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Article

Influence of Breastfeeding Time on Caloric Composition and IL-10 and TNF-α Cytokines, Fatty Acids, and Triacylglycerol in Human Milk Colostrum in Previous, Intermediate, and Posterior Milk

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The composition of human milk (HM) includes changes under the influence of factors such as genetics, stage of lactation, time of day, and even breastfeeding time. However, the authors are not aware of studies that evaluated the influence of breastfeeding time on fatty acid composition and cytokine. Thus, the objective of this study was to evaluate the influence of breastfeeding time (previous, intermediate, posterior milk) on kilocalorie concentration, fatty acid (FA) composition, and concentration of the cytokines interleukin-10 (IL-10), interferon factors for necrotic tumor (TNF-α) in HM colostrum. The results showed that the concentration of kilocalories was higher in posterior milk than in the previous, the composition of the main fatty acids found in the samples was influenced by the breastfeeding time, and the concentration of IL-10 and TNF-α was influenced in all samples with exception to mother 2. The principal component analysis explained 71% of the variances in the results. Thus, we concluded that the breastfeeding time is a factor that influences the concentration of kilocalories, and if it is necessary to use a more caloric milk, it is recommended to use the posterior milk; the FAs composition and the evaluated cytokines were also influenced by the breastfeeding time.

Keywords: human milk, GC-FID, lipids, lactation

Introduction

Human milk (HM) is a highly complex biological fluid, with several nutrients, bioactive components, and immunological factors that provide all the nutritional demands of the infant, ensuring adequate growth and development. Its composition includes changes under the influence of factors such as genetics, lactation stage, maternal diet, gestational age, habits of the breastfeeding woman, the volume of milk produced, time of day,[1] and can even vary in the same breastfeeding episode between the initially expressed milk (previous milk) and the last milking dairy (posterior milk).[2]

The various health benefits associated with breastfeeding are driven by the combined action of nutritional factors and bioactive components in HM.[3] In addition to containing epidermal growth factors, which are essential for maturation and healing intestinal, in the long term, it reduces respiratory infections, it also reduces the risk of developing intestinal hypersensitivity, and reduces the risks of hypertension, hypercholesterolemia, diabetes, asthma, and obesity.[4]

The HM composition is related to the stages of lactation, they occur under the influence of factors such as lactation stage-human milk colostrums (first seven days) rich in

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immunological factors,[5] human milk transitional (seven to fourteen days) rich in caloric and growth factors, and human milk mature (after 15 days) containing individualized nutrition.[6] The World Health Organization[7] recommends starting breastfeeding in the first 60 min of life and keeping it as a unique way of feeding until six months of age and, in a complementary way until the two years.

According to its composition in the HM, the lipids are present in the form of fat globules, consisting mainly of triacylglycerides (TAG) with 98% of its lipid composition being triacylglycerols, 1% phospholipids, and 0.5% sterols, surrounded by a phospholipids, cholesterol, proteins, and glycoproteins membrane. Milk TAG is formed in the endoplasmic reticulum from fatty acids (FAs) circulating or synthesized in glucose epithelial cells.[8,9] Consequently, the fatty acid composition of these constituents defines the nutritional and physicochemical properties of HM fat.[10]

Among the bioactive compounds of HM, cytokines stand out, described as polypeptides that act in an autocrine, paracrine or endocrine system through binding to specific cellular receptors, affecting the synthesis of cellular proteins and/or other cellular activities. The group of cytokines embraces interleukins, interferons factors for necrotic tumor (TNF) factor of growth beta transformation (TGF-β), chemokines, and lymphokines.[11]

Among the immunological factors of HM, it contains cytokines that have pro and anti-inflammatory activities and commands a complex network of immunity, which protects infants from infections and other diseases.[12] The tumor necrosis factor-alpha (TNF-α) participates in proinflammatory functions of HM, which is recognized as a cytokine-dependent inflammatory mediator, and it is increased during lactation.[13] The interleukin-10 (IL-10) and the transforming growth factor-beta (TGF-β) are anti-inflammatory. The IL-10 is found in the lipid layer of HM, it participates as a defense agent and the IL-10 is bioactive in a possible protected compartment. It is suggested that IL-10 in HM may have immunomodulatory and anti-inflammatory effects in the alimentary tract of the recipient infant.[14]

High levels of TNF-α have been associated with obesity, because the adipose tissue is the primary place of production of this cytokine, in addition to being, along with the IL-6, one mediator from macrophage, causing the inflammation of the tissue fat in obesity.[15,16]

One study[17] showed an association between the levels of cytokines and fatty acids polyunsaturated and saturated. This fact demonstrates that the inflammatory and nutrition factors are interrelated depending on the composition in fatty acids of HM. Main immunomodulatory cytokines identified in HM are adiponectin, immunoglobulin A (IgA), IL10, TNF-α.[17]

Studies[18,19] show that the concentration of fat in the milk increases in the final of breastfeeding with the emptying of the breast. This fact originated the terms "previous milk" and "posterior milk", the last one being referred to as the portion most caloric and rich in lipids.[20] Few studies assess the influence of breastfeeding time on the levels of cytokines and fatty acids.

Based on the above considerations, this study aimed to evaluate the influence of breastfeeding time on the concentration of kilocalories, fatty acid composition, TAG profile, and content of cytokines IL-10, TNF-α in human milk colostrum.

Experimental

This is an analytical and descriptive study of case series, with a quantitative approach. Number of samples = 5 donors (n = 5), being called: mother 1 (M1), mother 2 (M2), mother 3 (M3), mother 4 (M4), and mother 5 (M5).

Reagents

Chloroform, *n*-heptane, methanol, and sodium chloride (all analytical grade) were purchased from Synth (São Paulo, Brazil). Sodium hydroxide and potassium hydroxide (all analytical grade) were purchased from Dinâmica (São Paulo, Brazil).

Population and sample

The samples of this study was for convenience, constituted of mothers (breastfeeding women) admitted to the Gynecology and Obstetrics sector of two hospitals located in the Northwest of Paraná and they were followed until they were kept in exclusive breastfeeding under free demand.

The following inclusion criteria were used for lactating women: being in the process of healthy pregnancy and delivery, gestational age (GA) up to 37 weeks, regardless of the type of delivery, number of pregnancies and who lived at Northwest of the state of Paraná. The non-inclusion is given if the hospital has not been linked to the process of childbirth, pregnancy, and childbirth with comorbidities (disease hypertensive specific from pregnancy, diabetes mellitus, rich erythematosus, fetal distress, premature labor, etc.) and multiple births. Inclusion criteria for newborns: healthy, of any type of childbirth, AG greater than 37 weeks. Babies who did not present disorders (fever, diarrhea, atypical formation, jaundice, and/or congenital infections) were considered healthy.

Ethical aspect

The study was approved by the Research Ethics Committee (CEP in Brazil), number 3.098.157/2018, of the State University of Maringá (UEM, Maringá, Brazil).

Samples of human milk were collected. In this study, the milk from donors in the colostrum stage was analyzed. Milk was extracted using the manual milking technique at three different times: T1-before starting the feed, time zero (previous milk), T2-after 15 min of feeding (intermediate milk), and T3-after 30 min of feeding (posterior milk). The samples were stored in a test tube and refrigerated at 4 °C in a thermos box. Subsequently, 3 mL of each sample were aspirated and stored at −18 °C in a freezer for lipid composition analysis and 2 mL at −80 °C for cytokine analysis.

Kilocalorie analysis

For kilocalorie analysis, the hematocrit method was performed at the Human Milk Bank of the University Hospital of Maringá as described by Agência Nacional de Vigilância Sanitária (ANVISA),[21] 1 mL of each homogenized human milk sample was collected and heated in a water bath at 40 °C for 10 min. Three aliquots of 75 µL of each sample were extracted using 1.5/1.0 mm diameter micro-hematocrit capillary tubes (Perfecta®, São Paulo, Brazil) then one end was sealed with a Bunsen burner. The tubes were centrifuged in a microhematocrit centrifuge (Excelsa® Flex 3400, Fanem, Guarulhos, Brazil) at 4000 rpm for 15 min. After centrifugation, a millimeter ruler (mm) was used and the length of the cream column (mm) was analyzed.

