Published in final edited form as:

J Agric Food Chem. 2017 May 03; 65(17): 3523–3531. doi:10.1021/acs.jafc.6b05104.

Changes in Caprine Milk Oligosaccharides at Different Lactation Stages Analyzed by High Performance Liquid Chromatography Coupled to Mass Spectrometry

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Abstract

Changes of the abundance of caprine milk oligosaccharides (CMO) at different lactation stages have been evaluated by hydrophilic interaction liquid chromatography coupled to mass spectrometry (HILIC–Q MS) and nanoflow liquid chromatography–quadrupole-time-of-flight mass spectrometry (nano-LC-Chip–QTOF MS). Eight major oligosaccharides (OS) were quantified at different lactation stages by HILIC–Q MS, while the use of nano-LC-Chip–QTOF MS allowed expanding the study to forty-nine different OS by monitoring neutral non- and fucosylated species, as well as acidic species containing not only *N*-acetyl-neuraminic acid or *N*-glycolyl-neuraminic acid residues but also the combination of both sialic acids. Overall, the most abundant OS decreased with lactation time, whereas different trends were observed for minor OS. 6'-Sialyl-lactose was the most abundant acidic OS while galactosyl-lactose isomers were identified as the most abundant neutral OS. This is the first time that a comprehensive study regarding the changes of the abundance of CMO, both neutral and acidic, at different lactation stages is carried out.

Keywords

goat milk oligosaccharides; lactation stages; hydrophilic interaction liquid chromatography; nanoflow liquid chromatography–quadrupole-time-of-flight mass spectrometry

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Notes

The authors declare no competing financial interest.

INTRODUCTION

Human milk (HM) is considered the ideal food for neonates, not only because of its complex nutrients mixture, fulfilling infant's requirements, but also because of the presence of bioactive components, exerting important physiological effects for the neonate.¹ Among those, human milk oligosaccharides (HMOS) have been described to act as prebiotics and as potent inhibitors of bacterial adhesion to intestinal epithelial receptors.^{2,3} Moreover, as they are not digested and are absorbed only in a small proportion in the gastrointestinal tract, they may play a role in the local intestinal immune system of the breast-fed infants, thus acting in the prevention of infectious diseases.^{2–8}

HMOS are complex structures with different glycosidic linkages, degrees of polymerization, and monomeric composition, including hexoses (Hex, D-glucose, and D-galactose), *N*-acetyl-glucosamine (GlcNAc), L-fucose (Fuc), and sialic acid [*N*-acetyl-neuraminic acid (Neu5Ac)].⁹ More than 200 different HMOS have been described to be present in HM,^{10,11} with predominance of neutral fucosylated HMOS (50–70%) compared to acidic ones (10–25%).^{12,13} However, due to their specific structural composition diversity, at present, it is not feasible to totally replicate the HMOS profile by synthetic methods; only HMOS of lower molecular weight have been successfully produced by molecular engineering approaches.¹⁴ Therefore, there is great interest in finding alternative sources with similar bioactive properties to HMOS that can be exploited by the food or pharmaceutical industry on a large scale, either through direct consumption or as a functional ingredient in infant formulas, mimicking biological functions of oligosaccharides (OS) present in HM.^{15,16} In this sense, naturally occurring OS isolated from milk of animal species could be considered.^{17,18}

Goat milk is known to contain OS structurally similar to those present in HM^{4,19–21} and in higher concentrations than milks from other farm mammals.⁴ Recently, *in vitro* studies have revealed the potential prebiotic activity of OS from caprine whey.¹⁷ Moreover, it has been proved that OS isolated from goat milk reduce intestinal inflammation in rats with induced colitis,²³ and they can improve the barrier function of epithelial cell cocultures.²⁴ Therefore, goat milk could be a potentially promising functional food ingredient for improving intestinal health,²⁴ especially if supplemented in infant formulas.

Liquid chromatography (LC) is the most widespread technique for the analysis of OS from mammal milks. Although several LC operation modes, such as normal phase (NP)²⁵ and high performance anion exchange chromatography (HPAEC),^{4,14,22,23,26,27} have been widely used for the analysis of these carbohydrates, hydrophilic interaction liquid chromatography (HILIC) is considered an efficient operation mode for the analysis of complex OS mixtures,^{28–31} providing appropriate resolution between isomeric OS and good peak shapes. In a recent study from our group,²⁰ the main OS in different goat colostrals have been quantified by HILIC-Q MS. Additionally, up to 78 caprine milk oligosaccharides (CMO) structures were identified by nanoflow liquid chromatography–quadrupole-time-of-flight mass spectrometry (Nano-LC-Chip–Q-TOF MS). The combination of information provided by both techniques allowed obtaining comprehensive information, not provided in previous studies, about goat colostrals OS composition.

Changes of the OS abundance of human and bovine milks at different lactation stages have been widely studied, observing a decrease in their total content during lactation and a modification in the OS profile.^{28,32,33} However, little is known about the changes of CMO composition during lactation. A transcriptomic study of glycosylation-related genes in goat colostrum and milk obtained after 120 days of lactation has recently shown that most of them were more expressed in the former, which might be related to the higher OS concentration found in colostrum.³⁴ Recently, the content of three OS, namely 3'-sialyl-lactose (3'-SL), 6'-sialyl-lactose (6'-SL), and disialyl-lactose, in the colostrum and milks of two goat breeds sampled at different stages of lactation (up to 90 days) was reported.^{27,35} In general, a decrease of OS content was observed during lactation, although the 3'-SL contents increased in colostrum from 0 to 24 h. Formiga de Sousa et al.³⁶ also detected a decrease in sialic acid monomers, obtained after hydrolysis of CMO, at different lactation stages (85, 105, 125, and 145 days). However, to the best of our knowledge, a more comprehensive study of the evolution of both neutral and acidic CMO during lactation has not yet been carried out.

