Chemistry Research Journal, 2020, 5(6):180-188

Available online www.chemrj.org

Research Article

ISSN: 2455-8990
CODEN(USA): CRJHA5

Egyptian Mothers' Breast Milk Home Bank: Nutritional and Microbiological Study

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Abstract Human milk provides infant with the required nutrients for growth and development. Due to working mothers cannot breast feed their babies, storing milk is the answer to somewhat. The aim of this study was to assess the influence of different temperatures and storing durations on macro- and micronutrients contents of breast milk. The samples were also subjected to microbiological investigation. This research was an experimental study with a complete randomized design. Mature breast milk samples were obtained from 20 volunteer lactating mothers residing in Cairo, Egypt, at least 3 months after they delivered their babies. Samples were divided into three treatment-groups of storing temperatures: room (RT), refrigerator (4˚C) and freezer temperatures (-18: -20˚C). The measurement (protein, fat, lactose, vitamins as A, E, and C; and minerals as Zn content) were done on the 0 time (Fresh RT); 1st (refrigerator) and 15th day (Freeze) for micro-, macro nutrients and also were investigated for microbiological contamination. A non significant decrease in protein and fat were observed while lactose shows a significant decrease. Vitamins A, E and C and Zn showed a significant decrease but vitamin C is more affected where decrease reach 65%. Based on this research, breast milk can be safely stored up to 24 hours in the refrigerator or freezer temperature to keep the nutrient contents intact.

Keywords human milk, essential minerals

Introduction

Exclusive human milk feeding for the first 6 months of life, with continued breastfeeding for 1 to 2 years of life is the normal situation for infant feeding as the sole source of nutrition for infants in the first 6 mo [1-3]. Human milk is suited to the human infant feeding for its unique composition and valuable nutrition elements as it contain bioactive factors as anti-infectious and anti-inflammatory agents, growth factors, and prebiotics; and contribute to immune maturation, organ development, and healthy microbial colonization [4].

The use of expressed breast milk (fresh or frozen) has been noted to provide both nutritional and immunological benefits especially if its nutritional value can be conserved [5]. This becomes necessary when babies may be separated from mothers due to maternal employment or schooling. In the developing countries due to life style change and low economic of most populations, some women works and this sometimes can hinder their ability to breast feed their babies. As a way to resolve this problem breast milk can be stored and fed to babies while mothers are working, as long as the storing process is carried out properly, but still some mothers might not prefer milk that

has been previously stored either in refrigerator or frozen. The ability to store or keep human milk frozen for one's own child helps families around the world every day but still need to confirm, whether nutrients are lost or not in the process. Feeding infants with expressed human milk is increasing. Silvestre *et al.*, [6] consider freezing at < –80°C is the best for long-term freezing of breast milk since it minimizes the loss of its properties but still is too expensive for working mothers. Also there is a great fear of possible bacterial contamination and growth of pathogenic bacteria which makes stored milk harmful for babies feeding**.** Hygienic preservation of milk has been a major goal since the early days of milk banking [7].

Horwood *et al*., [8] and Isaacs *et al*., [9] stated that feeding the baby expressed milk rather than substitutes significantly improve various IQ parameters as measured at 7 – 8 years old.

In international guidelines the maximum period for freezing time at < -20°C recommended for breast milk is highly variable, ranging between 1 and 12 months [10].

Materials and Method

Informed consent was taken from all mothers participating in the study. This research is an experimental study with a complete randomized design. Mature breast milk samples of full term were obtained from 20 volunteer lactating mothers (n=20), at least 3 months after they delivered their babies; aged between 22 to 35 year-old in Cairo, and their babies 'ages 3 months. All mothers were of intermediate socioeconomic groups. Exclusion criteria included the presence of any significant maternal disease, malnutrition or the use of any medication during lactation. Before sampling, each mother gently washed both areolas with baby soap, rinsed them thoroughly with water, dried the skin, and collected the milk with a soft squeeze by her own effort. Samples were collected in sterile containers; divided into three groups (each in several small containers) of storing temperatures: room (RT), refrigerator (4˚C) and freezer temperatures (-18: -20˚C). The measurement of the contents of lactose, protein, fat, vitamin A, E, C; Zn, and microbiological investigation was performed in each sample on the 0 time (Fresh RT); 1st day (refrigerator) and 15th day (Freeze). Milk were collected between 10-12 O'clock, from both fore-and hind milk. The samples were transported in ice packs to the laboratory within 20–25 minutes. One of the 3 samples was analyzed immediately as the baseline fresh control one.

