



Physicochemical, Sensory And Microbial Properties Of Yoghurt Fortified With Casein-Chelated Iron

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Abstract:

Iron deficiency is one of the most prevalent micronutrient deficiencies worldwide, necessitating innovative strategies for iron fortification. Chelation is a process of binding of ions and their molecules to metal ions. It enhances the bioavailability of iron and minimizes the challenges of oxidative stability and sensory properties with traditional iron fortification. Casein, a major milk protein, has a high affinity for binding divalent metal ions, forming stable complexes that protect iron from premature reactions in the food matrix and gastrointestinal tract. Chelation of iron with casein improves the solubility of iron in aqueous environments.

Yoghurt, a widely consumed functional food, presents an ideal vehicle for nutritional fortification. The standard yoghurt was prepared according to the FSSAI specifications. The chelated iron was added into the boiled milk in the concentrations of 0.5g, 1.0g, 1.5g and 2.0g per 100ml milk respectively before fermentation. The four samples were analyzed for physicochemical, microbial, and sensory properties. Sensory analysis revealed that the yoghurt samples having of 0.5g and 1.0g and 1.5g levels of chelated iron were equal to the standard yoghurt in color, taste and texture compare to other concentrations. The iron content in 0.5, 1.0, 1.5, 2.0g yoghurt samples were control-0.5mg, 0.5-3.4mg, 1.0-5.2mg, 1.5-7.6mg and 2.0-9.8mg respectively was analyzed by ICP-OES method. The study highlights the potential of casein-chelated iron as an innovative fortification method, paving the way for nutritionally enriched dairy products.

Key Words: Iron, Fortification, Yoghurt, Fermentation, Casein chelated iron.

1. Introduction

Anemia remains a significant global public health concern, affecting a substantial portion of the population, particularly among children and women of reproductive age. This condition, characterized by a deficiency in the number or quality of red blood cells, impairs the oxygen-carrying capacity of the blood, leading to various health complications.

As of 2019, the World Health Organization (WHO) estimated that anemia affected 39.8% of children aged 6–59 months, equating to approximately 269 million children worldwide. Among women of reproductive age (15–49 years), the prevalence was 29.9%, with 29.6% in non-pregnant women and 36.5% in pregnant women. These figures have remained relatively stagnant since 2000, indicating a persistent global challenge. In 2021, the global number of individuals with anemia was reported at 1.92 billion, marking an increase of 420 million cases over three decades.

Iron deficiency in India affects 50% of the population, leading to poor pregnancy outcomes and 20% maternal deaths. Children aged 6–59 months experience the largest increase in anemia. According to the National Family Health Survey-5 (NFHS-5) (2019–21), 67.1% of Indian children between the ages of 6–59 months had anemia, which has seen a substantial rise from NFHS-4 survey. The prevalence of anemia has also increased among reproductive age women in India, rising from 53% in NFHS-4 (2015–16) to 57% during NFHS-5 (2019–21) IIPS.

Anemia continues to be a serious barrier to women's and children's health and development in low- and middle-income countries (LMICs), affecting 293.1 million children and 468.4 million nonpregnant women in LMICs. Anemia is a leading cause of infant and maternal death in the majority of these countries, and it is linked to an increased risk of low birth weight, cognitive impairment, greater susceptibility to infection, and delayed physical and mental development, as well as reduced ability to work. The key risk factors that are prominent in low- and middle-income nations include dietary inadequacies, iron deficiency, infection/inflammation, and hereditary haemoglobin abnormalities. Iron deficiency anemia (IDA) increases the risk of miscarriage, premature labour, placental abruption, and low birth weight in women of reproductive age. Lack of iron in diet has been connected to several awareness and behavioural issues (Yang et al., 2018).

