Cultivars of biofortified cowpea and sweet potato: Bioavailability of iron and interaction with vitamin A *in vivo* and *in vitro*

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Abstract: The objective of this study was to evaluate the interaction of pro-vitamin A-rich sweet potato on iron bioavailability of biofortified cowpeas, using *in vitro* Caco-2 cells and *in vivo* depletion–repletion rat model. Mixtures of conventional rice with cultivars of iron-biofortified (Aracê, Xiquexique, and Tumucumaque) or conventional (Guariba) cowpeas with or without sweet potato biofortified with pro-vitamin A carotenoids were evaluated. The ratio of ferritin/total protein in Caco-2 cells was used as the index of cellular Fe uptake in the *in vitro* assay. The animal study evaluated the hemoglobin gain, the relative biological value, and the gene expression of transferrin and ferritin proteins by reverse transcription polymerase chain reaction. In the *in vitro* study, Xiquexique cowpea presented higher bioavailability of iron in the absence of sweet potato, and no difference was observed between the other cultivars of cowpea with and without sweet potato. The *in vivo* bioavailability (relative biological value of hemoglobin regeneration efficiency) differed statistically only between Guariba groups added to sweet potato and Tumucumaque. Ferritin mRNA expression did not differ between the test and control (ferrous sulfate) groups. Regarding the transferrin mRNA expression, there was a difference between the test and control groups except for the Xiquexique group. The association of rice and beans with sweet potato rich in carotenoids favored the gene expression of proteins involved in the iron metabolism, as well as its bioavailability, corroborating beneficial effects of this mixture. Xiquexique cowpea was shown to be the most promising compared to the other cultivars, exhibiting higher iron content in the digestible fraction, better *in vitro* bioavailability of iron, and transferrin gene expression.

Keywords: antinutritional, bioavailability, biofortification, gene expression, iron

Practical Application: Data from the study indicated greater *in vitro* bioavailability of iron for Xiquexique cowpea and sweet potato mixtures, in addition to the greater regeneration efficiency of hemoglobin *in vivo* as the bioavailability of iron among biofortified beans, highlighting the promising benefits of biofortification.

1. INTRODUCTION

About 2 billion people worldwide, particularly preschool children and pregnant women, are affected by chronic deficiency of iron, zinc, and vitamin A, especially in less developed countries (Murgia, Arosio, Tarantino, & Soave, 2012; Singh, 2015; WHO, 2016).

Iron deficiency anemia has a great impact as high social cost, because it compromises work income, intellectual behavior and performance, immunity, and resistance to infections (Horton & Ross, 2003). In addition, it is argued that the low availability of dietary iron exerts more influence compared to low iron intake (Casgrain, Collings, Harvey, Boza, & Fairweather-Tait, 2010; Garcia et al., 2013; Mahlera, Shulerb, & Glahnc, 2009).

Furthermore, hypovitaminosis A also causes xerophthalmia, blindness, and death in thousands of children worldwide and is one of the major nutritional problems of populations in developing countries (Ambrosio, Campos, & Faro, 2006). Moreover, vitamin A deficiency may affect iron metabolism by up-regulating the mRNA of liver hepcidin and ferritin, which participate in iron mobilization mechanism, although the deficiency did not affect the expression of the genes involved in the iron absorption (Citelli, Bittecourt, Silva, Pierucci, & Pedrosa, 2012).

Biofortification of staple food crops is an ally in the fight against nutritional deficiencies of micronutrients, such as iron and vitamin A. This process consists of the development of foods with higher concentrations of micronutrients using conventional plant breeding and biotechnology techniques (Bouis, Hotz, Mcclafferty, Meenakshi, & Pfeiffer, 2011; Frano et al., 2014).

Because vitamin A acts on the gene expression of iron metabolism proteins, and hypovitaminosis A and anemia coexist in many population groups, the combined biofortified foods with iron and carotenoids can contribute to increase the content of these nutrients in the diet and improve the health of the population. However, there is a need to evaluate the effect of the interactions among biofortified foods consumed in the same diet, because the concomitant consumption of foods influences the nutrient bioavailability (Ameny, Raila, Walzel, & Schweigert, 2002; Casgrain et al., 2010).

Increased iron concentration in food crops does not necessarily translate into a proportional increase in absorbed iron because cultivars with high iron concentrations may have increased

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Table 1–Biofortified (A, T, X, and B) or conventional food mixtures (G).

