Stability of Antioxidant Capacity of Human Milk after Freezing and Pasteurization

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Abstract   Pasteurization and freezing are processes used in human milk banks that can affect some of the nutritional and biological properties of human milk. The aim of this study was to evaluate impact of the processing steps adopted by human milk banks on the contents of total phenolic compounds and antioxidant capacity in vitro. Experimental study was realized, in which 40 mL human milk were collected from 8 mothers registered at the Human Milk Bank of Ouro Preto, state of Minas Gerais. Samples were homogenized to form the pool. Human milk was divided into four portions that characterized the treatments: 1) immediately after milking; 2) pasteurization soon after the milking; 3) 7 days after freezing at -8.5 (± 2.8°C) and pasteurized; 4) 14 days after freezing at -8.5 (± 2.8°C) and pasteurized. The content of total phenolic compounds was determined, and the antioxidant capacity was assessed using the 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) and 2,2-diphenyl-1-picrylhydrazyl free radicals scavenging. The results were expressed as mean and standard deviation of six replications. There was a positive correlation between the evaluated methods and reduction in total phenolic content and total antioxidant capacity with slow pasteurization and according to freezing time. There was a negative effect of pasteurization and freezing on the total phenolic content and total antioxidant capacity, and the most significant reduction was observed within the first 7 days of storage.

Keywords: milk banks, heat treatment, antioxidant, phenolic compounds

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1. Introduction

Human milk (HM) consists all the nutrients required for the growth and development of infants, including carbohydrates, essential fatty acids, proteins, vitamins and minerals [1] and also provides immunological protection from soluble components such as lysozyme, lactoferrin, secretory IgA and other immunoglobulins, as well as cellular components, including, macrophages, neutrophils and T and B lymphocytes. Also, HM contains numerous bioactive compounds with antioxidant properties that protect against infections and reduce their severity. Among them, stand out the enzymes catalase, superoxide dismutase and glutathione peroxidase, vitamins E and C, retinol and β-carotene [2,3]. Compounds with antioxidant capacity present in HM are relevant for newborns due to situations of high oxidative stress, for example, at the moment of delivery, when there is the transition from a medium with low oxygen concentrations to a medium with concentrations considered normal [4]. These advantages are even more important in preterm newborns, because they have an immature antioxidant defense system, since it develops during the course of gestation [5]. In addition, they are often exposed to oxidative stress

caused by infections, mechanical ventilation, intravenous nutrition and blood transfusions [6]. Several studies have shown that the antioxidant capacity of HM is superior to infant formulas and bovine milk [2,6,7]. The production of free radicals caused by oxidative stress can lead to diseases both in the infancy and in the adult phase [5].

In situations in which mothers are unable to breastfeed, HM should be collected through donations and processed in human milk banks (HMB) [8]. However, for this milk to be used, it must undergo several procedures that ensure its microbiological and nutritional quality, so that it can be beneficial for the newborn that receive it [5]. The critical points for the preservation of the antioxidant capacity of HM include the heat treatment and storage. Pasteurization is a thermal treatment used in HMB to ensure the microbiological quality of milk, however, it can affect some of the nutritional and biological properties of HM [9]. In relation to pasteurization, Silvestre et al. [3] observed that the heat treatment of HM implied a decrease in its total antioxidant activity and important antioxidant components, such as the glutathione and glutathione peroxidase. They also verified that these losses may be to a lesser or greater extent, depending on the type of process adopted. Regarding storage, the studies to date are still not conclusive [4,5,6]. According to Akdag et al. [5], the total antioxidant capacity of preterm HM was preserved at -80

°C for 3 months. However, according to Sari et al. [4], the freezing samples of colostrum, transition and mature milk at -80 °C for two months did not preserve the antioxidant capacity. According to Hanna et al. [6], the antioxidant capacity of HM also decreased with the storage time and refrigeration was better than freezing to preserve it, because the freezing for 48 hours caused greater losses than the refrigeration for seven days.

