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Original Article

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Influence of refrigeration or freezing on human milk macronutrients and energy content in early lactation: Results from a tertiary centre survey

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Abstract

Background: Neonates with severe conditions that cannot be breastfed should receive fresh or preserved expressed human milk in addition to parenteral nutrition.
Objective: To identify the time during lactation when the macronutrients provide maximum energy and evaluate the effect of refrigeration and freezing.
Methods: We analyzed the composition of fresh milk, refrigerated at +4°C and frozen at −20°C, expressed by mothers of 60 preterm and 30 term infants from a level III maternity, in colostrum, transitional, and mature milk.
Results: In fresh milk, the protein level constantly decreases during lactation, with a significant difference after 3 weeks of lactation. Preterm milk of day 21 and day 30 had significantly lower protein than term milk (1.27 versus 1.43 g/dL, P=0.015 and 1.13 versus 1.28 g/dL, P=0.001). Refrigeration for 72 hours of term milk decreased protein content less than freezing. Preterm colostrum has significantly less protein after 48 hours of refrigeration or freezing. Preterm milk from day 60 lost carbohydrates if refrigerated 72 hours or frozen for 2 months. Lipids in preterm colostrum decrease after 8 weeks of freezing. Refrigeration for up to 72 hours did not change significantly the energy value of colostrum or transitional milk. Freezing preterm milk more than 2 weeks leads to significant loss of energy.
Conclusions: Milk frozen for more than 2 weeks contains less protein and energy than milk refrigerated for up to 72 hours. In the absence of milk bank access, in common settings, short-term refrigeration is preferable to long-term freezing.

Keywords: *Early lactation; Human milk; Macronutrients; Neonatal nutrition.*

Human milk is demonstrated to have the most appropriate composition for the growth and development of an infant. A better understanding of human milk composition and the potential impact of storage and pasteurization on milk components is important for the nutritional management of fragile, high-risk infants. For very preterm neonates, the protein content of breast milk is insufficient and requires supplementation for adequate growth support (1,2). With the progress of lactation,

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the concentration of lactose, lipids, and calories increases significantly, with the changes more pronounced in preterm than in term milk (3).

Critically-ill preterm infants are unable to breastfeed so they cannot benefit from the advantages of their mother's milk unless it is preserved by freezing to be administered later, as freezing guarantees its microbiological safety (4), but it affects milk composition (3,5,6).

Protein and lactose levels are increased after thawing, as it causes aggregation of the protein micelles, which may result in a variation of the protein content (7,8). Freezing and thawing can alter the physical and chemical properties of breast milk by generating micelles that may adhere to the recipient walls, resulting in the decrease of the lipid content (6).

Most neonatal units freeze expressed breast milk at −20°C. The maximum period for freezing at −20°C or lower recommended for both mother's own milk and donor milk (both pre- and postpasteurization) is highly variable according to international guidelines, ranging between 1 and 12 months (9–11).

Our study aims to: (a) identify the time during lactation when the macronutrients provide maximum energy for term and preterm neonates and (b) evaluate the effect of refrigeration and freezing in neonatal intensive care unit and household conditions on macronutrient and energy content of human milk in early stages of lactation.

SUBJECTS AND METHODS

Study design and setting

This prospective cohort study involved mothers with infants born between July 2015 and October 2016 in a level III maternity from the highest birth rate with a low economic level area.

Samples

The mothers were assigned to the following two predefined participant groups (Figure 1): Group 1—mothers who delivered at term (gestational age ≥37 weeks); Group 2—mothers who delivered prematurely (gestational age < 37 weeks).

Inclusion criteria were: mothers of premature or term infants whose lactation started in the first 2 to 4 days postpartum, aged 16 to 40 years. Exclusion criteria were: acute illness, treatment with drugs potentially excreted in human milk, chronic treatment for epilepsy, cancer, thyroid conditions, or arterial hypertension (systolic pressure ≥ 140 mmHg and/or diastolic pressure ≥ 90 mmHg), and localized breast infection.

The analysis was performed only on the complete sets of samples from participants who remained in the study up to day 60. Data regarding maternal age, type of delivery, parity, body mass index, and social status were recorded.

