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Short communication: Effect of refrigerated storage on the pH and bacterial content of pasteurized human donor milk

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ABSTRACT

Once pasteurized donor milk is thawed for its administration to a preterm or sick neonate, and until it is administered, it is kept refrigerated at 4 to 6°C for 24 h. After this time, unconsumed milk is discarded. This time has not been extended, primarily because of the concern of bacterial contamination. The aim of this study was to determine the changes in pH and bacterial count when pasteurized donor milk was kept under refrigeration for a prolonged period (14 d). In this prospective study, 30 samples of pasteurized donor milk from 18 donors were analyzed. Milk was handled following the regular operating protocols established in the neonatal unit and was kept refrigerated after thawing. pH measurements and bacteriology (on blood agar and MacConkey agar plates) were performed on each sample at time 0 (immediately after thawing) and then every day for 14 d. Changes in pH of samples over time were evaluated with linear mixed-effects regression models. A slow but gradual increase in milk pH was observed starting from the first day [mean (±SD) pH of 7.30 (±0.18) at time 0 and 7.69 (±0.2) on d 14]. No bacterial growth was observed in any of the samples throughout the complete trial except in one sample, in which *Bacillus flexus* was isolated. In conclusion, pasteurized human donor milk maintains its microbiological quality when properly handled and refrigerated (4–6°C). The slight and continuous increase in milk pH after the first day could be due to changes in the solubility of calcium and phosphate during refrigerated storage.

Key words: pasteurized donor milk, acidity, refrigerated, storage

Short Communication

Pasteurized donor milk is the first option for feeding premature or ill newborns when their mother's milk is not available (WHO, 2002; PATH, 2013). Although donor milk comes from healthy women, it is pasteurized to prevent the transmission of infectious microorganisms to an especially vulnerable population, such as preterm babies or infants admitted to the neonatal intensive care unit. The most common practice in human milk banks is to pasteurize donor milk according to the Holder method (62.5°C, 30 min; PATH, 2013). Holder pasteurization denatures viral proteins (Orloff et al., 1993; de Oliveira et al., 2009; Landers and Updergrove, 2010; Donalisio et al., 2014) and destroys all non-spore-forming viable bacteria in milk, although some strains of *Bacillus* spp. or other sporulated species can remain viable. In addition, some of the milk's beneficial properties are lost during pasteurization, such as the cellular components that confer immunologic properties (B cells, T cells, macrophages, and neutrophils); and immunologic proteins such as IgA, IgG, lactoferrin, lysozyme, and erythropoietin, are significantly reduced. Other components, such as oligosaccharides, remain intact (Akinbi et al., 2010; Ewaschuk et al., 2011; Gómez de Segura et al., 2012; Chang et al., 2013).

Unpasteurized mother's milk has bactericidal properties that confer protection against neonatal infections as well as against contamination during milk handling. This bactericidal activity remains unchanged during the

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first 72 h of refrigerated storage but is lost subsequently (Silvestre et al., 2006; Miranda et al., 2011). Holder pasteurization significantly reduces (50–70%) the bactericidal effect, but if the pasteurized donor milk is kept refrigerated, the residual bactericidal capacity remains stable for up to 72 h (Silvestre et al., 2006; Bertino et al., 2013).

After pasteurization, donor milk is typically stored frozen. Afterward, once it is thawed and, until it is administered to the recipient, it is kept refrigerated at 4 to 6°C for a variable period of time, usually 24 to 48 h (PATH, 2013). This period is not extended due to the concern of bacterial contamination (Frischknecht et al., 2010) and reductions in nutritive properties and biological activity (Meng et al., 2016).

Given that pasteurized donor milk is a scarce resource, studies have been conducted to determine whether this refrigeration time can be extended (Slutzah et al., 2010; Vickers et al., 2015; Meng et al., 2016). These studies have measured the pH or bacterial growth in refrigerated pasteurized human donor milk for varying periods. However, none of these studies has performed a quality assessment of pasteurized donor milk during refrigerated storage by both pH and bacterial count measurement in the same samples of milk for more than 7 d. Therefore, the aim of the current study was to determine the changes in pH and bacterial counts in pasteurized donor milk stored refrigerated up to 14 d. Our initial hypothesis was that pasteurized human milk acidifies when refrigerated, although more slowly than unpasteurized milk.

A prospective study was planned with the aim of determining the changes in pH and bacterial growth in pasteurized donor milk stored at 4 to 6°C. The study was performed at the Neo 12 Human Milk Bank, at University Hospital 12 de Octubre (Madrid, Spain). It was reviewed and approved by the Ethics Committee for Clinical Investigation (Comité Etico de Investigación Clínica) of University Hospital 12 de Octubre. Written informed consent was obtained from each donor during their first interview at the milk bank.

