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Ferrous sulfate microparticles as a food fortification strategy: Application in plant-based yogurt and bioaccessibility assessment☆

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ABSTRACT

This study evaluated the feasibility of using ferrous sulfate microparticles (FSM), produced through the combination of spray drying and spray chilling techniques, to fortify plant-based yogurt and increase dietary iron intake. The stability of FSM was assessed, and iron bioavailability was estimated using the standardized INFOGEST in vitro digestion method, followed by Caco-2 cell culture assays. FSM showed moisture content and water activity (Aw <0.3) comparable to control particles without iron (CP). Despite similar average particle sizes (D50), FSM exhibited lower luminescence and a more greenish-yellow hue than CP. The iron content in FSM and CP was 519 ± 23 mg/kg and 13.5 ± 0.8 mg/kg, respectively. FSM contained 309 mg of soluble iron/kg of particles, corresponding to 59.5 % bioaccessibility. After 180 days of storage, FSM showed slightly higher moisture, Aw, and color values compared to CP, but overall stability remained similar. Total and soluble iron contents did not significantly differ, confirming FSM's suitability for yogurt fortification. When both plant-based and dairy yogurts were fortified with FSM and isolated ferrous sulfate salt, total iron content ranged from 9.83 to 19.77 mg/kg, with bioaccessible iron ranging from 1.40 to 13.2 mg/kg. Bioaccessibility percentages varied depending on the type of iron (isolated vs. encapsulated), matrix origin (plant-based vs. dairy), and plant-based formulation. Notably, strawberry-flavored plant-based yogurt fortified with FSM showed higher bioavailability than yogurt fortified with isolated salt, emphasizing FSM's effectiveness in improving iron availability in plant-based yogurts.

1. Introduction

Consumers are becoming increasingly aware of the nutritional aspects and health impacts of their food choices. This shift in behavior has driven the growth of meat and dairy alternatives, catering to diverse dietary habits, religious preferences, and specific needs such as lactose intolerance and vegan diets (Klost & Drusch, 2019; Wang et al., 2024; Yang et al., 2020). Plant-based yogurts have emerged as an alternative to traditional dairy yogurts, being produced from plant sources such as legumes, cereals, nuts, and fruits (Levy et al., 2021; Montemurro et al., 2021). Although plant-based yogurts are similar to dairy yogurt in flavor and texture, they often face challenges in achieving optimal nutritional characteristics, particularly in terms of protein and mineral content. Therefore, they are commonly fortified to ensure adequate nutritional quality and to serve as viable substitutes for animal-derived products (Jeske et al., 2017; Mäkinen et al., 2016; Sethi et al., 2016). Rebellato, de Moraes, et al. (2023) evaluated the mineral composition of different plant-based yogurts and found variations in the following elements: calcium (Ca < LOQ–240), copper (Cu < LOQ–0.2), iron (Fe 0.1–1.5), potassium (K 27–226), magnesium (Mg 3.7–24), manganese (Mn 0.1–0.3), sodium (Na 2.8–165), phosphorus (P 7.8–190), and zinc (Zn < LOQ–1.1), expressed in mg/100 g, as well as selenium (Se < LOQ–2.2 μg/100 g). While these yogurts contain essential minerals, they still lack sufficient levels of certain micronutrients, such as iron (Nole-Jaramillo et al., 2024).

Micronutrient deficiency, primarily caused by inadequate dietary intake, is a widespread global public health issue recognized by the World Health Organization (WHO, 2020) and highlighted in the Global

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Nutrition Report (2022) as affecting billions of people worldwide. Iron deficiency anemia affects approximately 30 % of the global population and can have severe health consequences. It is diagnosed by measuring hemoglobin levels in the blood and is commonly associated with the low bioavailability of iron found in foods, particularly those of plant origin (Burayu & Degefa, 2024; WHO, 2020). To address this issue, Brazil has established mandatory fortification of wheat and corn flours with iron and folic acid in 2002, updating its regulations in 2017 to allow the use of more bioavailable iron salts, such as ferrous sulfate and ferrous fumarate, in either encapsulated or non-encapsulated forms (ANVISA, 2002, 2017). Other countries, including Peru and Ecuador, also implement food fortification programs with micronutrients (Nole-Jaramillo et al., 2024). Although these compounds are more soluble and bioavailable, they may lead to undesirable changes in color, aroma, and flavor of foods, as well as contribute to lipid oxidation due to interactions with other nutrients (Muñoz-More et al., 2023; Nole-Jaramillo et al., 2024).

One of the strategies to combat iron deficiency while preserving the sensory and nutritional characteristics of foods is the use of encapsulated micronutrients (Muñoz-More et al., 2023; Nimbkar et al., 2023; Wang et al., 2017). Microencapsulation is the process of enclosing solid, liquid, or gaseous materials in micron-sized particles, allowing the encapsulated core to be released in a controlled manner under specific conditions (Espinoza-Espinoza et al., 2024). This technique has broad applications and can be used to protect iron from oxidation — by forming an impermeable membrane that acts as a barrier to oxygen diffusion — to mask the unpleasant metallic taste of iron salts, and to enhance iron bioavailability (Espinoza-Espinoza et al., 2024; Gupta et al., 2015; Nimbkar et al., 2023). Several microencapsulation techniques can be employed, such as coacervation, spray drying, spray cooling (or spray chilling), and extrusion, among others. Also, the composition of the microparticles can vary depending on the intended application, using matrices based on proteins, pectins, gelatins, fats, and other components (Constantino & Garcia-Rojas, 2023; Espinoza-Espinoza et al., 2024; Gonçalves et al., 2022; Ribeiro et al., 2021).

