Research Article

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# Bioaccessibility and bioavailability of iron in biofortified germinated cowpea

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## Abstract

**BACKGROUND: Cowpea (*Vigna unguiculata* L. *Walph*) is predominantly consumed in the North and Northeast regions of Brazil, and its biofortification with iron seeks to reduce the high prevalence of iron deficiency anemia in these regions. It is commonly eaten cooked; however, in the germinated form, it can improve nutritional quality by reducing the antinutritional factors and consequently improving the bioavailability of elements. The present study aimed to determine the physico-chemical characteristics, bioaccessibility and bioavailability of iron in biofortified germinated cowpea.**

**RESULTS: There was no statistical difference between the germinated and cooked beans with regard to centesimal composition. Germinated beans had phytates and tannins similar to cooked beans. The phytate-iron molar ratio for all groups did not present a statistical difference (cooking 3.58 and 3.41; germinated 3.94 and 3.51), nor did the parameters evaluating *in vivo* iron bioavailability. Total phenolics was higher in the germinated group (cooking 0.56 and 0.64; Germinated 2.05 and 2.45 mg gallic acid $kg^{-1}$). *In vitro* bioaccessibility of iron of germinated beans presented higher values ($P \leq 0.05$) compared to cooked beans. There was higher expression of divalent metal transporter-1 in biofortified and germinated beans.**

**CONCLUSION: The iron bioavailability from the biofortified and germinated beans was comparable to ferrous sulfate. Germination can be considered as an alternative and efficient method for consuming cowpea, presenting good iron bioaccessibility and bioavailability.**


**Keywords:** bioavailability; bioaccessibility; germination; beans; biofortification; iron; antinutritional factors

## INTRODUCTION

Beans comprise a traditional part of the meal for Brazilians, supplying essential nutrients such as proteins, vitamins, carbohydrates and fibers, as well as elements such as iron, zinc, calcium and manganese. Among plant foods, they are considered the best source for iron and proteins, presenting a prominent alternative that improves the nutritional quality of the meal.[1–3]

As a result of high temperatures and high humidity or high temperatures and a semi-arid climate, Cowpea (*Vigna unguiculata* L. *Walph*) is predominant in the North and Northeast regions of Brazil with respect to production and consumption.[4]

Some antinutritional factors, however, are present that reduce the digestibility of this legume, such as phytic acid and tannins, with the antinutritional characteristics of these compounds being related to a capability to form insoluble complexes with proteins and elements, thus reducing their bioavailability.[5]

The human consumption of germinated seeds 'or sprouts', such as beans, soybeans, lentils and peas, amongst others, is well-known and appreciated in many countries such as China, Japan and the USA for being part of the consumption tradition in their populations. In Brazil, the consumption of cooked beans is more common but, in recent years, there has been a growing demand for the consumption of bean sprouts.[6,7]

Although beans are consumed by most populations when cooked, germinated, peeled and macerated beans are alternative processes that result in the beans being more easily digestible with an improved nutritional quality, leading to a decrease of some antinutritional factors and, consequently, a higher bioavailability of vitamins and elements. In addition, the germination process is a cost-worthy and very simple alternative.[7–9]

Studies have shown a significant increase in iron content for germinated foods.[9,10] According to Mubarak *et al.*,[11] bean germination significantly increased the protein content, ranging from 26.8% to 30.0%, and also decreased the content of antinutritional factors, resulting in a phytic acid reduction of 30.5%.

Other methods to improve the nutritional quality of foods are being extensively studied. These include biofortification, which is

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performed by means of genetic breeding, focusing on micronutrients such as iron, vitamin A and zinc, which are recognized by the World Health Organization as having a great impact on the health of populations when inadequately consumed. This strategy allows the population to consume the same foods that they are accustomed to, at the same time as improving the intake of important micronutrients for health. Currently, the biofortification of basic foods such as rice, beans, cowpea, cassava, sweet potatoes, corn, pumpkin and wheat is being developed.[12–14]

An investigation of the biofortified bean germination process as an alternative that improves iron bioavailability is thus important because it may bring benefits to populations affected by deficiencies related to malnutrition.

