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Article

COMPARATIVE ANALYSIS OF THE FATTY ACID COMPOSITION IN HUMAN, COW AND GOAT MILK AND THEIR INTERPOSITIONAL DISTRIBUTION IN TRIACYLGLYCEROLS

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Exploring the triacylglycerols composition in milk samples from different ruminant species is crucial for dairy product development. Identifying the fatty acid (FA) composition in human milk is essential and is usually performed using gas chromatography (GC). Heat maps are useful for visualizing these results, aiding in pattern identification and relationships between samples and variables. Additionally, nuclear magnetic resonance (NMR) spectroscopy provides important qualitative and quantitative information about fatty acids (FA). In the present study, we compare the fatty acid composition in samples of HM, cow milk, and goat milk, as well as to demonstrate their positional distribution in triacylglycerols (TAGs). Human milk contains 49.21% saturated fatty acids (SFAs), 34.32% monounsaturated fatty acids (MUFA), and 16.47% polyunsaturated fatty acids (PUFA), including 2.14% docosahexaenoic acid (DHA) and 13.02% linoleic acid (LA). On the other hand, cow and goat milk have higher proportions of SFAs (56.2 and 58.02%, respectively) and MUFAs (40.56 and 40.95%, respectively), but lower amounts of PUFAs (3.24 and 1.03%, respectively), with significantly lower quantities of DHA (0.03 and 0.43%, respectively) and LA (1.46 and 2.76%, respectively). Additionally, fatty acids in ruminant milk mainly consist of long-chain fatty acids (LCFAs), with oleic acid being the most abundant.

Keywords: lipids; milk; infant formula; gas chromatography.

INTRODUCTION

According to the World Health Organization (WHO), human milk (HM) is considered the gold standard for feeding newborns (NB). The WHO further recommends that babies be exclusively breastfed for the first six months of life to achieve optimal growth, development and health.[1]

Among the nutrients present in HM, fats contribute the majority (45-55%) of the energy required for proper growth and development of the newborn.[1,2] The various lipids provided by HM modulate gastrointestinal function, lipoprotein metabolism, membrane composition and functional and signaling pathways, thereby influencing infant development.[3,4]

When breastfeeding is not possible, the recommended alternative for newborns is commercial infant formula (IF). IFs are formulated with various fat sources, most of which contain a blend of commercial fat derived from skim milk of ruminants (cow and goat) supplemented with vegetable oils such as coconut, corn, soybean, palm, sunflower, peanut, and rapeseed oils. This blend aims to simulate the composition of fatty acids (FAs) found in human milk.[5-8]

About 98% of the total lipid content in human milk consists of TAGs, which are generally influenced by species, environment, and lactation period. TAGs are composed of three FAs attached to the sn-1, sn-2, and sn-3 positions on a glycerol molecule. The types and positions of FA attachments play important roles in infant nutrition and metabolism. Both human milk and bovine milk are rich in saturated fatty acids such as palmitic acid (C16:0). However, in human milk, palmitic acid is primarily found in the sn-2 position, which is important for absorption and metabolism, whereas in cow milk, it is found in smaller proportions at this position. There are no comparative studies in the literature investigating the positional distribution of FAs in the TAGs of human milk, cow milk, and goat milk.[9,10]

The hydrolysis of human milk fat with C16:0 in the sn-2 position in the TAGs results in monoacylglycerol that is digested and absorbed more efficiently by the baby.[11] In contrast, after hydrolysis, C16:0 present as free FAs tends to bind to calcium and become insoluble in the intestine, which can cause dry stools and constipation. In this context, research supporting the development of dairy products more similar to HM and ensuring better infant development are potentially important.[12]

The determination of the composition of HM FAs is commonly performed by gas chromatography (GC). GC coupled to the flame ionization detector (FID) is the most commonly used method for separation due to its lower cost and ease of maintenance. In GC, the carrier gas drags the analyte through the chromatographic column. Compounds are identified by analyzing the chromatogram, based on the elution order and retention time of analytes, and comparing them with standards.[13]

