

# Effects of storage on the physicochemical and antibacterial properties of human milk

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## Introduction

There is a conclusive body of evidence to suggest that breast-feeding protects the infant against a wide range of infectious and other diseases, especially in developing countries.<sup>1,2</sup> In the past few years, efforts have been made to identify various immune-active substances in human milk (HM) that would account for the observed protective effects. Specific antiviral antibodies, antibacterial antibodies, non-specific IgG, IgA and IgM, lactoferrin, lysozyme, different cytokines, lymphocytes, polymorphonuclear leucocytes and macrophages have been suggested.<sup>3,4</sup> Individual components of the complement system in the human milk also have been assayed.<sup>5</sup>

The need to store human milk for a limited period of time is often unavoidable, especially in neonatal units caring for sick and preterm newborn infants, and, increasingly, in the home. For preterm neonates, who are particularly susceptible to infection and often require prolonged intensive care in hospital, milk flow may be inadequate for the first few days due to poor suckling stimulation by the weak neonate. With increased risk of bacterial contamination associated with unhygienic preparation and improper storage of artificial formula feeds, many such preterm babies may need to be fed with donor human milk stored aseptically in the hospital if they are to have a fair chance of survival. This is often associated with attendant transportation problems, to and from the milk bank.<sup>6</sup>

Furthermore, some small-for-date babies – who can be excessively hungry – might require additional milk, while others may have mothers who are unwell after delivery and unable to express human milk. Also, increasing numbers of mothers, even in the developing world, have to go out to work soon after delivery,<sup>7</sup> but still want to exclusively breast-feed their children. Such mothers often need to express and store milk for use during their absence.

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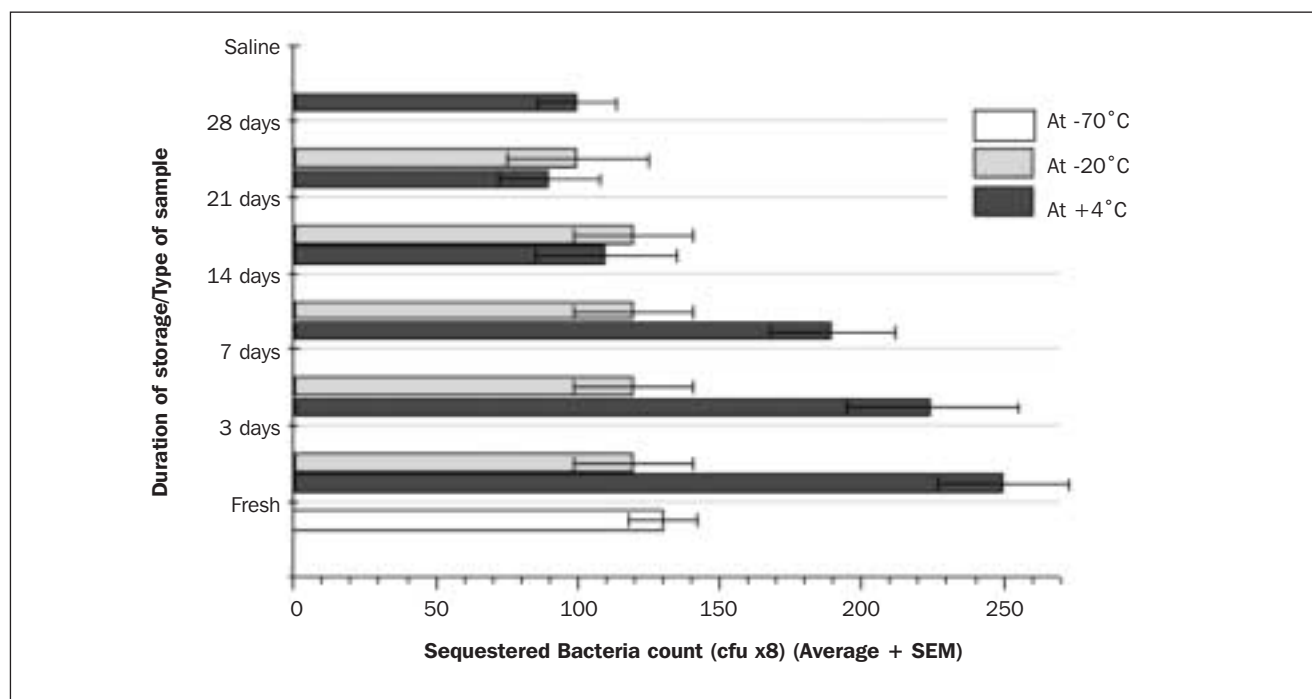
## ABSTRACT

