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ESTABILIDADE LIPÍDICA DO LEITE HUMANO SUBMETIDO AOS PROCESSOS DE ALTA PRESSÃO, PASTEURIZAÇÃO E LIOFILIZAÇÃO

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Maringá
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Tese apresentada à Universidade Estadual de Maringá, como parte das exigências do Programa de Pós-Graduação em Ciência de Alimentos, para obtenção do grau de Doutor em Ciência de Alimentos.

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BIOGRAFIA

Luciana Pelissari Manin nasceu no estado do Paraná, na cidade de Maringá. Possui graduação em Engenharia de Alimentos pela Universidade Estadual de Maringá, e mestrado em Ciência de Alimentos pela Universidade Estadual de Maringá. Tem experiência na área de Ciência de Alimentos atuando principalmente nos seguintes temas: processamento do leite humano, processos de liofilização, pasteurização e alta pressão hidrostática, estabilidade lipídica, composição em ácidos graxos por cromatografia em fase gasosa e perfil de triacilgliceróis por espectrometria de massas com ionização por *electrospray*.

Dedico

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APRESENTAÇÃO

Essa tese de doutorado está apresentada no formato de dois artigos científicos.

1. **Autores**: Luciana Pelissari Manin, Adriela Albino Rydlewski, Eloize Silva Alves, Isadora Boaventura Ponhozi, Matheus Campos Castro, Bruno Henrique Figueiredo Saqueti, Oscar Oliveira Santos, Jesuí Vergilio Visentainer.
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GENERAL ABSTRACT
ARTICLE 1

INTRODUCTION. Human milk (HM) is the best food for newborn nutrition. It is a dynamic fluid and changes over time, adapting itself to the needs of a baby. Lipids are an important macronutrient in HM. Besides energy, lipids play a fundamental role in neurological development; provide an important source of fatty acids, including long-chain polyunsaturated fatty acids (LC-PUFA), such as docosahexaenoic acid (omega-3) and arachidonic acid (omega-6), which are related to the child's cognitive development. Recent studies have shown that the fatty acid (FA) composition of HM varies with lactation period. Colostrum is the first liquid released by the mammary glands, in a small and sufficient volume to feed the newborn. It contains a high concentration of immunologically and physiologically active components to build a baby's immune system. From days 5 to 14, the mother secretes transitional milk. It has higher levels of calories than colostrum and is responsible for the baby's growth and development. Mature milk is the final stage of breast milk transition, important for the immune system, in addition to the development and well-being of the baby. It is produced from the fifteenth day until weaning. In the event of a mother being unable to breastfeeding her child, human milk banks (HMB) offer a safe alternative to provide HM for babies. The HMB are responsible for collecting, identifying, processing, and distributing donated milk. The donated HM is pasteurized using the Holder method to minimize the risk of transmission of infections. This method is carried out in a water bath at 62.5 ºC for 30 minutes, followed by rapid freezing at -18 ºC. However, new alternatives are being studied in order to preserve the properties of HM, such as lyophilization, considered an alternative to freezing applied to HM, which is a technology that aims to increase the shelf life, facilitate the storage and transport, and preserve the nutritional characteristics of the donated HM.

AIMS. The aim of this study was to analyze the acidity in Dornic degrees, the FA composition, and the triacylglycerol (TAG) profile of raw, pasteurized, and pasteurized lyophilized HM in the three lactation stages (colostrum, transitional, and mature) using gas chromatography with flame ionization detector and electrospray ionization mass spectrometry, in order to verify if the lyophilization and pasteurization processes are efficient at preserving the FA and TAG of HM, as compared to raw HM.

MATERIAL AND METHODS. The present study was approved by the Research Ethics Committee of the State University of Maringá, with approval number 2,797,476. Raw HM samples were collected from the three lactation phases (colostrum, transitional, and mature), separated into three pools with a final volume of 300 mL for each phase. A volume of 100 mL of pooled milk of each lactation phase was stored without processing. The remaining 200 mL underwent Holder pasteurization. An amount of 100 mL of pasteurized HM was frozen at -18 °C and the remaining pasteurized milk (100 mL) was lyophilized. Dornic acidity, fatty acid composition by gas chromatography with flame ionization detector, and the TAG profile by electrospray ionization mass spectrometry (ESI-MS) were determined in the raw HM, frozen pasteurized HM, and lyophilized pasteurized HM from the three lactation stages. TAG ions were assigned and estimated in percentage (%) using the LAMES Platform. The data obtained were subjected to analysis of variance (ANOVA) with 5% significance ($p < 0.05$), and the means compared by Tukey's test.

RESULTS AND DISCUSSION. For HM to be considered suitable for consumption by babies, the acidity needs to be in the range of 1 to 8 degrees Dornic (°D). This analysis is an indirect measure of the degree of HM contamination, since the fermentation of lactose by bacterial growth increases the concentration of lactic acid and, consequently, its acidity. The acidity values showed the following ranges: colostrum, from 3.66 to 4.33 °D; transitional,

from 3.66 to 4.33 °D; and mature, from 4.33 to 5.00 °D. The results did not show significant differences ($p < 0.05$) between them, that is, the acidity was not influenced by the applied treatments. Regarding the composition of FAs, twenty-six FAs were identified and determined in all samples. The results showed that the processes applied to the samples did not significantly influences the composition of FAs at the 5% significance level by Tukey's test ($p < 0.05$). The oleic acid (18:1n-9) and palmitic acid (16:0) were the FAs with the higher concentrations in all samples. The concentration of oleic acid, ranged from 30.62 to 32.14% in colostrum; in transitional between 26.64 to 26.91%; and in mature between 31.74 to 32.68%. Palmitic acid, showed values between 26.01 and 26.97% in colostrum; 23.23 to 23.56% in transitional; and 21.78 to 22.47% in mature. Results of ESI-MS presented that the higher peak (*m/z* 875 and 877) and TAG profile were similar among samples at their respective phase, indicating that the TAG profile did not change when pasteurization and lyophilization processes were used in the HM samples.
CONCLUSIONS. The results indicate that the pasteurization and lyophilization processes did not induce significant alterations in the fatty acids composition and TAG profile of HM. Therefore, the pasteurization used together with lyophilization can be a promising alternative to the freezing performed in HMBs, since it helps to reduce the storage volume and facilitates the transport of this food.
Keywords: fatty acid composition, triacylglycerol in human milk, human milk banks, Dornic acidity, human milk.

RESUMO GERAL
ARTIGO 1

INTRODUÇÃO. O leite humano (LH) é considerado um alimento ideal para os recém-nascidos, sendo um fluido dinâmico capaz de se adaptar e suprir às necessidades dos bebês. Os lipídios são componentes essenciais do LH, desempenham papel fundamental no desenvolvimento neurológico e atendem às altas necessidades energéticas da criança; além de serem fontes de vários ácidos graxos poliinsaturados de cadeia longa (AGPI-CL), como o ácido docosahexaenoico, da família ômega 3 e o ácido araquidônico, da família ômega 6, que estão relacionados ao desenvolvimento cognitivo da criança. Estudos recentes têm mostrado que a composição de ácidos graxos (AG) do LH varia com o período de lactação. O colostro é o primeiro líquido liberado pelas glândulas mamárias, em volume pequeno e suficiente para alimentar o recém-nascido. Ele contém uma alta concentração de componentes imunologicamente e fisiologicamente ativos para contribuir o sistema imunológico de um bebê. Do 5º ao 14º dia, o leite é considerado de transição, tem níveis mais elevados de calorias do que o colostro e é responsável pelo crescimento e desenvolvimento do neonato. O leite maduro é a etapa final do HM, importante para o sistema imunológico, além do desenvolvimento e bem-estar do bebê. É produzido a partir do décimo quinto dia até o desmame. No caso de uma mãe não conseguir amamentar seu filho, os bancos de leite humano (BLH) oferecem uma alternativa segura para fornecer LH para bebês. Os BLH são responsáveis por coletar, identificar, processar e distribuir o LH doado. O LH doado é pasteurizado pelo método de Holder, para minimizar o risco de transmissão de infecções. Este método é realizado em banho-maria a 62,5 ºC por 30 minutos, seguido de congelamento à -18 ºC. No entanto, novas alternativas estão sendo estudadas a fim de preservar as propriedades do LH, como a liofilização. Considerada uma alternativa ao congelamento aplicado ao LH, que é uma tecnologia que visa aumentar a vida de prateleira, facilitar o armazenamento e transporte e preservar a características nutricionais do LH doado.
OBJETIVOS. O objetivo deste estudo foi analisar a acidez em graus Dornic, a composição em AG e o perfil de triacilglicerol (TAG) do LH cru, pasteurizado e pasteurizado liofilizado,

nas três fases de lactação (colostro, transição e maduro), usando cromatografia gasosa com detector de ionização em chama e espectrometria de massa por ionização eletrospray, a fim de verificar se os processos de liofilização e pasteurização são eficientes em preservar os AGs e os TAGs do LH, em comparação ao LH cru.

MATERIAIS E MÉTODOS. O presente estudo foi aprovado pelo Comitê de Ética em Pesquisa da Universidade Estadual de Maringá, sob o número 2.797.476. Amostras de LH cru foram coletadas das três fases da lactação (colostro, transição e madura), separados em três pools, com volume final de 300 mL para cada fase. Um volume de 100 mL de leite de cada fase da lactação foi armazenado para as amostras sem processamento. E os 200 mL restantes foram pasteurizados por Holder. Uma quantidade de 100 mL de LH pasteurizado foi congelada à -18 °C e o restante do LH pasteurizado (100 mL) foram liofilizados. A acidez Dornic, a composição em AGs por cromatografia gasosa com detector de ionização em chama e o perfil de TAG por espectrometria de massa com ionização eletrospray (ESI-MS) foram determinados no LH cru, LH pasteurizado congelado e pasteurizado liofilizado, nas três fases da lactação. Os íons TAG foram atribuídos e estimados em porcentagem (%) usando a Plataforma LAMES. Os dados obtidos foram submetidos à análise de variância (ANOVA) com significância de 5% ($p < 0,05$), e as médias comparadas pelo teste de Tukey.

RESULTADOS E DISCUSSÃO. Para que o LH seja considerado adequado para consumo dos bebês, a acidez precisa encontrar-se na faixa de 1 a 8 graus Dornic (°D). Essa análise é uma medida indireta do grau de contaminação do LH, uma vez que a fermentação da lactose pelo crescimento bacteriano aumenta a concentração de ácido lático e, conseqüentemente, sua acidez. Os valores de acidez apresentaram as seguintes faixas: LH colostro, de 3,66 a 4,33 °D; LH de transição, de 3,66 a 4,33 °D; e LH maduro, de 4,33 a 5,00 °D. Os resultados não mostraram diferenças significativas ($p < 0,05$) entre eles, ou seja, a acidez não foi influenciada pelos tratamentos aplicados. Em relação à composição em AGs, vinte e seis AGs foram identificados e determinados em todas as amostras. Os resultados mostraram que os processos aplicados às amostras não influenciaram significativamente a composição em AGs, ao nível de significância de 5% pelo teste de Tukey ($p < 0,05$). O ácido oléico (18:1n-9) e o ácido palmítico (16:0) foram os AGs com maiores concentrações em todas as amostras. A concentração de ácido oléico variou de 30,62 a 32,14% no colostro; no LH de transição entre 26,64 a 26,91%; e no LH maduro entre 31,74 a 32,68%. O ácido palmítico apresentou valores entre 26,01 e 26,97% no LH colostro; 23,23 a 23,56%, no LH de transição; e 21,78 a 22,47% no LH maduro. Os resultados do ESI-MS mostraram que o pico mais alto (*m/z* 875 e 877) e o perfil de TAG foram semelhantes entre as amostras em suas respectivas fases, indicando que o perfil de TAG não mudou quando os processos de pasteurização e liofilização foram aplicados nas amostras de LH.

CONCLUSÃO. Os resultados indicam que os processos de pasteurização e liofilização não induziram alterações significativas na composição em AGs e no perfil de TAG do LH. Portanto, a pasteurização utilizada em conjunto com a liofilização pode ser uma alternativa promissora ao congelamento realizado em BLHs, pois auxilia na redução do volume de armazenamento e facilita o transporte deste alimento.

Palavras-chave: composição em ácidos graxos, triacilgliceróis em leite humano, bancos de leite humano, acidez Dornic, leite humano.

ARTICLE 1

Evaluation of the lipid composition of the three lactation phases of raw, pasteurized and lyophilized pasteurized human milk

Avaliação da composição lipídica das três fases de lactação do leite humano cru, pasteurizado e pasteurizado liofilizado

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Abstract

The aim of this study was to analyze the acidity in Dornic degrees, fatty acid (FA) composition, and the triacylglycerol (TAGs) profile of human milk (HM) from three lactation phases (colostrum, transitional, and mature) submitted to different treatments (pasteurization and pasteurization in conjunction with lyophilization) to verify if these processes applied to the HM samples can influence the characteristics of the analyzed components. This project was approved by the ethics committee of the State University of Maringá (Maringá, Paraná, Brazil). The HM was acquired at the Human Milk Bank (HMB) of the University Hospital of Maringá (HUM; Paraná, Brazil). The acidity analysis was performed using the titratable acidity method in Dornic degrees (ºD); the FA composition was determined using the Gas Chromatography with Flame Ionization Detector (GC-FID); and the TAG profile was evaluated using the Electrospray Ionization Mass Spectrometry (ESI-MS). Results showed

that the Dornic acidity and the FA composition of HM samples were not influenced by pasteurization and pasteurization combined with lyophilization processes ($p < 0.05$). The TAG profiles of HM submitted to pasteurization and pasteurization combined with lyophilization processes remained similar to raw samples at their respective phase. Therefore, the pasteurization technique in conjunction with lyophilization can be a promising alternative for HM storage and conservation in HMBs, as it guarantees the preservation of the evaluated components, in addition to reducing the storage volume and facilitating the transport of this HM.
Keywords: Gas chromatography; Human milk; Fatty acids; Triacylglycerol; Acidity.

Resumo

O objetivo deste estudo foi analisar a acidez em graus Dornic, a composição em ácidos graxos (AG) e o perfil de triacilglicerol (TAG) do leite humano (LH) nas três fases da lactação (colostro, transição e maduro) submetido a diferentes tratamentos (pasteurização e pasteurização em conjunto com a liofilização) para verificar se esses processos aplicados às amostras de LH podem influenciar nas características dos componentes analisados. Este projeto foi aprovado pelo comitê de ética da Universidade Estadual de Maringá (Maringá, Paraná, Brasil). O LH foi adquirido no Banco de Leite Humano (BLH) do Hospital Universitário de Maringá (HUM; Paraná, Brasil). A análise da acidez foi realizada pelo método da acidez titulável em graus Dornic (ºD); a composição em AG foi determinada utilizando a Cromatografia Gasosa com Detector de Ionização em Chama (GC-FID); e o perfil de TAG foi avaliado com auxilio da Espectrometria de Massa por Ionização por Eletrospray (ESI-MS). Os resultados mostraram que a acidez Dornic e a composição em AG das amostras de LH não foram influenciadas pelos processos de pasteurização e pasteurização combinada com a liofilização ($p < 0,05$). Os perfis de TAG dos LH submetidos aos processos de pasteurização e pasteurização combinada com a liofilização mantiveram-se semelhantes aos das amostras sem processamento em suas respectivas fases. Portanto, a técnica de pasteurização em conjunto com a liofilização pode ser uma alternativa promissora para armazenamento e conservação do LH em BLHs, pois garante a preservação dos componentes avaliados, além de reduzir o volume de armazenamento e facilitar o transporte deste LH.
Palavras-chave: Cromatografia em fase gasosa; Leite humano; Ácidos Graxos; Triacilglicerol; Acidez.

1. Introduction

Human milk (HM) is the best food for newborn nutrition. It is a dynamic fluid and changes over time, adapting its self to the needs of the newborn (Andreas et al., 2015). For this reason, exclusive breastfeeding during the first six months of life is recommended for the child's proper growth and development (Who, 2003).