Milk samples were 30 aliquots (120 mL each) of milk pasteurized in the Human Milk Bank following its regular procedures (no special pasteurizations were performed for the study). Milk was donated by healthy women; donor screening included a serological analysis for hepatitis B virus, hepatitis C virus, human immunodeficiency virus, and syphilis, and the women completed a questionnaire on life habits. Donors were instructed on hygiene measures for milk extraction (such as using cap and facemask, hand washing, and breast pump sterilization before expression). Immediately after expression, milk was frozen at home for a maximum of 15 d, and then transported frozen to the milk bank where it was kept at a controlled temperature (−20°C) for a maximum of 41 d. To pasteurize the milk, it was thawed in a shaking water bath at 37°C (Jeio Tech BS-21; Lab Companion, Seoul, Korea) until a central block of ice (approximately 50% of the container) was present, and then transferred to the fridge to finish thawing at 4°C. Batches of milk from the same donor were created. For each batch, nutrient composition (fat, protein, and lactose contents) was measured using a MilkoScan FT 2 (Foss Iberia S.A., Barcelona, Spain). Holder pasteurization was then performed in a shaking water bath (Jeio Tech BS-21; Lab Companion) by heating milk samples (120 mL) at 62.5°C for 30 min, and then rapidly cooling to 4°C. After pasteurization, a microbiological analysis of the milk was performed. In the event of any bacterial growth, the entire batch was discarded, unless <500 cfu/mL of *Bacillus cereus* had grown. Properly pasteurized samples were stored frozen at −20°C.

For the study, 30 aliquots of 120 mL were randomly chosen from the milk available at the Human Milk Bank; samples came from 18 donors. Each one of the samples was obtained from an individual donor but some donors provided more than one sample. For each sample, the following data were recorded: maternal age and nationality, type of milk, nutritional composition, days from expression to pasteurization, and days from expression to the beginning of the study.

The samples were partially thawed in a shaking water bath at 37°C until a central block of ice (approximately 50% of the container) was present, and transferred to a refrigerator at 4 to 6°C. Each 120-mL sample was divided into two 60-mL aliquots under the laminar flow hood, using 120-mL sterile glass containers with plastic stoppers. From this time forward, the samples were handled under the same conditions as commonly used in the neonatal unit to prepare feedings (nonsterile cap, facemask, gloves, and surfaces cleaned with alcohol), and in the same room, which was exclusively dedicated to human milk handling. Milk was kept in the neonatal unit refrigerator (5–6°C), not in the milk bank. The refrigerator used to store human milk in the neonatal unit does not have continuous temperature monitoring, because it is usually used to store milk for short periods. The temperature was set at 5°C and a complete count of the number of times the refrigerator was opened was performed for 72 h.

Term milk was considered milk from donors whose children were born ≥37 wk of gestational age, and preterm milk was milk from mothers of children born <37 wk of gestational age. Colostrum was milk expressed during the first 7 d after birth, intermediate milk was

Table 1. Donor milk composition (mean of all milks combined ± SD unless otherwise noted) analyzed before pasteurization

Composition	Value
Protein, g/dL	1.80 ± 0.10
Lactose, g/dL (median and IQR[1])	7.55 (7.31–7.94)
Fat, g/dL	2.90 ± 1.08
Energy, kcal/dL	63.5 ± 8.3

[1]IQR = interquartile range.

that expressed from d 8 to 21, and mature milk was that expressed from 22 d postpartum.

Initially (time 0) and then daily for the next 14 d, a 5.5-mL sample of refrigerated pasteurized donor milk was extracted from the 60-mL aliquot. Each aliquot was opened to extract samples a maximum of 7 times, simulating the maximum number of times containers are opened to prepare feedings for hospitalized infants.

We measured pH and performed bacterial counts to evaluate the quality of the pasteurized milk samples during refrigeration. The pH of each sample was measured twice using a calibrated pH meter (BASIC 20, Crison Instruments, Barcelona, Spain). Bacterial counts were determined by spreading 10 μL of milk onto blood agar and 10 μL onto MacConkey agar plates (Biomerieux, Lyon, France), which were incubated at 37°C in an aerobic environment for 48 h. These culture media were chosen because blood agar is an enriched medium that allows the growth of almost any type of bacteria, whereas MacConkey medium is specific for gram-negative and lactose-fermenting bacteria. If any bacterial growth was detected, colonies were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in collaboration with the Department of Microbiology at University Hospital 12 de Octubre.

