

Holder-Pasteurized Human Donor Milk: How Long Can It Be Preserved?

*Marita de Waard, †Elise Mank, ‡Karin van Dijk, *Anne Schoonderwoerd,
and *†Johannes B. van Goudoever

ABSTRACT

Objective: When own mother's milk falls short, pasteurized human donor milk is recommended as alternative feeding for preterm infants. Donor milk has to meet the highest safety standards, but its processing and storage is expensive. The recommended storage time of pasteurized donor milk is 3 months. The objective of the present study was to determine whether the frozen storage time of pasteurized donor milk can be extended beyond 3 months without compromising its safety and quality.

Methods: For this prospective observational study breast milk samples of 34 unique women, collected between November 2014 and June 2015, were provided by the Dutch Human Milk Bank. Samples were Holder pasteurized within 3 months after expression and stored at -20°C . Analysis of both bacterial growth (by inoculation of milk on a blood and a cysteine-, lactose-, and electrolyte-deficient agar) and fat, crude protein, carbohydrate and energy content of milk (analyzed by infrared spectroscopy) was done monthly during the first 6 months and every 2 months thereafter, up to 1 year postpasteurization.

Results: Thirty of 306 (9.8%) follow-up samples showed bacterial growth when cultured. None of the samples showed sequential contamination with the same strain up to 8 months of frozen storage. No significant decreases in macronutrients and energy content were observed over 8 months.

Conclusion: Pasteurized human donor milk can be stored safely for 8 months at -20°C , without compromising its macronutrient and energy content. This longer storage time will reduce disposal of expired donor milk and subsequently reduce costs.

Key Words: breast milk, macronutrients, microbial, milk bank, preterm

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From the *Department of Pediatrics, VU University Medical Center, the †Department of Pediatrics, Emma Children's Hospital-Academic Medical Center, and the ‡Department of Medical Microbiology and Infection Control, VU University Medical Center, Amsterdam, The Netherlands.

Address correspondence and reprint requests to Prof Johannes B. van Goudoever, MD, PhD, Department of Pediatrics, VU University Medical Center, Amsterdam, c/o Room ZH 9D-11, PO Box 7057, 1007 MB, Amsterdam, The Netherlands (e-mail: h.vangoudoever@vumc.nl); Marita de Waard, MD, Department of Pediatrics, VU University Medical Center, Amsterdam, The Netherlands (e-mail: ma.dewaard@vumc.nl).

The study was approved by the ethical committee of VU University Medical Center (Amsterdam, The Netherlands), and written informed consent was obtained from all participants.

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What Is Known

- Human donor milk has to meet the highest safety standards, but its processing and storage is expensive.
- National Institute for Health and Care Excellence guidelines recommend to store donor milk not >6 months after expression, and to pasteurize donor milk within 3 months after expression.

What Is New

- Human donor milk can be safely stored up to 8 months after Holder pasteurization, without compromising its macronutrient and energy content.
- This longer storage time will reduce disposal of expired donor milk, reduce costs, and facilitate to keep up adequate supplies.
- Possibly, this frozen storage period can be extended even further, but future studies with a longer follow-up period are then needed.

Given the well-known benefits of own mother's milk for the health, development, and growth of all newborn infants (1,2), leading health authorities such as the European Society for Pediatric Gastroenterology, Hepatology and Nutrition, the World Health Organization, and the American Academy of Pediatrics highly recommend own mother's milk as the preferred source of postnatal nutrition (2–4). When own mother's milk is not (or not in sufficient amounts) available, pasteurized donor breast milk is recommended as first alternative for preterm born infants (4,5). Pasteurization of donor milk is imperative, to limit transmission of pathogens. Currently, the most commonly used process for pasteurization of donor milk by human milk banks is heating the milk for 30 minutes at 62.5°C , followed by fast cooling (Holder pasteurization) (6,7). Although this heating process affects the milk quality by reduction of nutritional and bioactive components and inactivation of beneficial microbiota (5,8,9), it is thought to result in the best available compromise between safety and quality (7).