Treatments	Abbreviation	Mix Foods
1	G	Rice + Cowpea Guariba
2	A	Rice + Cowpea Aracê
3	T	Rice + Cowpea Tumucumaque
4	X	Rice + Cowpea Xiquexique
5	GB	Rice + Cowpea Guariba + Sweet potato
6	AB	Rice + Cowpea Aracê+ Sweet potato
7	TB	Rice + Cowpea Tumucumaque + Sweet potato
8	XB	Rice + Cowpea Xiquexique + Sweet potato

or decreased concentrations of inhibitors or enhancers of iron absorption (Ariza-Neto, Blair, Welch, & Glahn, 2007; Moura & Canniatti-Brazaca, 2006). For instance, Vaz-Tostes, Verediano, Mejia, and Costa (2016) found no difference between biofortified beans compared with their conventional counterparts in Caco-2 cells, rats, and pre-school children. On the other hand, Haas et al. (2016) evaluated the consumption of iron biofortified beans in women from Rwanda and concluded 4.2-g/L increase in hemoglobin; consumption of biofortified beans improved nutritional status in iron. Murray-Kolb et al. (2017) determined the efficacy of iron-biofortified beans in improving cognition in women with low-iron status; consumption of iron-biofortified beans improved cognitive performance, especially the efficiency of searching and the speed of retrieval on memory tasks, in women.

Efforts are necessary to improve iron bioavailability of foods targeted for biofortification. One approach is to combine biofortified foods rich in iron and pro-vitamin A in the same meal to evaluate whether the pro- vitamin A provided by biofortified food crops may affect iron metabolism. Thus, this study aimed to evaluate the *in vitro* and *in vivo* bioavailability of iron of mixtures of biofortified cowpea beans and sweet potato and their conventional counterparts and the gene expression of proteins involved on iron metabolism. Also, the study aimed to evaluate the micronutrient contents (iron, zinc, and pro-vitamin A), phytate, phenolic compounds, and dietary fiber of biofortified foods (sweet potatoes and cowpeas BRS Aracê, BRS Xiquexique, and BRS Tumucumaque) and conventional (Guariba cowpea) rice.

2. MATERIALS AND METHODS

The cowpea (*Vigna unguiculata* L. Walp.) cultivars BRS Aracê, BRS Tumucumaque, and BRS Xiquexique biofortified with iron and zinc were used; the sweet potato (Ipomoea batatas) cultivar Beauregard, biofortified with pro-vitamin A, the conventional Chorinho polished rice BRA 02535, and the cowpea BRS Guariba were supplied by the Brazilian Agricultural Research Corporation (EMBRAPA). Biofortified cowpea was provided by EMBRAPA Meio Norte (Teresina, PI, Brazil), harvested manually in October 20, 2016 and sun dried up to 10% to 11% moisture.

The rice was washed and cooked in a ratio of 1:2 (rice:water, w:v) for 20 min after boiling, and then dried with water in forced air circulation oven, at 60 °C, for 51 hr. The dried material was ground in a ball mill (MARCONI® Marconi Equipamentos para Laboratório, Piracicaba, SP, Brazil) and stored in metal packaging at 4 °C.

The cowpeas were washed and cooked in a pressure cooker in a ratio of 1:2 (cowpea:water, w:v). The grains and the remaining water were distributed in aluminum trays and oven dried with forced air circulation at 60 °C for approximately 48 hr. The dried beans were ground in a ball mill (MARCONI®) and stored at 4 °C.

The sweet potatoes were sanitized with deionized water for later manual peeling. Then, they were subdivided into small cubes and moistened in aluminum pan with enough water to cover the potatoes, and capped on low heat for 20 min. The cooked sample and the remaining cooking water were distributed in glass trays and oven dried with forced air circulation at 60 °C for 50 hr and stored in a dark packaging to protect from light at 8 °C.

From the raw material flours (cowpea, sweet potato, and rice), eight mixtures were obtained (described in Tables 1 and 2) to compose the diets of the *in vitro* and *in vivo* study. The proportion of experimental diets was based on the food intake of children from 2 to 5 years old and was adapted for the present experiment. There were 38%, 45%, and 17% of rice, beans, and potatoes, respectively.

Table 2–Composition of the experimental diets (g/kg).

