Extraction and Quantitative Analysis of Goat Milk Oligosaccharides: Composition, Variation, Associations, and 2′-FL Variability

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ABSTRACT: Human milk oligosaccharides (hMOS) are associated with health benefits for newborns. We studied the composition of goat MOS (gMOS) from colostrum up to the 9th month of lactation to conceive an overview of the structures present and their fate. Potential correlations with factors such as age, parity, and lifetime milk production were examined. An effective method for gMOS extraction and ultra-high-performance liquid chromatography coupled to fluorescence detection (UPLC-FLD) analysis was established, following 2-aminobenzamide gMOS labeling. Considerable biological variability was highlighted among the 12 quantified gMOS and the 9 non-quantified structures in the individual milk samples. Most characteristic, 2′-fucosyllactose was present in 73.7% of the milk samples analyzed, suggesting the possibility of a secretor/non-secretor goat genotype, similar to humans. Contributing factors to the observed biological variability were goat age, parity, lifetime milk production, and the kids’ sex. The results significantly contribute to the current understanding of (variations in) gMOS composition.

KEYWORDS: goat milk, UPLC-FLD, oligosaccharides, 2′-fucosyllactose, secretor

INTRODUCTION

One of the most abundant classes of macronutrients found in human milk are carbohydrates, mainly lactose. Among carbohydrates, the free milk oligosaccharides, referring to glycans that are not bound to glycoproteins, are suggested to have significant biological functions. Human milk oligosaccharides (hMOS) are present in relatively high concentrations in human milk (5–20 g/L).1 The diversity of the structures of these hMOS is high. It is mentioned that in human milk, 162 hMOS have been structurally identified and more than 240 compositional varieties have been annotated.2 These are all based on five monosaccharide units: galactose (Gal), glucose (Glc), N-acetylglucosamine (GlcNAc), fucose (Fuc), and N-acetylneuraminic acid (Neu5Ac). The typical length of these hMOS is between three and seven monosaccharide units. Composition of hMOS differs between individual mothers due to genetics, diet, and epigenetics, and within the mothers over the course of lactation.3–6 However, most variance is yet unexplained.

Recent studies have unraveled the importance of hMOS, providing a long list of functionalities.7,8 Besides the hMOS “prebiotic” character toward certain beneficial bacteria (e.g., Bifidobacterium longum subsp. infantis), it is reported that they inhibit pathogen adhesion to epithelial cells9 and have immune modulation effects.10 Protection of the neonate from certain diseases (e.g., necrotizing enterocolitis) has been reported in animal and human studies,11,12 while possible enhanced offspring brain development has been studied in animal studies.13,14 Potential protective effects of hMOS toward the breastfeeding mothers have been suggested, either by influencing the microbiota of the mammary gland or by contributing to its epithelial cell responses.8

The high structural diversity of hMOS has not been reported for MOS of domesticated animals (non-human mammals).15,16 In all studies of domesticated animals, less variety of structures is reported together with an overall lower amount of MOS. In hMOS, a high percentage of the compounds found are neutral and mostly (∼70%) fucosylated,17 while this is not the case for the MOS of domesticated dairy animals, which are mostly sialylated structures.17

Undoubtedly, human milk is the preferable source of oligosaccharides for neonates. However, not all mothers can or want to breastfeed. These mothers depend on infant formula, where bovine milk is the most chosen option for infant formula production. Several studies have reported on bovine MOS composition, reviewed recently.18 The differences between bovine milk and human milk in terms of MOS content and structural diversity have been highlighted by those studies.8,15

Goat milk has higher levels of MOS compared to other non-human mammals and a richer variety of structures.19 Goat milk-based infant formula is becoming increasingly popular. However, goat milk oligosaccharides (gMOS) studies are limited, especially those providing quantitative data. Some of those providing quantitative information are based on rather small sample sizes.20–22 Other gMOS studies cover a selection of predefined targets resulting in underestimation of the total gMOS content.23 Different analytical instruments and MOS
every month (one milk collection per month).

facilitating the understanding of the complete gMOS collections per day); during days 4

EXPERIMENTAL SECTION

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quantitation. Finally, some gMOS studies focus only on speci-

MOS from goat milk samples and their analysis with ultra-high-

structures (i.e., 3′-sialyllactose and disialyllactose23), not

focusing on the presence of the gMOS structures. The 57 goats of the study were divided over two

farms between February 2018 and January 2019. Goats were

produced, and milk characteristics, were studied.

composition. Moreover, associations between oligosaccharide

structures. (i.e., 3′-sialyllactose and disialyllactose23), not

aiming to provide average concentrations of the identi-

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cations before injection in the analytical

cation of the oligosaccharides before injection in the analytical

Materials. The standards of 3′-galactosyllactose (3′-GL), 4′-

galactosyllactose (4′-GL), 6′-galactosyllactose (6′-GL), 6′-sialyl-N-acetyllactosamine (6′-SLN), and lacto-N-neotetraose (LNNt) of

minimum 95% purity were purchased from Carbosynth (Berkshire, U.K.). 2′-Fucosyllactose (2′-FL), 3′-fucosyllactose (3′-FL), 3′-sialyllac-

tose (3′-SL), and 6′-sialyllactose (6′-SL) of >95% purity were from

isoSep AB (Tullinge, Sweden).

