

IRON FORTIFIED PASTEURIZED MILK: PHYSICO-CHEMICAL ATTRIBUTES AND EFFICACY AGAINST IRON DEFICIENCY ANAEMIA IN SPRAGUE DAWLEY RATS

N. Akhter, S. Iqbal, M. Nasir, A. Ashraf, U. Bacha and M. A. Khan*

Department of Food Science & Human Nutrition, University of Veterinary and Animal Sciences, Lahore-Pakistan

*Department of Epidemiology and Public Health, University of Veterinary and Animal Sciences, Lahore-Pakistan

Corresponding author Email: sanaullah.iqbal@uvas.edu.pk

ABSTRACT

Iron Deficiency Anaemia (IDA) is a serious public health issue involving large number of population around the globe especially from the developing countries. In Pakistan, the iron deficiency is a crucial nutritional problem and almost one fifth of country's women are suffering. For community based management of IDA, iron fortification in various food commodities is considered to be the best approach. Keeping in mind the importance of this issue, iron fortified pasteurized milk (IFPM) was prepared and its ability to reduce the IDA in Sprague Dawley rats was evaluated. Raw buffalo milk was fortified with FeSO_4 at a concentration of 0.0%, 0.04%, 0.06% and 0.08% of milk solids and vitamin C (30mg/100g of milk) followed by pasteurization at 75°C for 16sec.

Physico-chemical and sensory attributes of all treatments were not significantly different from control with the exception of vitamin C and iron contents. The improvement of haemoglobin concentration in rats after consumption of fortified milk was found to be statistically significant as compared to control ($P < 0.05$) and IFPM has increased the haemoglobin level from 9.84 ± 0.287 to 14.37 ± 0.325 g/dL in 56 days.

It can be concluded from this study that the iron fortification in pasteurized milk does not affect its sensory and physical characteristics and can be helpful to improve IDA.

Key words: Pasteurized Milk, Iron Fortification, Iron Deficiency Anaemia.

INTRODUCTION

The survival of human body without food and drinking water is three weeks and three days respectively, but without oxygen not more than three minutes (Wolchover, 2012). The supply of sufficient oxygen to each and every cell in the body is necessary for its survival and function as it provides key role in extraction of energy from food constituents. The same is the case with iron as it is a necessary agent of the molecule that carries oxygen i.e. Haemoglobin (Hb). The range of normal Hb level for men and women is 14-18 and 12-16 g/dL of blood, respectively and normal hematocrit for men and women 42-54% and 36-48%, respectively (Reid *et al.*, 2004).

Anaemia is one of wide spread public health problems and according to a survey conducted by WHO from 1993 to 2005 about 1.62 billion people which corresponds to around 25% of world population, are suffering from anaemia and more than 50% of the above are suffered from IDA (WHO/CDC, 2008). IDA is "the condition in which there is decline in Hb concentration (<11 and <12 g/dL in children and non-pregnant women, respectively) in blood due to iron deficiency" (WHO/CDC, 2008). According to recent statistics in Pakistan, 43.8% of children (<5 years of age), 26.8% of non-pregnant women and 37% of pregnant women are suffering from IDA (NNS, 2011).

Iron fortification is an effective approach to reduce iron deficiency but may result in organoleptic changes in the final product which involves undesirable colour and flavour. To avoid these problems and to make product more acceptable to consumer, the addition of antioxidant is the appropriate approach (Hurrell, 2002). When cost effectiveness, bioavailability and fortificant stability is under consideration; preference is given to ferrous iron in which ferrous sulphate and gluconate are mostly used (Zlotkin *et al.*, 2001). Cereal flours and other cereal-based products are good vehicle for iron fortification but the presence of phytic acid as an inhibitor is the main drawback which hinders iron absorption in addition to their sensitivity to fat oxidation (Hurrell, 2002). But on the other way, milk can be an effective vehicle for iron fortification with the addition of vitamin C as antioxidant (Bradley *et al.*, 1993) especially for Pakistan which is the fourth largest milk producer country in the world (FAO, 2011) with annual production of 49.5 thousand tons in 2012-13 (PES, 2013). In Pakistan, buffalo is the main source for milk production contributing 30% to the world buffalo milk production (FAOSTAT, 2013). Unfortunately buffalo milk is very poor source of iron having 0.42 to 1.5mg/L (Enb *et al.*, 2009; Ahmed *et al.*, 2013). Various studies have proved that fortification of iron (13-30mg/L) is helpful in reduction of IDA in human of different age groups (Salvador *et al.*, 2006; Hoa *et al.*, 2005). Similarly,