The cream content was calculated using the following calculation according to Lucas *et al.*:[22]

$$\text{Cream content (\%)} = \frac{\text{cream column (mm)} \times 100}{\text{total column (mm)}} \quad (1)$$

The kilocalorie content was calculated using the following calculation:

$$\frac{\text{kilocalorie}}{\text{litre}} = (\text{cream (\%)} \times 66.8 + 290) \quad (2)$$

Lipid extraction

The lipids were extracted according to Folch *et al.*,[23] 1 mL of human milk was pipetted, 20 mL of solvent (1:20) and chloroform:methanol ratio (2:1) were added and stirred for 15 min in a magnetic stirrer. In the end, the sample was centrifuged for 5 min in a centrifuge refrigerated at 25 °C and 6000 rpm to remove the precipitated protein. At the second moment, 4 mL of 0.9% sodium chloride solution (NaCl) were added, and the solution was stirred for 5 min and centrifuged (under the same condition as the previous step for phase separation occur). The lower phase was collected in a 250 mL flask, previously weighed, and the solvent evaporated at 37 °C using a rotary evaporator.

Esterification

The esterification followed the methodology proposed by the International Organization for Standardization-ISO No. 12966/2017,[24] in which, in a centrifuge tube, 100 mg of lipid sample were added to 2 mL of heptane, stirred for 2 min in a vortex, then were added 2 mL of the reagent esterifying potassium hydroxide (KOH) 2 mol L^{-1} in methanol. This solution was stirred for 3 min in a vortex again and carried to the refrigerator for 24 h to separate the phases, and the upper phase was analyzed on a gas chromatograph coupled with a flame ionization detector (GC-FID).

Fatty acid composition analysis

The fatty acid methyl esters (FAMEs) analyzes were performed using Thermo Scientific gas chromatography (GC) (Trace Ultra 3300, Waltham, USA) with a flame ionization detector (FID), capillary column CP-7420 (100.0 m size, 0.25 mm internal diameter and 0.25 µm cyanopropyl thin film as stationary phase) and split/splitless injector. Detector and injector temperatures were 250 and 230 °C, respectively. The GC-FID oven was programmed at 65 °C and kept for 4 min, then heated to 185 °C at 16 °C min^{-1} and held for 12 min, then heated to 235 °C at 20 °C min^{-1} and held for 9 min. The gas flow rates used were 1.4 mL min^{-1} to the carrier gas (H_2), 30 mL min^{-1} for nitrogen replacement gas (N_2), and 30 to 300 mL min^{-1} for flame gases (H_2 and synthetic air, respectively).

The split injection model was used at a ratio of 1:100 and the volume of sample injections was 2.0 µL. FAMEs were identified by comparing the retention times of the sample constituents with those of the analytical standards (FAME standard mixture, C4-C24, containing linoleic acid geometric isomers c9t11 and t10c12, Sigma-Aldrich, Saint Louis, USA). Peak areas were determined using the software ChromQuest™ 5.0, and the compositions of fatty acids were expressed as a relative percentage of FA total.[25] The analysis was performed in triplicate.

TAG assignment and estimation

The TAGs were assigned and estimated (%) via the LAMES Platform, which is based on the mathematical

algorithm that describes the distribution of FA in the TAG molecules[26] using the percentage of FA determined by GC-FID. With the Lipid maps® database,[27] it was possible to find a molecular formula of the TAGs.

Analysis of IL-10 and TNF-α cytokine

TNF-α and IL-10 were measured in the collected milk. The samples were evaluated in triplicate, without dilution.

The dosage of IL-10 was performed using the Human Custom Procartaplex 9-plex kit (Invitrogen™, Thermo Fisher Scientific, Inc., Burlington, Canada) following the manufacturer's recommendations. The cytometer of the flow Luminex® 100/200™ system was used for the reading fluorescence.

The TNF-α measurement was performed by enzyme-linked immunosorbent assay (ELISA). For this, the HumanTNF alpha ELISA kit (Invitrogen™, Thermo Fisher Scientific, Inc., Burlington, Canada) was used following the manufacturer's recommendations. The absorbance was read in an ASYS™, Holliston, USA, microplate reader, EXPERT PLUS model.

Statistical analysis

The compositional analysis data in fatty acids, kilocalories, and cytokines were submitted to analysis of variance (ANOVA) and Tukey's test ($p < 0.05$), using the Assistant software version 7.7.[28] The composition data in fatty acids, cytokines, and kilocalorie analysis were submitted to a multivariate exploration technique, the chosen technique was the principal component analysis (PCA), which is performed in the Rstudio software version 1.4.1106.[29]

Results and Discussion

Kilocalorie *per* liter of human milk colostrum

The kilocalorie *per* liter content of human milk colostrum from five different mothers (breastfeeding women) is shown in Figure 1 represented by M1-M5. The main factors described in the literature that modify the kilocalorie content of human milk are the duration of the lactation period,[30,31] body mass index (BMI),[32] times of day,[19] maternal age,[20,33] body composition and BMI,[34] and lactation stage.[6,35]

The crematocrit and kilocalorie *per* liter content in breast milk show the daily differences. Kociszewska-Najman *et al.*[36] reported that colostrum milk differs from the ripe milk of mothers of full-term newborns. In Figure 1, it is shown that sample M1 in T2 obtained the highest value (919.67 kcal L^{-1}), followed by M2 in T2 (1039.83 kcal L^{-1})

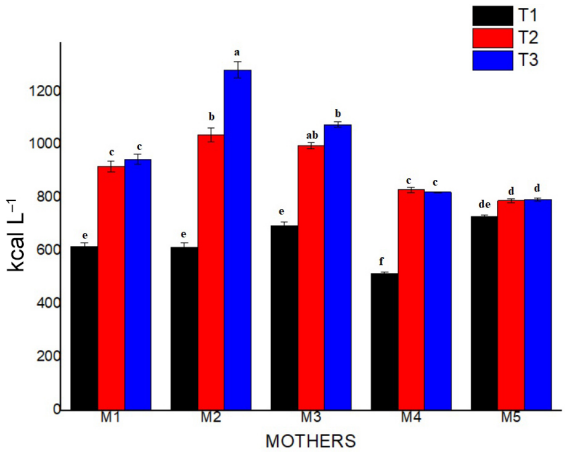

Figure 1. Influence of breastfeeding time on the kilocalorie *per* liter content of human milk colostrum. M1: mother 1, M2: mother 2, M-3: mother 3, M4: mother 4 and M5: mother 5. Differen letters in the columns indicate that they are significantly different ($p < 0.05$) by Tukey's test.

and T3 with 1284.42 kcal L^{-1}. While the lowest values of kilocalorie *per* liter in the samples were for M4 in T1 (516.07 kcal L^{-1}), T2, and T3 (831.65-823.19 kcal L^{-1}), respectively.

Human milk can be classified into normocaloric, hypercaloric and hypocaloric. Normocaloric is milk whose fat fraction is concentrated between 450-725 kcal L^{-1}, hypercaloric is that above 725 kcal L^{-1}, and low-calorie milk, less than or equal to 450 kcal L^{-1}.[37] It is noteworthy that all colostrums of the analyzed lactating mothers obtained hypercaloric milk in the intermediate and posterior phases.

Authors[6,19,31] affirm that the prior milk shows one content higher than water, with an appearance similar to coconut water, and is an important source of antibodies. In turn, the posterior milk contains one concentration of fat over high, which promotes greater satiety for the children. These variations support the importance of complete emptying of the breast during breastfeeding, to meet all nutritional needs of the child.

The results of this study indicated that the kilocalorie of the posterior milk was statistically significant when compared to the previous milk in all samples of human milk, the posterior milk is more caloric (Figure 1). The study at Showa University Hospital[18] showed evidence of an increase in kcal in milk. The increase in fat content from the fore-milk to the after-milk was related to the removal of fat globules adsorbed by the breast alveoli.[18] Thus, it can be suggested that when there is a need for high-calorie milk, offer posterior milk to the infant.