Storage of human milk for limited periods of time is unavoidable in neonatal units and also in the home where increasing numbers of mothers go back to work soon after delivery. Many mothers, convinced of the importance of prolonged exclusive or complementary breast-feeding, often express and store human milk for use during the period of separation. This study examines the effects of different storage methods on the pH and some antibacterial activities of human milk. Portions of milk and colostrum samples from healthy lactating mothers were stored at 4°C and -20°C for periods ranging from one day to 12 weeks. The stored milk samples were analysed for pH, bactericidal and bacteria sequestration activities against a serum-sensitive *Escherichia coli*, and compared with freshly collected samples, with and without EDTA. Milk became progressively more acidic during storage. While the bactericidal activities of refrigerated samples diminished rapidly, up to two-thirds of the original activity level was maintained by freezing for up to three months. The ability of milk fat globule membrane to adhere to suspended bacteria was gradually lost in frozen milk samples, while it was greatly enhanced during the first few days in refrigerated samples, before declining sharply. This study shows that loss of bactericidal activity in refrigerated milk is well compensated for by enhanced bacteria sequestration activity, and allays any fears that might arise concerning the suitability of stored human milk for infant consumption.

KEYWORDS: Complement. Infection.  
Milk banks. Milk, human.

The greatest fear that hinders the prospects of *in vitro* storage of human milk for any considerable period of time is the possibility of bacterial contamination and growth of infectious pathogens in the stored milk, thereby rendering them unsafe for human consumption. There is also fear that a fall in milk pH during storage might indicate excessive bacteria growth and deterioration of its protective activities.

Most contamination of stored milk is said to occur at collection, irrespective of the method used.<sup>8,9</sup> Freshly collected human milk is rarely sterile and normally contains bacteria originating from the maternal skin and nipple duct microflora. It may also contain potential pathogens, but these seem to produce no ill effects in the suckling infant.<sup>10-14</sup> Pooled human milk is also known to contain potentially pathogenic organisms, although the ability of these organisms to cause infection is uncertain and probably minimal.<sup>11</sup>

**Fig. 1.** Effects of storage temperature on the sequestration of bacteria by human colostrum samples ( $n=5$ ).**Table 1.** Effect of added PBS and EDTA on the pH of different human milk samples<sup>a</sup>.

	Fresh whole milk	Whole milk + PBS	After centrifugation	Whole milk + EDTA <sup>b</sup>	After centrifugation
Colostrum ( $n=5$ )	7.60 ± 0.20	7.63 ± 0.24	7.70 ± 0.29	6.80 ± 0.16 <sup>c</sup>	7.00 ± 0.33
Transitional milk ( $n=4$ )	7.44 ± 0.25	7.46 ± 0.23	7.66 ± 0.23	6.93 ± 0.12 <sup>d</sup>	7.12 ± 0.31
Mature milk ( $n=4$ )	7.29 ± 0.20	7.30 ± 0.27	7.40 ± 0.25	6.97 ± 0.18 <sup>e</sup>	7.05 ± 0.21

<sup>a</sup>Results expressed as average ± SEM<sup>b</sup>Difference between mean value for all categories of milk statistically significant in comparison to fresh milk samples ( $t = 3.15$ ;  $df 12$ ;  $0.001 < P < 0.01$ )<sup>c</sup> $t = 4.187$ ;  $df 4$ ;  $0.01 < P < 0.02$ <sup>d</sup> $t = 2.853$ ;  $df 3$ ;  $0.05 < P < 0.1$ <sup>e</sup> $t = 2.189$ ;  $df 3$ ;  $0.1 < P < 0.5$ **Table 2.** Preliminary assays comparing the bactericidal activities of human breast milk against two different strains of *E. coli*.