Before and after pasteurization, human milk banks typically keep the donor milk frozen at -20°C to guarantee microbiological safety. This condition, and the thawing processes both before pasteurization and before dispense, may additionally affect the composition of the milk. The exact effect of frozen storage time after pasteurization on human milk composition is not clear. This results in a wide diversity in procedures for handling donor milk by milk banks, with frozen storage times varying between 3 and 12 months after expression or pasteurization (7,10–12).

Recipients of human donor milk are in general preterm infants. Therefore, donor milk has to meet the highest quality and safety standards. On the other hand, processing and storage of human donor milk is expensive (leaving aside all efforts made by donors). A longer storage time would reduce the disposal of expired donor milk (~10 L/mo in the Dutch Human Milk Bank [DHMB]) and therefore reduce costs per liter of milk, whereas simultaneously facilitating to keep up adequate supplies for all patients in need of donor milk. The National Institute for Health and Care Excellence currently recommends to store donor milk for no longer than 6 months after the date of expression, and to pasteurize donor milk within 3 months after expression (13). In the DHMB raw donor milk is stored at -20°C for a maximum of 3 months before and a maximum of 3 months after pasteurization. To determine whether the frozen storage time of pasteurized donor milk can be extended beyond 3 months without compromising its safety and quality, we studied the effect of frozen storage during 12 months after Holder pasteurization on bacterial growth and macronutrient (fat, protein, and carbohydrate) and energy content in human donor milk.

METHODS

Sample Collection

Breast milk samples were collected between November 2014 and June 2015. Samples were provided by the DHMB (located at VU University Medical Center, in Amsterdam). Written informed consent was obtained from all donors before study enrollment. Donors were screened according to international guidelines (5), and milk samples were collected by standardized procedures (14). Donors collected breast milk samples (complete expression of all milk in one breast) at home, in disposable bisphenol A–free bottles (Sterifeed, Medicare Colgate Ltd, Devon, England), by use of a breast milk pump.

Immediately afterwards, the milk was stored in their home freezer at -18°C to -20°C . Milk was transported to the DHMB by carrier in a freezer at -20°C . There, the milk was stored at the same temperature for a maximum of 3 months postexpression, up on pasteurization.

Sample Preparation

Sample preparation was incorporated into routine procedures of the DHMB regarding donor milk preparation. Within 24 hours before pasteurization, milk was thawed in a fridge at 4°C . Milk from a single donor, collected at multiple days, was pooled under laminar flow. An amount of 125 mL of pooled milk per donor was reserved for the present study and poured into a separate bottle. All milk underwent Holder pasteurization through heating at 62.0°C to 62.5°C for 30 minutes, followed by fast cooling to $<4^{\circ}\text{C}$ (Sterifeed S180 pasteurizer with datalogger, Medicare Colgate Ltd), according to our milk bank protocols. After pasteurization, the study sample of each donor was divided into 20 aliquots: 10 aliquots of ~10 mL for macronutrient analysis and 10 aliquots of ~2.5 mL for microbial analysis. Nine samples of both amounts were stored at -20°C . The remaining 2 samples were used for baseline (T_0) measurements.

Sample Analysis

Immediately after pasteurization, a T_0 sample (10 mL) of each donor was homogenized by use of an ultrasonic processor (VCX130, Sonics & Materials Inc, Newtown, CT; 98% amplitude for 7.0 seconds), and then macronutrient analysis was performed using a commercially available human milk analyzer (MIRIS, Uppsala, Sweden). This instrument provides information on fat, crude protein (total amount of nitrogen including nonprotein nitrogen compounds, multiplied by a conversion factor of 6.25), total

carbohydrate and energy content of human milk in a single reading, by mid-infrared spectroscopy. Energy content (kcal/100 mL) was calculated by use of the formula: $9.25 \times \text{fat} + 4.40 \times \text{crude protein} + 3.95 \times \text{carbohydrate}$. The analyzer was used according to the manufacturer's recommendations. It was precalibrated by the manufacturer, and a daily internal calibration was done using the solution (MIRIS check) provided by the manufacturer.

