3dHex3	46.267	1875.6864	1875.68259	H+	18.0910	-	-	-	JCGG	

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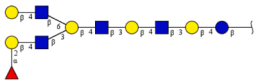
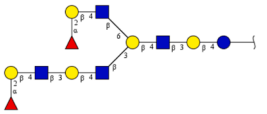
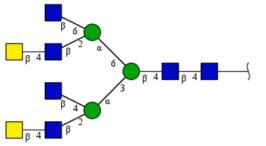
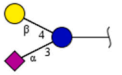
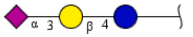
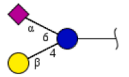
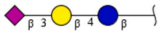
Table 1 (*continued*)

Hex6HexNAc4dHex1	45.582	1948.7028	1948.69822	H+	50.8347	-	-	-	JCGG	
Hex6HexNAc4dHex2	46.429	2094.7607	2094.76375	H-	178.9300	-	-	-	JCGG	
Hex3HexNAc8	46.503	2128.8042	2128.81064	H+	92.4640	-	-	-	JCGG	
Hex6HexNAc4dHex3	47.182	2240.8186	2240.82339	H-	54.2381	-	-	-	JCGG	

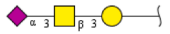
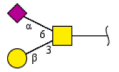
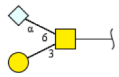
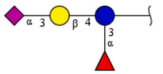
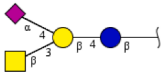
Glucose (Glc)
Mannose (Man)
Galactose (Gal)
Fucose (Fuc)
N-acetylglucosamine (GlcNAG)
N-acetylgalactosamine (GalNAC)

Table 2
Acidic OS identified and their peak area in various milk.

OS	RT (min)	Mass		Ion	Peak area ($\times 10^6$)				Database	Proposed Structure
		Found	Cal		Human	Bovine	Goat	Rat		
Hex2NeuAc1	36.965	633.2116	633.2111	H+	6471.9939	492.2184	832.7446	-	OS	
Hex2NeuAc1	34.053	633.2116	633.21236	H-	2302.2443	2188.0787	754.9434	-	OS	
Hex2NeuAc1	33.776	633.2116	633.21238	H-	1233.0567	1477.0897	524.2122	-	OS	
Hex2NeuAc1	39.609	633.2116	633.21017	H+	-	-	-	30.3712	JCGG	
Hex2NeuAc1	33.276	633.2116	633.21174	H-	-	-	-	18368.4979	OS	
Hex2NeuAc1	36.034	633.2116	633.21056	H-	-	-	-	8334.3654	OS	
Hex2NeuGc1	37.778	649.2065	649.20736	H-	-	-	573.3734	-	OS	
Hex2NeuGc1	39.257	649.2065	649.20671	H-	-	-	507.3002	-	OS	
Hex2NeuGc1	39.089	649.2065	649.20553	H+	-	-	-	36.3808	JCGG	
Hex2NeuGc1	36.962	649.2065	649.20665	H-	-	-	-	80.3261	OS	

(*continued on next page*)

Table 2 (*continued*)

Hex1HexNAc1NeuAc1	33.122	674.2383	674.23771	H+	12.1101	126.4251	189.3302	-	OS	
Hex1HexNAc1NeuAc1	34.109	674.2383	674.23795	H+	-	32.7590	55.8214	-	OS	
Hex1HexNAc1NeuGc1	36.418	690.2331	690.23344	H-	-	-	117.8462	-	OS	
Hex1HexNAc1NeuGc1	37.504	690.2331	690.2336	H-	-	-	40.5988	-	OS	
Hex1HexNAc1NeuAc1	26.533	692.2487	692.24687	H+	-	9.7071	-	-	JCGG	
Hex2NeuAc1dHex1	38.992	779.2694	779.27138	H-	65.1889	-	-	-	JCGG	
Hex3NeuAc1	39.8	795.2645	795.2664	H-	-	40.1941	16.8451	-	OS	
Hex3NeuGc1	40.757	811.2594	811.26171	H-	-	-	18.4559	-	OS	Unknown
Hex2HexNAc1NeuAc1	39.036	836.291	836.29114	H+/-	-	-	-	130.8981	JCGG	
Hex2HexNAc1NeuAc1	39.727	836.291	836.2894	H+	-	-	-	11.2019	OS	
Hex2HexNAc1NeuAc1	40.371	836.291	836.29285	H-	-	-	-	7.3831	OS	
Hex2NeuAc2	39.294	924.307	924.30875	H-	-	49.7459	30.9832	-	OS	

