Storage beyond Three Hours at Ambient Temperature Alters the Biochemical and Nutritional Qualities of Breast Milk

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ABSTRACT

The effect of storage on stability of human breast milk was investigated in 30 lactating mothers. Samples stored for 3, 6 and 24 hours at ambient temperature of 302K (29°) were analysed for protein, lactose, pH, and microbial content. There were significant ($p < 0.01$) decreases in protein, lactose and pH upon storage for 6 and 24 hours, compared with storage for 3 hours as control. The mean ± SEM values for protein for 6 and 24 hours were 15.56 ± 0.48 and 13.27 ± 0.50, compared with 17.26 ± 0.41 for 3 hours. For lactose, corresponding values for 6 and 24 hours were 0.08 ± 0.005 and 0.07 ± 0.006, compared with 3 hours (0.09 ± 0.005). The pH values were 6.1 ± 0.09, 5.9 ± 0.07 and 5.6 ± 0.07 in 3, 6 and 24 hour samples respectively. The skin floras investigated were *Streptococcus viridians, Straphylococcus aureus* and *Staphylococcus albus.* The microbial content increased with increase in storage time from 3 to 24 hours. The predominant bacterial specie was *S. Albus,* followed by *S. viridians* and *S. aureus.* A positive correlation ($r = 0.453$, $p < 0.01$) between lactose level and pH were obtained. These results suggest that breast milk is stable for 3 hours, beyond which significant changes occur in its biochemical composition and nutritional quality. The implications of these findings are discussed with respect to its consequences on their child's survival. (*Afr J Reprod Health* 2001; 5[2]:130-134)

RÉSUMÉ

La Conservation au-delà de trois heures à une température ambiante modifie les qualités biochimiques et nutritionnelles du lait maternel. L'effet de la conservation sur la stabilité du lait maternel humain a été étudié chez 30 mères allaitantes. Les échantillions qui ont été conservés pendant 3, 6 et 24 heures à une température ambiante de 302K (29°) ont été analysés pour déterminer la présence de la protéine, du lactose pH et du contenu microbien. Il y a eu des baisses importantes ($P < 0.01$) par rapport à la protéine, au lactose et pH quand ils sont conservés pour 6 et 24 heures en comparaison à la conservation de 3 heures comme cas témoin. Les valeurs moyennes ± SEM pour la protéine pour 6 et 24 heures étaient de 15, 56 ± 0,48 et 13, 27 ± 0, 50 comparée à 17, 26 ± 0, 41 pour 3 heures. En ce qui concerne le lactose, les valeurs correspondantes pour 6 et 24 heures étaient de 0,08 ± 0,005 et 0,07 ± 0,006,comparée à 3 heures (0,09 ± 0,005). Les valeurs pH étaient de 6,1 ± 0,09, 5,9 ± 0,07 dans les échantillons de 3,6 et 24 heures respectivement. Les flores entanées qui ont été étudiées étaient *Streptocoques viridians, Staphylocoques aureus* et *Staphylocoques blanc.* Le contenu microbien a augmenté au fur et à mesure que la durée de la conservation a augmenté de 3 à 24 heures. L'espèce de la bactérien prédominante était *S. albus,* suivie de *S. viridians* et *S. aureus.* Une corrélation positive ($r = 0,453$, $p < 0,01$) entre le niveau de lactose et pH a été obtenue. Ces résultats suggèrent que le lait du sein reste stable pendant 3 heures et qu'au-delà de cela il se produit des modifications importantes dans la composition biochimiques et dans la qualité nutritionnelle. Nous avons discuté les implications des ces résultats par rapport à leurs conséquences sur la survie de l'enfant. (*Rev Afr Santé Reprod* 2001; 5[2]:130-134)

KEY WORDS: *Breast milk, storage time, microbial content, biochemical composition, nutritional quality*

