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Novel application of a food ingredient based on soybean extruded-expelled meal containing probiotics for improving gluten free bread quality.

Running Title: Ingredients with probiotics for breadmaking

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ABSTRACT

There is an increasing demand of alternatives to improve nutritional and quality profiles of gluten-free bread, and to profit nutrients from vegetable by-products. The effect of food ingredient based on soy by-products containing *Lacticaseibacillus casei* (ATCC 393^{TM}), and iron sulphate salt addition on the physical, textural, and nutritional properties of a gluten-free bread formulation was studied. The system containing both, the food ingredient and the iron salt, showed the lowest pH (5.62±0.01) and the highest specific volume (2.3±0.2) cm^3.g^{-1}. In addition, a darker crumb (L*=66.2±0.6), higher aerated crumb area (110±6) mm^2 and iron bioaccessibility (85±5) % were obtained compared with systems without this addition, while no effect was observed on the texture nor moisture. The acid lactic fermentation along with iron salt addition, improved specific volume, crumb colour and aeration as well as nutritional profile of gluten-free bread. These results improve gluten-free bread quality and add value of soy by-products.

KEYWORDS

Soybean by-products, probiotics, gluten-free bread, quality improvement

INTRODUCTION

Soybean extruded-expelled meal (soybean-EEM) obtained after oil extraction is a good source of high biological value proteins, and dietary fibre, and therefore, it is used for animal feed. However, few technological advances have been performed for adding value to the supply chain of soy expellers (Castellanos-Fuentes *et al.*, 2022). In this sense, Genevois and de Escalada Pla (2021), proposed the addition of soybean-EEM for increasing dietary fibre and protein content of gluten-free bread (GFB), obtaining a more uniform crumb microstructure. While Castellanos-Fuentes *et al.* (2022) proposed a solid-state fermentation treatment of soybean-EEM with *L. casei* for improving its physicochemical and functional characteristics. The processes applied, reduced sugars and oligosaccharides, pH, and the bulk density, enhancing the functional characteristics of the vegetable matrix, as hydration and oil holding capacity. Moreover, *Lactobacillus* remain stable for 42-day storage at room temperature. Furthermore, the fermented soybean-EEM could be assayed as food ingredient (FI) in a porridge formulation for increasing the protein content and for incorporating probiotics in a vegetable meal (Castellanos-Fuentes *et al.* 2020, 2022).

Food products made from gluten-free grains have been created for a group of people who experience gluten-related disorder of the small intestine, such as wheat allergies, gluten ataxia, non-celiac gluten sensitivity, and the most well-known, celiac disease. There is an increasing demand of alternatives for improving nutritional and quality profiles of GFB. In the production of GFB, the lack of the gluten viscoelastic network hinders the baking process and decreases the quality of the final product in terms of technological quality, particularly about the volume and softness of the bread, and characteristics during storage (Cappa *et al.*, 2016). The most used ingredients in GFB are rice, corn, pseudocereals and starches of different origins (Masure *et al*. 2016). The large amount of starch in the formulation makes the product more prone to aging, compared to wheat-based breads, and reduces its shelf-life (Cappa *et al*. 2016). In addition, at earliest stages of diagnosis, voluntary or involuntary intake of gluten, produces malabsorption of nutrients, which leads to deficiencies of vitamin B12, folic acid, vitamins soluble fats and iron (Khairuddin and Lasekan, 2021). Although addition of vitamins and minerals is widely used in cereal flours, this regulation is not yet applied to

