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Influence of multiple micronutrients (vitamin A and iron) fortification on the physicochemical, microbial and sensory characteristics of pasteurized milk during storage

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Abstract
Widespread malnutrition problems are becoming a serious health concern across the globe. According to WHO Iron deficiency affects more than 2 billion people globally, likewise vitamin A deficiency (VAD) has been observed for the increasing cases of night blindness and diminishing vision besides serious impact on cellular and immune functions. Iron deficiency often manifested in the form of anaemia has affected children and women of reproductive age and responsible for morbidity and mortality. Food fortification by using the suitable fortificant without affecting the quality characteristics of finished product and ensuring higher bioavailability remain a challenging task. Milk is advocated as suitable base material for micronutrient fortification because of several benefits associated with it. Fortification of iron in food promotes development of oxidized off-flavour, discoloration, sedimentation and metallic taste especially in milk. Present investigation was carried out to evaluate the storage stability of vitamin A and iron for fortification of toned buffalo milk and monitor the various physico-chemical, nutritional, microbiological and organoleptic changes that occur during storage of HTST pasteurized milk. During storage of vitamin A and iron fortified pasteurized milk values for acidity, FFA, HMF, furosine, TBA, proteolysis increased whereas colour properties determined as L*, a*, b* and Whitening Index (WI) as well as pH dwindled continuously during storage. The levels of iron remained constant; however appreciable loss of vitamin A occurred during storage.

Keywords: Food Fortification, Malnutrition, Milk, Iron, Vitamin A

Introduction

Micronutrient malnutrition remained a major challenge before the world community, even in developed and developing economies. India ranks 105th out of 127 countries in the 2024 Global Hunger Index. According to an estimate it is posing a serious threat to the health and productivity due to micronutrient deficiencies, of more than two billion people worldwide of all ages, especially pregnant women and children younger than (Kiani AK *et al*., 2022) [12]. Vitamin A and iron, two vital micronutrient deficiencies are major public health concern in India. Iron is a mineral with a crucial role in oxygen transport and metabolic processes. Iron can be found in meats, fortified grains, and leafy vegetables. The RDA for adults is 8 to 18 mg/d (Morris AL *et al*., 2023) [14]. According to WHO iron deficiency, primarily due to inadequate dietary iron intake, is considered the most common nutritional deficiency leading to anaemia. Deficiencies in vitamin A, folate, vitamin B12 and riboflavin can also result in anaemia due to their specific roles in the synthesis of haemoglobin and/or erythrocyte production. (WHO, 2025) The problem is more severe in areas where the malaria, hookworm infection, HIV/AIDS, tuberculosis, schistosomiasis and other public health issues are prevalent. In India, the trends in malnutrition indicators have witnessed a paradigm shift (P. H. Nguyen *et al*. 2021) [15]. The malnutrition not only affected the human development Index, but also loss to national GDP to an estimate of 0.8-2.4%. Women and children are more vulnerable to MDD because of higher micronutrient needs for reproduction and sustainable growth. The MDD could be attributed to various causative factors including non-availability of adequate food, lack of diversity in our food basket, presence of inhibitory substances in staples and certain infectious or non-infectious diseases.

Thermal processing mainly pasteurization remains the major intervention adopted by the dairy industries world-side to deliver safe and long-life milk to consumers. The effects of thermal processes used in the dairy industry may be evaluated by determining several physico-chemical changes specifically related to such processes, either through monitoring the degradation of original milk components or formation of new compounds as a result of reactions at the high temperatures used. Two types of reactions commonly occurred due to heat treatment; which includes the denaturation, degradation and inactivation of heat-labile components like whey proteins, enzymes and vitamins and other reactions include the formation of substances which are not present, or only at trace levels, in the raw milk like lactulose, 5-hydroxymethylfurfural (HMF), furosine, etc (Pellegrino *et al*., 1995) [16]. These treatments are also being used for extending shelf life, to obtain milk that is fit for human consumption and has longer conservation time (Kameni *et al*., 2002) [9].

Materials and methods

Source of milk samples

During the course of this study, milk samples were collected from the herd of buffaloes maintained in the cattle yard of National Dairy Research Institute, Karnal, Haryana, India. Fresh buffalo milk was heated to (50-60ºC) and passed through a Cream separator milk was standardized to 3% fat and 8.5% solid-not-fat (SNF).

Vitamin A and Iron Source

Ferric ammonium citrate and ferrous gluconate hydrate were supplied by Merck Specialist Private Limited, Mumbai and Sigma-Aldrich, USA respectively as source of iron suppliment. Cold water soluble vitamin A acetate (potency 325000 IU/g) was procured from DSM Nutritional Products India Pvt. Ltd., Mumbai, India and cold water soluble vitamin A palmitate (potency 250000 IU/g) from Piramal Healthcare Ltd., Mumbai, India.

Packaging material

Sterilized air tight transparent PET bottle of 250 ml procured from Hi media were used to store the pasteurized fortified milk.

Addition of vitamin A and iron salts

During the screening experiments cold water soluble vitamin A acetate and vitamin A palmitate were used for fortification of buffalo toned milk at the level of 2000 IU, 2500 IU and 3000 IU per litre. One International unit (IU) of vitamin A is equivalent to 0.33 µg vitamin A. To prepare stock solutions of 10000 IU/ml vitamin A, 307.7 mg vitamin A acetate was dissolved in distilled water and volume was made up to 10 ml

The iron content of salt was calculated from their molecular weight for ferrous gluconate hydrate. Amount of salt to be added was calculated for increasing the iron content of milk by 15, 20 and 25 mg per 1000 ml.