Analysis

All analysis: macro-and micro nutrient, vitamins and minerals were done according to AOAC 2016 method [11].

Condition of Storing

Frozen human milk should be stored in the back of the freezer to prevent exposure to different temperature due to freezer door opening, and should be kept away from the walls of self-defrosting freezers. All containers with human milk should be well sealed to prevent contamination; filled to 2/3rd its volume to allow expansion with freezing and labeled with the date of milk expression. Storing human milk in several small containers such as 15mL is a convenient way to prevent repeated thawing human milk. Boiling is prevented before storage of milk.

Statistical Analysis

Data are expressed as means ± SEM. The distribution of data normality was confirmed using Shapiro-Wilk tests. Their statistical significance was assessed one way ANOVA followed by post hoc test Duncan using the statistical package SPSS 21.0 for Windows (SPSS, Chicago, IL). $P < 0.05$ was considered statistically significant.

Results and Discussion

The average age of the women included in the present study were 28 ± 4 years old (22-35y). Of the babies, 60% were girls and 40% were boys and the average birth weight for infants was 3.79 ± 0.56 kg.

Freezing means a series of physicochemical changes in the breast milk components: a rupture in the fat globule membrane; formation of casein precipitates and structure of whey proteins are altered [12].

I-Macronutrients

The macronutrient composition of human milk varies within mothers and across lactation [13].

Table 1: The level of protein, fat and lactose contents in human breast milk during different storage method and durations (Mean ± SEM)

Storage	**Duration**	**Fresh**	**24h**	**15 Days**
	Method	**Room Temp**	**Refrigerator 4˚C**	**Freezer -18: -20˚C**
Nutrients	Protein g/100ml	1.70±0.18[a]	1.62±0.22[a] -4.25	1.56±0.29[a] -8.08 -4.00
	Fat g/100ml	3.77±1.11[a]	3.48±0.96[a] -7.72	3.11±0.94[a] -17.63 -10.75
	Lactose g/100ml	5.17±0.86[a]	4.31±0.82 [b] -16.54	3.69±0.62[b,c] -28.56 -14.41

[abc]significantly different.

Table 1a: Macronutrient concentration in human milk as referred in the other literatures and from current study

	Current Study	**Perrin *et al.*, 2016**	**Glew *et al.*, 2008**	**Glew *et al.*, 2011**	**Butte *et al.*, 1990**	**Nommsen *et al.*, 1991**
Protein	1.70±0.18	1.55±0.19			0.92±0.21	1.14±0.15
Fat	3.77±1.11	3.83±1.94	3.27±2.27	4.53±1.84		3.58±0..82
Lactose	5.17±0.86	5.67±0.73			6.51±.0.20	7.22±0.17

Table 1 shows the level of protein, fat and lactose contents in human breast milk during different storage method and durations. Analysis of protein and fat contents on 24 h and 15 th day contents in refrigerator and freezer storage respectively showed insignificant decreased in comparable to the control (Fresh: 0 day, RT) except for lactose where it showed a significant decrease when compared with the fresh one but no significant change between storage at 24 h and 15 days. Despite the insignificant change there was a decrease in the content ranging -4 : -8.08% for protein; -7 : -17.63% for fat; while for lactose it reach -28.56% (significant decrease for lactose, (3.69±0.62 vs 5.17±0.86)). This study indicated that there was no significant effect between the storage temperature and the level of protein and fat content of mature breast milk.

Table 1a shows fresh macronutrient concentration in human milk as referred in the other literatures and from current study. Our result for protein, fat and lactose agree with Perrin *et al.*, [14] and disagree with Glew *et al.*, [15-16] for fat, and with Butte *et al.*, [17] & Nommsen *et al.*, [18] for protein and lactose.

