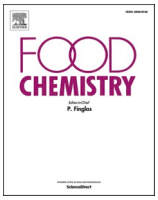
Food Chemistry 430 (2024) 136977

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



# Label-Free quantitation of milk oligosaccharides from different mammal species and heat treatment influence

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ARTICLE INFO

*Keywords:*
Milk oligosaccharides (MOs)
LC-ESI-MS/MS
Human milk
Domestic animal milk
Heat treatment

ABSTRACT

Milk oligosaccharides (MOs) exhibit significant variations in concentrations and patterns among different species. However, there is limited knowledge about milk oligosaccharides in domestic animals and the impact of heat treatment on them. Here, we developed an LC-ESI-MS/MS method to analyze 11 milk oligosaccharides in 7 distinct species simultaneously. The results showed that human milk presented a completely different composition pattern of milk oligosaccharides from animals. In detail, animal milk predominantly contained sialylated oligosaccharides, and human milk had high levels of fucosylated neutral oligosaccharides. Notably, sheep milk exhibited similarities to human milk in terms of oligosaccharides composition. Then, the milk samples from dairy cows were treated with two common industrial heat treatments. We found that 65 °C treatment had no significant effect on the concentration of milk oligosaccharides, whereas 135 °C heating was associated with their decline, suggesting that high temperatures should be avoided in the processing of oligosaccharides supplemented/enriched products.

## 1. Introduction

Breast milk is vital for the growth of newborns, serving as their primary source of nutrition (Azad et al., 2018). Human milk oligosaccharides (HMOs), the third most abundant solid component of breast milk, are complex carbohydrates (Bode, 2012). HMOs consist of various monosaccharide building blocks, including glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), and sialic acid (NeuAc), which determine their structure and function (Chaturvedi et al., 2001; Zivkovic et al., 2011). Based on the specific linkage and branching patterns of monosaccharides, HMOs are classified into fucosylated, sialylated, and non-fucosylated neutral HMOs (Ninonuevo et al., 2006).

These MOs are structurally diverse, and their concentration and composition can vary between species (Shi et al., 2021; Wang et al., 2020). Currently, previous research on milk MOs has mostly focused on a single breed of one species and its lactation regularity. Multiple studies have explored the concentration and composition of MOs in human milk, with fucosylated MOs being found to be the most abundant (Ma et al., 2018; Plows et al., 2021). As for domestic animals, sialylated MOs are identified as predominant in bovine and goat milk (Tao et al., 2009; van Leeuwen et al., 2020). Although there are some studies on MOs in other animal species, such as camels, yaks, and donkeys, they are mostly focused on elucidating MOs structures (Yan et al., 2018; Albrecht et al., 2014). Limited research has been conducted on the systematic comparison of MOs among different species, particularly to absolute quantitation. Only a few examples of absolute quantitation are available, such as the studies by Shi et al. (2021) and Wang et al. (2020). Shi et al. (2021) quantified 7 MOs in milk from Chinese humans, cows, goats, sheep, and camels by UPLC-MRM-MS, while Wang et al. (2020) compared the composition and concentration of 12 MOs between Chinese human and domestic animals (cows, goats, yaks, and donkeys) using HPAEC-PAD. However, the different analytical methods employed in these studies hinder the comparative analysis of MOs profiles. Moreover, the composition and concentration of MOs in horses and buffaloes remain largely unknown. Comparing the MOs profiles of

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https://doi.org/10.1016/j.foodchem.2023.136977
Received 8 May 2023; Received in revised form 7 July 2023; Accepted 15 July 2023
Available online 22 July 2023

**Table 1**
Optimal MRM conditions of each oligosaccharide.

| Compounds | Capillary (kv) | Cone (v) | Collision (v) | Parent (*m/z*) | Daughter iron 1 (*m/z*) | Daughter iron 2 (*m/z*) | Ion type |
|---|---|---|---|---|---|---|---|
| 2′-FL | 3.0 | 30 | 15/15 | 511.2 | 365.2 | 346.8 | $[M + Na]^+$ |
| 3′-FL | 3.0 | 30 | 38/34 | 511.2 | 365.2 | 346.8 | $[M + Na]^+$ |
| 3′-SL | 3.0 | 58 | 34/34 | 656.3 | 365.1 | 314.2 | $[M + Na]^+$ |
| 6′-SL | 3.0 | 60 | 34/36 | 656.3 | 365.1 | 314.2 | $[M + Na]^+$ |
| 3-GSL | 3.0 | 60 | 45/35 | 527.0 | 202.4 | 346.8 | $[M + Na]^+$ |
| LNT | 3.0 | 70 | 20/20 | 730.1 | 550.0 | 388.0 | $[M + Na]^+$ |
| LNnT | 3.0 | 70 | 50/50 | 730.1 | 550.0 | 388.0 | $[M + Na]^+$ |
| LNFP I | 3.0 | 30 | 20/20 | 854.2 | 512.2 | 366.2 | $[M + Na]^+$ |
| LNFP II | 3.0 | 60 | 52/60 | 876.1 | 550.0 | 730.3 | $[M + Na]^+$ |
| LNDFH I | 3.0 | 100 | 58/68 | 1022.4 | 387.8 | 876.2 | $[M + Na]^+$ |
| LNDFH II | 3.0 | 100 | 58/78 | 1022.4 | 549.9 | 876.2 | $[M + Na]^+$ |