The use of heatmaps to study TGAs is also a common practice for data visualization, as it encodes values with colors that simplifying the identification of patterns and relationships between samples and variables. Regions with similar colors highlight samples with similar profiles, while different colors indicate divergences in profiles. This representation facilitates data interpretation and makes the detection of associations and trends more efficient.[14,15]

Among the analyses that support this investigation, nuclear magnetic resonance spectroscopy (NMR) stands out, as a widely used tool in metabolomics. It provides qualitative and quantitative information about the composition of a multicomponent systems. This technique offers data related to the positional distribution of FAs in TAGs. Some authors[16] have successfully used NMR in the characterization of triacylglycerols in food matrices of both plant and animal origin.

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Furthermore, using heatmaps to study FAs is also a common practice in data visualization. It encodes values with colors that simplify the identification of patterns and relationships between samples and variables. Regions with similar colors highlight samples with similar profiles, while different colors indicate divergences in profiles. This visual representation facilitates data interpretation and enhances the efficiency of detecting associations and trends.[17,18]

Therefore, the objective of this study is to compare the fatty acid composition in samples of HM, bovine milk, and goat milk, as well as to demonstrate their positional distribution in triacylglycerols.

EXPERIMENTAL

Samples

This study has been authorized by the local Research Ethics Committee under protocol number 3,430,478, from the State University of Maringá (UEM, Maringá, Brazil). Pasteurized mature human milk samples were obtained from the Human Milk Bank (BLH) located at the University Hospital of Maringá (HUM, Maringá, Paraná, latitude: 23°25'38" S, longitude: 51°56'15" W). A pool of 30 samples (100 mL each) of mature human milk (HM) was collected and kept refrigerated (4 °C). They were transported in a thermal container with frozen thermogel packs and a digital thermometer attached for temperature control. Inclusion criteria stipulated that HM samples were obtained from donors with an average gestational age of 39 weeks and aged between 25 and 35 years.

The HM was subjected to drying using a mini-spray-dryer (Buchi, model B-191 (Flawil, Switzerland). Drying was carried out by lyophilization, with an inlet temperature of 175 °C and an outlet temperature of 103 °C, with vaporized water flow using 100% compressed air as recommended by Cavazos-Garduño *et al*.[17] The powdered milk was vacuum-packed in light-free aluminum bags and frozen at –18 °C, for further analysis.

Two liters of bovine milk (UHT milk) from the same batch were purchased at a local market in Maringá City (Paraná, Brazil). The milk was subsequently lyophilized, vacuum-sealed, and stored in a freezer at –18 °C. Similarly, a sample of goat milk was commercially acquired at the same location but in powdered form (400 g). Both were kept away from light until the moment of analysis.

Solvents and reagents

The methyl tricosanoate standard with high purity (≥ 95%) used as an analytical standard (23:0, Sigma-Aldrich, Saint Louis, USA). The solvents used were methanol (Labsynth, Diadema, Brazil), *n*-heptane (Neon, Suzano, Brazil), chloroform (Fmaia, Belo Horizonte, Brazil), potassium hydroxide (J.T. Baker, Mexico), and ultrapure Milli-Q water generated by a Millipore Type I water system (Merck, Germany).

Lipid extraction

The analysis was conducted according to the method described by Folch *et al*.[19] Triplicate samples of (HM), each consisting of 10 mL, were utilized to extract 125 mg of lipids for methylation. The fatty acid methyl esters (FAMEs) were then identified using GC-FID.

Transesterification reaction

The lipid transesterification reaction was conducted following the methodology described in ISO 12966-2:2017.[20] Initially, 100 mg of oil was measured in a test tube and 2.0 mL of *n*-heptane was added. The tube was shaken for 2 min. Subsequently, 2.0 mL of potassium hydroxide/methanol (2.0 mol L^{-1}) was added and stirred for another 2 min. Then, 500 µL of methyl tricosanoate standard were added and the test tubes and gently shaken. After complete phase separation, the organic portion was collected, and chromatographic analyses were carried out. The analyses were performed in triplicate.