Storage of human milk at 4˚C or -18: -20˚C had no significant effect on protein content since denaturation occurs only when there is factors as heat or alcohol treatment **().** Protein of human breast milk is not denatured at storage temperature of 4 °C for 48 h [19]. The storage of breast milk in the freezer does not have a major influence on the protein level, so that the milk can be stored safely for 72 hours at a temperature of 4-6 °C. The results of this study support the previous study stated that the temperature on different types of storage did not affect the levels of protein.

Storage duration influenced the changes in the fat content of breast milk. Breast milk contains the enzyme lipase (produced from lipolytic bacteria) which serves to digest fats into diglycerides then monoglycerides [20]. Another study also explained that lipolysis runs very fast starting from the first hour of storage and process reached 8% at 24 hours of storage [21].

Our results tend to agree with Pardou *et al.*, [22], who observed a non-significant decrease in total lipid content after refrigeration at 4–6 °C as well as after freezing at –20 °C for 8 days, which could be due to lipolysis occurring

during storage. They reported that this decrease did not exceed 2.0%–6.7% from the initial values of fat in breast milk samples.

Fat is the most highly variable macronutrient of milk. Hind milk, defined as the last milk of a feed, may contain two to three times the concentration of milk fat found in foremilk, defined as the initial milk of a feed [23]. A study of milk from 71 mothers over a 24- hour period found that the milk fat content was significantly lower in night and morning feedings compared to afternoon or evening feedings [24]. Another study found that ~25% of the variation in lipid concentration between mothers' milk may be explained by maternal protein intake [18]. Bertino *et al.*, [25] stated that lipid composition and lipase activity remained stable up to 96 hours in the refrigerator.

The principal sugar of human milk is the disaccharide lactose. The concentration of lactose in human milk is the least variable of the macronutrients, but higher concentrations of lactose are found in the milk of mothers producing higher quantities of milk. The other significant carbohydrates in breast milk are oligosaccharides (1 g/100 ml) [26]. Our results disagree with Iqbal *et al* [27] where they stated that the storage temperature has no effect on the level of lactose, protein and fat contents of breast milk and the duration of storage do not affect the lactose content of breast milk but affects the protein and fat content of breast milk.

Table 2: The level of vit A, E and C contents in human breast milk during different storage method and durations (Mean±SEM)

Storage	**Duration**	**Fresh**	**24h**	**15 Days**
	Method	**Room Temp**	**Refrigerator**	**Freezer**
Nutrients	Vit A	0.37±0.04 [a]	0.33±0.04[a,b]	0.27±0.05[c]
			-8.99	-25.34
				-17.96
	Vit E	1.39±0.07 [a]	1.33±0.08[a,b]	1.25±0.09[c]
			-4.10	-9.86
				-6.00
	Vit C	3.33±0.87 [a]	2.15±0.62[b]	1.14±0.52[c]
			-35.43	-65.95
				-47.26

[abc]Significantly different.

Table 2 shows the level of vitamin A, E and C contents in human breast milk during different storage method and durations. Analysis of vitamin A, E and C contents on 24 h in refrigerator showed insignificant decrease in comparable to the control (Fresh: 0 day, RT) except for vitamin C where it showed a significant decrease when compared with the fresh one where it reach -35.43%; but the opposite was noticed where a significant decrease was observed with freezer storage at -18: -20˚C for 15 day for vitamin A, E, and C where % decrease reach -25.34; -9.86 and -65.95 respectively. Vitamin C is the most vitamins that are affected dramatically. According to Bank *et al.,* [28] storage can result in the loss of components sensitive to oxidation, such as the physiologically relevant forms of vitamin C, ascorbic acid (AA) and dehydroascorbic acid (DHAA) with a Loss up to 40% of AA and 20% of total vitamin C.