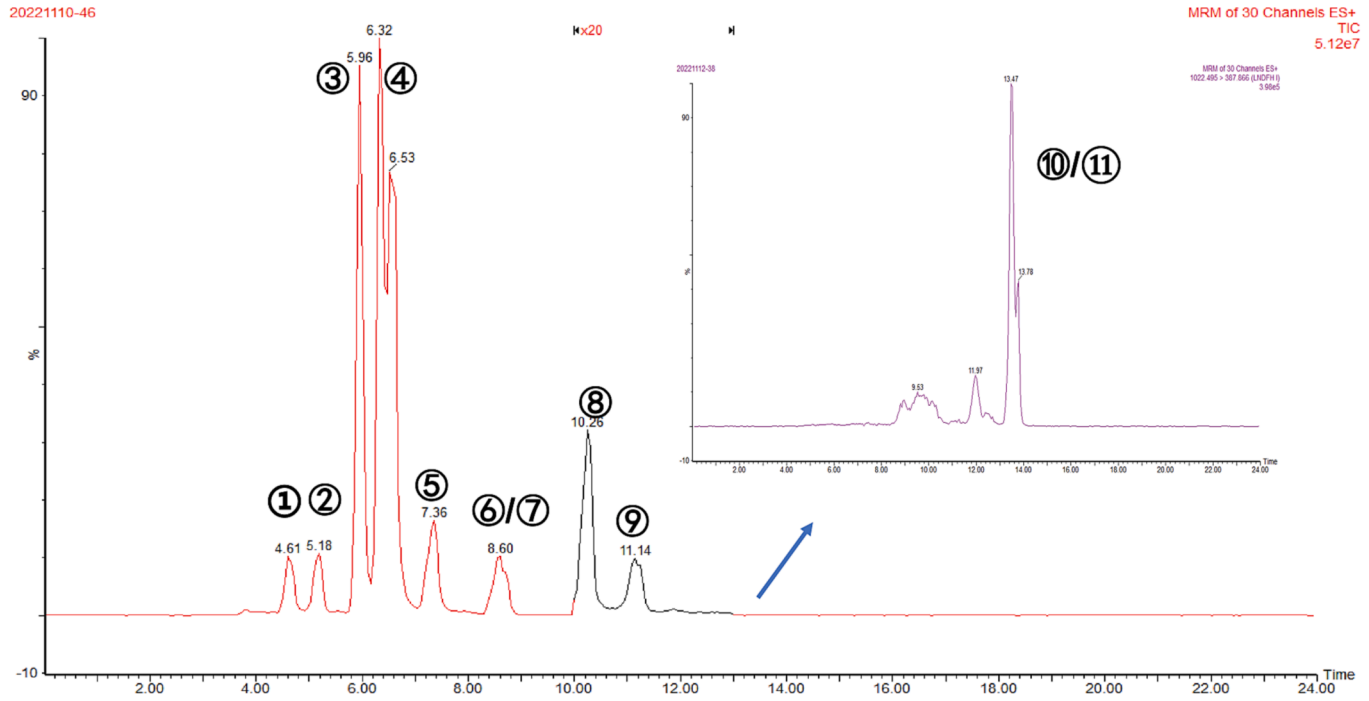


**Fig. 1.** Chromatogram of 11 MOs standards in MRM model. ①3′-SL; ②6′-SL; ③2′-FL; 3′-FL; ⑤3-GSL; ⑥/⑦LNT/LNnT; ⑧LNFP I; ⑨LNFP II; ⑩/⑪ LNDFH I/II.

different domestic animals can provide valuable insights into their nutritional value, including their potential as sources for infant formula.

Among domestic animals, milk from dairy cows is the most extensively consumed. Although some milk is still consumed as "untreated" or raw, the two primary heat treatments employed for milk are pasteurization (at 65 °C) and sterilization (UHT, at 135 °C) to reduce the microbial population (Lewis & Deeth, 2009). However, intense thermal processes can lead to severe degradation of water-soluble vitamins, certain fatty acids, and hormones present in milk (Kilic-Akyilmaz et al., 2022). But it is unclear whether MOs are sensitive to heat treatment.

Therefore, the study was carried out to obtain a comprehensive overview of MOs profiles in the milk of humans, cows, sheep, horses, yaks, buffaloes, and camels using the LC-ESI-MS/MS method combined with MRM scanning. Additionally, the effects of heat treatment on MOs were explored to gain a better understanding of their characteristics.

## 2. Materials and methods

### 2.1. Chemicals

Acetonitrile (LC-MS grade, purity ≥ 99.9%) was purchased from Honeywell Research Company (Charlotte, North Carolina, US). Ammonium acetate (CAS No. 631–61-8, purity ≥ 98.0%) was obtained from Merck Ltd (Beijing, China). Ultrapure water was purified from the Milli-Q124 purification system (Millipore, Bedford, MA, USA).

2′-fucosyllactose (2′-FL, Cat# 41263–94-9), 3′-fucosyllactose (3′-FL, Cat#41312–47-4), 3′-sialyllactose sodium salt (3′-SL, Cat#GY1143), 6′-sialyllactose (6′-SL, Cat#GY1144), 3′-galactosyllactose (3′-GSL, Cat#GY1093), lacto-*N*-tetraose (LNT, Cat#GY1145), lacto-*N*-neotetraose (LNnT, Cat#GY1146) and lacto-*N*-fucopentaose I (LNFP I, Cat#GY1147) were obtained from HuicH Biotech Co., Ltd. (Shanghai, China). The lacto-*N*-fucopentaose II (LNFP II, Cat#ZG-10049), lacto-*N*-difuco-hexaose I (LNDFH I, ZG-10060), and lacto-*N*-difuco-hexaose II (LNDFH II, Cat#ZG-10189) were purchased from ZZBIO Co., Ltd (Shanghai, China). Their structures were shown in Supplementary Figure S1.