	<i>E. coli</i> 0111	<i>E. coli</i> 0125	Significance
Colostrum ( $n=5$ )	89 ± 1.57	80 ± 1.25	$P < 0.001$
Transitional milk ( $n=4$ )	24 ± 0.9	20 ± 1.5	$P < 0.05$
Mature milk ( $n=4$ )	19 ± 0.8	12 ± 1.2	$P < 0.001$

Results expressed as % bacteriolysis ± SEM of selected whole milk samples

Many bacteriological studies in both tropical and temperate countries have shown that unheated human milk stored for up to eight hours at room temperature, 24–72 hours in a refrigerator or up to five days frozen at  $-20^{\circ}\text{C}$  is safe for human consumption.<sup>10–14</sup> Furthermore, most studies show a significant drop in bacterial colony count during the storage period in the majority of stored human milk samples. This is in sharp contrast to cow's milk, which shows significant increase in bacterial counts after just six hours of storage at

both room temperature and in the refrigerator.<sup>9</sup> It is claimed that freezing does not denature milk proteins but it does kill the cells present in the milk.<sup>11</sup>

This study aims to correlate pH changes in human milk with its antibacterial functions, particularly the bactericidal activities and adhesion of milk fat globule membrane (MFGM) to suspended bacteria, under different storage conditions.

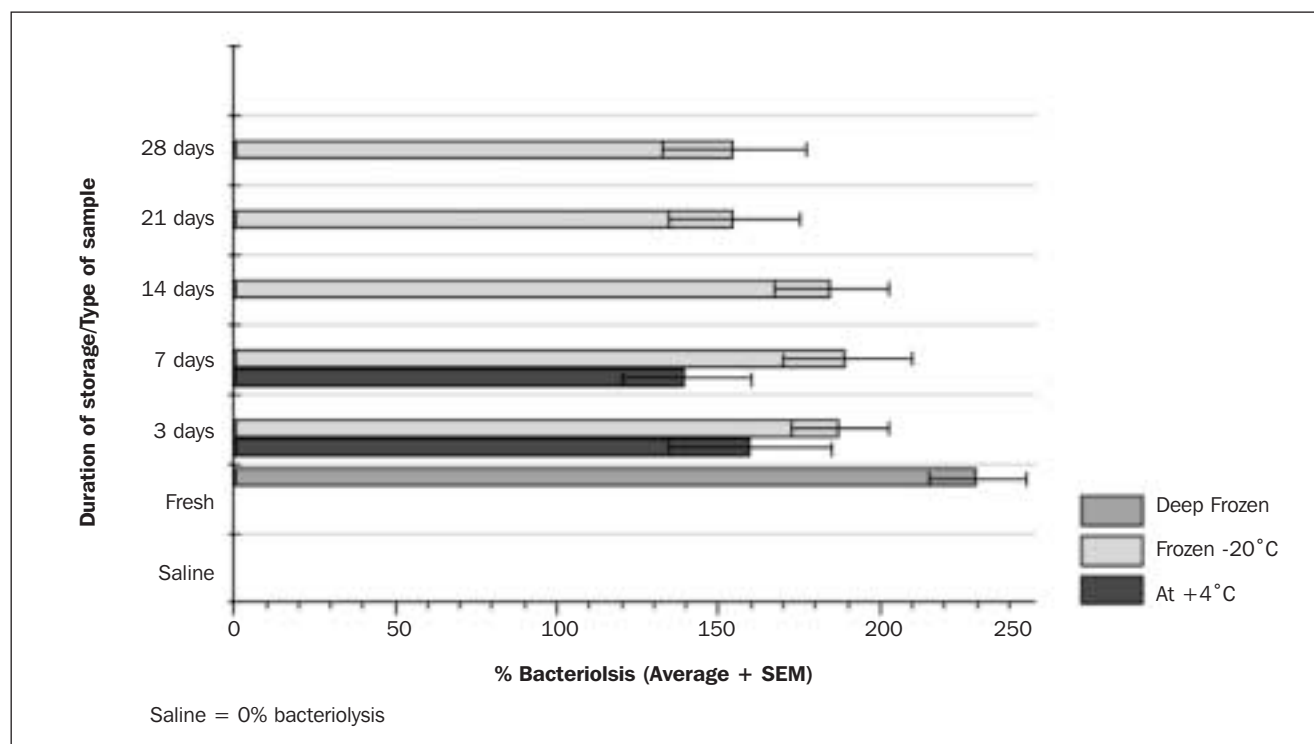
## Materials and methods

### Collection and storage

Human milk samples were donated by 13 healthy lactating mothers (who gave informed consent) with no clinical evidence of infection or inflammation by manual expression or a breast pump at various post-partum (PP) periods of lactation. Samples were placed in sterile plastic tubes and transported on ice to the laboratory and either processed immediately or stored in small aliquots at  $-70^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$  until use.