Therefore, the present work investigated the changes of the abundance of CMO during the first months of lactation in individual and pooled milks from goats of the same breed (Murciano-Granadina) by HILIC-Q MS and Nano-LC-Chip-Q-TOF MS.

MATERIALS AND METHODS

Chemicals and Reagents

Acetic acid was from Normasolv (Barcelona, Spain), ammonium acetate, and ammonium hydroxide were from Panreac (Barcelona, Spain), and ethanol of analytical grade was acquired from Lab-Scan (Gliwice, Poland). Acetonitrile (ACN) and formic acid (HPLC-MS grade) were obtained from Fisher-Scientific (Fair Lawn, NJ, USA). ESI-TOF Low Concentration Tuning Mix G1969-85000 was acquired from Agilent Technologies (Santa Clara, CA, USA). Nanopure water (18.2 MΩ·cm, 25 °C) was used throughout all experiments.

Analytical grade standards of 6'-sialyllactose (6'-SL) sodium salt, 3'-sialyllactose (3'-SL) sodium salt, 3'-sialyl-*N*-acetyllactosamine, 3'-galactosyl-lactose, 4'-galactosyl-lactose, and 6'-galactosyl-lactose were purchased from Carbosynth (Berkshire, UK), whereas 3 α ,4 β -galacto-triose was obtained from Dextra Laboratories (Reading, UK) and 2'-fucosyllactose (2'-FL) was from V-Laboratories (Covington, LA, USA). Nylon FH membranes (0.22 μ m; Millipore, Bedford, MA, USA) were used for filtering ACN:water (50:50, v:v) standard solutions before injection.

Milk Samples

Individual milk samples from one Murciano-Granadina goat (GM) were collected at an experimental farm at Estación Experimental del Zaidín (Granada, Spain). Samples were collected at days 2, 10, 20, 30, and 40 of lactation (GM2, GM10, GM20, GM30, and GM40, respectively). Moreover, milks kindly provided by Hermanos Archiduque farm (Granada, Spain) from 12 individual Murciano-Granadina goats (GP) were collected at days 1, 7, 30,

and 120 and pooled (GP1, GP7, GP30, and GP120, respectively). GM2 and GP1 samples were considered colostrum. Samples were immediately frozen at -80°C until their analysis. The Spanish guidelines for experimental animal protection (Royal Decree 53/2013) in line with the corresponding European Directive for animal care and handling (2010/63/EU) were followed. The Ethics Committee for Animal Research from the Animal Nutrition Unit approved the experimental protocol.

Fat and Protein Removal

Samples were defatted by centrifugation at $6500g$ for 15 min at 5°C and kept in an ice bath for 30 min. Whatman No. 1 filter paper was used for the removal of lipid layer supernatant.⁴

The removal of the protein fraction was carried out by the addition of two volumes of cold ethanol to the skimmed colostrum and milk samples. After shaking for 2 h in an ice bath, solutions were centrifuged at $6500g$ for 30 min at 5°C . Supernatants were collected, and ethanol was removed in a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) at 37°C . The carbohydrate fraction present in the aqueous solution was frozen and freeze-dried.

Chromatographic Analyses

HILIC-Q MS—CMO quantitation was performed on an Agilent 1200 series HPLC system (Hewlett-Packard, Palo Alto, CA, USA) equipped with an oven (Kariba Instruments, UK) and coupled to a quadrupole HP-1100 mass detector (Hewlett-Packard) with an electrospray ionization (ESI) source using the method previously optimized by Martín-Ortiz et al.²⁰ Samples were diluted with 50:50 (v:v) acetonitrile (ACN):water and filtered through a $0.22\text{ }\mu\text{m}$ filter (Millipore, Madrid, Spain), and $5\text{ }\mu\text{L}$ was injected.

An ethylene bridge hybrid with a trifunctionally bonded amide phase (BEH X-Bridge column; $150 \times 4.6\text{ mm}$; $3.5\text{ }\mu\text{m}$ particle size, $135\text{ }\text{\AA}$ pore size, Waters, Hertfordshire, UK) at a flow rate of 0.4 mL min^{-1} was used for LC experiments. For these assays, a gradient of ACN:water with addition of 0.1% ammonium hydroxide was used as follows: from 0 to 46 min a linear gradient from 15 to 50% aqueous phase was kept for 10 min at these conditions, and then ramped to the original composition in 1 min, and finally equilibrated for 15 min. The ESI source was operated under positive polarity using 4 kV capillary voltage at 300°C with a nitrogen drying gas flow of 12 L min^{-1} and a nebulizer (N_2 , 99.5% purity) pressure of 276 kPa; the fragmentor voltage was varied from 80 to 110 V. Adducts formed under optimal conditions were evaluated. Ions corresponding to $[\text{M} + \text{Na}]^{+}$ of the OS under analysis were monitored in SIM mode using default variable fragmentor voltages. HPChem Station software version 10.02 (Hewlett-Packard) was used for data processing.