The spray chilling technique is commonly used for encapsulating compounds like iron and closely resembles spray drying in terms of particle formation through spraying or atomization. The key distinction lies in the solidification process: in spray chilling, the system is cooled, resulting in lipid crystallization, whereas in spray drying, particle formation occurs via water evaporation (Alvim et al., 2016; Moraes et al., 2023). Both techniques provide effective protection for sensitive compounds and are economically viable for industrial-scale production. However, the selection of the wall material and the microencapsulation method must ensure the protection and proper delivery of the target compounds. Studies have reported the combined use of spray drying and spray chilling to offer enhanced protection to the particle core and to the food matrix as a whole (da Teixeira et al., 2025; Fadini et al., 2018, 2019).

The assessment of the bioavailable iron—i.e., the portion that can be effectively absorbed after digestion—is a crucial tool, given the essential role of this mineral in numerous physiological functions in the human body. In vitro gastrointestinal digestion methods, such as the standardized INFOGEST protocol, have been widely used as a valuable approach to estimate the soluble fraction of iron potentially available for absorption (Brodkorb et al., 2019). Furthermore, understanding the absorption behavior of iron released during digestion is key to validating its functional potential in the body. In this context, nutrient transport studies often employ Caco-2 cell cultures, due to their morphological and biochemical similarity to the epithelial cells of the small intestine. Under culture conditions, these cells undergo spontaneous differentiation, forming a monolayer with tight junctions and expressing typical transporters of mature enterocytes, such as PEPT1, making them widely used in models that simulate nutrient transport, including that of iron (Liang et al., 2022).

Despite the ongoing challenges in food fortification, particularly in plant-based products, this strategy remains essential for increasing iron intake and effectively combating iron deficiency anemia. In this context, the objectives of this study were: (i) to produce iron microparticles from isolated ferrous sulfate using a combination of spray drying and spray chilling methods for the fortification of commercial plant-based yogurt samples; (ii) to evaluate the stability of the resulting microparticles; (iii) to estimate the bioavailable fraction of iron using the INFOGEST protocol in conjunction with Caco-2 cell culture; and (iv) to assess the contribution of the fortification to the recommended daily iron intake. For comparison purposes, the fortification process was carried out using both encapsulated and non-encapsulated iron (isolated salt), as well as a dairy-based yogurt sample.

2. Materials and methods

2.1. Materials

For the analytical procedures, the following reagents and materials were used: purified water obtained by reverse osmosis with a resistivity lower than 18.2 MΩ·cm (Gehaka, São Paulo, Brazil); concentrated nitric acid purified using a sub-boiling system (Distillacid, Berghof, Eningen, Germany); 37 % hydrochloric acid and 30 % hydrogen peroxide (Merck, Darmstadt, Germany); α-amylase (code 10080), pepsin (P6887), bovine bile (B3883), and pancreatin (P7545), all purchased from Sigma-Aldrich (St. Louis, USA). All other reagents used in the preparation of the simulated salivary, gastric, and intestinal fluids were prepared in accordance with the specifications of the INFOGEST 2.0 protocol (Brodkorb et al., 2019). Human colorectal adenocarcinoma cell lines, Caco-2 (Rio de Janeiro Cell Bank – BCRJ, Rio de Janeiro, Brazil), Dulbecco-Modified Eagle Medium (Gibco, Waltham, MA), bovine fetal serum (Vitrocell Embriolife, Campinas-SP, Brazil), penicillin, and streptomycin (Sigma Aldrich, St. Louis, USA), trypsin-EDTA solution (Vitrocell Embriolife, Campinas-SP, Brazil), *MTT (*3-(4,5-Dimethylthiazole-2-yl)-2,5-iphenyltetrazolium Bromide, (*Sigma–Aldrich, St. Louis, Missouri, USA*)), phosphate-buffered saline solution (PBS, Gibco, Waltham, MA), dimethyl sulfoxide solution (DMSO, Sigma–Aldrich, St. Louis, Missouri, USA) were also used for the cell culture assays. The certified reference material (CRM) was ERM-BD 151 Skimmed Milk Powder (Joint Research Center, Geel, Belgium) and a 1000 mg/L iron standard solution (Specsol-Quimlab, Jacareí, Brazil) for iron quantification. In the preparation of the microparticles, dry ferrous sulfate (Synth, Brazil), vegetable fat (370B, Agropalma, Brazil), fully hydrogenated palm fat (A. Azevedo Óleos Vegetais, Brazil), gum Arabic (Instantgum AA, Nexira, Brazil), carboxymethylcellulose (CMC) (GELYCEL F1 2000 S, Amitex, Brazil), and surfactant Polysorbate 80 (Tween 80, Synth, Brazil) were used. For the fortification study, two plant-based yogurt samples (strawberry and vanilla coffee flavor) and one animal-based yogurt sample (banana, oat, and honey flavor) were purchased from the local market in Campinas-SP.