Simultaneously, a T_0 breast milk sample (2.5 mL) of each donor was used for microbial analysis. Breast milk (200 μL) was inoculated on both a blood and a cysteine-, lactose-, and electrolyte-deficient (CLED) agar and divided over the plates. Thereafter, both plates were incubated in carbon oxide at 35°C during 24 hours. In case of any bacterial growth at T_0 , the milk was excluded from the study and all remaining samples were discarded. This is in line with our normal milk bank procedures.

If the milk culture was negative at T_0 , analysis of both macronutrient content and bacterial growth was repeated monthly during the first 6 months (T_1 , T_2 , T_3 , T_4 , T_5 , and T_6) and every 2 months thereafter (T_8 , T_{10} , and T_{12}), up to 1 year postpasteurization. If a sample culture appeared to be positive at 1 of these 9 time points, bacterial isolates were selected and identified using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (Maldi-tof; Vitek MS, Biomérieux Inc, Durham, NC).

Data Analysis

Data including gestational age (GA) and sex of the infant, lactation stage at sample collection (time between the infants' date of birth and date of expression), and frozen storage time before pasteurization were collected. Variables were expressed as mean (standard deviation) or median (range), depending on their distribution, or as frequency. Because each milk sample consisted of pooled milk from a single donor collected at multiple days, average times were used for the variables "lactation stage at sample collection" and "frozen storage time before pasteurization".

For macronutrient analysis, all samples were measured 2-fold (with replacement of sample) by use of the homogenized sample mode of the human milk analyzer, and the average of the 2 results was used for further analyses. Assay reproducibility of the human milk analyzer was evaluated with use of intraclass correlation coefficients (95% confidence interval [CI]) for fat, protein, carbohydrate, and energy, by use of the 2-way random model. An average measure coefficient of ≥ 0.8 was considered strong.

Changes in macronutrient content over frozen storage time were analyzed with use of generalized estimating equations (GEEs). GEE adjusts for related breast milk samples, collected by the same donor at different times, by use of a correlation structure. We used an exchangeable correlation structure, in which 1 average within-subject correlation between samples over time is assumed. GEE results are presented as beta (95% CI). The variables "GA of the infant", "lactation stage at sample collection", and "frozen storage time before pasteurization" were investigated as potential confounders. A P value < 0.05 was considered statistically significant.

RESULTS

Breast milk samples of 41 donors were collected. At T_0 , milk of 7 donors showed bacterial growth, and their follow-up samples were therefore excluded from further analysis.

Thus, breast milk samples of a total of 34 unique women (without bacterial growth at T_0) were analyzed during 1-year postpasteurization. Mean age of donors was 34.0 (4.3) years. One donor (2.9%) gave birth to twins, and 5 women (14.7%) delivered prematurely, before 37 weeks GA. Median GA of infants was 39.6 (24.9–42.1) weeks and 14 infants (40%) were male. Median lactation

donor	Freezing time (-20 °C) after pasteurization (months)											
	1	2	3	4	5	6	8	10	12			
1												
2												
3												
4												
5												
6												
7												
8												
9												
10												
11												
12												
13												
14												
15												
16												
17												
18												

FIGURE 1. Overview of all milk samples with bacterial growth (in grey) at different freezing time points. White squares represent samples without bacterial growth at the different time points.

stage at collection was 130.5 (9.5–651.5) days and mean time between expression and pasteurization was 65.6 (10.7) days.

Average intraclass coefficients for fat, crude protein, carbohydrate, and energy content in the samples were 0.996 (0.995–0.997), 0.941 (0.926–0.953), 0.916 (0.895–0.933), and 0.993 (0.991–0.994), respectively.

