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The Effects of Fortifying Rayeb Milk with Different Forms of Iron on Its Physicochemical, Sensory, Antimicrobial, and Anticancer Properties

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ABSTRACT

Although Rayeb milk is regarded as the greatest and most complete diet, it lacks iron. However, most users may obtain iron from Rayeb milk fortification. To fortify such Rayeb milk, three distinct iron salts were utilized: ferrous fumarate, ferric hydroxide poly maltose and iron amino acid chelate). The chemical properties, pH, color, sensory properties, antimicrobial and anticancer activities were evaluated 1 day, 7 days, and 15 days after manufacturing. There were notable differences ($P < 0.05$) detected in the levels of dry matter, total protein, and lactose between the control group and the iron-fortified Rayeb milk samples using different forms of iron. Using different forms of iron for fortification did not affect the incubation time of Rayeb milk until it reached a pH of 4.5. Iron enrichment decreased the b* parameter, reducing the yellow hue of the Rayeb milk samples, as well as the luminosity (L). However, it caused a significant increase in the a* parameter, indicating a shift towards redness in the samples. There are no notable differences in the sensory characteristics between Rayeb milk fortified with iron-amino acid chelate or ferric hydroxide polymaltose and the unfortified Rayeb milk. Rayeb milk fortified with ferrous fumarate displayed the most substantial inhibition zone against microbial indicator strains among all treatments. All treatments had an IC_{50} rate greater than Doxorubicin, indicating that the inhibition rate for all treatments in both types of cancer cells was lower than the positive control.

Keywords: Antibacterial activity; cytotoxicity assay; Fermented milk, Function food, Iron salts

INTRODUCTION

Foods can be classified as 'functional' when they are whole, enriched, fortified, or enhanced, offering health benefits beyond basic nutrient provision (Dawood *et al*., 2021; Darwish *et al*., 2022a; Darwish *et al*., 2023c; Darwish *et al*., 2023d). The functional properties of numerous fruits or plants, particularly, those used in functional foods as new nutraceuticals are becoming known. It's interesting to note that dairy products are among the categories of functional foods that people choose (El Dessouky Abdel-Aziz *et al*., 2020; Elbermawi *et al*., 2022a; Elbermawi *et al*., 2022b; Khojah *et al*., 2022). They are regarded as the quintessential carriers of functional ingredients, and consequently, their beneficial properties have been extensively investigated (Abd El-Aziz and Darwish, 2014; Darwish, 2016; Darwish and Mostafa, 2016; Darwish and Taher, 2017; Nassib *et al*., 2018a; Nassib *et al*., 2018b; Darwish *et al*., 2022a; Darwish *et al*., 2023a; Darwish *et al*., 2023b; Darwish *et al*., 2023d; Samra *et al*., 2023).

Fermented milk products with traditional origins enjoy widespread consumption globally. These items serve as significant additions to local diets, offering essential nutrients for growth, overall well-being, and a desirable taste, as noted by Mohran *et al*. (2018). Lactic acid starter cultures play a distinctive role in both transforming and preserving milk by-products, functioning as specialized bio-converters of energy. Rayeb milk is prominently recognized as a frequently fermented dairy item within the Middle Eastern geographical expanse. The traditional method of making Rayeb milk involves allowing raw milk to naturally ferment, which is made possible by the enzymatic activity and presence of bacteria in the milk. After letting raw milk sit at room temperature for about 48 hours, the cream layer is separated to make butter and butter oil. The remaining milk, with its notably high acid content, tends to coagulate upon heating. In recent years, there has been a shift towards large-scale production of safe and standardized Rayeb milk in dairy plants utilizing starter cultures (El-Sharoud *et al*., 2012; Ryssel *et al*., 2014; Delorme *et al*., 2017; El-Menawy *et al*., 2023).

Iron is one element that is crucial to human nourishment. It is an essential part of heme that is necessary for the transport, storage, and use of oxygen in both hemoglobin and myoglobin. Anemia, poor mental development, weakened immunity, poor pregnancy outcomes, decreased cognitive capacities in children and reduced work capability in adults can all result from iron deficiency (Martınez-Navarrete *et al*., 2002). Dietary iron, such as that found in heme form in red meat, can be highly absorbable, though it might be financially inaccessible to many individuals. The iron found in other vegetable-based goods is non-heme and has limited bioavailability due to its interaction with dietary ingredients including phytates, polyphenols and tannins that prevent it from being absorbed. People from lower socioeconomic strata eat a lot of this type of food, making it impossible for them to achieve their iron intake requirements (van den Broek and Letsky, 2000). As a result, it is common in both regions with less industrialization and developing nations. Additionally, poor absorption of iron, inadequate dietary iron intake, or a combination of both can lead to iron