Quantitative analysis was performed in triplicate by the external standard method, using calibration curves within the range $5\text{--}100\text{ mg L}^{-1}$ for 3'-SL, 6'-SL, 3'-sialyl-*N*-acetyl-lactosamine, and 3'-galactosyl-lactose. Considering the absence of glycolyl-neuraminyl-lactose (Neu5Gc-lactose) as commercial standard, the responses of *N*-acetyl-neuraminic and *N*-glycolyl-neuraminic acids were evaluated. Negligible differences were observed between the responses of these mono-saccharides; therefore, Neu5Gc-lactose isomers were quantified

using 3'-SL calibration curves. The reproducibility of the method was estimated on the basis of the intraday and interday precision, calculated as the relative standard deviation (RSD) of retention times and concentrations of OS standards obtained in five independent measurements. Good precision values were obtained (RSD ranging 6–8%). The limit of detection (LOD) and limit of quantitation (LOQ) were calculated as three and ten times, respectively, the signal to standard deviation of the noise ratio (S/σ_N). Values ranging from 0.03–0.16 mg mL⁻¹ and 0.1–0.5 mg mL⁻¹ were obtained for LOD and LOQ, respectively.

Nano-LC-Chip-Q-TOF MS. Sample Preparation—Considering that the high content of lactose present in goat milk would cause interference with the analysis of lower abundance OS, this disaccharide was removed from milk samples by size exclusion chromatography (SEC) using a Bio-Gel P2 (Bio-Rad, Hercules, CA, USA) column (90 × 5 cm). Water was selected as the mobile phase at a flow of 1.5 mL min⁻¹ at 4 °C. The OS fraction was dissolved in water (20 wt %:v), and 25 mL was injected into the column. Fractions of 10 mL were collected. The degree of polymerization (DP) of the collected fractions was determined by ESI-MS (Agilent 1200 series HPLC system, Hewlett-Packard) at positive polarity, selecting the corresponding m/z values. Fractions with DP 3 were pooled and freeze-dried.

MS Analysis—Prior to MS analysis, purified and dried OS of GM and GP samples were reconstituted to a final concentration of 0.1 mg mL⁻¹ with nanopure water, with lacto-*N*-difucohexaose I (Sigma Aldrich) being added as internal standard (final concentration of 0.001g L⁻¹). MS analysis was carried out using an Agilent 6520 LC/QTOF MS with a microfluidic nanoelectrospray chip containing graphitized carbon enrichment and analytical columns (Agilent Technologies, Santa Clara, CA, USA), as previously described.²⁰ A binary gradient of 3% ACN/0.1% formic acid in water (solvent A) and 90% ACN/0.1% formic acid in water (solvent B) at a flow rate of 0.3 μL min⁻¹ for the nano pump and an isocratic method (100% A) at a flow rate of 4 μL min⁻¹ for the capillary pump were applied. Positive ionization mode with a mass/charge (m/z) range of 450–2500 was selected for data acquisition. Automated precursor selection was employed based on abundance, with up to 6 MS/MS per MS. A precursor isolation window of 1.3 m/z and a fragmentation energy set at 1.8 V/100 Da was used. Reference masses of m/z 922.009 and 1221.991 were selected for internal calibration (ESI-TOF Low concentration Tuning Mix G1969-85000, Agilent Technologies).

OS identification was performed using the Find Compounds by Formula function of Mass Hunter Qualitative Analysis Version B.06.00 (Agilent Technologies), which allowed matching the masses of OS with the caprine milk OS databases, and OS composition was confirmed by MS/MS as described in the literature.¹⁹ The abundance of each OS relative to the internal standard (Area OS/Area i.s.) was obtained in triplicate.

Statistical Analysis

Statistica version 7.1 (2005) by Statsoft Inc. (Tulsa, USA) was used for statistical analyses. A one-way ANOVA test followed by a Fisher test as a post hoc comparison of means were

used to determine significant differences ($P < 0.05$) in OS content among the lactation stages.

RESULTS AND DISCUSSION

To evaluate the changes in CMO abundance at different lactation stages and considering their high variability in colostrum, as previously reported by Martín-Ortiz et al.,²⁰ two different milk collection approaches using goats of the same breed were followed in this study: (i) milks of an individual goat (GM) and (ii) pools of milk from 12 different goats (GP). GP samples could be considered as representative of milks from the Murciano-Granadina goat breed.

Quantitation of the Main Caprine Milk OS at Different Lactation Stages by HILIC-Q MS

Figure 1 shows the extracted ion chromatographic profile (HILIC-Q-MS) of the main OS in GM obtained at 20 days of lactation. Peaks at retention times of 13.9 and 16.2 min were assigned as 3'-SL and 6'-SL by comparison with those of commercial standards. The peak eluting at 14.0 min was identified as sialyl-lactosamine, whereas three peaks with t_R at 13.7, 16.3, and 17.8 min were assigned to different isomers of glycolyl-neuraminyllactose. The peak eluting at 35.1 min could be a mixture of different galactosyl-lactose isomers by comparison with the corresponding standards (3'-galactosyl-lactose, 4'-galactosyl-lactose, and 6'-galactosyl-lactose), which eluted between 34.8 and 35.4 min, whereas the peak eluting at 36.0 min could correspond to another galactosyl-lactose isomer for which a standard was not available. The presence of 3'-galactosyl-lactose in goat milks had been previously reported;⁴ however, these milks can also be constituted by other different isomers of this carbohydrate.