Microbial Analysis

In total, 30 of 306 (9.8%) follow-up samples (T₁ to T₁₂) showed bacterial growth when cultured (Fig. 1). These samples

belonged to a total of 18 (52.9%) donors, whereas milk cultures of 16 (47.1%) donors were negative at all time points during the 12 months postpasteurization period. In 10 (29.4%) donors 1 sample culture was positive, whereas in the remaining 8 (23.5%) donors cultures were positive at 2 to 4 time points. In 2 of these donors with multiple contaminated samples, positive cultures occurred isolated as they were all followed by a negative culture at the consecutive time point. In 1 donor (donor 3), 3 positive samples (at T₂, T₃, and T₄) were followed by a negative one. The remaining 5 donors had a positive culture at T₁₂. In 2 of these donors (donor 1 and 17) sample cultures were positive at T₁₀ and at T₁₂, which was caused by an identical bacterial strain (*Staphylococcus epidermidis*) in 1 donor. This resulted in the statement that donor milk can be stored safely at –20°C for 8 months.

Isolated bacteria in the other samples included *Bacillus cereus*, *B mycoides*, *B thuringiensis*, *Corynebacterium tuberculosis*, *Neisseria elongata*, *Rothia dentocariosa*, *Rothia mucilaginoso*, *S capitis*, *S cohnii*, *S epidermidis*, *S hominis*, *S warneri*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus parasanguinis*, and *Streptococcus sanguis*.

Macronutrient Analysis

The distribution of fat, crude protein, carbohydrate, and energy content of the pasteurized milk during 1 year postpasteurization is shown in Figure 2 and Table 1. A significant increase over time was observed for protein, whereas changes of fat, carbohydrate, and energy content over 8 months' time (the longest microbiologically safe period found in the present study) were not significant (Table 1). Mean difference (95% CI) in protein content

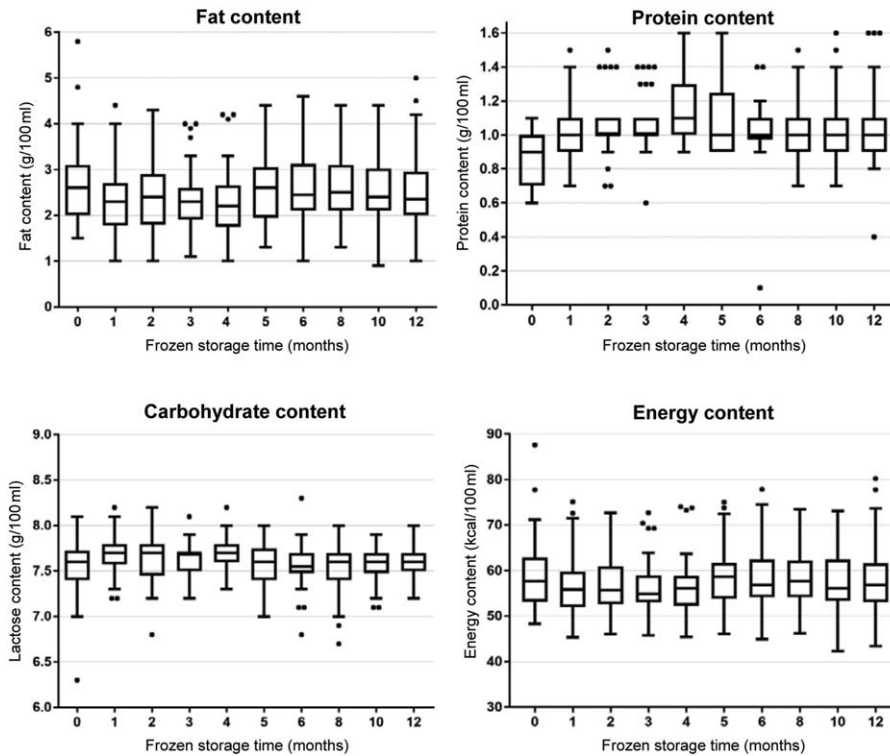


FIGURE 2. Distribution of fat, crude protein, carbohydrate, and energy content of pasteurized breast milk after 9^a frozen storage (–20°C) time points within 1 year postpasteurization. ^aThe first time-point was immediately postpasteurization. Tukey boxplots (boxes show the interquartile range [IQR] with the middle line being the median value. The end of the whiskers represents the values that correspond to the 25th percentile—1.5 × IQR and 75th percentile + 1.5 × IQR, respectively).