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deficiency (Gaucheron, 2000). Adding iron directly to dairy products might be a good approach to increase the amount of iron that the public consumes through food. Yogurt is a great source of proteins, minerals and vitamins, yet it contains minimal iron content, approximately 0.2 mg/kg (Gaucheron, 2000), This implies that the recommended daily intake for iron is not available for consumer. Hence, dairy products serve as a practical option for fortifying iron (El-Kholy *et al*., 2011) and are seen as a workable, long-term fix that is reasonably priced (Abbasi and Azari, 2011). Fermented milk products have been utilized to provide essential nutrients to the human diet as they are among the most widely eaten foods worldwide. Additionally, yogurt fortification is a wonderful approach to increase the amount of nutrients included in regular food items (Preedy *et al*., 2013). Dairy products fortified with iron might aid in the treatment of nutritional deficits. Yogurt with added iron has a comparatively high iron bioavailability (Vande Woestyne *et al*., 1991). But first, it's important to evaluate the results of adding iron to yogurt before carrying out any procedures, such fortification. It's crucial to determine the criteria, which include fat oxidation, flavor, shelf life, and microbial physiology, as well as the general acceptability and sensory quality of fortified yogurt (Zhang and Mahoney, 1989).

Adults need 19.3-20.5 mg of iron per day on average for men and 17.0-18.9 mg per day on average for women over the age of 19 (Ziena *et al*., 2019). Thirty percent of the various iron compounds used to strengthen formulations are bioavailable. Thus, the aim of the study was to supply about one-third of an adult's daily iron requirements by adding 20 mg of iron to a standard serving of yoghurt, considering that one-third of daily requirements is important availability (30%).

MATERIALS AND METHODS

Materials

Chemical and reagents

All chemicals and reagents utilized in this study were procured from Sigma Aldrich (St. Louis, MO, USA). Tryptone soya broth (TSB) and tryptone soya agar (TSA) were obtained from Thermo Fisher Scientific (Cairo, Egypt).

The standardized bovine milk, obtained from the local market in Mansoura City, contained 3% milk fat, 12% total solids (TS), 3.5% protein, and had a pH of 6.68.

Microbial strains and cell line

All microbial strains employed in this investigation were sourced from the stock strains collection at the food microbiology laboratory within the Dairy Department of the Faculty of Agriculture, Mansoura University, Egypt. Breast cancer cells (MDA-MB231) and colon cancer cells (LS-174T) were acquired from the American Type Tissue Culture Collection (ATCC). Tissue culture media and cell culture reagents were procured from Thermo Fisher Scientific (Cairo, Egypt).

Methods

Manufacture of Rayeb milk

The milk undergoes heating to 95°C for 10 minutes, followed by cooling to 50°C and partitioning into four portions. Iron amino acid chelate is added to the first portion, ferric hydroxide polymaltose to the second, ferrous fumarate to the third, while the fourth portion remains as plain Rayeb milk. Various iron forms are introduced to achieve a final concentration of 20 mg/kg in the milk. Subsequently, a 2% starter culture is introduced and incubated at 42°C until full coagulation. Following this, it is stored refrigerated at 4°C overnight. Each treatment is meticulously blended for 5 minutes, then divided into three segments for analysis. The first segment corresponds to Rayeb milk after one day of storage at 4°C, the second to Rayeb milk after 7 days at 4°C, and the third to Rayeb milk after 15 days at 4°C.

Extracting Rayeb Milk Supernatants

The Rayeb milk supernatants, which are essentially whey extracts, were prepared following established procedures (Abdel-Hamid *et al*., 2020). Briefly, samples underwent centrifugation at 20,000× g for 60 minutes at 4°C. After centrifugation, the supernatants were filtered through a 0.45 µm syringe filter and stored at −20°C until further analysis. These Rayeb milk supernatant filtrates were derived from both regular Rayeb milk (without added iron) and iron-fortified Rayeb milk prepared with various iron supplements. The supernatants obtained from plain Rayeb milk and iron-fortified yogurts were utilized for subsequent disc diffusion and MTT assays.

Chemical analysis of Rayeb milk samples

Chemical analysis was conducted by subjecting the samples to various tests. Total solids were measured using a hot air oven set at 105°C for 6 hours, following the method outlined in AOAC (Horwitz and Latimer, 1975). Protein, fat, and titratable acidity levels were determined based on the procedure outlined by Lin *et al*. (2016).

Physiochemical analysis

pH and acidity analysis

The pH was determined utilizing a digital pH meter (Hanna pH 210). The titratable acidity (TA) was calculated as the percentage of lactic acid following the method outlined in AOAC 947.05 (Darwish *et al*., 2021).

Color analysis

Color analyses of the Rayeb milk were performed on fresh products and after 7 and 15 days of refrigerated storage using a Hunter colorimeter (Hunter Ultra Scan VIS). The results were presented in terms of Hunter L, a, and b values. Specifically, L* represented the lightness value (ranging from 0 to 100, indicating dark to light), *a** indicated the degree of red and green color, with higher positive values suggesting more red, and *b** represented the degree of yellow and blue colors, with higher values indicating more yellow (Hunter and Harold, 1987).