	Repletion							
		With biofortified potato				Without biofortified potato		
Ingredients/diet	Ferrous sulfate (Control)	GB (Guariba)	AB (Aracê)	TB (Tumucumaque)	XB (Xiquexique)	G (Guariba)	A (Aracê)	T (Tumucumaque)	X (Xiquexique)
Beans test[a]	–	164.8	188.08	172.68	164.43	179.81	207.71	189.11	179.28
Sweet potato[a]	–	62.2	71	65.23	62.12	–	–	–	–
Conventional rice	–	139.16	158.73	145.75	138.78	139.16	158.73	145.75	138.78
Albumin[b]	200	86.99	77.68	89.02	99.66	42.09	39.86	42.62	45.11
Maltodextrin	132	132	85.35	120.48	132	58.92	58.92	58.92	58.92
Sucrose	100	100	100	100	100	100	100	100	100
Soy oil	70	70	70	70	70	70	70	70	70
Cellulose[c]	50	43.10	42.13	42.77	43.12	44.18	43.31	43.88	44.19
Mineral mix without iron	35	35	35	35	35	35	35	35	35
Vitamin mix	10	10	10	10	10	10	10	10	10
L-cystine	3	3	3	3	3	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Corn starch[d]	397.5	151.25	156.53	143.57	139.39	315.29	270.93	299.18	313.19
Ferrous sulfate (mg /kg)	59.57	–	–	–	–	–	–	–	–

[a]Amount sufficient to supply 12 mg Fe/kg of diet.
[b]Amount sufficient to supply 180 g protein/kg of diet.
[c]Amount sufficient to supply 50 g/kg of diet.
[d]Amount sufficient to supply 1 kg of diet.

2.1 Centesimal composition

The chemical analyses of contents of water, crude protein, ether extract, dietary fiber (soluble and insoluble), ash, and minerals were carried out in accordance with AOAC (2012). Carbohydrate content was obtained by the difference between the total sample (100%) and the levels of protein, lipid, dietary fiber, absorption, and ash, according to Brazil (2001).

2.2 Determination of total phenolic content

Estimation of the content of total phenolic compounds was determined by the spectrophotometric method according to Singleton, Orthofer, and Lamuela-Raventós (1999) using the Folin–Ciocalteu reagent. For the elaboration of the extracts, about 2 g of dry sample were added with 20 mL of methanol:water 60:40 (v/v). These were homogenized on an automatic shaker at 180 rpm for 2 hr at 25 °C. They were then centrifuged at 3,000 rpm for 15 min at 21 °C. The supernatant was transferred to a beaker and mixed with 20 mL of methanol:water solution. The extract was then packaged in an amber flask and stored at −18 °C until the time of analysis. A standard gallic acid curve was constructed and the results were in milligrams of Gallic Acid Equivalent (EqGA/g). Measurement was done by ELISA spectrophotometer (ThermoScientific®) at 760 nm.

2.3 Phytate

The determination of phytate was according to Latta and Eskin (1980). One gram of sample was weighed in a conical flask and 5 mL of 2.4% HCl were added for subsequent incubation in a water bath at room temperature and constant stirring for 12 hr. The samples contained in the conical flasks were filtered using vacuum pump and Whatman filter paper number 1. For the colorimetric method, sodium phytate was used as standard. A stock solution at a concentration of 1 mg/mL of phytate was made, and then diluted at concentrations of 100, 75, 50, 25, and 10 μg/mL. Then, 1 mL Wade reagent (0.03 g $FeCl_3 \cdot 6H_2O$ and 0.3 g sulfosalicylic acid diluted in 100 mL deionized water) was added to each 3 mL sample or standard. The reading was taken at 500 nm using deionized water as blank. Values were expressed in g/100g.

The molar ratio of phytate to iron (used to estimate relative mineral bioavailability) was calculated as millimoles of phytate present in the sample divided using molecular mass unit of 660.8 and atomic mass unit of 55.8 for iron.

2.4 *In vitro* study

The *in vitro* simulation of gastrointestinal digestion of foods was carried out according to the method described by Vaz-Tostes et al. (2016), for the analysis of iron of the digestible fraction.