healthy infant formulas based on milk containing 7.4 - 12.7mg/L of iron are appropriate for their normal growth and iron status (Bradley *et al.*, 1993). Due to high prevalence of IDA in Pakistan, this study aimed to give preventive approach in treating this condition. It was assumed that supplementing pasteurized milk could be a suitable approach in the dairy industry as compared to previously used fortification in cereal based products for the management of IDA.

MATERIALS AND METHODS

Procurement of Raw Materials: All chemicals and reagents were of highest purity and were purchased from Merck Pvt. Limited. The buffalo raw milk was procured from Animal Nutrition Center, Rakh Dera Chahal, Badian Road, Lahore. Ingredients for Sprague Dawley rat's diet were purchased from local market.

Development of Iron Fortified Pasteurized Milk (IFPM): Raw buffalo milk was fortified with FeSO₄ at a concentration of 0.0% (as a control), 0.04%, 0.06% and 0.08% level (w/w) (0, 15, 23, and 31mg of Fe/L, respectively) by following the procedure as described by Nakano *et al.*, 2007. After mixing, vitamin C (30mg/100g of milk) was added and mixed continuously for further 15 minutes. The IFM was pasteurized at 75°C for 16 sec at Food Analysis Lab., Department of Food Science and Human Nutrition, University of Veterinary and Animal Sciences (UVAS), Lahore.

Physico-chemical Analysis of IFPM: The IFPM was analyzed for fat, protein, solids-not-fat (SNF) by Lactoscan (Milk analyzer, serial No. 6429- Bulgaria) at Quality Operation Lab. UVAS, Lahore. The pH, acidity and total solids of milk samples were analyzed through AOAC (2006) methods and vitamin C contents by titrimetric method as described by Bosset *et al.*, 1991. The concentration of iron was determined through atomic absorption spectrophotometer (Perkin Elmer, AAnalyst 400, Massachusetts, USA).

Organoleptic Acceptability: IFPM was subjected to sensory evaluation by a semi-trained panel of 12 judges as described by Meilgaard *et al.*, 2007. Description of sensory attributes for the evaluation of IFPM was given to the panel. Evaluation was carried out using 15 cm unconstructed line for parameters of colour, aroma, taste, consistency and overall acceptability on a sensory evaluation proforma (Meilgaard *et al.*, 2007).

Efficacy Study: The efficacy studies of IFPM against anaemia was conducted on Sprague Dawley rats (n=14). They were housed in Animal Room, Department of Microbiology, UVAS, Lahore at a temperature of 25±2°C and relative humidity of 55±5% in a controlled light (12hr light-dark cycle) room. Iron deficient diet was

given for three weeks to make them iron deficient as described by Nakano *et al.*, 2007. After this period blood samples were taken from seven randomly selected rats & analyzed for Hb concentration. After developing anaemic conditions, rats were divided into two groups (Group A and B having 7 rats in each group). Group A was fed on iron deficient diet plus pasteurized milk and Group B on iron deficient diet plus IFPM. Blood sampling and analysis was carried out on fortnightly basis for a period of two months. The Hb level was determined by Sahlis method as described by Balasubramaniam and Malathi (1992).

Statistical Analysis: The characterization and chemical composition data for various treatments was presented in means. The data for product development i.e. control and three treatments of iron fortification, was analyzed through analysis of variance technique under CRD (Steel *et al.*, 1997). Mean values were compared for significance of difference with the Duncan's Multiple Range test. The *t*-test was applied to evaluate the difference of Hb level in two groups of Sprague Dawley rats. The statistical significance was defined as $P < 0.05$. CoHort software version CoStat 6.303 was used for all statistical analyses.