(*continued on next page*)

Table 2 (*continued*)

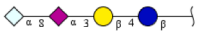
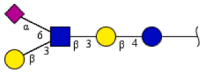
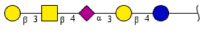
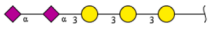
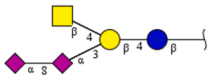
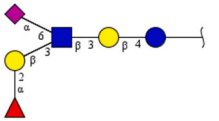
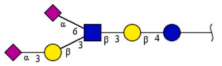
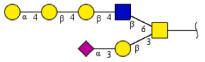
Hex2NeuAc1NeuGc1	40.339	940.302	940.30444	H-	-	-	25.6554	-	OS	
Hex2NeuGc2	41.14	956.2969	956.29852	H-	-	-	32.8689	-	OS	
Hex3HexNAc1NeuAc1	44.2	998.3438	998.3413	H+	9.3300	-	-	-	OS	
Hex3HexNAc1NeuAc1	41.234	998.3438	998.34363	H+	524.1972	-	-	-	OS	
Hex3HexNAc1NeuAc1	41.346	998.3438	998.34169	H+	-	-	-	369.8998	JCGG	
Hex3NeuAc2	41.97	1086.36	1086.3609	H-	-	90.3254	-	-	OS	
Hex2HexNAc1NeuAc2	41.339	1127.3863	1127.38447	H+	-	-	-	45.1631	JCGG	
Hex3HexNAc1NeuAc1dHex1	42.496	1144.4016	1144.40192	H-	290.5353	-	-	-	JCGG	
Hex4HexNAc1NeuAc1	42.862	1160.3965	1160.39668	H-	5.4335	-	-	-	JCGG	
Hex3HexNAc1NeuAc2	42.564	1289.4391	1289.43515	H+	22.9367	-	-	-	JCGG	
Hex4HexNAc2NeuAc1	43.299	1363.476	1363.47521	H-	248.4262	-	-	-	OS	

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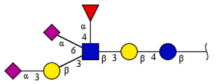
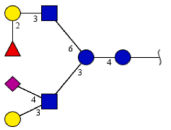
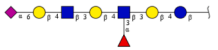
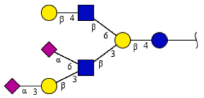
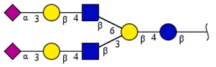
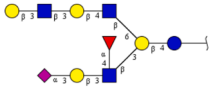
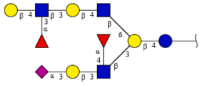
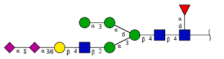
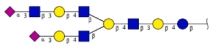
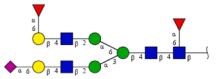
Table 2 (*continued*)

Hex3HexNAc1NeuAc2dHex1	43.282	1435.4969	1435.49868	H-	118.5892	-	-	-	JCGG	
Hex4HexNAc2NeuAc1dHex1	43.574	1509.5338	1509.53482	H-	35.3805	-	-	-	JCGG	
Hex4HexNAc2NeuAc1dHex1	44.083	1509.5338	1509.53278	H-	1286.2825	-	-	-	JCGG	
Hex4HexNAc2NeuAc2	43.753	1654.5713	1654.57302	H+	33.4184	-	-	-	JCGG	
Hex4HexNAc2NeuAc2	44.354	1654.5713	1654.57206	H-	141.9871	-	-	-	JCGG	
Hex5HexNAc3NeuAc1	44.584	1728.6082	1728.6105	H-	16.3572	-	-	-	JCGG	
Hex5HexNAc3NeuAc1dHex1	45.266	1874.666	1874.66881	H+	67.6713	-	-	-	JCGG	
Hex5HexNAc3NeuAc2	44.909	2019.7035	2019.70694	H-	9.0853	-	-	-	JCGG	

(*continued on next page*)

Table 2 (*continued*)