The antimicrobial effects of supernatant from iron enriched Rayeb milk.

The antimicrobial efficacy was assessed using the disc diffusion method, as outlined previously by Darwish *et al*. (2022b). In summary, the test involved various indicator microorganisms: Gram-positive bacteria (including. *Staphylococcus aureus*, *Bacillus cereus*, *Staphylococcus epidermidis*, and *Enterococcus faecalis*), Gram-negative bacteria (such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterobacter cloacae* and *Proteus vulgaris*), and yeast species (including *Candida albicans*, *Candida parapsilosis*, *Candida krusei*, *Candida tropicalis*, and *Candida glabrata*). Overnight cultures of bacteria (100 µL) were streaked onto nutrient agar synthetic

media, while Sabouraud dextrose agar was used for fungal cultures. The filtrates from supernatant of iron fortified Rayeb milk were then added to the wells, followed by an incubation period at 37°C for 24 hours. The diameter of the inhibition zone was measured using a Vernier caliper as an indicator of antimicrobial activity.

Sensory properties analysis

A group of trained experts from the Dairy Science Department at the Faculty of Agriculture, Mansoura University, assessed the occurrence of undesirable flavors like oxidized, metallic, or bitterness in Rayeb milk fortified with various iron compounds. The evaluation was conducted at intervals of 1, 7, and 15 days of storage at 4°C. Each assessor received four samples simultaneously and rated the intensity of bitter, oxidized, and metallic off-flavors using a nine-point scale, ranging from 1 (not noticeable) to 9 (very strong) (Simova *et al*., 2008).

Cytotoxicity Assay of iron fortified Rayeb milk

The breast cancer cells (MDA-MB231) and colon cancer cells (LS-174T) were cultured as a single layer in DMEM medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 2 mM L-glutamine. The potential cytotoxic effects of iron-fortified Rayeb milk supernatants on breast cancer cells (MDA-MB231) and colon cancer cells (LS-174T) were assessed concurrently with regular Rayeb milk, using an MTT assay following procedures outlined by Darwish *et al*. (2022b). The assay was conducted after exposing the cells to diluted supernatants of iron-fortified Rayeb milk for 24 hours.

Statistical Analysis

Each test was replicated three times. An ANOVA test, with a significance level set at $p < 0.05$, was employed to analyze alterations in chemical composition, physiochemical attributes, antimicrobial effects, cytotoxicity, and sensory characteristics. Results were expressed as mean ± standard deviation. Significant disparities among values were assessed using Duncan's multiple range tests.

RESULTS AND DISCUSSION

Chemical properties of Rayeb milk samples

The chemical analysis results indicated resemblances in the composition between the control and experimental batches of Rayeb milk (refer to Table 1). There were significant differences ($P < 0.05$) observed in the levels of dry matter, total protein, and lactose between the control group and the iron-fortified Rayeb milk samples utilizing various forms of iron. These outcomes are consistent with the findings reported by Simova *et al*. (2008). The significance of fat levels in Rayeb milk was notably high ($P<0.05$) over the duration of storage. Throughout the 15-day storage period, fat levels in all Rayeb milk samples decreased, likely attributed to enzymatic lipolysis. The findings regarding fat content align with prior research. Salih and Hamid (2013) explored the compositional qualities of fortified yogurt, echoing similar results. Kavas and Kavas (2016) investigated the nutritional and compositional aspects of set-type yogurt with different thickening agents, noting a decrease in fat content after 10 days of storage. Additionally, other studies by Huma *et al*. (2003), Dublin-Green and Ibe (2005) and Khaliq *et al*. (2022) also documented a decreasing trend in yogurt fat content over storage duration.

The statistical examination indicated a high significance ($P<0.05$) in protein contents due to both iron supplementation and storage duration (Table 1). Protein levels declined from day 1 to day 15 of storage (Table 1). Introducing iron-amino acid chelate in fortified Rayeb milk resulted in increased protein levels compared to other treatments. However, the addition of ferric hydroxide polymaltose and ferrous fumarate did not affect the protein concentration in Rayeb milk. These findings align with those of Ziena *et al*. (2019), who noted a rise in protein levels in iron-amino acid chelate-fortified yogurt.

The findings regarding protein content coincide with those of El Owni and Mahgoub (2012), who noted fluctuations in protein levels during the storage investigation of goat milk yogurt. Eissa *et al*. (2010) similarly observed a decrease in protein content of yogurt while examining its microbiological, sensory, and compositional characteristics using goat milk. The results are also consistent with those of Sah *et al*. (2016), who explored the compositional and rheological aspects of probiotic yogurt fortified with pineapple peel during refrigerated storage.

The statistical analysis shows that lactose levels were notably influenced ($P<0.05$) by fortifying yogurt with Ferric hydroxide poly maltose, while the other iron forms had no significant effect. However, the duration of storage significantly affected lactose content. The decrease in lactose content in iron-fortified yogurt is attributed to its conversion by lactic acid bacteria (LAB) into lactic acid, the main product of fermentation. These latest findings align with earlier studies that have also observed a decrease in lactose content during the storage of yogurt produced from milk obtained from different animals (Saccaro *et al*., 2009; Egwaikhide and Faremi, 2010; Salih and Hamid, 2013).