TABLE 1. Changes of fat, protein, carbohydrate, and energy content of pasteurized breast milk during 12 months of frozen storage (n = 34)*

Macronutrient	Frozen storage time in months (at -20°C)												β^{\dagger} (95% CI)	P
	0 (n = 30)	1 (n = 30)	2 (n = 30)	3 (n = 31)	4 (n = 32)	5 (n = 29)	6 (n = 26)	8 (n = 34)	10 (n = 34)	12 (n = 34)				
Fat (g/100 mL), mean (SD)	2.72 (0.96)	2.33 (0.83)	2.39 (0.78)	2.37 (0.78)	2.33 (0.80)	2.61 (0.84)	2.63 (0.84)	2.63 (0.76)	2.51 (0.83)	2.56 (0.93)	0.010 (-0.007-0.027)	0.257		
Protein (g/100 mL), mean (SD)	0.90 (0.16)	1.05 (0.20)	1.06 (0.19)	1.07 (0.19)	1.13 (0.21)	1.09 (0.21)	1.02 (0.23)	1.02 (0.20)	1.04 (0.22)	1.04 (0.25)	0.006 (0.000-0.013)	0.037		
Carbohydrate (g/100 mL), mean (SD)	7.54 (0.33)	7.67 (0.24)	7.62 (0.28)	7.62 (0.21)	7.67 (0.21)	7.56 (0.22)	7.54 (0.29)	7.52 (0.26)	7.53 (0.21)	7.60 (0.18)	-0.008 (-0.018-0.002)	0.125		
Energy (kcal/100 mL), mean (SD)	58.87 (8.91)	56.51 (7.45)	56.85 (6.58)	56.75 (6.74)	56.84 (6.90)	58.73 (7.53)	58.72 (7.56)	58.56 (6.58)	57.56 (7.65)	58.21 (8.92)	0.095 (-0.074-0.263)	0.270		

CI = confidence interval; SD = standard deviation.

*Macronutrient content could not be determined for 27 (8%) samples due to a break-down of the analyzing device during the study period.

[†]Change during 8 months' storage time, which was the longest microbiologically safe period found in this study; adjustment for gestational age of the infant at birth, lactation stage at sample collection, and frozen storage time before pasteurization did not significantly modify the outcomes of the generalized estimated equation (GEE) analyses.

between directly postpasteurization (T_0) and 8 months (T_8) post-pasteurization was 0.12 (0.03-0.21) g/100 mL. The corresponding relative increase was 13.4%.

DISCUSSION

In this observational study, the effects of frozen storage time (-20°C) on bacterial growth and macronutrient and energy content of human donor milk during 1 year post-pasteurization were investigated. Our results showed that storage of pasteurized donor milk for 8 months at -20°C was safe and did not result in a clinical important decrease in macronutrient or energy content.

During the year of frozen storage, 30 (9.8%) samples showed bacterial growth, even though follow-up samples were only analyzed when milk was sterile directly postpasteurization (at T_0). A vast majority of the positive cultures occurred isolated, because they were followed by a negative culture at the consecutive time point. Although possible causes for the bacterial growth in the follow-up samples could include the presence of micro-damages in the aliquots through which bacteria could enter the stored milk, the spore-forming capacity of certain bacteria, or false-negative cultures at T_0 (ie, due to the agar specificity of $<100\%$), consistent bacterial growth at all consecutive time points following a positive culture and/or less diversity in identified bacteria would then be expected. Therefore, the positive cultures at follow-up were most likely caused by contamination during handling of the milk, instead of being a consequence of the prolonged frozen storage at -20°C . Contamination could have occurred either when pouring pasteurized milk into separate aliquots (despite use of a laminar flow cabinet), or at culturing. Both steps are also applied to donor milk that is used for feeding preterm infants, although during routine procedures decanting of milk over syringes takes place at a later stage, just before administration. Nevertheless, contamination during this step could have clinical consequences for preterm infants, whereas contamination during culturing results in falsely discarding milk. The relatively high number of contaminated samples in this study is thus an important finding, and milk banks should be aware of the ease with which milk gets contaminated, even when following protocols.