One of essential micronutrient in human nutrition is iron. It is also a component of heme in hemoglobin and myoglobin in which it plays important role in the transport, storage and utilization of oxygen. Iron in food may be highly bioavailable as is the case in the iron found in the heme which is found in red meat, but the cost of these products may be high for many people. The iron present in other products of vegetable origin, is non-heme and has the disadvantage of interacting with substances in food that inhibit

its absorption such as tannins, phytates, and polyphenols hence it has low bioavailability. Much of this kind of food is consumed by people in the lower socioeconomic classes, who thus cannot meet their physiological needs for iron.

Iron can be supplied in salts, elemental iron, metal chelates, and iron complexed to proteins or peptides. Iron salts, especially ferrous sulphate, are widely used to prevent and cure anaemia, but their bioavailability is low. Food-based approaches may be an alternative way to improve iron bioavailability. Iron-chelated peptides could increase the stability, absorption and bioavailability of iron (Bielecka et al., 2022). However, bovine milk proteins are inhibitors of iron absorption unless they are hydrolysed. Milk peptides, released during proteolytic digestion, have been shown to have considerable binding ability for divalent iron, which can promote iron absorption and improve iron bioavailability. Among metal-binding peptides, caseinophosphopeptides (CPPs) have been widely studied. They contain binding sites, phosphoseryl and carboxyl, for different minerals. The ideal fortification provides high bioavailability iron while without altering the sensory characteristics of a particular food. Food scientists came up with a variety of molecules to improve the iron content, including chelated versions (Anusha et al., 2016). Casein, a major milk protein, can chelate iron, forming a stable complex that enhances iron absorption while minimizing sensory changes in fortified foods.

Dairy products are an important group in human nutrition. Direct addition of iron to dairy product might be effective way to increasing the dietary intake of iron to the general population. One of the most well-known fermented milk products produced worldwide is yogurt. The most common fermented dairy food is yoghurt, which has a high concentration of probiotics and nearly all of the necessary nutrients, easily digestible proteins. Because of its high nutritional value and the positive benefits on health that come from the presence of living bacteria like *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus*, it is of significant economic importance throughout the world (Seckin et al., 2012). It can be easily digested even by people who are lactose intolerant because it has a low lactose content.

Yoghurt is excellent source of vitamins, minerals and proteins but its iron concentration is low (approximately 0.2 mg/kg which makes it impossible to meet iron Recommended Daily Allowance (RDA). Therefore, dairy products are logical carrier for iron fortification and considered as practical and cost-effective long-term solution (Sobotka et al., 2019). Since fermented milk products are among highly-consumed food in the world, they have been used to deliver nutritional components into human diet. Furthermore, fortification of these products such as yoghurt is a good way to improve nutrient intake in daily food products. Fortification of dairy products with Fe would help nutritional deficiencies. Iron-fortified yoghurt has a relatively high iron bioavailability (Marciniak et al., 2019).