The statistical assessment concerning the ash levels of Rayeb milk, prepared using different forms of iron, did not yield significance ($P<0.05$, Table 1). Additionally, the impact of storage duration was deemed insignificant ($P>0.05$, Table 1). The ash content findings are consistent with those presented by El Owni and Mahgoub (2012), who examined yogurt characteristics over storage periods. Similarly, Kavas and Kavas (2016) investigated the properties of set-type yogurt and found no significant changes in ash content during their storage analysis. The statistical analysis reveals that both total solids and solids not fat were significantly influenced ($P<0.05$) by both the type of iron used and the duration of storage (Table 1).

The percentage of both total solids and solids not fat in Rayeb milk fortified with various forms of iron was higher than that in the control sample. The decline in solids not fat could be attributed to alterations in lactose and protein levels, as discussed earlier, which are typically regarded as biochemical changes such as proteolysis and saccharolytic behavior in Rayeb milk resulting from fermentation by lactic acid bacteria during storage. These findings are corroborated by other researchers who noted a decrease in solids not fat during yogurt storage studies, considering them as part of total (Hematyar *et al*., 2012; Al-Otaibi and El-Demerdash, 2013; Sakandar *et al*., 2014). It was demonstrated that the targeted iron fortification levels were achieved in the experimental Rayeb milk batches.

Table 1. The chemical composition of various iron-fortified Rayeb milk samples under storage conditions at 4°C ± 1°C

Parameters	Treatments	Storage periods (day)		
		1	7	15
Total solids	Control	12.75 ± 0.08 ^{Ac}	12.38 ± 0.12 ^{Bc}	12.05 ± 0.05 ^{Cb}
	T1	13.12 ± 0.06 ^{Aa}	12.65 ± 0.14 ^{Ba}	12.24 ± 0.11 ^{Ca}
	T2	13.17 ± 0.12 ^{Aa}	12.60 ± 0.18 ^{Ba}	12.20 ± 0.22 ^{Ca}
	T3	12.88 ± 0.24 ^{Ab}	12.48 ± 0.27 ^{Bd}	12.1 ± 0.18 ^{Cb}
Total protein	Control	3.5 ± 0.12 ^{Ab}	3.41 ± 0.05 ^{Bb}	3.32 ± 0.07 ^{Cb}
	T1	3.83 ± 0.11 ^{Aa}	3.64 ± 0.09 ^{Ba}	3.51 ± 0.05 ^{Ca}
	T2	3.56 ± 0.04 ^{Ab}	3.41 ± 0.12 ^{Bb}	3.32 ± 0.06 ^{Cb}
	T3	3.55 ± 0.13 ^{Ab}	3.44 ± 0.09 ^{Bb}	3.34 ± 0.05 ^{Cb}
Fat (%)	Control	3.53 ± 0.05 ^{Aa}	3.38 ± 0.08 ^{Ba}	3.26 ± 0.1 ^{Ca}
	T1	3.57 ± 0.08 ^{Aa}	3.42 ± 0.11 ^{Ba}	3.28 ± 0.04 ^{Ca}
	T2	3.58 ± 0.07 ^{Aa}	3.44 ± 0.1 ^{Ba}	3.28 ± 0.03 ^{Ca}
	T3	3.56 ± 0.06 ^{Aa}	3.45 ± 0.13 ^{Ba}	3.29 ± 0.05 ^{Ca}
Lactose (%)	Control	4.67 ± 0.08 ^{Ab}	4.54± 0.14 ^{Bb}	4.42 ± 0.12 ^{Cb}
	T1	4.72 ± 0.12 ^{Ab}	4.55 ± 0.12 ^{Bb}	4.41 ± 0.07 ^{Cb}
	T2	4.92 ± 0.11 ^{Aa}	4.72 ± 0.06 ^{Ba}	4.55 ± 0.06 ^{Ca}
	T3	4.71 ± 0.05 ^{Ab}	4.52 ± 0.05 ^{Bb}	4.43± 0.21 ^{Cb}
Ash (%)	Control	1.05 ± 0.05 ^{Aa}	1.05 ± 0.04 ^{Aa}	1.05 ± 0.05 ^{Aa}
	T1	1.05 ± 0.12 ^{Aa}	1.04 ±0.03 ^{Aa}	1.04 ± 0.03 ^{Aa}
	T2	1.06 ± 0.08 ^{Aa}	1.03 ±0.05 ^{Aa}	1.05 ± 0.04 ^{Aa}
	T3	1.06 ± 0.04 ^{Aa}	1.06 ± 0.02 ^{Aa}	1.04 ± 0.02 ^{Aa}
SNF (%)	Control	9.22 ± 0.05 ^{Ac}	9.00 ± 0.04 ^{Bb}	8.79 ± 0.08 ^{Cb}
	T1	9.55 ± 0.08 ^{Aa}	9.23 ± 0.11 ^{Ba}	8.96 ± 0.07 ^{Ca}
	T2	9.59 ± 0.12 ^{Aa}	9.16 ± 0.12 ^{Ba}	8.92 ± 0.12 ^{Ca}
	T3	9.32 ± 0.05 ^{Ab}	9.03 ± 0.05 ^{Bb}	8.81 ± 0.10 ^{Cb}
Fe (mg/Kg)	Control	0.76 ± 0.12 ^{Aa}	0.74 ± 0.12 ^{Aa}	0.75 ± 0.12 ^{Aa}
	T1	20.02 ± 0.13 ^{Aa}	20.05 ± 0.14 ^{Aa}	20.08 ± 0.13 ^{Aa}
	T2	20.05 ± 0.08 ^{Aa}	20.03 ± 0.13 ^{Aa}	99.99 ± 0.12 ^{Aa}
	T3	19.98 ± 0.09 ^{Aa}	20.01 ± 0.06 ^{Aa}	19.97 ± 0.05 ^{Aa}