For each donation, one sample was collected in 20 mmol/L EDTA solution and the other was collected in an equivalent volume of phosphate-buffered saline (PBS) only. Samples were classified according to post-partum period of lactation as follows:<sup>4</sup> colostrum (1–4 days PP,  $n=5$ ), transitional milk (5–30 days PP,  $n=4$ ) and mature milk ( $>30$  days PP,  $n=4$ ).

Fig. 2. Effects of different storage temperatures on the bactericidal activities of human colostrum samples ( $n=5$ ).



Samples of the human milk were stored at 4°C and -20°C for different times, following which they were collected and either processed immediately or stored at -70°C or -80°C in small aliquots until processed.

#### Separation of fat from milk samples

De-fatted (skimmed) milk samples were obtained by dividing whole milk samples into smaller portions and centrifuging them consecutively at 500 xg and 3000 xg at 4°C for 15 min, aspirating the aqueous phase of the milk each time. During centrifugation, the milk separates into three layers: a cell pellet, a middle aqueous layer and an upper fat layer. Aqueous layers were collected each time and processed immediately or stored as aliquots at -70°C or -80°C. The rate of centrifugation was selected from those used in pilot trials to reduce the level of milk fat content to less than 25% of the original, while minimising sedimentation and loss of milk proteins.

#### Measurement of pH values

pH values were taken using a glass electrode with combined sensing and reference elements, connected to a research pH meter (Radiometer PHM84; Radiometer, Copenhagen, Denmark) that was capable of measuring temperature-dependent pH values to three decimal places. The instrument was calibrated daily, both before and after sample measurements, by measuring its response to buffers of known pH. Results were expressed as average values and standard error of the mean ( $\pm$  SEM) for each group of milk samples.

#### Complement inactivation

Samples of de-fatted (skimmed) and whole human milk were heated in a water bath at 56°C for 30 min to inactivate the complement system.

#### Bactericidal assay – viability test

A serum-sensitive strain of *Escherichia coli* NCTC 8007, serotype 0111 K58 (B4) H2 was used, in comparison with another *E. coli* strain (0125).<sup>15</sup> Bacteria were cultured overnight on blood agar, suspended in sterile normal saline and adjusted to a turbidity equal to that of a McFarland<sup>16</sup> No. 1 standard (approximately  $3 \times 10^8$  colony-forming units [cfu] per mL). Samples (20  $\mu$ L) of the adjusted bacteria were added to round-bottom microtitre wells (Nunc A/S, Roskilde, Denmark) with 80  $\mu$ L of the milk sample to be tested. The trays were thoroughly mixed on an orbital mixer before and after incubation at 37°C for 2 hr.

Samples (20  $\mu$ L) were taken from each well before and after incubation to assess the viable count by a poured-plate method.<sup>17</sup> Degree of bacteriolysis was calculated as the difference between bacterial counts before and after the incubation period, expressed as a percentage of the initial count.

Whole milk and skimmed milk samples, stored at -70°C or -80°C, were also examined. Each sample was tested in duplicate and the results were expressed as the mean of average values obtained for each sample  $\pm$  SEM.

#### Bacterial sequestration assay

Bacterial sequestration assay was used as a surrogate estimate of the ability of MFGM to adhere to suspended bacteria. Only whole-milk samples and the serum-sensitive *E. coli* (serotype 0111) were used for this assay. Bacteria were cultured overnight on blood agar and 20  $\mu$ L of the bacteria suspended in sterile normal saline adjusted to  $3 \times 10^8$  cfu per mL was added to 80  $\mu$ L of the milk or colostrum sample to be tested, in round-bottom microtitre wells (Nunc A/S, Denmark). The trays were thoroughly mixed on an orbital mixer for 5 min. Samples (20  $\mu$ L) were then taken from each well for bacterial count to assess the number trapped or