Ultrapure water (18.2 MΩ) was generated by a Milli-Q water system

(Millipore Merck).

Methanol (MeOH) and acetonitrile (MeCN) (HPLC gradient

grade) were from Boom BV (Meppel, the Netherlands). Anhydrous

acetic acid (glacial, 100%) was from Merck (Darmstadt, Germany).

2-Methylpyridine borane complex (95%), 2-aminobenzamide (2-

aminobenzamide (2-AB, ≥98%), dimethyl sulfoxide (DMSO, ≥99.5%), and cellulose

microcrystalline powder were from Sigma-Aldrich (Steinheim, Germany).

Trifluoroacetic acid (TFA, 99%) was purchased by Acros Organics

(New Jersey).

The 150 mg/4 mL porous graphitic carbon (PGC) solid-phase

extraction (SPE) cartridges (Extract-Clean SPE Carbo) for the removal of

excessive amounts of lactose were purchased from Aurora Boareis Control BV (Schoonebeek, the Netherlands). The 96-well plate filters

used for the removal of the excessive labeling reagent and the final purification of the oligosaccharides before injection in the analytical

system were the AcroPep 96-well 0.2 µm GHP from Pall Corporation

(Puerto Rico). The Amicon Ultra-15 spin-filters with a 10 kDa cutoff

were from Millipore.

Goat Milk Samples. Goat milk was obtained from two Dutch goat

farms between February 2018 and January 2019. Goats were

individually milked using a bucket milking system. Full milkings were

homogenized, and samples of ~10 mL were collected from 57 Dutch Saanen (Melkgeit) goats. The samples were stored at −20 °C at

the farms until collection and then stored at −80 °C in the laboratory until further

analysis. The kidding and, hence, the offset of sample collection fell within a 44-day span for all goats of farm #1 (25 February to 11 April

2018) and within 14 days for farm #2 (26 February to 13 March 2018). The two farms followed different feeding regimes, farm #1 using

homogenized (pelletized) feed at libitum and farm #2 using diverse feeding based on crop excess in the local area, supplemented by

pelletized feed. The sample collection was performed at multiple time

points per lactation period (Figure 1). For colostrum milk, three

samples per day were collected in the first 3 days of lactation (day 1

(D1), day 2 (D2), and day 3 (D3)). For the next 7 days, one sample was

collected per day, and after this, one sample per week till the end of the

first month (day 31 (D31)) of lactation. The sample collection was

continued for the next 8 months, collecting one sample per month. A
total of 28 samples per goat were collected from the 57 goats of the two

farms. Moreover, tank (pooled) mature milk of all farms providing milk to Ausnutria BV and pooled colostrum milk from one farm was used as

reference milk during the optimization of the extraction method and as a

quality control (QC) sample during the analysis of the samples of the

individual goats.

Optimization of Oligosaccharide Extraction and Lactose

Removal. Optimization experiments for the efficient extraction of

gMOS were executed with the pooled sample of colostrum and the

pooled sample of mature goat milk (reference milk). First, direct

labeling to 20 μL of reference goat milk was performed, following the

Austin et al. approach for human milk.25 Moreover, direct

labeling to 20 μL of reference goat milk and reference

gMOS were executed with the pooled sample of colostrum and the

samples were centrifuged (4000 g for 30 min) of reference goat milk and

reference goat colostrum was attempted, prior to the labeling of the

defatted extracts. The same tests were repeated after dilution of the

above-mentioned samples with Milli-Q water in a 1:1 ratio.

Additional experiments were performed at larger scale to obtain an

enriched gMOS profile. For this purpose, precipitation of the caseins by

lowering the pH to 4.6 was attempted. For this test, a volume of 10 mL

of reference mature goat milk was employed, diluted 1:1 with Milli-Q

water. The pH was reduced to 4.6 by gradual addition of 1 M HCl. The

samples were centrifuged (4000g for 30 min at 4 °C), and the

supernatant was collected and applied to spin-filters with a 10 kDa

cutoff. Subsequently, the deaceminated fraction was further processed to

gMOS analysis as described below.

Another larger-scale method aimed to “salt out” the proteins by the

slow addition of ammonium sulfate. To 10 mL of reference goat milk

diluted 1:1 with Milli-Q water, 10 g of ammonium sulfate was added. The

samples were centrifuged (4000g for 30 min at 4 °C), and the

supernatant was collected. The deproteinated sample was processed as
described below to analyze gMOS.

Since the above experiments highlighted the problem of lactose

interference with the obtained gMOS profiles, further tests were

performed to achieve sufficient and selective removal of lactose.