Fatty acid composition

In the samples analyzed in this research, a total of

Table 1. Fatty acid composition (percentage of relative area) of colostrum milk at different times of feeding

Fatty acid / %	T1					T2					T3				
	M1	M2	M3	M4	M5	M1	M2	M3	M4	M5	M1	M2	M3	M4	M5
4:0	0.49 ± 0.02^{ef}	0.63 ± 0.09^{de}	0.48 ± 0.02^{ef}	0.71 ± 0.03^{cd}	0.88 ± 0.01^{bc}	0.48 ± 0.03^{ef}	1.07 ± 0.13^{a}	0.75 ± 0.06^{cd}	0.39 ± 0.02^{f}	0.94 ± 0.02^{ab}	0.51 ± 0.00^{ef}	0.97 ± 0.08^{ab}	1.01 ± 0.05^{ab}	1.06 ± 0.09^{a}	0.94 ± 0.04^{ab}
6:0	0.96 ± 0.03^{bc}	0.47 ± 0.02^{d}	1.15 ± 0.12^{b}	0.95 ± 0.08^{bc}	0.45 ± 0.02^{de}	0.94 ± 0.02^{c}	0.18 ± 0.01^{f}	0.92 ± 0.07^{c}	0.26 ± 0.00^{ef}	0.46 ± 0.01^{de}	0.97 ± 0.07^{bc}	0.16 ± 0.00^{f}	1.52 ± 0.18^{a}	0.45 ± 0.01^{de}	0.46 ± 0.06^{de}
8:0	0.16 ± 0.01^{de}	0.48 ± 0.01^{b}	0.21 ± 0.01^{de}	0.89 ± 0.09^{a}	0.34 ± 0.00^{c}	0.16 ± 0.01^{de}	0.18 ± 0.01^{de}	0.16 ± 0.01^{de}	0.23 ± 0.01^{d}	0.35 ± 0.02^{c}	0.14 ± 0.00^{e}	0.16 ± 0.01^{de}	0.32 ± 0.02^{c}	0.32 ± 0.02^{c}	0.33 ± 0.04^{c}
10:0	0.17 ± 0.01^{cde}	0.12 ± 0.00^{cde}	0.24 ± 0.01^{bc}	0.65 ± 0.02^{a}	0.13 ± 0.00^{cde}	0.17 ± 0.00^{cde}	0.04 ± 0.00^{e}	0.18 ± 0.00^{bcd}	0.11 ± 0.00^{cde}	0.11 ± 0.00^{cde}	0.16 ± 0.01^{cde}	0.05 ± 0.00^{de}	0.31 ± 0.01^{b}	0.54 ± 0.02^{a}	0.19 ± 0.01^{bc}
12:0	2.74 ± 0.05^{d}	4.31 ± 0.03^{b}	2.43 ± 0.23^{de}	3.46 ± 0.18^{c}	2.28 ± 0.16^{e}	2.77 ± 0.17^{d}	3.96 ± 0.27^{b}	2.45 ± 0.04^{de}	4.07 ± 0.14^{b}	3.31 ± 0.04^{c}	2.37 ± 0.18^{de}	4.20 ± 0.05^{b}	2.36 ± 0.11^{de}	5.00 ± 0.07^{a}	3.38 ± 0.17^{c}
14:0	6.16 ± 0.09^{bc}	6.84 ± 0.18^{a}	3.82 ± 0.18^{fg}	4.19 ± 0.24^{f}	5.96 ± 0.54^{cd}	5.99 ± 0.08^{cd}	6.54 ± 0.21^{abc}	3.74 ± 0.08^{fg}	5.30 ± 0.26^{e}	4.21 ± 0.05^{f}	5.48 ± 0.21^{de}	6.77 ± 0.08^{ab}	3.37 ± 0.09^{g}	5.10 ± 0.18^{e}	4.33 ± 0.28^{f}
14:1	0.14 ± 0.01^{cd}	0.16 ± 0.00^{c}	0.15 ± 0.00^{c}	0.16 ± 0.00^{c}	0.12 ± 0.00^{d}	0.14 ± 0.00^{cd}	0.15 ± 0.01^{c}	0.16 ± 0.02^{c}	0.12 ± 0.00^{d}	0.09 ± 0.00^{e}	0.14 ± 0.01^{cd}	0.16 ± 0.01^{c}	0.19 ± 0.01^{b}	0.30 ± 0.01^{a}	0.09 ± 0.00^{e}
15:0	0.07 ± 0.00^{cd}	0.07 ± 0.00^{cd}	0.09 ± 0.00^{cd}	0.12 ± 0.00^{bcd}	0.18 ± 0.01^{b}	0.08 ± 0.00^{cd}	0.06 ± 0.00^{cd}	0.10 ± 0.01^{bcd}	0.08 ± 0.00^{cd}	0.04 ± 0.00^{d}	0.08 ± 0.01^{cd}	0.06 ± 0.00^{cd}	0.12 ± 0.00^{bcd}	1.17 ± 0.11^{a}	0.13 ± 0.00^{bc}
15:1	0.27 ± 0.02^{cde}	0.26 ± 0.01^{cde}	0.34 ± 0.02^{a}	0.19 ± 0.01^{fg}	0.30 ± 0.04^{abc}	0.27 ± 0.01^{cde}	0.24 ± 0.00^{def}	0.33 ± 0.02^{ab}	0.22 ± 0.01^{efg}	0.18 ± 0.00^{g}	0.26 ± 0.02^{cde}	0.26 ± 0.01^{cde}	0.35 ± 0.02^{a}	0.28 ± 0.01^{bcd}	0.19 ± 0.01^{fg}
16:0	26.99 ± 0.17^{b}	26.77 ± 0.28^{bc}	27.34 ± 0.29^{ab}	20.57 ± 0.14^{f}	27.90 ± 0.23^{a}	26.72 ± 0.29^{bc}	26.50 ± 0.18^{bc}	26.04 ± 0.45^{c}	20.40 ± 0.27^{f}	20.47 ± 0.21^{f}	26.75 ± 0.32^{bc}	26.54 ± 0.19^{bc}	24.64 ± 0.32^{d}	21.85 ± 0.21^{e}	20.48 ± 0.32^{f}
16:1n-7	0.36 ± 0.02^{bcd}	0.36 ± 0.01^{bcd}	0.28 ± 0.01^{ef}	0.48 ± 0.05^{a}	0.38 ± 0.02^{b}	0.36 ± 0.01^{bcd}	0.36 ± 0.05^{bcd}	0.32 ± 0.03^{bcdef}	0.29 ± 0.01^{def}	0.30 ± 0.01^{cdef}	0.34 ± 0.02^{bcde}	0.38 ± 0.02^{b}	0.26 ± 0.02^{f}	0.37 ± 0.02^{bc}	0.31 ± 0.02^{bcdef}
16:1n-9	1.41 ± 0.09^{abcd}	1.69 ± 0.11^{ab}	1.55 ± 0.17^{abc}	1.25 ± 0.09^{cd}	1.14 ± 0.09^{d}	1.43 ± 0.11^{abcd}	1.71 ± 0.08^{a}	1.59 ± 0.11^{abc}	1.36 ± 0.15^{bcd}	1.45 ± 0.09^{abcd}	1.44 ± 0.17^{abcd}	1.74 ± 0.15^{a}	1.30 ± 0.09^{cd}	1.42 ± 0.09^{abcd}	1.47 ± 0.05^{abcd}
17:0	0.38 ± 0.02^{bcde}	0.32 ± 0.01^{def}	0.41 ± 0.02^{abc}	0.33 ± 0.02^{cdef}	0.44 ± 0.03^{ab}	0.40 ± 0.02^{abcd}	0.30 ± 0.02^{ef}	0.32 ± 0.04^{def}	0.39 ± 0.05^{abcd}	0.32 ± 0.04^{def}	0.40 ± 0.03^{abcd}	0.29 ± 0.02^{f}	0.47 ± 0.03^{a}	0.40 ± 0.03^{abcd}	0.33 ± 0.01^{cdef}
17:1	0.16 ± 0.01^{fg}	0.11 ± 0.00^{i}	0.27 ± 0.01^{b}	0.23 ± 0.02^{c}	0.14 ± 0.00^{gh}	0.16 ± 0.01^{fg}	0.12 ± 0.01^{hi}	0.21 ± 0.00^{cd}	0.18 ± 0.01^{ef}	0.13 ± 0.00^{hi}	0.19 ± 0.01^{de}	0.12 ± 0.00^{hi}	0.31 ± 0.02^{a}	0.16 ± 0.00^{fg}	0.12 ± 0.00^{hi}
18:0	7.36 ± 0.23^{b}	6.42 ± 0.21^{c}	5.56 ± 0.18^{de}	5.40 ± 0.18^{e}	7.95 ± 0.15^{ab}	7.57 ± 0.32^{ab}	6.11 ± 0.16^{cd}	5.32 ± 0.16^{e}	5.27 ± 0.05^{e}	5.66 ± 0.22^{de}	8.05 ± 0.19^{a}	5.85 ± 0.31^{cde}	5.73 ± 0.21^{de}	3.95 ± 0.17^{f}	5.79 ± 0.26^{de}
18:1n-7	1.41 ± 0.08^{ef}	1.21 ± 0.07^{f}	2.53 ± 0.17^{b}	1.99 ± 0.11^{cd}	1.73 ± 0.19^{de}	1.47 ± 0.09^{ef}	2.31 ± 0.09^{bc}	2.96 ± 0.05a	2.00 ± 0.08^{cd}	1.75 ± 0.12^{de}	1.36 ± 0.11^{f}	2.58 ± 0.02^{b}	3.16 ± 0.18^{a}	1.87 ± 0.17^{d}	1.72 ± 0.02^{de}
18:1n-9	30.69 ± 1.12^{ab}	29.35 ± 0.97^{ab}	31.26 ± 1.09^{ab}	24.01 ± 0.67^{c}	24.37 ± 0.68^{c}	31.25 ± 1.18^{ab}	29.44 ± 0.86^{ab}	31.93 ± 1.02^{a}	24.96 ± 0.75^{c}	25.73 ± 0.76^{c}	32.12 ± 1.21^{a}	28.74 ± 0.95^{b}	30.40 ± 0.99^{ab}	25.13 ± 0.87^{c}	25.89 ± 0.56^{c}
18:2 n-6	14.57 ± 0.18^{hi}	14.98 ± 0.43^{gh}	16.23 ± 0.19^{ef}	26.56 ± 0.16^{ab}	17.45 ± 0.22^{d}	14.27 ± 0.12^{hi}	15.47 ± 0.34^{fg}	16.69 ± 0.17^{de}	26.84 ± 0.43^{ab}	27.39 ± 0.19^{a}	13.91 ± 0.23^{i}	15.66 ± 0.54^{fg}	18.38 ± 0.34^{c}	26.15 ± 0.24^{b}	26.98 ± 0.17^{ab}