Processing Treatments

The fortified toned milk was heated to a temperature of 60±1ºC and subjected to homogenization in two stage batch type of homogenizer (GOMA Private Ltd., Mumbai) using a pressure of 2000 psi in first stage and 500 psi in second stage. This homogenized toned milk was fortified with iron salt and vitamin A sample and then subjected HTST pasteurization (83º C for 16 seconds).

Sensory evaluation

Sensory evaluation was done by a panel of eight trained judges. Composite scoring card for sensory analysis of pasteurized milk as suggested by BIS (IS: 7768 1975) was used, with slight modifications.

Physico-chemical and microbiological evaluation

pH and titratable acidity of control and fortified milk samples, was determined by pH meter (Thermo Scientific, Singapore) as per the method described in IS: SP:18, part XI (1981). TBA value of milk was determined as per the method described by King (1962) [13]. TBA value of samples were expressed as optical density (OD) at 532 nm.

The heat stability of control and fortified samples were determined as heat coagulation time (HCT) according to the method of Davies and White (1966) [4] as modified by Jairam *et al.* (1976) [8]. Method of Kazmi *et al.* (2007) [10] was adopted for extraction of vitamin A from control and fortified milk. Vitamin A was measured and reported as IU/L. Iron was analyzed using atomic absorption spectrophotometer (AAS) by AOAC (2005) using dry digestion method. Free fatty acid (FFA) content of milk was determined by the method of Deeth *et al* (1975) [5].

$$\text{FFA}\ \left(\mu\ \text{equi}/\text{mL}\right) = \frac{\text{Titre value} * \text{N} * 10^3}{3 * \text{proportion of upper layer of aliquote}}$$

where, N =normality of KOH

Hunter colorimeter was used to measure the colour of fortified, thermally processed milk samples and to monitor degree of change in colour during storage produced by addition of iron salts to milk. The colour coordinates of this meter were L=whiteness; a=redness to greenness and b=yellowness to blueness. Whitening index of the samples were calculated as per the equation suggested by Sheen (1990)

$$\text{WI} = 100 - \sqrt{(100 - \text{L})2 + \text{a}2 + \text{b}2}$$

HMF content was determined following the spectrophotometric method described by Keeney and Bassette (1958) [11]. Furosine was estimated by the method described by Birlouez-Aragon *et al.* (1997) [2]. The viscosity of the milk samples was determined by capillary flow Ostwald viscometer.

Pasteurized fortified milk samples were examined for microbiological parameters according to the methods described in IS: SP 18 (Part I, 1980). Samples were evaluated for SPC, coliform, yeast and mold count.

Statistical Analysis

The research data obtained during study were analyzed statistically using ANOVA method in SPSS tool (IBM SPSS Statistics Version 20) as function of Duncan Post Hock Test.

Results and discussion

Effect of multiple micronutrients (vitamin A and iron) and HTST pasteurization on physico-chemical properties of fortified milk during storage

On the basis of performed investigation Table 1(a),(b),(c),(d) show the trend in physico-chemical changes of vitamin A and iron fortified pasteurized milk from 0^{th} day to day 5. Acidity of fortified HTST pasteurized milk increased during the storage under refrigerated conditions. Similar trend was noted in case of control samples. Significant increase in acidity was observed on or after 3^{rd} day of storage in control as well as fortified milk samples. Fortification did not influence the present acidity of HTST pasteurized milk and no significant ($p>0.05$) difference was observed among the control and fortified milk samples.

pH of fortified samples decreased significantly ($P<0.05$) beyond addition of 15 ppm of iron salt. This might be due to release of micellar bound H^+ ions. Saini *et al.* (1987) [18] and Rosenthal *et al.* (1993) [17] reported that there was decrease in pH of milk samples containing iron. pH of control as well as fortified samples decreased significantly ($P<0.05$) on each day of storage period.

Free Fatty Acid (FFA) content of control and fortified milk samples increased significantly during storage. Initial FFA value of 1.07 increased to 6.04 and 5.69 in control and fortified milk samples. Increase in FFA value of pasteurized milk is generally attributed to microbial lipases. The control and fortified milk samples differ significantly for the FFA content during storage. Our results are similar to Celestino *et al.* (1996) [3] who recorded a value of FFA 1.6 μeq/ml after 2 days of pasteurized milk storage. Vassila *et al.* (2002) [19] reported FFA values between 1.9 and 2.7 μeq/ml ml for milk packaged in various pouch materials. Similarly Zygoura *et al.* (2003) [21] observed FFA values increased from 1.5 to 2.1 μeq/ml after storage of 6 days and small differences in degree of lipid degradation, between samples stored in the five packaging materials.

Unlike hydrolytic rancidity expressed FFA, oxidative rancidity of milk fat determined as Thiobarbituric acid value and expressed as Optical Density (OD) at 532 nm, enhanced during storage of HTST pasteurized control and fortified milk. Increase in oxidative rancidity was significantly affected by storage period and iron content. Enhancing the level of iron for milk fortification resulted in rapid rise in TBA value during storage.