Lipids are an important nutrient in HM. Besides energy, lipids play a fundamental role in neurological development; in the modulation of immune responses in health and disease (Koletzko, 2016); provide a source of long-chain polyunsaturated fatty acids (LC-PUFA), such as docosahexaenoic acid (DHA), and arachidonic acid (AA), which are related to the child's cognitive development. DHA is the most abundantomega-3 fatty acid in the brain and has impact on cognitive and behavioral performances. AA, an omega-6 fatty acid, is fundamental for brain growth. It plays an important role in cell division and signaling (McCann & Ames, 2005).

Recent studies suggest that several factors, including lactation phase, maternal diet, mode of feeding, geographical location, infant gender influence the composition of the HM (Delplanque et al., 2015; Deng et al., 2018;Wang, 2020; Floris et al., 2020). Colostrum is the first fluid released by the mammary glands, in a small and sufficient volume to feed the neonate. It contains a high concentration of immunologically and physiologically active components to build a baby's immune system (Pang & Hartmann, 2007). From days 5 to 14, the mother secretes transitional milk. It has higher levels of calories than colostrum and is responsible for the baby's growth development. After two weeks, human milk is characterized as mature milk, important for the immune system, in addition to the development and well-being of the baby (Ballard & Morrow, 2013).

In the event of a mother being unable to breastfeeding her child, human milk banks (HMB) offers a safe alternative to provide HM for babies (Who, 2003). The HMB are responsible for collecting, identifying, processing, and distributing donated milk. The donated HM is pasteurized using the Holder method to minimize the risk of transmission of infections. This method is performed in a water bath at 62.5 ºC for 30 minutes, followed by rapid freezing at -18 ºC (Ballard & Morrow, 2013). However, new alternatives are being studied in order to preserve the properties of HM, such as lyophilization, which is a technology that aims to increase the shelf life, facilitate the storage and transportation, and preserve the nutritional characteristics of donated HM (Lozano et al., 2014; Cortez & Soria, 2016; Martysiak-Żurowska et al., 2020).

Thus, the aim of this study was to analyze Dornic acidity, the FA composition, and TAG profile of raw, pasteurized, and pasteurized lyophilized HM in the three lactation stages

(colostrum, transitional, and mature) using gas chromatography with flame ionization detector (GC-FID) and electrospray ionization mass spectrometry (ESI-MS), in order to verify if the lyophilization and pasteurization processes are efficient at preserving the FA and TAG of HM, as compared to raw HM.

2. Materials and Methods

Samples

HM samples were collected in the HMB of the University Hospital of Maringá (HUM; Maringá, Paraná), separated according to the availability, with the approval number of the Human Research Ethics Committee 2,797,476. Raw HM samples were collected from the three lactation phases (colostrum, transitional, and mature), separated into pools according to their respective phases, mixed until total homogenization of all samples, with a final volume of 300 mL for each phase. A volume of 100 mL of pooled milk of each lactation phase was stored without processing.

Holder pasteurization

The remaining 200 mL HM from each lactation phase underwent Holder pasteurization that was performed in accordance with the current Brazilian HMBs standard. A bottle containing the HM was heated in a water bath at 62.5 ºC for 30 minutes, with manual stirring every five minutes. Then, it was cooled to a temperature of 4 °C, and frozen in a domestic refrigerator at -18 °C (Almeida, Guimarães & Novak, 2005).

Lyophilization

An amount of 100 mL of pasteurized HM from each lactation phase was lyophilized, in aluminum forms during 36 hours in a Lyophilizer (Alpha 1-2 LD Plus, model 101522), at approximately -50 °C and 0.023 mbar. The pasteurized lyophilized HM was vacuum-packed, in aluminum bags, stored at -18 °C free of moisture, oxygen, and light for further analysis. The pasteurized lyophilized HM was reconstituted with distilled water to a total volume of 100 mL.

Determination of acidity in Dornic degrees

Raw HM, pasteurized HM, and pasteurized lyophilized HM, of the colostrum, transitional and mature lactation phases were submitted to the acidity analyzes, in Dornic

degrees (º D), according to the Instituto Adolfo Lutz (2008). The titrant solution (Dornic solution) was N/9 sodium hydroxide in the presence of phenolphthalein indicator. Each 0.01 mL spent of Dornic solution to neutralize 1.0 mL of HM is equivalent to 1 °Dornic. All samples were analyzed in triplicate.

Extraction and methylation of total lipids

The extraction of total lipids of raw HM, pasteurized HM, and pasteurized lyophilized HM, in the three lactation phases, was performed according to Folch et al. (1957), in which a mixture of chloroform and methanol 2:1 (v v^{-1}) is used.

The fatty acid methyl esters (FAMEs) was prepared according to the methodology proposed by ISO 5509 (2000). In 100 mg of lipid, 2 mL of n-heptane and 2 mL of KOH/methanol solution (2 mol L^{-1}) are added. Shake for 2 min and collect the organic phase.

Fatty Acid Composition by GC-FID

The FA composition of the raw HM, pasteurized HM, and pasteurized lyophilized HM in the three lactation phaseswere analyzed by GC-FID. The FAMEs were separated according to the methodology proposed by Simionato et al. (2010), using a gas chromatograph (GC) TRACE™ Ultra Thermo Scientific™ (Thermo Scientific™, USA), with flame ionization detector (FID) and a fused silica column (100 m x 0.25 mm internal diameter, 0.25 µm cyanopropyl, CP-7420). Gas flows were 1.4 mL min^{-1} for Hydrogen carrier gas (H_2), 30 mL min^{-1} for Nitrogen auxiliary gas (N_2), and 30 and 300 mL min^{-1} for Hydrogen (H_2) and synthetic air gases, respectively. A sample volume of 2 µL was injected in triplicate with a sample division of 1:100. The column temperature was raised to 65 °C for 4 min, followed by a heating ramp from 16 °C min^{-1} to 185 °C, maintained for 12 min. After that, a new ramp of 20 °C min^{-1} was applied up to 235 °C, and maintained for 14 min. The total analysis time was 40 min.

The FAMEs were identified by comparing the retention times with the relative analytical standards (FAME Mix, C4-C24, Sigma-Aldrich). Results were expressed in relative percentage of the total fatty acids (%), automatically processed using the Chromquest software^{TM} 5.0.

Determination of triacylglycerols by ESI-MS

The TAG profiles of raw HM, pasteurized HM, and lyophilized pasteurized HM of the three lactation stages were analyzed by ESI-MS. Samples were injected directly into an

XEVO TQ-D mass spectrometer (Waters, Massachusetts, United States) with an electrospray ionization source (ESI) operating in positive mode, comprising the range of *m/z* 100-1200, in triplicate.

The preparation of the samples for analysis by ESI-MS was carried out in accordance with Silveira et al. (2017). An amount of 50.0 µL HM lipid was diluted in 950.0 µL chloroform. Then, 5.0 µL of this solution was added to 1.0 mL methanol/chloroform (9:1) and 20.0 µL of 0.10 $mol.L^{-1}$ ammonium formate solution (prepared in methanol). This last addition was performed to form predominantly ammonium adducts. The operating conditions of the equipment were: desolvation gas flow of 450 $L\ h^{-1}$, desolvation temperature of 250 °C, source temperature of 130 °C, a capillary voltage of 3.00 kV and cone voltage of 35.0 V. The samples were injected with a continuous flow of 50.0 $\mu L\ min^{-1}$.

TAG assignment and estimation

TAG ions were identified and their percentage (%) was estimated via LAMES Platform and Lipid maps® database. The LAMES Platform is based on the mathematical algorithm that describes the distribution of FAs in TAG molecules (Filho, Mendes, & Lanças, 1995), using the FA percentage determined by GC-FID. The Lipid maps® database was used to determine the molecular formula and *m/z* of TAGs.

Statistical analysis

The results of acidity measurement and FA composition were analyzed using the Assistat software version 7.7 (Silva & Azevedo, 2008). The analyses of variance (ANOVA) were performed with 5% significance level and the means were compared by the Tukey test.

3. Results and Discussions

Dornic Acidity

Table 1 shows the results of Dornic titratable acidity of raw HM, pasteurized HM, and lyophilized pasteurized HM of the three lactation stages. The acidity values in Dornic degrees (°D) presented the following ranges: colostrum, from 3.66 to 4.33 °D; transitional, from 3.66 to 4.33 °D; and mature, from 4.33 to 5.00 °D. The results showed no significant differences ($p<0.05$) between samples, that is, the acidity was not influenced by the treatments applied.

Table 1. Dornic acidity of raw HM, pasteurized HM, and lyophilized pasteurized HM of the three lactation stages (colostrum, transitional, and mature).

Samples	Dornic degrees (º D)		
	Raw	Pasteurized	Pasteurized Lyophilized
Colostrum	3.66 ± 0.57^{A}	4.33 ± 0.57^{A}	4.33 ± 0.57^{A}
Transitional	3.66 ± 0.57^{A}	4.33 ± 0.57^{A}	4.33 ± 0.57^{A}
Mature	4.33 ± 0.57^{A}	4.66 ± 1.15^{A}	5.00 ± 1.00^{A}

Results expressed as mean ± standard deviation of the triplicate. Values with different letters on the same line are significantly different ($p < 0.05$) by the Tukey test. Source: Authors.

In raw HM, there is no formation of lactic acid and its acidity is considered original, with values ranging between 1 and 4 °D (Anvisa, 2008), similar to the values found for raw HM in colostrum and in the transitional phases of this work. The storage time of HM favors elevation conditions of the microbiota, which produces lactic acid and, consequently, increases acidity (Várquez-Román et al., 2016).

The increase of acidity causes a decrease in nutritional quality of HM, inducing the destabilization of serum proteins and casein micelles, resulting in their coagulation. In addition, it increases osmolarity and reduces the availability of minerals, including calcium and phosphorus. It also causes a sensory change in odor and taste of HM and decreases its immunological properties (Pereira, Dametto, & Oliveira, 2016). Dornic acidity values accepted by Brazilian HMB, according to Anvisa (2008), comprise the range of 1 - 8 °D. Therefore, the studied samples before and before treatments were appropriate for consumption, because the Dornic acidity varied between 3.66 to 5.00 °D.

Fatty acid composition by GC-FID

Tables 2, 3, and 4 show the results for the FA composition of the raw HM, pasteurized HM, and pasteurized lyophilized HM in the three lactation phases (colostrum, transitional, and mature), obtained by GC-FID analysis.

Table 2 - Fatty acid composition of raw colostrum human milk and submitted to pasteurization and pasteurization combined with lyophilization processes.

Fatty acid composition	Colostrum (%)		
	CR	CP	CL
10:0	0.43 ± 0.01^{A}	0.42 ± 0.07^{A}	0.43 ± 0.01^{A}
12:0	3.32 ± 0.06^{A}	3.30 ± 0.16^{A}	3.20 ± 0.12^{A}

14:0	5.89 ± 0.11^{A}	5.97 ± 0.10^{A}	5.79 ± 0.07^{A}
14:1n-9	0.07 ± 0.01^{A}	0.06 ± 0.00^{A}	0.06 ± 0.01^{A}
15:0	0.29 ± 0.01^{A}	0.30 ± 0.00^{A}	0.29 ± 0.00^{A}
15:1n-9	0.07 ± 0.00^{A}	0.07 ± 0.00^{A}	0.07 ± 0.00^{A}
16:0	26.21 ± 0.26^{A}	26.97 ± 0.57^{A}	26.01 ± 0.33^{A}
16:1n-9	1.48 ± 0.06^{A}	1.45 ± 0.07^{A}	1.48 ± 0.05^{A}
17:0	0.41 ± 0.03^{A}	0.45 ± 0.01^{A}	0.43 ± 0.01^{A}
17:1n-9	0.19 ± 0.03^{A}	0.18 ± 0.01^{A}	0.17 ± 0.01^{A}
18:0	7.00 ± 0.07^{A}	6.88 ± 0.20^{A}	7.00 ± 0.07^{A}
18:1n-9	31.69 ± 0.22^{A}	30.62 ± 0.35^{A}	32.14 ± 0.17^{A}
18:2n-6	17.95 ± 0.35^{A}	18.51 ± 0.47^{A}	17.93 ± 0.03^{A}
18:3n-3	1.07 ± 0.10^{A}	1.05 ± 0.05^{A}	1.04 ± 0.03^{A}
20:0	0.20 ± 0.04^{A}	0.19 ± 0.03^{A}	0.20 ± 0.00^{A}
20:1n-9	0.54 ± 0.08^{A}	0.51 ± 0.06^{A}	0.53 ± 0.07^{A}
21:0	0.68 ± 0.10^{A}	0.69 ± 0.03^{A}	0.70 ± 0.03^{A}
20:3n-6	0.81 ± 0.06^{A}	0.84 ± 0.15^{A}	0.85 ± 0.03^{A}
20:3n-3	0.13 ± 0.01^{A}	0.12 ± 0.05^{A}	0.15 ± 0.02^{A}
20:4n-6	0.18 ± 0.02^{A}	0.16 ± 0.00^{A}	0.15 ± 0.04^{A}
22:0	0.50 ± 0.03^{A}	0.46 ± 0.04^{A}	0.48 ± 0.03^{A}
20:5n-3	0.09 ± 0.01^{A}	0.11 ± 0.00^{A}	0.13 ± 0.02^{A}
22:1n-9	0.15 ± 0.03^{A}	0.13 ± 0.02^{A}	0.13 ± 0.02^{A}
24:0	0.14 ± 0.01^{A}	0.12 ± 0.01^{A}	0.15 ± 0.02^{A}
24:1n-9	0.21 ± 0.02^{A}	0.18 ± 0.04^{A}	0.22 ± 0.02^{A}
22:6n-3	0.27 ± 0.03^{A}	0.24 ± 0.00^{A}	0.28 ± 0.02^{A}
ΣSFA	45.09 ± 0.47^{A}	45.76 ± 0.71^{A}	44.68 ± 0.58^{A}
ΣMUFA	34.39 ± 0.50^{A}	33.21 ± 0.95^{A}	34.80 ± 0.74^{A}
ΣPUFA	20.52 ± 0.53^{A}	21.03 ± 0.37^{A}	20.53 ± 0.76^{A}
Σ n-3	1.57 ± 0.13^{A}	1.52 ± 0.09^{A}	1.59 ± 0.05^{A}
Σ n-6	18.95 ± 0.28^{A}	19.51 ± 0.58^{A}	18.93 ± 0.05^{A}

Results expressed as mean ± standard deviation of the triplicate. Values with different letters on the same line are significantly different ($p < 0.05$) by Tukey test. SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids; CR– Raw colostrum milk; CP - Pasteurized colostrum milk; CL - Lyophilized pasteurized colostrum milk. Fatty acids composition: Capric acid (10:0); Lauric acid (12:0); Myristic acid (14:0); Physeteric acid (14:1n-9); Pentadecylic acid (15:0); 9-Pentadecenoic acid (15:1n-9); Palmitic acid (16:0); 7-hexadecenoic acid (16:1n-9); Margaric acid (17:0); 9-Methylundecanoic acid (17:1n-9); Stearic acid (18:0); Oleic acid (18:1n-9); Linoleic acid (18:2n-6); Linolenic acid (18:3n-3); Arachidic acid (20:0); Eicosenoic acid (20:1n-9); Eicosatrienoic acid (20:3n-3); Dihomo-gamma-linolenic acid (20:3n-6); Arachidonic acid (20:4n-6); Eicosapentaenoic acid (20:5n-3); Heneicosylic acid (21:0); Behenic acid (22:0); Erucic acid (22:1n-9);

Docosahexaenoic acid (22:6n-3); Lignoceric acid (24:0); Nervonic acid (24:1n-9); omega-3 (n-3); omega-6 (n-6). Source: Authors.

Table 3 - Fatty acid composition of raw transitional human milk and submitted to pasteurization and pasteurization combined with lyophilization processes.