2.2. Methods

2.2.1. Production of ferrous sulphate microparticles

The ferrous sulfate microparticles were produced using two encapsulation processes. The first involved the formation of lipid microparticles with ferrous sulfate (MPL) using spray chilling, followed by a second encapsulation step through spray drying (Fadini et al., 2019), aiming to improve homogenization and dispersion in yogurt samples. Microparticles without the addition of ferrous sulfate (control) were also produced for comparison purposes. The amount of encapsulated ferrous sulfate used in the yogurt fortification was calculated based on the Brazilian Normative Instruction (IN 75/2020), which sets the recommended daily value of 15 % for a food to be considered a “source” of iron.

The coating material consisted of 70 % palm oil and 30 % hydrogenated vegetable oil. The lipids were weighed in a glass beaker, melted

in a microwave oven, and kept on a heating plate until use. Ferrous sulfate was added to the lipid mixture and homogenized by mechanical stirring (IKA-Werke, RW 11 Lab egg – stirrer, Staufen, Germany) on the heating plate. The final mixture was subjected to an ultrasonic bath (750 A, MaxiClean Unique, Indaiatuba, São Paulo, Brazil) to improve the dispersion of the ferrous sulfate in the melted fat, and then transferred to the heated (80 °C) reservoir of the spray chilling system. The equipment consisted of a laboratory-scale mini spray dryer B-290 (Büchi, Flawil, Switzerland) equipped with a cooling module (Dehumidifier B-296) for operation as a spray chiller. The spraying occurred through a dual-fluid atomizer nozzle with a diameter of 0.7 mm, and the chamber temperature was maintained at 7 ± 2 °C. The operating conditions were air pressure of 50 mbar, 70 % aspirator rate, and air flow rate of 500 L/h. Then, 20 % of the lipid microparticles containing ferrous sulfate were dispersed in a solution composed of 15.5 % gum Arabic, 0.5 % CMC, 80 % water, and 0.04 % tween 80. The mixture was then transferred back to the spray dryer reservoir (B-290, Büchi, Flawil, Switzerland). The microparticles obtained from each process were collected and stored under refrigeration for subsequent characterization.

2.2.2. Characterization of the microparticles

The microparticles were characterized for the following parameters: moisture content (IAL, 2008); water activity, measured with the Aqualab meter model 4TE (Decagon, Pullman, Washington, USA); color evaluation using the CIELab* system, with the L*a*b*parameters, where L* represents luminosity, a* ranges from −red to +green, and b* from −yellow to +blue (MiniScan XE, Hunter Associates Laboratory, VA, USA); average diameter and particle size distribution, determined by light scattering (laser diffraction) using the LA 950 V2 device (Horiba, Kyoto, Japan). For analysis, the microparticles were dispersed in a 0.5 % (*w*/w) polysorbate 20 (Tween 20) solution and placed in the reading chamber of the device, using ultrapure water as the dispersion medium (adapted from Alvim et al., 2016). The average diameter of the microparticles was expressed as the diameter corresponding to 50 % of the cumulative distribution (D50), and polydispersity was determined by the span index, calculated using the formula: span = (D90 – D10)/D50, where D10, D50, and D90 correspond to the diameters associated with 10 %, 50 %, and 90 % of the cumulative distributions, respectively. Six replicate measurements were performed for each sample. The total iron content of the microparticles and the estimate of the bioaccessible fraction were evaluated according to Sections 2.2.4 and 2.2.5, respectively. All analyses were performed at the initial time and after 180 days of preparation, in triplicate.

2.2.3. Analytical control

A certified reference material (skimmed milk powder, ERM-BD 151) and a plant-based yogurt sample were used to evaluate the analytical method for iron determination. The performance characteristics evaluated were linearity, precision, accuracy, detection limit, and quantification limit (AOAC, 2016; INMETRO, 2020).

Linearity was assessed using analytical curves with five equidistant points of increasing concentration. The analytical curve, ranging from 0.001 to 1.0 mg/100 mL of Fe, exhibited a linearity with $R^2 > 0.99$. The tendency/recovery was evaluated using certified reference material, showing a recovery percentage of 99 %. Precision was determined by performing 7 repetitions of a sample, with a variation of 6 %, meeting the coefficient of variation (CV) specifications set by INMETRO (2020). The limits of detection (LOD: 0.02 mg/100 g) and quantification (LOQ: 0.04 mg/100 g) were estimated based on the analyte concentration corresponding to the blank's average, using 3 and 5 times the standard deviation, respectively.

2.2.4. Preparation of the samples

The yogurts were fortified with iron to provide 2 mg in a 170 g serving, which corresponds to 15 % of the recommended daily value of Fe for adults (Brasil, 2020). The analyses were conducted briefly after the yogurt samples were fortified with iron, in triplicate.