**Table 3.** Effect of fat clearance on the bacterial sequestration activities of human milk

	Whole milk	Skimmed milk	Significance	Difference between whole milk and saline	Significance
Colostrum ( <i>n</i> =5)	130 ± 12.3	110 ± 13.5	0.01 < <i>P</i> < 0.05	30 ± 5.7	<i>P</i> < 0.001
Transitional milk ( <i>n</i> =4)	140 ± 15.2	95 ± 12.9	<i>P</i> < 0.001	40 ± 6.8	<i>P</i> < 0.001
Mature milk ( <i>n</i> =4)	130 ± 16.4	100 ± 15.6	0.001 < <i>P</i> < 0.01	30 ± 8.5	0.01 > <i>P</i> > 0.001

Results expressed as average sequestered bacterial count (cfu x 8) ± SEM

sequestered from suspension by MFGM in whole milk and colostrum, using a poured-plate method.<sup>17</sup>

A short incubation period was used to preclude the initiation of complement-mediated bacteriolysis or other enzyme-mediated and time-dependent effects on the suspended bacteria. Any significant difference between the levels of bacterial count in skimmed milk (or colostrum) samples was compared with the level in whole milk samples, and confirmed the assumption that the higher bacterial count in whole milk samples was an estimate of bacteria adhering to MFGM.

The bacterial count in skimmed milk (or colostrum) samples was compared to the level in whole milk samples, to estimate the level of bacteria adhering to MFGM. Bacterial count in saline suspension was used as a further control of the assay. Each sample was tested in duplicate and the results were expressed as mean of average values obtained for each sample and the SEM for each group of milk samples.

#### Statistical analysis

Mean pH values were compared between fresh milk samples and milk samples with added PBS and EDTA, with and without centrifugation, using matched-pairs Student's *t*-test analysis (Table 1). A similar test was used to compare the mean bactericidal activities of different milk types against two strains of *E. coli* (Table 2) and to compare the levels of suspended bacteria between skimmed and whole milk samples, while independent groups *t*-test was used to compare the levels of suspended bacteria between whole milk samples and the saline control (Table 3).

Pearson correlation tests were used for serial pH values of various stored milk samples against the duration of storage (Table 4a/b), using Epi Info 2000 Statistical Software Release 1.1.2 (CDC, Atlanta, USA). Correlation coefficients were expressed as averages for all milk samples in each group at both 4°C and -20°C. *P* < 0.05 was considered statistically significant.

## Results

#### Physicochemical changes in breast milk during storage

EDTA was used to prevent ongoing activation of the complement system and lipolysis in the human milk samples. Addition of EDTA to milk led to an immediate significant fall in pH (0.001 < *P* < 0.046), compared to when a comparable volume of PBS buffer was added. Although EDTA is a weakly acidic compound, the drop in pH was in excess of what would have been expected of it, and the drop was partly reversed by defatting the human milk by centrifugation (Table 1).

The normal pH of the colostrum averaged 7.60, while the transitional and mature milk samples were slightly more acidic, averaging 7.44 and 7.29, respectively. Defatting fresh milk led to a slight increase in the pH. PBS caused no significant change in the pH of the human milk samples (Table 1). The pH of the milk progressively fell during storage at both 4°C and -20°C. The progressive fall in pH was significant for all the milk types as evidenced by relatively high average correlation coefficients greater than 0.94 at both temperatures (Table 4a/b).

#### Effect of defatting on bacterial sequestration

Sequestration of suspended bacteria, as measured by bacterial count in whole milk suspensions, was consistently higher than corresponding levels in defatted milk and colostrum. Human milk defatted by centrifugation contained a reduced bacterial count recovered from suspension to the levels comparable to that of the saline control (Table 1).

The difference in suspended bacterial level between skimmed and whole milk samples was statistically significant in mature milk, transitional milk and colostrum. This difference reflected the ability of MFGM to adhere to suspended bacteria in milk by trapping or sequestering them.<sup>18</sup> Further assays to determine the effects of different storage temperatures were carried out using the whole milk samples.

#### Increased sequestration of suspended bacteria by refrigerated human milk

It was observed that the refrigerated colostrum samples sequestered significantly more viable bacteria from suspension within the first three to seven days of storage, compared with their corresponding fresh milk samples. This was followed by a progressive fall, gradually approaching the level cultured from the normal saline control after three weeks (Figure 1). Similar observations were made for the transitional and mature whole milk samples.