GF products in many countries. For instance, in Italy, Di Nardo *et al*. (2019) reported that only 5% of GFB contained all four essential fortified nutrients (calcium, iron, niacin, and thiamine), while 28% were fortified with only calcium and iron. In different works, Kiskini *et al*. (2010) investigated the effects of different iron compounds on the physical and sensory characteristics of fortified wheat or GFB and reported similar effects of these iron compounds on both types of bread. In general, they observed changes of crumb colour due to the iron addition as well as higher crumb firmness. The first step towards mineral bioavailability comprises bioaccessibility, that is, the fraction of a compound released from its matrix in the gastrointestinal tract, and that is available for intestinal absorption (de Escalada Pla *et al*. 2020). In this sense, probiotic strains were used in dairy and vegetable matrices to improve iron bioavailability. In some cases, the positive effect on iron absorption was the result of the live cells, and not the fermentation *per se* (Hoppe *et al.*, 2015). In other cases, metabolites generated during lactic acid fermentation, also called posbiotics, were responsible for this improvement (de Escalada Pla *et al.*, 2020, Vinderola *et al.*, 2022). For example, the authors reported in a previous work, reduction on phytic acid content in a fermented dairy-free dessert based on soy milk with *L. casei* (ATCC®393™) (Genevois *et al.*, 2018), due to the phytases production during fermentation. Iron and other minerals could be released from vegetable matrix during *L. casei* fermentation, improving their solubility and therefore their bioaccesibility (Genevois *et al.*, 2017). Khodaii *et al.* (2019) reported that lactic acid fermentation of bread with *Lactobacillus acidophilus*, either with or without iron addition, increases ferritin formation in the intestinal cells, which may be due to the production of acid by lactic acid bacteria as well as phytase activity of the probiotic.

The aim of this work was to study the FI based on soy by-products containing *Lacticaseibacillus casei* (ATCC™ 393), as an additive to improve benefits during fermentation in GF dough, and nutrients such as dietary fibre and protein. The effects of FI and/or Fe salt incorporation on the physical and texture properties of GFB as well as on the iron bioaccessibility were analysed. Searching for new specific applications of the FI could also contribute to add value to supply chain of soybean.

MATERIALS AND METHODS

The rice flour, coarse, bran and soybean extruded-expelled meal (soybean-EEM) were provided by the Cooperativa Arrocera (Villa Elisa, ER, Argentine) and R-Mix SRL (Urdinarrain, ER, Argentine), respectively. Other food-grade ingredients were used: pregelatinized cassava starch (PGS; Lorentz 681; Alphatrade S.R.L., Argentine); hydroxypropyl-methylcellulose (HPMC; Dow Chemical Methocel™, USA); salt, sucrose, glycerol monostearate (Palsgaard, Denmark) and dehydrated commercial yeast LEVEX™; Lesaffre, Argentine). Ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) was analytical reagent (Biopack™, BA, Argentine). The commercial strain *Lacticaseibacillus casei* (ATCC®393™) (Microbiologics, St Cloud, Minnesota, USA), was chosen due to its good tolerance to stress factors encountered in food processing and its probiotic activity previously reported (Reale *et al.*, 2015).

Food ingredient based on extruded expelled meal (FI)

The FI was prepared as previously detailed in Castellanos-Fuentes *et al*. 2020; 2022. Briefly, milled soybean-EEM were mixed with dehydrated cheese whey and hydrated with distilled water, sterilised, cooled and inoculated with 2 mL of a suspension containing ≈8.5.10^3 CFU/mL of *L casei*. The system was incubated and following that, submitted to centrifugation and washing with sterilised water, centrifugated and finally vacuum dried for 36 h. Thereafter, the dried product was ground and sieved in the ASTM 40 mesh. More details can be found in supplementary data (**ANNEX 1**).

Gluten free bread making

Based on formulations previously optimised by Genevois and de Escalada Pla (2021), small individual doughs and breads were prepared. The systems studied in the elaboration of GFBs were soy flour (SF), soy flour with added iron (SF+Fe), food ingredient with added iron (FI+Fe) and food ingredient without added iron (FI). A table detailing the formulation of the systems is provided in supplementary data (**ANNEX 2**). The dry ingredients were weighed, sifted, and placed in a stainless-steel container. The dry yeast was reactivated with a portion of the total water content. The Fe salt solution was prepared separately under magnetic agitation for 5 min at room temperature with the rest of water content and added to dry ingredients. While mixing began, the reactivated yeast was incorporated. The dough was kneaded in a professional standard mixer (Planetaria, Moulinex, Brazil) provided with a dough hook at 20±2°C for 2 min at speed 1 (on a scale of 1–5 of the mixer), until a homogeneous and easy-to-mix dough was obtained. Dough pieces (~45 g) were placed into disposable aluminium pans (total volume: 146 mL), fermented in a proofing chamber (25°C, 75% relative moisture) for 6 h and finally were baked at 180°C for 20-30 min. The loaves were cooled for 1 h at room temperature (18±1°C) before performing analytical measures.