Table 1 (a): Effect of micronutrients (vitamin A and iron) and HTST pasteurization on physico-chemical properties of fortified milk during storage

Storage Days	**pH**				**Acidity (%LA)**			
	C	**FG15+A25**	**FG20+A25**	**FG25+A25**	**C**	**FG15+A25**	**FG20+A25**	**FG25+A25**
Day 0	6.773 ± 0.05^{Aa}	6.773 ± 0.04^{Aa}	6.759 ± 0.05^{Ab}	6.729 ± 0.03^{Ac}	0.135 ± 0.01^{Aa}	0.135 ± 0.05^{Aa}	0.138 ± 0.05^{Aa}	0.141 ± 0.05^{Aa}
Day 1	6.744 ± 0.03^{Ba}	6.743 ± 0.02^{Ba}	6.709 ± 0.01^{Bb}	6.703 ± 0.02^{Bc}	0.138 ± 0.05^{Aa}	0.138 ± 0.05^{Aa}	0.141 ± 0.05^{Aa}	0.141 ± 0.05^{Aa}
Day 2	6.713 ± 0.01^{Ca}	6.711 ± 0.01^{Ca}	6.702 ± 0.02^{Cb}	6.690 ± 0.02^{Cc}	0.141 ± 0.05^{Ba}	0.141 ± 0.05^{Ba}	0.144 ± 0.05^{Ba}	0.147 ± 0.05^{Ba}
Day 3	6.657 ± 0.03^{Da}	6.653 ± 0.01^{Da}	6.630 ± 0.01^{Db}	6.622 ± 0.01^{Dc}	0.144 ± 0.00^{Ca}	0.144 ± 0.05^{Ca}	0.147 ± 0.05^{Ca}	0.153 ± 0.05^{Ca}
Day 4	6.622 ± 0.01^{Ea}	6.622 ± 0.01^{Ea}	6.618 ± 0.01^{Eb}	6.604 ± 0.04^{Ec}	0.147 ± 0.05^{Da}	0.150 ± 0.05^{Da}	0.150 ± 0.05^{Da}	0.153 ± 0.05^{Da}
Day 5	6.598 ± 0.01^{Fa}	6.596 ± 0.02^{Fa}	6.590 ± 0.01^{Fb}	6.583 ± 0.01^{Fc}	0.153 ± 0.01^{Ea}	0.156 ± 0.05^{Ea}	0.156 ± 0.05^{Ea}	0.159 ± 0.05^{Ea}
Storage Days	FFA (μequivalent/ml)				HMF (μmole/L)			
	C	FG15+A25	FG20+A25	FG25+A25	C	FG15+A25	FG20+A25	FG25+A25
Day 0	1.07 ± 0.05^{Aa}	1.07 ± 0.05^{Aa}	1.07 ± 0.05^{Aa}	1.07 ± 0.05^{Aa}	2.15 ± 0.05^{Aa}	2.22 ± 0.37^{Aa}	3.94 ± 0.20^{Ab}	4.57 ± 0.45^{Ac}
Day 1	1.78 ± 0.61^{Ba}	1.96 ± 0.30^{Ba}	1.78 ± 0.61^{Ba}	1.96 ± 0.30^{Ba}	2.94 ± 0.27^{Ba}	3.08 ± 0.10^{Ba}	3.84 ± 0.07^{Bb}	4.37 ± 0.10^{Bc}
Day 2	1.60 ± 0.53^{Ba}	1.42 ± 0.61^{Ba}	1.78 ± 0.30^{Ba}	1.96 ± 0.30^{Ba}	3.57 ± 0.43^{Ca}	4.07 ± 0.09^{Ca}	4.47 ± 0.09^{Cb}	4.90 ± 0.38^{Cc}
Day 3	4.27 ± 0.01^{Ca}	4.62 ± 0.61^{Ca}	4.27 ± 0.01^{Ca}	4.98 ± 0.61^{Ba}	4.89 ± 0.09^{Da}	5.16 ± 0.10^{Da}	5.82 ± 0.20^{Db}	6.55 ± 0.09^{Dc}
Day 4	4.62 ± 0.30^{Ca}	4.44 ± 0.30^{Ca}	4.98 ± 0.61^{Ca}	4.98 ± 0.30^{Ca}	6.42 ± 0.20^{Ea}	6.48 ± 0.56^{Ea}	7.77 ± 0.57^{Eb}	7.97 ± 0.36^{Ec}
Day 5	6.04 ± 0.61^{Da}	5.69 ± 0.61^{Da}	5.69 ± 0.61^{Da}	5.69 ± 0.61^{Ca}	6.61 ± 0.11^{Ea}	6.65 ± 0.19^{Ea}	7.87 ± 0.36^{Eb}	8.04 ± 0.12^{Ec}

Data are presented as means±SEM (n=3).$^{a\text{-}b}$ Means with the same superscript in a column do not differ significantly ($P<0.05$) from each other.

Colour attributes of control and fortified milk were determined as L* (Whiteness), a* (Redness) and b* (yellowness) using COLOURFLEX showed that lightness value did not follow a definite pattern in fortified milk. L* value decreased in all the samples during the storage period and the lowering in whiteness value was significantly different. However increasing the iron content as fortificant did not cause any significant difference in L* value, when compared to control samples, however 25 ppm FG addition lowered the L* value significantly. Lowering in L* value of HTST pasteurized micronutrient fortified milk may be partially due to heat induced non-enzymatic browning reactions and could be because of colour imparted by iron and vitamin A esters.