Chromatographic analysis

The fatty acid methyl esters were separated using a TRACE™ Ultra Thermo Scientific™ gas chromatograph (Thermo Scientific™, USA) equipped with a flame ionization detector and a fused silica column (100 m × 0.25 mm inner diameter, 0.25 µm cyanopropyl, CP-7420). The gas flows were set at 1.2 mL min^{-1} for the carrier gas (H_2), 30 mL min^{-1} for the auxiliary gas (N_2), and 30 and 300 mL min^{-1} for the flame gasses (H_2) and synthetic air, respectively. A sample volume of 1 µL was injected, in triplicate with a sample split ratio of 1:40. The injector and detector temperature was maintained at 235 °C. The column temperature was programmed to start at 165 °C, increased to 185 °C at a heating ramp of 4 °C min^{-1} for 7 min, and then held constant for 3 min. Then, the temperature was raised to 235 °C at a heating ramp of 6 °C min^{-1} and maintained constant for 2.67 min, resulting in an analysis time of 26 min. Identification of fatty acid methyl esters was achieved by comparing retention times with relative analytical standards (FAME Mix, C4-C24, Sigma-Aldrich), and the results expressed in mg g^{-1} of total fatty acids (Equation 1), processed automatically using the Chromquest TM 5.0 software.[21]

$$FA = \frac{A_X M_P F_{CT}}{A_P M_X F_{CAE}} \times 100 \quad (1)$$

where: FA is the concentration in mg of fatty acids *per* g of total lipids; A_X is the peak area (fatty acids); A_P is the peak area of the internal standard (IS) - methyl tricosanoate (23:0me); M_P is the mass of IS added to the sample; M_X is the mass of the sample; F_{CT} is the theoretical correction factor and F_{CAE} is the necessary conversion factor to express the results in mg of fatty acids.

Analysis of the distribution of FAs in the positions of TAGs by nuclear magnetic resonance (NMR)

The NMR analyses were conducted following the procedure outlined by Tang *et al*.,[22] with some modifications. Approximately 200 mg of milk was added into 1.5 mL Eppendorf microtubes and dissolved in 400 µL of deuterated chloroform ($CDCl_3$, 99.8% D, Acros Organics, New Jersey, USA) containing 0.03% tetramethylsilane (TMS) as an internal reference. The solutions were then transferred to 5 mm NMR tubes and stored in refrigerators until the analysis was conducted.

The equipment used was a Bruker Avance III HD (Bruker, Karlsruhe, Germany) nuclear magnetic resonance spectroscope operating at 75 MHz for the ^{13}C nucleus. For data acquisition, the parameters established for ^{13}C were 256 scans (NS), 131 k data points (TD), with a spectral width (SWH) of 245 ppm, acquisition time (AQ) of 3.5 s, repetition delay (D1) of 2 s and pulse angle of 30°. The MestReNova software[23] was used to process the spectra.

Statistical analysis

In this study, analyses were conducted in triplicate to ensure the accuracy and reliability of the obtained results. Fatty acid composition results were presented as mean ± standard deviation. To assess the

significance of the results, we applied analysis of variance (ANOVA), followed by Tukey's test for mean comparison using the Assistat software.[24]

Regarding multivariate analyses, R version 4.3.0 software[25] was used to design graphs and heatmaps to identify differences among the analyzed samples, variations in the extraction method, and potential patterns in the samples.

RESULTS AND DISCUSSION

Composition of FAs

The fatty acids (FAs) were determined by identifying fatty acid methyl esters, comparing retention times with relative analytical standards (FAME Mix, C4-C24, Sigma-Aldrich). In this way, the concentration (%) was calculated based on relative area. The fatty acid composition and the total amount of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) present in human milk, cow's milk, and goat's milk were visually represented using a heatmap, as exemplified in Figure 1, where the fatty acids are listed in rows and the samples are arranged in columns.

