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EFEITO DE MÉTODOS DE CONSERVAÇÃO EM NUTRIENTES E COMPONENTES BIOATIVOS DE LEITE HUMANO MADURO

ISADORA BOAVENTURA SÁ PONHOZI

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2022

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Dissertação apresentada ao programa de Pós Graduação em Ciência de Alimentos da Universidade Estadual de Maringá, como parte dos requisitos para obtenção do título de mestre em Ciência de Alimentos

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BIOGRAFIA

Isadora Boaventura Sá Ponhozi nasceu no Paraná, na cidade de Maringá. Possui graduação em Engenharia de Alimentos pela Universidade Estadual de Maringá. Tem experiência na área de Ciência de Alimentos, atuando principalmente com processamento de leite humano, análise de composição centesimal, composição de ácidos graxos por cromatografia em fase gasosa e análise sensorial de alimentos.

Dedico

Aos meus pais, que sempre me incentivaram, acreditaram em mim e possibilitaram essa realização.

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APRESENTAÇÃO

Esta dissertação de mestrado está apresentada na forma de um artigo científico:

Autores: Isadora Boaventura Ponhozi, Patrícia Magalhães de Souza, Luciana Pelissari Manin, Bruno Henrique Figueiredo Saqueti, Joana Maira Valentini Zacarias; Christyna Beatriz Genovez Tavares, Jane Martha Graton Mikcha, Oscar Oliveira Santos, Jeane Eliete Laguila Visentainer, Jesui Vergilio Visentainer.

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GENERAL ABSTRACT

INTRODUCTION. In exceptional situations where the neonate cannot be breastfed or receive their mother's own milk (MOM), among the main recommendations is human milk (HM) donated from the Human Milk Bank (HMB). In Neonatal Intensive Care Units (NICU), MOM can be extracted and given for immediate use, without the quality control requirement. In order to reduce the risks of neonatal infection, the most common treatment in HM is Holder Pasteurization (HoP, 62.5 °C for 30 min). When HM is heated in temperatures higher than its physiological temperatures, the nutritional and immunological properties could be changed. Thus, in HMB, freeze-drying is seen as a promising alternative in the storage and preservation of HM, being effective in preserving nutritional properties.

AIMS. This study aiming at comparing the effect of three methods of conservation (pasteurization, freeze-drying, and pasteurization followed by freeze-drying) into the HM composition. Furthermore, evaluating the possibility of using freeze-dried raw HM when donated MOM, as well as using HM that would be discarded for scientific purposes.

MATERIAL AND METHODS. Mature HM was donated by the HMB. Subsequently, a pool was made and divided into 4 treatments: control, untreated human milk (HMC), pasteurized human milk (HMP), freeze-dried human milk (HMF), pasteurized and freeze-dried human milk (HMPF). HoP (62.5 °C for 30 min) process was performed on the raw HM to obtain the HMP and HMPF. HMF and HMPF were obtained with a SLH-50 lyophilizer, under vacuum of up to 50 μHg, at -55 °C condenser temperature and heating plate temperature of 40 °C. Subsequently, the powdered HM was reconstituted. The moisture, crude protein and total ash determinations were performed according to AOAC (1995). Total lipid content was analyzed according to Folch et al. (1957), and carbohydrate content was obtained by difference. The energy value was calculated by the equation of Fischer Fumeaux et al. (2019). The detection of coliform bacteria was performed according to Novak and Almeida (2002). The extraction of fatty acid methyl esters (FAME) from HM was performed by direct methylation according to the methodology described by Cruz-Hernandez et al. (2013). Subsequently, the FAME were separated using a gas chromatograph (GC) and a flame ionization detector (FID), and the identification was performed by comparing retention times with analytical standards. In relation to cytokines, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α contents were determined using the ProcartaPlex 10-plex Human Custom Kit. The results were subjected to statistical analysis of variance (ANOVA), and the triplicates of the samples were compared by Tukey's Test ($P < 0.05$) probability level.

RESULTS AND DISCUSSION. The data presented in this study indicate that pasteurization (HMP) and freeze drying (HMF), compared to raw HM (HMC), do not affect the macronutrient value and energy value. In the present research, proteins were less preserved with heat treatment followed by freeze-drying (HMPF), with a significant reduction ($P < 0.05$). Regarding the analysis of coliform bacteria, HMC and HMF were positive for total coliforms, while HMP and HMPF were negative. The results showed that the use of the equipment maintained the microbiological quality in relation to total coliforms of HM before the process. Thus, in case coliforms are detected, as in the study, it is necessary to perform heat treatment before processing, as done in HMPF. About the FA composition, oleic acid was the majority FA, with values ranging from 633.38 to 910.02 mg 100 g^{-1} of HM. The results indicate that pasteurization and pasteurization followed by freeze-drying did not significantly alter the concentration of any FA ($P < 0.05$). However, the fact that one sample had been pasteurized before freeze-drying (HMPF) and another sample had been freeze-dried only (HMF) may have caused changes in the internal structures and, consequently, in the physical properties of the fat molecules. According to the results, freeze-drying at 40 °C shelf temperature destabilized the fat and caused the separate and adherence to the bottle when reconstituted. About

cytokines, temperature, storage and freeze-thaw cycles are factors that can affect the stability of them, since some cytokines are more unstable to these factors. Thus, the data suggest that the removal of water through the freeze-drying (HMF) did not affect the biological structure, being able to maintain the cytokine content. Moreover, the storage of HM in powder form also proved favourable in the conservation of cytokine content, as occurred with IL-4 and TNF-α in HMPF, while when submitted to the HoP process (HMP), it caused a significant decrease in IL-6, IFN-γ, TNF-α and IL-4 ($P < 0.05$).

CONCLUSIONS. This study is the first to evaluate the effects of each treatment on HM composition by comparing the effect of freeze-drying on raw HM. According to the results, each treatment caused a change in the composition of raw HM, with a significant decrease in total protein (HMPF), fatty acids (HMF) and some cytokines (HMP and HMPF). In relation to microbiological results, it is suggested that pasteurization remains the most viable alternative. Although MOM submitted only to freeze-drying maintains important bioactive compounds for the neonate, further studies using different shelf plate temperatures would be necessary to find optimal process conditions that maintain the FAs composition. In addition, it would be necessary to ensure that milking and handling of MOM were performed free of microbiological contamination, following the microbiological quality standards of HMB and with result of absence of coliform bacteria.

Key words: breast milk, processing, macronutrient, fatty acid, total coliforms.