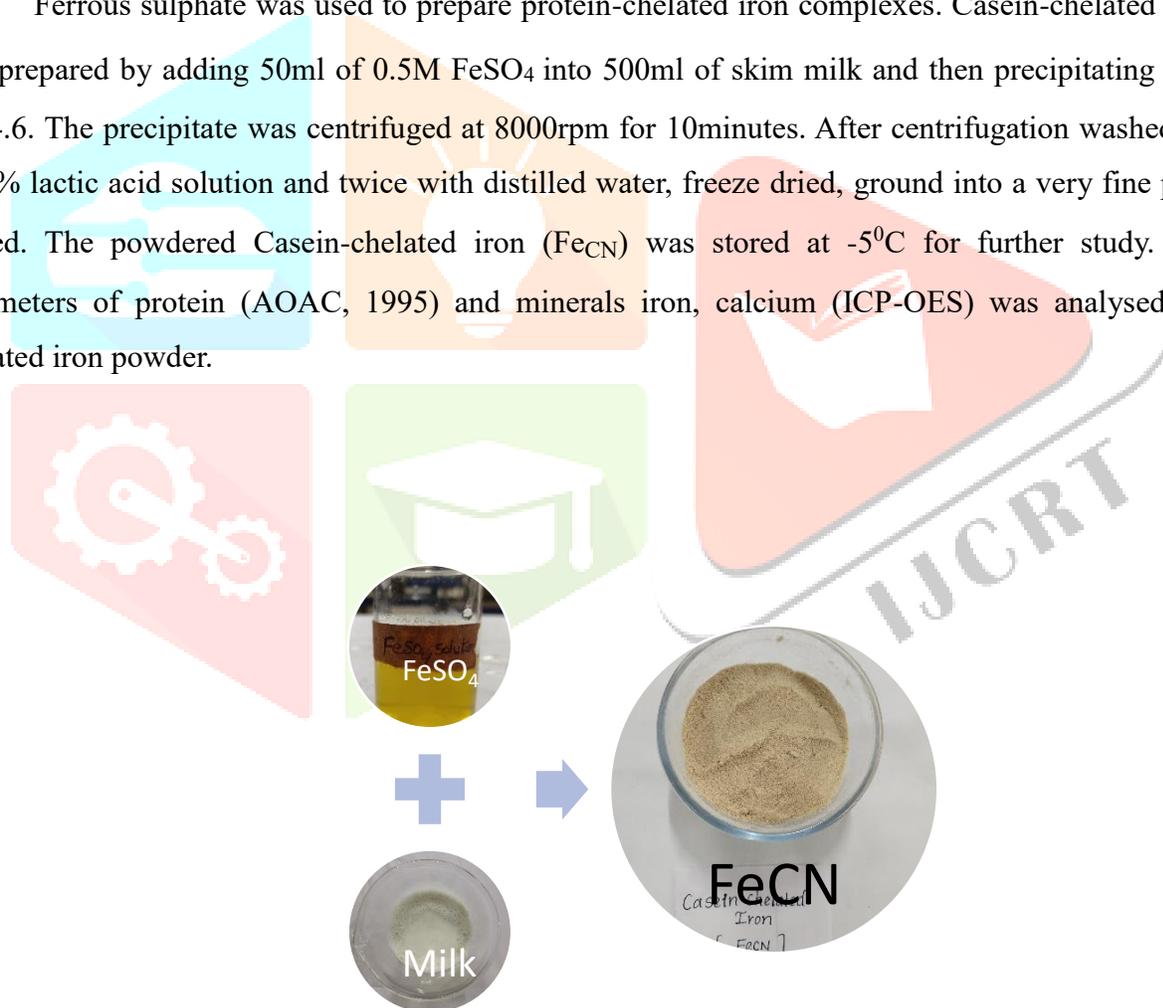
2. Materials and Methods

Ferrous sulphate, Lactic acid solution was purchased from the SD fine chemicals Mumbai and Frozen Culture of Yoghurt Mix Culture (*Lactobacillus delbrueckii* spp. *Bulgaricus*, *Streptococcus thermophilus*) was purchased from the National Collection of Dairy Cultures (NCDC) at ICAR- National Dairy Research Institute (NDRI) in Kernal, Haryana.

Skim milk powder, full cream milk was purchased from the local market in authorized milk store Tirupati. MRS agar, M17 broth, Violet red bile agar and Yeas glucose chloramphenicol agar was purchased from the Hi media Laboratories company pvt.ltd.

2.1 Preparation of Casein Chelated iron

Ferrous sulphate was used to prepare protein-chelated iron complexes. Casein-chelated iron (Fe_{CN}) was prepared by adding 50ml of 0.5M $FeSO_4$ into 500ml of skim milk and then precipitating the Fe_{CN} at pH 4.6. The precipitate was centrifuged at 8000rpm for 10minutes. After centrifugation washed once with 0.25% lactic acid solution and twice with distilled water, freeze dried, ground into a very fine powder and sieved. The powdered Casein-chelated iron (Fe_{CN}) was stored at $-5^{\circ}C$ for further study. Nutritional parameters of protein (AOAC, 1995) and minerals iron, calcium (ICP-OES) was analysed in casein-chelated iron powder.