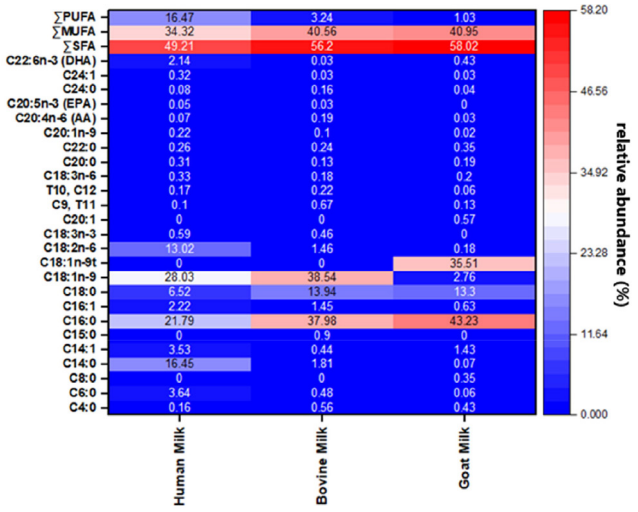

Figure 1. *Heatmap illustrating the composition of fatty acids in samples of human milk, cow's milk, and goat's milk*

Each cell in the heatmap represents the relative abundance (%) of fatty acids in a specific milk sample. The numerical values in each cell indicate the proportion of fatty acids present, with a color scale ranging from blue (0.000) to red (58.20). In this scale, darker shades of blue signify lower relative abundance of fatty acids, whereas darker shades of red indicate higher relative abundance.

In human milk, a total of 21 fatty acids were identified, with the largest quantities attributed to saturated fatty acids (SFA), representing 49.21% of the total, followed by monounsaturated fatty acids (MUFA) with 34.32% and polyunsaturated fatty acids (PUFA) with 16.47%. These findings align with the results reported by Ahmed *et al*.,[26] who also identified similar proportions of saturated fatty acids, followed by unsaturated acids, predominantly monounsaturated, and noted the presence of polyunsaturated fatty acids, aligning closely with our results.

In cow's and goat's milk, the quantities of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) were higher compared to human milk, representing 56.2 and 58.02% for SFA and 40.56 and 40.95% for MUFA, respectively. However, the main discrepancy lies in polyunsaturated fatty acids (PUFA), as in cow's milk and goat's milk, the amounts of PUFA were significantly lower, representing only 3.24 and 1.03%, respectively, compared to human milk. Wang *et al*.[9] also observed this variation, identifying PUFA concentrations in the range from 3 to 5% in ruminant milk, while values greater than 20% were observed in human milk and infant formulas.

Approximately 70% of the polyunsaturated fatty acids (PUFAs) found in breast milk originate from reserves accumulated in the mother's body during pregnancy, mainly in adipose tissues. The remaining 30% come from the mother's diet and the use of nutritional supplements.[27]

In human milk, the most prominent SFAs include palmitic acid (C16:0) representing 21.79% of the total, followed by myristic acid (C14:0) and stearic acid (C18:0) with 16.45 and 6.52%, respectively. In cow's milk, of the 52.60% SFA content, 37.98% is attributed to palmitic acid (C16:0) and 13.94% to stearic acid (C18:0). Goat's milk stands out with a higher concentration of palmitic acid (C16:0) compared to human milk and cow's milk, reaching 43.23%. Additionally, it contains 13.30% stearic acid (C18:0). However, it is important to highlight that myristic acid (C14:0), although present in cow's and goat's milk, is found in much smaller quantities, just 1.81 and 0.07%, respectively. This differs significantly from human milk, potentially impacting the nutritional composition and needs of newborns on fed formulas based on cow's milk and goat's milk. Full-term newborns already have significant amounts of 16:0, due to its production internally during fetal development. Breast milk is responsible for providing 10 to 12% of dietary energy in the form of 16:0, especially present in triglycerides. This underscores the importance of 16:0 for infants, as imbalances could potentially impact their development and health of newborns.[12]

It is important to note that pentadecanoic acid (C15:0), an odd-chain fatty acid, was found exclusively in cow's milk, comprising less than 1% of its composition. This fatty acid plays an important nutritional role, especially forinfantgrowth.[28-30] Jenkins *et al*.[31] and Wei *et al*.[32] observed its significant presence in human milk fat. However, within the scope of this study, we did not detect C15:0 in the lipid profile of the human milk analyzed , which may be related to how these fatty acids are distributed in human milk triglycerides.