The average of three repeated measurements indicated in the same manuscript (small letters within columns, capital letters within rows) show no significant difference at a significance level of $p \leq 0.05$. T1: Iron amino acid chelate; T2: Ferric hydroxide poly maltose; T3: Ferrous fumarate.

Physiochemical Analysis

pH and acidity

The use of various iron forms in fortification did not impact the incubation time of Rayeb milk until reaching pH 4.5, as all batches reached pH 4.5 ± 0.1 within 3 hours (Figure 1).

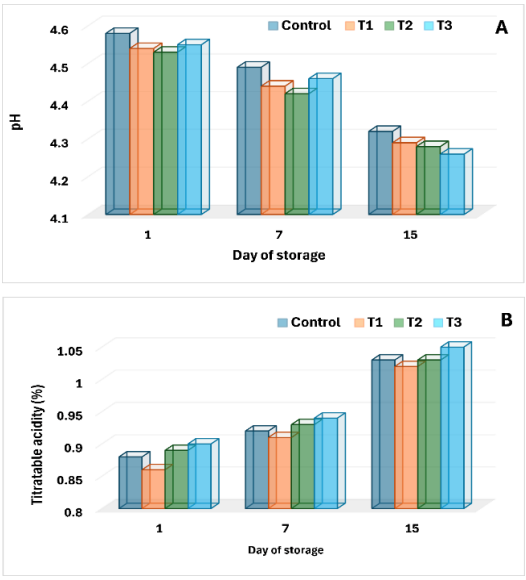

Figure 1. Variations in the pH and total acidity (TA) of Rayeb milk fortified with various iron forms were monitored over a 15-day storage period at 5°C. T1 refers to Rayeb milk supplemented with iron amino acid chelate, T2 denotes Rayeb milk enriched with ferric hydroxide polymaltose, and T3 signifies Rayeb milk fortified with ferrous fumarate.

This finding aligns with findings reported earlier (Hekmat and McMahon, 1997; Darwish *et al*., 2021). Figure 1 illustrates the variations in pH and TA values of iron products over a 15-day cold storage period. The findings indicated no notable distinctions between treatments either upon initial product assessment or after various storage durations.

However, all products exhibited a significant decline ($p \leq 0.05$) in pH values after 15 days, ranging from 4.32 to 4.26. A contrasting pattern was noted in the total acidity (TA) of all products, which steadily rose throughout the storage period until the 15th day. There were no significant differences in the TA among all treatments.

Color

The iron-fortified samples displayed noteworthy variances ($P < 0.05$) when compared to the control group. Iron enrichment resulted in diminished luminosity (L), a marked elevation in the *a** parameter indicating a tendency towards redness, and a decrease in the *b** parameter, indicating a reduction in the yellow hue of the Rayeb milk samples. (Figure 2). This aligns with findings by Ramírez-Sucre and Velez-Ruiz (2013), who noted an elevation in the *a** parameter. Specifically, they reported values of -2.97 for the control group and 4.26 for samples with a high concentration of caramel and fiber in the yogurt formulation.

Sensory evaluation

The outcomes of the sensory evaluation (as displayed in Figure 3) revealed that there was no rise in the occurrence of oxidized, metallic, or bitter off-flavors in Rayeb milk fortified with various iron formulations. The expert panel did not identify any notable distinctions in the

overall quality between milk fortified with iron-amino acid chelate or ferric hydroxide polymaltose, in comparison to unfortified Rayeb milk. However, Rayeb milk fortified with ferrous fumarate received the lowest score across all sensory attributes when compared to other treatments. There were no observable alterations in sensory parameters or consumer acceptance after 7 and 15 days of refrigerated storage in products fortified with different iron forms, in contrast to fresh Rayeb milk. Our findings coincide with those of Simova *et al*. (2008), who similarly observed no escalation in oxidized, metallic, or bitter off-flavors in yogurts fortified with 8, 15, and 27 mg of iron per kilogram of yogurt.