### 2.2. Samples

Human milk (n = 50) was collected from mothers who gave birth to healthy full-term infants and had no metabolic disease. The volunteer's information is shown in Table S1 with a mean lactation value of day 90, the earliest at day 7, and the longest at day 270.

Milk samples (n = 20, day 100–120 post-partum) from Holstein cows were obtained at Zhongdi Dairy Farm (Beijing, China). Camel milk (n = 20, day 180 post-partum), horse milk (n = 10, day 120–150 post-

**Table 2**
The regression equations of 11 MOs.

| Compounds | Precursor ion | Calibration curves | $R^2$ | Range (mg/mL) | LOD (μg/mL) | LOQ (μg/mL) |
|---|---|---|---|---|---|---|
| 2′-FL | 511.2 | y = 637356x-472869 | 0.993 | 0.4–40 | 1.04 | 3.49 |
| 3′-FL | 511.2 | y = 1002576x-1342568 | 0.995 | 0.4–40 | 1.12 | 3.72 |
| 3′-SL | 656.3 | y = 48567x + 1575 | 0.998 | 0.1–10 | 100 | 333.3 |
| 6′-SL | 656.3 | y = 231918x + 46102 | 0.999 | 0.1–10 | 0.94 | 3.13 |
| 3′-GSL | 527.0 | y = 178044x-212974 | 0.998 | 0.4–40 | 12.01 | 40.0 |
| LNT + LNnT | 730.1 | y = 216190x-9862834 | 0.990 | 0.4–40 | 7.90 | 25.40 |
| LNFP I | 854.2 | y = 13263x-1625 | 0.991 | 0.4–10 | 21.8 | 72.72 |
| LNFP II | 876.1 | y = 35317x-1486 | 0.990 | 0.4–10 | 40.0 | 133.3 |
| LNDFH I | 1022.4 | y = 16057x-3714 | 0.996 | 0.4–10 | 125 | 416 |
| LNDFH II | 1022.4 | y = 12792x-4184 | 0.994 | 0.4–10 | 100 | 250 |

**Table 3**
The regression equations of 11 MOs (n = 3).

| Compounds | Spiked levels | | | | | |
|---|---|---|---|---|---|---|
| | 5 mg/L | %CV | 20 mg/L | %CV | 40 mg/L | %CV |
| 2′-FL | 91.3 | 3.90 | 92.4 | 6.42 | 99.2 | 3.94 |
| 3′-FL | 83.8 | 5.74 | 82.5 | 9.05 | 83.5 | 2.47 |
| Compounds | 2.5 mg/L | %CV | 10 mg/L | %CV | 20 mg/L | %CV |
| 3-GSL | 83.8 | 15.42 | 87.4 | 11.81 | 82.2 | 4.18 |
| LNT + LNnT | 91.6 | 12.13 | 85.3 | 1.68 | 96.1 | 2.79 |
| Compounds | 1 mg/L | %CV | 5 mg/L | %CV | 10 mg/L | %CV |
| 3′-SL | 83.1 | 7.59 | 99.4 | 5.64 | 102.3 | 14.40 |
| 6′-SL | 88.8 | 13.42 | 85.7 | 9.68 | 75.7 | 3.92 |
| LNFP I | 77.3 | 3.60 | 80.4 | 3.68 | 94.2 | 6.59 |
| LNFP II | 75.6 | 3.77 | 82.9 | 11.84 | 96.7 | 6.70 |
| LNDFH I | 98.2 | 4.34 | 91.9 | 7.59 | 107.3 | 4.13 |
| LNDFH II | 102.2 | 2.49 | 107.4 | 3.84 | 97.1 | 10.68 |

**Table 4**
The concentration of MOs in different species.

| MOs | Human | Dairy cow | Camel | Yak | Buffalo | Sheep | Horse |
|---|---|---|---|---|---|---|---|
| Fucosylated neutral MOs (g/L) | | | | | | | |
| 2′-FL | 2.705 | 0.010 | 0.012 | 0.009 | 0.011 | 0.244 | 0.010 |
| 3′-FL | 1.546 | 0.023 | 0.043 | 0.016 | 0.042 | 0.024 | 0.021 |
| LNFP I | 0.183 | ND | 0.003 | ND | 0.004 | 0.003 | ND |
| LNFP II | 0.416 | ND | 0.002 | ND | 0.003 | 0.001 | 0.001 |
| LNDFH I | 0.089 | ND | ND | ND | ND | ND | ND |
| LNDFH II | 0.098 | ND | ND | ND | ND | ND | ND |
| Non-fucosed neutral MOs (g/L) | | | | | | | |
| LNT + LNnT | 0.628 | 0.049 | 0.050 | 0.050 | 0.054 | 0.050 | 0.049 |
| 3-GSL | 0.189 | 0.032 | 0.055 | 0.021 | 0.093 | 0.075 | 0.165 |
| Sialylated MOs (g/L) | | | | | | | |
| 3′-SL | 0.163 | 0.347 | 0.194 | 0.431 | 0.346 | 0.245 | 0.511 |
| 6′-SL | 0.268 | 0.075 | 0.040 | 0.061 | 0.146 | 0.190 | 0.062 |
| SUM | 6.285 | 0.535 | 0.399 | 0.589 | 0.699 | 0.832 | 0.818 |

ND, the concentration was lower than LOQ.

partum), sheep milk (n = 20, days 150 post-partum), yak milk (n = 10, days 90 post-partum) and buffalo milk (n = 30, days 120–150 post-partum) were obtained from Hebei, Xinjiang, Shanxi, Gansu, and Guangxi provinces, separately. All milk samples were put into 50 mL sterile polypropylene tubes and stored at − 80 °C. This study was approved by the Committee on the Ethics of Animal Experiments of the Chinese Academy of Agricultural Sciences (Beijing, China; permission number: IAS2022-124).