An *in vitro* digestion model was used with cultures of Caco-2 cells in passage 29 to evaluate the bioavailability of Fe. Cells were seeded at densities of 50,000 cells/cm^2 in 6-well plates (Costar Corp, Cambridge, MA, USA). The integrity of the monolayer was checked by optical microscopy. The cells were cultivated at 37° C in an incubator with 5% CO_2/95% air atmosphere at constant humidity, Dulbecco's modified Eagle's medium plus 1% antibiotic/antimycotic solution, 25 mmol/L HEPES, and 10% fetal bovine serum was changed every 2 days. Then, 48 hr before the experiment, the growth medium was removed from the culture wells, the cell layer was washed, and the growth medium was replaced with minimal essential medium (MEM) at pH 7.0. The MEM was changed every 48 hr and supplemented with 10 mmol/L PIPES, 1% antibiotic/antimycotic solution, 4 mg/L hydrocortisone, 5 mg/L insulin, 5 μg/L selenium, 20 μg/L triiodothyronine, and epidermal growth factor of 20 μg/L. This enriched MEM contained less than 80 μg Fe/L.

Cells were used in the Fe absorption experiment at 13 days after seeding. On the day of the experiment, 1.5 mL of the digested sample was added to the upper chamber of the insert and incubated for 2 hr. The pellets were removed and 1 mL of MEM was added. Cell cultures were incubated for 22 hr at 37° C.

The growth medium was removed from the culture by aspiration and the cells were washed twice with a solution containing 140 mmol/L NaCl, 5 mmol/L KCl, and 10 mmol/L PIPES (pH 7.0). Cells were harvested by adding an aliquot of deionized water and placing them in a sonicator (Lab-Line instruments, Melrose Park, IL, USA).

The concentrations of ferritin and total protein were determined in an aliquot of the cell suspension harvested with one stage of the immunoradiometric assay (FERIRON II Ferritin Assay, Ramco Laboratories, Houston, TX, USA) and a colorimetric assay (Bio-Rad DC Protein assay, Bio-Rad , Hercules, CA, USA), respectively. Caco-2 cells synthesize ferritin in response to increases in intracellular Fe concentration. Therefore, we used the ratio of ferritin/total protein (expressed as ng ferritin/mg protein) as the index of cellular Fe uptake.

2.5 *In vivo* study

The Animal Breeding Center of the Federal University of Espírito Santo (CCS/UFES) provided 72 male Wistar weanling rats (*Rattus norvegicus*), with average initial body weight of 74 g. The animals were divided into nine groups with eight animals each and kept in stainless steel cages with light–dark cycle of 12 hr at 23 °C. This study was approved by the Committee on Ethics in the Use of Animals (CEUA) of the Federal University of Espírito Santo (UFES), protocol no. 72/2016.

The experimental groups received the following diets: rice + cowpea BRS Guariba (G); rice + cowpea BRS Aracê (A); rice + cowpea BRS Tumucumaque (T); rice + cowpea BRS Xiquexique (X); rice + cowpea BRS Guariba + biofortified sweet potato (GB); rice + cowpea BRS Aracê + biofortified sweet potato (AB); rice + cowpea BRS Tumucumaque + biofortified sweet potato (TB); rice + cowpea BRS Xiquexique + biofortified sweet potato (XB); and ferrous sulfate (FS; control group).

During the depletion period, all animals were fed AIN-93 G (Reeves, Nielsen, & Fahey, 1993) iron-deficient diet *ad libitum* for 21 days to induce iron deficiency. During the repletion period, the animals were pair-fed (16 to 18 g/day) with their respective experimental diet. The animals received deionized water *ad libitum* throughout the experimental periods. At the end of the depletion and repletion periods, blood samples from the tail end were obtained, and hemoglobin (Hb) concentration was assessed via the cyanide Hb method (AOAC 1984).

The relative values of bioavailability of the test foods were calculated considering the control group (ferrous sulfate) with bioavailability equal to 100%.

2.6 Hemoglobin regeneration efficiency

The hemoglobin regeneration efficiency (HRE) was calculated using the formula:

HRE (%) = [100 × (mg of final Hb Fe − mg of initial Hb Fe)]/Fe consumed, according to (Haro-Vicente, Rez-Conesa, Braqueh, & Ros, 2009), where final Hb is the final hemoglobin (end of repletion period) and initial Hb is the initial hemoglobin (end of depletion period).

Table 3–Nutritional composition of the flours for each sample on a dry weight basis.