Redness value (a*) of control and fortified milk increased by 0.8-0.9 unit from the initial day to final day of storage. Slight increase in a* value on fortification and also during storage was significant ($P<0.05$). Milk with added iron @ 15 and 20 ppm with 2500 IU of Vitamin A acetate exhibited similar redness values, which was statistically different both form control and 25ppm iron added milk.

Yellowness (b*) value of fortified milk samples were significantly higher than the unfortified milk during the initial two days of storage. Yellowness value (b*) increased linearly upto 2^{nd} day, decreased again on 3^{rd} day and finally reached to maximum value of 8.1 on 4^{th} day of storage. FG used as fortificant is yellowish grey to yellowish green water soluble salt of iron recommended for fortification of powdered milk, infant formula and wheat flour products (Ellis *et al.*, 2013). Hence addition of FG was expected to impart its own effect on colour of fortified milk.

Whitening index of control and fortified milk did not differ significantly which showed that fortification with iron and Vitamin A acetated had no major influence on whitening properties of fortified milk, if subjected to HTST pasteurization. Irrespective to level of fortification, whitening index decreased significantly during storage of 5^{th} day under refrigeration.

Table 1 (b): Effect of multiple micronutrients (vitamin A and iron) and HTST pasteurization on colour profile of fortified milk during storage at 4 °C

Storage	L* (Lightness or whiteness)				Whitening Index			
Days	**C**	**FG15+A25**	**FG20+A25**	**FG25+A25**	**C**	**FG15+A25**	**FG20+A25**	**FG25+A25**
Day 0	86.58±0.10^{Aa}	86.56±0.26^{Aa}	86.55±0.53^{Aa}	86.46±0.07^{Ab}	84.8±0.03^{Aa}	84.5±0.05^{Ab}	84.6±0.04^{Ab}	84.5±0.04^{Ac}
Day 1	86.11±0.10^{Ba}	86.03±0.21^{Ba}	86.96±0.15^{Ba}	85.93±0.01^{Bb}	84.2±0.01^{Ba}	84.0±0.03^{Bb}	83.9±0.08^{Bb}	83.8±0.01^{Bc}
Day 2	85.17±0.78^{Ca}	85.28±0.40^{Ca}	85.57±0.00^{Ca}	85.12±0.02^{Cb}	83.3±0.08^{Ca}	83.3±0.01^{Cb}	83.6±0.07^{Cb}	83.2±0.07^{Cc}
Day 3	85.02±0.10^{Da}	85.13±0.02^{Da}	85.02±0.12^{Da}	84.93±0.02^{Db}	83.2±0.01^{Da}	83.3±0.02^{Db}	83.1±0.04^{Db}	83.0±0.01^{Dc}
Day 4	84.31±0.21^{Ea}	84.27±0.19^{Ea}	84.15±0.15^{Ea}	84.18±0.04^{Eb}	82.2±0.03^{Ea}	82.1±0.10^{Eb}	82.0±0.00^{Eb}	82.1±0.00^{Ec}
Day 5	84.25±0.15^{Ea}	84.26±0.02^{Ea}	84.26±0.31^{Ea}	84.24±0.08^{Eb}	82.3±0.01^{Fa}	82.2±0.01^{Fb}	82.3±0.03^{Fb}	82.3±0.08^{Fc}
Storage	a* (Redness or Greenness)				b* (Yellowness or Blueness)			
Days	C	FG15+A25	FG20+A25	FG25+A25	C	FG15+A25	FG20+A25	FG25+A25
Day 0	-3.0±0.01^{Aa}	-2.9±0.06^{Ab}	-2.8±0.06^{Ab}	-2.8±0.06^{Ac}	6.4±0.56^{Aa}	7.1±0.25^{Ab}	7.0±0.21^{Ab}	7.0±0.46^{Ab}
Day 1	-2.7±0.05^{Ba}	-2.7±0.03^{Bb}	-2.8±0.15^{Bb}	-2.6±0.10^{Bc}	7.0±0.15^{Ca}	7.3±0.36^{Cb}	7.3±0.29^{Cb}	7.5±0.32^{Cb}
Day 2	-2.6±0.04^{Ca}	-2.6±0.02^{Cb}	-2.5±0.21^{Cb}	-2.5±0.10^{Cc}	7.3±0.21^{Ba}	7.3±0.31^{Bb}	7.4±0.26^{Bb}	7.4±0.10^{Bb}
Day 3	-2.7±0.01^{Da}	-2.7±0.01^{Db}	-2.7±0.21^{Db}	-2.6±0.42^{Dc}	7.2±0.25^{Ca}	7.2±0.50^{Cb}	7.3±0.36^{Cb}	7.4±0.36^{Cb}
Day 4	-2.6±0.01^{Da}	-2.6±0.01^{Db}	-2.6±0.32^{Db}	-2.5±0.10^{Dc}	8.1±0.53^{Da}	8.1±0.20^{Db}	8.1±0.44^{Db}	8.1±0.87^{Db}
Day 5	-2.1±0.01^{Ea}	-2.1±0.05^{Eb}	-2.0±0.35^{Eb}	-2.0±0.29^{Ec}	7.8±0.10^{Ea}	8.0±0.15^{Eb}	7.8±0.15^{Eb}	7.8±0.31^{Eb}

Data are presented as means±SEM (n=3).$^{a-b}$ Means with the same superscript in a column do not differ significantly (P<0.05) from each other.