CLA C9.T11	0.81 ± 0.02^{b}	0.46 ± 0.08^{cd}	0.86 ± 0.05^{b}	0.46 ± .02^{cd}	0.88 ± 0.07^{ab}	0.78 ± 0.01^{b}	0.48 ± 0.01^{cd}	0.37 ± 0.02^{d}	0.56 ± 0.03^{c}	0.49 ± 0.02^{cd}	0.74 ± 0.05^{b}	0.47 ± 0.01^{cd}	1.02 ± 0.14^{a}	0.52 ± 0.05^{cd}	0.43 ± 0.02^{cd}
CLA T10.C12	0.41 ± 0.01^{f}	0.50 ± 0.03^{ef}	0.73 ± 0.04^{bc}	0.88 ± 0.03^{a}	0.63 ± 0.08^{cd}	0.40 ± 0.02^{f}	0.49 ± 0.03^{ef}	0.58 ± 0.05^{de}	0.86 ± 0.04^{a}	0.83 ± 0.04^{ab}	0.39 ± 0.01^{f}	0.48 ± 0.05^{ef}	0.58 ± 0.01^{de}	0.66 ± 0.01^{cd}	0.87 ± 0.04^{a}
18:3 n-3	1.11 ± 0.06^{cde}	0.77 ± 0.02^{fg}	0.87 ± 0.06^{efg}	1.75 ± 0.15^{b}	1.41 ± 0.12^{c}	1.06 ± 0.02^{def}	0.80 ± 0.02^{fg}	0.93 ± 0.07^{ef}	2.05 ± 0.09^{ab}	2.05 ± 0.21^{a}	1.00 ± 0.02^{ef}	0.80 ± 0.05^{fg}	0.57 ± 0.06^{g}	1.32 ± 0.14^{cd}	2.02 ± 0.08^{ab}
20:0	0.76 ± 0.07^{cd}	0.80 ± 0.08^{bcd}	0.50 ± 0.01^{e}	0.20 ± 0.01^{f}	1.14 ± 0.07^{a}	0.75 ± 0.03^{cd}	0.67 ± 0.05^{d}	0.91 ± 0.08^{b}	0.77 ± 0.02^{cd}	0.88 ± 0.03^{bc}	0.75 ± 0.02^{cd}	0.68 ± 0.03^{d}	0.74 ± 0.05^{d}	0.15 ± 0.01^{f}	0.76 ± 0.01^{cd}
20:3n-3	0.54 ± 0.00^{gl}	0.52 ± 0.04^{d}	0.69 ± 0.05^{c}	0.52 ± 0.01^{d}	1.27 ± 0.09^{a}	0.52 ± 0.01^{d}	0.53 ± 0.04^{d}	0.46 ± 0.05^{d}	0.83 ± 0.04^{b}	0.86 ± 0.04^{b}	0.51 ± 0.03^{d}	0.52 ± 0.02^{d}	0.53 ± 0.03^{d}	0.24 ± 0.00^{e}	0.81 ± 0.03^{b}
20:4n-6 (AA)	0.59 ± 0.04^{bc}	0.63 ± 0.05^{bc}	0.24 ± 0.01^{e}	0.86 ± 0.03^{a}	0.21 ± 0.01^{e}	0.56 ± 0.00^{c}	0.67 ± 0.03^{b}	0.65 ± 0.04^{bc}	0.41 ± 0.01^{d}	0.26 ± 0.00^{e}	0.59 ± 0.01^{bc}	0.65 ± 0.07^{bc}	0.63 ± 0.05^{bc}	0.26 ± 0.02^{e}	0.23 ± 0.01^{e}
20:5n-3(EPA)	0.19 ± 0.01^{e}	0.23 ± 0.00^{bc}	0.20 ± 0.01^{de}	0.23 ± 0.01^{bc}	0.25 ± 0.01^{b}	0.22 ± 0.01^{bc}	0.12 ± 0.00^{f}	0.25 ± 0.01^{b}	0.25 ± 0.01^{b}	0.19 ± 0.01^{e}	0.24 ± 0.01^{bc}	0.12 ± 0.00^{f}	0.22 ± 0.01^{cd}	0.29 ± 0.01^{a}	0.20 ± 0.00^{de}
22:2 n-6	0.36 ± 0.04^{fg}	0.32 ± 0.01^{g}	0.50 ± 0.08^{def}	0.96 ± 0.18^{a}	0.26 ± 0.01^{g}	0.36 ± 0.02^{fg}	0.37 ± 0.01^{efg}	0.51 ± 0.01^{def}	0.83 ± 0.02^{ab}	0.68 ± 0.04^{bc}	0.41 ± 0.02^{efg}	0.36 ± 0.04^{fg}	0.58 ± 0.04^{cd}	0.53 ± 0.03^{cde}	0.69 ± 0.03^{bc}
24:0	0.32 ± 0.01^{de}	0.71 ± 0.02^{b}	0.47 ± 0.02^{c}	0.86 ± 0.02^{a}	0.95 ± 0.05^{a}	0.31 ± 0.01^{de}	0.70 ± 0.03^{b}	0.48 ± 0.01^{c}	0.34 ± 0.01^{de}	0.36 ± 0.01^{de}	0.35 ± 0.01^{de}	0.76 ± 0.08^{b}	0.37 ± 0.04^{d}	0.27 ± 0.1^{e}	0.36 ± 0.02^{de}
24:1n-9	0.13 ± 0.00^{h}	0.15 ± 0.01^{fgh}	0.23 ± 0.01^{cd}	0.44 ± 0.02^{a}	0.29 ± 0.01^{b}	0.13 ± 0.00^{h}	0.18 ± 0.01^{ef}	0.24 ± 0.01^{c}	0.20 ± 0.00^{de}	0.14 ± 0.00^{gh}	0.15 ± 0.00^{fgh}	0.17 ± 0.01^{efg}	0.23 ± 0.02^{cd}	0.22 ± 0.01^{cd}	0.13 ± 0.01^{h}
22:6n-3 (DHA)	0.28 ± 0.01^{ef}	0.36 ± 0.02^{cde}	0.39 ± 0.02^{bc}	0.69 ± 0.09^{a}	0.64 ± 0.03^{a}	0.28 ± 0.01^{ef}	0.26 ± 0.01^{f}	0.46 ± 0.04^{b}	0.46 ± 0.03^{b}	0.37 ± 0.05^{bcde}	0.29 ± 0.01^{def}	0.32 ± 0.01^{cdef}	0.38 ± 0.02^{bcd}	0.16 ± 0.01^{g}	0.37 ± 0.01^{bcde}
ΣSFA	46.61 ± 0.76^{b}	49.24 ± 0.69^{a}	42.71 ± 0.76^{c}	37.44 ± 0.12^{e}	47.91 ± 0.76^{ab}	46.38 ± 0.57^{b}	46.29 ± 0.93^{b}	41.38 ± 0.51^{cd}	38.08 ± 0.21^{e}	37.42 ± 0.57^{e}	46.07 ± 0.48^{b}	46.48 ± 0.36^{b}	41.18 ± 0.32^{cd}	40.81 ± 0.92^{d}	37.80 ± 0.57^{e}
ΣMUFA	34.57 ± 0.54^{d}	32.69 ± 0.53^{e}	36.61 ± 0.28^{ab}	28.75 ± 0.18^{fg}	28.46 ± 0.45^{g}	35.20 ± 0.17^{cd}	34.51 ± 0.78^{de}	37.74 ± 0.41^{a}	29.33 ± 0.42^{fg}	29.78 ± 0.32^{f}	36.00 ± 0.23^{bc}	34.15 ± 0.47^{d}	36.20 ± 0.19^{bc}	29.75 ± 0.31^{f}	29.92 ± 0.19^{f}
ΣPUFA	18.81 ± 0.16^{ghi}	18.08 ± 0.29^{j}	20.68 ± 0.18^{f}	31.82 ± 0.38^{b}	23.68 ± 0.28^{d}	18.42 ± 0.14^{hi}	19.20 ± 0.18^{gh}	20.87 ± 0.12^{f}	32.61 ± 0.51^{a}	32.81 ± 0.17^{a}	18.03 ± 0.22^{i}	19.38 ± 0.21^{g}	22.68 ± 0.21^{e}	29.48 ± 0.29^{c}	32.28 ± 0.26^{ab}
Σn-3	2.12 ± 0.09^{e}	2.64 ± 0.05^{d}	2.15 ± 0.02^{e}	4.53 ± 0.04^{c}	5.08 ± 0.09^{a}	2.08 ± 0.07^{e}	2.69 ± 0.08^{d}	2.10 ± 0.04^{e}	5.02 ± 0.05^{a}	4.79 ± 0.04^{b}	2.05 ± 0.03^{e}	2.70 ± 0.05^{d}	1.70 ± 0.02^{f}	2.79 ± 0.09^{d}	4.71 ± 0.06^{b}
Σn-6	16.69 ± 0.18^{e}	15.44 ± 0.16^{g}	18.53 ± 0.01^{d}	27.29 ± 0.17^{b}	18.60 ± 0.23^{d}	16.34 ± 0.03^{fg}	16.51 ± 0.24^{fg}	18.77 ± 0.19^{d}	27.59 ± 0.18^{ab}	28.02 ± 0.26^{a}	15.98 ± 0.11^{fg}	16.67 ± 0.11^{e}	20.99 ± 0.14^{c}	26.69 ± 0.12^{b}	27.57 ± 0.54^{ab}
EPA+DHA	0.47 ± 0.02^{de}	0.39 ± 0.03^{ef}	0.60 ± 0.03^{c}	0.92 ± 0.01^{a}	0.89 ± 0.02^{a}	0.50 ± 0.04^{g}	0.38 ± 0.01^{f}	0.71 ± 0.03^{b}	0.70 ± 0.01^{b}	0.57 ± 0.02^{c}	0.54 ± 0.06^{cd}	0.44 ± 0.03^{ef}	0.60 ± 0.05^{c}	0.45 ± 0.01^{ef}	0.57 ± 0.02^{c}
n-6/n-3	7.89 ± 0.07^{d}	5.85 ± 0.12^{efg}	8.61 ± 0.15^{c}	6.02 ± 0.15^{ef}	5.66 ± 0.03^{fg}	7.84 ± 0.03^{d}	6.14 ± 0.15^{e}	8.93 ± 0.07^{c}	5.50 ± 0.12^{g}	5.85 ± 0.34^{efg}	7.80 ± 0.08^{d}	6.16 ± 0.05^{e}	12.35 ± 0.16^{a}	9.57 ± 0.15^{b}	5.86 ± 0.08^{efg}
PUFA/SFA	0.40 ± 0.02^{ef}	0.37 ± 0.02^{f}	0.48 ± 0.04^{cde}	0.85 ± 0.02^{a}	0.49 ± 0.05^{cde}	0.40 ± 0.01^{ef}	0.41 ± 0.03^{def}	0.50 ± 0.03^{cde}	0.86 ± 0.03^{a}	0.88 ± 0.03^{a}	0.38 ± 0.02^{f}	0.42 ± 0.01^{def}	0.55 ± 0.03^{c}	0.72 ± 0.04^{b}	0.85 ± 0.06^{a}

