Bactericidal activity of human milk: stability during storage

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ABSTRACT

Human milk provides infants with defensive factors against many illnesses. This study aims to analyse global bactericidal activity in fresh human milk and evaluate its stability in relation to milk manipulation and its possible alteration following refrigeration. Nineteen milk samples (mature milk) from 19 healthy women are analysed. Viability testing involving a strain of *Escherichia coli* NCTC 9111, serovar O111:K58(B4):H– was used to determine the bactericidal effect of human milk. Degree of bacteriolysis is calculated as the difference between *E. coli* counts in controls and in milk samples, expressed as a percentage of the control sample counts. An evaluation of the effect of refrigeration at 4–6°C after 24, 48 and 72 hours, and at –20°C for seven days on bactericidal capacity is made. Bactericidal activity was detected in all milk samples analysed (77.33±15.14%). This activity persisted after refrigeration for 48 hours and after freezing for 10 days, but showed a significant decrease after refrigeration for 72 hours. In conclusion, maternal milk has bactericidal capacity, providing defence and protection against infection for newborn infants. This property can be altered during the storage of milk. Consequently, if storage in excess of 48 hours is required, freezing is preferable to refrigeration.

KEY WORDS: Bactericidal activity.
Biological specimen banks.
Milk, human.

Introduction

In addition to nutrients, maternal milk provides infants with a large number of biocomponents with as yet undefined functions (e.g., cells, oligosaccharides, proteins, enzymes, immunoglobulins, growth factors, hormones and nucleotides) that specifically or non-specifically[1] protect the infant against digestive and respiratory infections and afford defensive action against many illnesses.[2–19] Some of these agents have been isolated and identified, although their true number, potency and importance are probably underestimated.

The beneficial effects of these biocomponents are based on their antioxidant and anti-inflammatory action, their immune response-favouring properties, and their bactericidal or bacteriostatic action on different microorganisms. Goldman and Goldblum[11] classified these actions as follows: a) agents with direct antimicrobial action; b) promoters of the growth of protective microorganisms (probiotic and prebiotic effects); c) leucocytes; d) anti-inflammatory agents; and e) immunostimulants.

The components in maternal milk that possess bactericidal capacity are numerous and have not all been identified to date,[8] but they comprise all those agents that exert direct action as antimicrobials. Identification of the components involved and their mechanism of action are clearly of interest, and has been the subject of many studies. The components that have been identified to date include lactoferrin (antimicrobial activity),[1,7,8] lysozyme (bacteriostatic action),[6,8] oligosaccharides,[7,10] lipid digestion products,[1] neuraminic acid, gangliosides and complement components (C3 and C4).[6]

This protective capacity of maternal milk is particularly beneficial to low weight and/or preterm infants, and in other pathological situations affecting the newborn infant or child (e.g., infectious diseases, certain immunological deficiencies, recurrent diarrhoea, etc.). Under these circumstances, the infant may be unable to ingest milk directly and previously collected and stored milk must therefore be used.[9] The collection and storage of milk prior to ingestion is increasingly common in both the hospital setting and in the home, where mothers resort to this method when the return to work or other reasons do not allow direct administration.

Conditions selected for milk storage affect its contents, and many studies have investigated milk stability[21,23–27,28] and the consequences of storage on microbiological quality.[20,22,23,27,28] In order to preserve the microbiological quality of milk during manipulation, its microbial load must not be allowed to increase (fresh milk is rarely sterile and usually contains bacteria originating from maternal skin and nipple duct microflora[23]) and its constituents and properties must be preserved.

Prior to the identification of the protective components in milk, the present study analyses the bactericidal capacity of human milk, with quantification of its possible alteration following refrigeration (4–6°C for 24, 48 and 72 h) or freezing at –20°C for seven days. Comparisons are also made with values found in fresh milk.

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Materials and methods

Subjects
A total of 19 samples of human milk were collected from 19 healthy, well-nourished women attending a healthcare centre in Meliana, 6 km north-west of Valencia (Spain), between April and September 2005. Prior to sampling, all donors gave informed consent to participation in the study.

Sample collection
Sampling was carried out using an electric breast pump fitted with a vacuum regulator (Mamilat S.M. 122), which was connected directly to polypropylene containers in which the samples were collected, thereby eliminating the risk of accidental contamination. The total sample volume collected (approximately 10 mL) was divided into five aliquots: 2 mL for analysis fresh; 2 mL each for refrigerated storage (4–6°C) at 24 h, 48 h and 72 h, followed by analysis; and 2 mL for storage frozen (–20°C) for seven days, followed by analysis.

Bactericidal assay – viability testing
A strain of *Escherichia coli* NCTC 9111 serovar O111:K58(B4):H– was used to determine the bactericidal effect of human milk. Bacteria were cultured overnight on blood agar, suspended in sterile normal saline and adjusted to a turbidity equal to that of a McFarland No. 1 standard (approximately 3 x 10^8 colony-forming units [CFU]/mL).