Fatty acid composition	Transitional (%)		
	TR	TP	TL
10:0	1.93 ± 0.05^{A}	1.97 ± 0.04^{A}	1.95 ± 0.05^{A}
12:0	10.42 ± 0.10^{A}	10.34 ± 0.14^{A}	10.06 ± 0.13^{A}
14:0	9.42 ± 0.06^{A}	9.44 ± 0.02^{A}	8.97 ± 0.08^{A}
14:1n-9	0.05 ± 0.00^{A}	0.05 ± 0.00^{A}	0.05 ± 0.00^{A}
15:0	0.16 ± 0.00^{A}	0.16 ± 0.00^{A}	0.16 ± 0.00^{A}
15:1n-9	0.03 ± 0.00^{A}	0.03 ± 0.00^{A}	0.03 ± 0.00^{A}
16:0	23.23 ± 0.14^{A}	23.56 ± 0.20^{A}	23.52 ± 0.03^{A}
16:1n-9	1.67 ± 0.02^{A}	1.72 ± 0.02^{A}	1.72 ± 0.02^{A}
17:0	0.24 ± 0.01^{A}	0.25 ± 0.01^{A}	0.26 ± 0.00^{A}
17:1n-9	0.13 ± 0.01^{A}	0.13 ± 0.00^{A}	0.14 ± 0.00^{A}
18:0	4.80 ± 0.01^{A}	4.80 ± 0.21^{A}	4.87 ± 0.06^{A}
18:1n-9	26.64 ± 0.26^{A}	26.87 ± 0.06^{A}	26.91 ± 0.27^{A}
18:2n-6	17.88 ± 0.09^{A}	17.66 ± 0.07^{A}	18.12 ± 0.22^{A}
18:3n-3	0.93 ± 0.02^{A}	0.97 ± 0.04^{A}	1.07 ± 0.09^{A}
20:0	0.10 ± 0.01^{A}	0.10 ± 0.01^{A}	0.11 ± 0.00^{A}
20:1n-9	0.40 ± 0.01^{A}	0.33 ± 0.05^{A}	0.43 ± 0.06^{A}
21:0	0.54 ± 0.10^{A}	0.51 ± 0.06^{A}	0.52 ± 0.01^{A}
20:3n-6	0.55 ± 0.07^{A}	0.48 ± 0.11^{A}	0.52 ± 0.03^{A}
20:3n-3	0.12 ± 0.01^{A}	0.10 ± 0.02^{A}	0.09 ± 0.04^{A}
20:4n-6	0.09 ± 0.01^{A}	0.08 ± 0.02^{A}	0.10 ± 0.01^{A}
22:0	0.15 ± 0.01^{A}	0.16 ± 0.02^{A}	0.16 ± 0.00^{A}
20:5n-3	0.04 ± 0.00^{A}	0.04 ± 0.01^{A}	0.06 ± 0.01^{A}
22:1n-9	0.06 ± 0.00^{A}	0.05 ± 0.02^{A}	0.07 ± 0.01^{A}
24:0	0.04 ± 0.01^{A}	0.06 ± 0.01^{A}	0.06 ± 0.01^{A}
24:1n-9	0.06 ± 0.00^{A}	0.08 ± 0.01^{A}	0.09 ± 0.00^{A}
22:6n-3	0.16 ± 0.02^{A}	0.13 ± 0.03^{A}	0.17 ± 0.01^{A}
ΣSFA	51.21 ± 0.16^{A}	51.35 ± 0.26^{A}	50.37 ± 0.26^{A}
ΣMUFA	29.28 ± 0.23^{A}	29.26 ± 0.33^{A}	29.78 ± 0.48^{A}
ΣPUFA	19.51 ± 0.18^{A}	19.46 ± 0.18^{A}	19.85 ± 0.32^{A}
Σ n-3	1.27 ± 0.03^{A}	1.24 ± 0.03^{A}	1.30 ± 0.05^{A}
Σ n-6	18.24 ± 0.15^{A}	18.22 ± 0.15^{A}	18.55 ± 0.26^{A}

Results expressed as mean ± standard deviation of the triplicate. Values with different letters on the same line are significantly different ($p < 0.05$) by the Tukey test. SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids; TR – Raw

transitional milk; TP - Pasteurized transitional milk; TL - Lyophilized pasteurized transitional milk. Fatty acids composition: Capric acid (10:0); Lauric acid (12:0); Myristic acid (14:0); Physeteric acid (14:1n-9); Pentadecylic acid (15:0); 9-Pentadecenoic acid (15:1n-9); Palmitic acid (16:0); 7-hexadecenoic acid (16:1n-9); Margaric acid (17:0); 9-Methylundecanoic acid (17:1n-9); Stearic acid (18:0); Oleic acid (18:1n-9); Linoleic acid (18:2n-6); Linolenic acid (18:3n-3); Arachidic acid (20:0); Eicosenoic acid (20:1n-9); Eicosatrienoic acid (20:3n-3); Dihomo-gamma-linolenic acid (20:3n-6); Arachidonic acid (20:4n-6); Eicosapentaenoic acid (20:5n-3); Heneicosylic acid (21:0); Behenic acid (22:0); Erucic acid (22:1n-9); Docosahexaenoic acid (22:6n-3); Lignoceric acid (24:0); Nervonic acid (24:1n-9); omega-3 (n-3); omega-6 (n-6). Source: Authors.

Table 4 - Fatty acid composition of raw mature human milk and submitted to pasteurization and pasteurization combined with lyophilization processes.

Fatty acid composition	**Mature (%)**		
	MR	**MP**	**ML**
10:0	1.03 ± 0.05^{A}	1.02 ± 0.04^{A}	0.99 ± 0.03^{A}
12:0	6.90 ± 0.10^{A}	6.95 ± 0.27^{A}	6.63 ± 0.02^{A}
14:0	7.55 ± 0.10^{A}	7.40 ± 0.29^{A}	7.19 ± 0.06^{A}
14:1	0.08 ± 0.01^{A}	0.08 ± 0.00^{A}	0.09 ± 0.01^{A}
15:0	0.21 ± 0.01^{A}	0.21 ± 0.01^{A}	0.21 ± 0.00^{A}
15:1	0.06 ± 0.00^{A}	0.06 ± 0.00^{A}	0.06 ± 0.00^{A}
16:0	22.47 ± 0.24^{A}	22.23 ± 0.88^{A}	21.78 ± 0.13^{A}
16:1n-9	1.62 ± 0.12^{A}	1.68 ± 0.07^{A}	1.70 ± 0.03^{A}
17:0	0.36 ± 0.01^{A}	0.34 ± 0.01^{A}	0.33 ± 0.03^{A}
17:1	0.20 ± 0.02^{A}	0.20 ± 0.01^{A}	0.19 ± 0.01^{A}
18:0	7.57 ± 0.14^{A}	7.40 ± 0.29^{A}	7.36 ± 0.06^{A}
18:1n-9	31.74 ± 0.59^{A}	32.05 ± 1.26^{A}	32.68 ± 0.44^{A}
18:2n-6	17.29 ± 0.23^{A}	17.91 ± 0.71^{A}	17.64 ± 0.17^{A}
18:3n-3	1.04 ± 0.03^{A}	1.09 ± 0.04^{A}	1.08 ± 0.03^{A}
20:0	0.18 ± 0.03^{A}	0.21 ± 0.01^{A}	0.22 ± 0.01^{A}
20:1n-9	0.33 ± 0.05^{A}	0.35 ± 0.01^{A}	0.39 ± 0.02^{A}
21:0	0.34 ± 0.02^{A}	0.39 ± 0.02^{A}	0.37 ± 0.01^{A}
20:3n-6	0.43 ± 0.07^{A}	0.45 ± 0.02^{A}	0.52 ± 0.03^{A}
20:3n-3	0.11 ± 0.00^{A}	0.11 ± 0.00^{A}	0.09 ± 0.03^{A}
20:4n-6	0.07 ± 0.02^{A}	0.08 ± 0.00^{A}	0.08 ± 0.00^{A}
22:0	0.10 ± 0.01^{A}	0.08 ± 0.00^{A}	0.10 ± 0.01^{A}
20:5n-3	0.04 ± 0.01^{A}	0.05 ± 0.00^{A}	0.04 ± 0.00^{A}
22:1n-9	0.03 ± 0.01^{A}	0.02 ± 0.00^{A}	0.04 ± 0.00^{A}
24:0	0.02 ± 0.00^{A}	0.02 ± 0.00^{A}	0.01 ± 0.00^{A}

24:1n-9	0.11 ± 0.02^{A}	0.08 ± 0.00^{A}	0.11 ± 0.00^{A}
22:6n-3	0.13 ± 0.01^{A}	0.13 ± 0.01^{A}	0.13 ± 0.01^{A}
ΣSFA	46.73 ± 0.35^{A}	46.26 ± 1.82^{A}	45.18 ± 0.21^{A}
ΣMUFA	34.17 ± 0.52^{A}	34.53 ± 1.36^{A}	35.24 ± 0.39^{A}
ΣPUFA	19.10 ± 0.34^{A}	19.83 ± 0.78^{A}	19.58 ± 0.20^{A}
Σ n-3	1.32 ± 0.03^{A}	1.38 ± 0.05^{A}	1.34 ± 0.05^{A}
Σ n-6	17.79 ± 0.30^{A}	18.44 ± 0.73^{A}	18.23 ± 0.16^{A}

Results expressed as mean ± standard deviation of the triplicate. Values with different letters on the same line are significantly different ($p < 0.05$) by the Tukey test. SFA - saturated fatty acids; MUFA - monounsaturated fatty acids; PUFA - polyunsaturated fatty acids; MR - raw mature milk; MP - Pasteurized mature milk; ML - lyophilized pasteurized mature milk. Fatty acids composition: Capric acid (10:0); Lauric acid (12:0); Myristic acid (14:0); Physeteric acid (14:1n-9); Pentadecylic acid (15:0); 9-Pentadecenoic acid (15:1n-9); Palmitic acid (16:0); 7-hexadecenoic acid (16:1n-9); Margaric acid (17:0); 9-Methylundecanoic acid (17:1n-9); Stearic acid (18:0); Oleic acid (18:1n-9); Linoleic acid (18:2n-6); Linolenic acid (18:3n-3); Arachidic acid (20:0); Eicosenoic acid (20:1n-9); Eicosatrienoic acid (20:3n-3); Dihomo-gamma-linolenic acid (20:3n-6); Arachidonic acid (20:4n-6); Eicosapentaenoic acid (20:5n-3); Heneicosylic acid (21:0); Behenic acid (22:0); Erucic acid (22:1n-9); Docosahexaenoic acid (22:6n-3); Lignoceric acid (24:0); Nervonic acid (24:1n-9); omega-3 (n-3); omega-6 (n-6). Source: Authors.

A total of twenty-six FAs were identified in raw HM and HM submitted to pasteurization and pasteurization combined with lyophilization processes in the three lactation phases (colostrum, transitional, and mature). No statistically significant differences were observed ($p < 0.05$) between FA composition of raw mature HM and HM submitted to pasteurization and pasteurization combined with lyophilization processes of the same lactation phase (Tables 2, 3, and 4).

As shown in Tables 2, 3, and 4, oleic acid (O, 18:1n-9) and palmitic acid (P, 16:0) were the most abundant FA in all samples. In colostrum HM, 18:1n-9 varied from 30.62 to 32.14%; in transitional HM from 26.64 to 26.91%; and in mature HM from 31.74 to 32.68%. This FA is the main energy source of HM. It is strategically positioned at the ends of the TAG, favoring the attack of digestive enzymes, enhancing its absorption, as well as the mineral calcium (Rydlewski et al., 2020). P (16:0) was found in the range of 26.01 to 26.97% in colostrum HM; 23.23 to 23.56% in transitional HM; and 21.78 to 22.47% in mature HM.

This FA has analgesic effects by raising the levels of anandamide, a neurotransmitter, which can produce sedation in neonates (Mayo et al., 2020).

The essential FAs, linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) presented values between 17.29 to 18.51%, and 0.93 to 1.09%, respectively. These results are similar to other studies found in the literature (Kuipers et al., 2012; Manin et al., 2019). These both FAs have important functions to newborn, helping to form the immune system and the central nervous system (Koletzko, 2017).

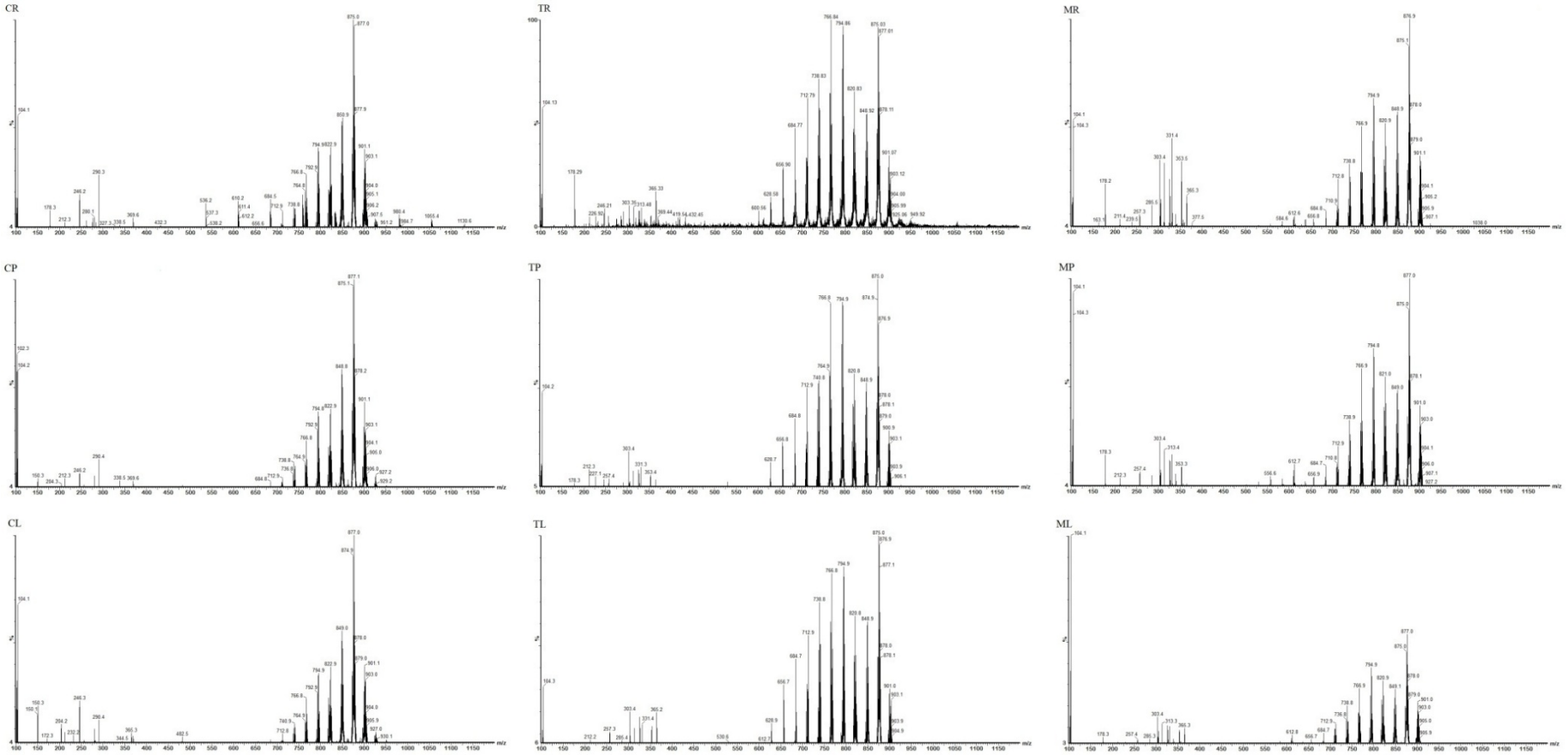
The polyunsaturated fatty acids (PUFA) ranged from 19.10 to 21.03%; monounsaturated fatty acids (MUFAs) from 29.26 to 35.24%; and saturated fatty acids (SFAs) from 44.68 to 46.73%. These results are according to those obtained by Lubetzky et al. (2016), who evaluated the FA composition in HM of Brazilian lactating mothers and obtained the percentages of PUFAs, MUFAs, and SFAs of 20.6, 38.0 and 43.0%, respectively.