Results expressed as mean ± standard deviation (SD) of triplicate. Values with different letters on the same line are significantly different ($p < 0.05$) by Tukey's test. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; M1: mother 1; M2: mother 2; M3: mother 3; M4: mother 4; M5: mother 5; T1: before starting the feed, time zero (previous milk); T2: after 15 min of feeding (intermediate milk); T3: after 30 min of feeding (posterior milk); CLA: conjugated linoleic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; AA: arachidonic acid.

29 fatty acids were identified in all samples, the data obtained are shown in Table 1.

Analyzing Table 1, for all samples, the major fatty acid was oleic acid (18:1n-9), followed by palmitic acid (16:0) for M1, M2, and M3. For M4 and M5 it was linoleic acid (18:2n-6) in greater proportion. This variation in the concentration of fatty acids can be attributed to the difference in the diet between the analyzed breastfeeding women. Food intake is one of the main reasons for the change in the concentration of fatty acids, this result corroborates what was found by Alves *et al*.[38]

The oleic acid (18:1n-9), the predominant one in all samples, did not present a significant difference when the breastfeeding times of each infant were compared. This fatty acid is a monounsaturated fatty acid, used by newborns as the main energy source, favors the absorption of fat and calcium by the small intestine, and also acts in the myelination of axons.[9]

The palmitic acid (16:0) belongs to the class of saturated fatty acids (SFA), the second most abundant in milk samples from M1, M2, and M3, and between M1 and M2 there was no statistically significant difference when comparing the time of feeding. However, for the other samples, the difference was statistically significant, that is, M3 and M5 at T1 resulted in higher values and for M4 it was at T3. This fatty acid is extremely important for the neonate, as it is responsible for increasing the concentration of anandamide, a neurotransmitter that produces an analgesic effect, improving intestinal discomfort and reducing cramps. This benefit is related to the position that palmitic acid occupies in position in the TAG molecule, according to studies,[8,39] 70% of palmitic acid is in the Sn2 position, the central position in the TAG molecule, thus it is more easily absorbed by the body.

Among the polyunsaturated fatty acids (PUFA), linoleic acid (18:2n-6) was the one with the highest concentration. When comparing the breastfeeding time of M1, M2, and M4, the concentration of linoleic acid did not show any statistical difference between the milk samples, but for M3 in T3 and M5 in T1 there was a statistical difference.

This PUFA is the main representative of the omega 6 series, is considered a strictly essential fatty acid, that is, it can only be acquired through food, and is a precursor of arachidonic acid (AA, 20:4n-6) also found in this work. The values verified between M1, M2 and M5 were not statistically significant when comparing the times of breastfeeding. However, in T1 of M3 and M4, there was a statistical difference, and in both cases, T1 was the one with the highest value.

Another strictly essential fatty acid is identified as alpha-linolenic acid (18:3n-3). Feeding times did not influence the concentration of this fatty acid in M1 and M2. For M3, M4 and M5 there was a statistical difference between the means, and in T3 of M3 and M4 there was a lower concentration and in M5 there was a greater amount than in T1.