Human milk and colostrum frozen at -20°C and stored for four weeks demonstrated levels of bacterial sequestration which was less than those of the freshly collected samples but higher than that of the saline control (Figure 1).

#### Effect of storage on bactericidal activity

Bactericidal activity of the colostrum against the serum-sensitive *E. coli* decreased during storage at both 4°C and -20°C. Loss of activity was, however, more rapid in the refrigerated samples than in the frozen samples (Figure 2). While the bactericidal activity in most refrigerated whole mature or transitional milk was lost rapidly within 48 hours of storage (results not shown), activity in colostrum fell more slowly, with at least half still measurable up to seven days.

**Table 4.** pH changes during storage of different human milk samples at (A) 4°C and (B) -20°C <sup>a</sup>.

(A)						
	Duration of storage (days)					
	0	3	7	14	21	28
Colostrum* (n=5)	7.60 ± 0.20	7.35 ± 0.18	7.05 ± 0.16	6.80 ± 0.23	6.55 ± 0.27	6.40 ± 0.22
Transitional milk** (n=4)	7.44 ± 0.25	7.10 ± 0.16	6.95 ± 0.13	6.75 ± 0.21	6.50 ± 0.18	6.35 ± 0.26
Mature milk *** (n=4)	7.29 ± 0.20	7.25 ± 0.18	6.95 ± 0.12	6.61 ± 0.10	6.28 ± 0.21	6.37 ± 0.16

<sup>a</sup> Results expressed as average ± SEM

\* Average value of correlation coefficients = -0.984 ± 0.006

\*\* Average value of correlation coefficients = -0.974 ± 0.011

\*\*\* Average value of correlation coefficients = -0.945 ± 0.015

(B)				
	Duration of storage (weeks)			
	0	4	8	12
*Colostrum (n=5)	7.60 ± 0.20	7.30 ± 0.11	7.01 ± 0.18	6.53 ± 0.16
**Transitional milk (n=4)	7.44 ± 0.25	7.42 ± 0.16	6.82 ± 0.15	6.60 ± 0.08
***Mature milk (n=4)	7.29 ± 0.20	7.12 ± 0.23	7.00 ± 0.22	6.57 ± 0.10

<sup>a</sup> Results expressed as average ± SEM

\* Average value of correlation coefficients = -0.987 ± 0.007

\*\* Average value of correlation coefficients = -0.955 ± 0.014

\*\*\* Average value of correlation coefficients = -0.947 ± 0.011

Most activity was abolished by heating at 56°C for 30 minutes, or by addition of EDTA. Centrifugation of the milk samples also led to some loss of bactericidal activity, suggesting the involvement of MFGM in the activation of the milk complement (Figure 3).

Similar but less distinctive patterns of changes were observed when the *E. coli* 0125 strain was used (Table 3).

## Discussion

Storage of human milk, at least for a limited period, is hard to avoid because of social circumstances and also the particular needs of some preterm babies. The need to keep it safely for the infant's consumption gains in importance, both in industrialised countries and in urbanised developing communities, and storage by freezing or refrigeration, with and without heating, has been recommended.<sup>11</sup>

Lipid content of human milk consists mainly of triglycerides enveloped in complete tri-laminar biological cell membranes – the MFGM – ranging from 4 µm to 20 µm in diameter. This is derived from the apical region of the mammary gland epithelial cell and formed around the milk lipids as they are being secreted.<sup>19</sup> Electron microscopic studies of the outer layer of human MFGM shows the presence of numerous thin filaments, many of which appear branched, oriented radially around the membrane into the aqueous phase of milk. They are said to be absent from the MFGM of other mammals;<sup>20</sup> however, their functions and physiological significance have not been clearly identified.