Tables 1 and 2 show the content (mg L^{-1}) of the main OS found in GM and GP samples, respectively, at the different lactation stages. In general, the concentration of OS quantified in GP colostrum (488.0 mg L^{-1}) was higher than in GM colostrum (199.1 mg L^{-1}). Differences in OS concentration would be expected considering the variability of individual goats, as previously reported by different authors.^{20,27} In both samples, the galactosyl-lactose isomer eluting at 35.1 min was the most abundant OS, followed by 6'-SL. These results agree with those found in a previous work for colostrum of Murciano-Granadina goats.²⁰ However, Claps et al.²⁷ reported a higher content of 3'-SL compared to 6'-SL in colostrum from two Italian goat breeds (Maltese and Garganica). These dissimilarities could be attributed to the different breeds studied. Other authors have also found significant differences in OS composition (neutral, fucosylated, and sialyl-OS) of different bovine milks depending on the breed,^{37,38} reporting a higher proportion of 6'-SL in Friesian bovine colostrum and higher proportions of 3'-SL in Jersey one.

Differences in total OS concentration were observed along lactation, with higher values in colostrum and decreasing amounts during the studied period (3.3 times lower at 40 days of lactation in GM and 4.3 times lower at 120 days of lactation in GP). However, a slight increase of CMO content was observed in GM between 10 and 20 days of lactation (from 128.8 to 162.8 mg L^{-1}), a trend previously described by Tao et al.³⁹ in porcine milk OS.

Regarding neutral CMO, a significant decrease was observed in galactosyl-lactose isomers in GP during lactation, although the content of these OS increased from 30 to 120 days of lactation (Table 2). Nevertheless, neutral OS present in GM samples varied differently along lactation: while the major galactosyl-lactose isomer with t_R at 35.1 min decreased with time, the minor one eluting at t_R 36.0 min experienced an initial increase (colostrum to day 20), decreasing at further lactation stages (Table 1). To the best of our knowledge, there are not previous data regarding changes of neutral CMO abundance during lactation stages in the literature.

When acidic OS were evaluated, trends were dependent on the OS considered. Sialyl-lactosamine content steeply decreased during lactation in both types of samples. Although the concentration of this OS increased at 20 days in GM, it decreased below the LOQ at 40 days of lactation (Table 1), and it was present at very low levels ($<2 \text{ mg L}^{-1}$) from the seventh day of lactation onward in GP sample (Table 2). A different behavior was observed for 3'-SL and 6'-SL concentration in both samples. Although both OS decreased during lactation, an increase was observed at day 30 in GP and at day 20 in GM samples. It is worth noting that 6'-SL showed a more noticeable decrease than 3'-SL; no significant differences between these OS contents at the end of the lactation period in both types of samples were observed. Concentrations of glycolyl-neuraminyl-lactose isomers slightly increased in GP and GM samples at early stages of lactation to further progressively decrease up to the end of the studied period (Tables 1 and 2). Furthermore, the ratio between acetyl-neuraminyl- and glycolyl-neuraminyl-OS was noticeably higher in colostrum (that is, 6.4-fold in GP1 and 3.0-fold in GM2) than in any of the samples corresponding to more advanced lactation period.

In general, sialyl-OS, which have been reported to show several physiological functions in human and other mammalian infants,¹ are more abundant in colostrum than in mature or late lactation milk. These data are in agreement with results obtained for human and bovine milks and colostrum.^{32,35}

Identification and Changes of Abundance of Caprine Milk OS Fraction during Lactation by Nano-LC-Chip–Q-TOF MS

To evaluate the abundance variation of minor CMO during lactation, GM and GP samples were loaded onto a SEC column to remove the high content of lactose, which could interfere in further analyses. The lactose concentration was reduced up to 99.9%, although a partial loss of trisaccharides, mainly galactosyl-lactose, was also observed in these fractions (data not shown). Considering the high sensitivity, identification capacity, and small consumption of sample provided by Nano-LC-QToF MS, this technique was selected to study minor OS.

Tables 3 and 4 show the individual OS identified by nano-LC-QTOF MS in goat milks (GM and GP, respectively). The OS are cited according to their sequential monomeric composition (in order): Hex_HexNAc_Fuc_Neu5Ac_Neu5Gc, indicating the number of units that they contain. A total of 49 unique OS structures were detected. Neutral nonfucosylated OS showed the greatest diversity with 23 different structures, followed by Neu5Ac-OS with 11 structures, Neu5Gc-OS with 9, neutral fucosylated OS with only 4 different structures, and Neu5Ac-Neu5Gc-OS with 2 structures. This distribution resembles

bovine and porcine milk, where neutral and acidic OS show the greatest structural diversity with only a few fucosylated OS detected.^{10,11,40,41}

Figure 2 shows the relative abundances (percentages) of these CMO classes in GM and GP, as well as their changes during lactation. It can be observed that, independently of the type of sample considered, the variation of OS profile during lactation is similar. By considering the OS fraction, which remained unaffected during SEC purification, the most abundant CMO present in colostrum were Neu5Ac-OS, followed by those containing Neu5Gc and neutral OS, with fucosylated OS contributing in minor proportion. As lactation advances, Neu5Ac-OS significantly decreased until the end of the period evaluated. On the contrary, the relative abundance of Neu5Gc-OS and fucosylated OS increased with lactation. A similar behavior has also been reported for fucosylated OS of porcine milk during the first week of lactation.⁴²

The evolution of relative abundances of individual OS during lactation is shown in Tables 3 and 4. Differences in the evolution of neutral OS were observed not only among the different lactation stages, but also between both samples (GM and GP). Moreover, in general, different trends were observed for isomers of the same OS. In this sense, six different isomers of 3_0_0_0_0 were detected. Whereas the relative abundances of most of these isomers were kept constant during lactation in GM, a significant decrease at 7 and/or 30 days of lactation was observed in GP. Some exceptions were observed, such as the significant increase found in the isomer eluting at t_R 16.35 min at the end of lactation in GP and at 20 days in GM. In general, a similar trend in the evolution of 4_0_0_0_0 OS was observed during lactation in both milks, except for the isomer with t_R 19.97 min. This OS remained constant from GM2 to GM30 and significantly increased in GM40, whereas it decreased in GP at 7 days of lactation.