Alpha linolenic fatty acid is a precursor of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). For EPA, all milk samples showed statistical differences between breastfeeding times, with M1 and M2 at T1, M5 at T1, and T3 and M3 at T2 with the highest concentration.

Regarding DHA, the breastfeeding time between M1 and M3 showed no statistical difference. The highest concentration of DHA was found in T2 of M1 and T1 of M4 and M5.

Therefore, all essential and strictly essential fatty acids (linoleic and alpha-linolenic) were identified in the samples. These fatty acids are important, as they maintain cell membranes, brain functions under normal conditions, participate in the transfer of atmospheric oxygen to the blood plasma, and are also the precursors of AA, EPA, and DHA that were similarly found in this work. These fatty acids play an important role in the cognitive, visual, brain, and immune development of the newborn, in addition to protecting and reducing allergies, asthma, and childhood obesity.[40,41]

Conjugated linoleic (CLA) were identified in the samples. The concentration of T9 and t11 of mothers M1, M2 and M4 do not show statistical difference when compared to breastfeeding time, the mother M3 in T3 was the one with the highest concentration. M3 at T1, M4 at T3, and M5 at T1 and T3 were the ones with the highest concentration for t10, C12 except for mothers M1 and M2 did not show any statistical difference when compared to the breastfeeding time, and for mothers M4 and M5, T3 was the one with the highest concentration and for M3 the T1 was the one with the highest concentration. These conjugated linoleic fatty acids are extremely important for the neonate, as they are linked to the reduction of body fat, modulation of the immune system, improvement in bone mineralization, as well as antidiabetic and anticarcinogenic effects.[42]

Thus, it was possible to observe that, except for the samples of M1 and M2, which showed differences in the concentration of FA in the EPA, the other mothers showed significant differences in the concentration of the main fatty acids, when compared to those concerning breastfeeding times. This is because studies indicate that the concentration of milk fat increases at the end of the feed with the emptying of the breast,[18,19] thus affecting the concentration of fatty acids.

The ideal ratio according to Simopoulos[43] for n6/n3 is 5 to 10, almost all samples met this parameter, that is, the values were between 5 to 10 except M3 in T3, which

was greater than 10, a possible imbalance, which suggests an inflammatory process and may contribute to the onset of diseases such as atherosclerosis and obesity. This relationship is important to be kept within the standard, as these FA compete for the metabolic pathways of elongation and desaturation, causing a possible imbalance in the concentration of strictly essential fatty acids.

Determination of triacylglycerol (TAG) from human milk colostrum

Table 2 presents the 21 highest *m/z* ratios, with their respective TAGs, found in the region between 792 and 904 *m/z*, with 29 different TAGs. The platform used (LAMES) was developed for the random configuration of TAGs.[26] However, for human milk, the order of FAs in the TAG (Sn-1, Sn-2, Sn-3) is not in agreement with the literature; because the palmitic acid (16:0) for this matrix (human milk) is mostly in the Sn-2 position, while unsaturated FAs, such as oleic acid (18:1n-9) and linoleic acid (18:2n-6) are preferably esterified at the Sn-1,3 positions.[44,45]

The highest estimates in percentage of TAGs identified in sample M1 were: *m/z* 876 POO, in T1 12.769%, T2 13.105% and T3 13.684%; *m/z* 874 PLO at T1 12.265%, T2 12.090% and T3 11.888%; *m/z* 850 POP in T1 11.341%,

Table 2. Estimate of TAG ions defined by the LAMES platform from human milk colostrum at different times of feeding

				TAG ions / %													
				Sample													
				T1					T2					T3				
				M1	M2	M3	M4	M5	M1	M2	M3	M4	M5	M1	M2	M3	M4	M5
$C_{49}H_{90}O_6$	46:2	792	PLLa	–	1.721	–	2.322	–	–	–	–	2.477	2.000	–	1.785	–	3.054	2.032
$C_{49}H_{92}O_6$	46:1	794	POLa	2.268	3.373	2.116	2.119	–	2.367	3.146	2.208	2.310	1.876	2.053	3.217	2.001	2.937	1.949
$C_{49}H_{94}O_6$	46:0	796	PMP	2.313	2.431	–	–	2.671	2.185	2.312	–	–	–	1.970	2.439	–	–	–
$C_{51}H_{90}O_6$	48:4	816	LaLL	–	–	–	–	–	–	–	–	–	–	–	–	–	1.820	–
$C_{51}H_{92}O_6$	48:3	818	LaLO	–	1.888	–	2.706	–	–	1.840	–	3.035	2.507	–	1.906	–	3.500	2.567
$C_{51}H_{94}O_6$	48:2	820	LaOO	–	1.850	–	–	–	–	1.739	–	–	–	–	1.718	–	–	–
$C_{51}H_{94}O_6$	48:2	820	PLM	2.501	2.722	1.728	2.787	3.331	2.340	2.704	–	3.202	2.545	2.043	2.890	–	3.115	2.570
$C_{51}H_{96}O_6$	48:1	822	POM	5.208	5.334	3.350	–	4.671	5.073	5.112	3.268	2.987	2.387	4.704	5.209	2.835	2.995	2.465
$C_{51}H_{98}O_6$	48:0	824	PPP	3.357	3.194	3.487	2.543	4.139	3.240	3.142	3.119	–	–	3.199	3.168	2.767	1.870	–
$C_{53}H_{94}O_6$	50:4	844	MLL	–	–	–	–	–	–	–	–	2.103	–	–	–	–	1.856	–
$C_{53}H_{96}O_6$	50:3	846	MLO	2.816	2.986	1.988	3.247	2.913	2.717	2.990	2.099	3.924	3.191	2.440	3.086	2.121	3.570	3.247
$C_{53}H_{98}O_6$	50:2	848	PLP	5.447	5.363	6.208	6.834	7.745	5.207	5.512	6.011	6.162	6.211	4.978	5.630	6.208	6.688	6.126
$C_{53}H_{98}O_6$	50:2	848	MOO	2.932	2.926	1.926	–	2.043	2.945	2.826	2.005	–	–	2.808	2.781	–	–	–
$C_{53}H_{100}O_6$	50:1	850	POP	11.341	10.512	12.033	6.237	10.860	11.287	10.420	11.481	5.748	5.826	11.460	10.149	10.257	6.431	5.876
$C_{53}H_{102}O_6$	50:0	852	SPP	2.761	2.288	2.146	–	3.561	2.767	2.169	1.908	–	–	2.901	2.080	1.923	–	–
$C_{53}H_{102}O_6$	50:0	852	OVcL	–	–	–	–	–	–	–	–	–	–	–	–	1.996	–	–
$C_{55}H_{98}O_6$	52:4	872	PLL	2.945	3.002	3.684	8.725	4.830	2.789	3.224	3.861	8.095	8.301	2.582	3.336	4.643	7.970	8.068
$C_{55}H_{100}O_6$	52:3	874	PLO	12.265	11.767	14.281	15.924	13.546	12.090	12.190	14.749	15.102	15.573	11.888	12.026	15.343	15.330	15.479
$C_{55}H_{100}O_6$	52:3	874	PVcO	–	–	2.204	–	–	–	1.809	2.650	–	–	–	1.991	2.668	–	–
$C_{55}H_{104}O_6$	52:2	876	POO	12.769	11.532	13.840	7.266	9.498	13.105	11.521	14.087	7.044	7.303	13.684	10.838	12.675	7.371	7.424
$C_{55}H_{104}O_6$	52:2	876	SLP	2.986	2.562	2.547	3.583	4.441	2.964	2.538	2.450	3.202	3.454	3.009	2.632	2.877	2.443	3.466
$C_{55}H_{104}O_6$	52:1	878	SOP	6.216	5.021	4.937	3.270	6.228	6.426	4.797	4.681	2.987	3.240	6.927	4.443	4.753	2.349	3.325
$C_{57}H_{98}O_6$	54:6	896	LLL	–	–	–	3.713	–	–	–	–	3.545	3.699	–	–	–	3.166	3.542
$C_{57}H_{100}O_6$	54:5	898	OLL	3.316	3.293	4.237	10.165	4.224	3.238	3.565	4.737	9.920	10.407	3.083	3.562	5.738	9.135	10.194
$C_{57}H_{103}O_6$	54:4	900	OLO	6.905	6.454	8.213	9.276	5.923	7.019	6.739	9.048	9.254	9.761	7.097	6.421	9.481	8.785	9.778
$C_{57}H_{102}O_6$	54:4	900	SLL	–	–	–	2.287	–	–	–	–	2.103	2.308	–	–	–	–	2.283
$C_{57}H_{104}O_6$	54:3	902	OOO	4.792	4.217	5.306	2.822	2.769	5.072	4.246	5.761	2.877	3.052	5.446	3.858	5.221	2.816	3.127
$C_{57}H_{104}O_6$	54:3	902	SLO	3.361	2.810	2.929	4.174	3.884	3.441	2.806	3.007	3.924	4.330	3.593	2.632	3.555	2.800	4.379
$C_{57}H_{106}O_6$	54:2	904	SOO	3.500	2.754	2.839	–	2.723	3.730	2.652	2.872	–	2.031	4.136	2.372	2.937	–	2.101