We assessed macronutrients and energy content at six different times after birth: 3 ± 1 days (colostrum) (D3), 7 ± 1 days (transitional milk) (D7), 14 ± 1 days (D14), 21 ± 1 days (D21), 30 ± 1 days (mature milk) (D30), and 60 ± 1 days (D60). Breast milk was expressed in sterile plastic bottles, between 8:00 and 11:30 a.m., using an electric breast pump (Medela Symphony Plus, McHenry, IL, USA) and gently homogenized to prevent the difference between foremilk and hindmilk. Each sample was divided into nine aliquots of 2 mL placed in plastic Eppendorf tubes: one for fresh milk (FM), three for refrigeration at 4°C, and five for freezing at −20°C. Fresh milk was analyzed the same day, within 2 hours after expression. Refrigerated milk was analyzed the next 24, 48, and 72 hours (R24, R48, R72), after rewarming to room temperature. Samples of frozen milk were thawed at room temperature 1, 2, 4, 8, and 12 weeks after sampling (F1, F2, F4, F8, F12) the same way milk is thawed for feeding infants in our neonatal intensive care unit. For each patient, 54 aliquots were analyzed.

Written informed consent was obtained from each participant. The study was approved by the University's Ethical Committee.

Measurements

For determination of energy and macronutrient content, we used the Miris AB Human Milk Analyser (Uppsala, Sweden). The sample was brought to room temperature, transferred to a sterile capillary plastic tube, shaken for homogenization, and introduced into the device where it was warmed at 40°C and analyzed using spectrophotometry.

Data analysis

Statistical analysis was performed by SPSS v.24.1 (IBM) software (Supplementary Material). Data were expressed as means (95% confidence interval [CI]). A paired-samples t test was used for comparison of means at different times. Statistical comparisons between all groups were made by parametric (t-student, analysis of variance) and nonparametric (Kruskal–Wallis or Mann–Whitney U) tests. The choice of tests used considered verification of distribution of values (Kolmogorov–Smirnov or

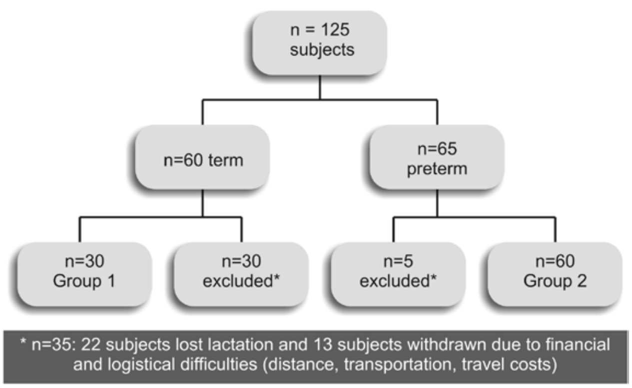

Figure 1. Study subjects distribution

Shapiro–Wilk) and of variances homogeneity (Levene's test). Statistical significance was defined as P<0.05.

RESULTS

The study groups were homogenous except for mother's age, social status, and financial status (P=0.033, P=0.009, P=0.031, respectively) (Table 1).

Proteins

In fresh milk, the protein level decreased constantly during lactation, with a significant difference between the two groups starting after 3 weeks of lactation (Table 2). Preterm milk of day 21 and day 30 has significantly lower protein than term milk (1.27 versus 1.43 g/dL, P=0.015 and 1.13 versus 1.28 g/dL, P=0.001). After day 60, protein continued to decrease, but with no significant difference between term and preterm samples (1.04 versus 1.10 g/dL, P=0.129).

Refrigeration for 72 hours of term milk (group 1) decreased protein content less than freezing, although not significantly so. Preterm colostrum (day 3) and preterm mature milk from day 60 lose protein by refrigerating more than 24 hours. Freezing more than 1 week affects protein from preterm milk significantly more than from term milk.