Extraction of gMOS with acetone and methanol at a sample-to-solvent

ratio of 1:2 was applied prior to porous graphitic carbon (PGC) solid-

phase extraction (SPE). Also, direct application of undiluted milk (1

Figure 1. Milk sample collection schedule per goat during lactation. The collection was performed as follows: during the first 3 days (three milk

collections per day); during days 4–10 (one milk collection per day); every week (one milk collection per week for the rest of the first month); and
every month (one milk collection per month).
Table 1. Oligosaccharides Identified and Quantified in the 57 Goat Milk Samples of the Study and Their Concentrations (mg L⁻¹) at Three Time Points: Colostrum of the First and Second Days and Mature Milk of the 31st Day of Lactation

| Abbreviation | Name | MMOS | Structure | Retention time (Da) | Mean conc. mg/L | Min | Max | Mean conc. mg/L | Min | Max | Mean conc. mg/L | Min | Max |
|--------------|------|------|-----------|-------------------|----------------|-----|-----|----------------|-----|-----|----------------|-----|-----|
| 2′-FL | 2′-Fucosyl-lactose | Y | Glc: blue circle, Neu5Ac: pink diamond, NeuSgc: turquoise diamond filled | 609.2 | 13.1 | 41.41 | 21.44 | 268.05 | 62.92 | 0.58 | 236.92 | 5.12 | 0.35 |
| 3′-GL | 3′-Galactosyl-lactose | N | Glc: blue circle, Neu5Ac: pink diamond, NeuSgc: turquoise diamond filled | 625.2 | 19.1 | 147.94 | 62.67 | 300.60 | 51.56 | 22.63 | 123.82 | 31.69 | 13.62 |
| 6′-GL | 6′-Galactosyl-lactose | Y | Glc: blue circle, Neu5Ac: pink diamond, NeuSgc: turquoise diamond filled | 753.4 | 21.7 | 198.92 | 89.21 | 482.48 | 72.53 | 2.48 | 242.18 | 43.62 | 0.42 |
| 3′-SL | 3′-Sialyl-lactose | Y | Glc: blue circle, Neu5Ac: pink diamond, NeuSgc: turquoise diamond filled | 795.3 | 23.9 | 107.2 | 3.1 | 14.88 | 3.69 | 14.25 | 45.32 | 6.03 |
| 6′-SL | 6′-Sialyl-N-acetyllactosamine | N | Glc: blue circle, Neu5Ac: pink diamond, NeuSgc: turquoise diamond filled | 770.3 | 24.4 | 75.71 | 6.8 | 17.46 | 41.88 | 3.69 | 14.25 | 45.32 | 6.03 |
| 6′-SL | 6′-Sialyldolylactosamine | N | Glc: blue circle, Neu5Ac: pink diamond, NeuSgc: turquoise diamond filled | 751.3 | 24.6 | 75.71 | 6.8 | 17.46 | 41.88 | 3.69 | 14.25 | 45.32 | 6.03 |
| 3′-NL | 3′-N-glycolyl-neuraminyl-lactose | N | Glc: blue circle, Neu5Ac: pink diamond, NeuSgc: turquoise diamond filled | 811.3 | 25.8 | 48.29 | 2.39 | 188.48 | 19.88 | 0.65 | 100.20 | 2.98 | 0.37 |
| 6′-NL | 6′-N-glycolyl-neuraminyl-lactose | N | Glc: blue circle, Neu5Ac: pink diamond, NeuSgc: turquoise diamond filled | 770.3 | 27.6 | 11.93 | 3.02 | 29.57 | 45.59 | 9.34 | 665.50 | 30.29 | 5.84 |
| OSL | Oslactosyl-lactose | N | Glc: blue circle, Neu5Ac: pink diamond, NeuSgc: turquoise diamond filled | 1045.6 | 28.3 | 66.6 | 7.2 | 209 | 3.39 | 0.26 | 11.29 | 2.19 | 0.14 |
| 9′HL | 9′-Sialylglactosyl-lactose | N | Glc: blue circle, Neu5Ac: pink diamond, NeuSgc: turquoise diamond filled | 916.3 | 28.3 | 47.38 | 14.77 | 130.63 | 3.67 | 0.10 | 11.58 | 2.46 | 0.08 |
| 9′NOL | 9′-N-acetyllactosaminyl-dolylactosyl-lactose | N | Glc: blue circle, Neu5Ac: pink diamond, NeuSgc: turquoise diamond filled | 990.4 | 33.1 | 25.47 | 4.03 | 67.67 | 6.50 | 0.48 | 17.07 | 0.69 | 0.38 |
| Total OS | 1102.95 | 438.31 | 271.25 | | | | | | | | | |
| Total Acidic OS | 822.46 (75%) | 302.49 (69%) | 175.70 (70%) | | | | | | | | | |
| Total Neutral OS | 254.45 (25%) | 135.16 (21%) | 55.57 (22%) | | | | | | | | | |
| Total Fucosylated OS | 41.52 (2%) | 6.29 (14%) | 2.54 (5%) | | | | | | | | | |
| Total 8-Sialylated OS | 382.46 (75%) | 302.49 (69%) | 175.70 (70%) | | | | | | | | | |

Analysis of Oligosaccharides. The labeled and purified oligosaccharides were analyzed using an Ultimate 3000 UHPLC system (Thermo Fisher Scientific) coupled to an FP-920 fluorescence detector (Jasco, Inc.). The detector was set for excitation and emission at 330 and 420 nm, respectively.