Hex5HexNAc3NeuAc1dHex2	46.099	2020.7239	2020.72648	H-	40.6350	-	-	-	JCGG	
Hex6HexNAc4NeuAc1	45.65	2093.7404	2093.74112	H-	19.7642	-	-	-	JCGG	
Hex5HexNAc3NeuAc2dHex1	45.337	2165.7615	2165.76309	H-	47.8446	-	-	-	JCGG	
Hex6HexNAc4NeuAc1dHex1	46.319	2239.7981	2239.79769	H-	50.6278	-	-	-	JCGG	
Hex5HexNAc4NeuAc2	46.342	2240.7935	2240.80313	H-	30.9354	-	-	-	JCGG	
Hex5HexNAc4NeuAc1dHex2	47.184	2241.814	2241.82583	H-	46.2592	-	-	-	JCGG	

- Glucose (Glc)
- Mannose (Man)
- Galactose (Gal)
- Fucose (Fuc)
- N-acetylglucosamine (GlcNAG)
- N-acetylgalactosamine (GalNAC)
- N-acetylneuraminic acid (NeuNAc)
- N-Glycolylneuraminic acid (NeuGc)

had been detected in other three milks. All the milk samples contained Hex3, Hex4 and Hex2HexNAc1 and their isomers. Hex1HexNAc1Fuc1 at *m/z* 529.2007 and its isomers were detected in both human and goat milk. Hex2Fuc1, known as fucosyllactose, had the highest abundance in HMOs in present study, which was followed by Hex2NeuAc1 and Hex3HexNAc1. The finding was in consistent with previous reports that Hex2Fuc1 (*m/z* 488.1741) was one of the most abundant OS in human milk (Moreno & Sanz, 2014). It was worth noting that Hex3HexNAc1 (*m/z* 707.2484) and Hex4HexNAc2 (*m/z* 1072.3810) had been proved to have prebiotic activity, which can be preferentially consumed by some Bifidobacterium strains (LoCascio, Ninonuevo, Freeman, Sela, Grimm, Lebrilla, et al., 2007). As the third most abundant OS in HMOs, Hex3HexNAc1 at *m/z* 707.2484 and its isomers was found in human milk, but there was a slight amount of Hex3HexNAc1 present in rat milk. Hex4HexNAc2 at *m/z* 1072.3810 was also detected either in human milk or rat milk with a small abundance. However, Hex3HexNAc1 and Hex4HexNAc2 were not observed in bovine and goat milk. Hex1HexNAc1Fuc1 (*m/z* 529.2007), Hex4 (*m/z* 666.2219), Hex3HexNAc3dHex1 (*m/z* 1259.4650), Hex3HexNAc8 (*m/z* 2128.8042) and their isomers have not been detected in human milk in a previous study (Porfirio, Archer-Hartmann, Moreau, Ramakrishnan, Haque, Kirkpatrick, et al., 2020). Of all the 5 neutral OS identified in bovine milk of this study had the same compositions with HMOs. Hex4 was first found in human milk (Table 1). 6 fucosylated OS were identified in 23 GMOs and 3 fucosylated OS were detected in 24 RMOs. The most abundant OS in BMOs and GMOs was Hex4. All the neutral GMOs described in present work are reported in other studies (Lu, et al., 2020; Martín-Ortiz, et al., 2016) and also had the same compositions with HMOs. 11 of 14 neutral OS identified in rat milk were present in human milk. Hex3dHex (*m/z* 814.2953), Hex6 (*m/z* 1008.3379), Hex3HexNAc4dHex3 (*m/z* 1754.6602) were only observed in rat milk (Table 1). Hex3dHex2 and Hex3HexNAc4dHex3 were of particular interest that they were for the first time identified in milk. All the neutral OS were reported for the first time in rat milk.