Table 1. Clinical and demographic characteristics (N=90)

Clinical and demographic characteristics	Group 1 GA ≥ 37 weeks n=30	Group 2 GA <37 weeks n=60	95% CI
Age			
<25 years	13 (43.3%)	13 (21.7%)	**P=0.03253***
≥25 years	17 (56.7%)	47 (78.3%)	
Delivery mode			
Vaginal	16 (53.3%)	27 (45%)	P=0.45561
C-section	14 (46.7%)	33 (55%)	
Median gestational age (weeks)	38.0 (37–39)	32.5 (30.5–33.5)	P<0.001
Newborn gender			
Male	20 (66.7%)	33 (55%)	P=0.28579
Female	10 (33.3%)	27 (45%)	
Parity			
Primipara	19 (63.3%)	37 (61.7%)	P=0.87782
Multipara	11 (36.7%)	23 (38.3%)	
BMI (body mass index)			
<25 kg/m^2	9 (30%)	26 (43.3%)	P=0.22127
>25 kg/m^2	21 (70%)	34 (56.7%)	
Residence			
Urban	17 (56.7%)	37 (61.7%)	P=0.64808
Rural	13 (43.3%)	23 (38.3%)	
Educational level			
Primary school	4 (13.3%)	10 (16.7%)	P=0.18334
High school	10 (33.3%)	30 (50%)	
Graduates	16 (53.3%)	20 (33.3%)	
Social status			
Single	3 (10%)	0 (0%)	**P=0.00911***
Married	27 (90%)	60 (100%)	
Financial level**			
Low income	14 (46.7%)	12 (20%)	**P=0.03110***
Medium income	10 (33.3%)	29 (48.3%)	
Good income	6 (20%)	19 (31.7%)	

95% CI 95% confidence interval; IQR Interquartile range.

*P-value <0.05 was considered to be statistically significant;

**Low income: < 100 €/family member/month; medium income: 100–200 €/family member/month; good income: > 200 €/family member/month.

Table 2. Mean values and confidence intervals for protein in fresh, refrigerated and frozen milk in term and preterm human milk (N=90)

Protein milk (g/dL) mean (95% CI)		FM†	R24†	R48†	R72†	F1†	F2†	F4†	F8†	F12†
Group 1 (Term)	**D3**	**2.03 (1.90–2.12)**	2.12 (1.86–3.28)	2.03 (1.86–2.21)	2.02 (1.85–2.2)	2.01 (1.8–2.22)	1.91 (1.69–2.13)	1.83 (1.61–2.05)	1.89 (1.65–2.13)	1.84 (1.65–2.13)
	P		0.431	1.000	0.921	0.843	0.279	0.077	0.201	0.094
	D7	**1.68 (1.63–1.73)**	1.71 (1.59–1.83)	1.66 (1.55–1.76)	2.02 (1.54–2.11)	1.69 (1.59–1.79)	1.7 (1.59–1.81)	1.72 (1.56–1.88)	1.63 (1.51–1.76)	1.6 (1.51–1.76)
	P		0.611	0.734	0.000	0.865	0.734	0.497	0.497	0.235
	D14	**1.39 (1.36–1.42)**	1.54 (1.39–1.70)	1.61 (1.39–1.84)	1.79 (1.16–1.86)	1.34 (1.28–1.41)	1.28 (1.22–1.34)	1.17 (1.08–1.26)	1.19 (1.16–1.22)	1.02 (1.16–1.22)
	P		0.004	0.000	0.000	0.412	0.041	0.000	0.000	0.000
	D21	**1.43 (1.37–1.50)**	1.47 (1.29–1.65)	1.39 (1.24–1.54)	1.33 (1.18–1.49)	1.41 (1.25–1.58)	1.38 (1.26–1.5)	1.26 (1.2–1.31)	1.13 (1.05–1.22)	0.91 (1.05–1.22)
	P		0.666	0.565	0.196	0.773	0.472	0.021	0.000	0.000
	D30	**1.28 (1.24–1.32)**	1.26 (1.16–1.35)	1.31 (1.22–1.4)	1.44 (1.13–1.76)	1.27 (1.18–1.36)	1.26 (1.15–1.36)	1.21 (1.13–1.29)	1.06 (0.94–1.17)	1.03 (0.94–1.17)
	P		0.742	0.622	0.014	0.869	0.742	0.299	0.001	0.000
	D60	**1.1 (1.07–1.13)**	1.1 (1.02–1.18)	1.12 (1.05–1.2)	1 (0.92–1.08)	1.07 (0.99–1.14)	1 (0.88–1.12)	1.09 (1.13–1.29)	0.89 (0.81–0.97)	0.86 (0.81–0.97
	P		1.000	0.619	0.026	0.456	0.026	0.804	0.000	0.000
Group 2 (Preterm)	**D3**	**1.97 (1.94–2.00)**	1.89 (1.82–1.96)	1.84 (1.77–1.91)	1.88 (1.81–1.95)	1.77 (1.7–1.84)	1.81 (1.73–1.88)	1.75 (1.13–1.29)	1.73 (1.66–1.8)	1.55 (1.66–1.8)
	P		0.755	0.003	0.034	0.000	0.000	0.000	0.000	0.000
	D7	**1.6 (1.58–1.63)**	1.62 (1.57–1.68)	1.61 (1.56–1.67)	1.88 (1.32–1.98)	1.58 (1.52–1.63)	1.58 (1.52–1.63)	1.55 (1.13–1.29)	1.48 (1.41–1.54)	1.44 (1.41–1.54)
	P		0.505	0.802	0.000	0.404	0.404	0.080	0.000	0.000
	D14	**1.35 (1.33–1.38)**	1.50 (1.40–1.60)	1.43 (1.35–1.5)	1.41 (1.35–1.46)	1.31 (1.24–1.37)	1.24 (1.18–1.3)	1.23 (1.13–1.29)	1.21 (1.14–1.27)	1.23 (1.14–1.27)
	P		0.000	0.038	0.000	0.209	0.003	0.000	0.000	0.000
	D21	**1.27 (1.25–1.30)**	1.24 (1.19–1.29)	1.28 (1.23–1.32)	1.3 (1.26–1.35)	1.16 (1.11–1.22)	1.14 (1.1–1.18)	1.16 (1.13–1.29)	1.14 (1.08–1.2)	1.01 (1.08–1.2)
	P		0.213	0.848	0.292	0.000	0.000	0.000	0.000	0.000