There are no studies in the literature that reveal the impact of the processing steps adopted by HMB on the content of bioactive compounds and the antioxidant capacity of HM. The Agência Nacional de Vigilância Sanitária (ANVISA) regulated the processing of HM by providing standardized procedures for donor selection, collection, domestic storage, transport, storage, heat treatments (thawing, pasteurization and cooling), quality control, distribution and administration [7]. And pasteurization must occur within a maximum period of 15 days after the first collection [10].

Given the known importance of bioactive compounds and antioxidant capacity for the development and health of children [3,4,5,6] and the limited literature that evaluate the real conditions of the processing stages of HM by the Network of Human Milk Banks in Brazil, it is important to conduct studies to evaluate the stability of the antioxidant capacity of HM after the procedures adopted. Therefore, this study evaluated the interference of the freezing time and the pasteurization of HM on the content of total phenolic compounds and antioxidant capacity in vitro.

2. Methodology

This is an experimental study performed in the laboratories of the School of Nutrition of the Federal University of Ouro Preto with the support of the HMB of Santa Casa da Misericórdia in Ouro Preto, state of Minas Gerais. Samples were from the pool of HM from 8 volunteer donors registered in the service. As an exclusion criterion we adopted age below 20 years. The Research Ethics Committee of the Federal University of Ouro Preto approved the study.

Data from the donors were obtained through a questionnaire to characterize the mothers regarding their age, occupation, schooling, family income, type of housing, health plan, parity, pregestational body mass index (BMI), gestational weight gain, lactation phase and data of the infant, such as age, gender, gestational age, birth weight and breastfeeding practice. In relation to the sample collected from each donor, this was 40 mL of HM, extracted by milking performed by the volunteer herself, following procedures recommended by the HMB. Samples were transported under a cold chain not exceeding six hours.

The milk of each donor was divided into four aliquots of 10 mL, which received different procedures, characterizing the treatments of the study, as follows: 1) immediately after milking; 2) Pasteurization soon after the milking; 3) 7 days after freezing at -8.5 (± 2.8°C) and pasteurized; 4) 14 days after freezing at -8.5 (± 2.8°C) and pasteurized. For each treatment, samples from the 8 donors were homogenized to form the pool.

The heat treatment and the verification of its effectiveness were conducted in accordance with the Brasil [8]. Samples were thawed in a water bath at 40°C and pasteurized at 62.5°C for 30 minutes after the preheating time. Immediately after the heat treatment, the HM was cooled to below 5°C.

In order to evaluate the effectiveness of the thermal processing to which HM was subjected, we used the microbiological quality control method described by Brasil [8], which aims to detect total coliform microorganisms. Thus, after pasteurization, four aliquots of 1 mL of pasteurized HM were inoculated into 10 mL of 50 g/L (5% w/v) bright green bile broth with Durham tubes inside. Subsequently, the samples were incubated at 36±1°C and evaluated after 48 hours for turbidity and presence of gases inside Durham tubes, which characterizes a positive result and, consequently, the non-effectiveness of pasteurization [10].

The total phenolic contents were determined according to the adapted Folin-Ciocalteu method [11]. HM was diluted in a proportion of 3:1 with phosphate buffer pH 7.4 and a 0.5 mL aliquot was added to 2.5 mL Folin-Ciocalteu reagent (10%) and 2 mL o sodium carbonate solution (4%). The mixture was vortexed for 5 seconds and held at room temperature for 90 minutes in the dark. Subsequently, it was centrifuged at 8000 rpm for 10 minutes and rested again for 20 minutes. Absorbance readings were performed using a spectrophotometer at a wavelength of 750 nm. Aqueous solutions of gallic acid (10 to 80 μg/mL) were used to determine the standard curve and the results were expressed as gallic acid equivalents (GAE mg/L).