Our results agree with Romeu-Nadal *et al.,* [29] to somewhat as they found that total vitamin C content at 4°C (6 h), −20°C (8 mo) and −80 °C (12 mo) was significantly decreased while vitamin E levels did not change at either refrigeration temperature (under 24 h) or at freezing temperatures. Also it agrees with Buss *et al.,* [30]; and Ahrabi *et al.,* [31] where they stated that freezer storage affects nearly all vitamins and minerals in human milk. Level of vitamin C in this study differs from other studies as that of Buss *et al.,* [30] (3.33±0.87 vs 5.31±1.66). The % decrease was higher in Buss *et al.,* [30] study than this study (65 vs 35) for storage for 24 h. In this study vitamin C loss is higher with increasing storage time; the decrease increase from -35.43 to -65.95 with increasing time of storage from 24h to 15 days and this agree with Buss *et al.,* [30] where they found that vit C decrease may reach 90-100% of the initial concentration. The losses of vitamin C are partly due to lactoperoxidase activity [30].

Table 3: The level of Fe and Zn contents in human breast milk during different storage method and durations (Mean±SEM)

Storage	**Duration**	**Fresh**	**24h**	**15 Days**
	Method	**Room Temp**	**Refrigerator**	**Freezer**
Nutrients	Zn mg/100mL	0.32±0.09 [a]	0.25±0.07[a,b] -20.71	0.20±0.06[b] -35.55 -18.73

[abc]Significantly different.

Table 3a: Element concentration in human milk as referred in the literature and from current study

	Current Study mg/100mL	**Li *et al.*, 2016 Micmol/L**	**Ezz El Din *et al.*, 2004**	**Curran & Barness 2000**	**Yamawaki *et al.*, 2005**	**Shi *et al.*, 2011**	**Björklund *et al.*, 2012**	**Andrade *et al.*, 2014**	**Līva *et al.*, 2018**
Zn mg%	0.32±0.09	20.0±11.9	0.11±0.01	0.17–3.02	0.10±0.06	0.20±0.10	0.35±0.1	0.36±0.02	0.01-0.34 0.10 (0.05-0.15)

Table 3 shows the level of Zn contents in human breast milk during different storage method and durations. Analysis of Zn contents on 24 h in refrigerator showed insignificant decrease in comparable to the control (Fresh: 0 day, RT); but the opposite was noticed where a significant decrease was observed with freezer storage at -18: -20˚C for 15 day for Zn where % decrease reach -35.55 for Zn.

Table 3a shows fresh element concentration in human milk as referred in the literature and from current study. Our obtained zinc content (0.32 mg /100mL) in breast milk was higher compared to data from Poland (0.16) ([5, 13, 32-33]; Table 3a). Similar zinc content (approximately 0.11 mg /100 mL) was detected in mature breast milk samples among lactating women from Portugal ([34-36]**:** Table 3a). Līva *et al.,* [37] stated that Zn level range 0.01-0.34. Lower zinc content (0.07 and 0.05 mg /100 mL) has only been reported by Domellöf *et al.,* [38] for breast milk samples from mothers in Honduras and Sweden, respectively. Overall, it is difficult to compare data between studies due to differences in sampling plans, time postpartum, and analytical methods used. The difference between our obtained lower zinc results and others could be explained by the fact that we collected both hind- and foremilk samples. Doneray *et al.,* [39] observed that zinc content in hind milk was approximately 0.2 mg lower than in foremilk; however, other researchers [40] have not observed variations in zinc content between fore- and hind milk. Alam *et al.,* [41] identified that mutation in the protein ZnT2 (SLC30A2) that transports zinc can lead to low content of this element in breast milk (<0.09 mg/ 100 mL).

Panel Test

Some of the samples of the stored human milk have a different odor from fresh ones. On a scale of 10 it reaches 8. Refrigerated and frozen human milk may have an odor different from fresh milk due to lipase-mediated triglyceride breakdown, releasing fatty acids. The odor likely comes from oxidation of these fatty acids [42]. This lipolysis process has antimicrobial effects preventing the growth of microorganisms in thawed refrigerated milk [43].

