Cariogenic Potential of Stored Human Milk—An *In-Vitro* Study

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Human milk samples collected from ten lactating mothers in the K. S. Hegde Medical Hospital, Mangalore were divided into five different parts and stored at different temperatures for varying durations. The pH, buffer capacity and growth of Streptococcus mutans were assessed in each of these samples. There was a fall in pH of human milk stored at various temperatures. The buffer capacity of human milk increased with duration of storage. There was an increase in Streptococcus colony count in stored human milk proportional to the duration of storage and it increased more rapidly in case of milk stored at higher temperatures (0ºC - 4ºC) compared to the milk stored in the freezer (-19ºC). Milk samples stored at room temperature for 6 hours and in the freezer at -19ºC for 2 weeks were found to be relatively safe.

Key Words: *Human milk, Storage, Cariogenic potential, pH, Buffer capacity, Streptococcus mutans.*

List of Abbreviations: *HBM- Human breast milk; Sm- Streptoccus mutans*

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INTRODUCTION

Exclusive breastfeeding is the preferred method of infant feeding in the first six months of life, and continued breastfeeding throughout the first year and into the second year carries with it a multitude of advantages for both the mother and the infant.[1,2,3,4,]

The numerous advantages offered by breast milk when compared to animal milk and formulas include nutritional superiority, cost effectiveness, rapid growth and development of infant, immune protection and protection from allergies.[5]

Human milk most often goes directly from producer to consumer. However, the need to store breast milk for at least limited periods of time is unavoidable in a neonatal unit taking care of sick and preterm new-born infants. In preterm neonates, the mother's milk may not be adequately consumed due to inadequate suckling stimulation by the weak neonate.[6] In addition, some mothers may not be able to nurse their babies due to reasons pertaining to mental or physical illness, marital discord or other personal reasons. In all such cases, storage of breast milk followed by frequent feeding becomes important. Milk may be either stored in milk banks or stored at home or workplaces in refrigerators following specific storage guidelines.[7]

Nursing caries is a frustrating condition which is difficult to treat in infants and very young children. It can be painful, is infectious, and results in impairment of nutrition and esthetics with accompanying psychological problems.[8] Prolonged and excessive breastfeeding has been suspected as a causative factor in early childhood caries.[1,9,10,11,12,13,14,15,16]

Freshly expressed human milk was found to have pH in the range of 6.56 and 7.2.[6,17] It was reported that pH of human breast milk progressively falls during storage.[6] Buffer capacity of any liquid is the quantity of strong acid or base that must be added to change the pH of one liter of solution by one pH unit.[18] Buffer capacity of HBM was found to be very poor.[19]

Many bacteriological studies have concentrated on examining the changes occurring in the breast-milk stored by refrigeration.[6] Little or no research has been done on the cariogenic potential of the stored human milk. Hence, the present study was undertaken to investigate the cariogenic potential of human milk stored at various temperatures by studying the pH, buffer capacity and microbial growth.

MATERIALS AND METHODS

Consent was obtained from ten healthy lactating mothers in KSHEMA Hospital, Mangalore, who had deliveries 5 to 7 days previously. Mothers who were diagnosed with systemic diseases and mothers who were under medications were excluded from the study.

The nipples and the tissue around it were cleaned with 0.2% chlorhexidine solution using sterile cotton swab with gloved hands to prevent contamination.[20,21] It was wiped off

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with sterile water and breast milk samples were collected by gentle manual expression of breast tissue using the necessary precautions[22] and sealed immediately after collection.

Each sample was divided into five parts[7] and stored in sterile containers at room temperature for 30 min, room temperature for 6 hours, in refrigerator at 0º to 4ºC for 24 hours, in refrigerator at 0º to 4ºC for 8 days and in freezer at -19ºC for 2 weeks respectively.