When neutral *N*-acetylated OS (mainly 2_1_0_0_0 and 3_3_0_0_0) were considered, an increase in their percentage was observed in GP with lactation, whereas these compounds either decreased or remained constant in GM. Regarding fucosylated OS (3_1_1_0_0), a significant increase of the isomer eluting at 12.87 min was observed during the studied period in both samples, whereas the other isomers remained constant.

Lower changes were observed during lactation stages on acidic OS, and more similar trends were observed in both milk samples. In general, Neu5Ac OS either continuously decreased or remained constant, with the exception of the isomer 4_2_0_1_0 (t_R 26.55 min), which experienced a significant increase at 20 or 30 days for GM or GP, respectively, and the isomer 2_0_0_2_0 (t_R 25.61 min), which significantly increased at 10 days of lactation in GM. On the contrary, most OS with Neu5Gc were constant or increased during lactation. Moreover, 2_0_0_1_1 OS (t_R 24.92 min), which contained both Neu5Ac and Neu5Gc, showed a significant increase during lactation in both samples.

It is worth noting that, although the same OS were detected in both the individual and the pool milk samples, different trends during lactation, mainly for neutral OS, were observed. Previous studies have evidenced the changes of abundance of OS of other mammal milks during lactation^{20,30} and the influence of goat breed on the composition of some CMO (3'-

SL, 6'-SL, and disialyllactose).^{24,25} However, our results reveal that CMO composition could be affected not only by the time of lactation, but also by the individual variation in goats belonging to the same breed.

In conclusion, this is the first time that a comprehensive study regarding the changes of abundance of both neutral and acidic CMO during lactation is carried out. In general, the most abundant OS decreased with lactation time, whereas different trends were observed for minor OS. Regarding acidic OS, both HILIC-Q MS and nano-LC-Chip-Q-TOF MS demonstrated that 6'-SL was the most abundant acidic OS at the first lactation stages followed by 3'-SL; however, smaller differences between these OS contents were observed at the end of the lactation period. Galactosyl-lactose isomers were the most abundant neutral OS. Milks from Murciano-Granadina goats could be considered as an attractive source of OS with potential beneficial effects for human health and could be a promising ingredient for infant formula.

Acknowledgments

The authors thank I. Martín –García for providing milk samples and L. Ruiz-Aceituno for her technical support.

Funding

This work has been funded by Junta de Andalucía (project AGR2011-7626), Ministerio de Economía, Industria y Competitividad (AGL2016-80475-R, AEI/FEDER, UE), Comunidad Autónoma de Madrid (Spain), European funding from FEDER program (AVANSECAL-CM S2013/ABI-3028,) and Fundación Ramón Areces. This work was also supported by the National Institutes of Health awards R21AT006180, R01AT007079, and R01 AT008759.

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Figure 1. HILIC-Q MS profile of main oligosaccharides of GM at 20 days of lactation. See Tables 1 and 2 for peak assignment.

Figure 2.

Relative abundance of OS classes and evolution during lactation. * Neutral OS fraction could be underestimated because the partial loss of trisaccharides during SEC purification.

Table 1

Concentration (mg L^{-1}) of Oligosaccharides (OS) in Goat Milk GM Analyzed by HILIC-Q MS^a

Peak number	Carbohydrate	t_R (min)	Lactation period (days)				
			GM2	GM10	GM20	GM30	GM40
1	Glycolyl-neuraminyl-lactose (isomer 1)	13.7	7.1 (0.3) ^a	–	–	–	–
2	3'-Sialyl-lactose	13.9	17.7 (3.0) ^a	19.4 (3.7) ^a	25.7 (0.8)b	12.2 (1.6)c	11.2 (1.7)c
3	Sialyl-lactosamine	14.0	28.8 (1.1) ^a	6.6 (1.0)b	13.3 (2.0)c	5.7 (0.2)b	–
4	6'-Sialyl-lactose	16.2	36.4 (5.1) ^a	26.6 (2.8)b	27.4 (1.0)b	12.6 (1.1)c	11.3 (1.3)c
5	Glycolyl-neuraminyl-lactose (isomer 2)	16.3	10.4 (1.0) ^a	13.2 (2.2)b	19.0 (0.1)c	6.7 (0.5) d	12.1 (0.4) ^d b
6	Glycolyl-neuraminyl-lactose (isomer 3)	17.8	10.3 (1.7) ^a	13.6 (3.7)b	9.5 (0.1)c	4.4 (0.4)c	–
7	Galactosyl-lactose (isomer 1)	35.1	84.1 (11.0) ^a	43.0 (1.8)b	53.9 (2.2)c	14.9 (0.8) d	19.2 (0.8) d
8	Galactosyl-lactose (isomer 2)	36.0	4.3 (0.2) ^a	6.4 (1.3)b	14.0 (0.3)c	2.4 (0.3) d	5.5 (0.4)b
	Total CMO		199.1	128.8	162.8	58.9	59.3
	Acidic OS		110.7	79.4	94.9	41.6	34.6
	Neutral OS		88.4	49.4	67.9	17.3	24.7

^aStandard deviations are presented in parentheses. Dashes (–) indicate content below LOQ (0.1–0.5 mg L^{-1}). Different letters indicate significant differences ($P < 0.05$) among the lactation stages for each OS.