2.2 Preparation of Yoghurt mix culture

Mother Culture: Frozen Culture of Yoghurt Mix Culture (*Lactobacillus delbrueckii* spp. *Bulgaricus* and *Streptococcus thermophilus*) was purchased from the National Collection of Dairy Cultures (NCDC) at ICAR- National Dairy Research Institute (NDRI) in Kernal, Haryana. Two portions of skim milk were prepared by reconstituting skim milk powder in deionized water (11%) and autoclaved. Frozen Culture of

Yoghurt Mix Culture was inoculated into sterile Skim milk, after that incubated anaerobically (Bacteriological incubator) for 15 hours at 41°C, and stored at -70°C.

2.3 Standardization of Yoghurt preparation: Full cream milk of FSSAI approved brand with SNF-9%, Total solids- 12-13% and Fat 6% was purchased from the local authorized store tirupati. 100ml of full cream milk was heated to 90°C for 10minutes and cooled to 45°C followed by inoculation with the yoghurt mix culture (1% - 2%). The inoculated yoghurt mixture was incubated at 40°C for 6 hours. After fermentation stored at refrigerator 4 ± 1° C for further analysis. This yoghurt sample used as standard sample.

2.4 Yoghurt fortification with FeCN: Four experimental yoghurt samples were designed by adding 0.5, 1.0, 1.5, and 2.0g of FeCN to 100ml milk. The four milk samples with casein-chelated iron fortified was inoculated with culture and then filled in cups and sealed. The milk samples are fermented at 35° to 40°C for 6 hours. After curd formation the yoghurt subjected to physical, chemical, microbial and sensory analysis.



2.5 Physico-chemical analysis

The pH was measured using pH meter. Titratable acidity was measured as per (Jayarathna et al. 2020). The total solids content was determined as described by AOAC (2005). The moisture content was determined by indirect method as described by AOAC (2005). Water holding capacity (WHC) were determined following the technique of (Chen at al., 2023)

The syneresis is measured following Arab et al. (2020) centrifugation of 10 mL of yogurt samples at 2500rpm in 10 min was followed. Then, the clear supernatant was weighed and expressed as a percentage of the initial weight of the yogurt sample.

$$\text{syneresis}\% = \frac{\text{Weight of the liquid}}{\text{Initial sample weight}} \times 100$$

The proximal composition of both fortified and standard yoghurt was determined by the methods approved by Association of Official Analytical Chemists (AOAC, 2001) for protein, and ICP-OES for iron, calcium, phosphorous.

Microbial analysis

Viable numbers of starter culture of *Lactobacillus delbrueckii* ssp. *bulgaricus* was counted on MRS agar (Hi Media laboratory) pour plates in yoghurt were determined. *Streptococcus thermophilus* was counted on M17 broth (Hi Media laboratories) pour plates. Yeast and Mold were counted on Yeas glucose chloramphenicol agar (Hi Media laboratories Mumbai) *Escherichia coli* was counted on violet red bile agar (Hi Media laboratories Mumbai).

Sensory Evaluation

Sensory evaluation of the standard and fortified yoghurt were done by trained panelists of the Department of Home Science. The evaluation was conducted using the hedonic scale method applied by, Wichchukit and Mahony (2022).

Results

The Protein, iron and calcium content in Casein-chelated iron (FeCN) was 31.34g, 9175mg and 6252mg per 1kg. So 1g of FeCN contains 0.31g protein, 9.17mg iron and 6.25mg calcium (Table no.1).

Table 1 Nutritive Values of Casein-chelated iron/1kg

S. No	Sample	Protein (g)	Iron (mg)	Calcium (mg)
1.	Casein-chelated iron (FeCN)	31.34	9175	6252

Physicochemical properties of iron fortified yoghurt

The data of the physicochemical properties of iron fortified yoghurt is analysed and presented in table 2. The iron fortified yoghurt samples prepared from the different concentrations of FeCN, i. e S I – 0.5g, S II- 1.0g, S III- 1.5g, S IV- 2.0g.