Nessel et al. (2019) researched the effects of processing conditions on donated HM, concluding that Holder pasteurization did not significantly change the FAs composition in HM, showing that the technique is effective for the conservation of this nutrient. Cavazos-Garduño et al., (2016) studied the effect of pasteurization and lyophilization applied on HM and these processes did not change the profile of FAs. Martysiak-Żurowska et al. (2020) and Manin et al. (2019) evaluated the effect of lyophilization on the FA composition of lyophilized HM, and when compared to raw HM, the results did not presented significant difference. Thus, it was possible to conclude that lyophilization can be a promising method because it allows a longer storage time of HM, being a viable alternative to freezing, currently used in HMBs.

Determination and identification of the Triacylglycerols (TAG) by ESI(+)-MS

TAGs were detected by ESI(+)-MS as adducts of ammonium $[M + NH_4]^+$ in HM samples and identified by LAMES platform (Filho et al., 1995) and Lipid maps® database. Figure 1 presented the mass spectra of HM in the different phases (colostrum, transitional and mature) submitted to different treatments (raw, pasteurized, pasteurized lyophilized) carried out by ESI-MS, in the range of *m/z* 100 to 1200.

Figure 1. Mass spectra of human milk from different lactation phases (colostrum, transitional, mature) submitted to different treatments (raw, pasteurized, pasteurized and lyophilized) obtained by ESI-MS.



CR - Raw colostrum milk; CP - Pasteurized colostrum milk; CL - Lyophilized Pasteurized colostrum milk; TR - Raw transitional milk; TP - Pasteurized transitional milk; TL - Lyophilized Pasteurized transitional milk; MR - Raw mature milk; MP - Pasteurized mature milk; ML - Lyophilized Pasteurized mature milk. Source: Authors.

This analysis is important to obtain the TAG profile of HM in different stages of lactation (colostrum, transitional and mature), unprocessed (raw) and submitted to pasteurization and lyophilization combined with pasteurization. In Figure 1, it was observed that the TAG profile was similar before and after the processes, indicating that the lipid profile was not altered with the applied processes.

Table 5 shows the main TAGs identified by ESI-MS, LAMES Platform (Filho et al., 1995) and Lipid maps® database of the raw, pasteurized, and pasteurized and lyophilized HM in different stages of lactation.

The most intense ion peak of colostrum, transitional, and mature in HM was present between *m/z* 875 [TAG+NH_4] PLO and 877 $[TAG+NH_4]^+$ POO. These results corroborate with Manin et al. (2019) and Rydlewski et al. (2019), in which, analyzed the lipid profile of TAG in HM in the different stages of lactation, in addition to being in accordance with Table 2, 3, and 4, the FAs. O: oleic acid (18:1n-9); P: palmitic acid (16:0) and L: linoleic acid (18:2n-6) showed higher concentrations in the samples analyzed by GC-FID and the TAGs found through the *m/z* ratio by ESI(+)-MS were composed mainly of these three FAs.

Table 5 - [M+ NH4]$^+$ ions and estimated concentration (%) determined by ESI-MS, LAMES Platform, Lipid maps® database of the raw, pasteurized, and pasteurized and lyophilized HM in different stages of lactation.

					Estimated concentration (%)								
Molecular		**Ionizatio**		**TAG**	**Colostrum**			**Transitional**			**Mature**		
formula	**CN/DB**	**n**	***m/z***	**assigment**	**CR**	**CP**	**CL**	**TR**	**TP**	**TL**	**MR**	**MP**	**ML**
$C_{45}H_{86}O_6$	42:0	$[M+NH_4]^+$	740	PMLa	0.310	0.321	0.292	2.067	2.095	2.005	0.699	0.690	0.622
$C_{47}H_{86}O_6$	44:2	$[M+NH_4]^+$	764	LaLM	0.213	0.220	0.199	1.595	1.580	0.537	0.537	0.556	0.502
$C_{47}H_{86}O_6$	44:1	$[M+NH_4]^+$	766	LaOM	0.366	0.364	0.360	2.396	2.352	2.261	0.994	0.979	0.932
$C_{47}H_{90}O_6$	44:0	$[M+NH_4]^+$	768	PLaP	0.695	0.722	0.659	2.550	2.630	2.617	1.483	1.509	0.941
$C_{49}H_{90}O_6$	46:2	$[M+NH_4]^+$	792	PLLa	0.955	0.989	0.900	3.936	3.967	4.032	2.281	2.433	2.230
$C_{49}H_{92}O_6$	46:1	$[M+NH_4]^+$	794	POLa	2.446	2.431	2.357	5.914	5.906	5.903	4.219	4.281	4.143
$C_{49}H_{94}O_6$	46:0	$[M+NH_4]^+$	796	PMP	1.851	1.950	1.733	2.305	2.400	2.332	1.612	1.595	1.507
$C_{51}H_{92}O_6$	48:3	$[M+NH_4]^+$	818	LaLO	1.680	1.666	1.610	4.563	4.455	4.546	3.244	3.452	3.345
$C_{51}H_{94}O_6$	48:2	$[M+NH_4]^+$	820	LaOO	0.970	0.927	1.453	3.429	3.316	3.328	3.000	3.038	3.108
$C_{51}H_{94}O_6$	48:2	$[M+NH_4]^+$	820	PLM	2.543	2.673	2.377	3.557	3.620	3.592	2.479	2.572	2.433
$C_{51}H_{96}O_6$	48:1	$[M+NH_4]^+$	822	POM	4.374	4.421	4.272	5.346	5.390	5.260	4.586	4.526	4.520
$C_{51}H_{96}O_6$	48:1	$[M+NH_4]^+$	822	SOLa	0.434	0.418	0.434	0.806	0.786	1.007	1.425	0.979	0.958
$C_{51}H_{98}O_6$	48:0	$[M+NH_4]^+$	824	PPP	2.772	2.925	2.609	1.896	2.009	2.030	1.612	1.595	1.521
$C_{53}H_{96}O_6$	50:3	$[M+NH_4]^+$	846	MLO	3.004	3.029	2.918	4.124	4.065	4.051	3.526	3.649	3.649
$C_{53}H_{98}O_6$	50:2	$[M+NH_4]^+$	848	PLP	5.711	6.013	5.347	4.390	4.545	4.690	3.719	3.858	3.683
$C_{53}H_{98}O_6$	50:2	$[M+NH_4]^+$	848	MOO	2.583	2.505	2.633	3.099	3.026	2.966	3.261	3.211	3.390
$C_{53}H_{100}O_6$	50:1	$[M+NH_4]^+$	850	POP	9.822	9.946	9.648	6.597	6.766	6.867	6.879	6.789	6.843
$C_{53}H_{100}O_6$	50:1	$[M+NH_4]^+$	850	SOM	0.776	0.760	0.787	0.728	0.717	1.094	1.549	1.509	1.534
$C_{53}H_{102}O_6$	50:0	$[M+NH_4]^+$	852	SPP	2.197	2.243	2.091	0.775	0.802	1.154	1.634	1.595	1.549

$C_{55}H_{98}O_6$	52:4	$[M+NH_4]^+$	872	PLL	3.922	2.772	3.653	3.387	3.428	3.612	2.860	3.111	2.973
$C_{55}H_{101}O_6$	52:3	$[M+NH_4]^+$	875	PLO	13.491	13.630	13.184	10.180	10.207	10.578	10.579	10.948	11.049
$C_{55}H_{103}O_6$	52:2	$[M+NH_4]^+$	876	SLP	3.017	3.074	2.857	1.816	1.849	1.956	2.512	2.572	2.500
$C_{55}H_{104}O_6$	52:2	$[M+NH_4]^+$	877	POO	11.601	11.273	11.895	7.649	7.598	7.743	9.784	9.633	10.264
$C_{55}H_{104}O_6$	52:1	$[M+NH_4]^+$	878	SOP	5.189	5.084	5.156	2.730	2.752	2.864	4.647	4.526	4.646
$C_{57}H_{100}O_6$	54:5	$[M+NH_4]^+$	898	OLL	4.633	4.670	4.504	3.927	3.849	4.074	4.067	4.414	4.460
$C_{57}H_{103}O_6$	54:4	$[M+NH_4]^+$	900	OLO	7.968	7.724	8.126	5.902	5.730	5.964	7.523	7.767	8.287
$C_{57}H_{104}O_6$	54:3	$[M+NH_4]^+$	902	OOO	4.567	4.259	4.888	2.956	2.844	2.911	4.638	4.556	5.132
$C_{57}H_{104}O_6$	54:3	$[M+NH_4]^+$	902	SLO	3.564	3.483	3.522	2.106	2.076	2.206	3.573	3.649	3.751
$C_{57}H_{106}O_6$	54:2	$[M+NH_4]^+$	904	SOO	3.064	2.881	3.178	1.583	1.545	1.615	3.305	3.211	3.484

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); L: linoleic acid (18:2n-6); CN: total number of acyl carbons; DB: total number of double bonds; CR– Raw colostrum milk; CP - Pasteurized colostrum milk; CL - Lyophilized Pasteurized colostrum milk; TR - Raw transitional milk; TP - Pasteurized transitional milk; TL - Lyophilized Pasteurized transitional milk; MR - Raw mature milk; MP - Pasteurized mature milk; ML - Lyophilized Pasteurized mature milk. Source: Authors.

The major TAGs in the colostrum lactation phase were PLO (*m/z* 875), which varied between 13.184 and 13.630 %; followed by POO (*m/z* 877) ranging from 11.273 to 11.610; and POP (*m/z* 850) varying from 9.648 to 9.822. In the transitional lactation phase, the main TAGs were PLO (*m/z* 875) with values ranging between 10.180 and 10.578; POO (*m/z* 877) varying from 7.598 and 7.649; and POP (*m/z* 850) that varied between 6.597 and 7.743. The main TAGs identified in mature lactation stage were PLO (*m/z* 875), POO (*m/z* 877), and OLO (*m/z* 900), varying from 10.579 to 11.049; 9.633 to 10.264; 7.752 to 8.287. Observe that TAGs were major composed by oleic acid (O, 18:1n-9) and palmitic acid (P, 16:0). These results were similar to the work by Manin et al. (2019), who evaluated the possible TAGs in HM from different stages of lactation analyzed after being subjected to pasteurization and pasteurization + lyophilization processes. Haddad et al. (2011) determined the composition of molecular TAG in mature milk and also obtained the POO (13.587%), PLO (3.045%), and POP (9.790%) as main TAGs.

Observing Table 5, it was possible to verify that the estimated concentration results were similar between processing and raw HM. Manin et al. (2019) analyzed pasteurized and lyophilized HM in the three different stages of lactation for a period of six months of storage. Authors observed that the possible combinations of TAGs in the samples remained similar after the lyophilization process, confirming that the lipid profile did not change with the application of the processes or with the passage of time.

2. Conclusions

The results of this study indicate that the pasteurization and lyophilization processes do not induce significant negative changes in the lipid quality of HM, because the Dornic acidity, FA composition, and the TAG profile remain preserved after the treatments. Results of the FA composition of raw, pasteurized and lyophilized pasteurized HM in different lactation phases (colostrum, transitional, and mature), revealed that oleic acid, palmitic acid, and linoleic acid were the ones with the higher quantities. In addition, no statistically significant differences were observed ($p<0.05$) between FA composition of raw mature HM and HM submitted to pasteurization and pasteurization combined with lyophilization processes of the same lactation phase. All samples were major composed by TAGs PLO and POO. Therefore, pasteurization in conjunction with lyophilization, can be a promising alternative, aiming to improve the processes of quality and conservation of HM lipids in HMBs, in addition to reducing the storage volume and facilitating transport of this food.

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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GENERAL ABSTRACT
ARTICLE 2

INTRODUCTION. Human milk (HM) contains numerous benefits for the child's health. It is considered an essential and exclusive source of nutrition up to the first six months of life. Among the HM components, lipids have the second highest concentration of nutrients and are responsible for providing 50% of the total caloric value, acting as the main source of energy for the newborn. The fatty acids (FA) present in HM are essential for children's physical, mental, and cognitive growth and development, acting as structural elements of cell membranes, which make up several cells, including neurons. When the mother has some difficulty in breastfeeding her child, human milk banks (HMB) present an alternative to provide HM for babies from donors. All HM donated to the HMB must undergo pasteurization and freezing processes to ensure microbiological quality and increase shelf life. Pasteurization is carried out in a water bath at a temperature of 62.5 °C for 30 minutes, followed by freezing at -18 °C. Despite the benefits of these processes, studies show that these processing techniques can modify the nutritional, structural and biological composition of HM. High-pressure processing, considered an alternative to pasteurization, is responsible for eliminating pathogenic microorganisms of HM, in addition to providing foods without nutritional, sensory, and original chemical composition alterations, since it is considered a fast technique, which may not involve high temperatures. In this way, research has been performed in partnership with HMBs, for the development of other techniques that contribute to microbial inactivation but preserve the HM in relation to its chemical and structural composition.

AIMS. The present study aimed to evaluate the influence of high-pressure processing on macronutrients (proteins, lipids, carbohydrates, ash, and moisture), acidity in Dornic degrees, total coliforms, FA composition, and triacylglycerol (TAG) profile of HM, in different conditions, comparing with Holder's pasteurization and with unprocessed HM.

MATERIAL AND METHODS. The present study was approved by the Research Ethics Committee number 2,797,476, of the State University of Maringá. Mature HM samples were collected from twenty different mothers at the HMB of the University Hospital of Maringá. The total volume collected from each mother was 120mL. Samples were mixed and homogenized. After pooling, 400 mL of the HM pool (Control HM) were separated and stored at -18 °C before analyses were performed. An amount of 400 mL of HM pool were submitted to pasteurization processing (HM HoP) and 1600 mL to high-pressure processing. The pressures used were 300 (HM HPP1), 400 (HM HPP2), 500 (HM HPP3), and 600 MPa (HM HPP4). The composition of macronutrients (moisture, ash, proteins, lipids and carbohydrates), acidity in Dornic degrees, FA composition by gas chromatography, TAG profile by electrospray ionization mass spectrometry, and total coliforms were analyzed. Statistical analysis of the macronutrient composition, Dornic acidity and FA composition was performed on the data obtained, using analysis of variance (ANOVA), and the means were compared by Tukey's test at a significance level of 5%. Hierarchical cluster analysis was also performed to explore and highlight similarities and discrepancies in the TAG intensity dataset.

RESULTS AND DISCUSSION. Dornic acidity values and concentrations of moisture, ash, proteins, lipids, and carbohydrates did not vary significantly between processed and unprocessed HM. HM samples that presents an acidity greater than 8 °Dornic are discarded by the HMB before pasteurization, as it is an indirect measure of the presence of bacteria in the HM. The Control HM acidity value was similar to that of the HM HPP1 whose value was 3.33 °Dornic. The acidity of HM HoP, HM HPP2, HM HPP3, and HM HPP4 were higher

than Control HM (3.67 to 4.00 °Dornic). Moisture concentrations varied from 88.20 to 89.07%; ash, 0.15 to 0.17%; lipids, 3.23 to 3.31%; protein, 1.12 to 1.21%; and carbohydrates, 6.40 to 6.99%. Regarding the FA composition, thirty-four FAs were identified and determined by gas chromatography in the studied samples. Among the results, 50.40 to 53.26% were composed of saturated FAs, 17.89 to 19.07% of polyunsaturated FAs and 28.85 to 30.53% of monounsaturated FAs. Palmitic acid had the highest concentration, with values ranging between 24.45 and 27.46%, followed by oleic acid that varied between 24.45 and 26.56%. Regarding the processing carried out, it was observed that the FAs did not differ significantly ($p < 0.05$) when compared to the HM without processing. The TAGs present in HM are essential to ensure the healthy growth of babies. TAGs were detected by electrospray ionization mass spectrometry and identified by the Lipid Maps database in conjunction with the LAMES platform. The results showed that the estimated concentrations and the TAG profile of samples submitted to high-pressure processing and Holder pasteurization were similar to the control HM. That is, HM HoP, HM HPP1, HM HPP2, HM HPP3, and HM HPP4 maintained the characteristics of the Control HM TAG. Finally, the analysis of total coliforms was performed because it is the standard microbiological analysis in the HMBs. In the HM Control, the coliform group was present, and for the treated samples (HM HoP, HM HPP1, HM HPP2, HM HPP3 and HM HPP4), this class of microorganism was found absent, showing that the processes were effective in its elimination.
CONCLUSIONS. Results showed that high-pressure processing offers a viable alternative to standard pasteurization in the treatment of HM, since the processes carried out eliminated the microorganisms of the coliform group. The FA composition, the concentration of macronutrients (proteins, lipids, carbohydrates, ash, and moisture) and Dornic acidity did not present significant difference in relation to the evaluated processes. The estimated concentrations and the TAG profile obtained for the control HM sample were similar to those obtained for the HM HoP sample and all the high-pressure processing samples, indicating that the lipid profile did not change with the processes.
Keywords: fatty acid composition, direct infusion mass spectrometry, human milk banks, total coliforms, high-pressure processing.