## MATERIALS AND METHODS

### Raw material

Cowpea (*Vigna unguiculata* L. *Walph*), conventional BRS Nova Era cultivar and the BRS Tumucumaque biofortified cultivar were used. Both were provided by the Brazilian Agricultural Research Corporation (EMBRAPA), from the city of São Raimundo das Mangabeiras, Maranhão, Brazil. BRS Nova Era cowpea and BRS Tumucumaque are white-colored cultivars with a smooth-skinned and black-hued grain.[15]

### Germination and cooking of beans

The bean were kept in deionized water for 4 h at a 1:2 ratio (w/v). Subsequently, the excess water was removed. Then, the germination was performed by keeping beans in polyethylene trays in an incubator (CLIMATEC, Ledbury, UK) at 28 ± 2 °C for 72 h.[16,17] The grains were moistened twice a day, during the morning and the late afternoon to ensure the moisture required for germination to occur.[17,18] For cooking, 1:2 deionized water (w/v) was added and cooked in pressure cookers previously rinsed with deionized water for 20 min under a low heat after the pressure began to increase.[19]

### Acquirement of bean flour

After germination or cooking, the beans were subjected to oven drying (CIENLAB - Equipamentos Científicos Ltd,CE220/480,Campinas, SP, Brazil) with air circulation at 60 °C until a constant weight was achieved. The cooked beans were dried together with the water used in cooking. After drying, the beans were ground in a blender to obtain a fine and homogeneous flour and, later, packed in laminated containers and kept under refrigeration until use.

### Centesimal composition

The moisture content, protein, lipids and ash were determined according to the standard procedure described by the Association of Official Analytical Chemists (AOAC).[20]

Soluble and insoluble fibers were quantified via the enzymatic-gravimetric method using thermoresistant $\alpha$-amylase, protease and amyloglucosidase (Sigma-Aldrich, St Louis, MO, USA) for enzymatic hydrolysis. Total fiber was determined by the sum of soluble and insoluble fibers.[21]

Carbohydrate was calculated by difference, subtracting the sum of the contents of lipids, proteins, moisture, ashes and fibers by 100.

Iron was determined by flame atomic absorption spectrometry (model ICE 3000 Series; Thermo Scientific, Waltham, MA, USA), with the digestion being conducted with nitric acid in a microwave (MARS 6; CEM Corporation, Matthews, NC, USA).[22]

The content of total phenolic compounds was determined by a spectrophotometric method using Folin–Ciocalteau reagent.[23] The extracts were obtained via extraction with 70% methanol (v/v) (Sigma-Aldrich). The results were expressed as mg gallic acid equivalents 100 $g^{-1}$ sample.

Tannins were analyzed according to the methodology by Price *et al.*,[24] whereas phytic acid content was analyzed according to the methodology described by the AOAC,[25] as adapted by Latta and Eskin.[26]

### Determination of the phytate:iron molar ratio

From the iron and phytate contents of the beans, the phytate:iron molar ratio was calculated according to:

$$\text{Molar ratio phytate:iron} = [\text{Phytate (g)}/659.91]/[\text{Iron (g)}/55.8]$$

where the phytate molecular weight is 659.91 and the iron atomic weight is 55.8.

### *In vitro* study

*Iron bioaccessibility*

*In vitro* digestion was performed using the enzymes pepsin and pancreatin, simulating gastrointestinal digestion.[27] Briefly, deionized water was added to the samples and the pH was adjusted to 2.0 with 0.1 mol $L^{-1}$ HCl. Pepsin was then added to simulate gastric digestion. Subsequently, the samples were incubated in a water bath at 37 °C under agitation for 2 h. As a way of simulating intestinal digestion, the pH was adjusted to 7.5 with 0.1 mol $L^{-1}$ NaOH and pancreatin activated in a basic medium was added. The samples were again incubated in a water bath at 37 °C under agitation for 2 h. Then, for enzymatic inactivation, the samples were incubated at 75 °C for 20 min in a water bath. The samples were centrifuged and the supernatants collected and lyophilized for 48 h and stored at −20 °C, remaining until the analysis was conducted for determination of iron, in accordance with the methodology described in above[22], and bioaccessibility was calculated based on the total iron content in the sample and the ratio of the iron content found in the digested fraction.

### Biological assay: iron bioavailability

*Ethical approval*

The experiment with animals was approved by the Animal Research Ethics Committee of the Federal University of Espírito Santo, protocol No. 72/2016.

*Experimental design*

The evaluation of iron bioavailability was performed with use of the AOAC depletion-repletion method, adapted to 21 days of depletion and only a single level of repletion (12 mg Fe $kg^{-1}$).[25] In total, 40 male Wistar rats weaned at 21 days of age and with an average initial weight of 80 g, as obtained from the Central Animal Breeding of the Health Sciences Center of the Federal University of Espírito Santo (CCS/UFES), were used. The animals were distributed into individual stainless steel cages under a 12:12 h light/dark cycle at 23 °C.

During the depletion period (21 days), all animals received an iron-free diet (Table 1), adapted from AIN-93G for rodents,[28] as well as deionized water *ad libitum* with the objective of inducing iron deficiency anemia.