Microbiological Investigating

To the best of our knowledge, only few studied deals with microbiological profile of human milk and they have been mainly focused on the identification of pathogenic bacteria [44]. Martín *et al*. [45] considered breast milk a sterile environment which is in contrast with the finding of Deodhar and Joshi [46] where they stated that freshly collected milk is not sterile since it contain bacteria either pathogenic or from nipple duct and maternal skin. Some of these pathogenic bacteria could damage antimicrobial proteins or convert amino acids into toxic amines [47]. Recently Martín *et al*. [45] considered isolated bacteria from human milk as staphylococci, streptococci, and lactic acid bacteria considered as the natural microbiota of breast milk rather than contaminant bacteria. Some of the

bacterial strains transferred to the infant gut through breast-feeding have a high probiotic potential and have a protective effect against infectious diseases [48].

Table 4: Microbial count of the fresh and stored breast milk samples (CFU: Colony forming units)

Sample / Microbial Count	Fresh cfu/ml	Cooling after 24 hour (cfu/ml)	Freezing after 15 day (cfu/ml)
Total bacterial count	3.0×10^2	3.0×10^2	<10
Coliform	-ve	-ve	-ve
Staph oureus	1.0×10^2	1.0×10^2	<10
Mold and yeast	5.0×10^2	7.0×10^2	<10

Table (4) shows mean of microbial count of the fresh and stored breast milk samples. It can be seen from the table that we found growth of bacterial as noticed from bacterial count, *staph. aurous* and mold & yeast in the fresh samples and in the samples which stored at cooling degree after 24 h, while almost no growth of these microorganisms was found in the samples which stored at freezing degree after 15 days. Bacterial, *staph, aurous* and mold & yeast count was decreased after storing in freezer for 14 days. No coli form was found in either or stored samples.

Our results agree with Lund [49]; Archer [50]; La Leche League International USA [51]; and Martinez-Costa *et al.*, [52] who stated that cold storage is an excellent way to preserve bacteria although the final variability depend on several factors as bacterial species, medium, concentration, temperature, and time. It is clear that the longer milk is stored the greater the risk of deterioration of its essential constituents and of an increase in bacterial contamination, even though because human milk is a living tissue, it has numerous immunologic properties that protect it from contamination.

In general: this study agrees with other studies as that of Zinn [53], Lawrence and Lawrence USA [54]; and Tully [55] which have been carried out under optimum conditions of storage and they noted that expressed breast milk could last up to 3-8 days in the refrigerator.

Conclusion

In conclusion our results reveal that milk can be stored safely in refrigerator for 24 h and for 2 weeks at -18: -20 in the freezer despite the loss of some macro & micronutrients which agree to somewhat with [10] despite they study longer duration as seen in table 5 and notes.

Table 5: Guidelines for healthy full-term babies [10]

Type	Location of storage	Temperature	Maximum recommended storage duration	This Study
Fresh	Room temperature	16–29˚C	4 hours optimal 6–8 hours acceptable under very clean conditions	Fresh
Stored	Refrigerator	4˚C	4 days optimal 5–8 days under very clean conditions	24 h
Stored	Freezer	<-18--20˚C	6 months optimal 12 months acceptable	15 days

Storage of Human Milk

1. Freshly expressed human milk may be stored safely at room temperature (18–29˚C) for 4 hours; 6–8 hours for very clean expressed milk with very low bacterial counts. Warmer ambient temperatures are associated with faster growing bacterial counts in stored milk [56-57].
2. Milk can be stored safely in refrigerator (4˚C) due to significant decline of bacterial capacity [52, 58-59] for 24-48h.
3. Milk can be stored safely in freezer (-18-20˚C) for up to 3 mo since thawed human milk, previously frozen for at least 6 weeks at -20˚C has the same bacterial viability and diversity as it did when it was freshly expressed [60]. Freezer storage affects nearly all vitamins and minerals in human milk [30, 61].

Financial support and sponsorship: Nil.
Conflicts of interest: There are no conflicts of interest.