La: lauric acid (12:0); M: myristic acid (14:0); P: palmitic acid (16:0); S: stearic acid (18:0); O: oleic acid (18:1n-9); Vc: vaccenic acid (18:1n-7); L: linoleic acid (18:2n-6); TAG: triacylglycerides; M1: mother 1; M2: mother 2; M-3: mother 3; M4: mother 4; M5: mother; T1: before starting the feed, time zero (previous milk); T2: after 15 min of feeding (intermediate milk); T3: after 30 min of feeding (posterior milk).

T2 11.287% and T3 11.460%; *m/z* 900 OLO in T1 6.905%, T2 7.019% and T3 7.097% and *m/z* 878 SOP in T1 6.216%, T2 6.426% and T3 6.927%. Among the other samples (M2 to M5) the majority of TAG is presented by *m/z* 874 PLO. Furthermore, samples M2 and M3 are similar among the attribution of their five highest TAGs, according to *m/z* 874 PLO, *m/z* 876 POO, *m/z* 850 POP, *m/z* 900 OLO, and *m/z* 848 PLP, respectively. In sample M4, the highest TAGs have new formation, being expressed by *m/z* 874 PLO, *m/z* 898 OLL, *m/z* 900 OLO, *m/z* 872 PLL, and *m/z* 876 POO.

Differently from the others, the estimate of TAGs between the feeding times T1, T2, and T3 in sample M5 had differences as compared to the sample in T1, being *m/z* 874 PLO, *m/z* 850 POP, *m/z* 876 POO, *m/z* 848 PLP and *m/z* 878 SOP. In T2 and T3 they were *m/z* 874 PLO, *m/z* 898 OLL, *m/z* 900 OLO, *m/z* 876 POO, and *m/z* 848 PLP, respectively.

The TAG percentage estimation results in this study are similar to the TAGs identified in the research carried out by Manin *et al*.,[46] who analyzed pasteurized and lyophilized human milk in its three stages of lactation. In Table 2, TAGs that do not appear in percentage were observed, this is because the evaluated percentage is below 1% of estimate in the samples. This is a factor used to select the main TAGs in the LAMES Platform, that is, TAGs that present a percentage below 1% do not appear in the estimate.

Fat is the main energy component of human milk and comprises a complex mixture of different lipid species, with a quantitative predominance of TAGs. [8] Human milk TAGs are formed in the endoplasmic reticulum from fatty acids removed from the circulation or synthesized again in the mammary epithelial cells from glucose.[47] Neutral lipids including TAG, diacylglycerols, monoacylglycerols, and sterol esters contribute 98% (m/m) or more to total milk fat, and about 95% of these are provided by fatty acids.[48]

An important point to consider is the positioning of palmitic acid in the glycerol structure, in contrast to most TAG synthesized by mammals, the 16:0 in human milk is mainly positioned at Sn2 in the TAG, the central carbon atom of the glycerol structure.[45] Dietary distribution of triacylglycerol fatty acids can alter lipoprotein metabolism in infants.[49] However, the mammary gland can modulate bioactive lipid profiles present in different stages of lactation, from colostrum to mature milk.[50]

IL-10 and TNF-α concentration in colostrum human milk

The concentration of cytokines in colostrum at different times of feeding, anterior, intermediate, and posterior were evaluated, the concentration data are presented in Table 3.

Table 3. The concentration of cytokines TNF-α and IL-10 in colostrum milk at different times of breastfeeding

Sample	IL-10 / (pg mL^{-1})	TNF-α / (pg mL^{-1})
M1T1	36.12 ± 0.98^{b}	14.27 ± 0.67^{b}
M2T1	8.24 ± 0.42^{c}	2.96 ± 0.12^{d}
M3T1	7.66 ± 0.12^{cd}	13.88 ± 0.38^{b}
M4T1	3.32 ± 0.15^{e}	–
M5T1	5.87 ± 0.15^{cde}	5.93 ± 0.32^{c}
M1T2	152.45 ± 5.12^{a}	1.41 ± 0.04^{e}
M2T2	5.26 ± 0.28^{cde}	3.11 ± 0.31^{d}
M3T2	8.81 ± 0.17^{c}	15.85 ± 0.43^{a}
M4T2	8.24 ± 0.19^{c}	–
M5T2	3.98 ± 0.17^{de}	–
M1T3	32.98 ± 0.87^{b}	15.90 ± 0.29^{a}
M2T3	5.86 ± 0.19^{cde}	2.68 ± 0.19^{d}
M3T3	5.26 ± 0.22^{cde}	6.37 ± 0.21^{c}
M4T3	7.66 ± 0.16^{cd}	3.29 ± 0.18^{d}
M5T3	5.25 ± 0.26^{cde}	5.65 ± 0.28^{c}

Values were represented as mean ± standard deviation obtained from triplicates; values with different letters in the same column are significantly different ($p < 0.05$) by Tukey's test. IL-10: cytokines interleukin-10; TNF-α: tumor necrosis factor-alpha; M1: mother 1, M2: mother 2, M-3: mother 3, M4: mother 4; M5: mother; T1: before starting the feed, time zero (previous milk); T2: after 15 min of feeding (intermediate milk); T3: after 30 min of feeding (posterior milk).

The two cytokines were identified in all samples evaluated, but TNF-α had a value below the curve's limit of detection for M4 at times T1 and T2, and for M5 at T2.

Cytokines operate in networks and produce a cascade of effects that contribute to the orchestration, development, and functions of the immune system. Furthermore, cytokines mediate inflammatory responses often associated with the immune response. The ability of human milk to provide passive protection and actively modulate the development of the systemic and mucosal immune response is closely linked to the presence of substances with antimicrobial, anti-inflammatory, and immunomodulatory activities.[51]

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine and is recognized as an important mediator in many cytokine-dependent inflammatory events.[52] In this study, it was possible to observe that for M2 the breastfeeding time did not influence the concentration of this cytokine, however for the others it did. For M1 and M4 at T3 the concentration was higher, but in the milk sample from M3 at T2 (15.85 ± 0.43) and M5 at T1 (5.93 ± 0.32) it stood out with the highest value.

Studies show that IL-10 is present not only in the aqueous phase of milk but also in the lipid layer. Its bioactive properties were confirmed through a study that found that HM inhibited the proliferation of blood lymphocytes using an anti-IL-10 antibody.[53]

According to Garofalo,[12] interleukin-10 (IL-10) is an anti-inflammatory cytokine that exerts an immunomodulatory and anti-inflammatory activity. In the present study, the M2, M3, and M5 when compared to breastfeeding times did not show a significant difference in the concentration of IL-10. However, for M1 and M4, the concentration of IL-10 was higher at time 2, and both mothers had their babies vaginally.

Thus, the amount of IL-10 verified in all samples confirms the importance of this cytokine in human milk to maintain the baby's survival, due to its immunomodulatory, anti-inflammatory action, in the regulation of intestinal inflammation and the responses to the microbiome.[17,54]

Therefore, it is possible to observe that, except for M2, for the other mothers, the duration of breastfeeding influenced the concentration of IL-10 and TNF-α.