	Rice Chorinho	Cowpea Guariba	Cowpea Aracê	Cowpea Tumucumaque	Cowpea Xiquexique	Sweet potato Beauregard
Moisture (g/100)	$5.59^{c} \pm 0.13$	$2.08^{a} \pm 0.35$	$3.21^{b} \pm 0.39$	$5.3^{c} \pm 0.19$	$7.72^{d} \pm 0.15$	$12.15^{e} \pm 0.02$
Ashes (g/100)	$0.36^{a} \pm 0.01$	$3.79^{d} \pm 0.01$	$4.19^{e} \pm 0.02$	$3.7^{cd} \pm 0.02$	$3.69^{c} \pm 0.02$	$3.49^{b} \pm 0.07$
Fat (g/100)	$0.58^{a} \pm 0.16$	$1.57^{c} \pm 0.06$	$1.91^{cd} \pm 0.18$	$1.95^{d} \pm 0.09$	$1.97^{d} \pm 0.13$	$0.94^{b} \pm 0.10$
Protein (g/100)	$10.34^{b} \pm 0.45$	$30.05^{e} \pm 0.10$	$27.86^{d} \pm 0.50$	$27.64^{d} \pm 0.69$	$24.96^{c} \pm 0.12$	$5.77^{a} \pm 0.16$
Total dietary fiber (g/100)	6.49	35.9	23.36	43.05	25.69	19.89
Soluble fiber	2.28	3.32	4.74	25.57	7.45	8.54
Insoluble fiber	4.21	32.58	18.62	17.48	18.24	11.34
Carbohydrates (g/100)	$83.13^{c} \pm 0.49$	$62.51^{ab} \pm 0.29$	$62.83^{ab} \pm 0.79$	$61.40^{a} \pm 0.81$	$69.98^{abc} \pm 0.04$	$77.66^{bc} \pm 0.05$
Total phenolic (mg of EqGA/g)	$0.26^{a} \pm 0.18$	$1.01^{b} \pm 0.04$	$0.91^{b} \pm 0.03$	$1.02^{b} \pm 0.09$	$1.09^{b} \pm 0.09$	$1.53^{c} \pm 0.03$
Phytate (g/100g)	$1.37^{a} \pm 0.02$	$1.82^{b} \pm 0.03$	$1.79^{ab} \pm 0.05$	$1.8^{b} \pm 0.03$	$1.75^{ab} \pm 0.02$	$1.59^{ab} \pm 0.02$
Minerals						
Iron (mg/100g)	$0.0^{a} \pm 0.58$	$6.67^{d} \pm 0.28$	$5.78^{c} \pm 0.23$	$6.35^{cd} \pm 0.04$	$6.69^{d} \pm 0.01$	$1.60^{b} \pm 0.50$
Zinc (mg/100g)	$1.69^{b} \pm 0.02$	$4.38^{c} \pm 0.02$	$4.51^{c} \pm 0.11$	$4.39^{c} \pm 0.03$	$4.63^{c} \pm 0.13$	$0.33^{a} \pm 0.12$
Molar ratio						
Phytate:iron	0.0	22.87	26.36	23.96	22.0	82.85

Note. Data presented as mean and standard deviation. Averages in lines followed by different lowercase letters differ from each other by Tukey's test ($P \leq 0.05$). $N = 3$.

The iron content in hemoglobin is estimated to be [body weight (g) × Hb (g/L) × 0.335 × 6.7]/1,000.

This variable was calculated assuming that total blood volume equals 6.7% of the body weight of the rat and the body iron in the hemoglobin content as 0.335 g/L.

The relative biological value of HRE (RBV-HRE) was calculated as follows:

RBV-HRE = HRE of each animal/Mean of HRE for Ferrous Sulfate Control Group.

2.7 Biomolecular analysis

2.7.1 Total mRNA extraction of liver. For the protein dosages in the liver, the total RNA was extracted of samples (100 mg) with TRIzol reagent, quantified at 260 nm in a spectrophotometer (Ultrospec 3000 UV-Visible, Pharmacia Biotech), and the degree of purity determined by the optical density ratio 260/280 nm.

2.7.2 Determination of ferritin and transferrin gene expression. The expression of mRNA levels in liver proteins involved in iron metabolism was analyzed by reverse transcription polymerase chain reaction. The markers comprised SYBR Green, PCR mastermix from Applied Biosystems (Foster City, CA, USA) and analyses were performed on the ABI Prism 5700 Sequence Detection System using the SYBR-Green fluorescence quantification system and Primer Express software (Applied Biosystems). The relative expression of mRNA levels was normalized by endogenous glyceraldehyde 3-phosphate dehydrogenase control for rats. All steps occurred under RNase-free conditions.