RESUMO GERAL

INTRODUÇÃO. Em situações excepcionais em que o recém-nascido não pode ser amamentado ou receber o leite da própria mãe (LPM), entre as principais recomendações está o LH doado pelo Banco de Leite Humano (BLH). Nas Unidades de Cuidados Intensivos Neonatais (UCIN), o LPM pode ser extraído e doado para uso imediato, sem a exigência de avaliação de controle de qualidade. Para reduzir os riscos de infecção neonatal, o tratamento mais comum em LH é a Pasteurização Holder (PHo, 62,5 °C durante 30 min). Quando LH é aquecido em temperaturas superiores às suas temperaturas fisiológicas, as propriedades nutricionais e imunológicas podem ser alteradas. Assim, em BLH, a liofilização é vista como uma alternativa promissora no armazenamento e preservação do LH, sendo eficaz na preservação das propriedades nutricionais.
OBJETIVOS. Este estudo tem como objetivo comparar o efeito de três métodos de conservação (pasteurização, liofilização e pasteurização seguida de liofilização) na composição do LH. Além disso, avaliar a possibilidade de utilizar LH cru liofilizado quando LPM, bem como usar LH que seria descartado para fins científicos.
MATERIAL E METODOS. LH maduro foi doado pelo BLH. Posteriormente, foi realizado um *pool* e dividido em 4 tratamentos: controle, leite humano não tratado (LHC), leite humano pasteurizado (LHP), leite humano liofilizado (LHL) e leite humano pasteurizado e liofilizado (LHPL). O processo HoP (62,5 °C durante 30 min) foi realizado no LH cru para obter o LHP e LHPL. LHL e LHPL foram obtidos com um liofilizador SLH-50, sob vácuo de até 50 μHg, à temperatura do condensador de -55 °C e temperatura da placa de aquecimento de 40 °C. Posteriormente, o LH em pó foi reconstituído. As determinações de umidade, proteína bruta e cinzas totais foram realizadas de acordo com AOAC (1995). O teor total de lipídios foi analisado de acordo com Folch et al. (1957), e o teor de carboidratos foi obtido por diferença. O valor energético foi calculado pela equação de Fischer Fumeaux et al. (2019). A detecção de bactérias coliformes foi realizada de acordo com Novak e Almeida (2002). A extração de ésteres metílicos de ácidos graxos (EMAG) de LH foi realizada por metilação direta de acordo com a metodologia descrita por Cruz-Hernandez et al. (2013). Posteriormente, os EMAGs foram separados usando um cromatógrafo a gás (CG) e um detector de ionização de chama (DIC), e a identificação foi realizada comparando os tempos de retenção com os padrões analíticos. Em relação às citocinas, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 e TNF-α, o conteúdo foi determinado usando o Kit Personalizado Humano ProcartaPlex 10-plex. Os resultados foram submetidos à análise estatística de variância (ANOVA), e as triplicatas das amostras foram comparadas pelo teste de Tukey ($P < 0,05$).
RESULTADOS E DISCUSSÃO. Os dados apresentados neste estudo indicam que a pasteurização (LHP) e a liofilização (LHL), comparadas ao LH cru (LHC), não afetam o valor de macronutrientes e valor energético. Na presente pesquisa, as proteínas foram menos preservadas com tratamento térmico seguido de liofilização (LHPL), com redução significativa ($P < 0,05$). Em relação à análise das bactérias coliformes, LHC e LHL foram positivos para coliformes totais, enquanto LHP e LHPL foram negativos. Os resultados mostraram que o uso do equipamento manteve a qualidade microbiológica em relação a coliformes totais presentes no LH antes do processo. Assim, caso seja detectado presença de coliformes, como no estudo, é necessário realizar um tratamento térmico antes do processamento, como feito em LHPL. Sobre a composição de ácidos graxos (AG), o ácido oléico foi o AG majoritário, com valores variando de 633,38 a 910,02 mg 100 g^{-1} de LH. Os resultados indicam que a pasteurização e pasteurização seguida de liofilização não alteram significativamente a composição dos AG ($P < 0,05$). Entretanto, o fato de uma amostra ter sido pasteurizada antes da liofilização (LHPL) e outra amostra ter sido apenas liofilizada (LHL) pode ter ocasionado em mudanças nas estruturas internas e, conseqüentemente, nas propriedades físicas das moléculas de gordura. De acordo com os resultados, a liofilização com temperatura de prateleira de 40 °C desestabilizou a gordura e causou a separação e aderência ao recipiente quando reconstituída.

Sobre as citocinas, a temperatura, armazenamento e ciclos de congelamento-descongelamento são fatores que podem afetar a estabilidade das mesmas, já que algumas citocinas são mais instáveis à exposição a estes fatores. Assim, os dados sugerem que a remoção da água por meio da liofilização (LHL) não afetou a estrutura biológica, sendo capaz de manter o conteúdo de citocinas. Além disso, o armazenamento de LH na forma de pó também se mostrou favorável na conservação do conteúdo de citocinas, como ocorreu com IL-4 e TNF-α em LHPL, enquanto quando submetido ao processo PHo (LHP), causou diminuição significativa em IL-6, IFN-γ, TNF-α e IL-4 ($P < 0,05$).
CONCLUSÕES. Este estudo é o primeiro a avaliar os efeitos de cada tratamento na composição de LH, comparando o efeito da liofilização no LH cru. De acordo com os resultados, cada tratamento causou uma alteração na composição do LH cru, com diminuição significativa em proteínas totais (LHPL), ácidos graxos (LHL) e algumas citocinas (LHP e LHPL). Em relação aos resultados microbiológicos, sugere-se que a pasteurização continua sendo a alternativa mais viável. Embora o LPM submetido apenas à liofilização mantenha compostos bioativos importantes para o recém-nascido, estudos adicionais usando diferentes temperaturas de prateleira seriam necessários para encontrar condições ideais de processo que mantenham a composição de AGs. Além disso, seria necessário garantir que a ordenha e o manuseio do LPM fossem realizados sem contaminação microbiológica, seguindo os padrões de qualidade microbiológica do BLH e com o resultado da ausência de bactérias coliformes.
Palavras chaves: leite humano, processamento, macronutrientes, ácido graxo, coliformes totais.

ARTICLE

Effect of conservation methods on nutrients and bioactive components of mature human milk

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ABSTRACT

The study compared the effect of methods of conservation (pasteurization, freeze-drying, and pasteurization followed by freeze-drying) into the centesimal composition, fatty acids (FA), cytokines and microbiological quality of human milk (HM). Pasteurization (HoP) maintained the centesimal composition and FAs, but altered some cytokines, while freeze-drying maintained the centesimal composition and cytokines, but altered FAs. Already, HoP followed by freeze-drying maintained FAs, but altered total proteins and some cytokines. Oleic acid was the majority FA, with values ranging from 633.38 to 910.02 mg 100 g^{-1} of HM. According to the results, HoP remains the most viable microbiological alternative. Although mother's own milk (MOM) subjected to freeze-drying maintains important bioactive compounds to neonate, further studies would need to achieve optimal process conditions and ensure that milking and handling of MOM were performed free of microbiological contamination, following the microbiological quality standards of Human Milk Bank (HMB), with absence of coliform bacteria.

Keywords: breast milk, processing, macronutrient, fatty acid, total coliforms.

1. Introduction

Human milk (HM) is the ideal food for infants and the best source of nutrition for the neonate until at least six months. It is capable of undergoing adaptations in its composition throughout lactation, so that it provides the necessary nutrients and perfectly meets the needs of the neonate (Garwolińska et al., 2018; Guo, 2021).

It has a wide variety of bioactive components, as the cytokines that function by binding to specific cell receptors, capable of operating by mediating and regulating the inflammatory responses that are often associated with the body's immune response (Garofalo, 2010; Lyons, Ryan, Dempsey, Ross, & Stanton, 2020). Furthermore, the lipid composition of HM is essential in infant brain development and central nervous system structure (González & Visentin, 2016; Guo, 2021; Meng, Uniacke-Lowe, Ryan, & Kelly, 2021), and the main mechanism for obtaining energy from HM is through fatty acids (FA) (Meng, Uniacke-Lowe, Ryan, & Kelly, 2021; Qi et al., 2018).

In exceptional situations where the neonate cannot be breastfed or receive their mother's own milk (MOM), among the main recommendations is donated HM provided by the Human Milk Bank (HMB) (DeMarchis, Israel-Ballard, Mansen, & Engmann, 2017; Nessel, Khashu, & Dyall, 2019; Wesolowska, Sinkiewicz-Darol, et al., 2019). In Neonatal Intensive Care Units (NICU) in Brazil, MOM can be extracted and given, without the quality control requirement, for immediate use or within two hours, or kept under refrigeration and administered within twelve hours (Gianini et al., 2018; Grazziotin, Grazziotin, Vidal, Freire, & da Silva, 2016). There are species of HMB installed in hospitals, which are organized to collect, store, and distribute MOM to infants hospitalized in the same place (Picaud & Buffin, 2017).

In order to reduce the risks of neonatal infections caused by the use of the HM delivered by the HMB, it goes through a quality control protocol, being discarded when it does not meet the

established standards due to the possible presence of contaminating microorganisms (ANVISA, 2006; Grazziotin et al., 2016; Picaud & Buffin, 2017).

Holder pasteurization (HoP, 62.5 °C for 30 min), is the most common practice for processing raw HM. Although HoP offers microbiological safety, attention is needed regarding the nutritional quality of donated HM in order to ensure optimal nutrition to the newborn, since during processing, heat treatments are factors that can alter the composition of HM as it is a food with many sensitive components (Hård et al., 2019; Moro et al., 2019; Rodríguez-Camejo et al., 2020). When HM is heated in temperatures higher than its physiological temperatures, the nutritional and immunological properties could be changed (Bransburg-Zabary, Virozub, & Mimouni, 2015; Hård et al., 2019; Moro et al., 2019).

In HMB, freeze-drying is seen as a promising alternative in the storage and preservation of HM, being effective in preserving nutritional properties in the long term, increasing shelf life, as well as reducing storage volume and facilitating its transport (Manin et al., 2020; Martysiak-Żurowska, Rożek, & Puta, 2020; Meng, Uniacke-Lowe, Ryan, & Kelly, 2021).

More evidence is needed on the effect of processing on HM composition, and this information is necessary in order to establish guidelines on the optimal processing of HM on the health of infants (Fang, Grummer-Strawn, Maryuningsih, & Biller-Andorno, 2021). That is, to date there are few researches in the literature that has compared the effect of each processing including the effect of freeze-drying on raw HM.

This study aiming at comparing the effect of three methods of conservation (pasteurization, freeze-drying, and pasteurization followed by freeze-drying) into the HM composition. Furthermore, evaluating the possibility of using freeze-dried raw HM when donated MOM, as well as using HM that would be discarded for scientific purposes. The study of these processes can help

HMB make decisions and help mothers who are unable to breastfeed for some reason, as well as encourage studies on the effect of freeze-drying on raw HM.