3.2. Identification and analysis of acidic OS in various milk

Table 2 showed the full list of acidic OS at each peak which analyzed by two databases. The proportion of identified acidic OS in human milk was 22.39 %. Among the acidic HMOs detected, Hex1HexNAc1NeuAc1, Hex2NeuAc1dHex1, Hex5HexNAc3NeuAc1, Hex5HexNAc4NeuAc2 and Hex5HexNAc4NeuAc1dHex2 were newly identified in human milk comparing with other literature (Porfirio, et al., 2020). Hex2NeuAc1dHex1, Hex3HexNAc1NeuAc1dHex1 and some complex OS with large mass (greater than1000), such as Hex4HexNAc1NeuAc1, Hex3HexNAc1NeuAc2, Hex4HexNAc2NeuAc1, Hex3HexNAc1NeuAc2dHex1, Hex4HexNAc2NeuAc1dHex1, Hex4HexNAc2NeuAc1dHex1, Hex4HexNAc2NeuAc2, Hex4HexNAc2NeuAc2, Hex5HexNAc3NeuAc1, Hex5HexNAc3NeuAc1dHex1, Hex5HexNAc3NeuAc2, Hex5HexNAc3NeuAc1dHex2, Hex6HexNAc4NeuAc1, Hex5HexNAc3NeuAc2dHex1, Hex6HexNAc4NeuAc1dHex1, Hex5HexNAc4NeuAc2 and Hex5HexNAc4NeuAc1dHex2 were exclusively detected in human milk. It was particular Hex1HexNAc1NeuAc1 at *m/z* 674.2383, which is described in animal (e.g. bovine and goat) milk (Lu, et al., 2020; Sunds, Bunyatratchata, Robinson, Glantz, Paulsson, Leskauskaite, et al., 2021). Hex3HexNAc2 (*m/z* 692.2487) and Hex3NeuAc2 (*m/z* 1086.3600) were only present in bovine milk, as well as Hex2HexNAc1NeuAc1, Hex2HexNAc1NeuAc2 and their isomers were only present in rat milk. Hex1HexNAc1NeuGc1 (*m/z* 690.2331), Hex3NeuGc1 (*m/z* 811.2594), Hex2NeuAc1NeuGc1 (*m/z* 940.3020) and Hex2NeuGc2 (*m/z* 956.2969) and their isomers had only been identified in goat milk in present study. All the milk samples contained Hex2NeuAc1 and their isomers with *m/z* of 633.2116. The Hex2NeuAc1 was absolutely quantified as 3′-SL or its isomer 6′-SL in other reports (Lu, et al., 2020; Macias Rostami, Bénet, Spears, Reynolds, Satyaraj, Sprenger, et al., 2014), which was the most abundant acidic OS in human, goat, bovine and rat milk, and the results were in agreement with some previous studies (Barile, Marotta, Chu, Mehra, Grimm, Lebrilla, et al., 2010; Li, et al., 2021; Lu, et al., 2020; N Tao, DePeters, Freeman, German, Grimm, & Lebrilla, 2008). Hex1HexNAc1NeuAc1 at *m/z* 674.2383 and its isomers were identified in three milk samples except rat milk. Hex2NeuGc1 at *m/z* 649.2065 and its isomers were detected in both rat and goat milk, which was similar to Hex3NeuAc1 (*m/z* 795.2645) and Hex2NeuAc2 (*m/z* 924.3070), that were detected in both bovine and goat milk. Hex2NeuGc1, Hex3NeuGc1, Hex2NeuAc1NeuGc1 and Hex1HexNAc1NeuGc1 can be found in bovine milk previously (N Tao, DePeters, Freeman, German, Grimm, & Lebrilla, 2008), whereas these OS had not been identified in bovine milk in this work. Up until now, merely 15 acidic OS were identified in rat and mouse milk, which included three sulphated OS (Li, et al., 2021). However, there was no sulphated OS detected in current study. Among these 24 RMOs identified, 3 acidic OS, including Hex2NeuGc1 and its isomer and Hex2HexNAc1NeuAc2 were reported for the first time in rat milk. Noticeably, Hex2HexNAc1NeuAc1 (*m/z* 836.2910) was only detected in rat milk in present study, which have been identified in bovine and goat milk (Lu, et al., 2020; N Tao, DePeters, Freeman, German, Grimm, & Lebrilla, 2008). Hex3HexNAc1NeuAc1 (*m/z* 998.3438) was only detected in both human and rat samples in current research, which can be detected in bovine and goat milk previously (Barile, et al., 2010; Lu, et al., 2020). Hex2HexNAc1NeuAc2 at *m/z* 1127.3863 was particularly interest that it was first identified in rat milk, which is described in bovine milk before (Sunds, et al., 2021).