Table 2 Physicochemical properties of Iron fortified yoghurt

S. No	Samples	pH	Acidity (%)	Total Solids (%)	Moisture (%)	Syneresis (%/mL)	WHC (%/g)
1.	Control *	4.5	0.7	11	89	60	4.5
2.	Sample I *	4	0.76	12	88	56	4.5
3.	Sample II *	4.2	0.84	13.3	86	54	5
4.	Sample III *	4	0.93	12	88	50	4.8
5.	Sample IV *	4	0.74	13.8	86	50	5.5

*Control indicated without addition of FeCN, *Sample I-0.5g of FeCN, *Sample II-1.0g of FeCN, *Sample III-1.5g of FeCN, *Sample IV-2.0g of FeCN

The pH of the yoghurt should be 4.0 to 4.6 as per FSSAI. The pH of the control sample was 4.5. the pH of the fortified yoghurt ranged from 4 - 4.5 (table 2). The Casein-chelated iron yoghurt samples are slightly acidic than standard. This may be due to the reduction in pH and increase in titratable acidity can be attributed to the stimulatory effect of iron on lactic acid bacteria (LAB) metabolism. Iron can enhance the growth of lactic acid bacteria (LAB), especially *Lactobacillus* spp., by acting as a co-factor in enzymatic reactions. This can speed up fermentation and increase lactic acid production (Hurrell & Egli, 2010).

The total solids and moisture content values of the fortified yoghurt samples revealed that when concentration of the FeCN increases, total solids content of the samples increase compared to control. This increase may be due to the addition of iron-casein complexes, which contribute to the dry matter content of the yoghurt. Moisture content decrease when concentrations of the FeCN increased (table 2), It is found from the values that the moisture content of fortified yoghurt samples decreased with the increase in the concentration of FeCN. The reduction in moisture may reflect improved water binding by protein-iron interactions and the inclusion of solids from fortifying agents (Sandberg, 2002).

The syneresis (whey separation) of the control sample was 60%. The syneresis of the fortified yoghurt ranges from 50% to 56% (table 2), the syneresis was reduced with small variations by incorporating more concentrations of FeCN into the yoghurt. This decrease indicates improved gel network formation, likely due to protein cross-linking or increased protein-protein interactions facilitated by iron chelation (Sah et al., 2016). Reduced syneresis enhances yoghurt stability and is a desirable trait for acceptability.

Water holding capacity (WHC) improved in all fortified samples, with Sample IV exhibiting the highest value (5.5%) compared to the control (4.5%). Enhanced WHC can be attributed to a denser protein matrix, which better retains water. Iron, when chelated with casein, may form more stable protein complexes, enhancing the yoghurt's ability to trap moisture within its structure (Demirci et al., 2017).

Nutritional properties of iron fortified yoghurt

Table 3 Nutritive values of Iron fortified yoghurt (per 100ml of sample)

S.No	Test Parameters	Protein (g)	Iron (mg)	Calcium (mg)	Phosphorous (mg)
1.	Control	2.9	0.5	180	150
2.	S-I	3.2	3.4	183	180
3.	S-II	3.1	5.2	190	194
4.	S-III	3.5	7.6	186	300
5.	S-IV	2.9	9.8	192	320

The nutritional analysis of standard and fortified yoghurt samples are shown in table 3. The protein content in S-I, II, and III was 3.2g, 3.1g, and 3.5g respectively. This may be due to the increased presence of casein incorporated during the iron chelation process. Casein is a major milk protein and is often utilized to bind iron, increasing both protein and mineral content (Hurrell & Egli, 2010). In control 2.9g and S-IV – 2.9g of protein was recorded, which might suggest a different chelation ratio or processing variation that did not significantly alter the protein level.

Iron content in fortified yoghurt samples was S I- 3.4mg, S II- 5.2mg, S III- 7.6mg, and S IV- 9.8mg and in standard 0.5mg. The casein-chelated iron contains 91.75mg of iron per 100g. So 1g of casein-chelated iron contain 9.17mg. Hence addition of casein-chelated iron increased iron content in fortified samples in compared to standard sample. The use of casein-chelated iron enhances iron stability and bioavailability, while reducing metallic taste and reactivity with other components (Hurrell, 2002).