2. Material and methods

2.1. Sample collection

HM samples (n = 20) were donated by the HMB of the Hospital Universitário de Maringá (HUM, Maringá, Paraná, Brazil), with approval number from the Committee for Ethics in Research with Human Beings (COPEP) of 2.797.476. The samples were from healthy women residing in Maringá (Paraná, Brazil), between 3 and 46 weeks after the baby's birth, in the mature lactation phase. The HM was collected by the mothers in sterile containers, according to standard hygiene requirements, and stored at a temperature of 4 ºC. After being delivered to the HMB, they were immediately stored at -18 ºC, then thawed at a final temperature of up to 5 ºC (ANVISA, 2006) and subsequently a pool of 150 mL of each sample was cooled and pre-stored in a freezer at -36 ºC. The HM pool was divided into 400 mL per treatment, which were subjected to the following conditions evaluated in this study:

(a) control, untreated human milk (HMC),

(b) pasteurized human milk (HMP),

(c) freeze-dried human milk (HMF),

(d) pasteurized and freeze-dried human milk (HMPF).

2.2. Heat Treatment

According to the current HMB's standard (ANVISA, 2006), the HoP process was performed on the raw HM to obtain the HMP and HMPF samples, with heating in a water bath and manual stirring until the temperature in the center of the flask reached 62.5 °C for 30 min. After the treatment, the samples were stored in plastic bottles at -36 °C until the analyses were performed.

2.3. Freeze-drying process

In the freeze-drying process the HM was distributed in vials with a volume of 80 mL, which were refrigerated in a vertical ultra freezer (Terroni, São Carlos, São Paulo, Brazil) at -36 °C for a minimum time of 24 h. A freeze-drying system suitable for use in HMB was used, in a sterile environment that allows handling within the standards required by the RDC nº 171 (ANVISA, 2006). The samples were placed inside the drying chamber of a SLH-50 lyophilizer (Terroni, São Carlos, São Paulo, Brazil), working according to the manufacturer's recommendations, under vacuum of up to 50 µHg, at -55 °C condenser temperature (condenser separate from the drying chamber), and at a heating plate temperature of 40 °C until the end of the process, corresponding to a maximum time of 72 h, determined based on preliminary experiments. The samples from each freeze-drying process were stored in vacuum-sealed metallic containers at freezing temperature -36 ºC until the analyses were performed.

2.4. Reconstitution of freeze-dried milk

A pool was made with the powdered HM, which was reconstituted with an equivalent volume of ultrapure distilled water to reach the initial volume of the HM before the freeze-drying

process, at a temperature of 36 ºC (0.126 g mL^{-1} for HMF powder and 0.139 g mL^{-1} for HMPF powder).

2.5. Determination of the centesimal composition

The moisture, crude protein and total ash determinations were performed according to AOAC (1995). Moisture analysis was performed in an oven at 105 ºC until a constant weight was reached. The crude protein analysis was determined by the semi-micro Kjedahl method, and the results were expressed using the conversion factor 6.38. The determination of total ash by heating in a muffle furnace at 550 °C.

Total lipid content was analyzed according to Folch et al. (1957). All analyzes were performed in triplicate. Carbohydrate content was obtained by difference with other components according to equation (1) by MacLean et al. (2003):

Carbohydrate (g 100 mL^{-1}) = 100 – [(water, g 100 mL^{-1}) + (protein, g 100 mL^{-1}) + (fat, g 100 mL^{-1}) + (ash, g 100 mL^{-1})]. (1)

The energy value was calculated by equation (2), according to Fischer Fumeaux et al. (2019):

Energy value (kcal 100 mL^{-1}) = [4.40 x (protein, g 100 mL^{-1}) + 9.25 x (fat, g 100 mL^{-1}) + 4.00 x (carbohydrate, g 100 mL^{-1})]. (2)

2.6. Determination of microbiological quality

The detection of coliform bacteria in the HMC, HMP, HMF and HMPF samples was performed according to Novak and Almeida (2002). Each HM sample was inoculated in bright green lactosate broth at 50 g L^{-1}, then the tubes were incubated at 36 ºC for 48 h. The presence of gas inside the Durham tube was evaluated, and this indicated a positive result. To confirm the positive results, seeding was performed in 40 g L^{-1} lactosate bright green bile broth. After incubation under the same conditions, the presence of gas confirmed the presence of coliform bacteria.

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

The extraction of fatty acid methyl esters (FAME) from HM was performed by direct methylation according to the methodology described by Cruz-Hernandez et al. (2013), with modifications. Subsequently, the FAME were separated using a gas chromatograph (GC), model GC-2010 Plus (Shimadzu), flame ionization detector (FID) and a CP-7420 fused silica capillary column (Select FAME, 100.00 m x 0.25 mm inner diameter, 0.25 μm and cyanopropyl stationary phase). The gas flows were 1.2 mL min^{-1} for the Hydrogen (H_2) carrier gas, 30 mL min^{-1} for the Nitrogen (N_2) replacement gas, and 35.0 and 350.0 mL min^{-1} for the H_2 and synthetic air flame gases, respectively. A sample volume of 1 μL was injected in triplicate in split mode and 1:40 ratio. The column temperature was raised to 65 ºC for 4 min, followed by a heating ramp of 16 °C min^{-1} to 185 ºC, maintained for 12 min. Subsequently, a further ramp of 20 °C min^{-1} was applied up to 235 °C, and held for 9 min, for a total analysis time of 35 min.

Identification of FAME was performed by comparing retention times with relative analytical standards (FAME Mix, C4-C24, Sigma-Aldrich). Theoretical FID correction values were applied according to Visentainer (2012), and results were expressed as mg of fatty acid per 100 g of HM.

2.8. Determination of cytokines

GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α contents were determined using the ProcartaPlex 10-plex Human Custom Kit (Invitrogen™, Life Technologies Corporation, Austria) according to the manufacturer's instructions, without dilution, and subsequently analyzed using a Luminex® 100/200™ Instrument System (Luminex Corporation, Austin, Texas). HMC and HMP in liquid samples, and HMF and HMPF in powder samples were stored for 15 days at -36 ºC until analysis. The results were expressed as pg per mL of HM.

2.9. Statistics Analysis

The results of the analyses of the centesimal composition, fatty acid composition, and cytokines were subjected to statistical analysis of variance (ANOVA), and the triplicates of the samples were compared by Tukey's Test ($P < 0.05$) probability level using Assistat Software Version 7.7 (Francisco & Carlos, 2016).

3. Results and Discussion

3.1. Determination of the centesimal composition

205 The results of the centesimal composition of HMC, HMP, HMF, and HMPF are presented in
206 Table 1. To the best of our knowledge, this study is the first to evaluate and compare the effect of
207 freeze-drying on raw HM on centesimal composition.

208 After each freeze-drying process, moisture analysis was performed on each sample, and
209 subsequently a pool of HMF and HMPF was obtained. The results of the water content of the
210 powders are presented in Table 1, in which the powders presented values of 1.64% and 1.56%, for
211 HMF and HMPF respectively and showed no significant difference between them ($P < 0.05$), since
212 the freeze-drying conditions were the same and freeze-drying occurred similarly in both HMC and
213 HMP samples. The results were close to those of Castro-Albarrán et al. (2016), in which the freeze-
214 drying process performed at a hotplate temperature of 40 ºC obtained water content of
215 approximately 1.75%.

216 According to the legislation RDC nº 171 (ANVISA, 2006), the moisture values for freeze-
217 dried HM need to be in the range of 4-5% and can be stored for one year at room temperature. In
218 the tests performed with moisture values of HM in the range presented, it was noted that the product
219 presented powder agglomerates that melted when in contact with ambient air humidity, resulting in
220 a pasty product that was not stable at room temperature. Thus, it was decided to use a lower
221 humidity than that presented in the legislation.

222 The water loss was determined based on the difference between the moisture content of the
223 liquid HM samples and after the freeze-drying process. The freeze-drying process was able to
224 reduce the amount of water by 87.55% of raw HM and 87.13% of pasteurized HM, values close to
225 that found by Martysiak-Żurowska et al. (2020), which obtained 86.51% water reduction in relation
226 to liquid HM.

227 Regarding the moisture content of the samples, it was noted that the freeze-dried samples
228 did not differ statistically ($P < 0.05$) from the respective samples before the freeze-drying process.