Table 2. *Continued*

Protein milk (g/dL) mean (95% CI)		FM†	R24†	R48†	R72†	F1†	F2†	F4†	F8†	F12†
D30		**1.13**	1.14	1.16	1.26	1.12	1.08	1.05	0.96	0.92
		(1.11–1.14)	(1.10–1.18)	(1.13–1.2)	(1.17–1.35)	(1.08–1.16)	(1.04–1.12)	(1.13–1.29)	(0.92–1)	(0.92–1)
	P		0.589	0.130	0.000	0.745	0.040	0.002	0.000	0.000
D60		**1.04**	1.05	1.00	0.93	1.01	0.94	0.96	0.83	0.71
		(1.03–1.06)	(1.02–1.09)	(0.96–1.04)	(0.9–0.97)	(0.98–1.05)	(0.89–0.98)	(0.91–1)	(0.79–0.87)	(0.79–0.87)
	P		0.507	0.047	0.000	0.185	0.000	0.000	0.000	0.000

FM fresh milk; R24, R48, R72: refrigerated for 24, 48, and 72 h; F1, F2, F4, F8, F12: frozen for 1, 2, 4, 8, and 12 weeks.
†Data given as mean (95% Confidence interval for means).
*P-value <0.05 was considered to be statistically significant.

In group 1, we observed that milk samples from D3 (colostrum) and D7 (transitional milk) are not significantly lower in protein after 12 weeks of being frozen. Those from D14 significantly loose protein after 2 weeks of freezing, D21 after 4 weeks, D30 and D60 (mature milk) after 8 weeks.

Carbohydrates

Carbohydrates in fresh milk increased constantly over the first 2 months of lactation (Supplementary Material). They were significantly higher in preterm than term milk, especially in D21 (7.11 versus 6.82 g/dL, P=0.014) and D30 (7.16 versus 6.91 g/dL, P=0.000) samples.

The carbohydrate content of term milk did not drop significantly regardless of storage method. Only preterm milk from D60 lost carbohydrates if refrigerated 72 hours or frozen for 2 months.

Lipids

In fresh milk from term mothers, the lipid content increased constantly during the first month, followed by a slight decrease over the next 30 days to a value similar to that on day 21 of lactation (Supplementary Material).

For preterm milk, the lipid content significantly increased during the first 2 to 3 weeks, followed by a slight decrease at the end of the first month and an increase during the second month. Also, it has a constantly higher fat content than term milk; the maximum concentration was reached on day 60 for preterm and on day 30 for term samples.

Mature fresh milk from day 30 shows significant differences in terms of lipid content between term and preterm (3.89 versus 3.36 g/dL, P=0.029).