For the evaluation of total antioxidant capacity, the 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (DPPH) and 2,2-diphenyl-1-picrylhydrazyl (ABTS) free radical scavenging methods were used. The scavenging capacity of DPPH was measured using the method proposed by Brand-Williams et al. [12] with modifications proposed by Zarban et al. [13], 50 μL of each HM sample was added with 1 mL DPPH in ethanol solution (0.06 mM). After homogenization, the mixture was allowed to stand for 30 minutes in a water bath at 37°C. 0.5 mL chloroform was added and centrifuged at 8000 rpm for 5 minutes. The absorbance of the solution was determined in a spectrophotometer at 517 nm. The solution of DPPH in ethanol (0.06 mM) was used as a control and the percentage of the DPPH radical scavenging activity was calculated according to the following equation:

$$Scavenging\ activity\ (\%) = \left[\frac{control\ absorbance - sample\ absorbangce}{control\ absorbance}\right] *100 \quad (1)$$

The antioxidant capacity equivalent to the synthetic antioxidant Trolox was estimated according to the procedure proposed by Turoli et al. [14], with some modifications. The ABTS radical was prepared from the reaction of 5 mL stock solution of ABTS (7 mM) with 88μL potassium persulfate (40 mM), leaving the mixture at room temperature for 16 hours in the absence of light. Then, the ABTS solution was diluted with ethanol to obtain an absorbance of 0.70 ± 0.05 at 734 nm. HM samples were diluted with phosphate buffer pH 7.4 at the following concentrations: 1000mL/L, 200mL/L and 100mL/L. Aliquots of 20 μL of each dilution were added with 2 mL

ABTS solution and, after homogenization, remained standing for 10 minutes in the dark. Subsequently, they were centrifuged at 8000 rpm for 5 minutes. Absorbance was read using a spectrophotometer at a wavelength of 734 nm. The antioxidant capacity was calculated using the Trolox standard curve (100, 500, 1000, 1500 and 2000 µM) and there respective inhibition percentages, and the results of the test were expressed in mmol Trolox equivalent per liter (mmol TE/L).

The results were expressed as mean and standard deviation of six replicates. The Kolmogorov-Smirnov and Shapiro-Wilk tests were applied to test the normality of the data. In the case of normal distribution, the results were analyzed by analysis of variance (ANOVA). For the analyses that were significant ($p \leq 0.05$), the Tukey's test ($p \leq 0.05$) was applied to compare the means. The Pearson correlation test ($p \leq 0.05$) was run to measure the correlation between the total phenolic compounds content and the antioxidant capacity by the different methods used. Statistical analyses were performed using the software Statistical Package for Social Sciences (SPSS - version 18.0).

3. Results

The characteristics of the donors and their infants are listed in Table 1. Among the participants, all lived in the municipality of Ouro Preto, state of Minas Gerais, had an employment relationship and were producing mature milk. Among the mothers, 62.5% were over 30 years of age and 37.5% were overweight or had pre-gestational obesity. Regarding the birth weight, 62.5% were born with adequate weight, that is, between 3,000 and 4,000 g. [15]

As for the HM pasteurization processes performed after the different storage times, these being 0,7 and 14 days of frozen storage at -8.5°C (± 2.8), after the adopted procedure it was observed the absence of total coliforms. This indicates the effectiveness of pasteurization as to the microbiological aspects of HM.

With respect to the total phenolic compounds (Figure 1a), the raw human milk pool obtained a higher concentration of these compounds (574.18 ± 77.95 mg GAE/L). There was a significant decrease of 30.29% (173.95 mg GAE/L) after pasteurization of HM on the day of milking. Regarding the effect of freezing at -8.5°C (± 2.8), there was no significant change up to 7 days. However, there was a significant reduction of 33.99% (136.05 mg GAE/L) at 14 days of storage.