The bacteria found growing in the samples were either typical skin commensals, bacteria that are part of the normal flora of the human oropharynx, or species that are ubiquitous in nature. Since milk banking protocols are already very strict, these contaminants may be inevitable. Nevertheless, wearing a surgical face mask when handling donor milk may be 1 option to try and reduce contamination, even when working under laminar flow.

From the microbial analyses, we concluded that donor milk can be stored safely at -20°C for 8 months after pasteurization. Up till that time-point, all positive cultures were (eventually) followed by a negative one. Because of the positive samples of 2 donors at 10 and 12 months postpasteurization (Fig. 1), and more importantly because of the growth of an identical bacterial strain at both time points in the milk of 1 of the 2 donors, our recommendation could not be extended beyond 8 months storage.

Macronutrient and energy content of the T_0 samples varied widely among donors, which is in accordance with previous studies (15,16). The observed differences in fat, carbohydrate, and energy content of pasteurized human milk during the 8 months storage period were trivial, variable, and not constant (Table 1). The decreases were not significant, nor clinically relevant. In contrast, García-Lara et al (15) described a significant decrease in fat, lactose, and energy content of pasteurized human milk during 6 months of frozen storage. Our findings did not change when including the results from the first 6 months only (data not shown). The disparity in results may be caused by the difference in statistical methods between the studies, because the relative decreases between 0 and 6 months were comparable (3.3%, 0.0%, and

0.3% in the present study vs 2.8%, 1.7%, and 2.2% in the study by García-Lara et al, for fat, lactose, and energy content, respectively). In addition to García-Lara et al, other previous studies have described a reduction in fat content of human milk during frozen storage, either with or without a pasteurizing step (16–18). This reduction was ascribed to the oxidation of free fatty acids during frozen storage, since oxidized fatty acids cannot be quantified by the human milk analyzer. An increase in free fatty acids would thereby be a direct result of the freezing and thawing processes that alter the fat globule membrane, and thus facilitate lipolysis by lipase. Although Holder pasteurization (partially) inactivates lipase (17), it is also thought to decrease the overall antioxidant capacity of the milk (19), resulting in oxidation of the already formed free fatty acids during frozen storage. Nevertheless, our results did not support this theory.

During the 8 months of frozen storage a significant increase in crude protein content was found, which can be subscribed to the relatively low baseline (T_0) protein content as compared to all other time points. The cause for this lower baseline measurement is unclear. Nevertheless, García-Lara et al described an increase in total nitrogen as well, although this increase was not significant and of a much less lower magnitude (15). The authors proposed suboptimal homogenization of the milk samples as a possible explanation for this increase. Especially after prolonged periods of frozen storage, a precipitation reaction tends to occur, due to destabilization of casein micelles and abnormalities in the protein structures proteins (20). Although, in our study, an ultrasound device was used to homogenize all samples, the 7 seconds per sample may not have been optimal (21).

Our study holds several strengths and limitations. The major strength is that it was incorporated into routine procedures of the DHMB, and thus represented real-life practice. Limitations included the possible suboptimal homogenization and that the study was limited to macronutrient changes whereby nothing can be said about the possible modification of specific elements of macronutrients during frozen storage, nor about changes in micronutrients. Currently, studies assessing the effects of frozen storage on micronutrient levels are scarce. There is some evidence that frozen storage of human milk reduces levels of vitamin C, but not of vitamin B (biotin, niacin, and pantothenic acid) (22,23). This evidence is, however, dated and inconsistent (7,24,25).

From our study, it can be concluded that human donor milk can be safely stored up to 8 months after Holder pasteurization, without compromising its macronutrient and energy content. It may be possible to extend the frozen storage period after pasteurization even further, but future studies with a longer follow-up period are then needed.

Milk banks should be aware of the ease with which milk gets contaminated during regular milk banking procedures, even when following protocols.

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