The study was performed in 3 phases, with 10 samples analyzed in each phase. The first phase was conducted with 10 samples, and we analyzed pH and bacterial count in order to determine the sample size. As the sample size needed was 20 samples (assuming a mean difference of 0.10, SD of 0.25, a correlation of 0.80, and a 95% confidence level), the second phase was conducted with another 10 samples. The third phase was planned to confirm the pH results, and microbiological analyses were not performed because bacterial growth was not detected in previous phases.

Characteristics of the patients and samples were described using means and standard deviations or absolute and relative frequencies. The normality of the data distribution was tested with the Shapiro–Wilk test. Changes in the pH of the samples over time were evaluated with linear mixed-effects regression models (Hedeker and Gibbons, 2006). The data were analyzed using Stata 10 software (StataCorp, College Station, TX), and significant differences were defined as $P < 0.05$.

Table 2. pH values during the study period (n = 30)

Day	Mean pH	SD	Minimum	Maximum	*P*-value
0	7.31	0.18	6.81	7.55	
1	7.39	0.19	6.83	7.66	0.00
2	7.39	0.22	6.84	7.79	0.57
3	7.40	0.23	6.80	7.68	0.44
4	7.45	0.22	6.87	7.77	0.02
5	7.56	0.19	7.09	7.91	0.00
6	7.58	0.19	7.06	7.77	0.16
7	7.60	0.18	7.22	7.87	0.49
8	7.61	0.18	6.96	7.83	0.36
9	7.59	0.24	6.90	7.92	0.22
10	7.65	0.24	6.90	7.86	0.00
11	7.65	0.20	7.00	7.92	0.91
12	7.65	0.21	7.09	7.96	0.88
13	7.66	0.19	7.11	7.93	0.52
14	7.69	0.21	7.11	7.99	0.00

Overall, 77.8% (14/18) of the donors were Spanish. Median age at the time of the study was 37 yr (34–37 yr). In total, 77.8% of the samples were mature, term milk and 22.2% were mature, preterm milk. In Table 1, the composition of the milk analyzed before pasteurization is shown. The mean number of days from expression to pasteurization was 26.4 ± 9.6 d and from expression to the first day of the study was 48.9 ± 18 d. The neonatal unit's refrigerator was opened an average of 58 times per day.

We observed a slight increase in pH over time (Table 2). The mean pH of the milk samples at room temperature (23 ± 2.5°C) was 7.30 (±0.18) at time 0 and 7.69 (±0.21) at d 14 (Figure 1), with a statistically

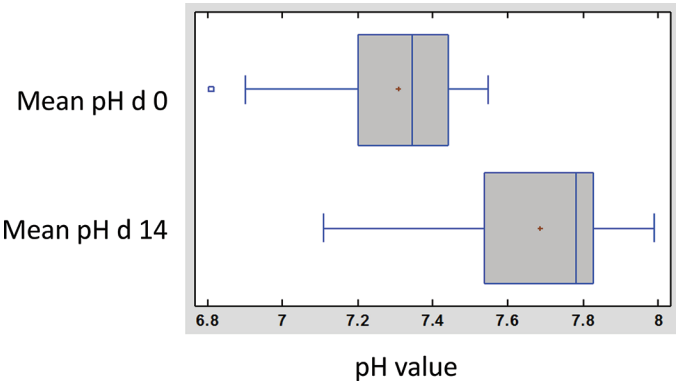

Figure 1. Mean pH values at time zero and at d 14 ($P = 0.00$). The central rectangle represents the SD and the line and the cross inside the rectangle show the median and the mean, respectively; the whiskers indicate the maximum and minimum values; and the black dots outside the rectangles are suspected outliers (>1.5 interquartile range). Color version available online.

significant difference ($P = 0.000$). The pH increased significantly from d 0 to 1 ($P = 0.000$), and then remained stable until d 4, when the difference was again significant ($P = 0.02$).

We detected no relationships between the days from expression to pasteurization or the days from expression to the first day of the study and the difference in pH over time, the composition of the milk, or the sociodemographic characteristics (mean age and country of origin) of the donors.

We observed bacterial growth in only 1 of the 20 samples analyzed. In the 14 cultures performed on this sample, *Bacillus flexus* was identified (100 cfu/mL). We did not detect any bacterial growth in the remaining samples.

The pH of pasteurized human milk increased slightly when stored refrigerated at 4 to 6°C for 14 d, and no bacterial contamination was observed. Our initial hypothesis was that pasteurized human milk would acidify when refrigerated, although more slowly than unpasteurized milk. As we have shown, the results found were the opposite of our initial hypothesis.