Both the microparticle samples and the yogurt samples were mineralized as described by Rebellato, Fioravanti, et al. (2023), with modifications. For mineralization, 0.5 g of sample and 4 mL of HNO_3 were added to a glass tube and left to rest overnight. Then, the samples were mineralized in a digestion block at 110 °C (Tecnal, Brazil) for 2 h, followed by cooling to room temperature. After this step, 2 mL of H_2O_2 were added and the samples were subjected again to the digestion block at 130 °C for 2 h. Upon completion of the process, the samples were cooled (to room temperature), diluted to 20 mL with ultrapure water, and filtered using a 0.45 μm PTFE filter (Agilent Technologies, Tokyo, Japan). All mineralization procedures were performed in triplicate, including the analytical blank.

Iron was quantified by inductively coupled plasma optical emission spectrometry (ICP OES) (5100 VDV, Agilent Technologies, Tokyo, Japan) under optimized operating conditions: RF power of 1200 W; Ar and auxiliary Ar flow rates of 12.0 and 1.0 L/min, respectively; dual cyclonic double-pass nebulization chamber; seaspray nebulizer at 0.70 L/min and a wavelength of 259.940 nm for Fe.

2.2.5. Simulated digestion in vitro

Bioaccessibility: the bioaccessibility and digestion in vitro were determined according to the INFOGEST 2.0 protocol (Brodkorb et al., 2019), with modifications. Enzymatic activities were assessed before starting the assays. Gastric lipase was not used due to unavailability, and the protocol was adjusted to use 2.5 g of sample. At the end of the intestinal phase, the samples were centrifuged at 3500*g* at 4 °C for 30 min, and the supernatant was transferred to digestion tubes, which were then placed in an oven at 100 °C overnight. Mineralization was carried out as described in Section 2.2.4. All analyses were performed in triplicate.

Cell Culture: Human colorectal adenocarcinoma cell lines, Caco-2 (Rio de Janeiro Cell Bank – BCRJ, Rio de Janeiro, Brazil) were cultured in 75 cm^2 culture flasks with Dulbecco-Modified Eagle Medium (Gibco, Waltham, MA) supplemented with 10 % Bovine Fetal Serum (Vitrocell Embriolife, Campinas-SP, Brazil), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma Aldrich, St. Louis, USA). Incubated in an atmosphere of 5 % CO_2 and 37 °C with medium replaced every 48 h. The numbers of passages were between 38 and 42. When 80 % confluency was reached, the cells were subcultured after treatment with trypsin-EDTA solution (Vitrocell Embriolife, Campinas-SP, Brazil), according to Stefos et al. (2024).

Toxicity test: For feasibility experiments, Caco-2 cells were seeded at a density of 3×10^5 cells cm^{-2} in 24-well plates and incubated for 48 h at 37 °C. After confluency, the medium was removed, and the cells were exposed for 2 h to various concentrations of the sample. Diluted in DMEM without serum supplemented and subsequently incubated with MTT (3-(4,5-Dimethylthiazole-2-yl)-2,5-iphenyltetrazolium Bromide, (Sigma–Aldrich, St. Louis, Missouri, USA) at 5 μg/mL in phosphate-buffered saline solution (PBS, Gibco, Waltham, MA) added to the wells for 30 min before the end of treatment. After the incubation period, the medium was removed and the volume of 300 μL of dimethyl sulfoxide solution (DMSO, Sigma–Aldrich, St. Louis, Missouri, USA) was added to the wells to dissolve the formazan crystals formed during the treatment. Absorbance was measured at 560 nm and 650 nm on a microplate reader. Cell viability was calculated as a percentage in relation to the control (untreated cells), according to de Espindola et al. (2023).

Caco-2 monolayer integrity and transport study: The transepithelial transport of iron was conducted as described by (Liang et al., 2022) with adaptations. Caco-2 cells were seeded (1×10^5 cells/cm^2) in 12-well filter support inserts with polyethylene terephthalate membranes (1.13 cm^2 and pore size of 0.4 μm). Before starting the transepithelial transport experiments, the cells were cultured for 21 days or until spontaneous differentiation was allowed.

During the cultivation period, the integrity of the cell monolayers was evaluated by transepithelial electrical resistance (TEER)

Table 1
Characterization of the microparticles.

Parameter	Time	Control microparticle			Fe-Microparticle		
Moisture (%)	Ti	7.33	±	0.15bA	7.35	±	0.17bA
	Tf	9.95	±	0.08aA	10.20	±	0.26aA
Aw	Ti	0.259	±	0.006bA	0.223	±	0.001bB
	Tf	0.382	±	0.007aA	0.394	±	0.002aA
Color							
L	Ti	94.68	±	0.12aA	93.73	±	0.32aB
	Tf	93.19	±	0.22bA	91.95	±	0.51bB
a	Ti	−0.12	±	0.01aA	−0.23	±	0.02aB
	Tf	−0.24	±	0.02bA	−0.27	±	0.03bB
b	Ti	6.07	±	0.14bA	6.16	±	0.28bA
	Tf	6.28	±	0.13aB	6.83	±	0.36aA
Particle distribution (μm)							
D10	Ti	3.01	±	0.36aA	3.32	±	0.04aA
	Tf	2.02	±	0.16bB	3.02	±	0.03bA
D50	Ti	8.36	±	0.27bA	8.42	±	0.14bA
	Tf	10.34	±	0.17aA	9.13	±	0.09aB
D90	Ti	15.89	±	1.48aA	20.62	±	2.83aA
	Tf	17.70	±	1.26aA	19.77	±	0.78aA
SPAN	Ti	1.54	±	0.17aA	2.16	±	0.36aA
	Tf	1.52	±	0.09aB	1.83	±	0.07aA
Fe content							
Total Fe (mg/kg)	Ti	18	±	1a	519	±	23a
	Tf	18.9	±	0.4a	509	±	7a
Bioacessible Fe fraction (mg/kg)	Ti	–			309	±	14a
	Tf	–			318	±	17a
Bioacessibility (%)	Ti	–			59.5a		
	Tf	–			62.5a		