Thus, both the HMC and HMF samples, and HMP and HMPF did not differ significantly ($P < 0.05$) from each other, evidencing that the reconstitution of HM powder is able to obtain close results in the water content of the sample before the processes. Furthermore, the moisture content of the samples ranged from 88.32-89.19 g 100 mL^{-1}, presenting values close to those found in the literature for mature HM of 86.6-90.43% (Butts et al., 2018; Martysiak-Żurowska et al., 2020).

As for macronutrients, the contents of proteins, lipids and carbohydrates ranged from 1.22-1.40 g 100 mL^{-1}, 3.71-4.07 g 100 mL^{-1}, 5.12-6.21 g 100 mL^{-1}, respectively, close to the average composition estimated by Binte Abu Bakar et al. (2021) of 0.9-1.3 g 100 mL^{-1}, 3.1-5.2 g 100 mL^{-1} and 5.6-7.4 g 100 mL^{-1} for proteins, lipids and carbohydrates, respectively. As reported in the literature, pasteurization of HM compared to raw HM does not affect proteins (Peila, Coscia, et al., 2016; Peila, Moro, et al., 2016; Picaud & Buffin, 2017), lipids (Cavazos-Garduño et al., 2016; Fidler, Sauerwald, Koletzko, & Demmelmair, 1998; Lepri, Del Bubba, Maggini, Donzelli, & Galvan, 1997) and carbohydrates (Espinosa-Martos et al., 2013). So does freeze-drying, which also does not affect protein (Cortez & Soria, 2016) and lipid (Cavazos-Garduño et al., 2016) contents.

Lipids are responsible for playing an important role in the nutrition and development of the neonate by being a source and storage of energy and facilitating the absorption of fat soluble compounds (Guo, 2021). Since HoP uses lower temperatures than the HM fat globule breakdown temperature, which occurs from 115 to 125 °C, minimal changes in total lipid content are expected (Binte Abu Bakar et al., 2021), as well as freeze-drying which uses lower temperatures than HoP.

As for the energy value, the samples presented values of 64.50-67.10 kcal 100 mL^{-1}, being within the values presented by Altomonte, Salari, Licitra, & Martini (2019) of 60.2-88.4 kcal 100 mL^{-1}. As presented by Ley et al. (2011), the pasteurization process did not affect the energy content of HM. The freeze-drying process and pasteurization followed by freeze-drying also did not change

252 the energy value of HM, since the calculation of energy content is most affected by the lipid content
253 present in the samples and the lipid content also did not change significantly ($P < 0.05$).
254 The proteins present in HM are responsible for providing important aminoacids for the
255 development of the neonate, as well as aiding in the solubility of essential nutrients and facilitating
256 digestion and absorption by the intestinal mucosa (Guo, 2021; Lönnerdal, 2003). In the present
257 study, it was observed that proteins were less preserved with heat treatment followed by freeze-
258 drying (HMPF), with a significant reduction ($P < 0.05$) in protein content compared to the other
259 samples. Czank et al. (2009), when quantifying the loss rate of some proteins present in HM, found
260 that there was a greater reduction of proteins in HoP (62.5 ºC for 30 min) than when subjected to 40
261 ºC, since at this temperature there was retention of the compounds at levels higher than 90%. Thus,
262 it is justifiable that the use of temperature in the processes caused damage and modified the
263 structure of proteins, since in the heat treatment, a temperature of 62.5 ºC was used for 30 min, and
264 subsequently in freeze-drying a shelf temperature of 40 ºC was used for up to 72 h.
265 Since HM has sensitive components, it becomes paramount that the processes performed do
266 not reach critical temperatures that induce denaturation and inactivation of bioactive proteins, as
267 long as heating HM above physiological temperatures affects the nutritional properties of HM
268 (Bransburg-Zabary et al., 2015).
269 The ash values present in the samples ranged from 0.18-0.21 g 100 mL^{-1}, close to that
270 reported by Altomonte, Salari, Licitra, & Martini (2019), with values between 0.17-0.21 g 100 mL^{-1}
271 for HM. Minerals present in HM contribute in physiological functions, as structural components of
272 tissues and biologically important molecules (Guo, 2021).
273 As shown in Table 1, none of the treatments significantly affected the carbohydrate
274 concentration ($P < 0.05$). The major carbohydrate in milk is lactose (Lyons, Ryan, Dempsey, Ross,
275 & Stanton, 2020; Saarela, Kokkonen, & Koivisto, 2007). It exerts a beneficial effect on the

intestinal absorption of calcium and other minerals important for the developing infant due to its conversion to lactic acid, which lowers the pH and increases the solubility of calcium. In addition, it is responsible for performing osmotic pressure regulation during HM production (Guo, 2021; Ziegler & Fomon, 1983).

3.2. Determination of microbiological quality

Inadequate conditions of hygiene and handling in HM can be evidenced by the excess of bacteria and high acidity of the sample, and one of the factors for discarding HMB is acidity (Meng et al., 2021; Vázquez-Román et al., 2013). In the present study, discarded HM was used in order to giving a destination to HM that would be discarded and test and evaluate the effectiveness of using the hygiene and handling procedures, and the use of ultraviolet (UV) irradiation for decontamination of the freeze-drying cabin. UV irradiation uses wavelength in the ultraviolet UV-C region (200-280 nm), and is effective in destroying the nucleic acids of microorganisms, in order to break DNA by radiation (Moro et al., 2019).

Regarding the analysis of coliform bacteria required by the RDC nº 171 (ANVISA, 2006), HMC and HMF were positive for total coliforms, while HMP and HMPF were negative. As expected, the results presented in Table 2 showed that freeze-drying did not eliminate microorganisms, but the use of the equipment maintained the microbiological quality in relation to total coliforms of HM before the process. Thus, in case coliforms are detected, as in the study, it is necessary to perform heat treatment before processing, as done in HMPF.

3.3. Fatty Acids (FA) Composition

300 The FAs composition of HMC, HMP, HMF and HMPF are contained in Table 3. Thirty-two
301 FAs were identified and quantified, of which eleven were saturated fatty acids (SFA), ten
302 monounsaturated fatty acids (MUFA), and eleven polyunsaturated fatty acids (PUFA).
303 Among the SFAs detected in the samples, the most abundant in all treatments was palmitic
304 acid (16:0), with values of 349.41-437.36 mg 100 g^{-1} of HM. Cruz-Hernandez et al. (2013) reported
305 for palmitic acid content of 840.11 mg 100 mL^{-1}, in average of HM, with the lowest value of 349.87
306 mg 100 mL^{-1}. This value is close to the value showed in Table 3.
307 Within the MUFAs detected, oleic acid (18:1n-9) was the most abundant and presented a
308 composition of 633.38-910.02 mg 100 g^{-1} of HM. The value found for mature HM in the literature
309 was, on average, 1233.76 mg 100 mL^{-1} of HM, close to that found for HMC (Cruz-Hernandez et al.,
310 2013).
311 Linoleic acid (LA, 18:2n-6) was the most abundant PUFAs, followed by α-linolenic acid
312 (ALA, 18:3n-3), with concentrations of 339.19-498.16 mg 100 g^{-1} and 24.03-34.84 mg 100 g^{-1} of
313 HM respectively. The values reported in the literature were 358.00-566.07 mg 100 mL^{-1} for LA and
314 26.00-40.04 mg 100 mL^{-1} for ALA, close to those found in the present study (Cruz-Hernandez et
315 al., 2013; Lepage & Roy, 1984). Total SFAs remained without significant difference in all
316 treatments ($P < 0.05$), with values ranging from 786.16-1020.69 mg 100 g^{-1}. Regarding the sum of
317 MUFA quantified, the values found were 690.41-988.26 mg 100 g^{-1}, and for PUFA 404.26-581.79
318 mg 100 g^{-1}. The values were slightly lower than those found by Thakkar et al. (2013), of 1520 mg
319 100 mL^{-1}, 1470 mg 100 mL^{-1} and 680 mg 100 mL^{-1}, respectively for the sum of SFAs, MUFAs and
320 PUFAs.
321 According to Table 3, the data indicate that pasteurization and pasteurization followed by
322 freeze-drying did not significantly alter the concentration of any FA ($P < 0.05$), keeping the FAs
323 composition statistically similar to raw HM. The results are in agreement with the literature

regarding the effect of processing on FAs in HM, which show that pasteurization (Delgado, Cava, Delgado, & Ramírez, 2014; Manin et al., 2020; Wesolowska, Brys, et al., 2019), and pasteurization followed by freeze-drying (Manin et al., 2020) did not significantly alter the relative composition of FAs when compared to raw HM ($P < 0.05$).