Milk acidity is a quality indicator (Novak and Cordeiro, 2007; Vázquez-Román et al., 2013). Milk can acidify by lipolysis (transformation of triglycerides in free fatty acids) or by the production of lactic acid by lactose-fermenting bacteria. Acidic milk is of poorer quality because it is more osmolar and has poorer bioavailability of phosphorus and calcium (Erickson et al., 2013). Milk acidity can be measured using 2 methods: Dornic acidity and pH. When milk acidifies, the pH decreases but the degree of Dornic acidity increases (Escuder-Vieco et al., 2016). Milk with Dornic acidity >7 (pH 6.57) is discarded because it is considered to be of poor quality (Clark et al., 1984; Hedeker and Gibbons, 2006).

Human milk has 2 types of lipases (Clark et al., 1984; Bertino et al., 2013): bile salt–dependent lipase, which seems to be active in the newborn, helping the immature pancreas to digest and absorb fat, and lipoprotein lipase, which is active in the lactating mammary gland (where it is involved in the uptake of circulating fatty acids) but not in the infant. In unpasteurized milk, the lipases remain active when refrigerated or frozen (Penn et al., 2014). Previous studies have shown that if unpasteurized human milk is kept refrigerated, acidification of the milk will occur because of 2 factors: lipolysis by the lipases (and thus the release of fatty acids into the medium) and the production of lactic acid by lactose-fermenting bacteria (Rona et al., 2008; Slutzah et al., 2010; Ghoshal et al., 2012; Ahrabi et al., 2016). However, when milk is pasteurized, lipases are destroyed and the bacteria present in the milk are eliminated. Thus, in the current study these 2 processes cannot be implicated (Ghoshal et al., 2012; Penn et al., 2014; Ahrabi et al., 2016; Baro et al., 2011); therefore, the change in milk pH likely due to other factors.

A study published in 2015 (Vickers et al., 2015) analyzed 42 samples of pasteurized donor milk. The samples were thawed and kept refrigerated for 9 d. None of the cultures conducted grew any microbes. As part of a larger study on unpasteurized milk (35 samples of unpasteurized milk), Slutzah et al. (2010) analyzed 5 samples of pasteurized milk that were kept refrigerated for 96 h; they observed that neither the pH nor the concentration of fatty acids changed, and there was no bacterial growth. In a previous study conducted in our unit (Tobío-Gimeno et al., 2016), we analyzed the pH of 30 samples of unfrozen pasteurized donor milk kept in a refrigerator, and the pH remained unchanged for 4 d. A more recently published study analyzed bacterial growth in refrigerated pasteurized milk and found no bacterial growth in 7 d (Meng et al., 2016).

In this study, we found the opposite result to what we had expected: we observed a slight increase in milk pH during refrigerated storage of pasteurized donor milk. This change was not related to bacterial growth, given that postprocessing contamination was not evident; thus, some milk component could be involved in this pH shift. Human milk has a complex mineral composition that plays a crucial role in sustaining normal growth and development of the newborn, but this composition also has an important influence on many milk properties. Certain milk salts are soluble, whereas others, such as calcium phosphate, exist partly in soluble form and partly in the colloidal phase associated with casein micelles. Changes in temperature modify the equilibrium between the soluble and colloidal phases. Moderate heating, such as Holder pasteurization, decreases the solubility of calcium phosphate due to the formation of colloidal calcium phosphate, which is accompanied by a concomitant decrease in milk pH. Subsequent cooling and keeping the milk at low temperature restores the original equilibrium by increasing both the solubility of calcium phosphate and the milk pH (Gaucheron, 2005; Lucey and Horne, 2009).

Bacterial growth was detected in only one sample: *Bacillus flexus* grew in all aliquots of the same sample (from d 1 to 14), with no increase in the number of colonies over refrigeration time, and never exceeding the acceptance limit of the milk bank. *Bacillus* spp. are sporulated microbes whose spores are highly resistant to heat and disinfectants. They can therefore remain as surface contaminants in laboratories and are difficult to eradicate. Occasional contamination of pasteurized milk with *Bacillus* spp. is a common problem in virtu-

ally all human milk banks and in the food industry (Hanson et al., 2005; Landers and Updergrove, 2010; Gómez de Segura et al., 2012).

In this study, alkalization of human milk was observed. Further studies are required that focus on understanding the processes that pasteurized milk undergoes when refrigerated and the potential consequences of these processes on the absorption of macronutrients and micronutrients and the activity of elements with biological value. We have found no previous references on the alkalization of human milk.

The main limitation of the study was that refrigerator temperature (and its variation) was not monitored during the 14 d of study, given that we planned the study with the intention of imitating the conditions under which human milk is stored in neonatal units.

In conclusion, it appears that pasteurized human donor milk, if kept refrigerated and handled with appropriate hygiene measures, does not become contaminated. Based on these results, we believe that, from a microbiological point of view, the validity period of refrigerated donor milk could be safely extended to 48 h, given that it is a scarce resource. The alkalization process that human milk undergoes needs further study to determine its consequences on milk quality.

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