As for the antioxidant capacity evaluated by the DPPH radical scavenging method (Figure 1b), the pasteurization process did not interfere significantly. However, there was a significant decrease of 52.73% with freezing time at -8.5°C (± 2.8), at 14 days of storage. It should be noted that the freezing for 7 days decreased the antioxidant capacity of HM by 45.99%, evaluated by this method.

It was possible to observe a significant reduction of 30.49% (1.93 mmol TE/L) in the antioxidant capacity by the ABTS radical scavenging method in the pasteurization process and 41.81% (1.84 mmol TE/L) in freezing at 7 days storage (Figure 1c). Between 7 and 14 days of freezing, the antioxidant capacity did not suffer significant interference, when evaluated by this method.

Table 1. Characterization of donors of human milk and their infants, Ouro Preto, state of Minas Gerais, 2016

|	Range	Mean ± SD
Mothers		
Age (years)	20 - 38	32 ± 6.3
Pregestational BMI[a] (kg/m²)	20.90 - 33.78	25.25 ± 3.84
Gestational weight gain (kg)	5.5 - 24	11.9 ± 6.87
Parity (number of pregnancies)	1 - 2	1 ± 0.52
Education (high school/college)	3/5	
Housing (own/rented)	6/2	
Family income (< 3 MW[b] / > 3 MW)	4/4	
Health Plan (yes/no)	7/1	
Infants		
Age (weeks)	3 - 19	8.25 ± 6.82
Gender (male/female)	2/6	
Breastfeeding (exclusive/supplemented)	6/2	
Gestational age (weeks)	37 - 41	38.62 ± 1.50
Weight at birth (kg)	2.585 - 3.400	3.080 ± 0.25

[a] Body mass index
[b] Minimum wage

The correlations between the evaluated methods, ABTS, DPPH and total phenolic compounds, tested by the Pearson Coefficient were significant ($p \leq 0.01$), all parameters being directly correlated (Table 2).

Table 2. Pearson correlation between total phenolic content and different methods evaluated for the total antioxidant capacity of human milk

Correlations	Pearson correlation coefficient (r)
ABTS x DPPH	0.766*
ABTS x Phenolic content	0.732*
Phenolic content x DPPH	0.747*

*Significant values ($p \leq 0.01$)
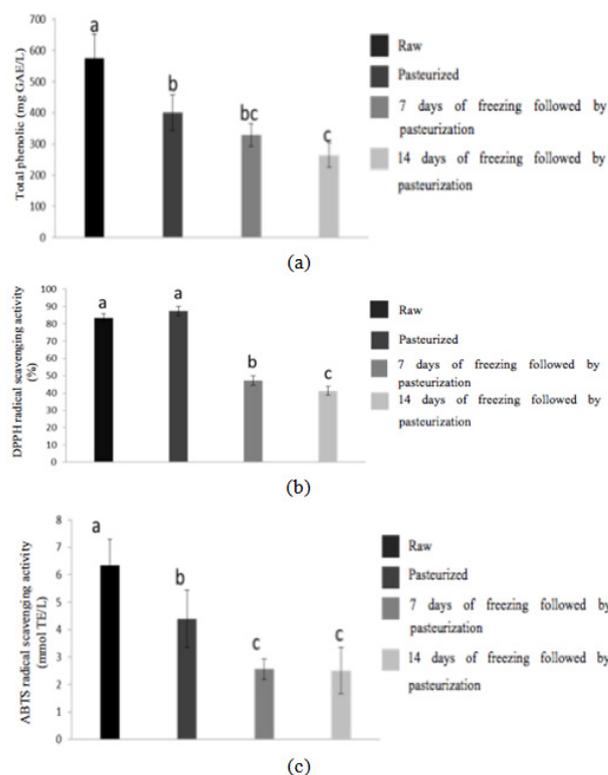
GAE = Gallic acid equivalent;
TE = Trolox equivalent
Treatments followed by different letters indicate significant difference at 5% by Tukey's test.

Figure 1. Content of total phenolic compounds (a), antioxidant capacity by DPPH free radical scavenging (b) and ABTS free radical scavenging (c) in different stages of human milk processing.

