Storage of unfed and leftover pasteurized human milk

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Abstract:

Objective: To determine the impact of storage on bacterial growth and immunological activity of pasteurized human milk and leftover pasteurized human milk that has been exposed to the microflora in an infant's mouth. ***Materials and Methods:*** Eighteen mother-infant dyads participated in two separate studies. Mother's milk was pasteurized, and each baby was fed 1 to 2 ounces. Pasteurized and leftover pasteurized milk were stored at room (24°C) and refrigerated temperatures (4°C). After storage, milk was analyzed for bacteria, total protein, lysozyme activity, and secretory immunoglobulin A (SIgA) activity. ***Results:*** In pasteurized and leftover pasteurized milk stored in the refrigerator for 7 days, total aerobic bacteria do not increase significantly and total protein and bioactive proteins are stable. At room temperature, there is a significant increase in total aerobic bacteria in leftover pasteurized milk during 12 hours of storage ($p < 0.01$) and a significant decrease in total protein and SIgA activity in pasteurized milk during 12 hours of storage ($p = 0.02$ and $p = 0.03$, respectively). ***Conclusions:*** When stored in the refrigerator, pasteurized and leftover pasteurized milk may be stored for at least 7 days when considering the variables studied. Caution should be used when storing pasteurized and leftover pasteurized milk at room temperature to prevent an increase in bacterial growth and a decrease in total protein and SIgA activity.

Keywords: human milk | leftover human milk | storage of human milk | NICU | bacteria | protein

Article:

Introduction

The use of pasteurized donor human milk is growing in the neonatal intensive care unit (NICU)[1] as an effective strategy for improving health outcomes[2] and reducing healthcare costs[3] among medically fragile infants. To ensure the safety of pasteurized human milk (PM), the Human Milk Banking Association of North America (HMBANA) and other global milk bank networks have issued guidelines, including appropriate storage temperatures and

durations.[4,5] Current best practices for the safe storage and handling of PM state that thawed milk should be stored at 4°C and should be used within 24 hours.[4]

Several studies have examined the impact of Holder pasteurization on the nutritive and immunological components of human milk immediately after pasteurization.[6–14] However, little attention has been paid to the immunological activity and microbial content of thawed PM during standard use. A recent study of PM that was thawed and dispensed in a clinical setting found that PM remained free of microbes when stored at 4°C for up to 9 days.[15] Extending the expiration time of thawed PM has the potential to reduce the unnecessary waste of a costly and limited resource, suggesting that further research into the immunological and microbial quality of thawed PM under various storage conditions is warranted.

The HMBANA primarily serves medically fragile infants that are in the NICU; however, outpatient children may also be the recipients of PM. In 2014, 23% of the PM distributed by HMBANA went to outpatients.[16] In an outpatient setting, it is more likely that infants are receiving PM through a bottle or cup than through a nasogastric tube, creating the potential for leftover milk that has been exposed to bacteria in the infant's mouth. The Academy of Breastfeeding Medicine recommends discarding leftover raw human milk within 1 to 2 hours of consumption for a full-term infant.[17] No research exists on safe storage practices of leftover pasteurized human milk (LPM), which is defined as PM that has been exposed to the microflora in an infant's mouth through bottle or cup feeding.

The objective of this pilot study is to examine the microbial content and immunological activity of PM and LPM during ambient and refrigerated storage conditions to inform storage recommendations.

Materials and Methods

North Carolina State University's Institutional Review Board approved the protocol and participants gave informed consent for themselves and their infants.

A preliminary study was conducted to inform the design of a more comprehensive study (described below) on storage of leftover human milk. In the preliminary study, five mother-infant dyads were recruited in the area of Raleigh, Durham, and Chapel Hill, North Carolina. Each mother was required to donate 3 to 4 ounces of freshly expressed milk. The milk was transported to the laboratory on ice and pasteurized at 62.5°C for 30 minutes, according to the Holder pasteurization method utilized in HMBANA milk banks.[18] After pasteurization, the chilled milk was returned to the mother's house and the infant drank 1 to 2 ounces of the milk from a clean bottle. The milk was transported back to the laboratory on ice and transferred into premarked sterile conical tubes and stored at 4°C for 0 hour, 2, 4, and 6 days, and at 24°C for 0, 3, 6, 9, and 24 hours. At the appropriate time point, the samples were immediately analyzed for total aerobic bacteria. Human milk within the preliminary study was never frozen.