Results expressed as mean ± standard deviation, $n = 3$ for moisture, Aw, and iron content, and $n = 7$ for color and size distribution. Ti: initial time, Tf: final time of 180 days. [a,b] Mean with different letters in the same column, for the same sample and same parameter, indicate a significant difference ($p < 0.05$) determined using one-way ANOVA and Tukey test at 95 % of confidence. [A,B] Mean with different letters in the same line, for different sample and same parameter, indicate a significant difference ($p < 0.05$) determined using one-way ANOVA and Tukey test at 95 % of confidence.

measurements using a Millicell® ERS voltmeter (EMD Millipore, Eschborn, Germany). The measurement was made at three different points of the inserts. The transport study only started when the monolayers reached TEER values higher than 250 Ω cm^2. On the day of treatment, TEER was evaluated and recorded, then the Caco-2 cell monolayers were gently rinsed twice with Hank's Balanced Saline Solution (HBSS) and incubated for 15 min in HBSS at 37 °C.

After the acclimatization period, the buffer was removed and 500 μL of HBSS transport medium supplemented with the sample was added on the apical side (AP) and 1500 μL of HBSS added on the basal side (BL). At the end of 2 h of treatment, 500 μL solutions of both AP and BL compartments were collected separately, placed in microtubes, and stored under freezing for analysis. Control was performed only with HBSS without sample supplementation. The analyses were performed in three distinct replicates of the strawberry-flavored plant-based yogurt fortified with iron.

2.3. Statistical analysis

The results were evaluated using descriptive statistics (mean and standard deviation estimation), subjected to F-test (normality and variance), one-way analysis of variance (ANOVA), and Tukey's test (95 % confidence level). For these analyses, Statistica 7.0 software was used (StatSoft, Tulsa, USA).

3. Results and discussion

3.1. Obtaining the microparticles containing ferrous sulfate

Preliminary tests were conducted using only lipid microparticles (MPL) obtained by spray chilling for fortifying plant-based yogurt. However, the particle distribution was not homogeneous, which led to an agglomeration of microparticles on the surface of the yogurt. Therefore, the MPL were coated with an additional layer through spray drying, using gum Arabic, carboxymethylcellulose (CMC), and Tween 80 as coating materials, aimed at a more homogeneous particle dispersion in the plant-based yogurt. Thus, a combined encapsulation method using spray chilling and spray drying was used to obtain ferrous sulfate microparticles, followed by the fortification of yogurt samples, aimed to prevent interactions between iron and the food matrix, avoiding organoleptic changes and oxidation.

The resulting ferrous sulfate microparticles appeared as a coarse powder, with a firm consistency, stable at room temperature, and light in color. The encapsulation efficiency (EE%), i.e., the percentage of iron retained after the encapsulation process relative to the initial amount, was 84 %. This result may be attributed to particle losses that adhered to the equipment compartments. Encapsulation efficiencies between 60 % and 90 % are considered adequate, indicating that the parameters used in the microparticle production did not cause significant changes in the active compound (Fadini et al., 2019; da Teixeira et al., 2025). An important consideration is the type of iron salt used in the microencapsulation process, as it can influence both the efficiency and yield of the process. Baldelli et al. (2023) reported encapsulation efficiencies of 87 % and 75 % for ferrous gluconate and sulfate, respectively. However, when using iron citrate or chloride, the efficiency is reduced due to the formation of different particles with low solubility. Ferrous sulfate is commonly used in food supplementation, which often leads to issues related to its high reactivity, such as the development of undesirable color and flavor in products (Muñoz-More et al., 2023), which emphasizes the importance of using microencapsulated iron in the food fortification process.

3.2. Characterization of the microparticles

Table 1 presents the results of the characterization of the control microparticles (without added iron) and iron-enriched microparticles.

The moisture content of the microparticles containing ferrous sulfate did not differ ($p > 0.05$) from that of the control microparticles, which showed values of 7.35 ± 0.17 % and 7.33 ± 0.15 %, respectively. The microparticles exhibited similar behavior after 180 days of storage, with increased moisture over time, not significantly different ($p > 0.05$). The determination of a material's moisture content is an important indicator for both chemical and microbiological stability. Despite the low moisture percentage of the samples studied, the evaluation was necessary, as variations in the relative humidity of the air during processing or storage, as well as the hygroscopicity of the active ingredient, can influence the microparticles' characteristics and, consequently, their protective capacity.