The pH of the milk samples stored at various temperatures was recorded using the portable digital pH meter with glass micro electrodes.[23] Before and after each reading, the electrode was cleaned with double distilled water, and wiped clean with blotting paper, and then placed in standard pH solution of 7.0.

Buffer capacity of the milk was then measured for each sample using a method similar to that used by Bullen and Willis in 1971.[24, 25] This was done by measuring the volume of added acid, (0.01M HCl) required to lower the pH when titrating milk from the starting pH to a drop in pH by one Unit.)

A standard strain of *S mutans* maintained by the Department of microbiology, K. S. Hegde Medical College, Mangalore was cultured[26] and added to samples of milk stored at various temperatures for different time intervals. Samples were taken and spread onto *Mutans Sanguis* agar (Hi Media, Mumbai) plates and incubated at 37ºC, 5% CO_2 for 72 hours.[27,28] Colony counts were made using the Quebec counter.

Results were subjected to statistical analysis using the ANOVA (Fisher's test) and the Tukey HSD test.

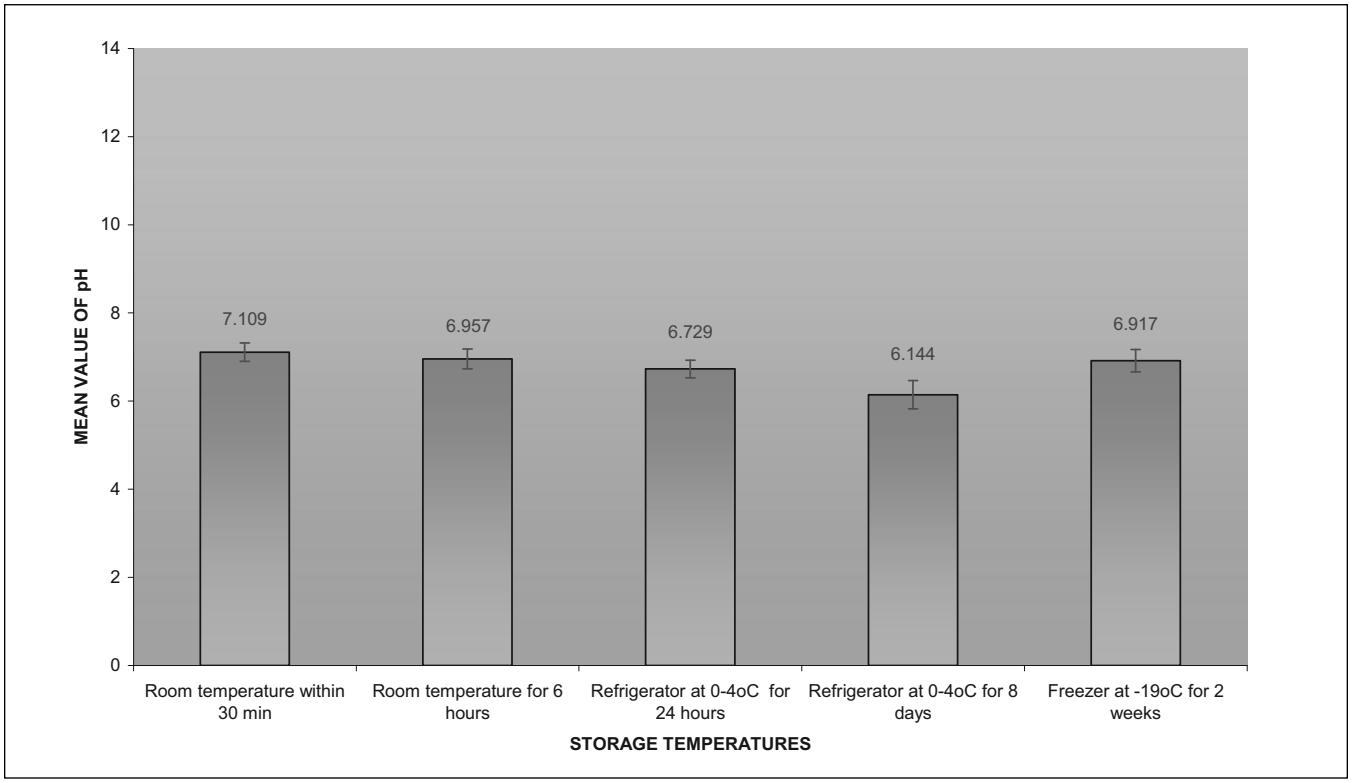
Table 1. Multiple comparisons of the pH of human milk stored at different temperatures using Tukey HSD test