Table 2
Concentrations (mg L⁻¹) of OS in the Goat Milk Pool GP Analyzed by HILIC-Q MS^a

Peak number	Carbohydrate	<i>t_R</i> (min)	Lactation period (days)			
			GP1	GP7	GP30	GP120
1	Glycolyl-neuraminyl-lactose (isomer 1)	13.7	1.7 (0.5) ^a ,b	3.9 (1.3) ^c	2.8 (1.3) ^{b,c}	0.7 (0.2) ^a
2	3'-Sialyl-lactose	13.9	23.6 (7.2) ^a	9.1 (0.3) ^b	20.0 (2.6) ^a	10.2 (2.9) ^b
3	Sialyl-lactosamine	14.0	18.3 (1.6) ^a	1.9 (0.1) ^b	1.2 (0.1) ^b	0.4 (0.1) ^b
4	6'-Sialyl-lactose	16.2	150.7 (99.3) ^a	45.0 (9.6) ^b	86.2 (8.3) ^{a,b}	17.7 (6.7) ^b
5	Glycolyl-neuraminyl-lactose (isomer 2)	16.3	11.5 (4.8) ^a	28.5 (8.1) ^b	19.5 (1.5) ^c	11.5 (3.2) ^a
6	Glycolyl-neuraminyl-lactose (isomer 3)	17.8	17.0 (6.9) ^{a,b}	23.2 (4.3) ^a	19.6 (2.9) ^a	9.0 (3.9) ^b
7	Galactosyl-lactose (isomer 1)	35.1	250.1 (16.2) ^a	65.8 (14.4) ^b	23.1 (3.1) ^c	53.4 (14.1) ^b
8	Galactosyl-lactose (isomer 2)	36.0	15.1 (2.9) ^a	13.0 (4.1) ^{a,b}	5.7 (0.8) ^c	9.5 (2.4) ^{b,c}
	Total CMO		488	190.4	178.1	112.4
	Acidic OS		222.8	111.6	149.3	49.5
	Neutral OS		265.2	78.8	28.8	62.9

^aStandard deviations are presented in parentheses. Different letters indicate significant differences ($P < 0.05$) among the lactation stages for each OS.

Table 3

Evolution of OS Detected by nano-LC-QTOF MS in Murciano-Granadina Goat Milk (GM) at Different Lactation Times: Colostrum (GM2), 10 days (GM10), 20 days (GM20), 30 days (GM30), and 40 days (GM40) of Lactation^a

#	<i>t_R</i>	Composition ^b	Formula	Neutral Mass	Verified by MS/MS	Lactation period (days)				
						2	10	20	30	40
1	16.50	1_1_0_0_1	C ₂₅ H ₄₂ N ₂ O ₂₀	690.232		+	+	-	+	+
2	18.03	1_1_0_0_1	C ₂₅ H ₄₂ N ₂ O ₂₀	690.233		+	+	+	+	+
3	13.85	1_1_0_1_0	C ₂₅ H ₄₂ N ₂ O ₁₉	674.238		+	+	+	+	+
4	16.74	1_1_0_1_0	C ₂₅ H ₄₂ N ₂ O ₁₉	674.238		+	-	-	-	-
5	18.34	1_1_0_1_0	C ₂₅ H ₄₂ N ₂ O ₁₉	674.239		+	-	-	-	-
6	16.69	2_0_0_0_1	C ₂₃ H ₃₉ NO ₂₀	649.207		+	++	+	+	+
7	18.04	2_0_0_0_1	C ₂₃ H ₃₉ NO ₂₀	649.206		+	+	+	+	+
8	22.42	2_0_0_0_1	C ₂₃ H ₃₉ NO ₂₀	649.207		+	+	+	+	+
9	23.75	2_0_0_0_1	C ₂₃ H ₃₉ NO ₂₀	649.206		+	++	+	+	+
10	25.28	2_0_0_0_2	C ₃₄ H ₅₆ N ₂ O ₂₉	956.296		+	+	+	+	+
11	16.99	2_0_0_1_0	C ₂₃ H ₃₉ NO ₁₉	633.211		+	+	+	+	+
12	23.25	2_0_0_1_0	C ₂₃ H ₃₉ NO ₁₉	633.212		+	+	+	+	+
13	24.92	2_0_0_1_1	C ₃₄ H ₅₆ N ₂ O ₂₈	940.303	×	+	++	+++	+	+
14	25.73	2_0_0_1_1	C ₃₄ H ₅₆ N ₂ O ₂₈	940.302		+	+	+	+	+
15	25.61	2_0_0_2_0	C ₃₄ H ₅₆ N ₂ O ₂₇	924.306		+	++	+	-	-
16	27.05	2_0_0_2_0	C ₃₄ H ₅₆ N ₂ O ₂₇	924.307		+	+	+	-	-
17	13.00	2_1_0_0_0	C ₂₀ H ₃₅ NO ₁₆	545.195		+	+	-	-	-
18	13.71	2_1_0_0_0	C ₂₀ H ₃₅ NO ₁₆	545.195		+	-	-	-	-
19	19.23	2_1_0_1_0	C ₃₁ H ₅₂ N ₂ O ₂₄	836.293		+	+	-	+	-
20	15.49	2_2_0_0_0	C ₂₈ H ₄₈ N ₂ O ₂₁	748.276	×	+	++	+	+	+
21	17.29	2_2_0_0_0	C ₂₈ H ₄₈ N ₂ O ₂₁	748.275		+	+	++	+	+
22	19.44	2_2_0_0_0	C ₂₈ H ₄₈ N ₂ O ₂₁	748.275	×	+	+	+	++	+
23	9.24	3_0_0_0_0	C ₁₈ H ₃₂ O ₁₆	504.169		+	+	+	+	+
24	11.94	3_0_0_0_0	C ₁₈ H ₃₂ O ₁₆	504.168		+	+	+	-	-