RESUMO GERAL
ARTIGO 2

INTRODUÇÃO. O leite humano (LH) contém inúmeros benefícios para a saúde da criança, considerado fonte essencial e exclusiva de alimentação até os seis primeiros meses de vida. Dentre os componentes do LH, os lipídios apresentam a segunda maior concentração de nutrientes e são responsáveis por fornecer 50% do valor calórico total, atuando como a principal fonte enérgica do recém-nascido. Os ácidos graxos (AG) presentes no LH são fundamentais para crescimento e desenvolvimento físico, mental e cognitivo das crianças, atuam como elementos estruturais de membranas celulares, que compõe diversas células, dentre elas, os neurônios. Quando a mãe possui alguma dificuldade na amamentação do seu filho, existem os serviços dos bancos de leite humano (BLH), que disponibilizam o LH de doadoras. Todo LH doado ao BLH deve passar por processamentos de pasteurização e congelamento, para garantir a qualidade microbiológica e aumentar o tempo de vida de prateleira. A pasteurização é realizada em banho-maria à temperatura de 62,5 °C por 30 minutos, seguida de congelamento a -18 °C. Apesar dos benefícios destes processamentos, estudos comprovam que essas técnicas de processamento podem modificar a composição nutricional, estrutural e biológica do LH. O processamento por alta pressão, considerado uma alternativa à pasteurização, é responsável por eliminar os microrganismos patogênicos do LH,

além de fornecer alimentos sem alterações nutricionais, sensoriais e em sua composição química original, visto que é considerada uma técnica rápida, que pode não envolver temperaturas elevadas. Desta forma, pesquisas vêm sendo realizadas em parceria com BLHs, para o desenvolvimento de outras técnicas que contribuam para a inativação microbiana, mas preservem o LH em relação a sua composição química e estrutural.

OBJETIVOS. O objetivo foi avaliar a influência do processamento de alta pressão nos macronutrientes (proteínas, lipídios, carboidratos, cinzas e umidade), acidez em graus Dornic, coliformes totais, composição em AG e perfil de triacilgliceróis (TAG) de LH, em diferentes condições, comparando com o a pasteurização de Holder e com o LH sem processamento.

MATERIAIS E MÉTODOS. O presente estudo foi aprovado pelo Comitê de ética em pesquisa, número 2.797.476, da Universidade Estadual de Maringá. Foram coletadas no BLH do Hospital Universitário de Maringá amostras de 120 mL de LH maduro de cada mãe, somando-se um total de vinte mães. As amostras foram homogeneizadas, 400 mL do pool de LH foi coletada para análise de LH sem processamento (Control HM), que foi congelado a -18 °C para análise posterior; 400 mL do pool de HM para o processamento de pasteurização (HM HoP) e 1600 mL para o processamento de alta-pressão.As pressões utilizadas foram 300 (HM HPP1), 400 (HM HPP2), 500 (HM HPP3) e 600 MPa (HM HPP4). Nas seis amostras foram realizados análises da composição de macronutrientes (umidade, cinzas, proteínas, lipídios e carboidratos), análise de acidez em graus Dornic, composição em AGs por cromatografia gasosa, perfil de triacilglicerol (TAG) por espectrometria de massas e análise microbiológica (coliformes totais). Nos dados obtidos foi realizado análise estatística da composição de macronutrientes, acidez Dornic e composição em AGs, utilizando análise de variância ANOVA e as médias foram comparadas pelo teste de Tukey a um nível de significância de 5%. Foi realizada também a análise de agrupamento hierárquico para explorar e destacar as semelhanças e discrepâncias no conjunto de dados da intensidade de TAG.

RESULTADOS E DISCUSSÃO. Os valores de acidez Dornic e as concentrações de umidade, cinzas, proteínas, lipídios e carboidratos não variaram significativamente entre os processamentos e o LH sem processamento. As amostras de LH que possuem acidez maior que 8 °Dornic, são descartadas pelo BLH antes da pasteurização, pois é uma medida indireta de presença de bactérias no LH.O valor de acidez Control HM foi semelhante ao do HM HPP1 cujo valor foi de 3,33 °Dornic. A acidez do HM HoP, HM HPP2, HM HPP3 e HM HPP4 foram superiores ao Control HM (3,67 a 4,00 °Dornic). As concentrações de umidade variaram entre 88,20 e 89,07%, cinzas entre 0,15 e 0,17%, lipídios entre 3,23 e 3,31%, proteína entre 1,12 e 1,21% e carboidratos entre 6,40 e 6,99%. Em relação à composição em AGs, trinta e quatro AGs foram identificados e quantificados por cromatografia gasosa nas amostras estudadas. Dentre os resultados, 50,40 a 53,26% foram compostos por ácidos graxos saturados, 17,89 a 19,07% por ácidos graxos poliinsaturados e 28,85 a 30,53% por ácidos graxos monoinsaturados. O ácido palmítico foi o de maior concentração, apresentando valores entre 24,45 e 27,46%, responsável pela absorção de cálcio no intestino do recém-nascido e por possuir efeito analgésico. Na sequência, o ácido oléico com valores entre 24,45 a 26,56%, atuando como fonte de energia para o recém-nascido e auxiliando na absorção de gordura pelo intestino do bebê. Com relação aos processamentos realizados, observou-se que os AGs não diferiram significativamente ($p < 0,05$) quando comparados ao LH sem processamento. Os TAGs presentes no LH são fundamentais para garantir o crescimento saudável dos bebês. Os TAGs foram detectados por infusão direta no espectrômetro de massas e identificados pela base de dados do Lipid Maps em conjunto com a plataforma LAMES. Os resultados mostraram que as concentrações estimadas e o perfil TAG dos principais TAGs das amostras lipídicas foram semelhantes para a amostra de LH controle, para a amostra HM HoP e todas as amostras de processamento de alta-pressão, indicando que o perfil lipídico não alterou. Ou seja, HM HoP, HM HPP1, HM HPP2, HM HPP3 e HM HPP4 mantiveram as características

do TAG do Control HM. E por fim, a análise de coliformes totais foi realizada por ser a análise microbiológica padrão nos BLHs. No Control HM, o grupo coliforme esteve presente, e para as amostras tratadas (HM HoP, HM HPP1, HM HPP2, HM HPP3 e HM HPP4), essa classe de microrganismo foi encontrada ausente, mostrando que os processamentos foram eficazes em sua eliminação.

CONCLUSÃO. Com esse estudo, foi possível concluir que o processamento de alta pressão oferece uma alternativa viável à pasteurização padrão no tratamento do LH, uma vez que os processos realizados eliminaram os microrganismos do grupo coliforme. A composição em AG, a concentração de macronutrientes (proteínas, lipídios, carboidratos, cinzas e umidade) e a acidez Dornic não sofreu diferença significativa em relação aos processos avaliados. E as concentrações estimadas e o perfil de TAG dos principais TAGs das amostras lipídicas foram semelhantes para a amostra de LH controle, para a amostra HM HoP e todas as amostras de processamento de alta-pressão, indicando que o perfil lipídico não alterou com os processamentos.

Palavras-chave: composição em ácidos graxos, espectrometria de massas por infusão direta, bancos de leite humano, coliformes totais, processamento de alta-pressão.

ARTICLE 2

Effects of pasteurization and high-pressure processing on the fatty acids, triacylglycerol profile, Dornic acidity and macronutrients in mature human milk

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Abstract

The effects of high-pressure processing (HPP; 300, 400, 500, and 600 MPa for 5 min) compared to Holder pasteurization treatments on the macronutrients, acidity in Dornic degrees, total coliforms, fatty acids (FA) composition, and triacylglycerol (TAG) profile of mature human milk (HM) were evaluated. The results showed that both processes eliminated the microorganisms present, and the concentration of macronutrients and Dornic acidity did not show significant differences between the processes. A total of 34 FAs were identified by gas chromatography with flame ionization detector, with palmitic and oleic acids having the highest concentrations. Regarding the TAG profile determined by electrospray ionization mass spectrometry, TAG PLO (P: palmitic acid; L: linoleic acid; and O: oleic acid) was the one with the highest estimated concentration (6.52 to 7.27%). The hierarchical clustering analysis results suggests that processing with lower pressures was more beneficial for HM due to the similarity of the intensities of TAGs identified in each sample compared to the control HM sample. Besides, the results obtained on the evaluated components suggests that HPP could be a promising alternative to HoP applied in human milk banks since it maintained the characteristics of HM and is faster than HoP.

Keywords: Fatty acid composition; Triacylglycerol profile; Pasteurization; High-pressure in human milk

ABBREVIATIONS

ANOVA - Analysis of variance;

CLA - Conjugated linoleic acid;

ESI-MS - Electrospray Ionization Mass Spectrometry;

FA – Fatty acid;

FAME – Fatty acid methyl esters;

GC-FID - Gas Chromatography with Flame Ionization Detector;

HCA - Hierarchical Clustering Analysis;

HM – Human Milk;

HMB - Human Milk Bank;

HoP - Holder pasteurization;

HPP - High-pressure processing;

MUFA - Monounsaturated fatty acids;

PUFA - Polyunsaturated fatty acids;

SFA - Saturated fatty acids;

TAG – Triacylglycerols.

1. Introduction

Human milk (HM) is considered an essential and exclusive source of nutrition for babies up to the first six months of their lives. In addition, it helps in adapting to the external environment in which they will live, by offering substances that contribute to the strengthening of immunity. Besides, HM has several nutrients that contribute to the healthy development of the newborn, such as bioactive compounds with antioxidant capacities, immune and growth factors, enzymes, and hormones (Ma et al., 2017; Matheson et al., 2012).

Among the components of HM, lipids have the second-highest concentration of nutrients and are responsible for providing 50% of the total caloric value, acting as the main energetic source for the newborn (Innis, 2013). For this reason, the National Health Surveillance Agency (ANVISA - Agência Nacional de Vigilância Sanitária) of Brazil, determined that, among the various services of the human milk banks (HMB), the donation of milk should be supported and encouraged for mothers with difficulties to breastfeed their children for some reasons (for example, infection by human immunodeficiency virus (HIV) or by human T cell lymphotropic virus (HTLV); use of some drugs incompatible with breastfeeding; and insufficient milk production). To supply the demand in these cases, the donated HM to the HMBs can remain frozen at -18 °C, for up to 15 days after collection (Almeida et al., 2008).

HMBs are responsible for maintaining the quality and conservation of all donated HM through the application of a mandatory pasteurization process, which ensures that HM becomes free of pathogenic microorganisms. According to ANVISA, Holder pasteurization (HoP) should be carried out in a water bath at a temperature of 62.5 °C for 30 minutes, and then frozen at -18 °C (Almeida et al., 2005; Peila et al., 2017). This process has beneficial effects concerning the reduction of microbial load, but it can modify the nutritional, structural, and biological composition of HM, causing the breakdown of lipid molecules and can degrade some biochemical components, such as IgA, IgG, and IgM (Romeu-Nadal et al., 2008; Vieira et al., 2011).

Thus, research has been performed in partnership with HMBs, for the development of other techniques that contribute to microbial inactivation and preserve HM in relation to its chemical and structural composition, such as, the application of high pressure.

High-pressure processing (HPP) may be considered an alternative to HoP since it is responsible for eliminating pathogenic microorganisms from HM, applying pressures from

300 to 800 MPa evenly for 5 to 10 minutes (Huppertz et al., 2006). HPP is considered rapid processing and does not need to involve high temperatures, so it provides food free of pathogens, without nutritional and sensory changes, and in its original chemical composition (Considine et al., 2008). It is different of processes that use heat as a form of preservation, such as pasteurization and sterilization, which slow growth or inactivate pathogenic and food-spoiling microorganisms, but can produce undesirable changes that affect taste, aroma, texture, and color of processed foods, in addition to destroying nutrients, especially vitamins (Pflanzer et al., 2008).

Therefore, the present study aims to evaluate the influence of HPP in the macronutrients (proteins, lipids, carbohydrates, ash, and moisture), acidity in Dornic degrees, total coliforms, FA composition, and the triacylglycerol (TAG) profile of HM, under different conditions, comparing with the HoP processing and unprocessed HM.

2. Material and Methods

2.1 Samples

The present study was approved by the Research Ethics Committee (CEP), number 2,797,476, of the State University of Maringá (Maringá, Paraná, Brazil). Mature HM samples were collected from twenty different mothersat the HMB of the University Hospital of Maringá, with a cooling temperature of 4 °C. The total volume collected from each mother was 120 mL. Samples were mixed and homogenized with a mixer (Viva Collection 400W RI136406, Philips Walita Ltd., Brazil) for 15 s to form a pool of HM (2400 mL) (Du et al., 2017). After pooling, 400 mL of the unprocessed HM pool (Control HM) were separated and stored at -18 °C before analyses were performed. An amount of 400 mL of HM pool were submitted to pasteurization processing (HM HoP) and 1600 mL to high-pressure processing.All processes were performed in triplicate.

2.2 High-pressure processing (HPP)

HPP was performed according to the methodology used by Moltó-Puigmartí et al. (2011), with some modifications. The high-pressure hydrostatic equipment used was the QFP 2L-700 (Avure Technologies, OH, USA). The storage chamber has a capacity of two liters, maximum pressure of 690 MPa, and a controlled temperature between 5 to 90 °C. The pressures used were 300 (HM HPP1), 400 (HM HPP2), 500 (HM HPP3), and 600 MPa (HM HPP4), with a fixed time of 5 min and initial temperature of 5 °C. The final temperature was calculated according to the formula $T_F = T_I + \alpha.P$ (Viazis et al., 2007), where T_F is the final

temperature (°C), T_I is the initial temperature (°C), α is the compression heating factor (3 °C/100 MPa) and P is the applied pressure (MPa). In total, four processes of HPP were carried out in the mature HM with a volume of 400 mL for each process. These samples were stored in plastic bags and vacuum-packed. After treatment, all 4 samples were quickly cooled to a temperature of 4 °C and frozen at -18 °C.

2.3 Holder pasteurization processing (HoP)

The Holder pasteurization is the current standard pasteurization process used in HMBs. So, it was applied in this study according to the literature guidelines (Arslanoglu et al., 2010). The pasteurization was performed with heating in a water bath, using a temperature of 62.5 °C for 30 minutes, with manual stirring every 5 minutes. Subsequently, the HM was cooled to a temperature of 4 °C, and frozen at -18 °C, as described by ANVISA (Almeida et al., 2005) and the Italian Association of Human Milk Banks (Arslanoglu et al., 2010). The HM sample subjected to HoP was named as HM HoP.