(I) Group	(J) Group	Mean Difference (I-J)	P
Room temperature within 30 minutes	Room temperature for 6 hours	.1520	.642 ns*
	Refrigerator 0ºC to 4ºC for 24 hours	.3800	.01 hs ***
	Refrigerator 0ºC to 4ºC for 8 days	.9620	.001 vhs****
	Freezer -19ºC for 2 weeks	.1920	.417 ns*
Room temperature for 6 hours	Refrigerator 0ºC to 4ºC for 24 hours	.2280	.249 ns*
	Refrigerator 0ºC to 4ºC for 8 days	.8130	.001 vhs****
	Freezer -19ºC for 2 weeks	.0400	.996 ns*
Refrigerator 0ºC to 4ºC for 24 hours	Refrigerator 0ºC to 4ºC for 8 days	.5850	.001 vhs****
	Freezer -19ºC for 2 weeks	-.1880	.438 ns*
Refrigerator 0ºC to 4ºC for 8 days	Freezer -19ºC for 2 weeks	-.7730	.001 vhs****

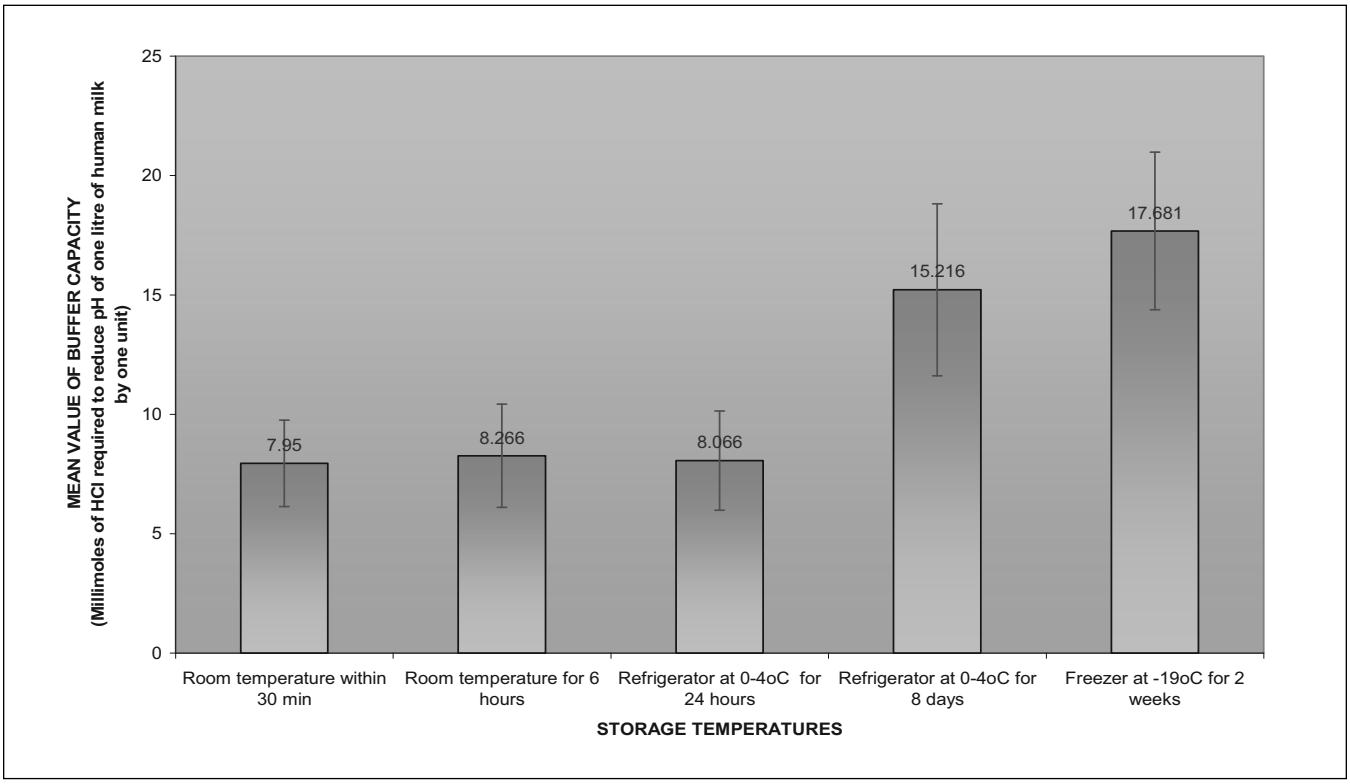
*- not significant * *- significant *** - highly significant
****-very highly significant