During the repletion phase (14 days), the anemic animals were divided into five groups with eight animals each, so that the mean



  

**Table 1.** Composition of the experimental diets of the depletion and repletion periods (g 1000 $g^{-1}$ diet)

| | | Repletion | | | | |
|---|---|---|---|---|---|---|
| Ingredients | Depletion | FS | CC | GC | CB | GB |
| Cowpea[a] | 0 | 0 | 254.46 | 254.26 | 224.70 | 190.26 |
| Albumin | 200 | 200 | 106.9 | 107.23 | 107.04 | 116.22 |
| Maltodextrin | 132 | 132 | 132 | 132 | 132 | 132 |
| Sucrose | 100 | 100 | 100 | 100 | 100 | 100 |
| Soyabean oil | 70 | 70 | 70 | 70 | 70 | 70 |
| Cellulose | 50 | 50 | 0 | 0 | 0 | 0 |
| Mineral mix without Fe | 35 | 35 | 35 | 35 | 35 | 35 |
| Vitamin mixture | 10 | 10 | 10 | 10 | 10 | 10 |
| L-cystine | 3 | 3 | 3 | 3 | 3 | 3 |
| Choline bitartrate | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| Maize starch | 397.50 | 397.44 | 286.14 | 286.01 | 315.76 | 341.02 |
| Ferrous sulfate | 0 | 0.05979[a] | 0 | 0 | 0 | 0 |
| Iron content (mg $kg^{-1}$) | 0 | 11.02 | 15.23 | 15.00 | 17.96 | 15.87 |

[a] Amount sufficient to supply a 12 mg Fe $kg^{-1}$ diet.
CB, cooked biofortified; CC, cooked conventional; FS, ferrous sulfate; GB, germinated biofortified; GC, germinated conventional.

hemoglobin (Hb) concentration was as close as possible between groups: Control Group Ferrous Sulfate (FS), Cooked Biofortified (CB), Germinated Biofortified (GB), Cooked Conventional (CC) and Germinated Conventional (GC). All groups received their respective experimental diets to recover Hb levels, during which they received deionized water *ad libitum* and a controlled dietary intake of approximately 18 g per day. The diets were prepared to provide a 12 mg Fe $kg^{-1}$ diet from ferrous sulfate ($FeSO_4$, control diet) and a 12 mg Fe $kg^{-1}$ diet from the beans (test diets) (Table 1).

The weights of the animals and their food consumption were monitored weekly during the two experimental phases. The Food efficiency ratio (FER) was determined by the expression that relates the total weight gain of the animals (g) to the total consumption of diet (g) × 100.

*Experimental diets*

The diets were prepared according to AIN-93G standards for rodents (Table 1).[28]

Albumin was used as a protein source. Initially, all ingredients were mixed manually and subsequently mixed in a semi-industrial mixer (model VBPS12NR; VENÂNCIO, Venancio Aires, RS, Brazil) for 20 min. The ready-made diets were packed in bags and stored in a refrigerator under 10 °C. All of the utensils used in the preparation of the diets were rinsed with deionized water.

For the control diet, ferrous sulfate was used as source of iron,[25] whereas, for the experimental diets, the beans were added in an adequate quantity to provide iron at 12 mg Fe $kg^{-1}$, and the remaining ingredients were handled according to the composition of the beans added so that the diets were similar.

*Determination of hematological parameters*

At the end of depletion (21 days) and repletion (14 days), blood samples were collected by dripping, after incision of the terminal tail portion of the animals to determine the Hb concentration. Hb dosage was performed in accordance with the cyanhemoglobin method, using the Bioclin® Hemoglobin Kit (LabTest, Lagoa Santa, MG, Brazil).[25] A 250-µL aliquot of each sample was pipetted into a 96-well microplate and read at 540 nm (MULTISKAN GO, FI01621; Thermo Fisher Scientific, Oy, Finland).

After the repletion period (14 days), the animals were anesthetized using ketamine combined with xylazine according to weight, inserted via the intraperitoneal route, and approximately 5 mL of blood was collected by cardiac puncture and centrifuged at 6000 × *g* for 10 min at 4 °C (Heraeus Megafuge 16R, EUA; Thermo Fisher Scientific) for plasma separation, which was used for further hepcidin analysis.

The Hb gain was calculated by the difference in Hb concentration between the final and initial periods of repletion.