Results of the preliminary study informed the storage conditions of a more comprehensive study in which 13 mother-infant dyads were recruited in the area of Raleigh, Durham, and Chapel Hill, North Carolina. Eligibility for participation included: currently breastfeeding a healthy term

infant aged 2–11 months; ability and willingness to express milk using a breast pump; confirmation of regular pump cleaning; and infant willing to drink milk from a bottle.

Mothers expressed milk as usual and froze the milk immediately after expression. No specific protocol was given for milk expression or pump cleaning; therefore, the results of the study may be applicable to the general population of mothers who express and handle milk using a variety of practices. Mothers expressed milk completely from one or both breasts and stored the milk from one pumping session into one container and refrigerated the bottle. Once a mother obtained 8 ounces of milk, it was frozen for no longer than 2 weeks and then delivered to the laboratory on ice. Milk was thawed under running water and divided into two sterile containers.

The milk was pasteurized at 62.5°C for 30 minutes, according to the Holder Pasteurization method utilized in HMBANA milk banks.[18] After samples were cooled, the pasteurized milk was aliquoted and stored in a freezer at −20°C. All milk samples went through one freeze/thaw cycle before pasteurization, and one freeze/thaw cycle after pasteurization, before they were subjected to the storage conditions. One frozen 4-ounce container of PM was delivered back to the original mother. The mother was instructed to thaw the milk as she usually did at home and to feed her baby 1 to 2 ounces of the milk with her own clean bottle. The directions given to the mothers for thawing and feeding the milk were not standardized so that the results could be applicable to those not following a specific protocol. However, all the mothers indicated that they heated the milk using warm water. The feeding process took 1 to 2 minutes and the leftover pasteurized milk was transferred into a new sterile container and delivered back to the laboratory in an ice-filled cooler. Each mother's milk was handled separately. The process of collecting a PM and LPM sample was completed within 1 month for each participating dyad.

All milk samples were aliquoted into premarked sterile conical tubes for further processing. The LPM samples were stored at 24°C for 0, 3, 6, and 9 hours and at 4°C for 1, 3, 5, and 7 days, respectively. The PM samples were stored at 24°C for 0, 3, 9, and 12 hours and 4°C for 1, 3, 5, and 7 days, respectively. The duration of storage was chosen based on results from the preliminary study. After the designated storage time, each sample was separated into four sterile tubes. One tube was sampled immediately for bacteriological analysis (PM tested for aerobic plate count; LPM tested for aerobic plate count and coliform count), and the other three tubes were stored in a freezer at −80°C until analysis of total protein, lysozyme activity, and secretory immunoglobulin A (SIgA) activity.

Analysis of total aerobic bacteria

Petrifilm™ (3M Company, St. Paul, MN) plates were used to examine the total aerobic bacteria and coliform counts in the milk in duplicate. Petrifilm is a ready-made culture medium system, which was found to give results with no significant differences compared to the standard agar plating method.[19,20]

Analysis of total protein and bioactive proteins

Total protein content was measured by the bicinchoninic acid assay (PI23227; Fisher Scientific, Waltham, MA).[21] Lysozyme activity was measured by a *Micrococcus lysodeikticus* based

turbidimetric assay. The method for analyzing lysozyme activity was adapted from Worthington Biochemical Corporation[22] to run in a 96-well plate.[23] SIgA activity was determined by a kinetic indirect enzyme linked immunosorbent assay, modified by Chen[24] and Viazis.[25] Assays were conducted in triplicate.