RESULTS

HBM samples stored at different temperatures showed an overall fall in the pH which was found to be statistically very highly significant. However, when multiple comparisons were made among the different groups, we observed that the change in the pH of milk stored at room temperature for 6 hours and milk stored in the freezer(-19º C) for two weeks was not statistically significant compared to the pH of milk measured within 30 minutes of expression. The fall in the pH of milk stored in the refrigerator at 0º C to 4º C for 24 hours



Graph 1. pH of human milk stored at different temperatures



Graph 2. Buffer capacities of human milk stored at different temperatures

and pH of milk stored in the refrigerator at 0º C to 4º C for 8 days was found to be statistically highly significant compared to the pH of milk measured within 30 minutes of expression. (Graph 1, Table 1)

The buffer capacity of HBM stored at different temperatures showed an over all increase which was found to be statistically very highly significant. However, when multiple comparisons were made among the different groups, we observed that the increase in buffer capacity of milk stored at room temperature for 6 hours and milk stored in the refrigerator at 0º C to 4º C for 24 hours was not statistically significant compared to buffer capacity of milk measured within 30 minutes of expression. The increase in buffer capacity of milk stored in the refrigerator at 0º C to 4º C for 8 days and in the freezer (-19º C) for two weeks were found to be statistically very highly significant compared to the buffer capacity of milk measured within 30 minutes of expression. (Graph 2, Table 2)

The *Streptococcus mutans* (*Sm)* colony count after incubating in HBM stored at different temperatures showed an overall increase which was found to be statistically very highly significant (Graph 3). However, when multiple comparisons were made among the different groups, we observed that (Table 3) the increase in *Sm* colony count after incubating in milk stored at room temperature for 6 hours was statistically significant compared to the colony count in HBM incubated with *Streptococcus mutans* within 30 minutes of expression. The increase in *Sm* colony count in milk stored in the refrigerator at 0º C to 4º C for 24 hours, in the refrigerator at 0º C to 4º C for 8 days at room temperature for 6 hours and in the freezer (-19ºC) for 2 weeks were statistically very highly significant compared to the colony count in HBM incubated with S mutans within 30 minutes of expression.