2.4 Determination of macronutrients composition (%) and acidity (°Dornic)

Macronutrients analyses were conducted for all samples (HM control, HM HoP, HM HPP1, HM HPP2, HM HPP3, and HM HPP4) in triplicate. The analyzes of moisture, ash, and protein were performed according to AOAC (1998). Moisture was determined by the gravimetric method with the use of heat, in which 3 g of each sample was subjected to heating at 105 °C until reaching constant weight. Ash was determined by submitting the samples to calcination in a muffle at 550 °C for 5 hours. Protein analyses were carried out by the Kjeldahl method, and the results were expressed in g 100 g^{-1} of HM, using 6.25 as the nitrogen-to-protein conversion factor. Lipids were determined by the method of Folch et al. (1957), using chloroform and methanol (2:1, v/v) as extractor solvents. Finally, carbohydrates concentrations were calculated by the difference between the total sample (100%) and the concentrations of the evaluated macronutrients (protein, fat, moisture, and ash), as described (Equation 1) (Terra et al., 2010).

[Carbohydrates] (%) = 100 – ([moisture] + [ash] + [proteins] + [lipids]) Equation (1)

The Dornic acidity was obtained with a titrating solution of sodium hydroxide 0.11 mol/L (Dornic solution) with phenolphthalein indicator and performed in triplicate (Almeida et al., 2008).

2.5 Fatty Acid (FA) Composition by Gas Chromatography with Flame Ionization Detector (GC-FID)

The fatty acid methyl esters (FAME) were prepared by methylation process according to ISO 5509 (2000). FAME analyzes were performed on a gas chromatograph coupled with a flame ionization detector (GC-FID, Shimadzu). The capillary column was a GC-2010 Plus (100.0 m, 0.25 mm i.d., and 0.25 μm). The detector and injector temperatures were at 250 and 240 °C, respectively. The GC-FID oven was programmed to 65 °C and held for 4 min, then heated to 185 °C at 15 °C min^{-1} and held for 12 min, then heated to 235 °C at 20 °C min^{-1} and held for 14 min. The gas flow rates used were 1.4 mL min^{-1} for carrier gas (H_2), 30 mL min^{-1} for make-up gas (N_2) and 30 and 300 mL min^{-1} for the flame gases (H_2 and synthetic air, respectively). Split injection mode was used with a 1:40 ratio, and the volume of sample injections was 2.0 μL (Simionato et al., 2010). FAMEs were identified by comparison of the retention times of the sample constituents with those of analytical standards (FAME standard mixture, C4–C24, Sigma-Aldrich, Product Number 18919-1AMP). The peak areas were determined using Labsolution main GC Solutions software, and the FAs compositions were expressed as a relative percentage of total FAs. All samples were analyzed in triplicate.

2.6 Triacylglycerol (TAG) Profile by Electrospray Ionization Mass Spectrometry (ESI-MS)

Lipid samples from the HM control, HM HoP, HM HPP1, HM HPP2, HM HPP3, and HM HPP4 were prepared based on Youzbachi et al. (2019) with modifications by Silveira et al. (2017). A volume of 50.0 μL of the total lipid from each sample was added to 950.0 μL of chloroform. Then, 5.0 μL of this solution were added to a 1.0 mL methanol/chloroform 9:1 (v v^{-1}) solution. After, 20.0 μL of 0.1 mol L^{-1} ammonium formate solution was added to the final solution. Ammonium formate was added to obtain mostly TAG ammonium adducts $[TAG + NH_4]^+$.

The prepared samples were directly injected into a Xevo TQD™ triple quadrupole mass spectrometer (Waters, Massachusetts, USA) equipped with an electrospray ionization source (Z spray™), operating in positive mode (ESI+), under the following conditions: injection flow rate of 50.0 μL min^{-1}, desolvation gas flow of 450 L h^{-1}, desolvation temperature of 250 °C, source temperature of 130 °C, a capillary voltage of 3.00 kV and cone voltage of 35.0 V. The TAG profiles were evaluated in the mass range from 100 to 1200 m/z. The TAG results were obtained using MassLynx™ software.

2.7 Microbiological analysis

The total coliforms analyses determined for all samples (HM control, HM HoP, HM HPP1, HM HPP2, HM HPP3, and HM HPP4) were performed according to Novak and Almeida (2002) and Almeida et al. (2008), whose result is described by the presence or absence of total coliforms, which consists of the inoculation of four aliquots of 1 mL of HM, in Durham's tubes, with 10 mL of bright green lactose bile broth (50 g L^{-1}), incubated for 48 h in an oven at 36 ± 1 °C. The presence of gas inside Durham's tube characterizes a positive result. Tubes with positive results are confirmed with the aid of a 0.05 mL calibrated bacteriological loop, using tubes containing bright green lactose bile broth (40 g L^{-1}). After incubating these tubes under the same conditions as the initial test, the presence of gas indicates the presence of microorganisms from the coliform group.

2.8 Statistics Analysis

The statistical analysis of macronutrients composition, Dornic acidity, and FA composition by GC-FID were conducted using one way ANOVA and the means were compared by Tukey's test at a significance level of 5%. Hierarchical clustering analysis (HCA) was conducted to explore the data structure and highlight the similarities and discrepancies in the data set. HCA was carried out with ESI-MS data (TAG intensity) and analysis was performed using Statistica 10.0 (Statsoft2011). Euclidean distance and single-linkage methods were used to plot the two-dimensional graph.

3. Results and discussion

3.1 HPP conditions

The HPP conditions chosen in this study were 300, 400, 500, and 600 MPa, with a fixed time of 5 min and an initial temperature of 5 °C. To date, none of the studies found in the literature have analyzed the TAGs of the lipid part of the HM, the composition of macronutrients, and the Dornic acidity under these conditions. Moltó-Puigmartí et al. (2011) evaluated FAs, vitamin C, and vitamin E; Permanyer et al. (2010) determined the HM IgA; Mateos-vivas et al. (2015) analyzed free nucleotide monophosphates; and Franch et al. (2010) evaluated the content of IgA, EGF, TGF-β1, TGF-β2, IL-6, IL-8, IL-10, IL -13, TNF-α, and TNF-RI. All these studies used the HM subjected to pressures of 400, 500, and 600 MPa, for 5 minutes, with an initial temperature of 12 °C. However, in this study, the initial temperature was 5 °C and pressure of 300 MPa/5 min was included.

3.2. Determination of macronutrients composition (%) and acidity (°Dornic)

Table 1 shows the results of macronutrient composition (%) and acidity values (°Dornic) of samples (Control HM, HM HoP, HM HPP1, HM HPP2, HM HPP3, and HM HPP4).

Table 1. Macronutrients composition (%) and acidity (°Dornic) of Control HM, HM HoP, HM HPP1, HM HPP2, HM HPP3, and HM HPP4.

Concentrations[a]	Control HM[a]	HM HoP[a]	HM HPP1[a]	HM HPP2[a]	HM HPP3[a]	HM HPP4[a]
Acidity (°Dornic)	$3.33^{A} \pm 0.58$	$4.00^{A} \pm 1.00$	$3.33^{A} \pm 0.58$	$3.67^{A} \pm 0.58$	$4.00^{A} \pm 1.00$	$4.00^{A} \pm 1.00$
Moisture (%)	$89.07^{A} \pm 0.35$	$88.20^{A} \pm 0.61$	$88.37^{A} \pm 0.31$	$88.70^{A} \pm 0.40$	$88.67^{A} \pm 0.25$	$88.77^{A} \pm 0.31$
Ash (%)	$0.15^{A} \pm 0.02$	$0.16^{A} \pm 0.01$	$0.15^{A} \pm 0.01$	$0.15^{A} \pm 0.02$	$0.17^{A} \pm 0.02$	$0.15^{A} \pm 0.01$
Protein (%)	$1.12^{A} \pm 0.07$	$1.15^{A} \pm 0.09$	$1.14^{A} \pm 0.06$	$1.13^{A} \pm 0.06$	$1.17^{A} \pm 0.07$	$1.21^{A} \pm 0.04$
Lipid (%)	$3.26^{A} \pm 0.15$	$3.24^{A} \pm 0.07$	$3.31^{A} \pm 0.11$	$3.24^{A} \pm 0.11$	$3.23^{A} \pm 0.07$	$3.23^{A} \pm 0.10$
Carbohydrate (%)	$6.40^{A} \pm 0.45$	$6.99^{A} \pm 0.81$	$6.87^{A} \pm 0.23$	$6.94^{A} \pm 0.25$	$6.65^{A} \pm 0.30$	$6.50^{A} \pm 0.23$

[a]Results expressed as means ± S.D (standard deviation) of three replicates. Values with the same uppercase letters (^{A}) in the same line are not statistically different ($p < 0.05$) by Tukey's test. HM subjected to Holder pasteurization (HM HoP); HM subjected to high pressure processing (HM HPP).

Dornic acidity values and the concentrations of moisture, ash, protein, lipid, and carbohydrate did not vary significantly between processing and unprocessed HM (Control HM) ($p<0.05$).

To the best of our knowledge, this study is the first to evaluate the effects of HPP on Dornic acidity. Dornic acidity is used as an indirect measure of the presence of bacteria in HM because the fermentation of lactose by the bacteria increases lactic acid, which increases the acidity of HM. Normally, HM samples that have acidity greater than 8 °Dornic, are discarded by HMB before pasteurization. As noted in Table 1, the values obtained in this study varied between 3.33 to 4.00 °Dornic and were between the reference value (1 - 8 °Dornic), therefore these samples are appropriate for consumption as required by the HMB (Vázquez-Román et al., 2013).

Fernández-Menéndez et al. (2020) studied ten samples of mature HM and the values of the Dornic acidity found were between 4.00 and 5.30 °Dornic. Manin et al. (2019) evaluated Dornic acidity in pasteurized HM and lyophilized pasteurized HM for six months. The values obtained for mature HM varied between 3.67 and 5.00 °Dornic. These two studies found values similar to those found in this present study.

The concentrations of carbohydrates in the present study were between 6.40 and 6.99%, obtained by calculating by difference, so it was influenced by the values of moisture, ash, protein, and lipid. The values found in this study were similar to the study by Meredith-Dennis et al. (2018). Authors evaluated three variations of HM processing, the first at 62.5 °C for 30 min, the second at 63 °C for 30 min and the third at 121 ºC for 5 min. They concluded that the process applied did not significantly change the carbohydrate content, which varied between 7.0 and 7.2%. HM carbohydrates are important for regulation of children's appetite; helping to reduce the presence of bacteria in the gastrointestinal tract and improving the absorption of some micronutrients, such as, calcium, magnesium, and phosphorus. The main HM carbohydrates found is lactose, that is important for maintaining the constant osmotic pressure in the baby (Gridneva et al., 2019).

The concentrations of moisture varied between 88.20 and 89.07%, similar to the results found by Castro-Albarrán et al. (2016) that obtained 88.43% of moisture in HM. Water is the most important component of HM, with a concentration of approximately 90% in relation to the total. Sousa and Silva (2010) monitored the quality of HM before and after pasteurization, the moisture after pasteurization and before freezing were 88.67% and 86.70%, respectively. This lower moisture concentration may indicate loss of solutes during HM processing.

Minerals are essential for the child's normal growth and development. They are important for brain development, bone health, and play a role in the metabolism of thyroid hormone (Sabatier et al., 2019). The concentrations of ash varied between 0.15 and 0.17%, showing the total amount of minerals present in the HM. The obtained results are according to Shi et al. (2011) and Sousa and Silva (2010), which got mineral concentration of 0.21% and 0.14 to 0.16%, respectively.

The concentrations of lipid varied between 3.23 and 3.31%, which are in accordance with 3.04% and 1.97 - 4.07% reported for the mature HM in other studies (Shi et al. 2011; Sousa and Silva, 2010). In HM, lipids are the main component that provide energy to the child. They are also important for providing essential FAs, fat-soluble vitamins, and specific bioactive compounds (Demmelmair & Koletzko, 2018).

The concentrations of protein varied between 1.12 and 1.21%. Results were close to that obtained by Shi et al. (2011) (1.27%). In HM, proteins play an important role in the child's growth, intestinal development, and with substances that act as anti-inflammatories (Lönnerdal, 2017).

Similarly with this study, Pitino et al. (2019) evaluated raw HM, HM subjected to HoP, and HM at HPP at 500 MPa for 8 minutes, and observed that the HM at HPP did not change the concentration of lipids and proteins, compared to both raw HM and HM HoP.

3.3 Fatty Acids (FA) Composition

The FAs composition of Control HM, HM HoP, HM HPP1, HM HPP2, HM HPP3, and HM HPP4 are shown in Table 2. Figure 1 shows the sum of the content of fatty acids (SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids).

Table 2. Fatty acid composition of Control HM, HM HoP, HM HPP1, HM HPP2, HM HPP3 and HM HPP4.