References

[1]. Mahan LK and Sylvia ES. 2004. Krause's: Food, Nutrition, & Diet Therapy 11th ed. Pennsylvania: Saunders.
[2]. Lönnerdal Bo. 2013. Bioactive Proteins in Breast Milk. Journal of Paediatrics and Child Health; 49 (Suppl. 1): 1–7.
[3]. Mosca F, and Gianni ML. 2017. Human milk: composition and health benefits. Pediatr Med Chir.; 39 (2): 155.
[4]. Horta B, Bahl R., Martines G. & Victora C. 2007. Evidence on the Long-Term Effects of Breastfeeding: Systematic Review and Meta-Analyses. WHO: Geneva, Switzerland.
[5]. Ezz El Din ZM, Abd El Ghaffar S, El Gabry EK, Fahmi WA and Bedair RF. 2004. Is stored expressed breast milk an alternative for working Egyptian mothers? Eastern Mediterranean Health Journal; 10 (6): 815-821.
[6]. Silvestre D, Miranda M, Muriach M, et al. 2010. Frozen breast milk at –20 degrees C and –80 degrees C: A longitudinal study of glutathione peroxidase activity and malondialdehyde concentration. J Hum Lact.; 26: 35–41.
[7]. Arnold LDW. 1992. Human milk storage and preservation in the early 1900s. J Hum Lact.; 8: 91–92.
[8]. Horwood LJ, Darlow BA, and Mogridge N. 2001. Breast milk feeding and cognitive ability at 7-8 years. Arch Dis Child Fetal Neonatal Ed. Jan; 84(1): F23- 72.
[9]. Isaacs EB, Fischl BR, Quinn BT, Chong WK, Gadian DG, and Lucas A. 2010. Impact of breast milk on intelligence quotient, brain size, and white matter development. Pediatr Res.; 67(4): 357–62.
[10]. WICBreastfeeding.fns.usda.gov www.cdc.gov/breastfeeding
[11]. AOAC 2016.
[12]. Nadia RG-L, Diana E-V, Oscar G-Al, Javier De la Cruz, David L, and Carmen P-A.2012. Effect of Freezing Time on Macronutrients and Energy Content of Breast milk. Breastfeeding Medicine; 7(4): 295-301.
[13]. Curran SJ and Barness LA. 2000. Nutrition: the feeding of infants and children. In: Behrman RE, Kliegman RM, Jenson HB, eds. Nelson textbook of pediatrics. Philadelphia, WB Saunders, P: 149–169.
[14]. Perrin MT, Fogleman AD, Newburg DS, and Allen JC. 2016. A longitudinal study of human milk composition in the second year postpartum: implications for human milk banking. Matern Child Nutr.; 13(1): e12239.
[15]. Glew RH, Wold RS, Herbein JH, Wark WA, Martinez MA, and Vanderjagt DJ. 2008. Low docosahexaenoic acid in the diet and milk of women in New Mexico. J Am Diet Assoc.; 108(10): 1693–1699.
[16]. Glew RH, Wold RS, Corl B, Calvin CD, and Vanderjagt DJ. 2011. Low docosahexaenoic acid in the diet and milk of American Indian women in New Mexico. J Am Diet Assoc.; 111(5):744–748.
[17]. Butte NF, Wong WW, Ferlic L, Smith EO, Klein PD, and Garza C.1990. Energy expenditure and deposition of breast-fed and formula-fed infants during early infancy. Pediatr Res.; 28(6): 631–640.
[18]. Nommsen LA, Lovelady CA, Heinig MJ, Lönnerda lB, and Dewey KG. 1991. Determinants of energy, protein, lipid and lactose concentrations in human milk during the first 12mo of lactation: the Darling study. Am J Clin Nutr.; 53(2): 457–465.
[19]. Ogundele MO. 2000. Techniques for the Storage of Human Breast Milk: Implication for Anti-microbial Function and Safety of Stored Milk. Eur J Pediatr.; 159: 759-797.