Bread characterisation

Supplementary file is provided for detailed methodology **(ANNEX 3)**. Briefly, specific volume (SV), moisture content, pH and total acidity were measured at least in duplicate. The colour determination in the crumb bread was performed using a portable colorimeter (MiniScan EZ HunterLAB, USA) in CIE L*a*b* space. The Texture Profile Analysis (TPA) was performed using a texturometer (Mod.3342, Instron, USA) and the alveolar structure of the breadcrumb was studied through the digital analysis of scanned images (1200 pixels per-dpi) (Kodak 1940 Scanmate). Measurements were performed at least in triplicate at room temperature.

Viability of LAB and nutritional characterisation

The viable cell count was performed by means of serial dilutions in peptone water 0.1% w/v; (Biokar Diagnostics, Beauvais, OI, Francia) of a homogenate of a dough sample taken after fermentation and prior to baking. Aliquots of 0.02 mL of the selected sample dilutions were plated on MRS agar (Biokar Diagnostics, Beauvais, OI, France) containing 0.2% potassium sorbate (Perales *et al*., 2003) followed by incubation at 37°C for 72h under aerobic conditions. The number of viable LAB cells was expressed as log (CFU/g dough db). Duplicate from independent samples was performed, reporting the mean value ± SD in the results.

Iron bioaccessibility

Iron content and bioaccessibility was analysed following the methodology described by Genevois *et al*. (2016) and detailed in the supplementary file (**ANNEX 3**). Measurements were performed at least in duplicate, and results are reported as mean value ± SD.

Statistical analysis

Analysis of the variance (ANOVA) was performed on the experimental data, with a level of significance, $p<0.05$ followed of a Fisher's test to describe the differences between the systems (Sokal and Rohlf, 1994), using Statgraphics Centurion XV program (V 2.15.06, 2007, Warrenton, VA, USA). Texture profile curves were analysed using Origin Pro-8 software (Origin Lab Corporation, USA).

RESULTS AND DISCUSSION

Physicochemical characteristics of GFB systems.

The results corresponding to the physicochemical characteristics of the different baked systems are summarised in **Table 1**. Specific volume (SV) is one of the most important visual characteristics of breads that greatly influences consumer choice. Therefore, it is a key parameter to consider when evaluating bread quality (Hager and Arendt 2013). GFB presented SV in a range from 1.4 to 2.3 $cm^3.g^{-1}$; being the highest value that from the formulation FI+Fe, followed by FI and SF+Fe systems; and lastly SF system presented the lowest one. Protein contributes to air entrainment and stabilisation in the dough, fluidity of the dough during the gas expansion stage, structural setting/coagulation, and transformation from a foam to a sponge structure (Ribotta *et al.*, 2004). As can be observed in **Table S1** (supplementary data **ANNEX 1**), FI presented higher ($p\leq0.05$) protein content compared with SF, that can explain, in part, the higher SV presented by systems containing FI. In addition, acidic medium conditions can have an effect in the dough-network formed by starch and proteins, influencing CO_2 retention and therefore, affecting the crumb texture (Irigoytia *et al.*, 2023). As can be observed in **Table 1**, systems containing FI, presented the lower pH and higher total acidity. Both total acidity and pH, refer to acid development in the ferment. However, pH is related to the dissociation of acids and depends on the strength of them, whereas total acidity measures the total amount of acid in a system. Lower changes in pH compared to total acidity is possibly due to buffering effect of the weak basic groups of proteins in the fermented dough (Borsuk *et al*. 2021). Besides, a significant ($p \leq 0.05$) reduction in pH was observed when Fe was added, in systems prepared with the FI; and a non-significant ($p>0.05$) tendency to lower pHs, in the case of systems prepared with SF. The decrease of pH was also observed in other food systems fortified with ferrous salts by different authors (Genevois *et al*. 2016). Probably, the acid hydrolysis of $Fe(H_2O)_6^{2+}$to Fe^{2+} ions contribute to this pH reduction according to the food matrix fortified. It must be highlighted that FI+Fe, resulted the system with the lowest pH and simultaneously, the highest ($p<0.05$) SV, between 21 and 64% more SV when comparing with other systems. In wheat bread, the increasing in loaf volume when preferments and/or sourdoughs are added, could be explained through the decrease in dough pH that affected the interaction among proteins from gluten (Lorenz and Bruemmer, 2003). In GF formulation, presence of proteins from other resources could present a similar behaviour when changes in dough pH occur. In GFB, the higher SV and crumb porosity when sourdough is applied, can also be explained by the metabolic activity of LAB that produces acidity, exopolysaccharides as well as enzymes like α-amylase, improving CO_2 generation and retention (Irigoytia *et al.*, 2023). The SV values observed herein were in the order of Genevois *et al*. (2020), that reported SV of GFB based on milling fractions from rice (1.22 to 1.7 $cm^3.g^{-1}$), and findings of Genevois and de Escalada Pla*,* (2021) for GFB (1.29 to 2.8 $cm^3.g^{-1}$) with added soybean-EEM by products and cassava starch.