Antimicrobial activity of iron enriched Rayeb milk

The hierarchical categories were separated into Ter1 and Ter2 groups based on the types of iron supplementation (plain Rayeb milk (P), iron amino acid chelate (T1), ferric hydroxide polymaltose (T2), and ferrous fumarate (T3)), along with the storage duration (1 day (1), 5 days (5), 10 days (10), and 15 days (15)). Ter1 consists of 2 subgroups: Ter1-A includes T1-10, T2-10, and T3-5, while Ter1-B comprises T3-10, T3-15, T2-15, and T1-15. Additionally, Ter2 group is divided into 2 subgroups: Ter2-A comprises P-1, P-5, and P-10, whereas Ter2-B denotes T2-1, T2-5, T3-1, T1-5, P-15, and T1-1. However, the microbial indicator strains were divided into two primary groups: MIS-1 and MIS-2. Within cluster MIS-1, there were two subcategories: MIS-1A comprised *C. parapsilosis* and *C. krusei*, whereas MIS-1B encompassed *C. albicans, C. tropicalis, C. glabrata, P. aeruginosa*, and *E. cloacae*. In MIS-2, subgroup MIS-2A included *E. faecalis, S. epidermidis, Staph. aureus, and P. vulgaris*, while MIS-2B comprised *K. pneumoniae, E. coli, B. cereus*, and *B. subtilis* (Figure 4). The findings showed that the antimicrobial activity of iron fortified Rayeb milk supernatants against indicator microorganisms remained potent across various storage periods, albeit to varying extents (Figure 4). There is a direct relationship between inhibition rate and storage period, as the inhibition zone diameter increases with longer storage durations. It was observed that inhibition rates were highest across all treatments after 15 days of storage at a temperature of 4°C. Rayeb milk fortified with ferrous fumarate displayed the most substantial inhibition zone against microbial indicator strains among all treatments. Conversely, the extract of plain Rayeb milk showed no inhibition zone against microbial indicator strains after being stored for 1 to 5 days at 5°C. The findings of the present research align with those of Gholami *et al*. (2020), who demonstrated that both ferric and ferrous iron ions, along with superparamagnetic iron oxide nanoparticles (SPIONs), exhibited noteworthy antimicrobial effects that were dependent on various factors. SPIONs displayed more pronounced inhibitory effects compared to ferrous and ferric ions when exposed to treated bacterial strains in anaerobic conditions, whereas under aerobic conditions, ferrous ions demonstrated the most potent antibacterial activity.

Cytotoxic Characteristics of iron-fortified Rayeb milk

All treatments had an IC_{50} rate greater than Doxorubicin, indicating that the inhibition rate for all treatments in both types of cancer cells was lower than the positive control. Regarding breast cancer cells (MDA-MB231), iron-fortified Rayeb milk using ferric hydroxide polymaltose (T2), had the lowest IC_{50} values ranging from 24.7-41.71 µg/mL (Figure 5), followed by Rayeb milk fortified with iron amino acid chelate (T1), which reached IC_{50} values from 24.7-41.71 µg/mL. Finally, Rayeb milk enriched with ferrous fumarate (T3), had IC_{50} values ranging from 54.8 to 72.2 µg/mL, indicating that iron fortification with ferric hydroxide polymaltose had the highest inhibition rate compared with other treatments (Figure 5). Concerning colon cancer cells (LS-174T), we found that the first treatment, iron-fortified Rayeb milk with iron amino acid chelate, had the lowest IC_{50} values ranging from 23.18-32.78 µg/mL (Figure 5), followed by the second treatment, iron-fortified Rayeb milk using ferric hydroxide polymaltose, with IC_{50} values ranging from 27.46-38.48 µg/mL. Finally, Iron-fortified Rayeb milk with ferrous fumarate, had IC_{50} values ranging from 50.45 to 64.91 µg/mL, indicating that iron fortification with iron amino acid chelate, had the highest inhibition rate compared with other treatments (Figure 5). It is also noticeable that there is an inverse relationship between IC_{50} and storage time, as the storage period increased, the cytotoxic activity of cancer cells in both types increased (Figure 5). The current study aligns with many previous studies regarding the ability of polysaccharides iron or iron amino acid chelate to act as anti-tumor agent (Feng and Zhang, 2020; Jing *et al*., 2022).