Table 2. Multiple comparisons of the buffer capacities of milk stored at different temperatures using Tukey HSD test

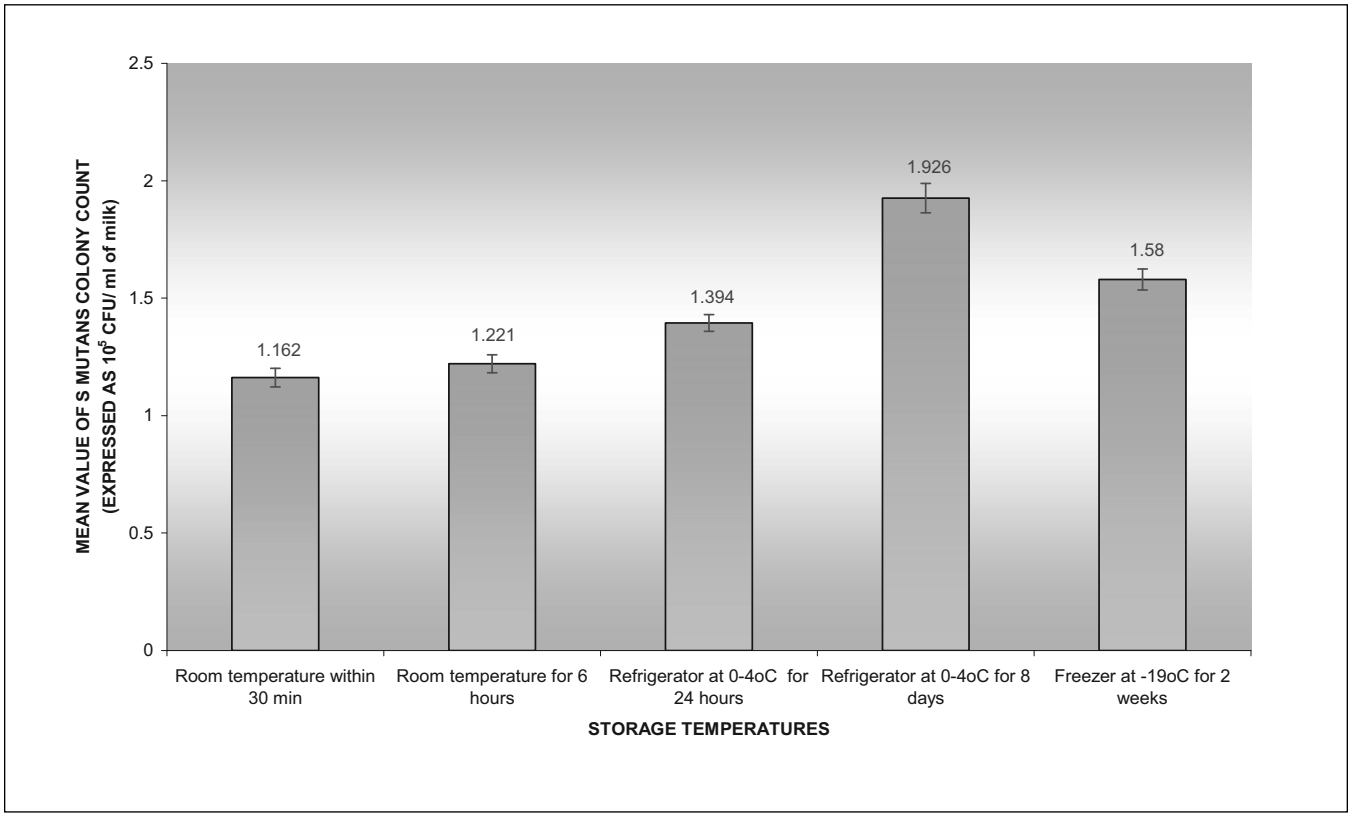
(I) Group	(J) Group	Mean Difference (I-J)	P
Room temperature within 30 minutes	Room temperature for 6 hours	-.3167	.999 ns*
	Refrigerator 0ºC to 4ºC for 24 hours	-.1167	1.000 ns*
	Refrigerator 0ºC to 4ºC for 8 days	-7.2667	.001 vhs****
	Freezer -19ºC for 2 weeks	-9.7310	.001 vhs****
Room temperature for 6 hours	Refrigerator 0ºC to 4ºC for 24 hours	.2000	1.000 ns*
	Refrigerator 0ºC to 4ºC for 8 days	-6.9500	.001 vhs****
	Freezer -19ºC for 2 weeks	-9.4144	.001 vhs****
Refrigerator 0ºC to 4ºC for 24 hours	Refrigerator 0ºC to 4ºC for 8 days	-7.1500	.001 vhs****
	Freezer -19ºC for 2 weeks	-9.6144	.001 vhs****
Refrigerator 0ºC to 4ºC for 8 days	Freezer -19ºC for 2 weeks	-2.4644	.259 ns*