Regarding the moisture content, no significant differences were observed among the systems, ranging from 53 to 58 g.100 g^{-1}(wb). As it was summarised in **Table S1** (**ANNEX 1**), FI presented higher ($p \leq 0.05$) protein, lipid, and lignin content than SF. Probably hydrophilic properties from proteins were compensated by hydrophobicity from lipids and lignin as can be observed through the water and oil holding capacity (WHC and OHC respectively). Therefore, there were no changes on the bread moisture content in the condition herein assayed.

Colour of the different baking systems

The colour characteristics of the bread systems are shown in **Table 1**. Slight but significant ($p<0.05$) higher colour difference (ΔE) was observed when comparing systems containing FI, with SF systems; while the addition of Fe did not show effects in the conditions herein assayed. The differences observed, were mainly related to the lightness, L* and the hue angle reduction ($p<0.05$). While the colour intensity, C* did not present differences among the systems, showing an average value of 23.3±0.5. According to the ferment or *Lactobacillus* used in breadmaking, reduction in crumb lightness was also reported in several cases (Borsuk *et al.* 2021;). Fermentation process by *L. casei* does not only produce lactic acid but simultaneously releases glucose from oligosaccharides or polysaccharides (Castellanos-Fuentes *et al.* 2022) that could act in Maillard reactions giving as result a darker crumb colour during baking process (Capuano *et al.* 2008). It must be highlighted that these slight colour differences, are hardly perceived by human eyes (**Table 1**), since ΔE values were lower than 3 (de Escalada Pla *et al.*, 2023).

Characterisation of the texture of GF bakery systems

The texture of the baked goods was characterised by a TPA test to simulate chewing conditions in the mouth through compression in two cycles. No significant differences ($p>0.05$) were observed among the formulated and analysed systems (Supplementary data **ANNEX 3**). The hardness values were found in the range of 13.1 to 14.0 N, being the SF+Fe system the one that tended to a highest value. The cohesiveness is a dimensionless parameter related to the forces that link the components of the matrix together (Paraskevopoulou *et al*. 2010). It presented the same trend in all systems (0.65-0.67). While chewiness (N) is related to the needing of keeping the bread portion in the mouth to achieve the enough wetting before swallowing (Nieto-Calvache *et al.*, 2022); and ranged between 7.5-7.8N. Elasticity or springiness, is a dimensionless related to the capacity to spring back to its initial form after being deformed, showing values between 0.85-1.00. It must be highlighted that, in general, the values obtained herein were in the order of reported in rice-based breads by Genevois and de Escalada Pla (2021).

Breadcrumb alveolus

In recent years, image analysis has been applied based on a wide variety of techniques for the characterisation of crumb bread (Lamacchia *et al*. 2021). **Table 2** shows the scanned images of the crumb from the GFB samples prepared with the different formulations. The systems that contained the FI presented a lower alveolar density, but at the same time a higher percentage of aerated area, which explains the larger average size of the alveoli. It must be highlighted that FI+Fe system presented the highest ($p<0.05$) total alveolar area and simultaneously the highest SV (**Table 1** and **2**). However, no substantial change was observed in the general honeycomb of the crumb of the different breads (**Table 2**). Demirkesen *et al*. (2010) reported that GFB containing chestnut flour could retain more air bubbles due to the fibre content, improving viscoelastic properties.