Significant changes occurring in the human milk during storage at temperatures at or above -20°C include enzymatic lipolysis with release of free fatty acids (FFA). The presence

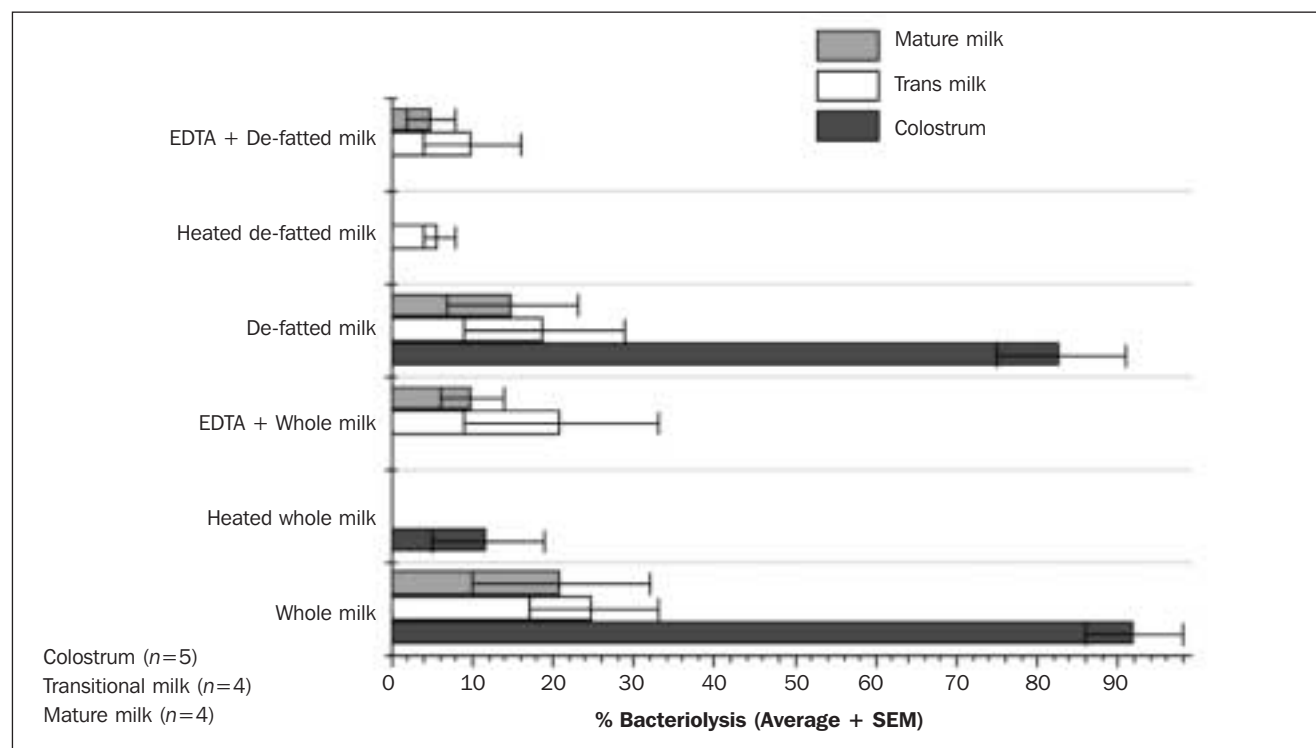
of FFA in stored milk is known to result in a reduction in pH and changes in the solubility of divalent ions.<sup>21</sup> This would explain the observed changes in pH during milk storage.

It is proposed that EDTA, a detergent, destroys MFGM and allows the triglyceride substrate to be released, thereby coming in contact with the lipoprotein lipase enzymes in the aqueous phase of milk. Subsequent release of FFA would account for the immediate and significant fall in pH of milk samples containing EDTA.

Interestingly, most bacteriological studies have demonstrated a decrease in the bacterial counts of human milk during storage both at 4°C and -20°C. This suggests that some antimicrobial processes are activated during this period. In addition to the well-documented lipolysis-induced cytolytic effects,<sup>22</sup> there is the possibility that other complement-dependent mechanisms are involved.<sup>23</sup> The involvement of the complement system in the bactericidal activity of human milk and colostrum was demonstrated by its abolition through heat inactivation or addition of EDTA. Activation of the alternative complement pathway is said to take place preferentially at 4°C and might suggest that this is involved in the bactericidal activity observed in stored human milk.<sup>24</sup>

The bacteria-adhesive role of high molecular weight mucus components of MFGM have been implicated in the prevention of colonisation and infection of epithelial mucosae by certain meningitis-causing bacterial strains in neonates.<sup>18</sup> The present study provides evidence that this adhesive property is enhanced during storage, by mechanisms that probable involve activated complement factors (particularly C3b). Refrigerated milk and colostrum samples trapped more bacteria from suspension than did corresponding fresh or frozen samples.