RESULTS AND DISCUSSION

Pakistan is the 4th largest milk producer country in the world (FAO 2011) and milk and/or other dairy products are used frequently in every home in one of the meals by persons of all ages but milk is the poor source of iron. To best of our knowledge, this is first effort in Pakistan to produce IFPM from buffalo. Some infant formulas, milk powders and UHT dairy products can be seen but liquid pasteurized milk is not available in Pakistan market.

Characterization of Raw Milk: The idea about nutritional status of any food ingredient is evaluated through its chemical /proximate composition. Proximate analysis of raw buffalo milk showed that the fat contents are 4.80±0.01%, for protein the value is 3.02±0.07%, the value for SNF is 8.22±0.06% and total solids are 13.23±0.06%. The results for acidity, vitamin C, pH and iron are 0.12±0.004 (% lactic acid), 1.50±0.89mg/100mL, 6.81±0.01 and 0.497mg/L, respectively. It is the normal composition of buffalo milk in the months of March/April but it can vary by several factors like month of year, animal feed, milking time, etc.

Composition and Chemical Properties of IFPM and Effect of Storage: The mean values for chemical attributes of various treatments of IFPM and results for their storage analysis are presented in Table 1 and 2, respectively. Fat content of fortified milk for various treatments showed non-significant effect. The highest

value for fat content was observed for 0.04% treatment level i.e. 4.63% while the lowest value 4.23% for 0.06% treatment. As the storage progressed there is no statistical difference on fat content of milk and it varies from 4.39% to 4.67% at 0 and 4 day, respectively. These findings are in line with Lavigne *et al.* (1989) who did not observe any change in fat content after storage of fortified milk.

Data regarding mean values for protein contents of different treatments showed that the mean value for control milk is 3.14% and there is no significant difference with iron fortification at 0.04, 0.06 and 0.08%. In another study, some different results have been shown and there is increase in protein content of control milk as compared to the ones which are supplemented by iron, zinc and vitamin C (Biringen *et al.*, 2003). This might be due to higher concentration of iron fortification in the above mentioned study as iron combines with casein and results in complex structures. As the storage progressed the protein content not differs significantly from 3.02% to 3.18% at 0 and 4 day, respectively. The SNF for IFPM showed statistically significant results between control and treatments ($P < 0.05$). The maximum value was observed for treatment 0.08% of FeSO_4 i.e. 8.82% and lowest for control, 8.21%. This might be due to treatment effect as increase in the concentration of iron proceeds from control to 0.08%, the SNF contents also increase.

The pH varied significantly among the treatments and increased as the storage progressed. This shows that addition of FeSO_4 has resulted in decrease in pH as highest level of fortification showed lowest value. This might be due to intentionally added vitamin C. These results are also supported by another study conducted by Rosenthal *et al.*, (1993) who reported the similar effect for decrease in pH as concentration of iron fortification and vitamin C increased. As the storage progressed, the pH decreases significantly from 6.74 at 0 day to 6.29 at 4 day, respectively. These findings are in line with Biringen *et al.*, (2003) which showed pH decreases during 5 day storage of iron and vitamin C fortified milk. Although all samples were pasteurized and placed at 04 °C where most of the bacteria become dormant but due to fluctuation in storage temperature, some microbes might be able to grow and convert lactose to lactic acid which ultimately reduce the pH and increase acidity. Another reason for variation of pH in control and treatments may be the addition of vitamin C/ascorbic acid. The acidity of the samples as a function of treatment varies significantly and maximum is observed for treatment 0.08% i.e. 0.15 and lowest for control, 0.12% lactic acid. With respect to storage analysis for acidity as storage time precedes acidity increases and pH reduces significantly.