3.3. Comparative analysis of OS in various milk

In the present investigation, 70 HMOs, 14 BMOs, 23 GMOs and 24 RMOs were detected in human and animal milks using UPLC-QE-HF-MS technique, respectively. 45 neutral and 25 acidic OS were identified in human milk, 5 neutral and 9 acidic OS were found in bovine milk, 9 neutral and 14 acidic OS were detected in goat milk and RMOs include 14 neutral and 12 acidic OS. By analyzing the mass of OS in Table 1 and 2, the neutral and acidic OS from bovine, goat and rat milk were mainly of short chain length comparing with HMOs, which was in agreement with a previous study (Albrecht, et al., 2014). It was obvious that the nature and number of OS were different between human and animal milk. The analysis of this study showed that many OS have relatively large amounts of their isomers. The abundance (peak area) and content of total, acidic and neutral OS in different milk were shown in Table 3. In current research, the numbers and abundance of HMOs were much higher than that of BMOs, GMOs and RMOs (Table 3). The concentrations and numbers in milk of most farm animals including cows, goats, sheep and pigs are much lower than that in human milk (Nannan Tao, Ochonicky, German, Donovan, & Lebrilla, 2010), which was in agreement with the results of present study. There were more than 200 different OS have been separated and described by HPLC-MS and at least 162 structures of HMOs have been determined (T. Urashima, Hirabayashi, Sato, & Kobata, 2018). The various analytical techniques, OS extraction methods, the genetic variation and decreased number and concentration of glycans during the course of lactation made the qualitative and quantitative composition of OS in animal milk variable and complex (Albrecht, et al., 2014; Barile, et al., 2010; N Tao, DePeters, German, Grimm, & Lebrilla, 2009). The number and composition of HMOs, BMOs and GMOs had been extensively studied before, and in

Table 3
The abundance (peak area) and relative content of total, acidic and neutral OS in different milk.

Milk	Total OS Abundance ($\times 10^6$)	Neutral OS Abundance ($\times 10^6$)	Content (%)	Acidic OS Abundance ($\times 10^6$)	Content (%)
Human	58630.9695	45500.6790	77.61	13130.2905	22.39
Bovine	15330.8885	10834.0522	70.67	4496.8363	29.33
Goat	12069.9302	8348.9513	69.17	3720.9789	30.83
Rat	46279.2930	18755.7231	40.53	27523.5699	59.47

current study, the number of OS identified in different milk samples were lower than previous studies. 71 HMOs, 14 BMOs and 23 GMOs have been detected in this work, which are much lower than 115, 40 (N Tao, DePeters, German, Grimm, & Lebrilla, 2009) and 64 (Lu, et al., 2020), respectively. These findings might be concerning with the applied databases, analytical techniques and the samples. The number of neutral OS was more than that of acidic OS in human and rat milk. There were more types of acidic OS than that of neutral OS in bovine and goat milk. As shown in Table 3, 77.61 % of HMOs was neutral OS, which was similar with the results reported by previous study (Albrecht, et al., 2014). Among all the milk samples investigated, human milk contained the highest percentage and the most abundant variety of neutral OS. The abundances of neutral OS were much higher than that of acidic OS in human, bovine and goat milk as depicted in Table 3. The content of acidic OS (59.47 %) was more than that of neutral OS (40.53 %) in rat milk (Table 3). To our knowledge, the acidic OS are the major components among the OS in animal milk (Li, et al., 2021). And the findings of this study were highly in contrast to previous studies that indicated the BMOs and GMOs contain higher percentage of acidic OS than neutral OS (Albrecht, et al., 2014; Lu, et al., 2020). It is particularly that a higher percentage of neutral OS observed to shift as time goes, especially for the enormous differences between colostrum and mature milk (Barile, et al., 2010). The controversial results of the abundance of BMOs and GMOs observed in present study might be result from the use of mature milk and the different variety of the animal. Furthermore, it was worth noting that the number and total abundance of HMOs and RMOs were greater than that of both BMOs and GMOs (Table 3). In present study, a total of 24 RMOs were detected, of which 14 neutral OS and 12 acidic OS. The rats share 90 % of the genome with humans (Dvorak, et al., 2004). In present work, 9 BMOs (included 5 neutral and 4 acidic OS), 11 GMOs (included 7 neutral and 4 acidic OS) and 13 RMOs (included 9 neutral and 4 acidic OS) had the same composition with HMOs. The composition and abundance of RMOs come more closest to that of HMOs comparing with BMOs and GMOs (Table 3), the results were in consistent with previous study reported that rat and mouse share more common OS with human when comparing with animals (such as cow, goat and sheep)(Li, et al., 2021). The similarity of composition and abundance between RMOs and HMOs might because of the dominance by gene in terms of evolution, which was deserved to be studied in the future.