In different types of milk, oleic acid (C18:1n-9) stands out as the predominant monounsaturated fatty acid (MUFA). However, it is in cow's milk that we find the highest amount, representing 38.54% of the total, surpassing other varieties. In human milk, it represents 28.03% of the total, while in goat's milk, it contributes 35.51% of MUFA.[28-30]

In human milk, linoleic acid (LA, C18:2n-6) stands out as the predominant polyunsaturated fatty acid (PUFA), contributing 13.02%. It is important to highlight that LA differs considerably from other types of milk; for example, in cow's milk, only 1.46% was identified, while in goat's milk, the presence of this fatty acid was 2.76%. As for α-linolenic acid (ALA, C18:3n-3), its contribution is 0.59% in human milk, 0.46% in cow's milk and it was not detected in goat's milk.[28-30]

These acids, linoleic and α-linolenic, are considered essential, since the human body cannot produce them. Both play fundamental roles as precursors to long-chain polyunsaturated fatty acids (LCPUFA). The relative ratio of LA and ALA in the diet influences the conversion of these precursors. When there is more LA compared to ALA in the diet, omega-6 fatty acids such as arachidonic acid (ARA) are more likely to be produced. On the other hand, a lower ratio of LA in relation to ALA favors the production of omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).[31]

Still in the context of polyunsaturated fatty acids (PUFA), it is important to highlight the significant presence of docosahexaenoic

acid (DHA, C22:6n-3) in breast milk, representing 2.14% of the total. This contrasts notably with the amounts found in cow's milk (0.03%) and goat's milk (0.43%), where DHA is substantially less prevalent. On the other hand, eicosapentaenoic acid (EPA, C20:5n-3) is found in much smaller concentrations, representing 0.05% in breast milk and 0.03% in cow's milk and is absent in goat's milk. Despite its lower abundance, DHA plays a crucial role in the development of the fetal brain, visual system, and baby's growth. The average amount of DHA in breast milk generally varies around 0.32 ± 0.22% of total fatty acids. However, our research revealed a significantly higher concentration of DHA, exceeding 1% of total fatty acids, which is associated with the consumption of marine foods.[32] These results highlight the importance of including LA and DHA into infant formulas made from cow's and goat's milk. Particularly, DHA, has become a mandatory addition in formulas intended to replace breast milk in Europe, in accordance with recently established regulations, which require a minimum of 20 mg of DHA *per* 100 kcal (or 4.8 mg *per* 100 kJ) for these formulas to be available on the European market.[33]

To compensate for the absence of ALA and EPA fatty acids in goat's milk, it would be advisable to consider including these fatty acids in infant formulas. This is justified by the fact that the analyzed human milk contains 0.05% EPA and 0.59% ALA, respectively. Furthermore, it is important to highlight that cow's milk can also be a viable alternative, as it has comparable levels of these fatty acids compared to human milk, thereby adequately addressing this deficiency.[34,35]

Short-chain fatty acids (SCFA) and medium-chain fatty acids (MCFA) are easily digestible in newborns with an immature digestive system, in contrast to long-chain fatty acids (LCFA). Goat's milk, generally, contains higher amounts of SCFA and MCFA compared to bovine milk. However, although we identified caprylic acid (C8:0) exclusively in goat's milk in our results, the combined concentrations of capric acid (C6:0) and butyric acid (C4:0) were higher in bovine milk. This was likely due dietary influences, which plays a significant role in fatty acid composition, however, neither cow's milk nor goat's milk surpassed the amount of SCFA and MCFA found in human milk, which was 3.8%.[34,35]

Distribution of FAs in the different TAGs positions

The analysis of the ^{13}C NMR spectra of the samples allowed determining the relative proportions of SFA, MUFA and PUFA in the TAGs of mature human, bovine, and caprine milk samples. For this purpose, the deconvolution technique was applied in the acyl carbons region (170-185 ppm), as described by Lopes *et al.*[36] In this region, two groups of acyl signals were identified, one referring to the sn2 position of TAGs and the other to the sn-1,3 positions. This technique was necessary due to peak overlap caused by the similar chemical characteristics of the acyl carbons in TAGs. A model illustrating how the deconvolution process was carried out is shown in Figure 2.[36]

In this context, the compositions of intrapositional FA, expressed as percentages, were compared among samples of mature human milk, cow's milk and goat's milk after analysis and interpretation of the results obtained by NMR, as shown in Figure 3.