Fatty Acid Composition[a]	Control HM[a](%)	HM HoP[a] (%)	HM HPP[a] (%)	HM HPP2[a](%)	HM HPP3[a](%)	HM HPP4[a](%)
4:0	$0.03^{A} \pm 0.01$	$0.03^{A} \pm 0.01$	$0.04^{A} \pm 0.00$	$0.04^{A} \pm 0.00$	$0.03^{A} \pm 0.00$	$0.03^{A} \pm 0.01$
6:0	$0.01^{A} \pm 0.01$	$0.01^{A} \pm 0.01$	$0.03^{A} \pm 0.01$	$0.02^{A} \pm 0.01$	$0.01^{A} \pm 0.00$	$0.01^{A} \pm 0.01$
8:0	$0.09^{A} \pm 0.00$	$0.07^{A} \pm 0.01$	$0.05^{A} \pm 0.01$	$0.04^{A} \pm 0.01$	$0.06^{A} \pm 0.01$	$0.11^{A} \pm 0.08$
10:0	$1.65^{A} \pm 0.07$	$1.69^{A} \pm 0.09$	$1.47^{A} \pm 0.08$	$1.65^{A} \pm 0.00$	$1.60^{A} \pm 0.03$	$1.56^{A} \pm 0.02$
12:0	$7.47^{A} \pm 0.64$	$8.51^{A} \pm 1.20$	$7.04^{A} \pm 0.57$	$6.55^{A} \pm 0.96$	$6.28^{A} \pm 0.88$	$6.16^{A} \pm 0.95$
14:0	$7.45^{A} \pm 0.23$	$8.29^{A} \pm 0.23$	$7.50^{A} \pm 0.23$	$7.65^{A} \pm 0.14$	$7.56^{A} \pm 0.05$	$7.68^{A} \pm 0.11$
14:1	$0.08^{A} \pm 0.01$	$0.08^{A} \pm 0.00$	$0.07^{A} \pm 0.00$	$0.07^{A} \pm 0.01$	$0.07^{A} \pm 0.00$	$0.08^{A} \pm 0.02$
15:0	$0.34^{A} \pm 0.05$	$0.28^{A} \pm 0.01$	$0.28^{A} \pm 0.01$	$0.27^{A} \pm 0.00$	$0.26^{A} \pm 0.01$	$0.32^{A} \pm 0.08$
15:1	$0.08^{A} \pm 0.01$	$0.07^{A} \pm 0.00$	$0.07^{A} \pm 0.00$	$0.07^{A} \pm 0.00$	$0.07^{A} \pm 0.00$	$0.08^{A} \pm 0.01$
16:0	$27.46^{A} \pm 1.34$	$24.45^{A} \pm 1.25$	$26.65^{A} \pm 1.05$	$26.71^{A} \pm 1.18$	$25.55^{A} \pm 0.83$	$25.86^{A} \pm 1.44$
16:1n-7	$0.28^{A} \pm 0.03$	$0.26^{A} \pm 0.00$	$0.23^{A} \pm 0.01$	$0.23^{A} \pm 0.01$	$0.23^{A} \pm 0.00$	$0.25^{A} \pm 0.04$
16:1n-9	$1.87^{A} \pm 0.16$	$1.69^{A} \pm 0.17$	$1.66^{A} \pm 0.11$	$1.64^{A} \pm 0.13$	$1.45^{A} \pm 0.12$	$1.41^{A} \pm 0.13$
17:0	$0.34^{A} \pm 0.05$	$0.30^{A} \pm 0.00$	$0.33^{A} \pm 0.00$	$0.33^{A} \pm 0.01$	$0.31^{A} \pm 0.00$	$0.33^{A} \pm 0.01$
17:1	$0.12^{A} \pm 0.02$	$0.10^{A} \pm 0.02$	$0.09^{A} \pm 0.01$	$0.09^{A} \pm 0.01$	$0.12^{A} \pm 0.02$	$0.10^{A} \pm 0.01$
18:0	$6.73^{A} \pm 0.36$	$6.67^{A} \pm 0.26$	$7.01^{A} \pm 0.41$	$6.94^{A} \pm 0.30$	$6.99^{A} \pm 0.26$	$7.06^{A} \pm 0.46$
18:1n-9	$24.45^{A} \pm 1.09$	$25.75^{A} \pm 1.09$	$25.40^{A} \pm 1.04$	$25.59^{A} \pm 1.25$	$26.56^{A} \pm 1.30$	$26.30^{A} \pm 1.14$
18:1n-7	$1.54^{A} \pm 0.13$	$1.40^{A} \pm 0.12$	$1.45^{A} \pm 0.11$	$1.49^{A} \pm 0.10$	$1.55^{A} \pm 0.11$	$1.59^{A} \pm 0.07$
18:2n-6	$16.20^{A} \pm 0.62$	$16.40^{A} \pm 0.31$	$16.50^{A} \pm 0.34$	$16.50^{A} \pm 0.59$	$17.16^{A} \pm 0.96$	$16.98^{A} \pm 1.11$
20:0	$1.01^{A} \pm 0.05$	$1.00^{A} \pm 0.05$	$0.95^{A} \pm 0.04$	$0.94^{A} \pm 0.04$	$0.99^{A} \pm 0.03$	$0.98^{A} \pm 0.03$
18:2; c9.t11	$0.13^{A} \pm 0.02$	$0.16^{A} \pm 0.01$	$0.19^{A} \pm 0.02$	$0.19^{A} \pm 0.02$	$0.19^{A} \pm 0.02$	$0.19^{A} \pm 0.02$
18:2; t10.c12	$0.19^{A} \pm 0.01$	$0.20^{A} \pm 0.00$	$0.24^{A} \pm 0.03$	$0.22^{A} \pm 0.00$	$0.22^{A} \pm 0.00$	$0.22^{A} \pm 0.00$
18:3n-3	$0.23^{A} \pm 0.03$	$0.25^{A} \pm 0.03$	$0.28^{A} \pm 0.03$	$0.28^{A} \pm 0.03$	$0.28^{A} \pm 0.02$	$0.27^{A} \pm 0.03$
18:3n-6	$0.06^{A} \pm 0.02$	$0.04^{A} \pm 0.01$	$0.04^{A} \pm 0.01$	$0.04^{A} \pm 0.01$	$0.04^{A} \pm 0.02$	$0.04^{A} \pm 0.01$
21:0	$0.31^{A} \pm 0.03$	$0.33^{A} \pm 0.01$	$0.37^{A} \pm 0.01$	$0.38^{A} \pm 0.01$	$0.38^{A} \pm 0.02$	$0.37^{A} \pm 0.04$
20:1n-9	$0.34^{A} \pm 0.02$	$0.37^{A} \pm 0.02$	$0.39^{A} \pm 0.01$	$0.38^{A} \pm 0.01$	$0.39^{A} \pm 0.00$	$0.37^{A} \pm 0.03$
22:0	$0.32^{A} \pm 0.03$	$0.35^{A} \pm 0.02$	$0.36^{A} \pm 0.01$	$0.37^{A} \pm 0.00$	$0.36^{A} \pm 0.00$	$0.36^{A} \pm 0.01$

20:2n-6	$0.03^{A} \pm 0.01$	$0.04^{A} \pm 0.01$	$0.06^{A} \pm 0.02$	$0.06^{A} \pm 0.02$	$0.06^{A} \pm 0.02$	$0.08^{A} \pm 0.03$
20:5n-3 (EPA)	$0.16^{A} \pm 0.02$	$0.17^{A} \pm 0.01$	$0.14^{A} \pm 0.00$	$0.14^{A} \pm 0.00$	$0.13^{A} \pm 0.00$	$0.15^{A} \pm 0.02$
20:3n-6	$0.02^{A} \pm 0.01$	$0.03^{A} \pm 0.01$	$0.04^{A} \pm 0.02$	$0.04^{A} \pm 0.02$	$0.04^{A} \pm 0.02$	$0.04^{A} \pm 0.02$
20:3n-3	$0.73^{A} \pm 0.12$	$0.75^{A} \pm 0.02$	$0.76^{A} \pm 0.02$	$0.77^{A} \pm 0.02$	$0.76^{A} \pm 0.02$	$0.76^{A} \pm 0.02$
20:4n-6 (AA)	$0.06^{A} \pm 0.01$	$0.07^{A} \pm 0.01$	$0.09^{A} \pm 0.01$	$0.09^{A} \pm 0.01$	$0.08^{A} \pm 0.01$	$0.08^{A} \pm 0.01$
24:0	$0.04^{A} \pm 0.01$	$0.04^{A} \pm 0.00$	$0.02^{A} \pm 0.02$	$0.02^{A} \pm 0.00$	$0.02^{A} \pm 0.00$	$0.02^{A} \pm 0.00$
24:1	$0.08^{A} \pm 0.00$	$0.09^{A} \pm 0.01$	$0.10^{A} \pm 0.02$	$0.10^{A} \pm 0.01$	$0.09^{A} \pm 0.00$	$0.08^{A} \pm 0.01$
22:6n-3 (DHA)	$0.08^{A} \pm 0.01$	$0.09^{A} \pm 0.01$	$0.11^{A} \pm 0.02$	$0.11^{A} \pm 0.02$	$0.11^{A} \pm 0.02$	$0.10^{A} \pm 0.02$
Σ SFA	$53.26^{A} \pm 1.35$	$52.01^{A} \pm 1.28$	$52.09^{A} \pm 1.13$	$51.90^{A} \pm 1.12$	$50.40^{A} \pm 1.16$	$50.83^{A} \pm 1.25$
Σ MUFA	$28.85^{A} \pm 0.98$	$29.80^{A} \pm 1.15$	$29.45^{A} \pm 0.73$	$29.67^{A} \pm 0.64$	$30.53^{A} \pm 1.29$	$30.27^{A} \pm 1.27$
Σ PUFA	$17.89^{A} \pm 0.60$	$18.19^{A} \pm 0.33$	$18.46^{A} \pm 0.39$	$18.43^{A} \pm 0.22$	$19.07^{A} \pm 0.27$	$18.90^{A} \pm 0.40$
Σ n-3	$1.20^{A} \pm 0.06$	$1.26^{A} \pm 0.05$	$1.29^{A} \pm 0.05$	$1.29^{A} \pm 0.06$	$1.27^{A} \pm 0.04$	$1.27^{A} \pm 0.04$
Σ n-6	$16.69^{A} \pm 0.37$	$16.93^{A} \pm 0.32$	$17.16^{A} \pm 0.38$	$17.14^{A} \pm 0.11$	$17.35^{A} \pm 0.76$	$17.62^{A} \pm 0.86$
Σn-6/ Σn-3	$13.90^{A} \pm 0.25$	$13.43^{A} \pm 0.78$	$13.30^{A} \pm 0.54$	$13.29^{A} \pm 0.43$	$13.66^{A} \pm 0.30$	$13.87^{A} \pm 0.21$

[a]Results expressed as means ± SD (standard deviation) of three replicates. Values with the same uppercase lettersin the same line are not statistically different ($p < 0.05$) by Tukey's test. Omega-3 (n-3); omega-6 (n-6); omega-6/omega-3 ratios (n-6)/(n-3); Saturated fatty acids (SFA); Monounsaturated fatty acids (MUFA); Polyunsaturated fatty acids (PUFA); Conjugated linoleic acid (CLA); HM subjected to Holder pasteurization (HM HoP); HM subjected to high pressure processing (HM HPP).

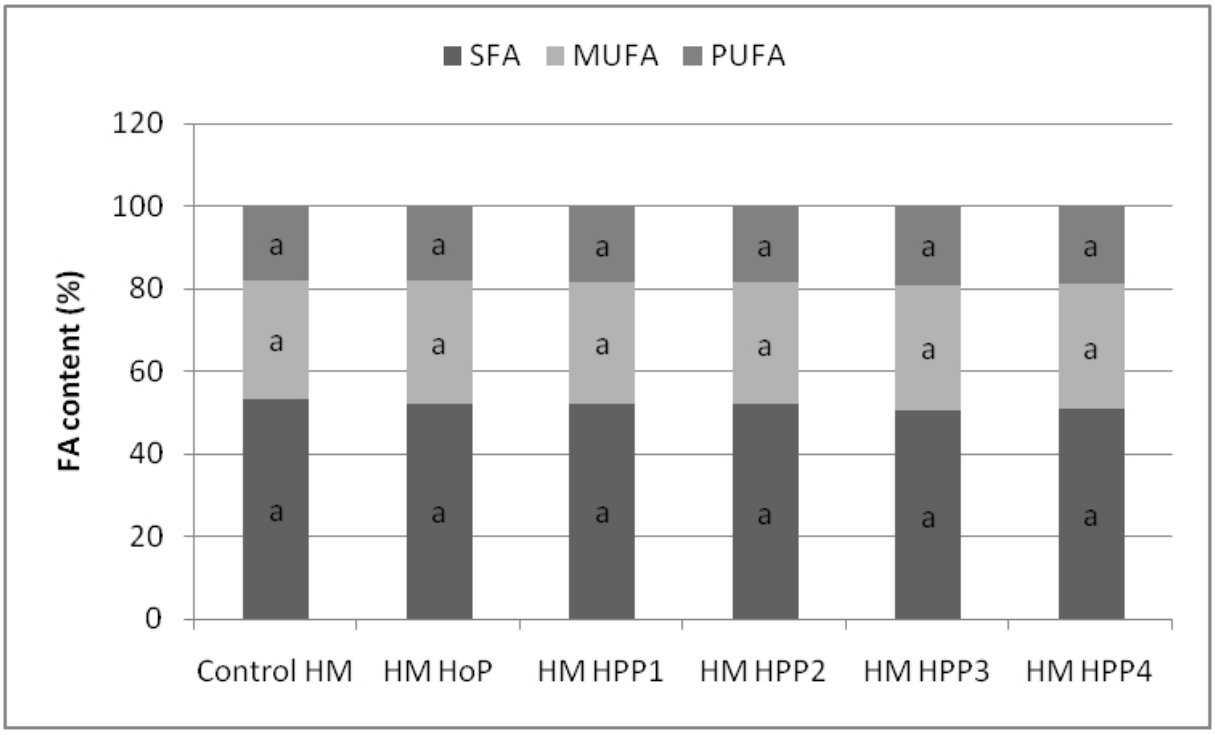

Figure 1. Content of fatty acids (SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids) in the samples (Control HM, HM HoP, HM HPP1, HM HPP2, HM HPP3, and HM HPP4). The same lower-case letters (for SFA, MUFA, and PUFA separately) indicate statistically significant similar values ($p < 0.05$).

Thirty-four FAs were identified and determined by GC-FID in the studied samples (Table 2). HM FAs analyzed showed 50.40 to 53.26% of saturated fatty acids (SFA), 17.89 to 19.07% of polyunsaturated fatty acids (PUFA), and 28.85 to 30.53% of monounsaturated fatty acids (MUFA) (Table 2 and Figure 1).

Among SFAs, palmitic acid (16:0) was the FA with higher abundance, presenting values between 24.45 and 27.46%, which is according to other studies (Demmelmair & Koletzko, 2018; Innis et al., 1994; Mayo et al., 2020). This FA (16:0) located in the TAG sn-2 region is important because it improves the absorption of calcium in the newborn's intestine and still has analgesic effects, as it increases levels of anandamide, a neurotransmitter that has analgesic effects (Visentainer et al., 2018).

Concerning MUFAs, the major FA was oleic acid (18:1n-9, 24.45 to 26.56%) that is according to Wu et al. (2010), which evaluated HM from Taiwanese donors, and found oleic acid values of approximately 27%. Oleic acid is much important to the baby. It is mainly responsible as a source of energy for the newborn, helps in the absorption of fat by the baby's intestine and acts as a structural component of the brain (Jensen, 1999).

About PUFA, linoleic acid (18:2n-6, 16.20 to 17.16%) and α-linolenic acid (18:3n-3, 0.23 to 0.28%), are considered essential, that is, they are not produced by human metabolism. Therefore, they need to be ingested by food. They have important functions in the child's immune system, growth, and maintenance of hair, and are still precursors of long-chain polyunsaturated fatty acids (LC-PUFA), such as arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3) (Claro et al., 2007; Koletzko et al., 2001).

In this study, AA, EPA, and DHA presented total FA abundance of 0.06 to 0.09%, 0.13 to 0.17%, and 0.08 to 0.11%, respectively. Duan et al. (2019) evaluated the FA compositions in HM of lactating mothers in Korea and obtained AA, EPA, and DHA values in mature HM of approximately 0.54%, 0.22%, and 0.77%, respectively. These higher abundance of the AA, EPA, and DHA are due to the Korean diet, that is rich in fish and seafood.

As expected, the FAs of the Control HM and HM HoP samples had no significant difference ($p<0.05$) (Table 2), because this heat treatment inactivates the enzyme lipase that degrades FAs in HM. Results are according to other studies, those compare the FAs of raw HM and pasteurized HM (Moltó-Puigmartí et al., 2011; Romeu-Nadal et al., 2008).

Regarding the HPP performed, it was also observed that the FAs did not differ significantly ($p < 0.05$) when compared to the control HM (Table 2). Besides, conjugated

linoleic acid (CLA, 18:2) cis-9, trans-11, and CLA trans-10, cis-12 also remained without significant difference ($p < 0.05$). The application of HPP did not cause isomerization of FA, being effective for the conservation of CLAs (Vieira et al., 2017).

Moltó-Puigmartí et al. (2011) studied the conditions of HPP in HM with pressures of 400, 500, and 600 MPa for 5 min and initial temperature of 12 °C, and observed that the applied processes did not change the FA composition.

Wesolowska et al. (2019) evaluated the FA composition in HM processed by HPP under the conditions of 600 MPa for 10 min; 100 MPa for 10 min with an interval of 10 min and 600 MPa for 10 min; 200 MPa for 10 min with an interval of 10 min and 400 MPa for 10 min; 200 MPa for 10 min with an interval of 10 min and 600 MPa for 10 min; and 450 MPa for 15 minutes. The authors also concluded that applied treatments did not change the composition of FAs.

Delgado et al. (2014) applied HPP in HM in different conditions: 400 and 600 MPa, both for 3 and 6 minutes. Results showed that the treatment of 600 MPa for 6 minutes, reduced the sum of PUFAs, however, the other processes did not differ significantly from the control, concluding that only the process of 600 MPa for 6 minutes would not be suitable for the conservation of HM.

Therefore, all HPP conditions used in the present study were effective in maintaining the FAs composition of the identified FAs in mature HM.

3.4 Triacylglycerol (TAG) Composition by ESI(+)-MS

TAGs present in HM are fundamental to ensuring the healthy growth of babies (Tu et al., 2017). They are the lipid component of HM with the highest concentration. Therefore, the TAG analysis of HM is extremely important.

TAGs were detected by ESI(+)-MS as adducts of ammonium $[M + NH_4]^+$in all HM samples (Control HM, HM HoP, HM HPP1, HM HPP2, HM HPP3, and HM HPP4) and identified by Lipid Maps® database in conjunction with the LAMES platform (Filho et al., 1995). With the LAMES platform, it is possible to obtain the estimated concentration (%) of each TAG and also the distribution of FAs in the TAG molecules using the relative percentage of the FAs obtained by the analysis of FA composition by GC-FID.