[20]. Roesli U. 2000. Mengenal ASI Ekslusif. Jakarta: Trubus Agriwidya.
[21]. Hamosh M, Lorie AE, Darren RP, Theresa RH and Paul H.1996. Breast-feeding and the Working Mother: Effect of Time and Temperature of Short-term Storage on Proteolysis, and Bacterial Growth in Milk. Pediatrics; 97: 492-498.
[22]. Pardou A *et al*., 1994. Human milk banking: influence of storage processes and of bacterial contamination on some milk constituents. Biology of the neonate; 65: 302-309.
[23]. Saarela T, Kokkonen J, and Koivisto M. 2005. Macronutrient and energy contents of human milk fractions during the first six months of lactation. Acta Paediatr.; 94(9): 1176–1181.
[24]. Kent JC, Mitoulas LR, Cregan MD, Ramsay DT, Doherty DA, and Hartmann PE. 2006. Volume and frequency of breast feedings and fat content of breast milk throughout the day. Pediatrics; 117(3): e387–e395.
[25]. Bertino E, Giribaldi M, Baro C, *et al*. 2013. Effect of prolonged refrigeration on the lipid profile, lipase activity, and oxidative status of human milk. J Pediatr Gastroentero Nutr.; 56: 390–396.
[26]. Morrow AL, Ruiz-Palacios GM, Jiang X, and Newburg DS 2005. Human-milk glycans that inhibit pathogen binding protect breast-feeding infants against infectious diarrhea. The Journal of nutrition; 135(5): 1304–1307.
[27]. Iqbal M, Lily AL, Weny K, and Nur LM. 2016. Effect of Temperature and Storage Duration on Lactose, Protein and Fat Content of Breast Milk. International Conference on Health and Well-Being (ICHWB) 2016.
[28]. Bank MR, Kirksey A, West K, and Giacoia G. 1985. Effect of storage time and temperature on folacin and vitamin C levels in term and preterm human milk. Am J Clin Nutr.; 41: 235–242.
[29]. Romeu-Nadal M, Castellote A, and Lopez-Sabater M. 2008. Effect of cold storage on vitamins C and E and fatty acids in human milk. Food Chem,; 106: 65-70.
[30]. Buss I, McGill F, Darlow B, *et al*. 2001. Vitamin C is reduced in human milk after storage. Acta Paediatr.; 90: 813–815.
[31]. Ahrabi A, Handa D, Codipilly C, *et al.* 2016. Effects of extended freezer storage on the integrity of human milk. J Pediatr.; 177: 140–143.
[32]. Winiarska-Mieczan A. 2014. Cadmium, lead, copper and zinc in breast milk in Poland. Biol Trace Elem Res., 157: 36–44.
[33]. Shi YD, Sun GQ, Zhang ZG, Deng X, Kang XH, Liu ZD, Ma Y, & Sheng QH. 2011. The chemical composition of human milk from Inner Mongolia of China. Food Chem.; 127: 1193 – 1198.
[34]. Matos C, Moutinho C, Balcão V, Almeida C, Ribeiro M, Marques FA, and Guerra A. 2009. Total antioxidant activity and trace elements in human milk: The first 4 months of breastfeeding. Eur. Food Res. Technol.; 230: 201–208.
[35]. Björklund KL, Vahter M, Palm B, Grandér M, Lignell S, & Berglund M. 2012. Metals and trace element concentrations in breast milk of first time healthy mothers: a biological monitoring study. Environ Health., 11: 92.
[36]. Andrade MTS, Del Ciampo LA, Del Ciampo IR, Ferraz IS, & Barbosa Junior, F. 2014. Breast Milk Micronutrients in Lactating Mothers from Ribeirão Preto (SP), Brazil. Food Nutr. Sci;. 5:1196 – 1201.
[37]. Līva A, Inga C, Dace Z, Konstantīns B, and Anastasija B. 2018. Zinc Content in Breast Milk and Its Association with Maternal Diet. Nutrients; 10 (10): 1438.
[38]. Domellöf M, Lönnerdal B, Dewey KG, Cohen RJ, and Hernell O. 2004. Iron, zinc, and copper concentrations in breast milk are independent of maternal mineral status. Am. J. Clin. Nutr. 2004; 79: 111–115.
[39]. Doneray H, Olcaysu E, Yildirim A, and Ozden A. 2017. The effect of the zinc concentration in breast milk on neonatal weight gain. J. Trace Elem. Med. Biol.; 41: 32–35.