Refrigeration of term milk for up to 72 hours does not decrease the fat content for colostrum and transitional milk. For preterm milk, however, only colostrum and transitional milk maintain an adequate lipid content after refrigeration for 72 hours. Freezing for more than 1 month for term mature milk (3.26 versus 3.68 g/dL, P=0.039) and more than 2 weeks for transitional (3.17 versus 3.48 g/dL, P=0.046) and mature preterm milk (3.34 versus 3.57 g/dL, P=0.040) generates a significant loss of lipids. Preterm colostrum may be frozen up to 8 weeks before decreasing lipid content (2.29 versus 2.56 g/dL, P=0.018).

Energy

The energy content increased steadily during lactation, but there were no statistically significant differences between term and preterm fresh milk samples (Supplementary Material).

Refrigeration for up to 72 hours did not change significantly the energy value of term colostrum or transitional milk; however, for term deliveries, mature milk maintained appropriate energy levels when refrigerated for up to 48 hours, and up to 24 hours in preterm mature milk.

Term colostrum and mature milk maintain stable energy content when frozen for 4 weeks, but significantly lose energy when frozen for 8 or 12 weeks. Freezing preterm milk more than 2 weeks leads to significant loss of energy at every moment of early lactation.

Predictive factors

Young mothers' milk had more protein (β=−0.103, P=0.008), fat (β=−0.435, P=0.028) and calories (β=−0.486, P=0.025) compared to those over 25 years of age, but lower levels of carbohydrate (β=0.315, P=0.022) (Table 3).

Mothers with better financial status had a higher level of protein (β=0.049, P=0.017), fat (β=0.175, P=0.001), and energy (β=1.370, P=0.005), but less carbohydrate (β=−0.074, P=0.038) in their milk.

Single mothers had higher levels of carbohydrate than those who were married (β=−0.363, P=0.047).

Table 3. Multiple linear regression method regarding the factors that influence the mean levels of macronutrients and energy in human milk

Dependent variable: protein Predictors	Standardized Coefficients Beta	T	P-value
Mothers' age	−0.104	−2.650	0.008
Social status	0.045	1.233	0.218
Financial status	0.087	2.393	0.017
Parity	−0.021	−0.581	0.561
BMI (body mass index)	0.163	4.379	0.000
Infants' gender	−0.107	−2.979	0.003
Multiple R=0.786, R^2=0.618, P<0.001			
Dependent variable: carbohydrates Predictors	**Standardized Coefficients Beta**	**T**	**P-value**
Mothers' age	0.009	0.225	0.022
Social status	−0.074	−1.991	0.047
Financial status	−0.077	−2.077	0.038
Parity	−0.027	−0.755	0.451
BMI	−0.060	−1.565	0.118
Infants' gender	0.008	0.217	0.828
Multiple R=0.560, R^2=0.314, P<0.001			
Dependent variable: lipids Predictors	**Standardized Coefficients Beta**	**t**	**P-value**
Mothers' age	−0.014	−0.348	0.028
Social status	0.003	0.086	0.932
Financial status	0.119	3.259	0.001
Parity	0.163	4.542	0.000
BMI	0.029	0.786	0.432
Infants' gender	−0.055	−1.524	0.128
Multiple R=0.726, R^2=0.528, P<0.001			
Dependent variable: energy Predictors	**Standardized Coefficients Beta**	**t**	**P-value**
Mothers' age	−0.312	−3.309	0.025
Social status	−0.009	−0.252	0.801
Financial status	0.102	2.797	0.005
Parity	0.184	5.129	0.000
BMI	0.054	1.430	0.153
Infants' gender	−0.054	−1.498	0.135

Multiple R=0.709, R^2=0.504, P<0.001.

The protein level in the breast milk was higher for overweight and obese mothers (β=0.140, P=0.001), confirming data from other studies (12–16). Carbohydrate, lipid, and energy levels were not influenced by the nutritional status of the mother.

Fat and energy contents were positively correlated with the number of pregnancies (β=0.360, P<0.001 and β=3.717, P=0.001, respectively), but parity had no statistically significant influence on protein and carbohydrate levels in breast milk.

The infant's gender did not influence the carbohydrate, fat, or energy content, but mothers of male infants had significantly more protein in their milk than those of female infants (β=−0.092, P=0.003).