### 2.3. Oligosaccharide extraction and analysis

MOs were isolated from milk samples according to the published approach with the modifications described below (Galeotti et al., 2012, Liu et al., 2014). Briefly, 1 mL milk samples were mixed with ultra-pure water (3 mL for animal milk and 9 mL for human milk) and then centrifuged at 12,000 × g for 20 min at 4 ℃. The 1 mL middle layer (containing oligosaccharides) was collected into a new 15 mL sterile polypropylene tube and diluted 3 times by adding 2 mL pure water. After homogenizing, the PestiCarb graphitized carbon column (GCB) (500 mg/3mL, S-GCB5003, Kangpu Xing Technology Co., Ltd, Beijing, China) was used to purify the rude oligosaccharides extraction. The GCB was activated by 10 mL acetonitrile and then balanced with 10 mL ultrapure water. After that, oligosaccharide extraction was added which would flow through the column under the action of gravity, and then the column was washed with 10 mL ultrapure water to remove excess salt and small molecules. Finally, 1 mL of 20%, 40%, and 80% acetonitrile were used to elute the oligosaccharide. The filtrates were filtered through 0.22 μm membranes before MOs components analysis.

### 2.4. LC-ESI-MS/MS analysis

The oligosaccharides in mammals were identified and quantified using a Waters UPLC XevoTQS (G-147) equipped with an ACQUITY UPLC BEH amide column (1.7 μm, 2.1 × 100 mm, Waters Corporation, Milford, MA, USA). A 24 min LC separation was performed using a binary gradient at 0.15 mL/min flow rate: solvent A of 10 mmol/L ammonium acetate; solvent B of acetonitrile (100%) and the injection volume was 1 μL. The optimized elution gradient was 70–56% solvent B for 0–21 min, 56–70% solvent B for 21–21.2 min, 70% solvent B for 21.2–24 min.

The multi-reaction monitoring (MRM) mode of the mass spectrometry (MS) was operated in positive mode, which can reduce background matrix interference and provide the best quantification sensitivity and accuracy. The following parameters were optimized for oligosaccharide analysis: source temperature 150 °C, desolvation temperature 650 °C, cone gas flow 152 L/Hr, desolvation gas flow 640 L/Hr, collision gas flow 0.13 mL/min. Individual standard solutions of the 11 MOs (2′-FL, 3′-FL, 3′-SL, 6′-SL, 3-GSL, LNT, LNnT, LNH, LNnH, LNFP I, LNFP II, LNDFH I, LNDFH II) at 10 or 50 μg/mL in 30% acetonitrile and were directly injected into the MS at 20 μL/min. For each MO, the capillary and cone voltage, cone gas flow, and desolvation temperatures were optimized and recorded.

### 2.5. Method validation

#### 2.5.1. Linear regression curves

For LC-MS detection, mixed standard solutions of 11 MOs with varied concentration gradients were prepared, and linear regression curves were established. The concentrations in this study were from 0.4 to 40 mg/mL for 2′-FL, 3′-FL, 3-GSL, LNT, and LNnT, from 0.4 to 10 mg/mL for 3′-SL, 6′-SL, LNFP I, LNFP II, LNDFH I and LNDFH II. The closeness of fit for the calibration curve was assessed using the coefficient of determination ($R^2$). The limit of detection (LOD) and the limit of quantitation (LOQ) of sensitivity were defined as the concentration with signal-to-noise ratio at 3 and 10, respectively. The signal-to-noise ratio (S/N) was calculated by MassLynx (Waters). The intra-day precision of

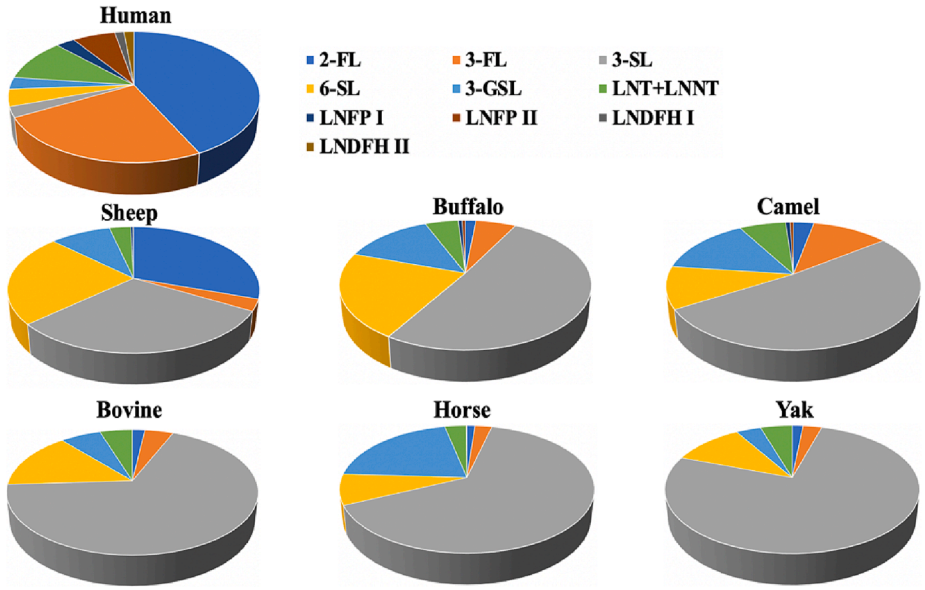


**Fig. 2.** The component pattern of 11 MOs in different species. The sample size was n = 20 for dairy cows and camels, n = 10 for horses and yaks, n = 20 for sheep, and n = 30 d for buffaloes.