Table 1 (c): Effect of multiple micronutrients (vitamin A and iron) and HTST pasteurization on physico-chemical properties of fortified milk during storage at 4 °C

Storage	Iron (ppm)				Proteolysis (Glycine µmole/ml)			
Days	**C**	**FG15+A25**	**FG20+A25**	**FG25+A25**	**C**	**FG15+A25**	**FG20+A25**	**FG25+A25**
Day 0	0.95±0.05^{Aa}	15.84±0.11^{Ab}	20.60±0.21^{Ac}	25.60±0.08^{Ad}	0.167±0.01^{Aa}	0.168±0.01^{Ab}	0.171±0.02^{Ac}	0.171±0.03^{Ad}
Day 1	0.91±0.04^{Aa}	15.80±0.25^{Ab}	20.39±0.03^{Ac}	25.51±0.17^{Ad}	0.171±0.05^{Aa}	0.172±0.05^{Ab}	0.173±0.05^{Ac}	0.177±0.06^{Ad}
Day 3	0.87±0.06^{Aa}	15.90±0.05^{Ab}	20.48±0.11^{Ac}	25.75±0.17^{Ad}	0.366±0.05^{Ba}	0.401±0.01^{Bb}	0.405±0.01^{Bc}	0.472±0.03^{Bd}
Day 5	0.86±0.06^{Aa}	15.47±0.19^{Ab}	20.21±0.13^{Ac}	25.38±0.04^{Ad}	0.939±0.09^{Ca}	0.987±0.08^{Cb}	1.203±0.33^{Cc}	1.250±0.03^{Cd}
Storage	TBA (abs at 532 nm)				Vitamin A (IU)			
Days	C	FG15+A25	FG20+A25	FG25+A25	C	FG15+A25	FG20+A25	FG25+A25
Day 0	0.021±0.02^{Aa}	0.026±0.01^{Ab}	0.029±0.01^{Ac}	0.032±0.03^{Ad}	682.96±5.97^{Aa}	2939.93±13.41^{Ab}	2941.50±1.55^{Ac}	2946.21±6.55^{Ad}
Day 1	0.028±0.01^{Ba}	0.030±0.01^{Bb}	0.034±0.02^{Bc}	0.037±0.01^{Bd}	606.59±2.87^{Ba}	2554.84±14.14^{Bb}	2553.31±11.41^{Bc}	2476.95±0.97^{Bd}
Day 3	0.037±0.02^{Ca}	0.039±0.01^{Cb}	0.043±0.01^{Cc}	0.046±0.02^{Cd}	393.04±0.03^{Ca}	1751.49±15.53^{Cb}	1822.33±12.44^{Cc}	1751.01±12.40^{Cd}
Day 5	0.068±0.01^{Da}	0.070±0.01^{Db}	0.075±0.01^{Dc}	0.079±0.01^{Dd}	298.91±0.03^{Da}	1299.33±23.16^{Db}	1275.67±8.08^{Dd}	1173.67±24.95^{Dd}

Data are presented as means±SEM (n=3).
$^{a-b}$ Means with the same superscript in a column do not differ significantly (P<0.05) from each other.
$^{A-B}$Means with the same superscript in a row do not differ significantly (P<0.05) from each other

Table 1 (d): Effect of multiple micronutrients (vitamin A and iron) and HTST pasteurization on physico-chemical properties of fortified milk during storage at 4 °C

Storage	Viscosity			
Days	**C**	**FG15+A25**	**FG20+A25**	**FG25+A25**
Day 0	1.58±0.014^{Aa}	1.58±0.014^{Ab}	1.59±0.012^{Abc}	1.58±0.017^{Ac}
Day 1	1.60±0.004^{Ba}	1.61±0.004^{Bb}	1.61±0.004^{Bbc}	1.61±0.004^{Bc}
Day 2	1.57±0.004^{Aa}	1.59±0.020^{Ab}	1.59±0.012^{Abc}	1.61±0.004^{Ac}
Day 3	1.63±0.007^{Ca}	1.63±0.004^{Cb}	1.63±0.004^{Cbc}	1.63±0.004^{Cc}
Day 4	1.67±0.014^{Da}	1.67±0.004^{Db}	1.68±0.004^{Dbc}	1.68±0.004^{Dc}
Day 5	1.66±0.007^{Da}	1.67±0.004^{Db}	1.69±0.010^{Dbc}	1.69±0.010^{Dc}
Storage	Furosine			
Days	C	FG15+A25	FG20+A25	FG25+A25
Day 0	7.70±0.194^{Aa}	7.85±0.121^{Ab}	8.16±0.102^{Ac}	8.81±0.068^{Ad}
Day 1	9.06±0.066^{Ba}	9.24±0.201^{Bb}	10.13±0.018^{Bc}	10.49±0.013^{Bd}
Day 3	10.18±0.054^{Ca}	10.45±0.001^{Cb}	10.79±0.033^{Cc}	11.07±0.030^{Cd}
Day 5	13.41±0.081^{Da}	14.23±0.130^{Db}	15.81±0.117^{Dc}	15.99±0.011^{Dd}

Data are presented as means±SEM (n=3).
$^{a-b}$ Means with the same superscript in a column do not differ significantly (P<0.05) from each other.
$^{A-B}$Means with the same superscript in a row do not differ significantly (P<0.05) from each other.