The chromatographic separation was conducted on an Acquity UPLC Glycan BEH Amide column (2.1 mm × 100 mm, 130 Å, 1.7 μm) and an Acquity UPLC Glycan BEH Amide VanGuard pre-column (2.1 mm × 5 mm, 130 Å, 1.7 μm) both from Waters. The column was maintained at 40 °C. The 10 times diluted sample (2 μL) was injected in the system, under gradient elution with a quaternary solvent system. The solvent system used consisted of acetone (solvent A), 250 mM formic acid in 10% acetonitrile in Milli-Q water (solvent B), and 10% acetonitrile in Milli-Q water (solvent C). The method used for the analysis was based on a gradient where solvent B was constant at 5%. The elution was performed with a gradient of 40 min moving from 5% to 27% solvent C. The gradient was followed by a cleaning step with 20% solvent B and 20% solvent C for 5 min and a subsequent equilibration step to the initial conditions of the analysis for 12 min, resulting in a total analysis time of 57 min. The flow applied for optimal chromatographic separation was 0.5 mL/min.

Quantification was performed using maltooligosaccharide as an internal standard (IS) and general calibrant, based on the study of Austin et al. This compound was selected, instead of the laminaritriose in Austin’s study, as it was eluting in an area of the chromatogram free of oligosaccharide peaks for both mature and colostrum milk samples. Commercial standards (2′-FL, 3′-GL, 6′-GL, 3′-SL, 6′-SL, and 6′-SLN) were used for identification of MMOS. For the identification of peaks not co-eluting with the analytical standards, the same chromatographic system and fluorescence detector were coupled inline with a time-of-flight mass spectrometer (MaXis Plus, Bruker). The MS was operated in positive-ion mode, scanning between m/z 300 and 2000, with collision energy 7 eV, collision RF 80 Vpp, and transfer time 100 μs.

Quality control (QC) samples were utilized to monitor the stability of the analytical system and the consistency of the sample preparation.
For this purpose, reference mature goat milk samples were treated in the same way as the individual goat’s milk samples, and were inserted in every analytical run every 10–15 samples.29,30

**Data Analysis.** For the statistical analysis of the two farms, pairwise comparisons were assessed via the Mann–Whitney and Welch’s test, depending on the outcome of the normality test. To check the normality, both D’Agostino and Pearson test and Shapiro–Wilk test were taken into consideration.

The descriptive parameters tested for potential comparisons with the gMOS levels were the age of the goats, milk production per day, lifetime milk production, % fat, % protein, number of pregnancies (parity), pseudopregnancy, and the outcome of pregnancy in terms of sex of the goat kid. Pearson and Spearman correlation tests were employed for the factors described by quantitative data, while Mann–Whitney and Welch’s tests were employed for the outcome of pregnancy factor. Pearson or Spearman test and Mann–Whitney or Welch’s tests were selected based on both D’Agostino–Pearson and Shapiro–Wilk normality tests.

**RESULTS AND DISCUSSION**

**Experimental Optimization.** Initial experiments, using direct labeling of the goat milk,25 resulted in poor oligosaccharide profiles both in reference mature milk and in the pooled colostrum samples. These were dominated by a lactose peak and almost no detectable oligosaccharides. Goat milk and cow milk differ in general composition from human milk. For example, the level of total protein in goat milk is almost 3.5 times higher than in human milk.31 Also, the lactose:MOS ratio in goat milk varies between 150 and 180, while in human milk, it is 8.5–13.6.32 The difference in lactose ratio may result in a very poor chromatogram. The higher protein levels may interfere with the labeling reaction. Moreover, the system setup used in this study does not allow their exchange with the solvent. Acetone extraction of oligosaccharides led to the formation of a pellet, and the extraction of gMOS structures in the supernatant remained poor. A possible explanation is that the precipitate of casein by acidification and salting out of proteins with ammonium sulfate. These approaches resulted in high-quality chromatograms clearly showing oligosaccharide peaks. However, these labor-intensive approaches would be prohibitive toward high-throughput sample handling, which was one of the requirements of this study.

Acetone extraction of oligosaccharides led to the formation of a pellet, and the extraction of gMOS structures in the supernatant remained poor. A possible explanation is that the pellet formed also caused precipitation of the carbohydrate structures, not allowing their exchange with the solvent. Methanol extraction resulted in a precipitate of the fat/protein content of milk, and the supernatant extract yielded a chromatogram rich in oligosaccharides. Direct application of 1 mL of milk to the PGC filters was not applicable, as the filters became clogged.

In view of the above results, extraction of oligosaccharides with methanol was chosen as the first step to remove most of the fat/protein content of the milk. After thawing overnight at 4 °C, 120 μL of milk was mixed with 240 μL of methanol. The samples were vortexed thoroughly and centrifuged for 15 min at 21 130g at 4 °C. The clear supernatant was collected, and the samples were dried using a Speedvac evaporator.

For the subsequent removal of the excessive amount of lactose interfering with the oligosaccharide analysis, filtration through PGC-SPE filters was applied. After initial conditioning, equilibration, and application of the samples on the SPE filters (see the Experimental section), different washing steps were tested. Optimal removal of lactose was achieved by washing the column with 4% MeCN/0.1% TFA; higher amounts of MeCN did not result in improved lactose removal and/or oligosaccharide recovery. Elution and drying of the samples resulted in extraction of purified gMOS.

After labeling of the oligosaccharides, excess of free label was removed by filtration in 96-well plate format with microporous cellulose. Four different ratios of the MeCN/Milli-Q mixture for the equilibration and washing of the samples were examined. The results showed that 100% MeCN was not sufficient to remove excess label, yielding a broad peak in the first minutes of the chromatogram and overloading the detector. The ratio of 80:20 MeCN/Milli-Q and 50:50 MeCN/Milli-Q resulted in a loss of oligosaccharides during the washing steps and, hence, in non-reproducible oligosaccharide profiles. Optimal conditions were achieved when equilibrating and washing with 90:10 MeCN/Milli-Q that resulted in maximal removal of the labeling mixture without detectable loss of oligosaccharides. Repeat analysis of the reference sample with the final method showed high reproducibility in results.