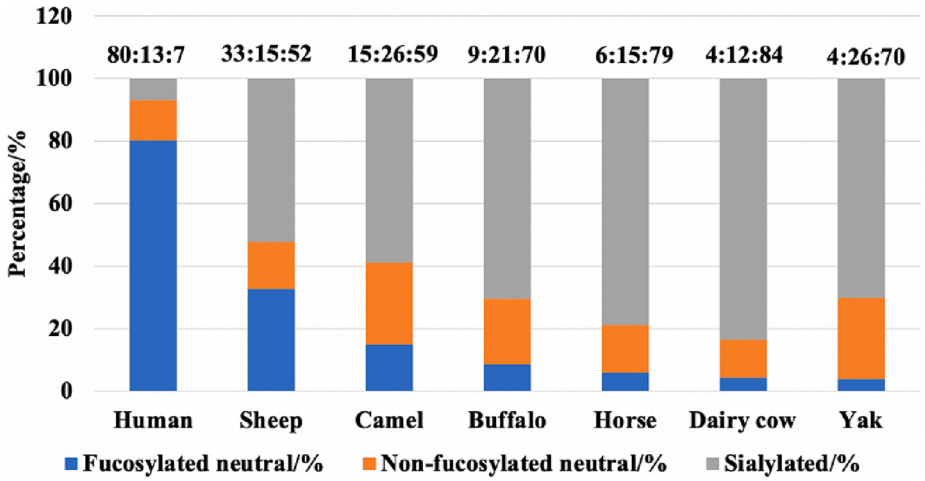


**Fig. 3.** Different type of MOs in 7 mammalian species. The sample size was n = 20 for dairy cows and camels, n = 10 for horses and yaks, n = 20 for sheep, and n = 30 d for buffaloes.

the method was determined by testing 4 parallel samples in one day, while the inter-day precision was analyzed by testing these samples once a day for 3 consecutive days.

#### 2.5.2. Recovery test

The recovery experiment involved spiking 11 MOs standards into a 25 × diluted sample at three concentration levels. Three concentration levels were 5, 20, and 40 mg/mL for 2′-FL and 3′-FL, 2.5, 10, and 20 mg/mL 3-GSL, LNT and LNnT, 1, 5, and 10 mg/mL for 3′-SL, 6′-SL, LNFP I, LNFP II, LNDFH I and LNDFH II. These particular concentrations were selected with the aims: a) to make the concentrations of middle levels closer to those of the real samples; b) to ensure those concentrations were within the linear ranges of corresponding standards.

### 2.6. Quantification of 11 MOs in milk samples

The 11 MOs in human milk, as well as 6 domestic animals, were identified and quantified according to the retention time and peak area. The corresponding concentration was calculated by standard curves. In human milk, the MOs concentrations were far above the detection range of the standard curves. Consequently, the samples were diluted 144 times and 72 times before the result was determined. Meanwhile, the other animal milk samples were diluted 8 times. Peak areas were obtained using Masslynx (Waters Corporation). The quantification results were analyzed using GraphPad Prism 9 (GraphPad Software) and expressed in mean.

### 2.7. Quantification of 7 oligosaccharides in heated dairy cow milk samples

Three parallel samples of Holstein cow's milk (n = 30) were collected simultaneously from Zhongdi Dairy Farm (Beijing, China). One of the three samples was without any heat treatment (raw milk), while another one was heated at 65 °C for 30 mins (DK-S22 electric thermostatic water bath, Jinghong Co. Ltd., Shanghai, China), then rapidly cooling the samples by immersing into cold water for 10 min. The other one was heated at 135 °C for 90 s (LLZ-225B Immersion Oil Bath, Semis Instrument Equipment Co. Ltd., Beijing, China), then rapidly cooled for 30

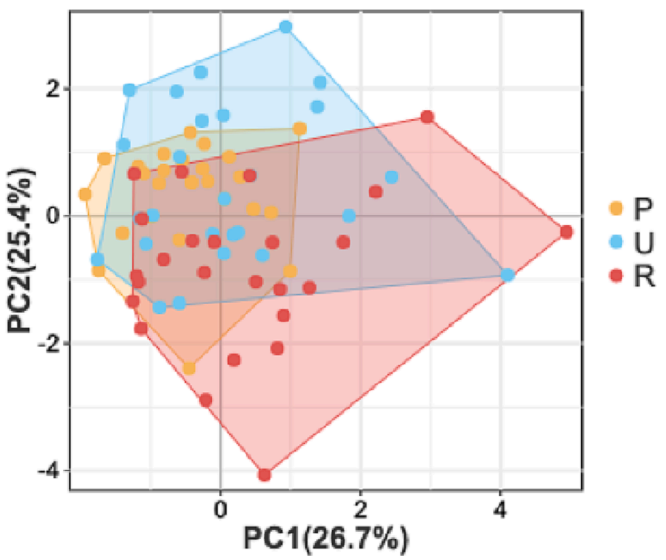


**Fig. 4.** Principal component analysis (PCA) diagrams of MOs composition in dairy cow milk at two different heat treatments (n = 30). R, Raw milk; P, Milk samples were treated at 65 ℃ for 30 min; U, Milk samples were treated at 135 ℃ for 60 s.

min. MOs were extracted and analyzed by LC-ESI-MS/MS method. The quantification results were analyzed by Ordinary One-Way ANOVA using GraphPad Prism 9 (GraphPad Software) and expressed in mean ± standard deviation (SD).