Regarding water activity, both particles showed values below 0.3 at the initial time, with an increase in Aw (< 0.4) after 180 days of storage, showing a significant difference between the times ($p < 0.05$). Although the microparticles (control and with ferrous sulfate) showed an increase in Aw, the values indicate a low risk of microbiological growth (Aw >0.6) (Jay, 2005) and demonstrate that the ferrous sulfate was not responsible for this change.

Regarding color parameters, the iron-containing microparticles exhibited lower luminescence, with a more greenish and yellow color compared to the microparticles without added iron. This result was expected due to the hue tone of the iron salt used in encapsulation, which contributes to the color changes of the particles. This observation can also be seen in the statistical analysis of the comparison between the

Table 2
Total and bioaccessible iron content and percentage of bioaccessibility in plant-based and animal yogurt.

Yogurt samples	Total Fe (mg/kg)	Bioacessible Fe (mg/kg)	Bioaccessibility (%)
IVC-S	13.60 ± 0.34a	7.70 ± 0.15a	56a
IVC-M	9.83 ± 0.51b	4.94 ± 0.14b	51b
IVM-S	18.56 ± 1.49a	1.40 ± 0.03a	8b
IVM-M	10.02 ± 0.13b	1.52 ± 0.02b	15a
INB-S	19.74 ± 0.34a	13.20 ± 0.18a	67b
INB-M	10.06 ± 0.31b	7.60 ± 0.39b	76a

Mean + standard deviation (n: 3) Samples: IVC: coffee-flavored vegan yogurt; IVM: strawberry-flavored vegan yogurt; INB: banana-flavored yogurt of animal origin. S: ferrous sulphate; M: microencapsulated ferrous sulphate. [a,b] Equal lowercase letters for the same sample do not differ significantly ($p > 0.05$).

particles. After 180 days of storage, the ferrous sulfate microparticles exhibited similar behavior when compared to the initial time, with lower luminescence and a more greenish and yellow color compared to the microparticles without added iron.

The microparticles with and without ferrous sulfate showed similar average diameters (D50) and did not differ in the initial time ($p > 0.05$). However, when analyzing the diameter variations, the microparticles without iron exhibited lower regularity in smaller sizes (D10) in the end time, while the microparticles with ferrous sulfate showed less regularity in larger sizes (D90) but there was no significant difference. The SPAN calculation showed a typical variation for materials obtained by the spraying technique, with the iron-containing microparticle having the highest polydispersity but without differing significantly from the control particle. Between the initial and final times (180 days), both the control and ferrous sulfate microparticles showed a significant difference in D10 and D50. However, no significant difference ($p > 0.05$) was observed for D90 and SPAN.

The iron content of the microparticles was 519 ± 23 mg/kg, while the control particle showed 18 ± 1 mg of iron/kg. A relative standard deviation below 6 % for total iron content indicates a homogeneous distribution among the particles, suggesting that the microencapsulation process was consistent and well controlled.

Regarding the bioaccessible fraction, the microparticles presented 309 ± 14 mg of soluble iron/kg, corresponding to 59.5 % bioaccessibility, while the control particles had no quantifiable bioaccessible content. The total and bioaccessible iron contents were also analyzed after 180 days of microparticle preparation, with no significant differences ($p > 0.05$) when compared to the initial time. Buyukkestelli and El (2019) evaluated the bioaccessibility of ferric chloride microparticles obtained by the double emulsion technique (A1/O/A2), resulting in 53 % for the 20:80 ratio, while Barbosa and Garcia-Rojas (2022) observed a bioaccessibility of 49.5 ± 5.5 % for ferrous sulfate microparticles obtained by the double emulsion technique (A/O/A).

3.3. Fortification and simulated digestion in vitro of yogurt samples with microencapsulated and non-encapsulated ferrous sulphate

The plant-based and animal-based yogurt samples were analyzed for total iron content and then fortified with isolated ferrous sulfate salt (2.86 mg ferrous sulfate in 100 mL yogurt) and microencapsulated ferrous sulfate (2.0 g microparticle in 100 mL plant-based yogurt). The results are shown in Table 2. Bioaccessibility tests were also carried out to estimate the available fraction of iron in plant-based and animal-based yogurt samples after the fortification process.

Although the microparticles showed greater difficulty in being incorporated into the yogurt samples, the total iron contents were similar across the samples, as the mass of microparticles was higher compared to the ferrous sulfate. This was not observed for the samples fortified solely with the iron salt, as the mass required to achieve 15 % of the recommended daily intake (RDI) was low, leading to challenges in weighing and adjustments in the final concentration. However, the objective of the study was achieved, as the intake of a 170 g serving of the fortified plant-based yogurt can contribute between 12 % and 24 % of the recommended daily value (RDV) for iron. These values cover approximately 15 % of the RDV for populations ranging from children aged 4 to 8 years to pregnant women, who require a higher iron intake, allowing the product to be classified as a source of iron (Brasil, 2020).

