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Hot topic: Holder pasteurization of human milk affects some bioactive proteins

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ABSTRACT

The aim of this research was to investigate the effect of Holder pasteurization (HoP; 62.5°C, 30 min) on the protein profile and activities of glutathione peroxidase (GPx) and lysozyme (LZ) in human milk. Over 6 mo of lactation, human milk samples were analyzed before (raw) and after HoP for GPx and LZ activity and electrophoresis protein profile. Holder pasteurization reduced human milk lactoferrin, immunoglobulin fractions, and GPx activity. In addition, GPx activity, which is high in colostrum and transitional milk, was naturally reduced over the 6-mo lactation period. In contrast, HoP did not affect human milk LZ activity. Besides its critical cellular antioxidant role in protecting the organism from oxidative damage, GPx decreases the redox potential of milk, stimulating the growth of anaerobic microorganisms, such as the probiotic *Bifidobacterium.* Considering the role of lactoferrin in infant health, we conclude that an important part of its function has been inactivated by pasteurization. These compounds should be replaced by human milk banks after the HoP step to recover lost functionality. Otherwise, an alternative technology to HoP that better retains human milk properties should be used by milk banks to eliminate the risk of transmission of infectious agents.
Key words: bifidogenic factor, probiotic, health

Hot Topic

Human milk is perceived not only as a food but also as a dynamic biologic system. This complex fluid provides nutrients, bioactive compounds, and immune factors such as immunoglobulins, lactoferrin (**Lf**), and lysozyme (**LZ**). Feeding human milk is the best method to nourish and protect the newborn from infectious diseases (Tobback et al., 2017).

Bioactive compounds from human milk, such as short-chain oligosaccharides, Lf, and LZ, promote the growth of beneficial gut microbiota, such as species of the genera *Bifidobacterium* and *Lactobacillus* (Thongaram et al., 2017). Sometimes, mothers discontinue breastfeeding soon after giving birth because milk is in short supply or unavailable and the infant cannot be nourished exclusively by this optimum source of nutrition (Kronborg et al., 2015). In this situation, human milk banks are essential, as they provide milk from donor mothers, which is a better alternative to infant formula. Safety of banked milk is ensured by Holder pasteurization (**HoP**), also called the LTLT method (low temperature, long time; 62.5°C for 30 min). The pasteurization step is required to inactivate pathogenic bacteria and viruses, and it reduces spoilage to an acceptable level when delivery to health-impaired individuals or medical institutions is considered. However, heat treatment may reduce the content of some bioactive compounds in human milk (Lima et al., 2017).

Human milk may prevent many illnesses and diseases, such as ear infections, gastrointestinal infections, severe lower respiratory tract infections, atopic diseases (allergies, hay fever, asthma, and dermatitis), obesity, cardiovascular diseases, childhood leukemia, and sudden infant death syndrome (Lönnerdal, 2017; Moukarzel and Bode, 2017). Governments usually spend money treating these diseases, and these resources could be better applied to other health sectors. Additionally, gut colonization by *Bifidobacterium* and *Lactobacillus* is largely related to a well-functioning immune system, especially the production of IgA, IgG, and IgM (Talja et al., 2014). Poor immunity is one of the main causes of childhood death. Improving the quality of the milk in human milk banks is essential to ensure a healthier life for the newborn and to reduce expenditure on disease treatment (Katke et al., 2015).

Unlike HTST treatment, HoP adversely affects bioactive compounds in human milk, including Lf (Klotz

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et al., 2017). Human milk is a dynamic enzymatic fluid, and pasteurization may cause protein denaturation (de Oliveira et al., 2016). Lactoferrin is an iron-binding glycoprotein, and its bacteriostatic and bactericidal effects on gram-positive and gram-negative bacteria are well known. It is known that some lactic acid bacteria, including probiotic bifidobacteria and lactobacilli, are resistant to its antibacterial effects. Moreover, Lf-binding protein in bifidobacteria is involved in growth stimulation mechanisms and it may play other roles (Oda et al., 2014).

Information about glutathione peroxidase (**GPx**) activity in prokaryotes is scarce. The *GPX* gene and GPx synthesis pathways have not been detected in anaerobes, including bifidobacteria. Lowering of the redox potential by GPx may stimulate the growth of bifidobacteria (Živković et al., 2015). Moreover, GPx may be depleted by heat treatment; therefore, pasteurization may also reduce the probiotic growth-promoting potential of human milk. Supplementation of glutathione to these bacteria may protect them from oxidative stress (Ladero and Sánchez, 2017); glutathione is also a sulfur and nutrient source.