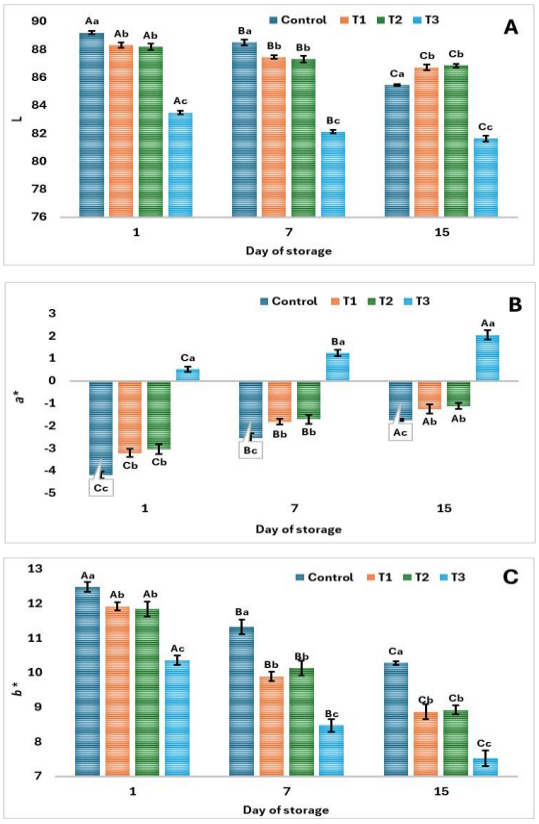

Figure 2. Parameters of color of iron-fortified Rayeb milk at 15 days of storage. T1 refers to Rayeb milk supplemented with iron amino acid chelate, T2 denotes Rayeb milk enriched with ferric hydroxide polymaltose, and T3 signifies Rayeb milk fortified with ferrous fumarate. The average of three replicates was taken. Significant differences (P < 0.05) are indicated by distinct letters. Lowercase letters denote variances between samples, while uppercase letters signify discrepancies observed during storage.

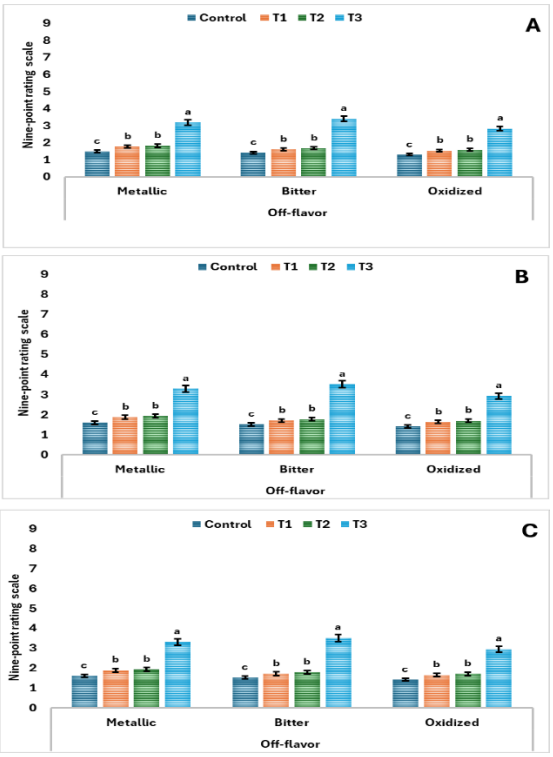

Figure 3. Evaluating the sensory traits of Rayeb milk in both its standard form and in experimental variations. T1 signifies Rayeb milk enriched with iron amino acid chelate, T2 denotes Rayeb milk fortified with ferric hydroxide polymaltose, and T3 represents Rayeb milk supplemented with ferrous fumarate. The average results from three replicates were considered. Notable variations ($P < 0.05$) are marked by different letters. Labels A), B), and C) correspond to fresh Rayeb milk, Rayeb milk stored for 7 days, and Rayeb milk stored for 15 days, respectively.

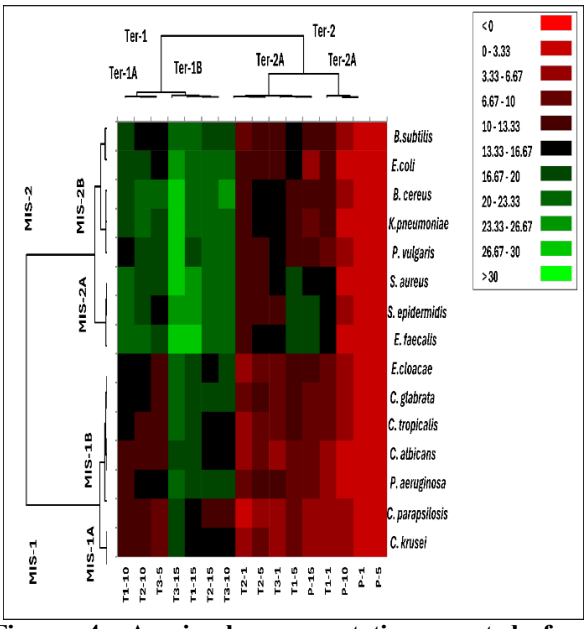

Figure 4. A visual representation created from quantitative data on antimicrobial activity across various treatments, displayed as a heat map. The color gradient shifts from red to green to indicate levels of antimicrobial effectiveness.