## 3. Results and discussion

To get stronger signals in the detector or increase ionization efficiency in MS, MOs are derivatized in some studies (Oursel et al., 2017; Plows et al., 2017). However, derivatization alters the natural structure of MOs resulting in the loss of their full biological activity. LC-MS/MS with multiple reaction monitoring (MRM) offers the possibility of a robust label-free method for quantifying MOs (Hong et al., 2014). In our study, the ionization and fragmentation conditions were developed for each MO compound, and the unique daughter ions were determined. After that, chromatographic separation of MOs was performed under optimal conditions. Each MO compound was identified based on its elution time and characteristic fragments.

### 3.1. Method validation

#### 3.1.1. Optimization of MS conditions

To increase the specificity and sensitivity, the MS conditions in positive ion mode were optimized. The high MRM sensitivity of precursor ions for each MO compound was obtained by optimizing the capillary and cone voltage. Due to the difficulty for oligosaccharides to fragment, collision energy was tuned to produce a stronger signal of daughter ions for each MO (Table 1). Table 1 displays the entire ion scans for the 11 standard oligosaccharides.

#### 3.1.2. Unique fragmentation-assisted MS for isomer analysis

The liquid chromatography equipped with an ACQUITY UPLC BEH amide column was used to separate the MOs. There are five pairs of isomers in our study, including 2′-FL and 3′-FL, 3′-SL and 6′-SL, LNT and LNnT, LNFP I and LNFP II, LNDFH I and LNDFH II. Due to their similar polarity, isomers are challenging to achieve chromatographic separation. After optimizing the separation condition, significant separation was achieved for three pairs of isomers: 2′-FL and 3′-FL, 3′-SL and 6′-SL, as well as LNFP I and LNFP II (Fig. 1). LNT and LNnT did not exhibit separation and displayed the same retention time (8.6 min), and neither did LNDFH I and LNDFH II (13.47 min). In LC-MS/MS, retention time is not the only way to identify the compounds. Some MOs isomers produce characteristic fragments that facilitate their identification by MS (Yan et al., 2018, Li et al., 2022). For example, although no chromatographic resolution could be obtained for LNDFH I and LNDFH, they could be successfully quantified using unique ion pair 1022 → 387 for LNDFH-I and 1022 → 550 for LNDFH-II. LNT and LNnT share the same precursor, iron products, and retention time due to their highly similar structure and polarity, making their differentiation challenging. Consequently, we quantified together LNT and LNnT (as LNT + LNnT) using LNT as a calibration standard, the same as Galeotti et al. 2012.

#### 3.1.3. Regression equations identification

A wide dilution range was established to cover all 11 MOs within the monitoring range as their concentrations in milk varied considerably. As shown in Table 2, the $R^2$ for each regression equation was more than 0.99, indicating the dependability of the equation to be suitably used for the calculation. Due to their great abundance, 2′-FL and 3′-FL required a wider linear range than the other MOs. The LODs and LOQs of 11 MOs in milk ranged from 0.94 μg/mL to 125 μg/mL and 3.13 μg/mL to 416 μg/mL, respectively. The more complicated structures of LNDFH I and LNDFH II make it challenging for them to ionize, resulting in higher LOQ of 416 μg/mL and 250 μg/mL, respectively. The intra-day CV varied from 2.99% to 8.86%, and the inter-day CV ranged from 3.6% to 8.8% based on the MOs in the milk samples (Table S2).

#### 3.1.4. Recovery tests

The recovery experiments were carried out to assess the accuracy of the method. The levels of the MOs detected in the non-spiked sample were subtracted from the amounts measured in the corresponding spiked sample before comparison with the amounts known for the added standards. As shown in Table 3, the recovery rate ranged from 75.6% to 107.4% for 11 MOs, with a few exceptions where the recoveries were below 80%. For example, in LNFP I and LNFP II, the recovery rate at low concentrations was 77.3% and 75.6%, respectively. This could be attributed to systemic losses, such as samples adhering to the tube walls during pre-treatment, which had a greater impact on low-level results compared to middle- and high-level results. For 3-GSL and 6′-SL, the CV at low concentrations was 15.42% and 13.42%, indicating that the operational errors (the noise) during pre-treatment processes had a greater influence on the S/N. Consequently, in sample testing, the concentrations of 3-GSL and 6′-SL should not be lower than 2.5 mg/L and 1 mg/L, respectively. For 3′-SL, the CV at high concentration was relatively high (14.40%) due to the possible matrix effect, indicating the high concentration of 3′-SL should be concerned in the sample test.

### 3.2. Mos in various mammalian milk

Using the above method, 11 MOs were detected in the milk of humans, dairy cows, sheep, yaks, buffaloes, horses, and camels. According to Table 4 and Fig. 2, human milk presented a distinct oligosaccharide composition pattern compared to milk from other animals, with a higher concentration and a greater number of detected MOs species (11 MOs). In general, the concentration of 11 MOs in human milk (6.285 g/L in total) is significantly higher than in other mammalian milk, with approximately 11.7,15.8, 10.7, 9.0, 7.6, 7.7 times more MOs than in dairy cow, camel, yak, buffalo, sheep, and horse milk, respectively. Studies have reported that the total MO concentration in mature human milk was approximately 5–15 g/L (Thurl et al., 2017). Among the MOs, 2′-FL was the most dominant component in human milk, consistent with previous findings (Li et al., 2022; Samuel et al., 2019).