Principal component analysis (PCA)

The principal component analysis was performed in order to find a similarity between the fatty acid, kilocalorie and cytokine composition of the samples, with that PCA1 and PCA2 together explained about 71% of the general variance of the results. The variables (fatty acid, kilocalorie and cytokines) are represented in the form of loadings, while the samples in the form of scores. This can be evidenced in Figure 2 below.

The variables on the right of PCA1 contribute positively to the separation of this principal component, as an example we have 18:1n-9 and 14:0, while the variables on the left contribute negatively to the expression of this principal component, as an example we have ∑ n-6 and ∑ n-3, and the PCA1 component explains the greatest variation in the results. For PCA2, which explains about 17.5% of the results, we have that those in the upper quadrant contribute positively to the expression of this PCA2, as an example we have 18:1n-7 and EPA + DHA, while those in the lower quadrant, contributes negatively, we can cite as an example 18:0 and 18:3n-3.

As already observed in the PCA analysis, PCA1 explained the greater similarity of the samples, explaining about 53.5%, it is possible to observe that the scores present some similarity with each other, this happens mainly between the samples that are the same lactating women in which change only the feeding time, with this it is possible to observe according to the PCA analysis and together with the results of FAs, kilocalories and cytokines, that each mother has a different concentration of these variables, but it is also possible to observe that the breastfeeding time influences the concentration of FAs, kilocalories and cytokines, as the samples are a little distant from each other, even though they belong to the same mother, this is quite evident for M5, which represents samples 13, 14 and 15 that are quite far apart.

The loadings do not have the same distance from the center of the graph, thus showing that they had a different significance when expressing the PCA.

In the upper left quadrant we have the presence of samples 10 referring to M4T1, sample 12 referring to

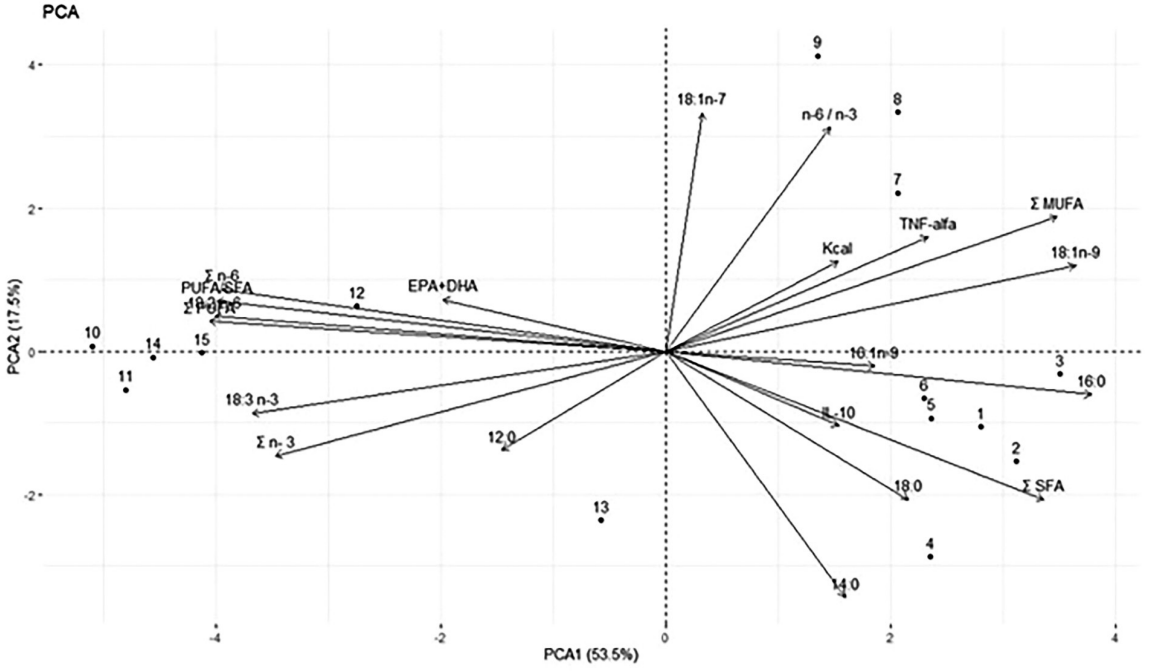

Figure 2. PCA biplot of fatty acid, kilocalorie, and cytokine composition of the samples. Samples: 1: M1T1; 2: M1T2; 3: M1T3; 4: M2T1; 5: M2T2; 6: M2T3; 7: M3T1; 8: M3T2; 9: M3T3; 10: M4T1; 11: M4T2; 12: M4T3; 13: M5T1; 14: M5T2; 15: M5T3.

M4T3 and sample 15 M5T3, this is due to the fact that these samples have a higher concentration of omega 6, and this variable as well as PUFA/SFA, ∑PUFA contribute more to the formation of this quadrant, and also these samples showed a higher value of EPA + DHA, however for this variable it contributes less to the formation of this quadrant. While in the upper left quadrant we have samples 7 referring to M3T1, sample 8 referring to M3T2 and sample 9 referring to M3T3, where the following variables appear contributing to this separation: 18:1n-7, n6/n3, monounsaturated fatty acid sum (∑ MUFA) and 18:1n-9, while the variables kcal and TNF-α, contribute less to the formation of this separation.

As for the lower quadrant, we have on the right the presence of most samples, 1 referring to M1T1, sample 2 referring to M1T2, sample 3 referring to M1T3, sample 4 referring to M2T1, sample 5 which refers to M2T2, sample 6 M2T3, in this quadrant we have the presence of the variables 16:0, ∑SFA, 14:0 among other variables. In the lower left quadrant we have sample 11 that refers to M4T2, sample 13 that refers to M5T1 and sample 14 that refers to M5T2, in this quadrant we observe the presence of the variables ∑ n-3 and 18:3n-3 which has the highest contribution to the quadrant expression, and also the 12:0 variable that has the lowest contribution to the quadrant expression.

Conclusions

With this we can determine that the highest concentration of kilocalories was in hind milk for all samples, for sample M1 the concentration of kilocalories in hind milk was 53% higher than in fore milk, for M2 it was 102%, for M3 it was 55%, for M4 it was 59%, and for M5 was 9%. Thus, when it is necessary to offer the lactating high-calorie milk, the use of hind milk is recommended.

Among the composition of FAs, it was possible to observe that, with the exception of samples M1 and M2, the other mothers showed differences in the composition of the main FAs, this can be explained by the concentration of milk fat that is increased in the posterior milk as a result of emptying of the breast, thus affecting the composition of FAs.

The highest percentages of TAGs identified in sample M1 were in the region *m/z* 876 (POO), *m/z* 874 (PLO), *m/z* 850 (POP), *m/z* 900 (OLO) and *m/z* 878 (SOP), respectively; among the other samples (M2 to M5) the majority TAG is presented by the *m/z* 874 PLO. In addition, samples M2 and M3 are similar in terms of the assignment of their five largest TAGs. In the M4 sample, the largest TAGs obtained new formation in their percentages. And different from the others, in the sample M5 it was obtained differences regarding the anterior milk (T1), and similarities between the intermediate milk (T2) and posterior milk (T3).

Breastfeeding time influenced the concentration of TNF-α in all mothers evaluated, with the exception of M2; for IL-10, only mothers M1 and M4 showed a statistical difference considering the breastfeeding time. Therefore, there is an influence on the duration of breastfeeding on the composition and concentration of kilocalories, on the concentration of some FAs, TAGs, and cytokines (IL-10 and TNF-α).

The PCA analysis explained 71% of the total variance of the results, showing that the concentration of FAs, kilocalories and cytokines, differ from one mother to another, the breastfeeding time is also evidenced a difference between the times studied and the variables analyzed.

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Author Contributions

Matheus C. Castro was responsible for investigation, formal analysis, data curation, writing- original draft, conceptualization, software; Francieli S. Oliveira for investigation, conceptualization, data curation, formal analysis, visualization; Eloize S. Alves for formal analysis, data curation, writing-original draft; Joana M. V. Zacarias for formal analysis, visualization; Josiane B. Alencar for formal analysis, visualization; Jeane E. L. Visentainer for resources, conceptualization, supervision; Jiuliane M. da Silva for writing-review and editing; Oscar Oliveira Santos for resources, supervision; Jesui V. Visentainer for resources, conceptualization, supervision; Sueli M. T. Ichisato for funding acquisition, supervision, writing-review and editing.

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