The aim of this research was to investigate the effect of HoP on the protein profile and GPx and LZ activities in human milk. Activity of GPx was assessed and SDS-PAGE performed on 21 samples of breast milk taken from a healthy mother over a period of 6 mo after term delivery, with 3 to 4 collections each month. Lysozime activity was measured in 30 samples from the Human Milk Bank of IFF-Fernandes Figueira Institute (Rio de Janeiro, Brazil), collected by hand expression, and 2 others donated from voluntary healthy individuals that were collected with a breast pump. On all occasions milk was collected after nipples were cleaned with saline solution and a paper towel. The samples were immediately frozen to −18°C and taken to the laboratory on ice. After thawing at 7°C for 9 h, the milk was transferred in equal volumes to sterile test tubes.

Breast milk samples (1 mL of each sample) were pasteurized by the HoP method, according to the Human Milk Bank technical standards (Almeida, 2005). Samples were heat treated by immersion in a thermostatically controlled, stirred water bath. When the water temperature stabilized at 62.5°C, the tubes were immersed in the bath so that the water level was 2 cm above the milk level. A tube containing the same amount of milk was used as a control, and a thermometer was inserted into the tube. The tubes were held in the water bath at pasteurization temperature for 30 min and shaken at regular intervals (3 min). Thereafter, samples were immediately cooled to 5°C in an ice bath and defatting by centrifugation at 21,000 × *g* (2K15, Sigma Laborzentrifugen, Osterode am Harz, Germany) for 10 min.

Glutathione peroxidase activity was measured in samples of raw and pasteurized milk (21 of each) from an individual donor by using a Glutathione Peroxidase Assay Kit (703102, Cayman Chemical, Ann Arbor, MI). A boiled milk sample was used as a negative control, in addition to the positive and negative controls provided with the assay kit. The reaction was started by addition of 20 μL of cumene hydroperoxide to all wells, and the absorbance was read by using a plate reader (Uniscience, Multiskan FC, Thermo Scientific, Waltham, MA) at 340 nm. After linear regression of time and absorbance values, the angular coefficient (ΔAC) was recovered and substituted into Equation [1] to calculate GPx activity:

$$\text{GPx activity} = \frac{\Delta AC}{0.00373} \times \frac{0.19}{0.02}, \qquad [1]$$

where 0.00373 μM^{-1} is the NADPH extinction coefficient adjusted for the path length of the solution in the well (0.6 cm) at 340 nm, 0.020 mL is the volume of diluted GPx (control) to 3 wells, and 0.19 mL is the final volume of the assay in all of the wells.

Defatted milk aliquots (100 μL) were mixed with sample buffer (200 μL) to obtain the protein electrophoresis profile and kept frozen (−18°C) until use. Proteins were identified by SDS-PAGE analysis in a Bio-Rad vertical Mini-Protean Tetra Cell (Bio-Rad Laboratories, Hercules, CA). Stacking and running gels were prepared by using 8% (wt/vol) acrylamide solution. A 100-V electric current was used throughout the running time (2.5 h). Gels were stained with Coomassie Brilliant Blue. High- and low-molecular-weight standards from Bio-Rad Laboratories were added to calculate the protein masses (Laemmli, 1970).

Lysozyme activity was measured using the lysoplate method, with *Micrococcus luteus* [ATCC 4698; INCQS 356, Instituto Nacional de Controle de Qualidade em Saúde (INCQS) Culture Collection, Rio de Janeiro, Brazil] as indicator. Melted de Man, Rogosa, and Sharpe agar (Himedia, Mumbai, India) was inoculated with a fresh suspension of the indicator microorganism to a final concentration of approximately 10^6 cfu/mL and plated in Petri dishes. Five wells were made in each plate with a sterile manual borer (6.8 mm diameter) and filled with 100 μL of milk sample. After incubation of the plates at 36°C for 48 h, the inhibition halo around each well's border was measured with a caliper.

Analysis of variance followed by Fisher test ($P > 0.05$) and Tukey test ($P > 0.05$) was used to analyze