LAB viability and Iron bioaccessibility

The LAB count in the dough did not present significant differences among the systems, showing values of 8.6±0.7 log(CFU.g^{-1} of dough) for SF, of 8.8±1.0 log(CFU.g^{-1} of dough) for SF+Fe, of 9.8±1.4 log(CFU.g^{-1} of dough) for FI+Fe, and 9.4±1.3 log(CFU.g^{-1} of dough) for FI (**Figure 1**). The values recorded for systems SF and SF+Fe correspond to the lactic acid bacteria present in the raw material. A non-significant tendence to higher *L. casei* count, in FI+Fe and FI, could be observed. This in turn allows explaining the lower pH and higher acidity recorded in these breads (**Table 1**). Acidification, proteolysis, and activation of a series of enzymes, as well as the synthesis of microbial metabolites, cause several changes during LAB fermentation, affecting the dough and the baked matrix and influencing the nutritional/functional quality (Irigoytia *et al.*, 2023). As can be observed in **Figure 1**, FI+Fe system presented a significantly ($p<0.05$) higher iron bioaccessibility, 85±5 %, than the control system SF, 76±3. Bioaccessibility is defined as the release of a mineral from food matrix when it is summited to gastrointestinal condition (de Escalada Pla *et al.*, 2020). The metabolism of the probiotic present in the FI, on the components of the GF dough matrix, could allow a better solubility of the mineral in the fermentation conditions (Genevois *et al.*, 2017). It must be highlighted that system FI+Fe presented 30 ± 3 mg.kg^{-1}, representing a contribution of 17% of the recommended daily intake of iron (18 mg.day^{-1}) for women 19-50 years old, with two serving size of GF bread (100 g total) (Institute of Medicine, 2006). In addition, SF or FI are source of high biological value proteins and dietary fibre, therefore its incorporation in gluten-free bread formulation would contribute to 18% and 27% of the daily value of protein (46 $g.day^{-1}$) and dietary fibre (25 $g.day^{-1}$) intake, enhancing 18-30%, respectively the intake of these nutrients compared to GF bread based on starch and rice flours (Genevois and de Escalada Pla, 2021).

CONCLUSIONS

It was possible to assay the addition of a FI based on soy by-products containing *L. casei*, and a Fe salt, in the preparation of GFB. The addition of the FI modified the bakery fermentation step obtaining breads with lower pH and higher total acidity. The system containing both, FI and Fe, showed the lowest pH and the highest specific volumes. In addition, FI+Fe system presented higher aerated crumb area than SF system, as well as a darkening of the breads and a decrease in the hue angle. The FI+Fe system increased iron bioaccessibility when compared with SF system. While the texture as well as moisture content of breads were not affected by the addition of the FI nor Fe. Two size portions of FI+Fe bread (100 g total) would contribute to the recommended daily intake of iron, protein and dietary fibre intake in 17%, 18% and 27%, respectively. These results show the potential of incorporating these ingredients to improve the functional and nutritional quality of GF baked goods. These results are important not only for GFB formulation but also for adding value to soy by-products. Further studies should be performed to complete a sensory evaluation that permits to validate the consumer acceptance of this proposal.

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The authors have no Conflict of Interest to declare.

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Figure Legends

Figure 1. LAB viability in fermented dough (light gray bars) and iron bioaccessibility of baked systems (dark gray bars). Different letters indicate significant differences between the iron bioaccessibility of the GFB systems.

Table 1. Physicochemical properties of the different gluten-free systems

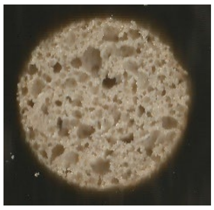
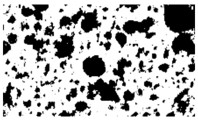
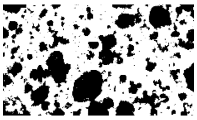
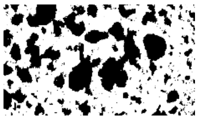
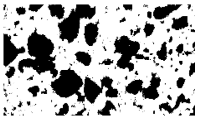
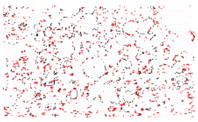
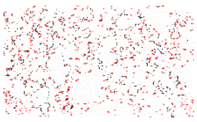
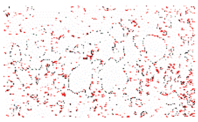
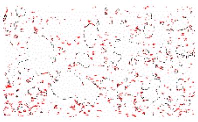
Systems	**SF**	**SF+Fe**	**FI**	**FI+Fe**
Specific Volume ($cm^3.g^{-1}$)	1.4±0.2 ^{a}	1.8±0.3 ^{b}	1.9±0.1 ^{b}	2.3±0.21 ^{c}
Moisture content (% wb)	56±1 ^{a}	53±5 ^{a}	53±1 ^{a}	57±6 ^{a}
Total Acidity g lactic acid.100 g^{-1}	0.26±0.02 ^{a}	0.29±0.01 ^{a}	0.39±0.01 ^{b}	0.35±0.02 ^{b}
pH	5.97±0.02 ^{c}	5.92±0.02 ^{c}	5.79±0.02 ^{b}	5.69±0.01 ^{a}
L*	68.0±0.8 ^{a}	68.0±0.5 ^{a}	66.7±1.0 ^{b}	66.2±0.6 ^{b}
C*	23.3±0.7 ^{a}	23.6±0.6 ^{a}	24.0±0.3 ^{a}	24.1±0.4 ^{a}
hue angle (°)	81.5±0.6 ^{a}	81.4±0.5 ^{a}	77.5±0.4 ^{b}	77.3±0.2 ^{b}
ΔE*	---	0.7±0.4 ^{a}	2.3±0.8 ^{b}	2.6±0.5 ^{b}