However, not all samples of animal milk contained the 11 MOs. Among them, dairy cow and yak milk presented 7 MOs (2′-FL, 3′-FL, LNT + LNnT, 3-GSL, 3′-SL, and 6′-SL), horse milk contained 8 MOs (2′-FL, 3′-FL, LNT + LNnT, 3-GSL, LNFP II, 3′-SL, and 6′-SL), and camel, buffalo, and sheep contained 9 MOs (2′-FL, 3′-FL, LNT + LNnT, 3-GSL, LNFP I,

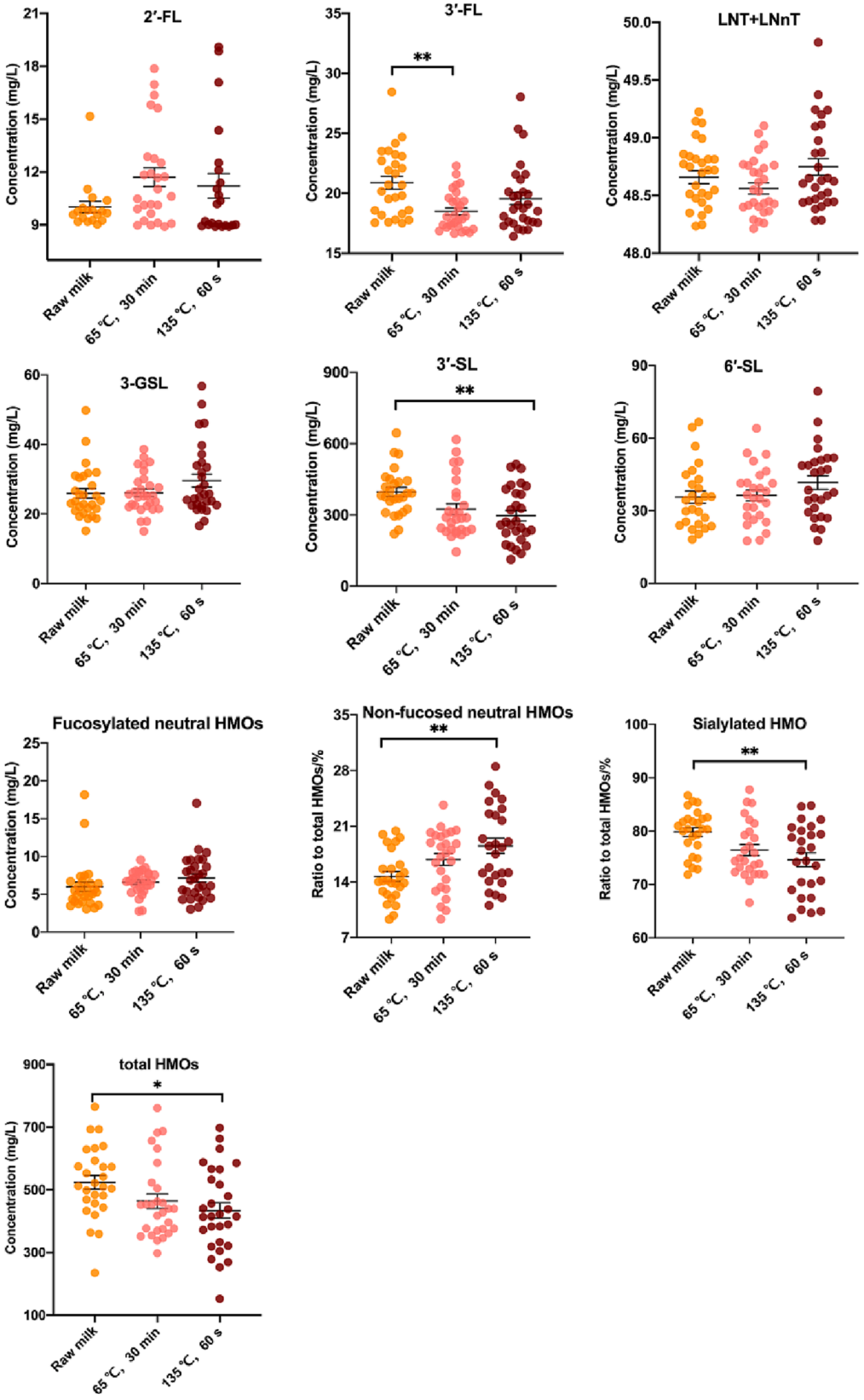


**Fig. 5.** Heat-treated effects on components of MOs in dairy cow milk (n = 30). Significance determined using One-Way ANOVA analysis. *, $P < 0.05$. **, $P < 0.01$.

LNFP II, 3′-SL, and 6′-SL). In contrast to human milk, 2′-FL concentration in animal milk was low, and 3′-SL was the most predominant MO, which has been confirmed by multiple studies (Liu, et al., 2014; Mariño et al., 2011). In sheep milk, the proportion of 3′-SL was approximately 30%, while in milk samples from the other 5 species, it exceeded 50% (Fig. 2). Additionally, 2′-FL in sheep milk accounted for about 30% of the total MOs, which was 20 times higher than in other animal milk. Compared to other animals, the MO composition in sheep is more similar to that of

human milk.

### 3.3. Different types of MOs in various mammalian milk

As shown in Table 4 and Fig. 3, there were 2 fucosylated neutral MOs detected in all milk samples: 2′-FL (2.705, 0.01, 0.012, 0.009, 0.011, 0.244 and 0.01 g/L in human, dairy cow, camel, yak, sheep, and horse milk, respectively) and 3′-FL (1.546, 0.023, 0.043, 0.016, 0.042, 0.024 and 0.021 g/L in human, dairy cow, camel, yak, sheep, and horses milk, respectively). Although all milk samples contained 3 non-fucosylated neutral MOs (3-GSL, LNT + LNnT) and 2 sialylated MOs (3′-SL and 6′-SL), their contents were much lower compared to human milk.