Colour changes during storage of thermally processed milk and milk products are mainly related to the occurance of maillard reaction. Boekel reviewed the physico-chemical phenomenon associated with heating induced maillard reactions in milk and suggested that determination of furosine and HMF in milk samples provide an estimate of early and advanced maillard reactions. Furosine content of control and fortified milks increased almost linearly during storage of HTST pasteurized milk. Addition of iron and increasing its concentration enhanced the furosine in thermally processed fortified milks. Lowest value of furosine was 7.70mg/ g protein in HTST pasteurized control milk immediately after normal treatment under as maximum value was observed in 25 ppm FG milk on 5th day of storage. Both storage period and concentration of iron significantly affected the furosine content. Similar trend was noticed for HMF content as well. Level of ferrous gluconate hydrate exerted a significant effect of HMF content of HTST pasteurized milk. Addition upto the level 15 ppm of ferrous gluconate hydrate resulted in HMF content similar to unfortified milk.

Proteolysis determined by calculating the free amino nitrogen and expressed as µm glycine per ml increased during storage of HTST pasteurized control and fortified milk. The proteolysis values remain constant in initial two days of storage, increased marginally on 3rd day and rapidly on 5th day of storage under refrigeration conditions.

Viscosity of milk samples was not much influenced by the fortification of milk with ferrous gluconate hydrate and Vitamin A acetate. Slight increase in viscosity in milk samples was noted after 4th day of storage.

Iron and Vitamin A were used as fortificants in present investigation. Addition of above mentioned micronutrients significantly in fortified milks. Iron content in control milk was less than 1 ppm enhanced to in the range of 15-25 ppm. There was no significant variation in the level of iron during storage and it remained constant. Compared to iron, Vitamin A level in control as well as fortified milks lowered during storage. Both storage period and iron content in milk significantly affected the iron and Vitamin A during storage. Maximum loss occurred in milk fortified with 25 ppm of iron, where the level of Vitamin A acetate decreased from and initial value of 2469 IU to 1173 IU on 5th day storage. Iron is well known pro-oxidant which promote the oxidation of lipid substance including fat soluble vitamins.

Effect of storage on the heat coagulation time (HCT) in HTST pasteurized milk

Heat stability as determined by the heat coagulation time/pH (HCT/pH) profile is, essentially, a measure of the temperature required to cause instantaneous coagulation (Miller and Sommer 1940). The heat stability (as heat coagulation time) of control and micronutrients fortified milk after processing and during storage at its natural pH and after adjustment of the pH between 6.4 to 7.0 at 0.1 unit intervals using disodium hydrogen phosphate and sodium dihydrogen phosphate was studied and the results are as shown in table 4.8.

The results revealed that heat coagulation time of control toned milk at 0th day at its natural pH (6.6) was 32.00 (140±1 °C) minutes and that of vitamin A and ferrous gluconate hydrate fortified milk (pH 6.6) at 15, 20 and 25 ppm was 31.00, 29.50 and 29.50 minutes respectively. HCT of control milk ranged from 23.50 minutes (pH 6.4) to 5.00 minutes (at pH 7.0) and the maxima in HCT/pH curve (at pH 6.6: 32.00 minutes) was on the natural pH. HCT of fortified milk at 15 ppm level ranged from 22.50 minutes (pH 6.4) to 4.00 minutes (at pH 7.0). Fortified milk samples with 20 and 25 ppm ferrous gluconate hydrate the HCT lie between 18 (pH 6.4) to 5.00 (pH7.00) and 19.00 (pH 6.4) to 5.00 (pH 7.00), respectively. Addition of iron salt caused reduction in heat coagulation time but the effect was negligible.

The heat coagulation time of control milk at 3rd day ranged from 20.00 minutes (pH 6.4) to 5.50 minutes (at pH 7.0) and the maxima was observed 30 minutes at pH 6.6. The maximum heat coagulation time for samples fortified with 15, 20 and 25 ppm ferrous gluconate hydrate was observed 29.00, 28.00 and 24.50 minutes. Similarly the heat coagulation time of control toned milk at 5th day of storage at its natural pH (6.6) was 24.25 (140±1 °C) minutes and that of vitamin A and ferrous gluconate fortified milk (pH 6.6) at 15, 20 and 25 ppm was 25.50, 25.00 and 24.50 minutes respectively.

Heat stability reduced with fortification of iron salt, the effect was not pronounced. The heat stability also reduced during storage. Maximum reduction in heat stability was observed with milk samples fortified with 25 ppm ferrous gluconate hydrate salt.

Table 2: Effect of multiple micronutrients (iron and vitamin A) and HTST pasteurization on HCT profile of fortified milk during storage at 4 °C

Day 1	**HCT (minutes) at 140ºC**			
pH	**C**	**FG15+A25**	**FG20+A25**	**FG25+A25**
6.4	23.50	22.50	18.00	19.00
6.5	28.00	27.00	26.00	24.00
6.6	32.00	31.00	29.50	29.50
6.7	21.00	20.50	20.50	20.00
6.8	9.00	8.50	6.50	7.00
6.9	8.50	7.50	6.00	6.00
7.0	5.00	4.00	5.00	5.00
Day 2	C	FG15+A25	FG20+A25	FG25+A25
6.4	20.00	20.00	20.00	19.00
6.5	27.00	26.00	25.00	24.00
6.6	30.00	29.00	28.00	24.50
6.7	21.00	18.00	19.00	20.00
6.8	8.00	7.50	7.00	7.00
6.9	8.50	7.50	7.00	6.00
7.0	5.50	4.00	5.00	5.00
Day 4	C	FG15+A25	FG20+A25	FG25+A25
6.4	18.50	18.50	17.00	17.00
6.5	23.50	23.50	23.50	23.00
6.6	24.25	25.50	25.00	24.50
6.7	22.50	22.00	22.00	20.00
6.8	7.00	6.50	6.50	6.50
6.9	7.00	6.50	6.50	6.50