N-acetylhexosamine (HexNAc) is regarded as a component of so-called bifidus factor (Barile, et al., 2010; GyÖRGY, Jeanloz, von Nicolai, & Zilliken, 1974). Of the colostrum samples analyzed in the present work, 26.30 %, 17.99 %, 7.51 %, and 15.86 % of the HMOs, BMOs, GMOs and RMOs contained HexNAc, respectively (Fig. 2B). Obviously, the human milk included more HexNAc of OS than animal milk, and rat milk had the second most abundant of HexNAc after human milk (Fig. 2A). According to the composition of literature data (Porfirio, et al., 2020), the dHex in Table 1 and 2 is speculated to represent Fuc in this work. In general, the main differences between HMOs and animal OS are that most HMOs are highly fucosylated OS (Mehra, Barile, Marotta, Lebrilla, Chu, & German, 2014). The neutral HMOs which containing *N*-acetylglucosamine and fucose monomers are regarded as substances for the development of the intestinal microbiota typical for breastfed infants (Simon, Goode, Mobasseri, & Zopf, 1997). Furthermore, the fucosylated HMOs are related to the lower risk of diarrhea and respiratory diseases in breast-fed infants (Stepans, Wilhelm, Hertzog, Rodehorst, Blaney, Clemens, et al., 2006). Different from neutral OS in animal milk, approximately 68.07 % of HMOs were fucosylated OS with large degrees of complexity (Fig. 2B). Numerous studies have revealed that no fucosylated species are described in BMOs (Mehra, Barile, Marotta, Lebrilla, Chu, & German, 2014; N Tao, DePeters, Freeman, German, Grimm, & Lebrilla, 2008; N Tao, DePeters, German, Grimm, & Lebrilla, 2009), and these findings were in consistent with the results of our study that there was no fucosylated OS detected in BMOs (Fig. 2). As shown in Fig. 2B, the contents of fucosylated OS in goat and rat milk were 4.17 % and 1.09 %, respectively, which were significantly lower than that in human milk.

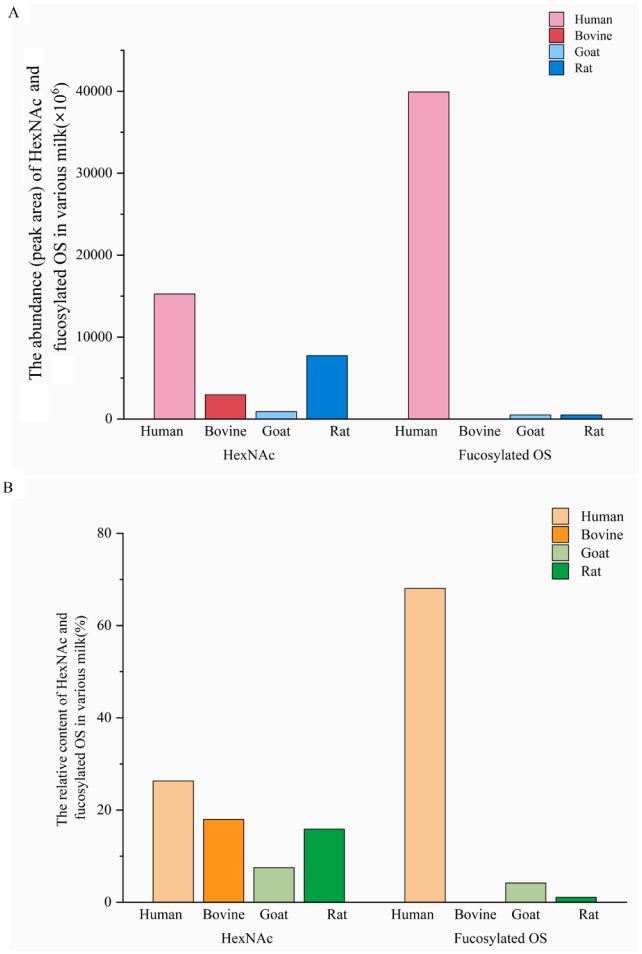


Fig. 2. The abundance (peak area) (A) and relative content (B) of HexNAc and fucosylated OS in various milk.