The total iron content ranged from 9.83 to 19.77 mg/kg across the samples, and the bioaccessible fraction ranged from 1.40 to 13.2 mg/kg. The results regarding bioaccessibility percentage showed variation depending on the type of salt used (microencapsulated or not) and the composition of the samples (animal or plant protein). The animal-based yogurt showed higher bioaccessibility compared to the plant-based yogurt, regardless of the type of iron used. For the plant-based yogurt, however, the composition of the samples was the factor influencing the bioaccessible content, as the IVC sample – coffee and vanilla flavor contained an oat base and other ingredients (oat base, chicory root fiber, coconut cream, instant coffee, taurine, powdered guarana, cocoa, cinnamon, vitamins (B6, B9, and B12), emulsifier (sunflower lecithin), natural-identical aromas, stabilizers (guar gum and gelana), and stevia sweetener), while the IVM sample – strawberry flavor contained ingredients such as: water, coconut cream, organic sugar, strawberry preparation (water, strawberry, maltodextrin, modified starch, natural colorants anthocyanin and urucum, flavorings, xanthan gum thickener, potassium sorbate preservative, and lactic acid acidulant), modified starch, soluble fiber, tricalcium phosphate, xanthan gum stabilizer, potassium sorbate preservative, and yeast. It is worth mentioning that the samples with the highest bioaccessibility percentages (both animal and plant-based) contained oats in the composition. Therefore, future studies are required to investigate the main factors that influence iron absorption in plant-based matrices.

In a previous study, Rebellato, Fioravanti, et al. (2023) reported a study of the mineral content of plant-based yogurts ($n = 17$) and one animal-based yogurt, and the authors observed a wide variation in the levels of the evaluated elements, including iron. Iron content ranged from 0.07 to 1.48 mg/100 g in the plant-based samples and was not detected in the animal-based yogurt. Zeinatulina et al. (2025) also evaluated commercial samples of plant-based yogurts ($n = 25$) and found that iron content ranged from 0 to 0.9 mg/100 g among lupin-, soy-, oat-, and coconut-based yogurts. Additionally, Buttriss (2003) reported low iron content (trace to 0.1 mg/100 g) in animal-based yogurts, from fruit-flavored low-fat versions to full-fat Greek yogurt. Regarding mineral bioaccessibility studies, Rebellato, Fioravanti, et al. (2023) found that only the plant-based samples showed a bioaccessible iron fraction (9–33 %), highlighting the importance of evaluating not only the total content of a given element but also its soluble fraction.

These data support the importance of fortification in this category of food products, especially with respect to iron, as the intake of this mineral is relatively low in vegan and vegetarian diets (Bickelmann et al., 2023; Neufingerl & Eilander, 2021). Furthermore, it is important to consider that the bioavailability of minerals in plant-based foods is compromised by the presence of antinutritional factors such as phytates, oxalates, dietary fibers, among others (Neufingerl & Eilander, 2021; Zeinatulina et al., 2025), meaning that vegans and vegetarians may require higher intakes to meet their nutritional needs. Additionally, according to product labeling, the addition of a variety of ingredients and additives is observed, which may also impact the mineral content of these products (Brazão et al., 2025; Di Salvo et al., 2023).

Due to the complexity of Caco-2 cell assays, as well as the involved in performing multiple dilution steps required for toxicity assessments, these tests were conducted exclusively on the fortified strawberry yogurt samples, both with iron salt and with microparticles. This targeted approach was adopted to ensure feasibility while still obtaining relevant insights into iron bioaccessibility.

Toxicity test: The MTT assay was used to assess cell viability in different dilutions of the digested sample (1:1, 1:2, 1:4, 1:8), with and

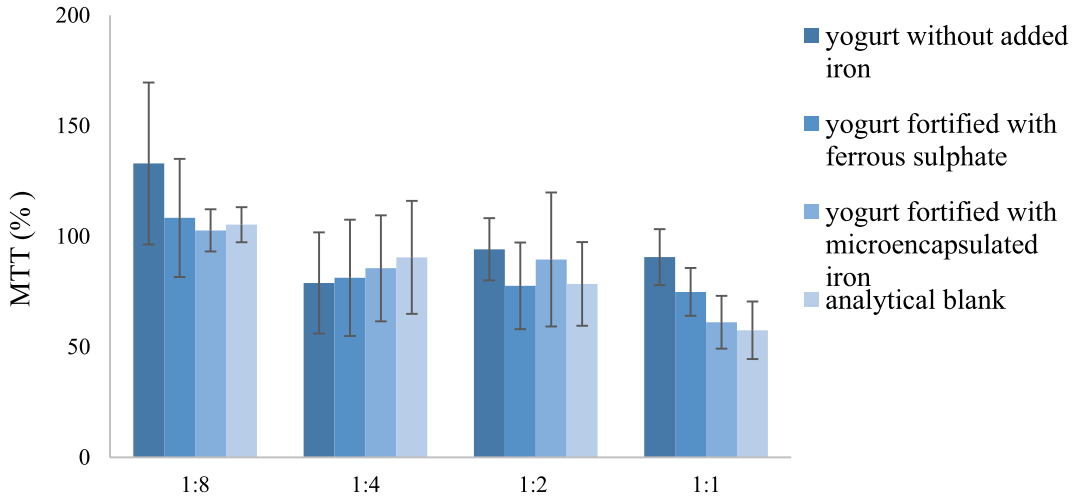

Fig. 1. Cell viability in different dilutions of the bioaccessible fraction.