Control, soy flour (SF). Soy Flour with added iron (SF+Fe). Functional Ingredient without added iron (FI) and Functional Ingredient with added iron (FI+Fe).

L* lightness. C* Chroma. ΔE* color difference taking SF system as reference.

Different letters in the same row indicate significant differences ($p \leq 0.05$) among systems.

Table 2. Parameters of the image analysis of the crumbs of gluten-free bread.

System	**SF**	**SF+Fe**	**FI**	**FI+Fe**
Image				
Area selected (binary image)				
Digitalised image				
Number of alveoli	1894±245 [a]	1893±11 [a]	1876±170 [a]	1903±279 [a]
Total Alveolar Area (mm^2)	86±13 [a]	85.3±0.7 [a]	106±13 [ab]	110±6 [b]
Average size (mm)	0.045±0.003 [a]	0.046±0.001 [ab]	0.05±0.01 [ab]	0.06±0.01 [b]
% Área	19.3±2.9 [a]	19.2±0.2 [a]	24±3 [ab]	25±1 [b]

Mean alveolus diameter (mm/alveolus)	0.535±0.005 [b]	0.539±0.003 [b]	0.522±0.004 [a]	0.534±0.003 [b]
$D_{4,3}$ (mm)	0.832±0.003 [a]	0.828±0.007 [a]	0.841±0.006 [b]	0.833±0.001 [ab]
Alveolar density (alveoli/mm^2)	22.1±1.5 [b]	22.2±0.3 [b]	19.9±2.7 [ab]	17.3±2.5 [a]

Different letters in the same row indicate significant differences ($p<0.05$) among systems.

Soy flour (SF). Soy Flour with added iron (SF+Fe). Functional Ingredient without added iron (FI) and Functional Ingredient with added iron (FI+Fe).

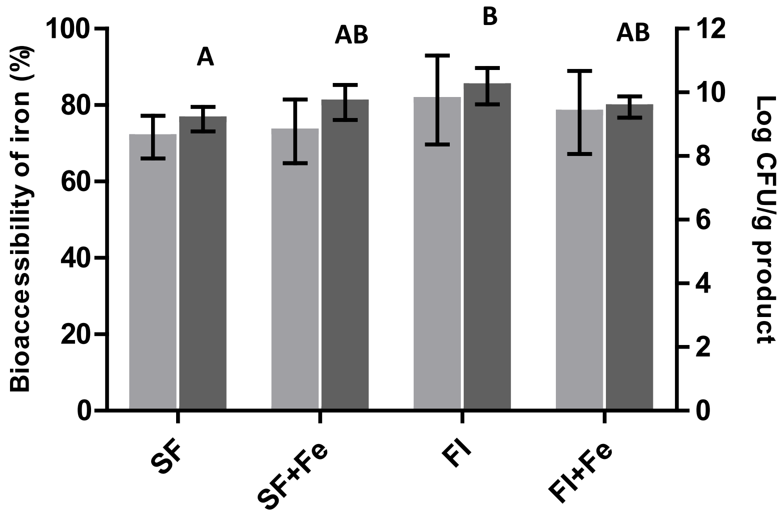


Figure 1. LAB viability in fermented dough (light gray bars) and iron bioaccessibility of baked systems (dark gray bars). Different letters indicate significant differences between the iron bioaccessibility of the GFB systems.