Two larger-scale experiments were performed, involving precipitation of casein by acidification and salting out of proteins with ammonium sulfate. These approaches resulted in high-quality chromatograms clearly showing oligosaccharide peaks. However, these labor-intensive approaches would be prohibitive toward high-throughput sample handling, which was one of the requirements of this study.

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Samples of different time points of different goats were analyzed in different batches of 60 to 65 samples. To monitor the performance of the analytical system and the repeatability of the extraction/labeling applied, two different controls were included in the analysis. Through the inclusion of quality control (QC) samples during the sample preparation of each batch, and their repetitive injection during the analytical run, both the batch-to-batch extraction efficiency and the analytical system stability were verified. The coefficient of variation (CV) for all of the structures detected in the QC samples (Table 1) did not exceed 15% within each batch and between different batches of analysis. The second control was the inclusion of an internal standard (IS) during sample preparation. The CV of maltolpentaoze (internal standard) did not exceed 10% within and between different controls.
batches. The final method is suitable for large sample sets and provides reproducible results.

**Oligosaccharide Composition and Variation.** Milk samples from the 57 goats participating in the study, early and later during the lactation period (colostrum and mature milk), were analyzed. From preliminary analysis performed with 22 goats for all time points until the second month of lactation, it was highlighted that the oligosaccharide profiles followed a similar pattern for all 22 goats; in the first days of lactation (colostrum), large variations occurred in the levels of oligosaccharides, followed by a gradual decrease over time. After the first few days, most structures were decreased to a minimum level (Figure S1). For the rest of the time points up until month 9, three goats from each farm were analyzed, showing that the profiles remained the same until the last sample point (9 months of lactation). Samples of all 57 goats were analyzed for selected time points, reflecting the colostrum sample profile (day 1—1st colostrum sample; day 2—4th colostrum sample) and the mature milk (day 31). Table 1 summarizes the 12 gMOS structures identified and quantified in the samples of all 57 goats and their mean concentrations in the selected colostrum and mature time points.

Peaks annotated as 2′-FL, 3′-GL, 6′-GL, 3′-SL, 6′-SL, and 6′-SLN were identified based on comparison of the retention times with commercial standards and verified by in-spiking with standards and UPLC-TOF-FLD mass spectrometric analysis.32 Peaks eluting at 24.4 and 27.4 min were identified as two isomers of N-glycolyl-neuraminyl-lactose (3′- and 6′-NGL, respec-
tively), based on the mass m/z 770.3 determined by the UPLC-TOF-FLD analysis. The peak at 25.6 min was characterized as N-glycolyl-neuraminyl-lactosamine (NGLN, m/z 811.3). Diallylactose (DSL, m/z 1045.4) and sialyl-galactosyllactose (SHL, m/z 916.3) were assigned to the peaks at 28 and 28.3 min respectively, while N-acetyl-glycosaminyl-dihexosyl-lactose (NADHL, m/z 990.4) was assigned to the peak at 33.1 min (Table 1). Based on previous observations of this structure and

Figure 5. Comparisons of goat milk MOS structures per farm and time point (D2 colostrum and D31 mature milk). The error bars indicate the minimum and maximum values and the stars the outcome of the pairwise comparison with the Mann–Whitney and Welch’s test, based on the normality test performed. The p-values are presented with stars: *<0.05, **<0.01, ***<0.001, and ****<0.0001.
the relative abundances reported, identification as NADHL appears more likely than other structures fitting the observed mass, e.g., α-galactosyl-lacto-N-neotetraose. Moreover, in some of the samples analyzed, a peak was identified at 24.7 min as lacto-N-neotetraose (LNnT) based on the detected mass (m/z 828.3) and by comparing with the retention time of the commercial standard. However, due to the very low abundance of the peak and its elution immediately after the high abundance peak of 6′-SL, it was not possible to quantify LNnT. It should be underlined that the 3-FL commercial standard was washed out during the PGC-SPE filtration step. This effect of the PGC filtration on 3-FL has been reported previously. Hence, information about the 3-FL structure cannot be provided for the analyzed samples. Figure 2 provides an overview of the structures identified in the current study and their relative abundances in colostrum (D2) and mature milk (D31).