#	t_r	Composition ^b	Formula	Neutral Mass	Verified by MS/MS	Lactation period (days)				
						2	10	20	30	40
25	12.71	3_0_0_0_0	C ₁₈ H ₃₂ O ₁₆	504.169		+	+	+	+	+
26	13.39	3_0_0_0_0	C ₁₈ H ₃₂ O ₁₆	504.169		+	+	+	+	+
27	14.37	3_0_0_0_0	C ₁₈ H ₃₂ O ₁₆	504.169		+	+	+	+	+
28	16.35	3_0_0_0_0	C ₁₈ H ₃₂ O ₁₆	504.169		+	+	++	+	–
29	24.82	3_0_0_0_1	C ₂₀ H ₄₀ NO ₂₅	811.260		+	+	+	+	+
30	25.38	3_0_0_0_1	C ₂₀ H ₄₀ NO ₂₅	811.260		+	+	+	+	+
31	25.23	3_0_0_1_0	C ₂₀ H ₄₀ NO ₂₄	795.266		+	+	+	+	+
32	25.85	3_0_0_1_0	C ₂₀ H ₄₀ NO ₂₄	795.264		+	+	+	+	+
33	12.26	3_1_0_0_0	C ₂₀ H ₄₅ NO ₂₁	707.248		+	–	–	–	–
34	15.27	3_1_0_0_0	C ₂₀ H ₄₅ NO ₂₁	707.249		+	+	+	+	+
35	16.19	3_1_0_0_0	C ₂₀ H ₄₅ NO ₂₁	707.249		+	+	+	+	+
36	16.80	3_1_0_0_0	C ₂₀ H ₄₅ NO ₂₁	707.249		+	+	+	+	+
37	17.34	3_1_0_0_0	C ₂₀ H ₄₅ NO ₂₁	707.248		+	++	++	+	+
38	12.41	3_1_1_0_0	C ₃₂ H ₅₅ NO ₂₅	853.305	×	+	+	+	+	+
39	12.87	3_1_1_0_0	C ₃₂ H ₅₅ NO ₂₅	853.305	×	+	+	+	++	++
40	15.93	3_1_1_0_0	C ₃₂ H ₅₅ NO ₂₅	853.305	×	+	+	+	+	+
41	16.74	3_1_1_0_0	C ₃₂ H ₅₅ NO ₂₅	853.305	×	+	+	+	+	+
42	20.20	3_3_0_0_0	C ₄₂ H ₇₁ N ₃ O ₃₁	1113.411	×	+	+	+	+	+
43	20.62	3_3_0_0_0	C ₄₂ H ₇₁ N ₃ O ₃₁	1113.411	×	+	+	+	+	+
44	13.21	4_0_0_0_0	C ₂₄ H ₄₂ O ₂₁	666.222		+	++	+	+	+
45	16.16	4_0_0_0_0	C ₂₄ H ₄₂ O ₂₁	666.222		+	++	–	–	–
46	19.97	4_0_0_0_0	C ₂₄ H ₄₂ O ₂₁	666.221		+	+	+	+	++
47	23.75	4_0_0_0_0	C ₂₄ H ₄₂ O ₂₁	666.222		+	+	+	+	+
48	21.92	4_2_0_0_0	C ₄₀ H ₆₈ N ₂ O ₃₁	1072.385	×	+	+	++	–	+
49	26.55	4_2_0_1_0	C ₅₁ H ₈₅ N ₃ O ₃₉	1363.486	×	+	–	++	–	+

^aSymbol + indicates the presence of OS in colostrum samples. Additional + symbols indicate a significant increase ($P < 0.05$) in their relative abundances. Symbol – indicates a significant decrease ($P < 0.05$) in their relative abundances.

^bComposition (in order): Hex_NAc_Fucose_Neu5Ac_Neu5GC.

Table 4

Evolution of OS Detected by nano-LC-QTOF MS in Murciano-Granadina Goat Milk Pool (GP) at Different Lactation Times: Colostrum (GP1), 7 days (GP7), 30 days (GP30), and 120 days (GP120)^a