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Introduction

Breast milk is a natural, complete and balanced food for the newborn, which is meant to serve as the sole source of nourishment for the growing infant for some time after birth.[1] It supplies nutrients such as protein, which promotes growth of children; calcium, which supports bone and teeth development; vitamins; as well as other substances in large quantities and in absorbable form.[1-3] Babies fed on infant formulas most often have problems of diarrhoea, respiratory and middle ear infection, which directly or indirectly slow down growth through malnutrition.[1] Compared with infant formula, the adequacy of breastfeeding in promoting growth and development of normal infants is now well established.[4-7]

In Nigeria, the period of maternity leave for working mothers lasts for three months only. Nursing mothers of high social status, particularly those who occupy top executive positions, are required to return to work after maternity leave, but are not permitted by law to carry their infants to their places of work. In line with the global trend in favour of breastfeeding for the first five or six months of life,[6,7] mothers returning to work have been encouraged to express their breast milk and store it in containers. The stored breast milk can then be fed to the babies while they are away. It has been suggested that such milk stored at room temperature can last for six to eight hours without significant change or putrefaction. This has, however, been a subject of controversy because microbial infection and alteration in the levels of protein have been reported during milk banking.[8,9] To allay the fears of many mothers, this study was designed to test the hypothesis that storage does not affect the biochemical and nutritional quality of human breast milk.

Materials and Methods

Collection of Breast Milk Samples

Samples of breast milk were collected from 30 lactating mothers who reported at the postnatal wards of the General Hospital and the University of Calabar Teaching Hospital (UCTH), both in Calabar, Cross River State, Nigeria. The consent of the mothers was sought before sample collection. The subjects were aged between 20 and 35 years, had been lactating for a period of three months or more, and had also not been placed on antibiotics for six weeks prior to sample collection. The samples were collected into clean sterile labeled plastic containers between 10.00 a.m. and 12 noon daily. Sample collection and analysis were completed within a period of three weeks. Each milk sample was divided into three portions and analysed after 3, 6, and 24 hours for lactose, protein, pH levels and microbial content at ambient temperature of 302K (29°C).

Determination of Lactose Level

Lactose levels in breast milk samples were determined using Benedict's quantitative reagent after fats, proteins and other interfering substances have been removed.[10] About 20ml of milk was diluted with 50ml of distilled water in a 100ml volumetric flask, and 5ml of 1% acetic acid added to precipitate fats and casein. The filtrate obtained, containing lactose and albumin, was separated from the precipitate and treated with 5ml of 1% Na_2Co_3 solution, added dropwise while heating. Albumin was coagulated from the solution and removed by filtration to give a clear filtrate containing lactose. This was titrated against 10ml Benedict's reagent to obtain the percentage of lactose in milk. Ten millilitres (10ml) of Benedict's reagent is equivalent to 27.12mg% of lactose.

Determination of Protein Level

The Association of Official Analytical Chemists (AOAC)[11] method was used for the determination of protein levels in breast milk. Briefly, the protein nitrogen of 10ml milk sample was digested in a Kjeldahl flask. The sample digest obtained was immediately treated with 10ml of 40% NaOH to liberate ammonia, which was removed by steam distillation and collected into a 10ml boric acid indicator solution contained in an Erlenmeyer flask. The distillate was titrated with 0.02N standard Hcl to a pink end point. A blank determination was carried out in similar manner. The total nitrogen obtained was multiplied by a factor of 6.25 to obtain the crude protein value.

Determination of pH

The pH meter (Mettler Digital, England) was used to determine pH levels of breast milk samples at 29°C.

Analysis of Microbial Levels

The determination of microbial content of breast milk was carried out as described by Fawole and Oso.[12] The bacteria were cultured on chocolate agar plates and incubated at 35°C to form colonies. Bacterial isolates from each colony were further emulsified in a drop of distilled water on clear slide and treated with gram staining reagents to establish whether it was gram positive or negative. Catalase test was used for pathogenicity and coagulase test was used for identification of organisms.

Statistical Analysis

Data obtained from the experiments on determination of protein, lactose and pH levels were expressed as mean ± SEM. Statistical analysis of the data was done using ANOVA, and differences at $p < 0.05$ were considered significant. The mathematical relationships between protein, lactose, or pH levels, and storage time were analysed using linear regression. Correlation analysis was employed to examine the relationship between lactose and pH.