Vitamin C: Mean values for vitamin C among different treatments showed that the values for control milk are 1.50mg/100mL and increases significantly with iron

fortification at 0.04, 0.06 and 0.08% which is 25.68, 26.01 and 25.26, respectively as compared to control. This is due to intentionally added vitamin C (30mg/100g of milk) in three treatments. Some studies have revealed that bioavailability of iron is directly proportional to vitamin C concentration up to the limit of acceptable sensory attributes of fortified milk. This is evidenced from two community based studies conducted in Chile in 1972 and 1974 (Stekel *et al.*, 1986). In the first study, 15mg of Fe/L (without ascorbic acid) in reconstituted milk powder was given to 3 month old infants and reduction in IDA from 35% to 13% was observed. In the 2nd study, vitamin C was added in the milk powder at the molar ratio of 2:1 in addition to Fe (15mg/L) which had resulted in IDA from 28% to less than 2%. Ascorbic acid concentration of 100 to 800mg/L of cow's milk is acceptable and enhances the bioavailability of iron from 5.9 to 11.3% in a study conducted on 396 infants of 5-10 months of age (Abraham *et al.*, 1986). Biringen *et al.* (2003) have fortified milk with various concentrations of vitamin C (30, 40, 45 and 50mg/100g), iron and zinc and stored this milk at 04 °C for 7 days and have concluded that the amount 30mg/100g is the most appropriate for good milk acidity and to avoid negative impact on sensory attributes. In the current study, theoretical values for vitamin C in all three treatments except control should be more than 30mg/100g but actual value is quite low. The reason is heat treatment i.e. milk pasteurization at 75 °C for 16 sec has reduced the concentration of vitamin C. As the storage progressed, the mean value for vitamin C decreases significantly from 22.7 to 18.5mg/100mL at 0 and 4 day, respectively. Rosenthal *et al.* (1993) have observed 30-35% losses of ascorbic acid after 7 days of refrigerated storage in pasteurized milk fortified with 100mg/100mL vitamin C and 5mg/100mL iron in the form of ferrous lactate. In another study, vitamin C contents have been decreased 35% of initial value after 3 days of storage at 04 °C. The fortified vitamin C to dairy products undergoes degradation during storage period and it depends upon type and intensity of heat treatment and also on the permeability of the packaging material to oxygen (Gliquem and Birloouez-Argon, 2005).

Sensory Evaluation of IFPM: The evaluation of different sensory parameters of fortified milk was done by semi-trained panel for the consumer response and results are given in Table 3. Sensory evaluation showed that before storage flavour, taste, consistency and overall acceptability scores were non-significant. The highest scores were given to 0.06% for colour, flavor, taste, overall acceptability except consistency parameter in which 0.04 % showed the highest value i.e. 9.54 ± 3.09 . The lowest scores were given to 0.08% for all the attributes except for colour but all treatments were not significantly different to each other. Supplementation of un-homogenized milk with iron salts, as the case in our

study, can enhance lipid oxidation which results in off-flavour but this problem can be minimized by adding an antioxidant like vitamin C. The statistical analysis for colour scores, flavour, consistency, taste, overall acceptability indicated non-significant effect of treatment and storage interaction (Figure 1). This means that addition of iron and the storage has not affected badly the colour, flavour, consistency, taste and overall acceptability of milk and FeSO₄ fortification with vitamin C can be used to produce various dairy products to improve IDA without compromising the sensory attributes of the products. In a study, iron fortified (10, 20 and 40mg of iron/Kg of yoghurt) low and non-fat yoghurt was prepared and trained panel of 11 judges was not able to differentiate the fortified and unfortified flavoured yoghurt in appearance, mouth feel, flavour and overall quality (Hekmat and McMahan, 1997).

Efficacy Study: The statistical analyses (*t*-test) regarding Hb content of control and IFPM group of Sprague Dawley rats with respect to study interval showed that both groups at 0 day are non significant (*P* 0.05) whereas at 14 and 28 days results are slightly significant (*P* 0.05) and further at 42 and 56 days quite significant results were shown (*P* 0.01). Data regarding mean values for Hb level of group B has shown progression from 0 Day to 56 Day and showed values of 9.84 ±0.287 and 14.37±0.325