Table 3. Spearman and Pearson Correlation Test Outcome of the MOS Studied for the Factors Age, Parity, and Lifetime Milk Production

| Abbreviation | Name | r parameter | Age D2 | Age D31 | Parity D2 | Parity D31 | Lifetime milk production D2 | Lifetime milk production D31 |
|--------------|------|-------------|--------|---------|-----------|-----------|-----------------------------|-----------------------------|
| 2′-FL | 2′-galactosyl-lactose | Spearman r | 0.1034 | 0.1661 | 0.0874 | 0.0508 | 0.1887 | 0.2237 |
| p value | 0.5300 | 0.3319 | 0.5967 | 0.7720 | 0.2500 | 0.1987 |
| 3′-GL | 3′-galactosyl-lactose | Spearman r | -0.0856 | 0.4170 | -0.1200 | 0.1874 | -0.1579 | 0.4429 |
| p value | 0.5910 | 0.0048 | 0.4167 | 0.2176 | 0.2891 | 0.0026 |
| 6′-GL | 6′-galactosyl-lactose | Spearman r | -0.2479 | 0.3410 | -0.3520 | 0.2397 | -0.4329 | 0.1440 |
| p value | 0.7337 | 0.0005 | 0.3514 | 0.0072 | 0.7012 | 0.0001 |
| 3′-SL | 3′-sialyl-lactose | Spearman r | -0.4936 | 0.0675 | -0.4989 | 0.0537 | -0.3992 | 0.1801 |
| p value | 0.1162 | 0.1246 | 0.0912 | 0.4333 | 0.1961 | 0.0472 |
| 5′-LN | 5′-sialyl-N-acetyllactosamine | Spearman r | -0.3159 | 0.0377 | -0.3167 | 0.0428 | -0.3023 | 0.1812 |
| p value | 0.0771 | 0.0235 | 0.0435 | 0.4148 | 0.2140 | 0.0255 |
| 3′-NOSL | 3′-N-glycosyl-neuraminyl lactose | Spearman r | 0.5096 | 0.8229 | 0.1657 | 0.8329 | 0.3779 | 0.7641 |
| p value | 0.0001 | 0.0003 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |
| 6′-SL | 6′-sialyl-lactose | Spearman r | -0.2733 | -0.1944 | -0.2465 | -0.2307 | -0.3048 | -0.2119 |
| p value | 0.0061 | 0.2061 | 0.0915 | 0.1274 | 0.0374 | 0.1639 |
| NGLN | N-glycosyl-neuraminyl lactosamine | Spearman r | 0.2873 | 0.7183 | 0.6443 | 0.3267 | 0.2486 | 0.7081 |
| p value | 0.3148 | 0.0032 | 0.0015 | 0.0349 | 0.3121 | 0.0011 |
| 6′-NOSL | 6′-N-glycosyl-neuraminyl lactose | Spearman r | 0.5257 | 0.7630 | 0.1192 | 0.6312 | 0.1897 | 0.6643 |
| p value | 0.0002 | 0.0002 | 0.0001 | 0.0004 | 0.0012 | 0.0135 |
| DSL | Disialyl-lactose | Spearman r | -0.2483 | -0.2274 | -0.2450 | -0.1779 | -0.2376 | -0.2285 |
| p value | 0.1041 | 0.1529 | 0.1947 | 0.2601 | 0.1204 | 0.4980 |
| SHL | Sialyl-galactosyl lactose | Spearman r | -0.3157 | -0.2669 | -0.3663 | -0.2956 | -0.2644 | -0.2272 |
| p value | 0.0285 | 0.0381 | 0.0109 | 0.0519 | 0.0725 | 0.1429 |
| NAHL | N-acetyl-glucosaminyl-dihexosyl-lactose | Spearman r | -0.3164 | 0.5028 | -0.7370 | 0.3045 | -0.3264 | 0.2633 |
| p value | 0.2997 | 0.4000 | 0.5276 | 0.4000 | 0.8175 | 0.5167 |

“The correlations were assessed in both colostrum (D2) and mature milk (D31). The MOS/factor highlighted with green are those for which significant correlations were found based on the p value (<0.05). Pearson or Spearman test was selected based on D'Agostino−Pearson and Shapiro−Wilk normality tests. S and P indications provide the data deriving from Spearman and Pearson test, respectively.”
important to highlight that the authors used only pooled mature goat milk from a non-explicitly mentioned number of goats \((n \geq 3, \) undefined breeds), and this could explain the differences in the levels of certain compounds when comparing with our findings. Concerning the extra structures identified in Albrecht’s study, the identification of structures took place after pre-fractionation into neutral and acidic oligosaccharide pools.17 The method suggested in our study provides a more high-throughput analysis of the most abundant gMOS structures.

Significant differences in the profiles of oligosaccharides were observed between individual goats, in agreement with variation between Murciano-Granadina goats reported previously.20 Four representative chromatograms of the study, belonging to milk samples of two goats of the same farm, following the same feeding regime, in two different time points colostrum of D2/4th collected sample and mature milk of (D31), are presented in Figure 3. Characteristic variations deriving from the time point of lactation (differences between D2 vs D31 for each goat) and biological factors (differences between the two goats) were observed. Figure 3 also shows the chromatogram of the tank milk (Ausnutria BV), which was used as a quality control sample during all performed analyses.

From colostrum (D1) to mature milk of the 9th month, almost all observed structures decreased in concentration in the milk samples for all of the studied goats. The only exception to this trend was the 3'-NGL structure, which remained high during later time points of lactation (D31, Figure 3, bottom chromatogram). Analysis of additional data points of later samples (up to the 9th month of lactation (M9)) showed that the levels of 3'-NGL even increased at later time points. The decrease in concentration of the identified gMOS is observed in the data presented in Table 1, while an indicative trend of 3'-NGL over the course of lactation is given Figure 4. In Figure 4, the trend of 3'-SL is also given for comparison purposes. Furthermore, it should be noted that the concentrations change very strongly the first days of lactation, but the difference between day 17 and day 31 post-partum for all goats was already small (Figure S2). After day 31, the month 2 sample showed no significant change with day 31 for 22 goats tested. A full analysis of all samples collected for six goats also showed no significant change across the 9 months; although observation of the means of all oligosaccharides suggested a downward trend, statistical significance was not observed, with the exception of 3'-NGL, which showed a continuous upward trend with a significance in change between day 31 and month 9, but not between day 2 and day 31.