#	<i>t_R</i>	Composition ^b	Formula	Neutral Mass	Verified by MS/MS	Lactation period (days)				
						2	7	30	120	
1	16.50	1_1_0_0_1	C ₂₅ H ₄₂ N ₂ O ₂₀	690.232		+	+	+	+	
2	18.03	1_1_0_0_1	C ₂₅ H ₄₂ N ₂ O ₂₀	690.233		+	+	+	+	
3	13.85	1_1_0_1_0	C ₂₅ H ₄₂ N ₂ O ₁₉	674.238		+	+	+	+	
4	16.74	1_1_0_1_0	C ₂₅ H ₄₂ N ₂ O ₁₉	674.238		+	-	-	-	
5	18.34	1_1_0_1_0	C ₂₅ H ₄₂ N ₂ O ₁₉	674.239		+	-	-	-	
6	16.69	2_0_0_0_1	C ₂₃ H ₃₉ NO ₂₀	649.207		+	+++	+++	++	
7	18.04	2_0_0_0_1	C ₂₃ H ₃₉ NO ₂₀	649.206		+	+++	+++	++	
8	22.42	2_0_0_0_1	C ₂₃ H ₃₉ NO ₂₀	649.207		+	+	+	+	
9	23.75	2_0_0_0_1	C ₂₃ H ₃₉ NO ₂₀	649.206		+	+	+	+	
10	25.28	2_0_0_0_2	C ₃₄ H ₅₆ N ₂ O ₂₉	956.296		+	+	+	+	
11	16.99	2_0_0_1_0	C ₂₃ H ₃₉ NO ₁₉	633.211		+	+	+	+	
12	23.25	2_0_0_1_0	C ₂₃ H ₃₉ NO ₁₉	633.212		+	+	+	+	
14	25.73	2_0_0_1_1	C ₃₄ H ₅₆ N ₂ O ₂₈	940.302		+	+	+	+	
15	25.61	2_0_0_2_0	C ₃₄ H ₅₆ N ₂ O ₂₇	924.306		+	-	-	-	
16	27.02	2_0_0_2_0	C ₃₄ H ₅₆ N ₂ O ₂₇	924.307		+	-	+	-	
17	13.00	2_1_0_0_0	C ₂₀ H ₃₅ NO ₁₆	545.195		+	+++	++	++	
18	13.71	2_1_0_0_0	C ₂₀ H ₃₅ NO ₁₆	545.195		+	++	+	+	
19	19.23	2_1_0_1_0	C ₃₁ H ₅₂ N ₂ O ₂₄	836.293		+	-	-	-	
20	15.49	2_2_0_0_0	C ₂₈ H ₄₈ N ₂ O ₂₁	748.276	×	+	+	-	+	
21	17.29	2_2_0_0_0	C ₂₈ H ₄₈ N ₂ O ₂₁	748.275		+	+	+	+	
22	19.44	2_2_0_0_0	C ₂₈ H ₄₈ N ₂ O ₂₁	748.275	×	+	-	-	+	
23	9.24	3_0_0_0_0	C ₁₈ H ₃₂ O ₁₆	504.169		+	+	+	+	
24	11.94	3_0_0_0_0	C ₁₈ H ₃₂ O ₁₆	504.168		+	+	-	+	
25	12.71	3_0_0_0_0	C ₁₈ H ₃₂ O ₁₆	504.169		+	-	-	+	

#	r_f	Composition ^b	Formula	Neutral Mass	Verified by MS/MS	Lactation period (days)				
						2	7	30	120	
26	13.39	3_0_0_0_0	C ₁₈ H ₃₂ O ₁₆	504.169		+	+	–	+	
27	14.37	3_0_0_0_0	C ₁₈ H ₃₂ O ₁₆	504.169		+	+	–	+	
28	16.35	3_0_0_0_0	C ₁₈ H ₃₂ O ₁₆	504.169		+	+	+	++	
29	24.82	3_0_0_0_1	C ₂₉ H ₄₉ NO ₂₅	811.260		+	+	+	+	
30	25.38	3_0_0_0_1	C ₂₉ H ₄₉ NO ₂₅	811.260		+	+	+	+	
31	25.23	3_0_0_1_0	C ₂₉ H ₄₉ NO ₂₄	795.266		+	+	+	+	
32	25.85	3_0_0_1_0	C ₂₉ H ₄₉ NO ₂₄	795.264		+	+	+	+	
33	12.26	3_1_0_0_0	C ₂₆ H ₄₅ NO ₂₁	707.248		+	+	+	+	
34	15.27	3_1_0_0_0	C ₂₆ H ₄₅ NO ₂₁	707.249		+	++	++	++	
35	16.19	3_1_0_0_0	C ₂₆ H ₄₅ NO ₂₁	707.249		+	+	+	+	
36	16.80	3_1_0_0_0	C ₂₆ H ₄₅ NO ₂₁	707.249		+	+	+	+	
37	17.34	3_1_0_0_0	C ₂₆ H ₄₅ NO ₂₁	707.248		+	+	++	++	
38	12.41	3_1_1_0_0	C ₃₂ H ₅₅ NO ₂₅	853.305	×	+	+	+	+	
39	12.87	3_1_1_0_0	C ₃₂ H ₅₅ NO ₂₅	853.305	×	+	++	++	+++	
40	15.93	3_1_1_0_0	C ₃₂ H ₅₅ NO ₂₅	853.305	×	+	+	+	+	
41	16.74	3_1_1_0_0	C ₃₂ H ₅₅ NO ₂₅	853.305	×	+	+	+	+	
42	20.20	3_3_0_0_0	C ₄₂ H ₇₁ N ₃ O ₃₁	1113.411	×	+	+	++	+++	
43	20.62	3_3_0_0_0	C ₄₂ H ₇₁ N ₃ O ₃₁	1113.411	×	+	+	++	+++	
44	13.21	4_0_0_0_0	C ₂₄ H ₄₂ O ₂₁	666.222		+	++	++	++	
45	16.16	4_0_0_0_0	C ₂₄ H ₄₂ O ₂₁	666.222		+	–	–	–	
46	19.97	4_0_0_0_0	C ₂₄ H ₄₂ O ₂₁	666.221		+	–	–	–	
47	23.75	4_0_0_0_0	C ₂₄ H ₄₂ O ₂₁	666.222		+	+	+	+	
48	21.92	4_2_0_0_0	C ₄₀ H ₆₈ N ₂ O ₃₁	1072.385	×	+	+	+	++	
49	26.55	4_2_0_1_0	C ₅₁ H ₈₅ N ₃ O ₃₉	1363.486	×	+	+	+++	++	

^aSymbol + indicates the presence of OS in colostrum samples. Additional + symbols indicate a significant increase ($P < 0.05$) in their relative abundances. Symbol – indicates a significant decrease ($P < 0.05$) in their relative abundances.

^bComposition (in order): Hex_HexNAc_Fucose_Neu5Ac_Neu5GC.