g/dL, respectively. At 14, 28 and 42 days values of Hb for group B are 10.99±0.406, 13.21±1.14 and 14.44±0.349g/dL, respectively (Figure 2). These findings are in line with the Zubillaga *et al.*, (1996) who fortified milk with FeSO₄ and improvement in Hb was determined. These results show that ~80% of the iron was consumed in blood followed by 11% in liver after 18 days of treatment with IFM. The use of pasteurized cow's milk has reduced the prevalence of IDA from 62.3% to 41.8% and 26.4% after 6 months and 1-year of study, respectively, in the children aged 6-42 months (Torres *et al.*, 1996). Eichler *et al.*, (2012) conducted a meta-analysis to evaluate the effect of micronutrient fortified milk and cereal based food on infants and children (6 months to 5 years of age) and revealed that IFM is a good vehicle to manage/reduce anaemia. In another study in Mexico, IFM was distributed on large scale in low-income young children (6 -12 months age) and concluded that this was an effective program to reduce iron deficiency and IDA (Rivera *et al.*, 2010). In another similar study in India, a micronutrient fortified milk containing 9.6mg of iron with other minerals and vitamins was given to 633 children of 1-4 years of age for a period of one year and concluded that milk is a quite effective vehicle for micronutrient fortification especially iron and zinc (Sazawal *et al.*, 2010).

Table 1. Mean values for chemical attributes of Iron Fortified Pasteurized Milk.

Treatments	Fat %	Protein %	SNF %	Total Solids %	pH	Acidity % (l.a)	Vitamin C (mg/100g of milk)	Iron (Fe) mg/L
0%	4.39 ^a	3.14 ^a	8.21 ^b	12.61 ^b	6.62 ^a	0.12 ^c	1.50 ^b	0.497 ^d
0.04%	4.63 ^a	3.15 ^a	8.73 ^a	13.37 ^a	6.51 ^c	0.14 ^{ab}	25.68 ^a	14.07 ^c
0.06%	4.23 ^a	3.22 ^a	8.66 ^{ab}	12.90 ^{ab}	6.55 ^b	0.14 ^{ab}	26.01 ^a	21.56 ^b
0.08%	4.35 ^a	3.21 ^a	8.82 ^a	13.17 ^{ab}	6.50 ^c	0.15 ^a	25.26 ^a	30.07 ^a

Means sharing the same letter in a column are not significantly different. IFPM was prepared by adding different concentrations of FeSO₄ and vitamin C (30mg/100g of milk). l.a., Lactic acid

Table 2. Mean values for chemical attributes of Iron Fortified Pasteurized Milk with respect to s storage.