The abundance of fucosylated OS in goat milk was similar with that in rat milk (Fig. 2A).

As seen in Fig. 3, all the acidic HMOs and BMOs only contained NeuAc, without NeuGc and NeuAcNeuGc. These findings were in consistent with some previous studies shown that no OS identified in both human and bovine milk contain NeuGc (Sunds, et al., 2021; Wu, Grimm, German, & Lebrilla, 2011). The NeuGc is not exist in human milk might due to the lack of ability to synthesize NeuGc arise from a mutation occurred in CMP-NeuAc hydroxylase gene which converts NeuAc to NeuGc (Lu, et al., 2020). It is mostly common that no OS contained monosaccharide NeuGc found in colostrum and early lactation milk (N Tao, DePeters, German, Grimm, & Lebrilla, 2009). Nevertheless, the absence of NeuGc are also been found in mid-lactation bovine milk (Robinson, Poulsen, Colet, Duchene, Larsen, & Barile, 2019) and mature (average lactation of 133 days) bovine milk (Sunds, et al., 2021). In present study, the bovine milk sample was mature milk, the result of the absence of NeuGc was might since the unique feature of the native breeds. The OS contained NeuGc are regarded as relationship with some negative health outcomes, such as possibly promoting inflammation and cancer progression (Samraj, Pearce, Läubli, Crittenden, Bergfeld, Banda, et al., 2015). Therefore, the absence of NeuGc in mature human and bovine milk was possible a beneficial trait. Out of 14 acidic GMOs identified in present study, 7 OS only contained NeuAc and 6 OS only contained NeuGc, while 1 OS contained both NeuAc and NeuGc (NeuAcNeuGc) with a slight abundance (Fig. 3A). In rat milk, 12 OS were acidic ones, in which 9 OS only contained NeuAc and 2 OS only contained NeuGc, while 1 OS contained both NeuAc and NeuGc