Moltó-Puigmartí et al. (2011) reported that HM donated by HMB provides key components, since the proportions of FAs, including PUFAs and CLA, are not affected by HoP. This is important due to the fact that the human body is unable to synthesize some FAs, such as the essential FAs LA and ALA making it essential to obtain them through HM (Guo, 2021).

These essential FAs are responsible for synthesizing important metabolites of the n-6 and n-3 series, such as arachidonic acid (ARA, 20:4n-6) synthesized by LA, and eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) synthesized by ALA (EFSA, 2014). EPA and DHA play important roles in retinal development and neurodevelopment, and are considered essential for the development of infant immune functions (Guo, 2021; Lee et al., 2018). Obtaining them through the conversion of LA and ALA is not sufficient for development, and it is also necessary to obtain them through HM (Martysiak-Żurowska et al., 2020).

Thus, it is essential that these FAs are provided through HM in sufficient amounts for the proper development of the infant. However, in this study, the concentration of LA, ALA, and DHA showed a significant reduction in the HMF sample compared to HMC ($P < 0.05$), as well as other FA that also had their concentrations significantly altered when subjected to the freeze-drying process ($P < 0.05$), as presented in Table 3.

The results of this research differed from the study by Martysiak-Żurowska et al. (2020), who also performed freeze-drying on raw HM, but showed no difference in the relative percentages of FA compared to raw HM.

When the freeze-dried samples were reconstituted, it was noted that the HMF powder was less miscible and with difficulty in solubilization, and presented some solids that did not solubilize and adhered to the bottle as a fatty layer. This problem was not observed in HMPF powder reconstitution. Despite both samples were subjected to the same freeze-drying process and reconstitution was performed under the same conditions.

Although the freeze-drying process is desirable because it minimally changes the composition of the HM and presents high storage stability, a difficulty of the process is to obtain the reconstitution of the sample without presenting undissolved granules. In addition, a possible improvement in the result of freeze-dried HM with the removal of fat before the freeze-drying process in order to improve the product (Friend, Shahani, Long, & Agel, 1983).

Nickerson et al. (1952) observed that the powders were not easily miscible in water when reconstituting freeze-dried milk because of the free fat, and the fat emulsion had been partially destabilized during processing, with a fatty film formed on the containers. They noted that the powder was most easily dispersed in hot water and with the use of a homogenizer to disperse the fat. Thus, the authors tested some processing techniques and adopted a procedure that reduced the destabilization of the fat emulsion, which involved pasteurization, condensation, homogenization, and rapid freezing the milk (Nickerson et al., 1952). In this research, pasteurization also supported the powders to be easily reconstituted without destabilizing the fat. Adaptations were not made to facilitate reconstitution, since the purpose was to compare the differences between the processes.

Milk is characterized as a natural emulsion of oil in water, in which milk fat globules (MFG) form an emulsion, composed internally of hydrophobic tracylglycerols (TAG), fatty acid esters and glycerol, and externally of an amphipathic layer composed of proteins, phospholipids, cholesterol, and enzymes (Jiang et al., 2020; Lopez, 2011; Martysiak-Żurowska, Puta, Rodzik, & Malinowska-Pańczyk, 2017). MFG allow hydrophobic TAG stay solubilized in the aqueous milk, since the

electrostatic repulsions of the components of the milk fat globule membrane (MFGM) contribute to the stability, avoiding coalescence and aggregation in milk (Lopez, 2011). Also, MFGM prevents lipases from acting on TAG because if MFG are damaged by heating, freezing or thawing, TAG become available for lipase action (Wardell, Hill, & d'SOUZA, 1981).

Moreover, the TAG molecules that are contained in MFG remain liquid at their physiological temperature (36-39 °C). However, crystallization and melting properties of MFG result from the composition of FA, the structure and polymorphism of TAG, the applied heat treatments, and the size of MFG. Thus, the procedures applied in milk can influence the structure and composition of MFGM and, consequently, the composition and size of MFG are also affected by processing (Lopez, 2011). Also, the stability of milk fat is affected at temperatures below 40 °C, due to the fat crystals that can perforate the membrane and cause changes and deformation, leading to aggregation and partial coalescence of MFG (Jukkola & Rojas, 2017).

Lopez et al. (2013) found that the final melting point of TAG from MFG was 39.8 ± 1.5 °C and that the crystallization and melting properties of TAG in HM are due to factors such as: diversity of TAG molecules with FA of various chain lengths and also by the number of unsaturations, in addition to the polymorphism of TAG.

Studies propose that thawing of HM should be performed by heating at body temperature (up to a maximum of 40 ºC) for a period of 20 min. In addition, heating milk at a temperature of 37 ºC reaches the melting point of lipids, changing from solid to liquid or oily phase, and may adhere to the container (Eglash et al., 2017; Thatrimontrichai, Janjindamai, & Puwanant, 2012).

The fact that one sample had been pasteurized before freeze-drying (HMPF) and another sample had been freeze-dried only (HMF) may have resulted in changes in the internal structures and, consequently, in the physical properties of the fat molecules. Thus, the data suggest that the

freeze-drying at 40 °C shelf temperature reached the melting point of the fat, destabilizing the HMF sample, and causing it to separate and adhere to the bottle when reconstituted.

Cavazos-Garduño et al. (2016) noted that the increase in pasteurization temperature (75 and 85 ºC) also caused rupture of the MFGM, causing instability in the MFM during the process, resulting in the separation and adherence to the container. In addition, to avoid lipid deterioration, they performed the FAs analyses under refrigeration temperature, and since the fat was solid, it remained attached to the container. In this study, the temperature used for reconstitution of the freeze-dried samples also kept the HMF fat adhered to the container.

According to Yao et al. (2016), the drying process results in different sizes of globules and, consequently, in changing the composition of their FAs. In this way, these globules can have different melting points and be in different states even at the same temperature. Therefore, the freeze-drying process in milk can alter physical, chemical and microstructural properties, which may influence the stability of the MFG and the way reconstituted milk is digested.

This work started the study on freeze-dried raw HM, however, more studies are needed to understand what the freeze-drying process causes in its fat. Thus, further research should be developed using a different shelf temperature than the one used, which was close to the melting point of the lipids, so that it is possible to evaluate whether freeze-drying is able to maintain the composition in FA as well as the other treatments. In addition, a possible alternative for reconstituting the HM powder would be to use water at a temperature at which it can be ingested by infants since it could solubilize the fat that was found adhered to the surface of the recipients, and to evaluate the effect of using higher temperatures in reconstituting HM in the fat.

3.4. Concentration of Cytokine

418 The result of cytokine composition of HMC, HMF, HMP and HMPF is presented in Table 4.
419 The intake of cytokines present in HM is able to influence the maturation and development of the
420 infant's immune system, regulating inflammatory responses associated with the immune response
421 and acting with antimicrobial activity (Field, 2005; Garofalo, 2010).
422 Cytokines are powerful signalling molecules, responsible for acting locally in
423 autocrine⁄paracrine form, sharing similar functions and also for having the ability to have functions
424 in several different cell types, which makes it difficult to generalize the effect of each cytokine
425 (Arango Duque & Descoteaux, 2014; Garofalo, 2010). So, the cytokines was represented based on
426 the division presented by Espinosa-Martos et al. (2013), in which in Figure 1 are the cytokines
427 related to the innate immunity: Interleukin 1β (IL-1β), Interleukin 6 (IL-6), Interferon gamma (IFN-
428 γ) and Tumour necrosis fator alpha (TNF-α), in Figure 2 the cytokines related to adaptive immune
429 response: Interleukin 2 (IL-2), Interleukin 4 (IL-4), Interleukin 5 (IL-5) and Interleukin (IL-10), in
430 Figure 3 the chemokine Interleukin 8 (IL-8) and in Figure 4 the cytokine related to the
431 haematopoietic stimulus Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF).
432 To the best of our knowledge, this is the first work to evaluate and compare the effect of
433 pasteurization, freeze-drying and pasteurization followed by freeze-drying on raw HM, including
434 the effect of storage on the cytokine concentration of liquid and powder HM. According to the
435 results presented in Table 4, regarding the cytokines related to innate development (Figure 1), IL-6
436 and TNF-α showed significant reduction ($P < 0.05$), as presented in other studies, which also
437 observed that HoP caused reduction in these compounds (Delgado et al., 2014; Franch et al., 2010).
438 In addition, it was observed that, not only pasteurization, but also storage was a factor that
439 influenced the reduction of TNF-α, since HMPF was also submitted by HoP, however it was stored
440 in powder form and preserved the compound without presenting significant difference compared to
441 HMC ($P < 0.05$). As presented by Ewaschuk et al. (2011), IFN-γ decreased significantly ($P < 0.05$)

after pasteurization, and in this work it was not possible to detect the compound in samples HMP and HMPF, demonstrating susceptibility to the temperature used in the process. As for IL-1β did not show significant difference ($P < 0.05$) in relation to HMC, in agreement with the results presented by Espinosa-Martos et al. (2013).