The estimated concentration and possible structure of the main TAGs are shown in Table 3. The typical TAG profile of samples are shown in Figures 2(a) to 2(f) in the region of 600 to 1000 *m/z*: 2(a) - Control HM; 2(b) - HM HoP; 2(c) - HM HPP1; 2(d) - HM HPP2; 2(e) - HM HPP3; and 2(f) - HM HPP4. To date, there are no studies on the lipid profile of HM

submitted to HPP in these conditions, and the composition of possible TAGs by ESI(+)-MS, compared with unprocessed HM and HM HoP.

Table 3. TAG ions and estimated concentration of the main triacylglycerols of Control HM, HM HoP, HM HPP1, HM HPP2, HM HPP3, and HM HPP4.

m/z $[M+NH_4^+]$	CN:DB	TAG main	Control HM (%)	HM HoP(%)	HM HPP1(%)	HM HPP2(%)	HM HPP3(%)	HM HPP4(%)
740	42:0	MPLa	0.928	1.041	0.851	0.873	0.770	0.773
768	44:0	LaPP	1.702	1.543	1.531	1.531	1.357	1.356
792	46:2	LaLP	2.104	2.133	1.928	1.928	1.722	1.707
794	46:1	LaOP	3.094	3.262	2.926	2.903	2.695	2.642
796	46:0	MPP	1.702	1.507	1.640	1.684	1.638	1.684
818	48:3	LaLO	1.913	2.254	1.842	1.928	1.709	1.663
820	48:2	MLP	2.104	2.083	2.066	2.121	2.078	2.121
820	48:2	LaOO	1.406	1.724	1.398	1.376	1.337	1.287
822	48:1	MOP	3.094	3.185	3.135	3.193	3.251	2.281
824	48:0	PPP	2.080	1.489	1.968	1.928	1.925	1.968
846	50:3	MLO	1.913	2.201	1.974	2.011	2.062	2.066
848	50:2	PLP	3.857	3.086	3.718	3.718	3.663	3.718
848	50:2	MOO	1.406	1.683	1.498	1.514	1.613	1.598
850	50:1	POP	5.672	4.720	5.642	5.599	5.732	5.752
852	50:0	SPP	1.520	1.216	1.531	1.531	1.508	1.553
872	52:4	PLL	2.384	2.133	2.341	2.341	2.324	2.341
874	52:3	PLO	7.013	6.524	7.105	7.050	7.271	7.243
876	52:2	POO	5.156	4.989	5.392	5.308	5.689	5.603
876	52:2	SLP	1.879	1.681	1.928	1.928	1.914	1.955
878	52:1	SOP	2.764	2.571	2.926	2.903	2.994	3.025
898	54:5	OLL	2.167	2.254	2.237	2.220	2.306	2.280
900	54:4	OLO	3.188	3.448	3.395	3.342	3.609	3.528
902	54:3	SLO	1.708	1.777	1.842	1.828	1.899	1.905
902	54:3	OOO	1.563	1.758	1.717	1.678	1.882	1.819
904	54:2	SOO	1.256	1.359	1.398	1.376	1.486	1.473

L: linoleicacid (18:2n-6); La: lauricacid (12:0); M: mystircacid (14:0); O: oleicacid (18:1n-9); P: palmiticacid (16:0); S: stearicacid (18:0). CN: total number of acyl carbons; DB: total number of double bonds. HM subjected to Holder pasteurization (HM HoP); HM subjected to high pressure processing (HM HPP).

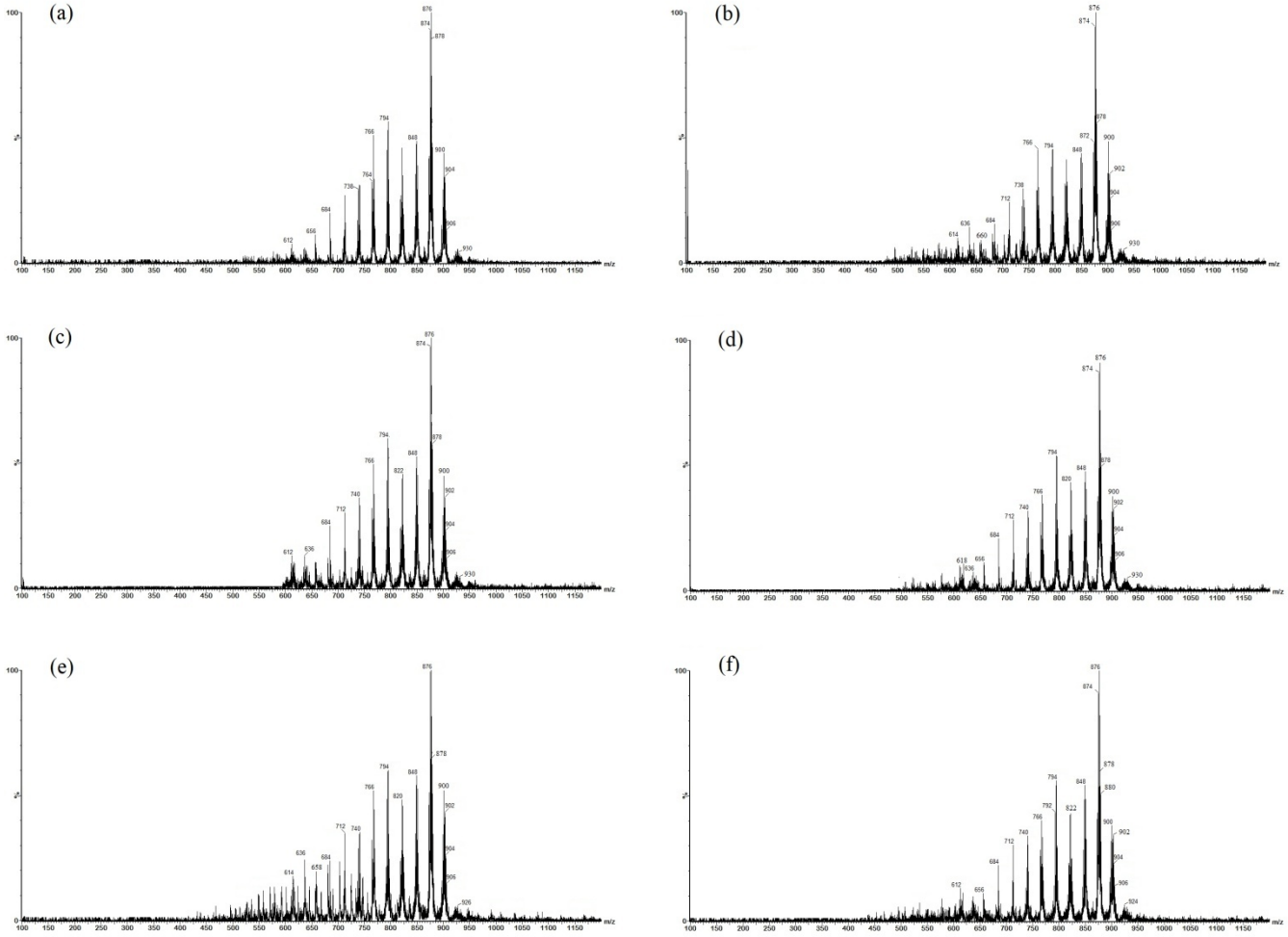

Figure 2. Lipid profile of (a) Control HM; (b) HM HoP; (c) HM HPP1; (d) HM HPP2; (e) HM HPP3; and (f) HM HPP4. HM subjected to Holder pasteurization (HM HoP); HM subjected to high pressure processing (HM HPP).

The main TAGs (Table 3) obtained in mature HM were PLP (848 *m/z*), POP (850 *m/z*), PLO (874 *m/z*), and POO (876 *m/z*). It is observed that these TAGs are composed mainly of palmitic (P, 16:0), oleic (O, 18:1n-9), and linoleic (18:2n-6) acids. These results are similar to those presented by Manin et al. (2019), which evaluated the lipid quality of lyophilized HM of the three phases (colostrum, transitional, and mature) for six months, and obtained that the most intense ratio of TAGs was found in the range of *m/z* 874-876.

Kallio et al. (2017) evaluated TAGs in HM samples from Finnish mothers and Chinese mothers and the main TAGs obtained for HM from Finnish mothers were POO, PLO, LaOP, SOP, MOO, POP, LaOO, and OOO; and for Chinese mothers were PLO, POO, PLL, OLL, SLP, OLO, POP, LaLO, and LaOP. These results are similar to those obtained in the present study.

It is observed that the four HPP conditions used in this study did not alter the TAG profile of the HM (Table 3 and Figures 2(a) to 2(f)). Lipids are the second highest constituent of HM, representing the largest source of energy for children, and providing essential nutrients, such as fat-soluble vitamins and PUFAs (Jensen, 1999). It is extremely important that the pasteurization applied by the HMBs does not modify the structure, composition, and initial concentration of the HM (HM Control). HPP is a technique that can use low temperatures, prevents some components from being modified. On the other hand, pasteurization applies high temperatures and may alter the flavor, odor and the structural composition of the HM (such as IgA, IgM, IgG, lactoferrin, some oligo-elements, and several vitamins) (Moltó-Puigmartí et al., 2011; Pflanzer et al., 2008).

Both analyzed HPP and HoP processes did not change the lipid profile, so concerning these components, HPP could be used as an alternative to pasteurization. As HPP is a promising technique, the perspective is that if the technique is effective in conserving the components of the HM and eliminating pathogenic microorganisms in the HM, it could be applied in HMBs. So, this study provides important results, suggesting HPP as a possible alternative for HoP in HMBs.

New studies have been developed with the aim of verifying the heat sensitive components in the HM after the HPP. Moltó-Puigmartí et al. (2011) evaluated the effect of HPP (400, 500 and 600 MPa, for 5 minutes) on vitamin C and tocopherols, showing that the HPP was effective in conservate these components. Delgado et al. (2014) compared HoP with HPP (400 MPa for 3 and 6 minutes, and 600 MPa for 3 and 6 minutes) on tocopherols and cytokines HM. They found that HoP and HPP at 600 MPa for 6 minutes decreased tocopherol levels. And for cytokines, HPP conditions did not affect the levels of IL-6, IL-8 and TNF-α, showing that HPP at 400 MPa (for 3 or 6 min) was more effective in conserving these nutrients in the HM.

3.5 Hierarchical Cluster Analysis (HCA)

The HCA employs an algorithm on ESI-MS data and builds a distance matrix with the intensity acquired for the TAGs identified for each sample. Then, the algorithm identifies the

sample set with the highest similarity and clusters them together. This iterative process continues until all samples are grouped into a big cluster formed by smaller clusters and the outcome is unveiled as a two-dimensional graph, namely a dendrogram.

What stands out in the dendrogram illustrated by Figure 3 is the increasing difference the processed HM samples have exhibited compared to Control HM. It can be seen from figure 4 that the data set related to HM HoP is highly different from the other samples since HM HoPis clustered with the other samples at the root of the dendrogram. Conversely, closer inspection of the dendrogram's layout shows an interesting pattern regarding HM HPP samples. The first cluster was formed with samples HM HPP1 and HM HPP2, which indicates that both displays the highest similarity among the samples. This first cluster is linked with Control HM sample. Another cluster with HM HPP3 and HM HPP4 led to significant changes in TAG intensity, due to the increase of the pressure employed for processing HM. Since Tukey's test of the macronutrient's composition, °Dornic acidity, and fatty acid composition results demonstrated that the evaluated processing methods caused no statistically significant difference in such parameters, evaluation of HCA results suggests that processing with lower pressures should be beneficial to HM due to the similarity among the intensities of the identified TAGs in each sample.

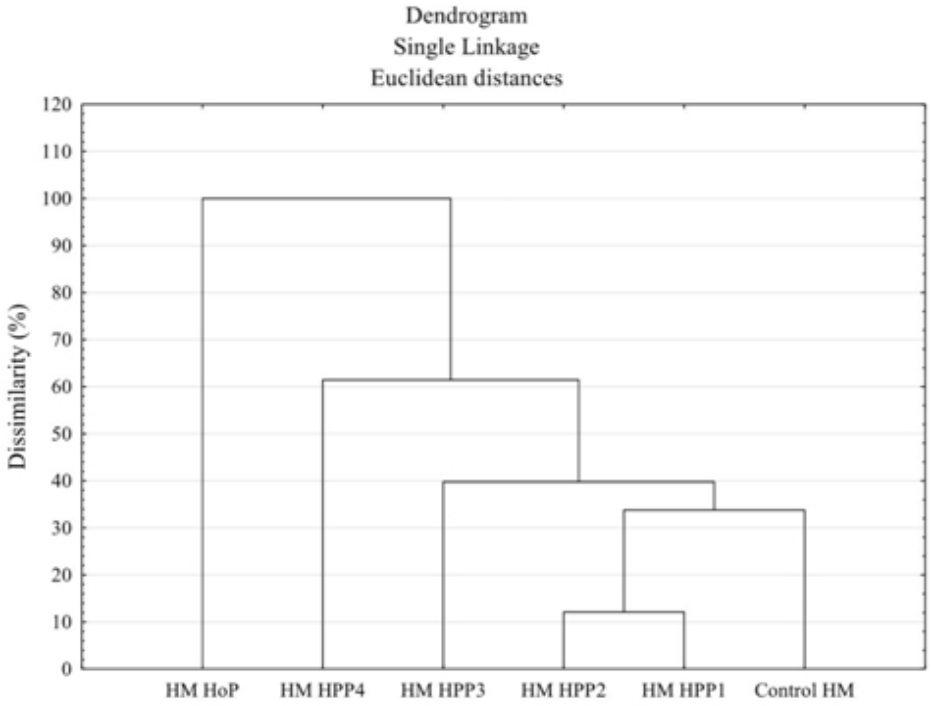

Figure 3. Dendrogram of ESI-MS data of the HM samples (Control HM, HM HoP, HM HPP1, HM HPP2, HM HPP3, and HM HPP4).

3.6 Microbiological analysis

The donated HM is considered as a gold standard, the HMBs must ensure their microbiological safety, and as standard the HoP is effective for this (Jarzynka et al., 2021). The analysis of total coliforms was performed because it is the standard microbiological analysis in HMBs (Almeida et al., 2008). In this analysis it is not possible to quantify the population of coliforms, therefore, the most likely number of these microorganisms in the HM is unknown, as the result is only the presence or absence.

In the control HM, the coliform group was present in the two stages of the analysis (bright green lactose bile broth, 50 and 40 g L^{-1}), and for the treated samples (HM HoP, HM HPP1, HM HPP2, HM HPP3, and HM HPP4), this class of microorganism was found absent, showing that processing was effective in its elimination. When the result is positive, it indicates that at some point in the collection of the HM, there was no good hygiene practices, so the treatments carried out in the HMBs aim to eliminate this through HoP(Novak & Almeida, 2002). Thus, as the result for the HM HoP and the HPP performed were negative, it was shown that the HPP technique can also eliminate this group.

4. Conclusions

The TAG profile in HM processed by HPP in different conditions using the ESI-MS technique presented in this study is the first found in the literature. Results obtained indicate that HPP offers a viable alternative to HoP in treatment of HM, since the processes carried out have eliminated the microorganisms of the coliform group and of the composition of FA, the concentration of macronutrients (proteins, lipids, carbohydrates, ash, and moisture) and acidity Dornic did not suffer significant difference in relation to the evaluated processes, comparing with the HM without processing and the HM submitted to HoP. It was possible to observe that in the HCA, the processes that employed lower pressures (HM HPP1 and HM HPP2) were the ones that presented the greatest similarity in relation to the Control HM sample. On the other hand, HM HPP3 and HM HPP4 indicated that the increase in the pressure used in the HM led to more significant changes in the intensity of the TAG, a result similar to the HM HoP. Thus, the evaluation of HCA results suggests that processing at lower pressures should be beneficial to HM due to the similarity between the intensities of the TAGs identified in each sample. These studies provide important results, but further investigations must be carried out in order to evaluate other HM compounds, such as proteins and the immunological part of HM, under these pressure conditions.

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