Statistical analysis

In the preliminary study, bacteria counts were measured in duplicate at each of several time points for milk samples assigned to two storage (temperature) treatments. Separate linear mixed models for the log of bacteria counts were fit for each temperature, using the MIXED procedure of the SAS software package (Cary, NC). These models included fixed factorial effects for time and random effects for sample and sample-by-time interaction to accommodate the repeated measures aspect of the design. In the model below, i, j, and k are index time, sample, and duplicate, respectively.

$$Y_{ijk} = \mu + \tau_i + S_j + (\tau S)_{ij} + E_{ijk}$$

In study two, separate models were fit for each temperature, which included fixed factorial effects for storage treatment and time along with random effects for sample and sample-by-treatment interaction. The design could be called a completely randomized split-plot design, with pasteurization treatment as a whole-plot factor and time as a split-plot factor, since each sample was measured repeatedly over time. Accordingly, random effects were included for sample, nested within the pasteurization treatment. Since technical replicate measurements were made for each sample at each time point, a random effect was included for sample-by-time interaction (also nested within pasteurization treatment). The model can then be written as follows:

$$Y_{ijkl} = \mu + \alpha_i + \tau_j + (\alpha\tau)_{ij} + S_{k(i)} + (\tau S)_{jk(i)} + E_{ijkl}$$

where i and j index the pasteurization treatments and time points, respectively, and k is an index for the milk sample (mother) and l is an index for the technical replicates. Greek symbols (α and τ) denote fixed and capital letters are used for random effects. Separate univariate models were fit for each of the several response variables (protein, lysozyme activity, and SIgA activity).

Results

Total aerobic bacteria

In the preliminary study ($n = 5$), leftover pasteurized milk that was never frozen did not have a significant increase in the total aerobic bacteria levels between 0 and 9 hours of 24°C storage ($p = 0.53$), but there was a significant increase between 9 and 24 hours ($p < 0.0001$) (Table 1). When stored at 4°C, bacteria did not increase significantly during the 6 days of storage ($p = 0.28$) (Table 1).

Table 1. Total Aerobic Bacteria in Leftover Pasteurized Human Milk ($n = 5$)

Temperature: 24°C	
Time (hours)	**Aerobic bacteria count (log CFU/mL)**
0	3.06
3	3.17
6	3.06
9	3.17
24	6.32
p-value	<0.0001
Temperature: 4°C	
Time (days)	**Aerobic bacteria count (log CFU/mL)**
0	3.06
2	2.87
4	4.10
6	3.05
p-value	0.28

p-values are for comparing rows within a column.

Table 2. Compositional Changes in Pasteurized and Leftover Pasteurized Milk During Storage ($n = 13$)

	Aerobic bacteria count (log CFU/mL)		**Total protein (mg/mL)**		**Lysozyme activity (units/mL)**		**SIgA activity (µg/mL)**	
	PM	**LPM**	**PM**	**LPM**	**PM**	**LPM**	**PM**	**LPM**
Storage time at 24°C (hours)								
0	0	2.8	12.51	12.16	41990	40599	721.95	697.62
3	0	2.8	12.19	12.30	41618	41354	716.71	704.05
6	NA	2.9	NA	12.40	NA	39813	NA	703.18
9	0	3.1	11.89	12.06	41905	40329	688.15	698.48
12	0	NA	11.71	NA	40406	NA	702.96	NA
SEM	NA[a]	0.3	0.46	0.46	6565.06	6564.17	68.93	68.93
p-value	NA[a]	<0.01	0.02	0.62	0.67	0.75	0.03	0.93
Storage time at 4°C (days)								
0	0	2.8	12.51	12.16	41990	40599	721.95	697.62
1	0	2.8	12.52	12.01	40898	39232	705.58	680.07
3	0	2.5	12.30	12.13	40036	38556	704.06	694.31
5	0	1.4	11.93	12.18	41219	39682	707.15	688.35
7	0	2.3	12.15	12.09	41559	40414	721.1	680.11
SEM	NA[a]	0.3	0.46	0.46	6756.79	6756.79	67.83	67.83
p-value	NA[a]	<0.001	0.27	0.95	0.77	0.66	0.49	0.60

p-values are for comparing rows within a column.
[a]Bacteria counts are essentially zero, with almost no variation at each of the time points for each of the temperatures. Therefore, *p*-values for tests of equality are unnecessary for this column in the table.
CFU, colony-forming units; LPM, leftover pasteurized human milk; PM, pasteurized human milk; SEM, standard error of the mean; SIgA, secretory immunoglobulin A.