Results

Table 1 shows the mean SEM values of protein (mg%), lactose (mg%) and pH in the 3, 6 and 24-hour breast milk samples. There were significant variations in protein ($F = -6.79$, $p < 0.001$), lactose ($F = 9.7$, $p < 0.001$) and pH ($F = +7.0$, $p < 0.001$). The mean ±SEM values of the 3, 6 and 24-hour samples were 17.26 ± 0.41, 15.26 ± 0.48 and 13.27 ± 0.50 for protein; 0.09 ± 0.005, 0.08 ± 0.005 and 0.07 ± 0.006 for lactose; and 6.1 ± 0.09, 5.9 ± 0.07, and 5.6 ± 0.07 for pH respectively. There were significant decreases in protein, lactose and pH upon storage for 6 and 24 hours, compared with storage for 3 hours. The linear regression plots (Figure 1) illustrate the pattern of changes in protein, lactose and pH over time in storage.

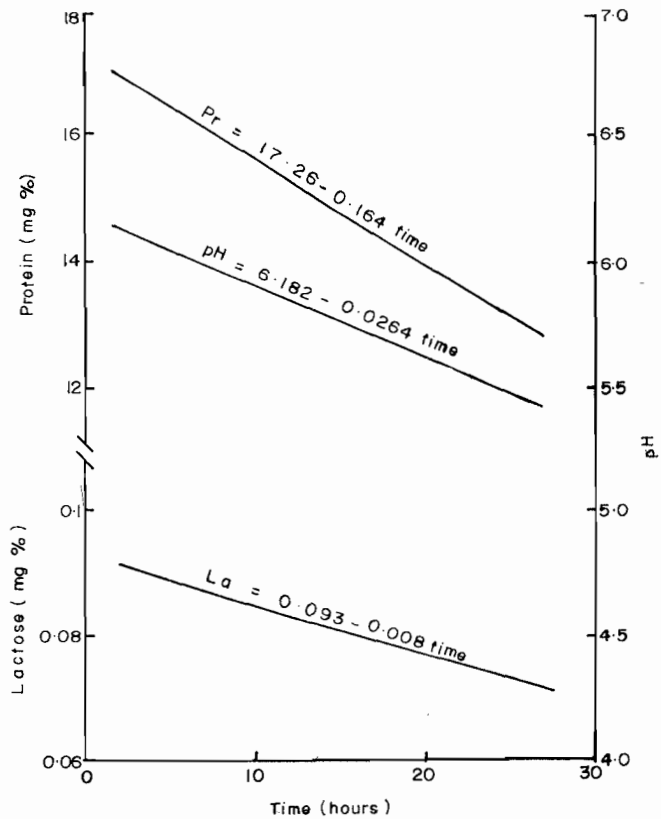

Figure 1 **Linear Regression Plots of Lactose (La), Protein (Pr) and pH against Storage Time of Milk Samples**

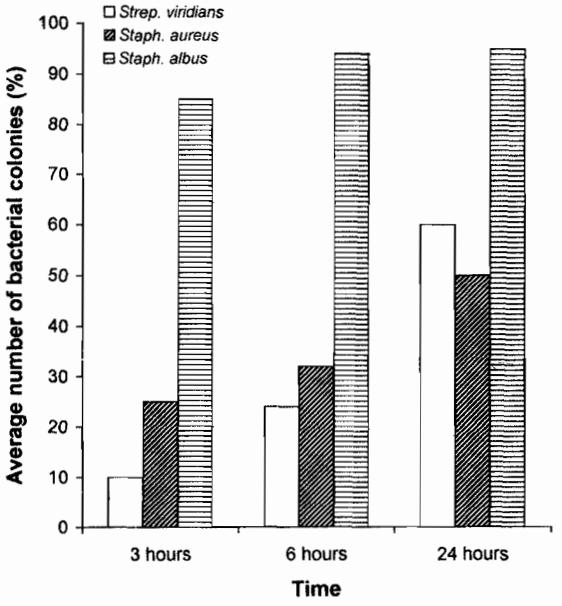

Figure 2 **Average Number of Bacterial Colonies after 3, 6 and 24 Hours of Storage at Ambient Temperatures**