4. Discussion

Studies evaluating the stability of the antioxidant capacity have established the storage temperature of the HM at -20°C [6,14,16,17], however this is not the reality. The Brazilian HMB network adopts 15 days for the pasteurization of human milk after the first collection and during this period, it remains most of the time stored in the freezer or the donor' domicile. According to Embrapa [18] inside a domestic refrigerator, the freezer reaches temperatures between -1°C to -4°C and in domestic freezers; temperatures are between -14°C and -17°C. These can be quite variable due to the state of conservation and the manipulation.

A study conducted by Bertino et al. [19], under controlled laboratory conditions, showed that even when the HM storage temperature was set at 5 °C, it oscillated and had a mean value of 6.88 °C ± 1.1 °C. This finding should be considered in the case of storage of HM, especially the domestic storage in which the temperature should oscillate even more due to the frequent entry of hot air by virtue of the manipulation.

The present study showed a positive correlation between total phenolic compounds content and total antioxidant capacity, measured by ABTS and DPPH free radical scavenging methods. Although the analysis showed the presence of phenolic compounds in HM and this was affected by both pasteurization and freezing for 14 days. Li et al. [20] found low levels of three types of phenolic acids, ferulic, p-hydroxybenzoic and p-coumaric acids. This indicates that the contribution of phenolic acids to total antioxidant capacity is limited in this food, mainly when compared with other bioactive compounds present, such as carotenoids.

As for the effect of HM pasteurization, the antioxidant capacity decreased when evaluated by the ABTS radical scavenging method, as verified by Silvestre et al. [3], who obtained a reduction of 66.67% (0.16 mMeq uric acid) due to the slow pasteurization process (63°C for 30 minutes). It was also observed the reduction of enzymes with specific antioxidant capacity, such as 46.12% for glutathione (GSH) and 62.71% for glutathione peroxidase (GPx) activity, which may have been denatured by heat treatment. According to this finding, Braga and Palhares [21] observed a decrease of 64.7% in IgA protein with the pasteurization process.

According to Lawrence and Lawrence [22] some components present in HM can be reduced with pasteurization. Meantime, different studies have divergent results for freezing, and the differences are not yet clear. This can be observed in studies such as Sari et al. [4], in which the freezing of HM at -80°C reduced by 56.25% the antioxidant capacity of mature HM stored at -80°C for two months by the ABTS free radical scavenging method. On the other hand, Akdag et al. [5] found no significant difference in the total antioxidant capacity of HM from mothers of preterm infants stored at -80°C for 3 months, also evaluated by the ABTS free radical scavenging method.

According to Turoli et al. [14], HM stored at -20°C for 2 months has a higher concentration of lipid peroxides. This fact can be explained by the activity of lipoprotein lipase during storage, which increases the concentration of free fatty acids and, consequently, lipid peroxides, which are the primary products of lipid oxidation. However, it was noted that this high content of lipid degradation compounds did not correspond to the reduction in total antioxidant capacity of the HM measured by the ABTS radical scavenging method.

Further, Miranda et al. [17] evaluated the activity of the enzyme glutathione peroxidase (GPx) and the content of a lipid peroxidation marker, malondialdehyde (MDA) in HM, and verified that GPx activity decreased significantly after cooling for 24 hours at 4°C and freezing for 10 days at -20°C relative to fresh HM. The MDA content, however, only had a significant increase in the cooled HM.

The variability of the results found in studies evaluating the effect of freezing under the same temperature conditions may be related to the freezing speed and crystal formation, because according to Fellows [23], the main effect of freezing on food quality is the growth of ice crystals leading to cellular damage. These are formed by a proportion of water that undergoes a change of its state and this occurs in both fast and slow freezing. In slow freezing, the formed ice crystals are larger and grow in the intercellular spaces, breaking the wall of adjacent cells. In addition, the ice crystals have a water vapor pressure lower than the intracellular one, causing the cells to dehydrate and suffer permanent damage due to higher concentration of solutes and the deformation and collapse of the cell structure. In the fast freezing, smaller ice crystals are formed in the intercellular and intracellular spaces, and no water vapor pressure gradients are developed, with minimal cell dehydration and little physical damage [23]. In freezing the HM in domestic freezers, there is no way to control the speed. Thus, the damage done is not yet well understood [23].