A noteworthy decrease in the concentration of most structures was observed, also between the colostrum samples (D1 and D2, Table 1). An exception to this trend is 2'-FL, which for some of the goats kept on increasing during D1 after parturition, reaching a peak during D2 and markedly decreasing during the next days of lactation. A very significant decrease in levels of sialylated structures has been reported previously for bovine colostrum.36 The decrease rates for the structures reported by Nakamura et al. are similar to the decrease rates we observed in our study of the gMOS for the first 24 h (3'-SL 63.5% decrease, 6'-SL 69.1% decrease, and 6'-SLN 74.5% decrease).

It is worth mentioning that the variability perceived in the first post-partum sample was very broad, adding to the biological variation and complexity observed also in later time points of lactation. The extremely high levels of oligosaccharides in the

![Figure 6](https://doi.org/10.1021/acs.jafc.1c00499)
first post-partum sample could be related to providing the goat lambs with key components contributing to their healthy development.

Previous studies on goat and human MOS [20–22] also reported an overall decrease in the levels of MOS throughout lactation. The increase of 3′-NGL, the only structure showing an average increase in later time points (Figure 4), may be associated with biological functions in the later stages of lactation. This observation contrasts with previous studies reporting a decline in the levels of both NGL isomers over time.23 The different trend that 3′-NGL follows over the lactation period was observed in our study already after 31 days. The difference with the above-mentioned study could be the fact that the time points studied by Martin-Ortiz et al. were up to 40 days, while in our case, the samples expanded up to 9 months after the delivery. Further investigation of the biosynthesis and the potential biological functionality of 3′-NGL is required. To conclude, the presented data indicate that most gMOS decrease in concentration during the first weeks of lactation, after which the concentrations become stable, with the exception of 3′-NGL (Figure S2).

The most characteristic example of the differences observed in the levels of gMOS between the goats is that of 2′-FL. More specifically, 2′-FL was detected only in 73.7% of the goats studied. Looking at each farm, 21 out of the 27 goats (77.8%) from farm #1 and 21 out of the 30 goats (70%) from farm #2 produce 2′-FL. When 2′-FL was absent in the colostrum samples, it also did not appear in samples of later time points. In Table 1, the mean, min, and max values presented for 2′-FL correspond only to those goat samples in which this structure could be detected (73.7%). These data present a similar phenotype division as observed for human secretor vs non-secretor individuals,6,33 which is the result of disabling mutations in the FUT2 gene. So far, there have been no studies showing such a phenotype division in non-human mammals.

Among the “secretor” goats, there is a 2′-FL concentration range of 0.6–236.3 mg/L in the D2 (4th collected) samples. Within this group of secretors, two subgroups were observed; at D2, 30 goats were found with 0.6–69.9 mg/L and 12 goats with 97.8–236.3 mg/L (5 goats between 97.8 and 157.1 mg/L, and 7 goats between 184.2 and 236.3 mg/L). At the D31 time point, the 2′-FL concentrations were less diverse, ranging from 0.4 to 14.9 mg/L, with only one goat having a level of 69.5 mg/L. A similar division in high and low 2′-FL producers has been observed in human milk.37,38 The absence of 2′-FL in milk samples of some goats may be caused by very low levels of this oligosaccharide in samples from all time points. However, the oligosaccharide enrichment in the PGC-SPE filtration step was a strategy used to improve the detection of oligosaccharides even when present in very low levels. Austin et al., in a similar analysis type, reported a LOD level for 2′-FL of 3.9 mg/L.23 Future studies focusing on genetic analysis could enlighten the genetic variance related with the very low levels/absence of 2′-FL in milk from some goats. It can be concluded that goat milk contains interesting levels of milk oligosaccharides, particularly in the first few days of lactation. It should be noted, however, that the concentrations are significantly lower than in human milk, which remains the best standard for infant nutrition.

**Comparison of Oligosaccharide Levels between Farms.** The differences in the levels of gMOS between the two farms were examined. Relevant data known for the goats and their milk characteristics included in this study are summarized in Table 2. The comparisons of the identified gMOS per farm at the D2 and D31 time points are presented in Figure 5. Going from colostrum (D2) to mature milk (D31), a significant reduction in the levels of gMOS is clear for all of the structures in milk samples from both farms, except 3′-NGL (Figures 3 and 5). The pairwise comparisons of the two farms in colostrum (D2) and mature milk (D31) highlighted that the 6′-SLN, DSL, and SHL structure concentrations were significantly different (corresponding p-values were p < 0.005 at both time points) between farms. It is noteworthy that DSL and SHL are two of the least abundant structures detected and identified in the present study. The concentrations of two other structures, 6′-SL and NGLN, were significantly different between the two farms only at D31 with p-values < 0.05 and <0.01, respectively.