2.8 Statistical analysis

The nine groups ($n = 8$) were compared using analysis of variance (ANOVA), followed by Tukey's test ($P < 0.05$), as well as the analysis of the flour chemical composition. Statistical analysis was done with SPSS Statistics Data Editor v19.0 (IBM SPSS Statistics Base, DMSS, SP, Brazil).

3. RESULTS AND DISCUSSION

3.1 Centesimal composition, phenolic compounds, phytate, and molar ratio

The composition of individual food is presented in Table 3. The flour of all cowpea cultivars presented higher protein content, whereas rice and sweet potato showed the lowest protein content ($P < 0.05$). Regarding the ashes, the highest values were observed for cowpea cultivars, followed by the sweet potato and rice, which was the sample with the lowest value among all ($P < 0.05$). The lipid content of cowpea cultivars varied from 1.57 (Guariba) to 1.97 (Xiquexique), and were higher than sweet potato and rice.

Concerning total phenolics, sweet potato had the highest concentration ($P < 0.05$) compared to the other flours. The cowpeas did not differ between each other ($P \geq 0.05$) and the lowest value was observed for rice among all ($P < 0.05$). Phenolic compounds are components that originate from the secondary metabolism of plants, essential for their growth and reproduction, and therefore are widely distributed in various plant foods. They have in their chemical composition an aromatic ring with one or more hydroxyl substituents, including their functional groups (Malacrida & Motta, 2005).

Except for its antioxidant activity, phenolics are known as antinutritional factors capable of reducing the organic utilization of some nutrients (Mohan, Tresina, & Daffodil, 2015). The sweet potato presented a higher concentration of total phenolics (1.53 mg of EqGA/g) compared to all other test samples used (Table 3). This component could, in addition to phytate, have affected the bioavailability of test beans, because the experimental groups that received sweet potato presented inferior bioavailability of iron *in vitro* to those who did not receive the source of carotenoids. However, Dias et al. (2015) reported higher bioavailability of iron in animals that received cowpea added with sweet potato or biofortified pumpkin with pro-vitamin A (127.11 mg of β-carotene/100 g of sample), but with low content (0.10 mg/100 g) and phytate:iron molar ratio compared to the values found in the present study (Table 3), which allows us to reaffirm the negative interaction of antinutritional factors on the bioavailability of minerals.

The content of phytate was similar between the four cowpea cultivars as well as sweet potato ($P \geq 0.05$). The rice showed lower phytate content ($P < 0.05$); however, it presented no statistical difference compared to cowpea BRS Aracê, BRS Xiquexique, and sweet potato ($P \geq 0.05$).

The iron content was similar among cowpea BRS Guariba, Xiquexique, and Tumucumaque ($P < 0.05$). This, in turn, also presented similarity to BRA Aracê ($P < 0.05$). The lowest mean iron content was observed in sweet potatoes, whereas rice did not present this mineral in its composition. With respect to zinc,

cowpea samples had similar levels to each other ($P < 0.05$) and higher than sweet potatoes and rice, whereas rice showed higher values compared to sweet potatoes ($P < 0.05$).

The prediction of the mineral bioavailability of phytate-containing foods is complicated by the complex interactions between minerals and phytate, phytase activity in the food and/or intestine, conditions in food processing, food digestibility, as well as the individual's physiological status, suggested that the phytate:mineral molar ratio is the best tool to predict mineral bioavailability (Ramirez Cardenas, 2006). To evaluate the effect of the phytate result present in food on the bioavailability of minerals (Fe), the phytate:mineral molar ratio has been investigated (Ma et al., 2005). Phytate begins to lose its inhibitory effect on iron when the phytate:iron molar ratios are less than 1 (Hallberg, Brune, & Rossander, 1989). This suggests that the phytate present in cowpea and sweet potatoes may interfere with the absorption of iron and zinc and contribute to the deficiency of these minerals.