In the comprehensive study ($n = 13$), PM and LPM went through one freeze/thaw cycle before pasteurization, and one freeze/thaw cycle after pasteurization, before they were subjected to the storage conditions. There were no significant changes observed in total aerobic bacteria in PM over 12 hours of 24°C storage or 7 days of 4°C storage (*p*-values unavailable as the values were

zero). As expected, the total aerobic bacteria in LPM were greater than the levels in PM at all points in time under both storage conditions. A significant increase in total aerobic bacteria ($p < 0.01$) was observed in LPM after 9 hours of 24°C storage. At 4°C, total aerobic bacteria in LPM showed a significant decrease over time ($p < 0.001$) from days 0 to 7. Changes in total aerobic bacteria in PM and LPM during room temperature and refrigerated storage are presented in Table 2. Coliforms were not detected in LPM, and total aerobic bacteria were not detected in PM throughout the storage conditions.

Total protein, lysozyme activity, and SIgA activity

At 24°C, there was a significant decrease in total protein content and SIgA activity in PM ($p = 0.02$ and $p = 0.03$, respectively) (Table 2). At 4°C, there were no significant changes observed in the total protein content, lysozyme activity, and SIgA activity in PM and LPM (Table 2). Pairwise comparisons (Table 3) indicate that levels of these proteins in PM and LPM are similar at each time point.

Table 3. Differences in Protein Content, Lysozyme Activity, and Secretory Immunoglobulin A Activity Between Pasteurized and Leftover Pasteurized Milk During Storage ($n = 13$)

		Total protein (mg/mL)		Lysozyme activity (units/mL)		SIgA activity (μg/mL)	
Effect	**Storage time at 24°C (hours)**	**Difference**	***p*-value**	**Difference**	***p*-value**	**Difference**	***p*-value**
PM minus LPM	0	0.34	0.60	1391.71	0.88	24.33	0.80
	3	−0.11	0.87	264.02	0.97	12.65	0.90
	9	−0.17	0.79	1576.63	0.86	−10.33	0.92
		Total protein (mg/mL)		**Lysozyme activity (units/mL)**		**SIgA activity (μg/mL)**	
	Storage time at 4°C (days)	**Difference**	**p-value**	**Difference**	**p-value**	**Difference**	**p-value**
PM minus LPM	0	0.34	0.60	1391.71	0.88	24.33	0.80
	1	0.51	0.43	1665.44	0.86	25.52	0.79
	3	0.17	0.80	1479.57	0.87	9.75	0.92
	5	−0.25	0.70	1536.83	0.87	18.79	0.85
	7	0.06	0.92	1144.89	0.90	40.99	0.67

LPM, leftover pasteurized human milk; PM, pasteurized human milk; SIgA, secretory immunoglobulin A.

Discussion

Storage of pasteurized human milk is unavoidable and necessary in milk banks, hospitals, and the home. Studies have analyzed the immunological activity of raw human milk during storage[26–29] or after Holder pasteurization[6,7,9–11,13]; however, none has examined the effect of storage on the immunological activity of thawed pasteurized milk.

This study is the first to examine the storage of partially consumed pasteurized human milk that has come into contact with an infant's oral microflora, and it provides analysis of total bacteria, coliform counts, and retention of bioactive proteins. Because this is the first study on the storage of LPM, we performed a preliminary study to inform the design of more comprehensive study. In the preliminary study, five mother-baby dyads participated, and it was shown that LPM could be stored at room temperature for 9 hours, and in the refrigerator longer than 6 days before total aerobic bacteria increased to an unacceptable level.

However, this “unacceptable level” of bacteria contamination has not been defined for human milk. No standards have been established to evaluate the bacteriological safety threshold for human milk provided to infants; therefore, there are two options in setting a threshold as follows: (1) One can set the maximum level of acceptance for total aerobic bacteria at 20,000 CFU/mL (4.30 log CFU/mL), as used in the Pasteurized Milk Ordinance (PMO) for Grade A pasteurized bovine milk,[30] or (2) One can set the maximum level of acceptance for total aerobic bacteria at the same level present when the infant immediately finishes consuming milk in the feeding container, which averaged 2.8 log CFU/mL in this study.