[40]. Neville MC, Keller RP, Seacat J, Casey CE, Allen JC, and Archer P. 1984. Studies on human lactation. I. Within-feed and between-breast variation in selected components of human milk. Am. J. Clin. Nutr.; 40: 635–646.
[41]. Alam S., Hennigar S.R., Gallagher C., Soybel D.I., and Kelleher S.L. 2015. Exome sequencing of SLC30A2 identifies novel loss- and gain-of-function variants associated with breast cell dysfunction. J. Mammary Gland Biol. Neoplasia.; 20:159-172.
[42]. Spitzer J, Klos K, and Buettner A. 2013. Monitoring aroma changes during human milk storage at +4C by sensory and quantification experiments. Clin Nutr.; 32: 1036–1042.
[43]. Handa D, Ahrabi AF, Codipilly CN, *et al.* 2014. Do thawing and warming affect the integrity of human milk? J Perinatol.; 34: 863–866.
[44]. Ng DK, Lee SYR, Leung LCK, *et al*. 2004. Bacteriological screening of expressed breast milk revealed a high rate of bacterial contamination in Chinese women. J Hosp Infect 2004; 58:146–150.
[45]. Martín R, Langa S, Reviriego C, *et al*. 2003. Human milk is a source of lactic acid bacteria for the infant gut. J Pediatr.; 143:754–758.
[46]. Deodhar L and Joshi S. 1991. Microbiological study of breast milk with special reference to its storagre in milk banks. J Postgrad Med.; 37: 14-16.
[47]. Björklund KL, Vahter M, Palm B, Grandér M, Lignell S, & Berglund M. 2012. Metals and trace element concentrations in breast milk of first time healthy mothers: a biological monitoring study. Environ Health., 11: 92.
[48]. Martín R, Olivares M, Marín ML, *et al*. 2005. Probiotic potential of 3 lactobacilli strains isolated from breast milk. J Hum Lact.; 21:8–14.
[49]. Lund BM. 2000. Freezing. In: Lund BM, Baird Parker TC, Gould GW, (eds). The Microbiological Safety and Quality of Food, Vol. 1. Gaithersburg, MD: Aspen Publishers; 2000, pp. 122–45.
[50]. Archer DL. 2004. Freezing: an underutilized food safety technology? Int J Food Microbiol 2004; 90:127–138.
[51]. La Leche League International. The breast feeding answer book. 3rd ed. Illinois, USA; 2003.
[52]. Martı´nez-Costa C, Silvestre M, Lo´pez M, *et al.* 2007. Effects of refrigeration on the bactericidal activity of human milk: A preliminary study. J Pediatr Gastroenterol Nutr.; 45: 275–277.
[53]. Zinn B. Supporting the employed breast feeding mother. J. Midwifery Women's Hlth. 2000; 45: 216-226.
[54]. Lawrence R and R Lawrence 2005. Breastfeeding: A Guide for the Medical Profession. Elsevier Mosby, Philadelphia, USA**.**
[55]. Tully MR. 2000. Recommendations for handling of mother's own milk. J. Hum. Lact.; 16: 149S-151S.
[56]. Eteng M, Ebong P, Eyong E, *et al.* 2001.Storage beyond three hours at ambient temperature alters the biochemical and nutritional qualities of breast milk. Afr J Reprod Health; 5:130–134.
[57]. Igumbor E, Mukura R, Makandiramba B, *et al.* 2000. Storage of breast milk: Effect of temperature and storage duration on microbial growth. Cent Afr J Med.; 46: 247–251.
[58]. Ogundele MO. 2002. Effects of storage on the physicochemical and antibacterial properties of human milk. Br J Biomed Sci.; 59 (4): 205-211.
[59]. Silvestre D, Lo´pez M, March L, *et al.* 2006. Bactericidal activity of human milk: Stability during storage. Br J Biomed Sci.; 63: 59–62.
[60]. Marı´n ML, Arroyo R, Jime´nez E, *et al.* 2009. Cold storage of human milk: Effect on its bacterial composition. J Pediatr Gastroenterol Nutr.; 49: 343–348.
[61]. Romeu-Nadal M, Castellote A, and Lopez-Sabater M. 2008. Effect of cold storage on vitamins C and E and fatty acids in human milk. Food Chem,; 106: 65-70.