About the cytokines related to adaptive immune response (Figure 2), as in the study of Espinosa-Martos et al. (2013), pasteurization caused no significant effect ($P < 0.05$) on IL-2, IL-5 and IL-10 compounds. It was observed that IL-2 showed a significant increase in the HMPF sample compared to the other samples ($P < 0.05$). According to Espinosa-Martos et al. (2013), the increase in cytokine concentration following pasteurization may be associated with cytokine release from the cell or else the fat fraction displaced to the aqueous part due to the use of heat. As for IL-4, the results were different from the work Ewaschuk et al. (2011), since in their study they found no significant difference in the pasteurized sample, while in this study there was a significant decrease in HMP when compared to the other samples ($P < 0.05$). However, as the HMPF sample was also subjected to HoP, and did not show a significant ($P < 0.05$) decrease in IL-4 compared to HMC, it indicates that powder storage showed benefit in preserving the compound.

The chemokine IL-8 (Figure 3), showed a significant increase ($P < 0.05$) in the samples submitted to pasteurization (HMP and HMPF), being consistent with other studies found in the literature that also detected the same effect (Delgado et al., 2014; Ewaschuk et al., 2011; Franch et al., 2010). According to Ewaschuk et al. (2011), what may have occurred was that pasteurisation caused separation of the bound protein and made IL-8 more susceptible to binding with the anti-IL-8 antibodies in the ELISA. And to GM-CSF (Figure 4) showed no change in any treatment performed ($P < 0.05$), unlike the work of Espinosa-Martos et al. (2013), which found a significant increase in the compound after pasteurization.

The study is consistent with that of Neia et al. (2021), which evaluated the cytokine profile after freeze-drying and spray-drying, and observed that it was able to maintain cytokines after processing. Thus, the data indicate that the exposure of HM to the temperature used on the shelf plate (40 ºC) did not cause a reduction in the concentration of any of the compounds analyzed, while when submitted to the HoP process, it caused a significant decrease in cytokines IL-6, IFN-γ, TNF-α and IL-4 ($P < 0.05$).

Temperature, storage and freeze-thaw cycles are factors that can affect the stability of cytokines, with an increase, decrease or maintenance in their levels. Some cytokines are more unstable to exposure to these factors, which may be due to the speed of degradation and the structure of the cytokine itself (Keustermans, Hoeks, Meerding, Prakken, & de Jager, 2013; Simpson, Kaislasuo, Guller, & Pal, 2020), since cytokines are proteins that have a tertiary or quaternary structure, and when exposed to inappropriate conditions may be susceptible to structural damage (Panicker, Meadows, Lee, Nisenbaum, & Unger, 2007). In this way, freeze-drying is able to maintain the structure of the material and minimize degradation reactions (Boss, Filho, & de Toledo, 2004). Thus, the results indicate that the removal of water through the process did not affect the biological structure, being able to maintain the cytokine content. Moreover, the storage of HM in powder form also proved favourable in the conservation of cytokine content, as occurred with IL-4 and TNF-α.

4. Conclusions

This study is the first to evaluate the effects of each treatment on HM composition by comparing the effect of freeze-drying on raw HM. According to the results, pasteurization did not significantly affect the centesimal and FAs composition of HM, but reduced the concentration of

some cytokines. The data indicate that the pasteurization process followed by freeze-drying resulted in a significant reduction in total protein content and concentration of some cytokines, but maintained the FA profile. The results also suggest that the freeze-drying process performed at 40 ºC shelf plate temperature maintained the centesimal and cytokine composition, but caused destabilization of fat with separation and adhesion to the vial when HMF was reconstituted, statistically changing the FA profile.

According to the microbiological results, pasteurization remains the most viable alternative. Although MOM submitted only to freeze-drying maintains important bioactive compounds for the newborn, further studies using different shelf plate temperatures would be necessary to find optimal process conditions that maintain the FAs composition. In addition, it would be necessary to ensure that milking and handling of MOM were performed free of microbiological contamination, following the microbiological quality standards of HMB and with result of absence of coliform bacteria.

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Conflict of Interest

513

514 The authors declare no conflict of interest.

515

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Table 1

Nutrients content of control (HMC), Holder pasteurized (HMP), freeze-dried (HMF) and Holder pasteurized freeze dried (HMPF) samples of human milk.[a]

		Treatments			
Nutrient	Units	HMC	HMP	HMF	HMPF
Moisture	%	-	-	$1.64^{a*} \pm 0.20$	$1.56^{a*} \pm 0.41$
Moisture	g 100 mL^{-1}	$88.98^{a} \pm 0.08$	$88.32^{b} \pm 0.20$	$89.19^{a} \pm 0.24$	$88.69^{ab} \pm 0.23$
Ashes	g 100 mL^{-1}	$0.19^{ab} \pm 0.01$	$0.21^{a} \pm 0.00$	$0.19^{ab} \pm 0.02$	$0.18^{b} \pm 0.01$
Proteins	g 100 mL^{-1}	$1.34^{a} \pm 0.04$	$1.37^{a} \pm 0.03$	$1.40^{a} \pm 0.05$	$1.22^{b} \pm 0.05$
Lipids	g 100 mL^{-1}	$4.07^{a} \pm 0.16$	$3.94^{a} \pm 0.48$	$4.11^{a} \pm 0.24$	$3.71^{a} \pm 0.71$
Carboidrates	g 100 mL^{-1}	$5.42^{a} \pm 0.25$	$6.15^{a} \pm 0.40$	$5.12^{a} \pm 0.26$	$6.21^{a} \pm 0.89$
Energetic value	kcal 100 mL^{-1}	$65.21^{a} \pm 0.66$	$67.10^{a} \pm 3.03$	$64.64^{a} \pm 1.99$	$64.50^{a} \pm 3.27$

[a] Values are means ± standard deviation of triplicate determinations. Different letters in the same row indicate statistically significant differences ($P < 0.05$).

[*] Results referring to samples before reconstitution.

Table 2

Determination of microbiological quality in control (HMC), Holder pasteurized (HMP), freeze-dried (HMF) and Holder pasteurized freeze dried (HMPF) samples of human milk.

	Treatments			
Coliform bacteria	HMC	HMP	HMF	HMPF
Result	Presence	Ausence	Presence	Ausence

Table 3

Fatty acid composition of control (HMC), Holder pasteurized (HMP), freeze-dried (HMF) and Holder pasteurized freeze dried (HMPF) samples of human milk.[a]