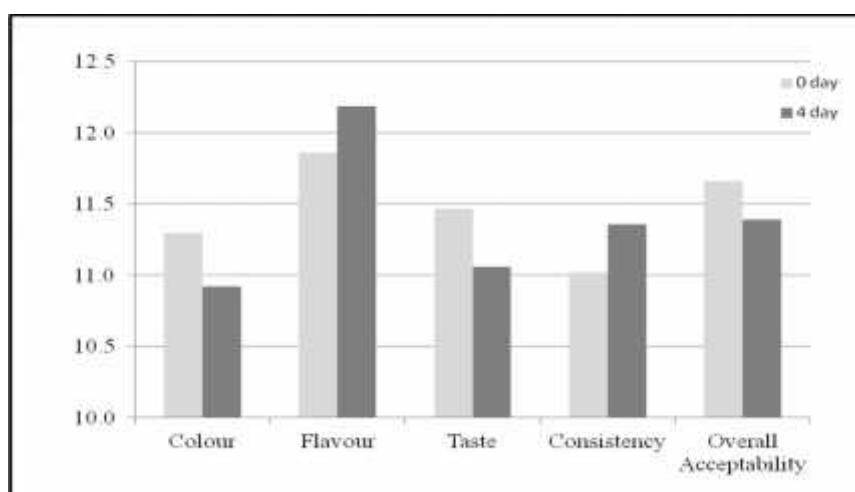
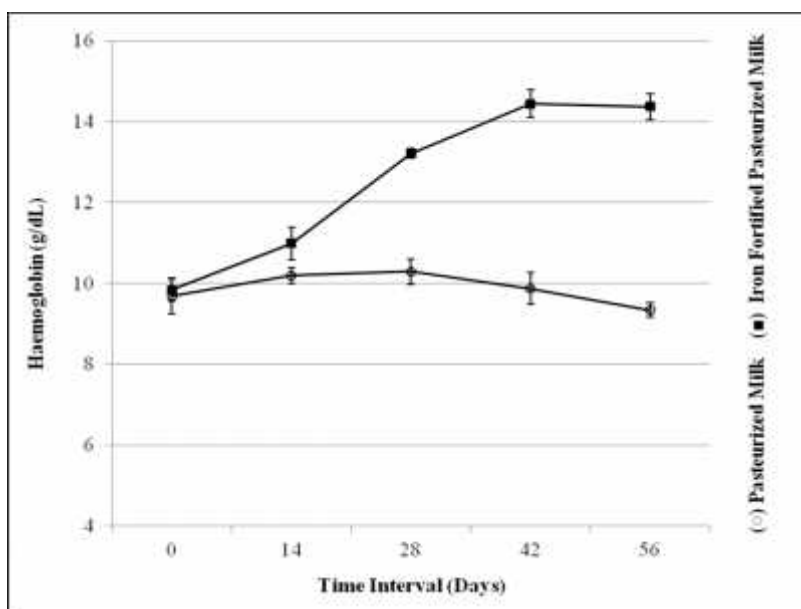
Days	Fat %	Protein %	SNF %	Total Solids %	pH	Acidity % (l.a)	Vitamin C (mg/100g of milk)	Iron (Fe) mg/L
0	4.67 ^a	3.02 ^a	8.50 ^{ab}	13.17 ^a	6.74 ^a	0.12 ^d	22.7 ^a	16.31 ^a
1	4.54 ^a	3.34 ^a	8.70 ^{ab}	13.25 ^a	6.67 ^b	0.12 ^d	21.0 ^b	---
2	4.42 ^a	3.02 ^a	8.22 ^b	12.22 ^b	6.55 ^c	0.13 ^c	20.1 ^{bc}	---
3	4.40 ^a	3.47 ^a	8.79 ^a	13.22 ^a	6.46 ^d	0.15 ^b	19.3 ^{cd}	---
4	4.39 ^a	3.18 ^a	8.81 ^a	13.22 ^a	6.29 ^e	0.17 ^a	18.5 ^d	15.91 ^a

Means sharing the same letter in a column are not significantly different. IFPM was prepared by adding different concentrations of FeSO₄ and vitamin C (30mg/100g of milk). The milk was stored at 4 °C for 5 days and changes in chemical composition and attributes were analyzed. l.a., Lactic acid

Table 3. Small-panel sensory evaluation mean scores of Iron Fortified Pasteurized Milk.

Treatment	Colour	Flavor	Taste	Consistency	Overall acceptance
0%	9.91±1.38 ^a	9.27±2.59 ^a	9.80±2.55 ^a	9.36±3.15 ^a	10.51±2.95 ^a
0.04%	10.15±1.85 ^a	9.03±2.96 ^a	9.69±2.82 ^a	9.54±3.09 ^a	10.58±3.27 ^a
0.06%	10.60±1.76 ^a	9.72±3.09 ^a	10.84±2.89 ^a	9.52±3.55 ^a	10.66±3.54 ^a
0.08%	10.05±2.16 ^a	8.85±2.55 ^a	9.41±2.56 ^a	9.33±3.27 ^a	9.79±3.41 ^a

Means sharing the same letter in a column are not significantly different. IFPM was prepared by adding different concentrations of FeSO₄ and vitamin C (30mg/100g of milk) and sensory attributes were analyzed by semi-trained panel.

**Figure 1. Small-panel sensory evaluation mean scores of Iron Fortified Milk and storage.****Figure 2. Means of Haemoglobin level of both A and B groups along with study interval**

Conclusion: This study shows that physico-chemical and sensory attributes of pasteurized buffalo milk fortified with ferrous sulfate (0.06% of total solids) and vitamin C (30mg/100g of milk) is acceptable and has capability to reduce IDA in Sprague Dawley rats. It can be concluded

that pasteurized milk can be an effective vehicle to fortify iron in combination with vitamin C without compromising sensory attributes and to combat IDA in Sprague Dawley rats. It can also be expected that iron fortification in milk will be quite effective for infants,

young children, pregnant and non-pregnant women and other iron deficiency anemic individuals.