*- not significant **- significant *** - highly significant
****-very highly significant

DISCUSSION

pH of stored human milk:

The average reported pH of human milk is 7.6 for first 1-4 days (colostrum), 7.44 for next 5-30 days (transitional milk)



Graph 3. *Streptococcus mutans* colony count after incubating in human milk at different temperatures

Table 3. Multiple comparisons of the *streptococcus mutans* colony count after incubating in milk stored at different temperatures using Tukey HSD test

(I) Group	(J) Group	Mean Difference (I-J)	P
Room temperature within 30 minutes	Room temperature for 6 hours	-.0590	0.042 sig**
	Refrigerator 0ºC to 4ºC for 24 hours	-.2320	.001 vhs****
	Refrigerator 0ºC to 4ºC for 8 days	-.7640	.001 vhs****
	Freezer -19ºC for 2 weeks	-.4180	.001 vhs****
Room temperature for 6 hours	Refrigerator 0ºC to 4ºC for 24 hours	-.1730	.001 vhs****
	Refrigerator 0ºC to 4ºC for 8 days	-.7050	.001 vhs****
	Freezer -19ºC for 2 weeks	-.3590	.001 vhs****
Refrigerator 0ºC to 4ºC for 24 hours	Refrigerator 0ºC to 4ºC for 8 days	-.5320	.001 vhs****
	Freezer -19ºC for 2 weeks	-.1860	.001 vhs****
Refrigerator 0ºC to 4ºC for 8 days	Freezer -19ºC for 2 weeks	.3560	.001 vhs****

*- not significant **- significant *** - highly significant
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and 7.29 after 30 days (mature milk).[6] Another study reported the average pH of human milk 10 months post delivery to be 7.4.[27]

Human breast milk has higher content of organic acids (two to three times than that in bovine milk). Phosphate and protein present in human breast milk are capable of buffering the free hydrogen ions associated with these organic acids. Thus, pH is maintained at near neutral until challenged by other acid sources.[19]

In our study, the mean pH of human milk within 30 minutes of expression ranged between 6.87 to 7.48 which was near neutral. This was more or less consistent with the pH reported by Morris FH in his study in 1986,[27] Erickson *et al* in 1999[19] and Ogundele in 2002.[6]

A fall in pH was observed on storage in all samples irrespective of the storage modality which was directly proportional to the time duration of storage. This was consistent with the results obtained by Ogundele who found that milk became progressively more acidic during storage in all the temperatures tested. However, unlike what was reported in the study, we found that the pH dropped by a bare minimum in case of storage in the freezer compartment at -19ºC.

The change in pH of human milk may be due to the lipolysis and release of free fatty acids occurring in the breast milk during storage at temperatures at or above -20ºC. The greater fall in pH with increase in duration of storage could be justified by the increased time available for lipolysis to occur.[28] This fall in pH renders the tooth exposed to stored human milk more susceptible to demineralization. [29]

We found that in three of our samples stored at 0º C -4º C for 8 days, there was a fall in the pH nearing the critical pH which may attribute more cariogenecity to this mode of milk storage.