The pool of iron in Hb was calculated assuming that the total blood volume was 6.7% of body weight, and also assuming that iron content in Hb was 0.335%, according to:

$$\text{Initial Hb-Fe} = [\text{initial weight (g)} \times \text{initial Hb (g dL}^{-1}) \times 6.7 \times 0.335]/1000$$

$$\text{Final Hb-Fe} = [\text{final weight (g)} \times \text{final Hb (g dL}^{-1}) \times 6.7 \times 0.335]/1000$$

Hb regeneration efficiency (HRE) and relative biological value (RBV) were estimated according to:[29]

$$\%\ \text{HRE} = \text{final mg Hb-Fe} - \text{initial mg Hb-Fe/consumed Fe (mg)}$$

$$\text{RBV} = 100 \times [\text{HRE (\%) test group/HRE (\%) control group}]$$

*Serum hepcidin*

The enzyme-linked immunosorbent assay (ELISA) kit (Quantikine; R&D Systems, Minneapolis, MN, USA) was used to determine serum hepcidin levels in accordance with the manufacturer's instructions and by verifying the ELISA reader at 450 nm (MULTISKAN GO, FI01621; Thermo Fisher Scientific).

*Divalent metal transporter-1 (DMT-1) in the duodenum by western blotting*

At the end of the *in vivo* experiment, the duodenum of the animals was collected. For protein extraction, 400 mg of duodenum samples from the animals were homogenized and extraction was performed with lysis buffer (0.25 mol $L^{-1}$ saccharose, 0.01 mol $L^{-1}$ Hepes, 0.002 mol $L^{-1}$ ethylenediaminetetraacacetic acid) and 1% protease inhibitor. Afterwards, the sample was centrifuged at 6000 × *g* for 15 min at 4 °C. The supernatant was collected and the



  

**Table 2.** Physico-chemical composition of the conventional cowpea (BRS Nova Era) and biofortified (BRS Tumucumaque) submitted to cooking and germination

| | CC | GC | CB | GB |
|---|---|---|---|---|
| Ash (g kg$^{-1}$ DW) | 5.54 ± 0.06 a | 5.12 ± 0.52 a | 5.65 ± 0.51 a | 5.00 ± 0.58 a |
| Moisture (g kg$^{-1}$ DW) | 3.27 ± 0.08 b | 3.39 ± 0.09 b | 3.92 ± 0.08 a | 3.98 ± 0.11 a |
| Protein (g kg$^{-1}$ DW) | 24.80 ± 0.85 b | 24.69 ± 0.44 b | 28.02 ± 0.33 a | 28.27 ± 0.23 a |
| Lipids (g kg$^{-1}$ DW) | 2.00 ± 0.05 a | 2.02 ± 0.13 a | 1.09 ± 0.46 b | 1.10 ± 0.36 b |
| Carbohydrates (g kg$^{-1}$ DW) | 46.30 ± 1.48 a | 46.76 ± 0.85 a | 44.19 ± 0.47 a | 43.74 ± 0.92 a |
| Total dietary fiber (g kg$^{-1}$ DW) | 18.09 | 18.02 | 17.14 | 17.91 |
| Soluble fiber (g kg$^{-1}$ DW) | 2.96 | 2.27 | 3.24 | 3.72 |
| Insoluble fiber (g kg$^{-1}$ DW) | 15.13 | 15.75 | 13.90 | 14.19 |
| Iron (mg kg$^{-1}$ DW) | 47.15 ± 0.06 b | 47.19 ± 0.39 b | 69.42 ± 1.94 a | 69.74 ± 0.79 a |
| Tannins (g catechin kg$^{-1}$ DW) | 0.51 ± 0.03 a | 0.52 ± 0.08 a | 0.50 ± 0.09 a | 0.52 ± 0.07 a |
| Phytic acid (g kg$^{-1}$ DW) | 0.20 ± 0.08 a | 0.22 ± 0.04 a | 0.28 ± 0.09 a | 0.29 ± 0.01 a |
| Total phenolics (mg gallic acid kg$^{-1}$ DW) | 0.56 ± 0.01 b | 2.05 ± 0.63 a | 0.64 ± 0.22 b | 2.45 ± 1.15 a |
| Phytate:iron molar ratio | 3.58 | 3.94 | 3.41 | 3.51 |

Data are expressed as the mean ± SD ($n = 3$). Different lowercase letters in the same row indicate a statistical difference ($P \leq 0.05$). CB, cooked biofortified; CC, cooked conventional; DW, dry weight; GB, germinated biofortified; GC, germinated conventional.

protein concentrations in the supernatant were evaluated using the Bradford method with bovine serum albumin as standard. Equal amounts of proteins (80 µg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%). The proteins were subsequently transferred to a 60 V nitrocellulose membrane in a wet blotting system. The standard molecular weight marker was used on each gel and membrane (1610375; Bio-Rad, Hercules, CA, USA). After transfer, the membranes were blocked with Tris, NaCl and 0.1% Tween-20 (TBST) and 5% skimmed milk powder for 2 h and then washed with TBST three times for 5 min each. The membranes were incubated for 24 h with anti-DMT-1 primary antibody (SAB2102164; Sigma-Aldrich) diluted at a concentration of 1:5000. After washing the membranes with TBST, they were incubated for 2 h with peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody (A0545; Sigma-Aldrich), diluted at 1:5000. The same membranes were used to determine $\beta$-actin expression using a mouse monoclonal antibody for $\beta$-actin, diluted at 1:2500 (A5441; Sigma-Aldrich) and the results were calculated using the density ratio of the proteins of interest, corrected by the intensity of the protein used as control ($\beta$-actin). The membranes were then washed three times in TBST solution and the bands were visualized with a NBT/BCIP reagent tablet (B5655; Sigma-Aldrich).