A volume of 0.2 mL of this solution of bacteria was added to Eppendorf tubes containing 0.8 mL of the respective milk samples. The tubes were mixed carefully before and after incubation at 37°C for 2 h. Control samples were prepared by mixing 0.2 mL bacterial solution with 0.8 mL peptone broth (Oxoid).

After incubation, *E. coli* counts were determined by inoculation of the contents of the Eppendorf tube on Violet Red Bile Agar (VRBA, Oxoid), which was incubated at 44.5°C for 24 h. Each sample was tested in duplicate. Degree of bacteriolysis was calculated as the difference between *E. coli* counts in the control and milk samples, expressed as a percentage of the control sample counts.

Statistical analysis
Statistical significance of the differences between values after each sample treatment was assessed using Student's *t*-test. Differences were considered to be statistically significant at $P<0.05$. The results were contrasted using analysis of variance (ANOVA).

Results

Evaluation of bactericidal capacity
Bactericidal capacity in fresh milk showed a normal distribution. The maximum and minimum values were 98.0% and 48.1%, respectively. All samples analysed showed bactericidal capacity, the overall mean value being 77.33±15.14%.

Alteration of bactericidal properties due to storage
Maximum values obtained for bactericidal capacity following refrigeration (4°C) for 24, 48 and 72 h were 100%, 99.9% and 87%, respectively. After frozen storage (–20°C) for seven days the figure was 99.8%. Minimum values for refrigeration were 0.0%, 18.1% and 0.0%, respectively, and for frozen samples was 9.8%.

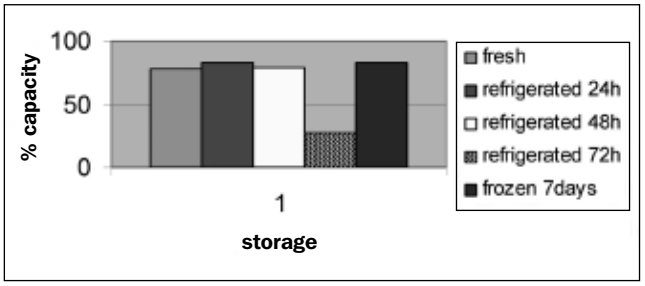

Fig. 1. Bactericidal capacity of human milk after different storage and periods. Mean values expressed as the percentage capacity related to control sample counts.

Figure 1 shows the mean values corresponding to each treatment. The statistical comparison of each treatment versus fresh milk showed that refrigeration at 4°C for 24 or 48 h did not result in a significant alteration to bactericidal capacity ($P>0.05$), with mean values of 82.1% and 78.9%, respectively. When refrigerated storage was extended to 72 h, almost 50% loss in bactericidal capacity (mean value: 26.61%) was recorded among the samples analysed. This was highly significant. ($P<0.01$).

Bactericidal capacity of milk frozen for seven days (mean value: 82.18%) was not significantly different to that recorded for fresh milk ($P>0.05$).

Effect of nursing stage on bactericidal capacity
The effect of nursing stage on bactericidal capacity was assessed in milk from 16 donors, as the date of delivery was unknown in three of the initial donors. Of the samples analysed, six were from nursing mothers between eight and 15 days post-partum (transition milk), and 10 were between 20 and 35 days post-partum (mature milk).

Mean bactericidal capacity for each period was 70.15±9.88% and 82.87±16.37%, respectively. Of note was the high variability of results in mature milk. ANOVA of the bactericidal capacity of the samples showed that nursing stage did not influence capacity significantly ($P=0.219$; Fig. 2).

Discussion

This study quantifies the combined action of all biocomponents found in maternal milk that have a

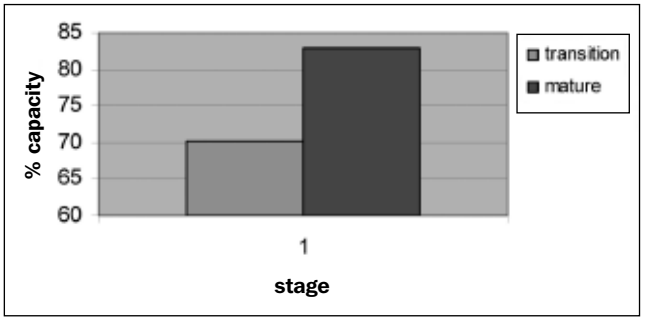

Fig. 2. Effect of nursing stage on bactericidal capacity. Mean values expressed as percentage capacity related to control sample counts.

bactericidal effect. An evaluation is made of the bactericidal action of maternal milk on a strain of *E. coli* NCTC 9111 serovar O111:K58(B4):H–, an enteropathogen known to cause infant gastroenteritis. The *E. coli* strain was inhibited in all the fresh milk samples, as growth of the microorganism was of a lesser magnitude than in control milk in all cases. Such action may be expected to reflect the cumulative effects of different components, some of which have already been identified and others remain to be characterised.