Effect of Micronutrients (vitamin A and Iron) and HTST Pasteurization on Sensory Properties of Fortified Milk during Storage

Micronutrient specially the iron had been reported to adversely affect the sensory characteristics of fortified foods. From the previous section, it is evident that iron addition influenced the various physico-chemical properties of fortified milk and HTST pasteurization further enhance the rate of some of these changes. The addition of iron is expected to affect the organoleptic quality of fortified milk; therefore milk samples were evaluated for sensory parameters namely flavour, colour & appearance, odour and mouthfeel using a structure composite scoring card by semi-trained panelists. The results obtained are discussed hereunder.

Fortification of micronutrients decreased the flavour score; however addition beyond 15 ppm ferrous gluconate caused a significant reduction in flavour scores. During the storage period of 5 days flavour scores of control as well as fortified milk lowered significantly from initial average scores in the range of 36-37 to final average scores of 30-32. The milk fortified with 20 or 25 ppm of iron did not differ significantly in their flavour scores. Mouthfeel scores too followed the similar trend, but the lowering in mouthfeel score was not much as in case of flavour on addition of fortificants. Milk fortified with 25 ppm of ferrous gluconate differed significantly for mouthfeel score from control and milk fortified with 15 and 20 ppm of ferrous gluconate. The odour score also decreased with increasing the level of ferrous gluconate from 15 to 25 ppm during the storage period. Like flavour and mouthfeel attributes, odour scores of control and 15 ppm iron-fortified milk were not significantly different, but were significantly higher than 20 or 25 ppm iron fortified milk. Thermal treatment might have resulted in formation of heat induced compounds that were not perceived by the panelist. Similarly, iron promote oxidation of lipids mainly the unsaturated fatty acids leading to enhancement in the concentration of aldehydes, ketones, acids and their interactive compounds which adversely affect the aroma of the milk during storage. Colour and appearance scores were in 9.00, 8.80, 8.77 and 8.43 for control, 15, 20 ad 25 ppm ferrous gluconate added milk on the initial days which decreased subsequently during storage to 7.33, 7.33, 7.00 and 7.00 respectively on the 5th day of storage. Like other sensory parameters lowering in colour and appearance scores could be attributed to continuous deteriorative chemical reactions occurred during storage including maillard browning reactions, fat oxidation. On the basis of sensory evaluation of control and fortified milk samples it can be inferred that addition of iron as ferrous gluconate hydrate up to 20 ppm in combination with 2500 IU of vitamin A acetate resulted in sensorial acceptable product, however 25 ppm iron content caused significant lowering in all sensory attributes and thus least acceptable product as well.

Table 3: Effect of multiple micronutrients (iron and vitamin A) and HTST pasteurization on sensory profile of fortified milk during storage at 4 °C

Storage Days	C & A (Max=10)				Odour (Max=20)			
	C	FG15+A25	FG20+A25	FG25+A25	C	FG15+A25	FG20+A25	FG25+A25
Day 0	9.00±0.01^{Aa}	8.80±0.10^{Aa}	8.77±0.15^{Ba}	8.43±0.28^{Ba}	18.33±0.57^{Aa}	18.33±0.29^{Aa}	18.00±0.01^{Ba}	17.83±0.29^{Ba}
Day 1	8.67±0.29^{Ab}	8.50±0.01^{Ab}	8.17±0.29^{Bb}	8.00±0.01^{Bb}	18.00±0.01^{Ab}	17.83±0.57^{Ab}	17.33±0.29^{Bb}	17.00±0.01^{Bb}
Day 3	8.00±0.01^{Ac}	7.83±0.29^{Ac}	7.83±0.29^{Bc}	7.67±0.29^{Bc}	17.00±0.01^{Ac}	17.00±0.01^{Ac}	16.83±0.29^{Bc}	16.67±0.29^{Bc}
Day 5	7.33±0.29^{Ad}	7.33±0.29^{Ad}	7.00±0.01^{Bd}	7.00±0.01^{Bd}	16.50±0.50^{Ad}	16.33±0.28^{Ad}	16.00±0.01^{Bd}	16.00±0.01^{Bd}
Storage Days	Flavour (Max=40)				Body (Max=30)			
	C	FG15+A25	FG20+A25	FG25+A25	C	FG15+A25	FG20+A25	FG25+A25
Day 0	37.67±0.57^{Aa}	37.67±0.57^{Aa}	36.67±0.57^{Ba}	36.33±0.57^{Ba}	28.00±0.01^{Aa}	28.00±0.01^{Aa}	27.67±0.57^{ABa}	27.50±0.50^{Ba}
Day 1	37.00±0.01^{Ab}	36.83±0.28^{Ab}	36.17±0.28^{Bb}	36.00±0.05^{Bb}	27.83±0.29^{Ab}	27.50±0.50^{Ab}	27.50±0.50^{ABb}	27.50±0.01^{Bb}
Day 3	35.00±0.01^{Ac}	34.83±0.29^{Ac}	34.67±0.28^{Bc}	34.50±0.50^{Bc}	27.00±0.01^{Ac}	27.00±0.01^{Ac}	27.00±0.01^{ABc}	26.50±0.001^{Bc}
Day 5	32.00±0.01^{Ad}	31.00±0.01^{Ad}	30.67±0.57^{Bd}	30.33±0.57^{Bd}	26.00±0.01^{Ad}	26.00±0.01^{Ad}	25.83±0.28^{ABd}	25.67±0.28^{Bd}