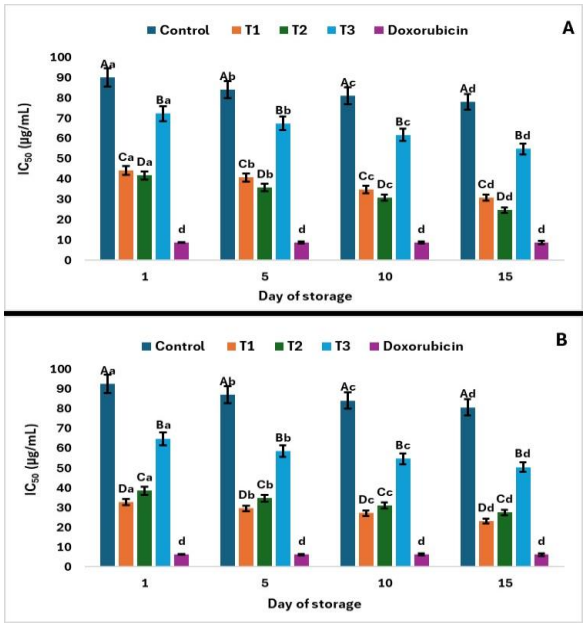

Figure 5. Comparison of the IC_{50} values of the iron-fortified Rayeb milk supernatants against human cancer cells. (A–B) for breast (MDA-MB231) and colon (LS-174T) cell lines, respectively. T1 refers to Rayeb milk supplemented with iron amino acid chelate, T2 denotes Rayeb milk enriched with ferric hydroxide polymaltose, and T3 signifies Rayeb milk fortified with ferrous fumarate. An average of three replicates was taken. Significant differences ($P < 0.05$) are indicated by distinct letters. Uppercase letters denote variances between samples, while lowercase letters signify discrepancies observed during storage.

CONCLUSION

In summary, the addition of iron to Rayeb milk in various forms has minimal impact on yogurt characteristics, with a preference for the first treatment (yogurt fortified with iron amino acid chelate) due to its superior levels of crude protein, ash content, sensory evaluation, color, and cytotoxicity assay results. Following this, iron-fortified Rayeb milk using ferric hydroxide polymaltose ranked next. However, iron fortification with ferrous fumarate exhibited the highest antibacterial activity after a 15-day storage period compared to the other treatments.

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تأثير تدعيم اللبن الرايب بصور مختلفة من الحديد على خواصه الفيزيائية والكيميائية والحسية والمضادة للميكروبات والمضادة للسرطان

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الملخص

على الرغم من أن اللبن الرايب يعتبر من الأغذية قريبة التكامل من حيث القيمة الغذائية، إلا أنه يفتقر إلى الحديد. ومع ذلك، يمكن لمعظم المستهلكين الحصول على الاحتياجات اليومية من الحديد من خلال اللبن الرايب المدعم بالحديد. ولتدعيم اللبن الرايب بالحديد، تم استخدام ثلاثة أملاح حديد متميزة: فومارات الحديدوز، هيدروكسيد الحديديك بولي مالتوز والحديد المرتبط بالأحماض الأمينية). تم تقييم الخواص الكيميائية ودرجة الحموضة واللون والخصائص الحسية والنشاط المضاد للميكروبات والخلايا السرطانية بعد يوم واحد و7 أيام و15 يومًا من التصنيع. هناك اختلافات ملحوظة ($P < 0.05$) في مستويات المادة الجافة والبروتين الكلي واللاكتوز بين الكنترول (اللبن الرايب غير المدعم بالحديد) وعينات اللبن الرايب المدعم بالحديد باستخدام صور مختلفة من الحديد. وكذلك وجد إن استخدام صور مختلفة من الحديد للتدعيم لم يؤثر على فترة التحضين للبن الرايب حتى وصوله إلى الرقم الهيدروجيني 4.5. أدى التدعيم بالحديد إلى انخفاض معامل b^*، مما قلل من اللون الأصفر لعينات اللبن الرايب، وكذلك اللمعان (L). ومع ذلك، فقد تسبب في زيادة كبيرة في المعلمة a^*، مما يدل على التحول نحو الاحمرار في العينات. لا توجد فروق ملحوظة في الخصائص الحسية بين اللبن الرايب المدعم بالحديد الأميني أو هيدروكسيد الحديديك بوليمالتوز واللبن الرايب غير المدعم. أظهر اللبن الرايب المدعم بفومارات الحديدوز قدرة تثبيطية عالية ضد السلالات المستخدمة لتقدير القدرة علي النشاط المضاد لنمو الميكروبات مقارنة بالمعاملات الأخرى. كان لجميع المعاملات معدل IC_{50} أكبر من الدوكسوروبيسين، مما يشير إلى أن معدل التثبيط لجميع المعاملات في كلا النوعين من الخلايا السرطانية كان أقل من معاملة الكنترول الإيجابية.

الكلمات الدالة : نشاط مضاد للبكتريا؛ فحص السمية الخلوية. الألبان المتخمرة، الأغذية الوظيفية، أملاح الحديد