Buffer capacity of stored human milk:

Buffer capacity can be defined as the quantity of strong acid or base that must be added to change the pH of one liter of solution by one pH unit.[27] The greater the buffer capacity,

(i.e. more acid required to drop the pH) means the solution is more resistant to alterations in pH.[18]

In our study we found that there was an increase in buffer capacity of stored human milk compared to freshly expressed human milk. There was only a minimal increase in buffer capacity of milk stored at room temperature for 4-5 hours and for milk stored in refrigerator at 0ºC to 4ºC for 24 hours compared to buffer capacity of freshly expressed milk. The buffer capacity of milk stored in the lower compartment of refrigerator at 0ºC to 4ºC for 8 days and for milk stored at – 19ºC in the freezer for 2 weeks increased markedly.

Touyz and Glassman (1981) conducted a study on citrus acids and stated that the increased acidity in frozen fruit candies occurs because freezing changes the physical state (liquid to solid phase) of residual juice and as solute concentrates(molecules being tightly packed) there is an increase in buffer capacity.[30] The strong buffering capacity of fruit juices with low pH resulted in a prolonged fall in pH in the oral environment and would require more than the usual volume of alkaline salivary buffering to raise and normalize the oral environmental pH.[30,31]

The freshly expressed milk, milk stored at room temperature for 4-5 hours, the milk stored in refrigerator for 24 hours as well as milk stored in the freezer compartment all had near neutral pH values, so even the high buffering capacity may not have any adverse effect (since milk pH is much above the critical pH).

However, the increased buffering capacity of stored human milk may result in a prolonged fall in pH since the salivary buffers especially bicarbonates are not able to raise the pH rapidly to neutrality. More of the base is required to neutralize the acidic milk which is most evident in the milk stored in the lower compartment of refrigerator (0ºC to 4ºC) for 8 days (which showed marked fall in pH along with increase in buffer capacity).

Bacterial growth and fermentation:

In our study we compared the incubation potential of stored human milk with freshly expressed breast milk with respect to *Sm*, a cariogenic bacterium. *Sm* was selected since it had been proved to be the principal bacteria responsible for dental caries.[32, 33,34,35] We found that the *Sm* colony count increased proportionate to the duration of storage. A trend towards gradual loss of inhibiting activity was noted with prolonged freezing of breast milk which was consistent with the results of the study by Hernandez.[36] It was also seen that bacterial growth increased more rapidly in case of milk stored at higher temperatures (0ºC -4ºC) compared to the milk stored in the freezer (-19ºC) consistent with the results obtained in the study by Ogundele in 2002.[6]

The loss in the antibacterial property of milk on storage might be due to the minimal loss in immunoglobulins,[37,38,39] and a marked loss in the total viable lymphocyte count[37] and complement mediated activity[6] seen after refrigeration. Ogundele in his study observed that there was loss in mainly complement-mediated bactericidal activities in refrigerated human milk but freezing could still be recommended, since up to two thirds of bactericidal activities are still preserved up to this period.[6]

Considering the three parameters tested namely – pH, buffer capacity and incubation potential for *S mutans*, we can say that stored milk may have more cariogenic potential compared to freshly expressed breast milk. Among the samples of human milk stored at the different temperatures, the milk stored at 0ºC to 4ºC in refrigerator for 8 days proved to be most cariogenic.

However, since milk is a complex fluid with innumerable components with protective factors, further studies on stored human milk like potential to dissolve enamel and changes in plaque pH need to be carried out before we can arrive at any conclusion.

The need to store breast-milk for at least limited periods of time may be unavoidable in case of working mothers who want to exclusively breast feed their infants. Storage at room temperature for 6 hours can be recommended. But, if storage for a longer duration is necessary, then the safest mode for storage keeping the health of teeth in mind would be in the freezer (-19ºC) up to 2 weeks.

CONCLUSION

1. There was a fall in pH of human milk stored at various temperatures. The milk sample stored in the refrigerator at 0ºC to 4ºC for 8 days showed marked fall in pH nearing the critical pH.
2. Buffer capacity of human milk increased with duration of storage irrespective of method of storage.
3. There was an increase in *Streptococcus mutans* colony count in human milk stored at various temperatures.

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