The formation of ice crystals immobilizes a certain amount of water in the food and, in addition, there is an increased concentration of several solutes present in the non-frozen water [24]. One of the immediate consequences is the acceleration of chemical reactions, such as oxidation, hydrolysis and protein denaturation, in this fraction of unfrozen water, mainly in the range between -5°C and -15°C [24]. This occurs because, in this temperature range, the speed of reactions does not decrease as much as expected at low temperatures [24]. Thus, the concentration of solutes increases at the speed of reactions, among them reactions of oxidation, hydrolysis and protein denaturation, in a proportional way [21].

Even at a temperature of -18°C, not all water will be frozen, the enzymes do not completely inactivate and the solutes present in the aqueous phase are concentrated, which can modify characteristics such as pH, ionic strength, redox potential, among others. As a consequence, some chemical and enzymatic reactions can continue even very slowly during freezing. Lipid auto-oxidation is one of the most important chemical reactions in frozen products, because even though slowly, it occurs even at -18°C [24]. The residual activity of the enzymes can cause loss of vitamin C and degradation of pigments [24], and the lower the freezing temperature, the slower the enzymatic activity, until the moment where the total interruption will occur [25].

According to Bertino et al. [19], HM refrigerated at 4°C for 96 hours maintained the initial and final content of

lipid peroxidation products, indicating that it had not undergone lipid oxidation within this period. It also maintained the antioxidant capacity and lipase content unchanged. This result may be due to non-formation of ice crystals and, therefore, there was better preservation of the antioxidant compounds. Meantime, the microbiological quality of HM was not evaluated and, because of its composition rich in several nutrients, this becomes an excellent culture medium for various microorganisms [26].

The differences found between the methods used to evaluate the total antioxidant capacity, ABTS and DPPH radical scavenging capacity can be explained by the degradation of certain compounds during the pasteurization and freezing processes, which may have different degrees of affinity for a particular radical. In agreement with Rufino et al. [27], who examined the antioxidant capacity of tropical fruit, verified a diversity of antioxidant substances present in a food matrix, and this complex can provide different responses in each method of determining antioxidant capacity in vitro.

Although the freezing and pasteurization process promotes the reduction of total phenolic compounds and antioxidant capacity, the World Health Organization [15] recommends HMB as the best option when it is not possible to offer the own mother's milk, because it contains all nutrients needed for of infants [28]. Hanna et al. [6] observed that the antioxidant capacity of HM is significantly higher than that of the formulas, and this difference persisted independently of storage duration and temperature evaluated (-20 °C and 4 °C for 2 and 7 days).

One of the limitations of the study was the infeasibility of conducting analyses at different times because of the amount of HM required for the dosages.

5. Conclusion

The present study evidenced a reduction in the total phenolic content and total antioxidant capacity with slow pasteurization in relation to raw human milk. In the simulation of domestic freezing for up to 14 days, there was a negative effect of the freezing at -8.5°C (± 2.8) on the total antioxidant capacity, and the most expressive reduction was found in the first 7 days of storage.

Therefore, it is suggested further studies to evaluate the effect of frozen storage immediately after freezing and at 24-hour intervals to determine more efficiently the effect of the formation of ice crystals on the HM quality.

Acknowledgements

List of Abbreviations

HM = Human milk
HMB = human milk banks
ANVISA = Agência Nacional de Vigilância Sanitária
DPPH = 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
ABTS = 2,2-diphenyl-1-picrylhydrazyl
ANOVA = analysis of variance

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