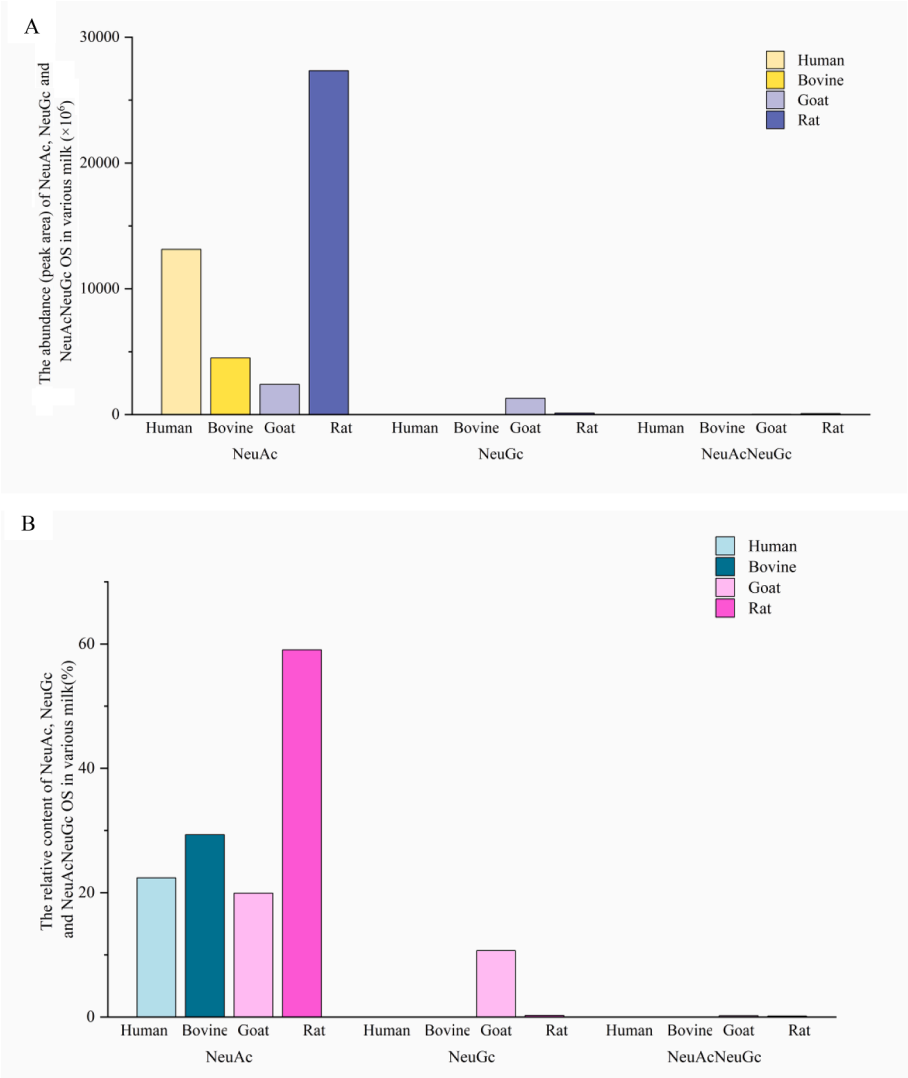


Fig. 3. The abundance (peak area) (A) and relative content (B) of NeuAc, NeuGc and NeuAcNeuGc OS in various milk.

(NeuAcNeuGc). The content of NeuGc-OS (0.25 %) and NeuAcNeuGc-OS (0.16 %) in rat milk were extremely small (Fig. 3B). The rat milk contained the most abundant NeuAc (Fig. 3A) with the content of 59.06 % (Fig. 3B), which was followed by human milk and bovine milk (Fig. 3A). Goat milk had the lowest abundance of NeuAc with the content of 19.92 % (Fig. 3B), comparing with other three milks. The sialylated OS (containing the monomer NeuAc) is benefit to preventing the adhesion of pathogenic bacteria to the intestinal epithelial surface (Simon, Goode, Mobasseri, & Zopf, 1997) and allergy (Eiwegger, Stahl, Haidl, Schmitt, Boehm, Dehlink, et al., 2010). On the other hand, sialylated OS is also shown to improve growth outcomes in undernourished infants and children (Charbonneau, O'Donnell, Blanton, Totten, Davis, Barratt, et al., 2016). As stated above, it was obvious to draw a conclusion that HMOs had the highest nutritional value among the OS in all the milk samples, and RMOs had higher nutritional value than BMOs and GMOs via analyzing the abundance of total OS, NeuAc OS, HexNAc OS and Fucosylated OS.

4. Conclusion

In conclusion, 70 HMOs, 14 BMOs, 23 GMOs and 24 RMOs were identified in human and animal milk via using UPLC-QE-HF-MS technique, respectively, which suggested that the method was effective and robust for detecting the OS in milks in present study. The number of identified OS in current research was relatively lower compared to previously reports might because of the applied databases, analytical techniques and breeds of samples. This work further verified the significant variations between the nature and number of OS in human and animal milk (e.g.bovine, goat and rat milk). Noticeably, many OS with distinct compositions, contents and abundances had been newly revealed in this work, in particular, the various contents of neutral and acidic OS in goat and bovine milk as well as 14 neutral OS and 3 acidic OS were found in rat milk for the first time. The compositions and abundances of RMOs were might more similar with that of HMOs comparing with BMOs and GMOs were of particular interests, which might due to the large similarity of genome with humans. The similarity between HMOs and RMOs might provide theoretical basis for better application of rats in biological/biomedical studies of HMOs as models. Based on the analysis of the abundances of total OS, NeuAc OS, HexNAc OS and Fucosylated OS, HMOs might have the highest nutritional value among the OS in different milk samples, which was followed by RMOs. Due to the existence of nutritional structure in BMOs and GMOs, the potential value of animal milk OS for promoting human health should be concerned. What's more, rat milk was able to be used for different mechanism studies, bovine and goat milk could be considered as source of bioactive OS which were able to be further applied in medical and functional foods due to the existence of functional structures in BMOs and GMOs.

CRediT authorship contribution statement

Rui Li: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Formal analysis. **Yalin Zhou:** Software, Visualization, Resources, Writing – review & editing. **Yajun Xu:** Data curation, Funding acquisition, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100705.

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