Furthermore, the ratio of different types of MOs in human milk and animal milk showed a significant difference. In detail, the fucosylated neutral MOs dominated in human milk, and the ratio of fucosylated neutral MOs: non-fucosylated neutral MOs: sialylated MOs was 80:13:7. Nevertheless, the sialylated MOs were the predominant type in animal milk, constituting more than 50% of the total MOs, which aligned with the previous studies (Shi et al., 2021; Albrecht et al., 2014).

### 3.4. Heat-treated effects on components of MOs

In fact, for various reasons, a considerable number of newborns are not available to breast milk. For them, instead of breast milk, the dairy milk-basis formula became the only source of nourishment. However, even though infant formulas are well-designed and recommended for early life, they do not provide an adequate quantity of desired components, such as cholesterol (Pietrzak-Fiećko and Kamelska-Sadowska, 2020), lipids (Sokol et al., 2015), and MOs (based on the above results). Therefore, supplemented MOs, including 2′-FL, 3′-FL, LNT, LNnT, 3′-SL and 6′-SL (approved by the United States Food and Drug Administration), are added to the formula. However, many procedures, including heat treatment, occur during the manufacturing process of infant formula, which may cause chemical changes in MOs. Currently, there is a lack of knowledge regarding the effects of heat treatment on the concentration and composition of MOs. Thus, in our study, we have chosen two common heat treatments in dairy milk production in order to carry out preliminary research on the influence of industrial heat treatment influence on the concentrations of MOs in cow's milk (2′-FL, 3′-FL, LNT + LNnT, 3-GSL, 3′-SL, and 6′-SL), to provide data for further processing of MOs supplemented/enriched products.

According to Fig. 4, the MOs composition in dairy milk after 65 °C heat treatment did not differ from that of raw milk samples, as indicated by the overlapping clusters in PCA analysis. In contrast, samples treated at 135 °C appeared to separate from raw milk. The concentration of each MO was further analyzed to determine which one was sensitive to high temperatures, and P values were calculated by analysis of variance (ANOVA).

It has been said that pasteurized donor breast milk (heated to approximately 65 ℃) provides a safe alternative and is regarded as the next best option when mother's milk is unavailable (Arslanoglu et al., 2010). According to our results, 3′-FL was significantly decreased after heating at 65 ℃ ($P < 0.05$, Fig. 5), while the concentration of other individual MO showed no significant changes ($P > 0.05$, Fig. 5). Although fucosylated neutral MOs are the dominant type in human milk, they were present in minimal amounts in dairy milk and 2′-FL was not detected in all dairy milk samples in this study. Therefore, a significant alteration in 3′-FL had little impact on the entire picture, as indicated by no significant difference in fucosylated neutral MOs (2′-FL, 3′-FL) between the control group and 65 ℃ heating group ($P > 0.05$, Fig. 5). Additionally, the other indices, including non-fucosylated neutral (3-GSL, LNT + LNnT) and sialylated MOs (3′-SL, 6′-SL), as well as total MOs, showed no remarkable changes in samples after heating at 65 ℃ ($P > 0.05$). Bertino et al. (2008) assessed how holder pasteurization (62.5 ℃ heating treatment for 30 min) affected the distribution of 24 MOs and found that the concentration or pattern of HMOs was not influenced by 62.5 ℃ treatment, which is consistent with the results of our study. Furthermore, other data supported our findings that pasteurization did not significantly influence the concentration of total HMO (Bertino et al., 2008; Hahn et al., 2019).

After heating at 135 ℃, the most dominant MO in dairy cow milk, 3′-SL, (constituting over 70% of the total MOs), showed a significant decrease ($P < 0.05$, Fig. 5), while other individual MO did not exhibit significant changes ($P > 0.05$, Fig. 5). And concentrations of non-fucosylated neutral MOs (3-GSL, LNT + LNnT) and sialylated MOs (3′-SL, 6′-SL), as well as total MOs, all declined ($P < 0.05$; $P < 0.01$). Meredith-Dennis et al. (2018) compared the composition of MOs between fresh human milk samples and retort sterilized ones (121 °C, 5 min) and observed a significant decrease in sialylated HMOs in the retort sterilized samples. One of the possible explanations for the low concentration of MOs in the retort sterilized samples could be attributed to the Maillard reaction, which involves a reaction between amino acids and reducing sugars at temperatures ≥ 120 °C (van Boekel, 1998).

## 4. Conclusion

We employed the LC-ESI-MS/ME method to simultaneously quantify 11 MOs in milk samples from 7 different species. Human milk exhibited the highest abundance of MOs, with 7.6 to 15.8 times more than other animal milk, and the fucosylated neutral MOs were predominant. The milk from dairy cows, camels, yaks, sheep, buffaloes, and horses showed similar MOs profiles, characterized by a richness of sialylated MOs. Compared to other animals, the MOs composition in sheep milk more closely resembled that of human milk. Furthermore, treatment at 65 °C had no significant effect on the concentration or distribution of MOs, whereas 135 °C heating was associated with their decline, suggesting more attention to temperature control is needed in milk product processing.

## CRediT authorship contribution statement

**Qianqian Yao:** Methodology, Investigation, Data curation, Writing – original draft. **Yanan Gao:** Methodology, Investigation. **Fengen Wang:** Data curation. **Veronique Delcenserie:** Writing – review & editing. **Jiaqi Wang:** Writing – review & editing. **Nan Zheng:** Funding acquisition, Supervision.

## 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.

## Acknowledgements

This study was supported by grants from the National Key R&D Program of China (2022YFD1600104), National Key R&D Program of China (2022YFD1600103), the Ministry of Modern Agro-Industry Technology Research System of China (CARS-36).

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136977.

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