### Statistical analysis

Analysis of variance followed by Tukey's test was performed to investihate differences in intergroup values. $P < 0.05$ was considered statistically significant. The analyses were performed using Prism, version 6.0 (GraphPad Software Inc., San Diego, CA, USA).

## RESULTS

### Centesimal composition

There was no statistical difference with regard to centesimal composition between the beans of the same cultivar compared to cooking and germination (Table 2).

The biofortified bean presented a higher protein percentage and alower lipid content compared to the conventional one ($P \leq 0.05$). All treatments presented similar iron, tannin and phytate contents.

**Table 3.** Iron bioaccessibility from cowpea

| Groups | Iron bioaccessibility (mg kg$^{-1}$) | Bioaccessibility percentage (%) |
|---|---|---|
| CC | 8.75 ± 0.34 d | 18.55 |
| GC | 10.78 ± 0.26 c | 22.84 |
| CB | 12.72 ± 0.42 b | 18.32 |
| GB | 15.01 ± 0.82 a | 21.52 |

Data are expressed as the mean ± SD ($n = 3$). Different lowercase letters in the same row indicate a statistical difference ($P \leq 0.05$). CB, cooked biofortified; CC, cooked conventional; GB, germinated biofortified; GC, germinated conventional.

Regarding total phenolic compounds, the germinated varieties presented statistically higher ($P \leq 0.05$) values compared to the cooked varieties.

### Phytate:iron molar ratio

As shown in Table 2, there was no statistical difference with regard to the phytate:iron molar ratio of the groups analyzed.

### Iron bioaccessibility

The germinated beans had values higher than the cooked ones ($P \leq 0.05$) with regard to iron bioaccessibility (Table 3).

### *In vivo* iron bioavailability study

*Weight gain and food consumption*

There was no difference ($P > 0.05$) in thw body weight of the animals between the experimental groups at both phases, depletion and repletion (Fig. 1).

Food intake (FI), FER and iron consumption did not show statistical differences between groups (Table 4).

*Iron bioavailability*

The Hb recovery occurred similarly between the groups. HRE and RBV showed no difference ($P > 0.05$) (Table 4).



  

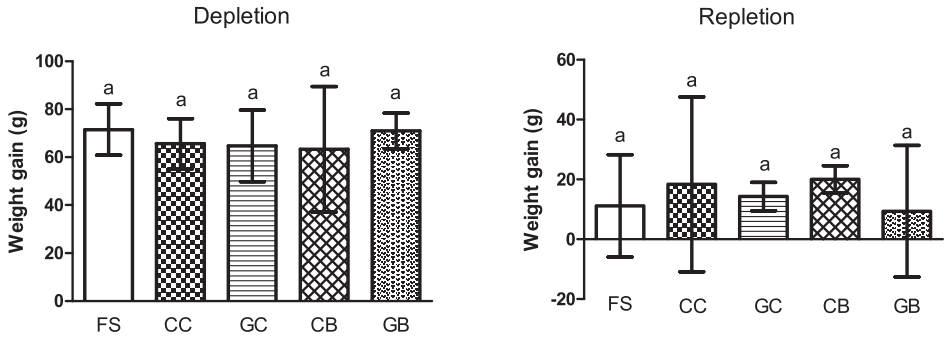


**Figure 1.** Weight gain (g) of the experimental groups in the depletion and repletion. Data are expressed as the mean ± SD ($n = 8$). Different letters indicate a statistical difference ($P \leq 0.05$). FS, ferrous sulfate; CC, cooked conventional; GC, germinated conventional; CB, cooked biofortified; GB, germinated biofortified.