Figure 3 reveals that the intrapositional composition of a FA is positively correlated with its total proportion in milk. Notably, there is a predominance of SFA in the sn-2 position of TAGs, while MUFA and PUFA have a more pronounced distribution in the sn-1 and sn-3 positions. These variations in stereospecific distribution may explain the improved efficacy in current lipid absorption of in human milk. The structure of TAGs also plays a role in determining the products resulting from lipase action, thereby influencing FA absorption control.[12,37] In the hydrolysis of breast milk, the formation of SFA

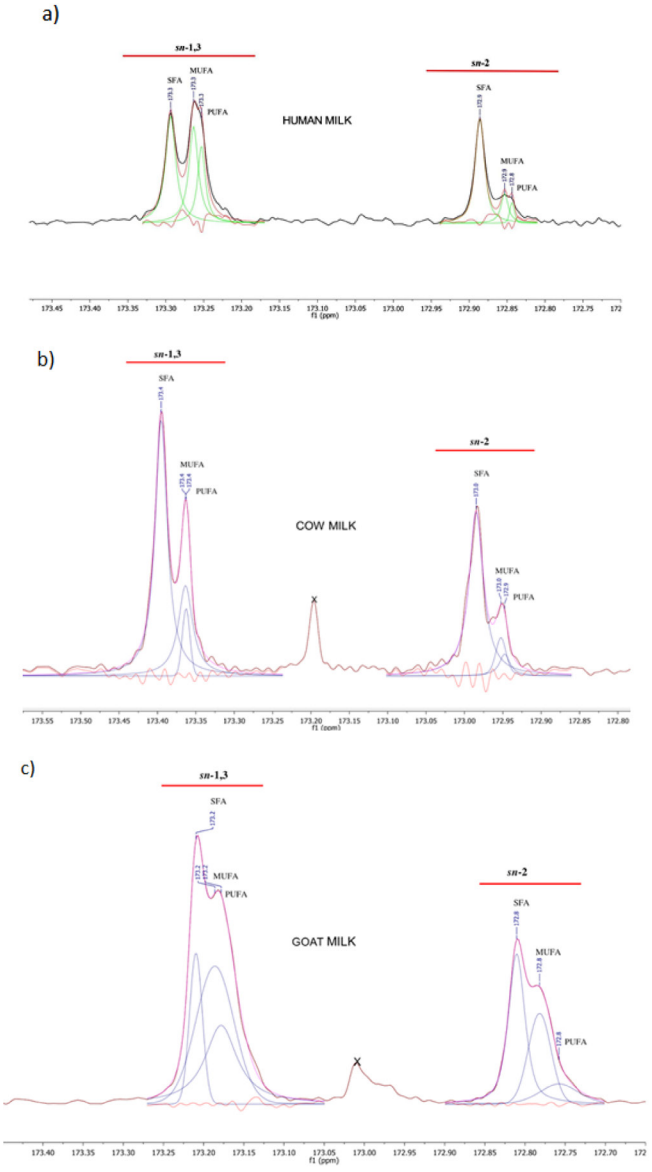

Figure 2. *Acyl carbons region ^{13}C spectra of (a) human; (b) cow and (c) goat milk after deconvolution. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids*

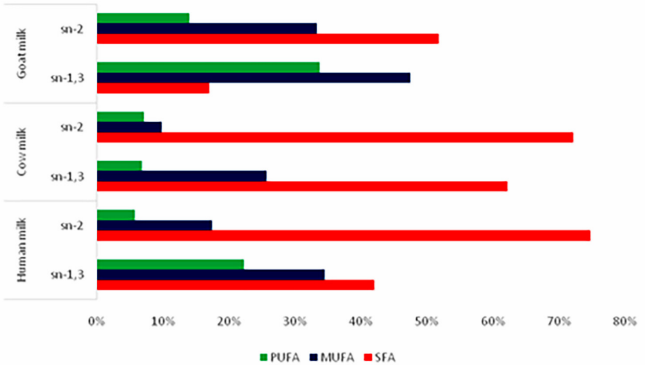

Figure 3. *Interpositional distribution graphs of GA in TAGs*

is predominantly observed in the form of monoacylglycerols in the sn-2 position, which are easily absorbed.