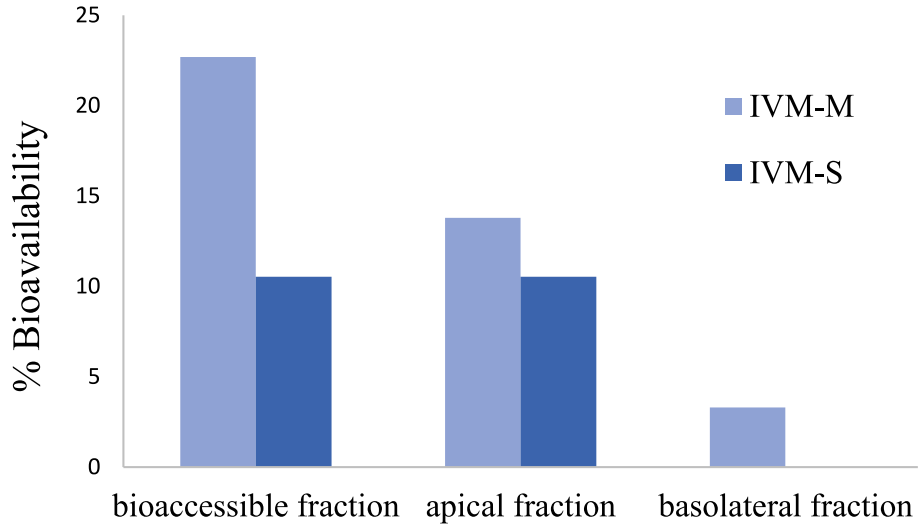

Fig. 2. Estimation of iron bioavailability in plant-based yogurt samples fortified with iron salt (IVM-S) and microencapsulated iron (IVM-M).

without microencapsulated iron. The results showed that exposure of Caco-2 cells to the 1:8 dilution of the digested sample did not indicate cell death, as the values obtained were close to 100 % when compared to the basal values. In the other dilution ratios, a reduction in MTT percentage was observed in relation to the basal values, indicating cell death (Fig. 1). Therefore, the cell assay was conducted using the 1:8 dilution of the bioaccessible fraction.

Bioavailability: Bioavailability tests were carried out on plant-based yogurt samples fortified with microencapsulated iron and with the isolated salt (ferrous sulphate). The bioaccessible fraction (INFOGEST 2.0, Brodkorb et al., 2019) was diluted in a 1:8 ratio and the results obtained in the cell culture tests (Caco-2 cells) are shown in Fig. 2.

The results of transport (basolateral fraction) confirmed the behavior observed in the bioaccessibility assays, where the highest bioaccessible fractions were found in strawberry-flavored vegan yogurt samples fortified with ferrous sulfate microparticles. Low bioavailability values should be interpreted with caution, as the cell culture model primarily measures transcellular transport, while other absorption mechanisms, such as paracellular and passive transport, are also present in the body (do Nascimento da Silva & Cadore, 2019; Milani et al., 2020; Peixoto, 2015).

Iron transport was approximately 3.3 % in the sample fortified with iron microparticles, which was not quantifiable in the sample fortified with the isolated salt, demonstrating that the fortification of plant-based yogurt with microencapsulated iron was more effective, offering greater availability for metabolic processes or storage in the body.

4. Conclusion

The results of the characterization of the microparticles showed that it was possible to obtain ferrous sulfate microparticles using a combination of spray chilling and spray drying encapsulation techniques for iron fortification in plant-based and animal-based yogurt samples. The INFOGEST 2.0 protocol was applied to estimate the bioaccessibility of iron in plant-based and animal-based yogurt samples, as well as in samples fortified with encapsulated and non-encapsulated iron. The animal-based yogurt showed higher bioaccessibility compared to the plant-based yogurt, regardless of the type of iron used. The composition of the plant-based yogurts also influenced the bioaccessible and bioavailable contents: the plant-based yogurt sample fortified with ferrous sulfate microparticles showed more effective results when compared to the sample fortified with the isolated iron salt, demonstrating the effectiveness of fortifying plant-based yogurt with microencapsulated iron. Furthermore, the aim of the study was achieved, as the intake of a 170 g serving of the fortified plant-based yogurt can be classified as an iron source. Future studies are needed to investigate the

interaction mechanisms between the chemical form of iron used to supplement foodstuff and the constituents of the food matrix, as well as to explore their effects on the processing and technological properties of plant-based yogurts.

CRediT authorship contribution statement

Ana Paula Rebellato: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **José Luan da Paixão Teixeira:** Validation, Methodology, Formal analysis. **Raquel Fernanda Milani:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Izabela Dutra Alvim:** Validation, Methodology, Formal analysis, Data curation. **Thalyne Mariane da Silva Santana:** Methodology, Formal analysis. **Fabiana Galland:** Writing – review & editing, Methodology, Formal analysis. **Marcelo Antonio Morgano:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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