Calcium content in fortified yoghurt samples was S I- 183mg, S II- 190mg, S III- 186, and S IV- 192mg and in standard 180mg. fortified samples generally showing slightly higher values. The increase may result from calcium present in caseinates used for iron chelation, or from milk solids added during formulation. However, there is no dramatic rise, suggesting that iron fortification did not significantly interfere with calcium levels a positive outcome since high iron concentrations sometimes inhibit calcium absorption or cause precipitation (Sandberg, 2002).

Phosphorous content in fortified yoghurt samples was S I- 180mg, S II- 194mg, S III- 300, and S IV- 320mg and in standard 150mg. Phosphorus content showed a substantial increase, especially in Sample III and Sample IV, compared to control. This rise is most likely due to phosphoproteins like casein, which are rich in phosphorus. When casein is used as an iron chelator, phosphorus content naturally increases (Wardlaw & Smith 2007). Additionally, the increased phosphorus may help maintain mineral balance with elevated iron levels, as phosphorus works synergistically with calcium in bone metabolism and buffering systems.

Microbial evaluation of iron fortified yoghurt

Yoghurt prepared from the different concentrations of casein-chelated iron were evaluated for microbial quality as total plate count, total coliform, yeast and mold count. The results obtained were recorded as colony forming unit per mL of sample i.e. cfu/mL for total plate count and yeast and mold count and most probable number per mL i.e. MPN/mL for total coliform count reported in table 4.

Values illustrated in table 4 indicated the growth of microorganisms in both control yoghurt and yoghurt fortified with FeCN. It is also observed that the total plate increased with an increase in FeCN concentrations. *L. delbrueckii ssp. Bulgaricus* for control and iron fortified yoghurt S I, S II, S III, and S IV were 90, 92, 89, and 86, control 83 respectively. Similarly, *S. thermophilus* counts were 76 and 81, 83, 80, 83 for control and S I, S II, S III, S IV respectively. No effect was found in bacterial growth that is *L.*

delbrueckii ssp. *Bulgaricus* and *S. thermophilus* when the source of iron used for fortification and level of concentrations (0.5g, 1.0g, 1.5g, and 2.0g of Fe/100ml). This slight enhancement can be attributed to the positive role of iron in bacterial metabolism. Coliforms are not detected in all variations of yoghurt samples. Thus, casein chelation of iron prior to its addition to the yoghurt mix had no beneficial effect on the growth of the yoghurt mix starter culture. It is quicker and easier to add the iron as FeSO₄.

Table 4 Microbial evaluation of fortified yoghurt

S. No	Samples	Total plate count (cfu/mL)		Yeast and mold count (cfu/mL)	Coliforms (MPN/mL)
		<i>L. delbrueckii</i> ssp. <i>Bulgaricus</i>	<i>S. thermophilus</i>		
1.	Control	83	76	27	Coliforms are not detected in all samples
2.	Sample I	90	81	20	
3.	Sample II	92	83	11	
4.	Sample III	89	80	15	
5.	Sample IV	86	83	19	

Sensory evaluation of iron fortified yoghurt

Yoghurt was evaluated for organoleptic properties (odor, color, taste, texture and overall acceptability) and the results are presented in table 5. Sample-I (0.5g) from the panelists no changes in terms of odor, color, taste, texture and overall acceptance score compared to control. Sample-II and Sample-III received a lower color score (3.5 and 3.5) compared to other concentrations. Sample-IV received a lower odor, color, taste, texture and overall acceptability score from the panelists compared to control and other concentrations.