It is important to highlight that the concentration of FA in the three positions of the TAG varies among different milk species . In human

milk, for example, the sn-1 and sn-3 positions are predominantly esterified by saturated fatty acids, such as 16:0 and 18:0, while the sn-2 position also presents a predominance of SFA. In contrast, the goat milk sample revealed that the sn-1 and sn-3 positions are mainly esterified by unsaturated fatty acids. Furthermore, FAs in ruminant milk mainly consist of long-chain fatty acids (LCFAs), with 18:1n-9 being the most abundant, followed by 18:0, 16:0, and 14:0. Such variations in the intrapositional compositions of the analyzed lipids may be related to differences in animal breeds and diets.

Understanding how the specific distribution of FA occurs within TAGs structures allows us to better comprehend certain processes related to the metabolization of lipids present in milk, such as the digestion and absorption process, as highlighted by Zhao *et al*.[38] The sn-2 position of monoacylglycerols (MAGs) enables efficient absorption and subsequent re-esterification of other fatty acids esterified in this position, making them highly bioavailable. Furthermore, FAs located at the sn-1 and sn-3 positions are closely related to the selectivity of gastric and pancreatic lipase, which confers additional nutritional relevance, as highlighted by Cossignani *et al*.[39]

In this way, with the analysis of the ^{13}C spectra of human, bovine and caprine milk obtained by NMR, it was possible to compare the stereospecific distribution of the different samples. Furthermore, NMR analysis is fast when compared to other analytical techniques and requires practically no sample preparation, making it important for studying the lipid profile of different types of milk and having great potential to assist in the formulation of new IF.

The study revealed significant differences in fatty acid composition between human, cow's and goat's milk, with important implications for infant nutrition. Human milk contains 49.21% saturated fatty acids (SFA), 34.32% monounsaturated fatty acids (MUFA) and 16.47% polyunsaturated fatty acids (PUFA), including 2.14% DHA and 13.02% of linoleic acid (LA). In contrast, cow and goat's milk have higher proportions of SFA (56.2 and 58.02%, respectively) and MUFA (40.56 and 40.95%, respectively), but lower amounts of PUFA (3.24 and 1.03%, respectively), with significantly lower levels of DHA (0.03 and 0.43%, respectively) and LA (1.46 and 2.76%, respectively).

The specific distribution of fatty acids in the sn-2 positions of TAGs has been identified as crucial for efficient lipid absorption , with human milk predominantly esterifying saturated fatty acids like16:0 and 18:0 at the sn-1 and sn-3 positions. These findings suggest that infant formulas should be supplemented with PUFA, especially DHA, and consider the intrapositional distribution of fatty acids to optimize digestion and nutrient absorption in newborns.

CONCLUSIONS

It was observed that saturated fatty acids (SFA) predominantly occupy the sn-2 position of TAGs, while monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) exhibit a more pronounced distribution in the sn-1 and sn-3 positions in human milk. In contrast, cow's milk samples reveal that the sn-1 and sn-3 positions are mainly esterified by unsaturated fatty acids. Furthermore, fatty acids in ruminant milk consist mainly of long-chain fatty acids (LCFAs), with oleic acid (38.5%) being the most abundant, followed by stearic (13.94%), palmitic (37.98%), and myristic acids. Such variations in intrapositional compositions of analyzed lipids may be related to differences in animal breeds and diets. Therefore, it is imperative to use supplementation in infant formulas that containing commercially available fat based on bovine milk in order to achieve greater similarity to HM.

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