**Table 4.** FI, FER, iron consumption (Fe cons) in the repletion period, Hb gain, HRE and RBV

| Groups | FI (g) | FER (%) | Fe cons (mg) | Hb gain (g dL$^{-1}$) | HRE (%) | RBV |
|---|---|---|---|---|---|---|
| FS | 223.48 ± 14.92 a | 26.23 ± 3.67 a | 2.46 ± 0.16 a | 1.63 ± 1.84 a | 60.88 ± 30.31 a | 100.00 ± 49.78 a |
| CC | 215.91 ± 29.71 a | 27.73 ± 4.93 a | 3.29 ± 0.45 a | 1.30 ± 1.52 a | 47.82 ± 12.46 a | 78.54 ± 20.47 a |
| GC | 224.26 ± 21.77 a | 29.44 ± 1.83 a | 3.36 ± 0.33 a | 1.23 ± 1.01 a | 50.32 ± 16.71 a | 82.65 ± 27.44 a |
| CB | 221.73 ± 20.25 a | 29.28 ± 3.21 a | 3.98 ± 0.36 a | 1.60 ± 0.86 a | 49.95 ± 11.88 a | 82.05 ± 19.52 a |
| GB | 225.49 ± 16.92 a | 27.52 ± 3.79 a | 3.58 ± 0.27 a | 1.14 ± 1.12 a | 43.49 ± 16.88 a | 71.44 ± 27.73 a |

Data are expressed as the mean ± SD ($n = 8$). Different lowercase letters in the same row indicate a statistical difference ($P \leq 0.05$). CB, cooked biofortified; CC, cooked conventional; FS, ferrous sulfate; GB, germinated biofortified; GC, germinated conventional.

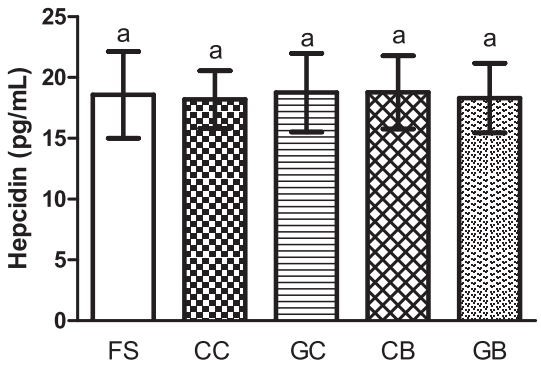


**Figure 2.** Serum hepcidin from the experimental groups. Data are expressed as the mean ± SD ($n = 8$). Different letters indicate a statistical difference ($P \leq 0.05$). FS, ferrous sulfate; CC, cooked conventional; GC, germinated conventional; CB, cooked biofortified; GB, germinated biofortified.

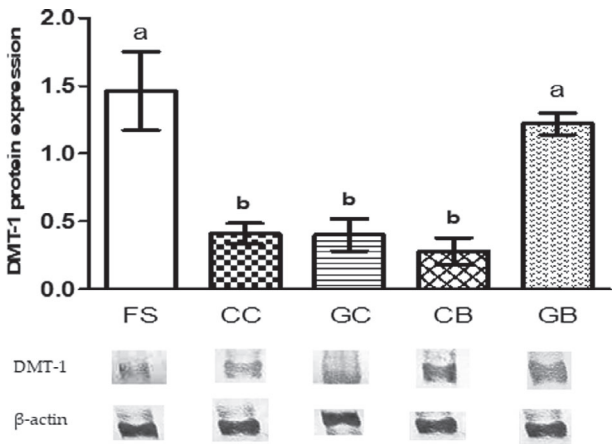


**Figure 3.** Protein expression levels of divalent metal transporter 1 (DMT1) in the duodenum, Protein levels were determined by western blot analysis and normalised to the levels of $\beta$-actin. Data are expressed as the mean ± SD ($n = 5$). Different lowercase letters indicate a statistical difference ($P \leq 0.05$). FS, ferrous sulfate; CC, cooked conventional; GC, germinated conventional; CB, cooked biofortified; GB, germinated biofortified.

*Serum hepcidin*

Serum hepcidin levels remained similar between experimental groups (Fig. 2).

*Duodenal DMT-1*

A higher expression of DMT-1 was present in the FS and GB groups, with the latter differing statistically from the other groups, as shown in Fig. 3.

## DISCUSSION

The results regarding centesimal composition did not demonstrate any statistical difference between the beans of the same cultivar with respect to cooking and germination, indicating that germination did not alter the composition. The results from previous studies corroborate those obtained in the present study, which showed a higher percentage of proteins and a lower lipid content in the biofortified beans compared to the conventional one ($P \leq 0.05$).[30–32]

The iron content (Table 2) from the conventional variety (BRS Nova Era) is close to the values reported in the literature of 50 mg kg$^{-1}$ beans. Compared to the biofortified variety (BRS Tumucumaque), lower levels were obtained than those reported in the literature (77 mg kg$^{-1}$).[33,34] However, these values remained significantly higher than the conventional variety ($P < 0.05$), as expected from biofortified varieties.