Lactoferrin is an abundant protein in human milk, with a concentration that increases after the second post-partum day to peak at 5–6 mg/mL, followed by a reduction to 1 mg/mL in mature milk.[13] Its concentration is greater in human milk than in milk from other species, with important variations in the course of lactation.[12] In effect, lactoferrin concentration in colostrum doubles the levels found in mature milk.

The intact protein and the derivatives of its digestion[14] possess antimicrobial activity as a result of their high affinity for iron. As a result, it is sequestrated and cannot be used by those microorganisms for which it is an essential requirement.[13,15] Susceptible bacteria also suffer alterations in membrane integrity and permeability, leading to cell death. Recent studies corroborate the bacteriostatic and bactericidal action of lactoferrin.[18]

The concentration of lysozyme in maternal milk (30–40 mg/100 mL)[15] is triple that found in cow milk. In contrast to other protective factors, its concentration increases with the course of lactation, and is found to be greater after three months than in the early stages of nursing. The bactericidal action of lysozyme is reinforced when added to the specific antibodies found in maternal milk, and particularly when associated with the presence of complement.[15]

The protective action of oligosaccharides against infections is associated with specific interactions with pathogenic bacteria, inhibiting their capacity to adhere to the host epithelial surfaces.

Antibacterial factors against *Staphylococcus epidermidis* and *E. coli* have been identified in the lipid fraction of the gastric contents of infants fed on maternal milk.[16] The digestion of triglycerides produces monoglycerides and fatty acids that have lytic effects on viruses and bacteria.

Complement components C3 and C4 are able to produce bacterial lysis on binding to specific antibodies such as IgA. Component C3 possesses chemotactic activity, as well as opsonin and bacteriostatic functions, against *E. coli*.[6,17]

Recently, human milk has been shown to inhibit the metabolic activity of *E. coli*. Such inhibition is dependent upon xanthine oxidase enzyme activity and the presence of nitrite, secondary to the generation of nitric oxide, which is a potent antibacterial agent.[19]

Storage of maternal milk usually involves refrigeration at 4–6°C for up to 72 h, or freezing at –20°C for 15–90 days.[9,20–23] Studies have been made of the effect of these temperatures on the different components of milk, particularly with regard to changes in its constituent nutrients (i.e., proteins,[25] lipids[24–26] vitamins,[24] etc), and in relation to different protective systems.[9,21,23,27]

References in the literature on the effect of storage on the bactericidal capacity of milk are limited. Of note are the studies conducted by Hernandez *et al*.[29] and, more recently, by Ogundele.[22,23] New information on this characteristic of milk is of interest in defining the optimum conditions for the manipulation and protection of human milk.

The present study aims to define the best conditions for the short-term storage of milk. As can be seen in Figure 1, refrigeration for less that 48 h does not modify the bactericidal capacity of human milk, and thus the protective properties for the nursing infant remain intact. However, if storage is extended beyond this time then bactericidal capacity decreases, and the loss of this protection is very significant statistically after 72 h ($P<0.01$).

It should be pointed out that this property of maternal milk is lost over a period of time in which other components remain stable and within the limits advised by usual protocols. Other authors report similar findings. Hernandez *et al*.[29] and Ogundele[23] report a reduction in the protective action of milk against bacterial growth after three days of refrigerated storage,[23] although data corresponding to periods of less than 72 hours are lacking.

When frozen storage is employed, milk stability is prolonged. After seven days, bactericidal action remains unaltered. Similar results have been reported by Ogundele,[23] who observed a faster loss of antimicrobial activity in milk stored at 4°C for three days than in milk frozen at –20°C for seven days. In contrast, Hernández *et al*.[29] reported a significant loss of antimicrobial activity, although they studied longer periods of frozen storage (21 days).

On examining the bactericidal capacity of milk in relation to nursing stage (i.e., transitional milk versus mature milk), no significant differences were observed between the two stages. Bearing in mind the high concentration of defensive proteins found in colostrum, this result was unexpected. However, it could be explained by a tendency for the bactericidal capacity of individual items to be cancelled out. For example, while peak concentration of lactoferrin occurs in the first post-partum week (followed by a reduction in mature milk), concentration of lysozyme shows the opposite tendency, reaching maximum values after approximately 30 days.

In conclusion, human milk possesses antibacterial activity that is probably attributable to a range of components that inhibit microbial growth. When milk storage is required, and in order to preserve this activity, refrigeration for no more than 48 h is recommended, as antimicrobial action is seen to diminish after this period of time. Frozen storage is an option when longer storage periods are needed. □

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