**Fig. 3.** Effect of centrifugation, heating and addition of EDTA on the bactericidal activities of human milk.

The enhanced ability of refrigerated whole milk, as opposed to that previously frozen at  $-20^{\circ}\text{C}$  or  $-70^{\circ}\text{C}$ , to adhere to suspended bacteria in the absence of EDTA, suggests the involvement of *in vitro* conditions that favoured complement activation. EDTA is a potent ion chelator that inhibits the activation of certain enzymatic processes *in vitro*, such as the complement system and lipolysis.<sup>25,26</sup>

Another possible explanation for this observation might be the effect of FFA released by ongoing lipolysis;<sup>21</sup> however, this seems unlikely since levels that should be higher in milk samples frozen at  $-20^{\circ}\text{C}$  than at  $-70^{\circ}\text{C}$  did not result in a corresponding enhancement of sequestration at this temperature. Failure of enhanced sequestration at  $-20^{\circ}\text{C}$  might also suggest that the freeze-thawing procedures lead to the destruction of MFGM, so that they are unable to bind to the bacteria in the milk samples. Irrespective of the presence of activated complement fragments, subsequent reduction in the sequestering ability of refrigerated milk samples might reflect the loss of intact MFGM due to ongoing *in vitro* lipolysis and the need for an intact MFGM to effect bacterial sequestration by milk samples.

Previous study confirmed the accumulation of complement split products, resulting from ongoing complement activation, in biological samples stored at temperatures below  $-70^{\circ}\text{C}$ .<sup>25</sup> Accumulation of these products in refrigerated or frozen human milk in the absence of EDTA has been documented recently, using established ELISA techniques. Rapid decline in bacteriolysis during the period of refrigeration (within the first 48 h in mature milk and within one week in colostrums) further attest to the ongoing loss of native complement components through non-specific activation (Figure 2).

As similar bactericidal FFA are released after the digestion of cow's milk-based formula feeds, the recognised differences between the occurrences of gastrointestinal

infections in breast-fed and formula-fed infants tends to throw doubt on the possible physiological significance of the antimicrobial effect of these lipids.<sup>22</sup> On the other hand, a deficiency of local complement components has been associated with an increased risk of developing mastitis in lactating mothers.<sup>27</sup> Skin sepsis in the suckling infant, as well as inflammation of the lactating breast (mastitis), has been associated with an increased level of complement secretion in the human milk.<sup>28</sup> Although there is no proof of a cause and effect relationship, these facts point to the possible physiological role of the complement system on the mucosal surface.

It has been suggested that the bactericidal effects of bovine colostrum and specific antibodies account for the favourable influence of breast-feeding on intestinal gut colonisation.<sup>17</sup> It is also possible that the physiological significance of the human milk complement system might contribute to the generally observed trend towards a reduced total faecal bacterial load in the breast-fed infant, particularly within the first week of life.<sup>29</sup> This would be another strong argument against the practice of heating stored human milk used for feeding nursing infants and neonates,<sup>11</sup> in order to preserve its immunologically active and heat-labile components and milk cells.

The potential ability of rapid freeze-thawing to disrupt MFGM<sup>30</sup> suggests that refrigeration is preferable for short-term storage. For periods of up to a month, however, freezing can still be recommended as two-thirds of the original bactericidal activity remained (Figure 2).

Fears arising from the increasing acidity of stored milk samples, which was thought to be due to formation of lactic acid by bacterial fermentation of milk sugars, proved to be unfounded, as this is mainly attributable to levels of FFA.<sup>31</sup> It can also be concluded that a fall in milk pH is not necessarily associated with either excessive contamination or

deterioration of its protective abilities.

It was interesting to observe that the loss in complement-mediated bactericidal activity in refrigerated milk is compensated by enhanced bacteria sequestration and trapping ability, suggesting that as inflammatory processes diminish, other protective mechanisms acquire greater importance. Several authors have proposed that the observed anti-inflammatory properties of human milk are attributable to some of its inherent basic components.<sup>32</sup> Although the exact mechanisms involved in the sequestration of bacteria by milk are not fully known, they may involve some unidentified non-anti-inflammatory protective mechanisms.

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