According to the data presented in this article, if one uses the PMO as the standard for setting the bacteriological threshold, pasteurized human milk that has gone through two freeze/thaw cycles may be stored accordingly: (1) PM for a minimum of 12 hours and 7 days at 24°C and 4°C, respectively, and (2) LPM for at least 9 hours and 7 days at 24°C and 4°C, respectively. PM and LPM consistently had a level of aerobic bacteria under 20,000 CFU/mL (4.30 log CFU/mL) (Table 2). If the maximum level of acceptance for total aerobic bacteria is set at the same level present immediately after partial consumption of the milk, LPM may be stored for no longer than 3 hours at 24°C, but may be stored at least 7 days at 4°C.

In the preliminary study, leftover pasteurized milk stored at room temperature exceeded 20,000 CFU/mL (4.30 log CFU/mL) at 24 hours of storage, but never exceeded this threshold when stored in the refrigerator. Results from the comprehensive study and the preliminary study indicate that total aerobic bacteria: (1) do not significantly increase during refrigerated storage for 6 to 7 days and (2) do not increase above 20,000 CFU/mL (4.30 log CFU/mL), unless stored at room temperature for 24 hours. In the preliminary study ($n = 5$), total aerobic bacteria in leftover pasteurized milk stored in the refrigerator did not significantly change during 6 days of storage. In the larger study ($n = 13$), there was a significant decrease in total aerobic bacteria stored in the refrigerator for 7 days. Results of both studies confirm that leftover pasteurized milk can be stored in the refrigerator longer than the current recommendations without an increase in total aerobic bacteria.

Pairwise comparisons (Table 3) indicate that total protein, lysozyme activity, and SIgA activity in PM and LPM are similar at each time point, which provides evidence that the concentration of protein and the activity of lysozyme and SIgA are not significantly altered when the infant partially consumes the serving of milk and it is stored under the conditions studied.

However, when PM is stored at room temperature for 12 hours, there was a significant decrease in total protein and SIgA activity ($p = 0.02$ and $p = 0.03$, respectively). When LPM is stored at room temperature, total aerobic bacteria levels exceed the initial level after consumption between 3 and 6 hours of storage. Therefore, storage of pasteurized milk at room temperature should be avoided when possible. It is not known why total protein and SIgA activity decreased during storage of PM at room temperature, but more research should be done to understand the changes in protein during storage of human milk.

Caution should be exercised when setting the bacteriological standards for PM because most of it is fed to fragile hospitalized infants. The bacteriological safety threshold for PM is likely to be

lower than the current threshold for pasteurized bovine milk (4.3 log CFU/mL) and may be different for infants in the NICU than for infants receiving milk in an outpatient setting.

Other studies have reported that bacteria levels in fresh milk remained stable or decreased at 4°C.[26,27,31] This study confirms these findings in pasteurized milk that has not been frozen, as well as in pasteurized milk that has gone through freeze/thaw cycles that mimic what would occur in a donor milk bank. Our findings validate those of others that thawed PM remains free of microbes when stored at 4°C for up to 7 days or more[15,32] and provides evidence that current milk bank storage guidelines could be extended beyond 24 hours. However, our study did not reflect actual NICU practices in the handling of pasteurized milk. The opening of a bottle of milk several times in a variety of hospital conditions (at the bedside or in a milk handling room) could impact the level of contamination and is an area for further research. In addition, research is needed to establish standards for bacteriological thresholds for the different populations that consume PM.

Conclusions

This study provides evidence that pasteurized donor human milk, including leftover milk, may be stored longer than the current recommendations, which could lead to less milk wasted and an increase in the availability of human milk for fragile infants. While the results may be generalizable because mothers were not instructed to follow a certain milk handling protocol, this study should be reproduced with a larger sample size and more diverse geographical representation.

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