Table 5 Sensory evaluation of fortified yoghurt

Hedonic rating test score card (Score points 5)

S.No	Sample	Sensory attributes				
		Odor	Color	Taste	Texture	Overall Acceptability
1.	Control	4	4	4	4	4.5
2.	S I	4	4	4	4	4.5
3.	S II	4	3.5	4	4	4.5
4.	S III	4	3.5	4	4	4
5.	S IV	3	3	3	3	3

The sensory scores indicated that adding FeCN (0.5g) formulation received higher scores of odor, color, taste, texture and overall acceptance i.e equal score of standard yoghurt compare to other concentrations. The FeCN (1.0g & 1.5g) formulation achieved the slightly lower score of color. The FeCN (2.0g) formulation was negatively associated with consumers liking of odor, color, taste, texture and overall

acceptance. While initial fortification levels (S-I to S-III) maintained consumer acceptability, S-IV showed a clear decline, indicating that there is a sensory threshold beyond which iron fortification becomes unacceptable. The moderate levels of iron fortification did not significantly alter the typical yoghurt sensory. However, high iron concentration in S-IV may have caused a slightly metallic or off-odor, which reduced its overall acceptance. Excessive iron fortification is known to interact with lipid and protein components, producing off-flavors or odors (Hurrell, 2002) importance of keeping iron concentrations below sensory detection thresholds to ensure palatability in fortified foods.

The sensory properties of the fortified yoghurt depend on the type of additives, their chemical compositions, % of addition, and the storage period of the fortified yoghurt (Ahmad et al., 2024).

Discussion

Fortification of yoghurt with casein-chelated iron significantly influences its physicochemical properties, particularly pH, acidity, total solids, moisture content, and water holding capacity (WHC). The pH of fortified samples tends to decrease slightly compared to the control, which may be attributed to enhanced metabolic activity of starter cultures like *Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus thermophilus* in the presence of bioavailable chelated iron, resulting in increased lactic acid production. Consequently, titratable acidity increases with higher iron concentrations due to greater fermentation efficiency and potential interactions of iron with the milk buffer system. Total solids content also rises in fortified yoghurt, primarily due to the additional casein introduced during the iron-chelation process, which contributes to protein content and overall solids. Correspondingly, moisture content shows a slight decline, inversely related to total solids, as the denser protein matrix holds less free water. The water holding capacity improves moderately with fortification, as casein enhances the protein network's ability to retain water, although excessively high iron levels could potentially destabilize this structure.

Iron fortification of yoghurt using casein-chelated iron enhances its nutritional profile, particularly in terms of protein, iron, calcium, and phosphorus content. Protein levels show a slight increase in fortified samples due to the additional casein used in the chelation process. Iron content rises significantly, demonstrating the effectiveness of casein as a carrier for improving iron bioavailability. Calcium and phosphorus levels also improve moderately, likely due to the mineral-binding capacity of casein, which helps retain these essential nutrients in the final product. Overall, the fortification process not only addresses iron deficiency but also supports a richer nutritional composition.

The microbial evaluation of iron-fortified yoghurt showed that total bacterial counts, particularly of beneficial cultures like *Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus thermophilus*, remained within acceptable ranges and slightly increased in fortified samples, indicating that casein-chelated iron did not inhibit the growth of starter cultures. Yeast and mold counts were low across all samples, suggesting good hygienic processing and stability of the product. Importantly, coliforms were

not detected in any of the samples, confirming the microbiological safety and sanitary quality of the fortified yoghurt.

The sensory evaluation of iron-fortified yoghurt indicated that samples up to a moderate level of fortification maintained comparable overall acceptability to the control (standard) yoghurt. Samples I to III received high scores for odor, taste, texture, and overall acceptability, showing that fortification with casein-chelated iron at lower levels did not negatively impact sensory attributes. However, Sample IV, with the highest iron concentration, showed a noticeable decline in all sensory parameters, particularly in color and taste, which may be due to metallic flavor or discoloration caused by excess iron. Thus, moderate fortification levels are well accepted, while higher concentrations may reduce consumer appeal.

Conclusion

Yoghurt is a daily consumable product in the meal in all economic levels of population. Hence Casein-chelated iron fortification in the yoghurt will be one of the best way to supplement iron in the community.

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