REFERENCES

- Abraham, S., M. Olivares, F. Pizarro, P. Chadud, I. Lopez, and M. Amar (1986). Absorption of fortification iron from milk formulas in infants. *Am. J. Clin. Nutr.* 43(6): 917-922.
- Ahmed, S., F. M. Anjum, N. Human, A. Sameen and T. Zahoor (2013). Composition and physico-chemical characteristics of buffalo milk with particular emphasis on lipids, proteins, minerals, enzymes and vitamins. *J. Anim. Plant Sci.* 23(1): 62-74.
- AOAC. (2006). The official methods of analysis of AOAC International, 18th Ed. The Association Official Analytic Chemist. Arlington, U.S.A.
- Balasubramaniam, P., and A. Malathi (1992). Comparative study of haemoglobin estimated by Drabkin's and Sahli's methods. *J. Postgrad. Med.* 38(1): 8-9.
- Biringen, L.G., M. Ugur, and M. Yildiz (2003). A partial supplementation of pasteurized milk with vitamin C, iron and zinc. *Nahrung.* 47(1):17-20.
- Bosset, J.O., P. Eberhard, and U. Butikofer (1991). Evaluation of criteria of milk deterioration after various heat and mechanical treatments as well as light exposure of different durations. Part I. Vitamin C. *Mitt. Geb. Lebensmittelunters. Hyg.* 82: 433-456.
- Bradley, C.K., L. Hillman, A.R. Sherman, D. Leedy, and A. Cordano (1993). Evaluation of two iron-fortified, milk-based formulas during infancy. *Pediatrics.* 91(5): 908-914.
- Eichler, K.S., I. Wieser, and U. U.B. Rütthemann (2012). Effects of micronutrient fortified milk and cereal food for infants and children: a systematic review. *BMC Public Health.* 12: 506-519.
- Enb, A., M.A. Abou Donia, N.S. Abd-Rabou, A.A.K. Abou-Arab, and M.H. El-Senaity (2009). Chemical composition of raw milk and heavy metals behavior during processing of milk products. *Global Veterinaria.* 3(3): 268-275.
- FAO (2011). Dairy production and products. Food and Agriculture Organization of the United Nations. [Accessed on 27th March 2014.] <http://www.fao.org/agriculture/dairy-gateway/milk-production/en/#.UzOmjfmSyCE>
- FAOSTAT (2013). Food and Agriculture Organization statistical year book 2013. Rome, Italy. [Accessed on 27th March 2014.] <http://www.fao.org/docrep/018/i3107e/i3107e00.htm>
- Gliquem, H., and I. Birloouez-Argon (2005). Effects of sterilization, packaging and storage on vitamin C, degradation, protein denaturation and glycation in fortified milks. *J. Dairy Sci.* 88(3): 891-899.
- Hekmat, S., and D.J. McMahon (1997). Manufacture and quality of iron-fortified yogurt. *J. Dairy Sci.* 80(12): 3114-3122.
- Hoa, P.T., N.C. Khan, C. van Beusekom, R. Gross, W.L. Conde, and H.D. Khoi (2005). Milk fortified with iron or iron supplementation to improve nutritional status of pregnant women: an intervention from rural Vietnam. *Food Nutr. Bull.* 26(1): 32-38.
- Hurrell, R.F. (2002). How to ensure adequate iron absorption from iron-fortified food. *Nutr. Rev.* 60(7 Pt.2): S7-15.
- Lavigne, C., J.A. Zee, and B.J. Beliveau (1989). Effect of processing and storage conditions on the fate of vitamins B₁, B₂, and C and on the shelf-life of Goat's milk. *J. Food Sci.* 54(1): 30-34.
- Meilgaard, M.C., G.V. Civille, and B.T. Carr (2007). Sensory evaluation techniques, 4th Ed., CRC Press, Boca Raton, Florida (USA).
- Nakano T., T. Goto, T. Nakaji, and T. Aoki (2007). Bioavailability of iron-fortified whey protein concentrate in iron-deficient rats. *Asian. Austral. J. Anim.* 20(7): 1120-1126.
- NNS (2011). National Nutritional Survey, Planning Commission, Planning and Development Division, Government of Pakistan. [Accessed date: 26th Aug. 2013] http://pakresponse.info/LinkClick.aspx?fileticket=scqw_AUZ5Dw%3D&tabid=117&mid=752
- PES (2013). Pakistan Economic Survey 2012-13. *In* Agriculture. P. 30. [Accessed date: 27th March 2014] http://finance.gov.pk/survey/chapters_13/02-Agriculture.pdf
- Reid, S.A., D.B. Speedy, J.M. Thompson, T.D. Noakes, G. Mulligan, T. Page, R.G. Campbell, and C. Milne (2004). Study of hematological and biochemical parameters in runners completing a standard marathon. *Clin. J. Sport Med.* 14(6): 344-353.
- Rivera, J.A., T. Shamah, S. Villalpando, and E. Monterrubio (2010). Effectiveness of a large-scale iron-fortified milk distribution program on anaemia and iron deficiency in low-income young children in Mexico. *Am. J. Clin. Nutr.* 91(2):431-438.
- Rosenthal, I., B. Rosen, and S. Bernstein (1993). Effects of milk fortification with ascorbic acid and iron. *Milchwissenschaft.* 48(12): 676-679.
- Salvador, V., T. Shamah, J.A. Rivera, Y. Lara, and E. Monterrubio (2006). Fortifying milk with ferrous gluconate and zinc oxide in a public nutrition program reduced the prevalence of