	Treatments			
Fatty acids	HMC	HMP	HMF	HMPF
8:0 (caprylic acid)	17.86^{a} ± 1.90	17.23^{a} ± 0.72	21.42^{a} ± 3.91	15.96^{a} ± 1.93
10:0 (capric acid)	62.69^{a} ± 4.77	62.11^{a} ± 4.77	41.09^{b} ± 4.14	53.67^{ab} ± 6.28
12:0 (lauric acid)	215.87^{a} ± 8.64	226.44^{a} ± 21.32	154.33^{b} ± 21.67	233.18^{a} ± 33.13
14:0 (myristic acid)	138.52^{ab} ± 2.89	146.78^{ab} ± 18.76	118.92^{b} ± 15.81	161.41^{a} ± 19.30
15:0 (pentadecanoic acid)	4.17^{a} ± 0.13	3.90^{a} ± 0.64	3.89^{a} ± 0.42	4.67^{a} ± 0.40
16:0 (palmitic acid)	389.09^{a} ± 3.77	393.17^{a} ± 52.37	349.41^{a} ± 43.29	437.36^{a} ± 42.47
17:0 (heptadecanoic acid)	3.35^{ab} ± 0.18	3.75^{ab} ± 0.30	3.05^{b} ± 0.34	4.16^{a} ± 0.41
18:0 (stearic acid)	91.17^{a} ± 0.40	99.57^{a} ± 8.01	83.00^{a} ± 10.15	102.38^{a} ± 8.21
20:0 (arachidic acid)	3.19^{b} ± 0.41	2.66^{b} ± 0.11	5.12^{a} ± 0.75	3.54^{b} ± 0.49
22:0 (behenic acid)	2.12^{a} ± 0.23	2.12^{a} ± 0.09	2.36^{a} ± 0.14	2.76^{a} ± 0.55
24:0 (lignoceric acid)	1.30^{b} ± 0.20	1.40^{b} ± 0.42	3.58^{a} ± 0.09	1.60^{b} ± 0.20
14:1n-5 (myristoleic acid)	1.62^{a} ± 0.10	1.56^{a} ± 0.38	1.20^{a} ± 0.12	1.62^{a} ± 0.28
15:1n-5 (pentadecenoic acid)	1.96^{b} ± 0.13	2.17^{b} ± 0.12	3.15^{a} ± 0.32	2.58^{ab} ± 0.32
16:1n-7 (palmitoleic acid)	53.62^{a} ± 3.80	51.44^{a} ± 7.03	35.78^{b} ± 4.43	54.57^{a} ± 5.79
16:1n-9 (7-hexadecenoic acid)	4.94^{ab} ± 0.64	4.77^{ab} ± 1.09	3.28^{b} ± 0.35	5.20^{a} ± 0.23
17:1n-7 (heptadecenoic acid)	3.71^{a} ± 0.19	3.51^{a} ± 0.41	3.03^{a} ± 0.42	3.79^{a} ± 0.65
18:1n-7 (vaccenic acid)	3.59^{a} ± 0.51	3.48^{a} ± 0.61	4.11^{a} ± 0.72	4.32^{a} ± 0.98
18:1n-9 (oleic acid)	910.02^{a} ± 24.47	866.39^{a} ± 107.81	633.38^{b} ± 79.63	875.83^{a} ± 68.89
20:1n-9 (eicosenoic acid)	4.01^{a} ± 0.12	4.02^{a} ± 0.15	2.99^{b} ± 0.22	3.98^{a} ± 0.16
22:1n-9 (erucic acid)	2.38^{a} ± 0.05	2.21^{ab} ± 0.05	1.92^{b} ± 0.19	2.18^{ab} ± 0.13
24:1n-9 (nervonic acid)	2.42^{a} ± 0.12	2.19^{a} ± 0.11	1.57^{b} ± 0.21	2.13^{a} ± 0.13
18:2n-6 (LA, linoleic acid)	498.16^{a} ± 13.15	458.11^{a} ± 62.22	339.19^{b} ± 40.95	473.60^{a} ± 40.94
18:2cis–9, trans–11 (CLA)	3.66^{ab} ± 0.23	3.98^{a} ± 0.65	2.93^{b} ± 0.26	4.00^{a} ± 0.30

18:2trans-10, cis-12 (CLA)	2.34^{b} ± 0.38	2.26^{b} ± 0.21	3.16^{a} ± 0.38	2.81^{ab} ± 0.25
20:2n-6 (eicosadienoic acid)	9.96^{a} ± 0.58	9.17^{a} ± 0.84	6.87^{b} ± 0.97	9.93^{a} ± 0.18
18:3n-3 (ALA, α-linolenic acid)	34.84^{a} ± 1.45	31.47^{a} ± 2.20	24.03^{b} ± 3.15	31.60^{a} ± 3.50
18:3n-6 (γ-linoleic acid)	5.40^{a} ± 0.34	5.19^{a} ± 0.03	4.63^{a} ± 0.52	5.22^{a} ± 0.59
20:3n-3 (eicosatrienoic acid)	2.30^{ab} ± 0.25	2.40^{a} ± 0.88	0.67^{b} ± 0.28	1.62^{ab} ± 0.81
20:3n-6 (eicosatrienoic acid)	4.13^{a} ± 0.18	4.52^{a} ± 0.88	3.96^{a} ± 0.29	4.31^{a} ± 0.27
20:4n-6 (ARA, arachidonic acid)	15.84^{a} ± 0.67	14.84^{a} ± 0.25	14.26^{a} ± 0.18	14.49^{a} ± 1.05
20:5n-3 (EPA, eicosapentanoic acid)	2.00^{a} ± 0.16	1.69^{a} ± 0.34	1.94^{a} ± 0.10	1.61^{a} ± 0.69
22:6n-3 (DHA, docosahexaenoic acid)	3.17^{a} ± 0.08	3.00^{ab} ± 0.21	2.63^{b} ± 0.16	2.86^{ab} ± 0.27
Σ SFA	929.31^{a} ± 21.36	959.12^{a} ± 106.25	786.16^{a} ± 100.69	1020.69^{a} ± 113.37
Σ MUFA	988.26^{a} ± 30.01	941.75^{a} ± 117.76	690.41^{b} ± 86.60	956.21^{a} ± 77.56
Σ PUFA	581.79^{a} ± 15.77	536.62^{a} ± 64.22	404.26^{b} ± 46.12	552.07^{a} ± 48.49
Σ PUFA n-3	42.31^{a} ± 1.66	38.56^{a} ± 1.45	29.26^{b} ± 2.93	37.70^{a} ± 5.27
Σ PUFA n-6	539.48^{a} ± 14.19	498.06^{a} ± 62.77	375.00^{b} ± 43.19	514.37^{a} ± 43.22
n-6 to n-3 ratio	12.76^{a} ± 0.21	12.89^{a} ± 1.17	12.80^{a} ± 0.21	13.71^{a} ± 0.71

[a] Abbreviations are: LA, linoleic acid; CLA, conjugated linoleic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Values (expressed in g 100 g^{-1} of human milk) are means ± standard deviation of triplicate determinations. Different letters in the same row indicate statistically significant differences ($P < 0.05$).

Table 4

Cytokines content of control (HMC), Holder pasteurized (HMP), freeze-dried (HMF) and Holder pasteurized freeze dried (HMPF) samples of human milk.[a]

	Treatments			
Cytokine	HMC	HMP	HMF	HMPF
IL-1β	246.82^{ab} ± 41.23	332.58^{a} ± 50.26	191.02^{b} ± 14.28	366.18^{a} ± 70.15
IL-2	617.59^{b} ± 73.60	603.43^{b} ± 107.33	638.45^{b} ± 78.24	899.18^{a} ± 89.62
IL-4	354.62^{a} ± 14.50	214.66^{a} ± 31.23	308.68^{a}	297.51^{a} ± 46.46
IL-5	299.25^{a} ± 6.64	328.65^{a} ± 12.64	455.18^{a} + 144.88	326.25^{a} ± 16.60
IL-6	255.89^{a} ± 26.75	148.15^{b} ± 12.19	227.51^{ab} ± 60.98	163.41^{b} ± 8.35
IL-8	2154.08^{b} ± 50.73	3062.91^{a} ± 114.29	2096.16^{b} ± 167.59	2973.86^{a} ± 103.35
IL-10	713.48^{a} ± 46.45	820.98^{a} ± 120.54	818.73^{a} ± 151.67	866.69^{a} ± 43.12
IFN-γ	48.97^{a} ± 26.61	n.d.^{b}	68.38^{a}	n.d.^{b}
GM-CSF	202.52^{a} ± 38.24	188.11^{a} ± 29.73	203.23^{a} ± 73.45	232.11^{a} ± 30.55
TNF-α	654.67^{a} ± 97.95	352.00^{b} ± 47.03	551.69^{ab} ± 102.16	469.39^{ab} ± 36.26

[a] Abbreviations are: IL, interleukin; IFN-γ, interferon gamma; GM-CSF, granulocyte-macrophage colony-stimulating fator; TNF-α, tumour necrosis fator alpha; n.d., not detected. Values (expressed in pg mL^{-1} of human milk) are means ± standard deviation of triplicate determinations, with the exception of the HMF sample for IL-4 and IFN-γ, which is the result of a single sample with values within the expected range. Different letters in the same row indicate statistically significant differences ($P < 0.05$).

Figure Captions

Fig. 1. Comparison of processing on control (HMC,■), Holder pasteurized (HMP,■), freeze-dried (HMF,■) and Holder pasteurized and freeze dried (HMPF,■) samples for evaluation of innate immunity cytokines. Abbreviations are: IL, interleukin; IFN-γ, interferon gamma; TNF-α, tumour necrosis fator alpha. Values (expressed in pg mL^{-1} of human milk) are means ± standard deviation. Different letters above the bars indicate statistically significant differences ($P < 0.05$).

Fig. 2. Comparison of processing on control (HMC,■), Holder pasteurized (HMP,■), freeze-dried (HMF,■) and Holder pasteurized and freeze dried (HMPF,■) samples for evaluation of cytokines of the adaptive immune response. Abbreviation is: IL, interleukin. Values (expressed in pg mL^{-1} of human milk) are means ± standard deviation. Different letters above the bars indicate statistically significant differences ($P < 0.05$).

Fig. 3. Comparison of processing on control (HMC,■), Holder pasteurized (HMP,■), freeze-dried (HMF,■) and Holder pasteurized and freeze dried (HMPF,■) samples for chemokine evaluation. Abbreviation is: IL, interleukin. Values (expressed in pg mL^{-1} of human milk) are means ± standard deviation. Different letters above the bars indicate statistically significant differences ($P < 0.05$).