Data are presented as means±SEM (n=3).
$^{a-b}$ Means with the same superscript in a column do not differ significantly (P<0.05) from each other.
$^{A-B}$Means with the same superscript in a row do not differ significantly (P<0.05) from each other

Effect of Multiple Micronutrients (Iron and Vitamin A) and HTST Pasteurization on Microbiological Quality of Fortified Milk during Storage

Microbial spoilage of pasteurized milk limits its shelf-life to 3-7 days under strict cold chain conditions and storage i.e. 4^{0}C or less. Time-temperature combinations used for pasteurization of milk are aimed at complete elimination of pathogens and substantial destruction of other forms of microbial life so that keeping quality of milk can be improved. Total plate counts expressed as log cfu per ml were in the range of 3.719-3.740 that increased to 5.118-5.196 on the 5th day of storage. The increase in SPC was expected and it is apparent from Figure that increasing the iron level as fortificant also caused slight increase in SPC. Iron is one of the essential elements required for various cellular functions of microbes and also protects them from the adverse conditions. Higher availability of iron in milk could have resulted in lower destruction of bacteria. However, coliform and yeast & mold counts were remained nil during the entire period of storage. Absence of coliforms particularly indicates that desired hygiene measures were applied during the entire production process and also pre and post production handling of milk. During the investigation cleaning-in-place (CIP) of modular pasteurizer was carried out as per the standard protocol using lye, hot water and finally it was flushed with steam. The all contact surfaces of equipment and packaging systems were sanitized by rinsing with iodophore and finally with steam.

Table 4: Effect of multiple micronutrients (iron and vitamin A) and HTST pasteurization on microbial properties of fortified milk during storage at 4 °C

Storage Days	SPC (log cfu/ml)				Coliforms (log cfu/ml)			
	C	FG15+A25	FG20+A25	FG25+A25	C	FG15+A25	FG20+A25	FG25+A25
Day 0	3.727±0.577^{Aa}	3.740±0.577^{Aa}	3.727±0.577^{Aa}	3.719±0.577^{Ac}	NIL	NIL	NIL	NIL
Day 1	4.195±0.577^{Aa}	4.261±0.577^{Aa}	4.255±0.577^{Aa}	4.425±0.577^{Ac}	NIL	NIL	NIL	NIL
Day 3	4.560±0.577^{Ba}	4.613±0.577^{Ba}	4.640±0.577^{Ba}	4.672±0.577^{Bc}	NIL	NIL	NIL	NIL
Day 5	5.118±0.577^{Ba}	5.169±0.577^{Ba}	5.183±0.577^{Ba}	5.196±0.577^{Bc}	NIL	NIL	NIL	NIL
Storage Days	Yeasts and Molds (log cfu/ml)							
	C	FG15+A25	FG20+A25	FG25+A25				
Day 0	NIL	NIL	NIL	NIL				
Day 1	NIL	NIL	NIL	NIL				
Day 3	NIL	NIL	NIL	NIL				
Day 5	NIL	NIL	NIL	NIL				

Data are presented as means±SEM (n=3).
$^{a-b}$ Means with the same superscript in a column do not differ significantly (P<0.05) from each other.
$^{A-B}$Means with the same superscript in a row do not differ significantly (P<0.05) from each other

Conclusion

The current study led to the development of multiple micronutrients (iron+ vitamin A) fortified toned milk with ferrous gluconate hydrate up to 15 ppm as iron source in combination with 2500 IU of vitamin A acetate. The fortified HTST pasteurized milk remained acceptable upto 5 days on the basis of physicochemical, biochemical and sensory parameters. This study showed that free Fatty Acid (FFA), furosine and acidity of control and fortified HTST pasteurized milk significantly ($P<0.05$) increased during the storage under refrigerated conditions. Increasing the iron content as fortificant did not cause any significant difference in L* value, when compared to control samples, however 25 ppm ferrous gluconate hydrate addition lowered the L* value significantly. Irrespective to level of fortification, whitening index decreased significantly during storage of 6th day under refrigeration. Colour related changes are partly due to formation of maillard reaction products on milk heating. Viscosity of milk samples was not much influenced by the fortification of milk with ferrous gluconate hydrate and Vitamin A acetate. Slight increase in viscosity in milk samples was noted after 4th day of storage. There was no significant variation in the level of iron during storage and it remained constant. Maximum loss in vitamin A content was occurred in milk fortified with 25 ppm of iron, where the level of Vitamin A acetate decreased from initial value of 2469 IU to 1173 IU on 6th day storage. Loss of vitamin A at higher iron content may be because of photo-oxidation of lipid compounds. Heat stability reduced with fortification of iron salt, the effect was not pronounced. The heat stability also reduced during storage. Maximum reduction in heat stability was observed in milk samples fortified with 25 ppm ferrous gluconate hydrate salt.

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