Figure 2 shows the average number of bacteria colonies present after 3, 6 and 24 hours respectively. The dominant bacterial specie was *S. albus,* followed by *S. aureus* and *S. viridians.* The three species were present in the 3-hour stored sample with 10% average growth for *S. viridians,* 25% for *S. aureus,* and 85% for *S. albus.* At 6 hours storage, *S. viridians* increased to 24%, whereas *S. aureus* increased to 32% and *S. albus* to 94%. At 24 hours storage, all bacteria species increased in growth but *S. viridians* had a higher (60%) increase, compared with *S. aureus* at 50%. *S. albus* remained the highest at 95%.

Table 1 **Mean SEM Values of Protein, Lactose and pH in 3 Hours, 6 Hours and 24 Hours Stored Human Breast Milk Sample**

Storage time (Hours)	Protein (mg%)	Lactose (mg%)	pH
3	17.26 ± 0.41	0.09 ± 0.005	6.1 ± 0.09
6	15.26 ± 0.48*	0.08 ± 0.005*	5.9 ± 0.07*
24	13.27 × 0.50*	0.07 ± 0.006*	5.6 ± 0.07*

*n = 30; *p < 0.001 (versus the 3 hour sample)*

Discussion

In this study, significant decreases in the levels of protein, lactose and pH in breast milk have been observed after storage for 6 and 24 hours, compared with storage after 3 hours (control). Also, an increase in growth of pathogenic microorganisms (*S.viridians, S. aureus,* and *S. albus)* with increase in storage time from 3 to 6, and to 24 hours has been demonstrated. The significant alteration in the level of protein, and the increase in microbial growth obtained agree with earlier reports by Bjorksten[9], and Lucas and Robert.[13]

A number of factors may explain the changes observed in this study. The presence and multiplication of pathogenic bacteria in milk samples may be as a result of direct contamination from skin flora.[13] Lactose, which is the sugar in milk and a major energy source, is also known to support growth of microbial flora.[14] As a major energy source, the microbes degrade the lactose to lactic acid by anaerobic glycolysis. This may explain the decrease in lactose and pH levels. The significant positive correlation between lactose levels and pH obtained from analysis of the data further indicates that the decrease in lactose and pH is as a consequence of microbial growth.

Despite the fact that the decrease in protein level upon storage of human breast milk reported

in this study agrees with earlier reports,[9,13] the exact mechanism by which this occurs has not been documented. DNA replication, coupled with transcription and translation, are the biochemical mechanisms underlying multiplication of bacteria. The degradation of protein in milk to generate amino acids, which are building units for protein synthesis in bacteria, may explain the decrease in protein levels observed in the study.

Generally, the results indicate that milk is stable within three hours of storage, beyond which significant changes in its biochemical and consequently nutritional quality occurs. These changes become greater with time and are linked to the presence and levels of pathogenic microbial flora. The consequences of degradation in the quality of milk upon storage beyond 3 hours with regards to the infant's health are serious. Energy deficits, lowered immunocompetence, and mental retardation may result from a decrease in lactose and protein levels. The overall picture is, therefore, that of protein energy malnutrition[1], retarded development, infection and disease.

The general recommendation that babies should be exclusively breastfed for the first four to six months has been adequately emphasised in recent times.[1,6,15] However, prolonged storage of milk at ambient temperature by working mothers is prone to incidents of microbial growth, and freezing, pasteurisation or autoclaving cause inactivation of milk lipase, thereby impeding fat absorption.[13,16,17] It is therefore suggested that if breast milk is stored, it should be done in sterilised containers, and during expression of breast milk for storage, nipples should be thoroughly cleansed. The expressed milk should not be stored beyond three hours at room temperature, otherwise significant deterioration would occur.

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