  

Thus, the biofortified cowpea, the target of this research, has not yet reached the recommended level of biofortification because its objective is the production of cultivars that reach a concentration of 50% higher than conventional cultivars.[33] However, according to the concentrations found, these are promising varieties for biofortification because, in the present study, a percentage of 46.8% more iron was obtained compared to the conventional variety; despite this, iron bioavailability is an important factor that must also be considered, and not just the percentage of increase in mineral content.

In the present study, there was no statistical difference between germinated and cooked beans, with all treatments having a similar iron content (Table 2). Contraditory results were found, demonstrating a higher iron content in germinated beans.[10] However, Lee and Karunanithy[35] confirmd the influence of germination on the chemical composition of beans on different days of germination (1, 3 and 5 days) and found that the iron content decreased until the third day of germination and increased on the fifth day. Hence, in the present study, the time used for germination may have been a determining factor with respect to not having found any difference in iron content because the time used was 3 days, with this comprising the germination period when the study demonstrated that a reduction of this mineral occurs.

The tannin values are consistent with expectations because the bean target of the present study is of a white variety and these compounds are mostly concentrated in the bean bark, being mainly present in beans with a dark-colored tegument, with the presenting white beans having very low tannin levels compared to red and black beans.[33,36]

In the present study, the beans underwent a maceration period with subsequent water disposal before the cooking and germination process, which may explain the low concentration of this antinutritional factor in the samples evaluated, in that maceration has an important role in the reduction of tannins present in the beans because they can migrate to the maceration water and the cooking broth.[19] Nevertheless, the beans also went through the drying process, and the temperature may have influenced the decrease in tannins because the results demonstrated that the heat treatment applied in the beans caused a reduction in the content of this compound.[19]

The biofortified cultivar had higher phytate values than the conventional one, although there was no statistical difference. It still maintained similar values between cooked and germinated beans compared to the same cultivar. Another study found higher values than the present study, with germinated cowpea having a phytate content of $^{0.48g\,100g-1}$.[37] Under natural conditions, phytate is negatively charged, and this causes it to have a great potential for binding to positively charged elements or molecules such as minerals and proteins, which may interfere with the use of these nutrients; however, it is also influenced by some factors such as pH, concentration and presence of minerals.[38]

The phytase enzyme, as present in several legumes including beans, activates during the germination process. Thus, a lower phytate content was expected in the germinated group but, despite this, such results were not observed because no statistical difference was obtained between groups.

Regarding total phenolic compounds, the varieties that were germinated for all cultivars presented statistical difference ($P \leq 0.05$), showing higher values than those of cooked beans. These higher values may be a result of the cooking process leading to greater loss of these compounds, which can reach losses of 39%.[19,39,40]

Based on the results, we confirm the positive results with regard to germination compared to cooking, demonstrating an efficacy in the cooking process with respect to maintaining levels at the same proportion for components capable of interfering in the nutritional value of the beans.

The phytate:iron molar ratio obtained for the beans in the present study is below the value considered harmful to iron bioavailability, demonstrating that both cooking and germination are efficient at reducing phytates so that they do not interfere in iron bioavailability.[41] Váz -Tostes *et al.*[42] obtained phytate:iron molar ratio values of 7.27 for biofortified beans and 8.53 for conventional beans, and these values did not interfere with iron bioavailability in the *in vivo* experiment.

Thus, the effects of germination on chemical composition, antinutritional compounds and biochemical constituents can vary greatly in accordance with the conditions used during the germination process, such as the time applied, temperature, humidity and the presence or absence of light, as well as the cultivar and the methodological analysis adopted.[7]

It was confirmed that germinated beans had higher iron bioaccessibility values ($P \leq 0.05$) than cooked beans. This result demonstrates the effectiveness of the germination and cooking process because bioaccessibility is able to indicate the amount of iron that can potentially be absorbed by the body after the gastrointestinal digestion process. As in the present study, research evaluating the impact of germination on iron bioaccessibility in different legumes, has achieved similar results, such as a significant increase in the bioavailable iron in germinated cowpea.[37]

*In vitro* digestion does not indicate the complexity of the human digestive process. However, this methodology has been used to provide preliminary information on the bioavailability estimation of certain nutrients as a result of its positive correlation with the *in vivo* models. Therefore, bioaccessibility comprises the quantity of a compound (iron) released from its matrix in the gastrointestinal tract that becomes available for absorption and bioavailability, including gastrointestinal digestion, absorption, metabolism, tissue distribution and bioactivity.[43–45]

The germinated beans showed greater iron bioaccessibility, contributing to the studies investigating different days of germination, as well as different methodologies, which indicated a greater iron bioavailability in germinated beans. It should also be taken into account that the iron bioavailability of beans depends on several factors, such as tegument color, processing type used and the amount of antinutritional factors. Previous studies have reported a greater bioavailability of iron in white-colored beans and shown that this bioavailability increases when removing the bark because most of the antinutritional factors are in the bean bark.[46–49]

By analyzing the results of the *in vivo* experiment, a similar FI was verified because the diet supply was controlled and the same amount was used for both groups (18 g day$^{-1}$). The same finding was verified for the FER, which correlates the weight gain of the animals with their diets. Thus, because dietary intake, weight gain and iron intake did not differ between groups, it is assumed that they may not have affected the biochemical variables and iron bioavailability.