The two farms were following different feeding regimes, which may play a role in the observed differences. Other factors, however, also may lead to the different levels of these structures, such as environmental or genetic ones. In human milk studies, the effect of the diet has been investigated with some studies reporting an interrelationship between hMOS levels and the diet of the mothers.39 It should be emphasized that the sample collection from both goat farms was performed during the same period of the year with only a few days difference from each other. Therefore, any differences derived from seasonal variations can most likely be excluded. The differences observed between the farms suggest that a feeding intervention study in goats may yield interesting insights into influences that diet can have on gMOS composition in goat milk. However, further differences that may exist between different farms also should be taken into consideration, such as age of goats, parity, and milk yield. To draw more solid conclusions, controlled intervention studies with standardized conditions should be considered. The current study provides several leads for interesting factors to study in such a controlled experiment.

**Correlations between gMOS and Available Goat Metadatas.** With the available metadata (Table 2), and the quantitative data collected from the gMOS milk fingerprinting, different correlations were assessed. Three parameters were found correlating with gMOS concentrations, namely, goat age, parity, and lifetime milk production. Table 3 provides information on the gMOS that were correlated with the above-mentioned factors. All three factors mainly correlated with the concentrations of two structures (3′-NGL and 6′-NGL), both in the colostrum samples (D2 time point of lactation) and in the mature milk (D31). These three factors are correlated with the structure 6′-GL only at D31 and with NGLN only at D2. The mechanisms involved remain to be studied. The average daily milk production, the average % fat and % protein content, and the appearance of pseudocyesis did not show any correlation with the gMOS fingerprints determined in the present study.

It should be emphasized that goat age, parity, and lifetime milk production are highly related to each other (p value < 0.0001 for age vs parity and age vs lifetime production comparisons), and it is not possible to conclude which has the most significant effect. Goat age is very strongly correlated with lifetime milk production and with the number of previous pregnancies of the goats (Figure S3). In the case of protein-bound glycosylation in human serum, it has been shown that age has an influence on the structural diversity of glycans.39 Therefore, it seems logical that age may be the dominant factor in the correlation with gMOS levels, but, to our knowledge, this is the first time that this is reported for goats. Age, also, has been associated with the levels of other hMOS structures (fucosyllacto-N-hexaese...
(FLNH) and of difucosyllacto-N-hexose (DFLNH).25 McGuire et al. studied also parity in humans,5 but no significant differences were reported in hMOS levels. Recently, another group reported that parity was associated independently with some hMOSs.41 The same was concluded by Claps et al. who underlined an association of goat parity with the structures 3′-SL, 6′-SL, and DSL.23 In our study, none of these structures correlated with parity.

For the lamb sex, pairwise comparison was performed among three groups (mothers of one female, mothers of one male, and mothers of both male and female lambs). Among the mothers that had lambs of both sexes, two goats had triplets of two daughters and one son and six goats had twins of one daughter and one son. The varied outcomes, female-only, male-only, or mixed-gender lambs, are distributed equally across the age ranges of the mother goats. Correlations were corrected for the age/parity effects mentioned earlier. For these comparisons, significant differences were highlighted for 3′-SL, NADHL, and DSL at the D2 time point and for 3′-SL, NGLN, and SHL at the D31 time point (Figure 6). Goat mothers of female lambs had significantly higher 3′-SL levels than mothers of both gender lambs at D2 (mean values, 96.2 and 32.6 mg/L, respectively; Mann–Whitney test, \( p = 0.016 \)) and at D31 (66.3 and 25.1 mg/L, respectively; Welch’s test, \( p = 0.002 \)). The same structure was found in significantly higher levels in the comparison of mothers with male and both gender lambs at D31 (52.9 and 25.1 mg/L, respectively; Welch’s test, \( p = 0.025 \)).

At D2, mothers of female lambs had higher NADHL levels compared to those of male lambs (10.2 and 8.3 mg/L, respectively; Welch’s test, \( p = 0.023 \)) and compared to both gender lambs (10.2 and 4.5 mg/L, respectively; Welch’s test, \( p = 0.003 \)). The milk of mothers of male lambs had significantly lower levels of DSL compared to both gender lambs at D2 (3.7 and 7.0 mg/L, respectively; Welch’s test, \( p = 0.017 \)). At D31, the milk of mothers giving birth to females contained lower levels of NGLN compared to mothers with male lambs (2.2 and 3.9 mg/L, respectively; Welch’s test, \( p = 0.012 \)) and to both genders’ pregnancy outcome (2.2 and 4.6 mg/L, respectively; Welch’s test, \( p = 0.024 \)). Comparing the pregnancy outcomes of female and both genders, SHL was also found significant at D31 (3.2 and 1.9 mg/L, respectively; Welch’s test, \( p = 0.013 \)).

A recent hMOS study also correlated the sex of the neonates with the concentration of specific structures, with a focus on 2′-FL, LNT, LNnT, and other neutral core hMOS.42 Our study showed that this correlation also may be found in the milk of goats, but in this case, mainly acidic gMOS are involved. It should be noted that, in contrast to human milk, in milk of goats and other domesticated animals, the acidic structures are predominant (Table 1).

## ASSOCIATED CONTENT

*Supporting Information*

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c00499.

HPLC-FLD chromatograms of gMOS (Figure S1); PCA analysis of gMOS data (Figure S2); and correlation graphs depicting the factors of age with parity and lifetime milk production (Figure S3) (PDF)

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