DISCUSSION

Although some early studies considered human milk composition as relatively homogenous (17), more recently, several studies revealed large variations in macronutrient composition within the same feeding, with duration of lactation, diurnally, between different populations (18–21), and maternal factors like age, weight, parity, diet (4), but also with expression method (22), pasteurization, storage container, freezing temperature, and thawing method (4). Macronutrient composition in particular differs between preterm and term milk, with preterm milk tending to be higher in protein and fat early in lactation (23). According to other studies, protein levels decrease in human milk over the first 4 to 6 weeks or more of life, regardless of the timing of delivery (24).

We studied a period of refrigeration of 72 hours and found that macronutrient and energy levels were preserved with minimal changes. This finding agrees with the results of Slutzah et al. (25), who studied milk composition over a 96-hour period and found little change in the composition of fresh milk refrigerated for 4 days at 4°C.

In practice, human term milk can be used after being refrigerated for 3 days without significant protein loss. Term colostrum may be kept frozen for 3 months, whereas milk from D14 to D21 for only 1 month; mature milk expressed after 30 days of lactation can only be kept for only 2 months.

Preterm milk of day 21 and day 30 has a significantly lower protein level than term milk. This finding is not consistent with other data (24) and may have been influenced by specific demographic aspects, ethnic differences, nutritional intake, or nutritional status.

Our data showed a significant influence of the infant's gender on protein content in their mother's milk, as also shown by Powe (26).

Results on breast milk fatty acid composition are not consistent (27). Aydin et al. (28) found increased alpha-linolenic acid in milk of mothers who deliver prematurely. Kumbhat (29) reported no significant difference in fat concentration between preterm and term milk. Gross (30) reported no variation in fat concentration with either duration of lactation or gestational age. Paul (31) observed a significant increase in lipid concentration with the progression of lactation but no significant difference between term and preterm milk. Genczel-Boroviczeny (32) did not find any differences on days 5, 10, 20, or 30 of lactation in mothers of preterm as compared to term infants. Controversies started when, contrary to others, Luukkainen (33) reported significantly higher contributions of C20:4 and C22:6 to the fatty acid composition of breast milk in mothers of preterm than term infants. We found an increased content of lipids in fresh milk during the first month of lactation, providing greater energy intake for the infants during this period.

Population type, geographical area, and nutritional habits generate wide differences between studies (3,23,29). The method used for determinations, type of containers (plastic or glass), and thawing process contribute also. Other measurable conditions like maternal BMI, age, financial status, and parity may also influence milk macronutrient composition. Sauer (34) observed high variability in macronutrient composition of expressed human milk, even when standardized samples that were not exclusively foremilk or hindmilk were used.

The practical implications are real and of large interest in neonatal practice and home care and nursing of either term or preterm infants. We emphasize the large variability of human milk composition related to different population and storage methods. Access to milk banks is rare or even impossible for large number of centres, so our results will be of real help in deciding the best strategy.

Due to the fact that the number of participants to this study was relatively small, it was impossible to perform a multivariate analysis to assess the potential factors that could explain why the storage method has a different impact on the macronutrient content of milk from different time periods. This work represents a pilot study and a preliminary stage of a larger prospective cohort study with a more complex analysis that could identify the causality.

Limitations

The major limitation of our study is the lack of information about the nutritional intake of the mothers; it would have been useful to examine how the mothers' intake of macronutrients and energy affected the levels of these nutrients in their milk. Human milk protein content from mothers who deliver twins or triplets (both males and females) was not addressed by our study but is worthy of further evaluation.

CONCLUSIONS

Protein content in fresh milk varies between term and preterm mothers in an inconsistent manner, influenced by multiple factors and conditions, mainly the mother's BMI, age, financial status, and the infant's gender. Fresh milk is the best option if available. Refrigeration for up to 72 hours is preferable to freezing for longer than 2 weeks, as our data showed that by freezing the protein content decreased more than by refrigeration. In the absence of milk bank access, in common settings, short-term refrigeration is preferable to long-term freezing.

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Author contribution: LP and MS conceived and designed the study; LP, ALA, and GIZ collected and analyzed the samples and drafted the article. All authors analyzed and interpreted the data and finally approved the version to be submitted.

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