Fig. 4. Comparison of processing on control (HMC,■), Holder pasteurized (HMP,■), freeze-dried (HMF,■) and Holder pasteurized and freeze dried (HMPF,■) samples for cytokine evaluation of haematopoietic stimulus. Abbreviation is: GM-CSF, granulocyte-macrophage colony-stimulating fator. Values (expressed in pg mL^{-1} of human milk) are means ± standard deviation. Different letters above the bars indicate statistically significant differences ($P < 0.05$).

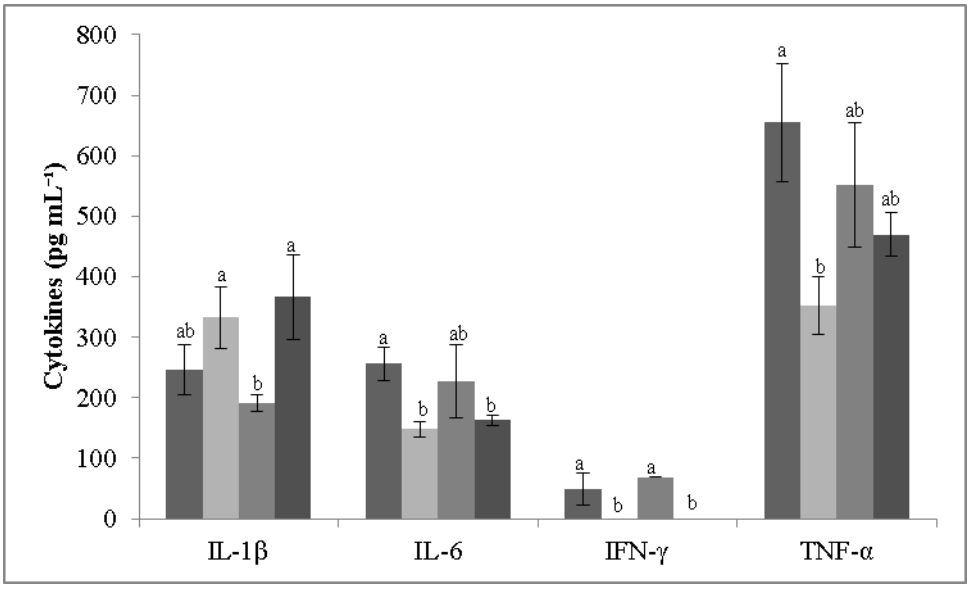
Cytokines (pg mL⁻¹)
800
700
600
500
400
300
200
100
0
ab
a
b
a
a
b
ab
b
a
b
a
b
a
ab
b
ab
IL-1β
IL-6
IFN-γ
TNF-α

Figure 1

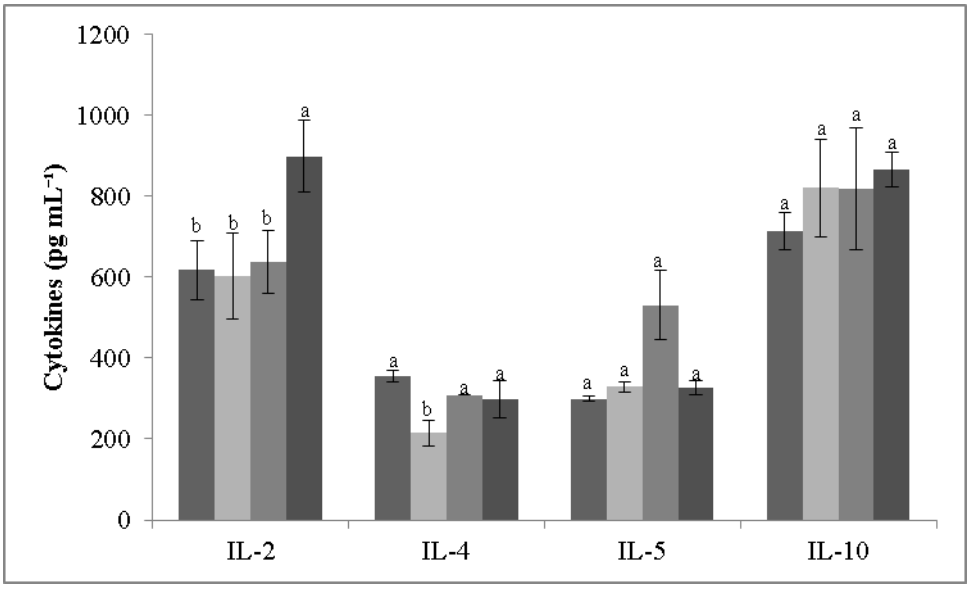
1200
1000
800
600
400
200
0
Cytokines (pg mL-1)
IL-2
IL-4
IL-5
IL-10
a
b
b
b
a
b
a
a
a
a
a
a
a
a
a
a

Figure 2

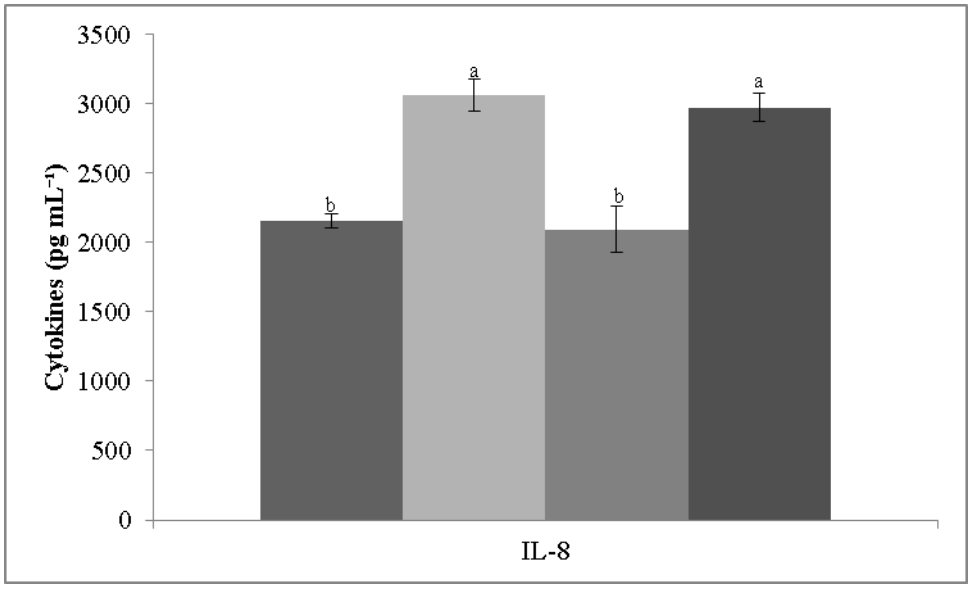

Figure 3

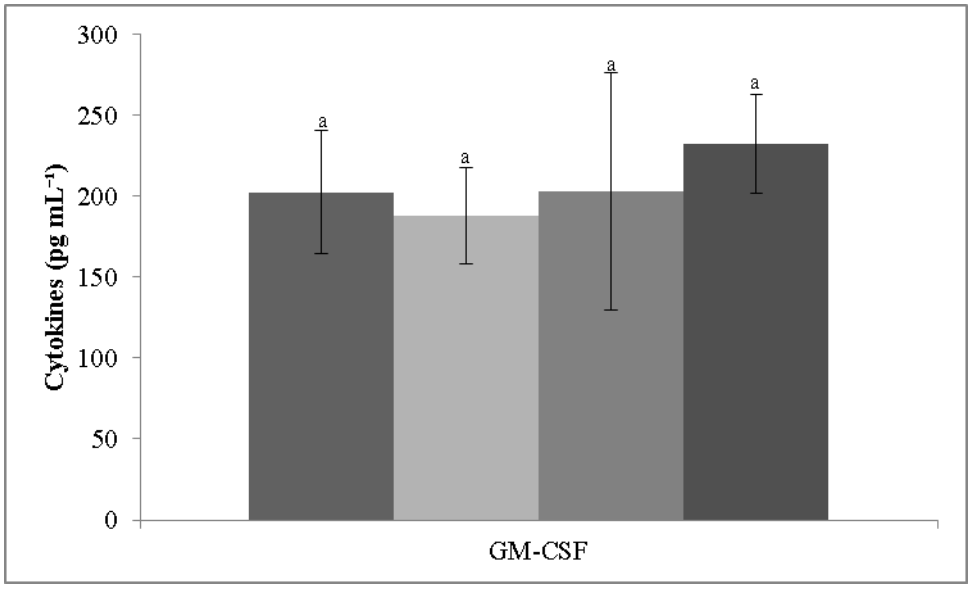

Figure 4