- anaemia in toddlers. *J. Nutr.* 136(10): 2633-2637.
- Sazawal, S., U. Dhingra, P. Dhingra, G. Hiremath, and A. Sarkar (2010). Micronutrient fortified milk improves iron status, anaemia and growth among children 1–4 years: A double masked, randomized, controlled trial. *PLoS ONE* 5(8): e12167. doi:10.1371/journal.pone.0012167.
- Steel, R.G.D., J.H. Torrie, and D.A. Dickey (1997). *Principles and procedures of statistics. A biometrical approach*, 3rd Ed. McGraw Hill Book Co Inc., New York.
- Stekel, A., F. Moncheberg, and V. Bayda (1986). *Combating iron deficiency in Chile: a case study: a report of the International Nutritional Anaemia Consultative Group (INACG)*, Nutrition Foundation, Washington, D.C.
- Torres, M.A.A., N.F. Lobo, and K. Sato (1996). Fortification of fluid milk for the prevention & treatment of iron deficiency anaemia in children under 4 years of age. *Rev. Saude. Publica.* 30:(4) 350-7.
- WHO/CDC, (2008). *Worldwide prevalence of anaemia 1993-2005*, WHO global database on anaemia. World Health Organization/ Centres for Disease Control and Prevention Atlanta. WHO Press, 20 Avenue Appia, 1211 Geneva 27, Switzerland. [Accessed on 23rd Aug. 2013.] http://whqlibdoc.who.int/publications/2008/9789241596657_eng.pdf
- Wolchover, N (2012). *Limits of the human body: How much sleep deprivation, radiation and acceleration can we survive?* Published on 13th August, 2012. [Accessed on 26th March, 2014.] http://www.huffingtonpost.com/2012/08/13/human-body-limits-survival_n_1773167.html
- Zlotkin, S., P. Arthur, K. Antwi, and Y. Yeung (2001). Treatment of anaemia with microencapsulated ferrous fumarate plus ascorbic acid supplied as sprinkles to complementary (weaning) foods. *Am. J. Clin. Nutr.* 74 (6): 791-795.
- Zubillaga, M., R. Caro, and J. Boccio (1996). New procedure to fortify cow milk with iron: metabolic and biochemical study in rats. *Nutr. Res.* 16(1):131-137.