Regarding the end of the repletion phase, in all experimental groups, Hb levels increased as expected as a result of the supply of experimental diets containing iron, although the Hb gain between groups was similar ($P > 0.05$). The HRE and RBV parameters were statistically similar between groups, indicating that both germination and cooking had high iron bioavailability because they presented values similar to those of the control group (ferrous sulfate).



  

Tako *et al.*[40] reported that biofortified beans exhibited greater iron bioavailability compared to conventional cultivars from the *in vivo* assay. By contrast, in the present study, there was no statistical difference between the biofortified and conventional beans, with both being comparable to the control group.

The similar hepcidin level values between groups, including the control group, demonstrate that this peptide involved in iron metabolism was not altered after recovery from animal anemia. This is a result of the analysis being performed at the end of the repletion phase, with the experimental groups recovering similar levels of Hb and thus not presenting any differences between them.

Hepcidin has been shown to be a potent biomarker for the detection of iron deficiency anemia in previous studies, having an association between low expression of hepcidin and iron-deficiency anemia because its production is defined according to iron concentrations and stock, suich that it is a negative regulator of iron metabolism.[50–52] Thus, the determination of serum hepcidin may be used in the future as an important tool in the diagnosis, classification and monitoring of individuals with pathologies related to iron metabolism, although consolidated reference values would be necessary.[53,54]

DMT-1 expression was higher in the GB group and similar to that in the control group (FS). This protein is a responsible for transporting iron from the lumen to the enterocyte and so, if more iron is bioavailable, there is a higher expression of this protein according to the *in vitro* results of the present study. These results differ from other studies with biofortified beans, which reported no difference in the expression of the DMT-1 protein in the duodenum of the groups that consumed biofortified beans, although they presented a higher iron bioavailability.[40,46]

The *in vivo* results did not corroborate with those obtained *in vitro* because the *in vitro* assay demonstrated increased iron bioaccessibility in the germinated groups ($P \leq 0.05$) and may predict an increased bioavailability *in vivo*; however, increased expression of DMT-1 in the germinated group may be indicative of a positive influence on more precise parameters, even though no significant gain in Hb was observed.

According to the results of the present study, a genetic improvement increasing iron content in beans alone may not reflect a proportional increase in its bioavailability because this is also influenced by other factors, such as the concentration of compounds that inhibit absorption, including antinutritional factors and other diet components.

## CONCLUSIONS

Germination did not alter antinutritional factors such as tannins and phytates compared to cooking. The biofortified cultivar presented higher levels of protein, ashes and lower lipids. Germinated beans had a higher content of total phenolics. The percentage of biofortification with iron was 46.8%, showing no difference in the content of this mineral between germination and cooking.

The *in vitro* bioaccessibility of germinated beans presented higher values, both conventional and biofortified, compared to cooking, demonstrating the efficiency of germination with respect to releasing iron from the food matrix of the bean.

The germinated biofortified bean can be considered as a promising vehicle for bioavailable iron because its bioavailability was comparable to that of ferrous sulfate when using the *in vivo* method, as well as with respect to the expression of DMT-1.

The higher content of total phenolics and other factors not analyzed may have affected iron bioavailability given that germination promoted greater bioaccessibility, although this iron release from the food matrix did not affect the higher absorption and/or utilization of iron for Hb synthesis.

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## CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

## AUTHOR CONTRIBUTIONS

CTS and NMBC were responsible for the study conception. CTS, PAT, EFM and TCR were responsible for the study methodology. CTS and EFM were responsible for the formal analysis. CTS, PAT and TCR were responsible for the investigations. NMBC was responsible for study resources. CTS was responsible for data curation. CTS was responsible for writing an original draft preparation. CTS, NMBC, EFM and MGVT was responsible for writing, including review and editing. NMBC and MGVT were responsible for study supervision. NMBC was responsible for project administration. NMBC was responsible for funding acquisition.

## ETHICAL APPROVAL

This study was approved by the Animal Research Ethics Committee of the Federal University of Espírito Santo – UFES, protocol No. 72/2016 on 4 July 2017.

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