Gomes et al. (2017), when analyzing experimental diets containing vitamin A-biofortified sorghum flour and sweet potato, observed an increase of iron bioavailability in these mixtures, because the phytate:iron molar ratio of the diets added with sweet potato was lower. In the present study, this ratio was higher in potatoes compared to all biofortified beans analyzed, which may have influenced the iron bioavailability of experimental diets with the presence of Beauregard potato. Human studies have shown that the phytate:iron molar ratio of 4 to 30 can significantly inhibit iron absorption (Petry et al., 2012).

3.2 *In vitro* study: Digestible fraction of iron and Caco-2 and gene expression

The term "bioavailability" is defined as the proportion of a nutrient ingested in food that is absorbed and used through normal metabolic pathways, with influences from dietary and individual factors (Hurrell, 2002; Mohan, Tresina, & Daffodil, 2015). Studies have shown an increase in the bioavailability of iron in biofortified foods, such as beans (Haas et al., 2005; Petry et al., 2012; Tako, Blair, & Glahn, 2011; Tako, Hoekenga, Kochian, & Glahn, 2013).

In order to verify the concentration of iron available to be absorbed after its passage through the gastrointestinal tract, the iron content in the digestible fraction of the experimental samples was evaluated in isolation (Table 4). BRS Xiquexique presented the highest available iron content and was similar to the values found for biofortified sweet potato and BRS Guariba cowpea ($P < 0.05$). The lowest values were observed for the BRS Aracê and Tumucumaque, both of which presented no statistical difference between them ($P < 0.05$). However, these resemble BRS Guariba and sweet potatoes, but not Xiquexique ($P < 0.05$).

Table 4–The *in vitro* analyses and gene expression of ferritin and transferrin.

	Iron content of the digestible fraction (mg)	Bioavailability of iron *in vitro* (ferritin/protein)	Gene expression (*in vivo*)	
			Ferritin	Transferrin
FS	–	77.87 ± 5.82^{d}	1.00 ± 0.00	1.00 ± 0.00^{b}
G	25.77 ± 1.71^{bc}	5.71 ± 0.32^{bc}	0.39 ± 0.11	0.17 ± 0.12^{a}
A	18.23 ± 1.61^{ab}	5.00 ± 0.31^{bc}	1.37 ± 0.74	0.06 ± 0.02^{a}
T	17.04 ± 2.90^{ab}	5.73 ± 0.75^{bc}	1.12 ± 1.04	0.34 ± 0.39^{a}
X	30.46 ± 8.58^{c}	6.53 ± 1.08^{c}	1.14 ± 0.95	1.36 ± 0.54^{b}
BB	24.50 ± 2.51^{bc}	–	–	–
GB	–	4.38 ± 0.99^{abc}	1.75 ± 0.96	0.17 ± 0.11^{a}
AB	–	3.72 ± 1.59^{ab}	0.35 ± 0.32	0.06 ± 0.02^{a}
TB	–	4.08 ± 0.50^{ab}	2.00 ± 1.80	0.08 ± 0.03^{a}
XB	–	2.16 ± 0.35^{a}	0.46 ± 0.20	0.09 ± 0.03^{a}

Note. Data presented as mean and standard deviation. Averages in the columns followed by different lowercase letters differ by Tukey's test ($P \leq 0.05$). $N = 3$.
Abbreviations: FS, ferrous sulfate; GB, rice + BRS Guariba + sweet potatoes; AB, rice + BRS Aracê + sweet potato; TB, rice + BRS Tumucumaque + sweet potato; XB, rice + BRS Xiquexique + sweet potato; G, rice + BRS Guariba; A, rice + BRS Aracê; T, rice + BRS Tumucumaque; X, rice + BRS Xiquexique; BB, biofortified sweet potato.

In vitro bioavailability analysis revealed a higher mean value for the control group (FS) ($P < 0.05$). The groups containing sweet potatoes (AB, XB, TB, and GB) did not differ among them, a result also observed for the mixtures without the addition of sweet potato (A, X, T, and G) ($P < 0.05$). The Xiquexique group (X) was the only one that differed from its added counterpart of sweet potato, presenting smaller values when added of the same ($P < 0.05$). In addition, it is suggested that the greater iron bioavailability of Xiquexique occurred due to the higher digestibility of this cowpea cultivar.

Groups A, T, and G on *in vitro* bioavailability were similar to all sweet potato mixtures, except for Xiquexique (XB), which resembled only sweet potato ($P < 0.05$) groups. The X mixture was similar only to the GB group ($P < 0.05$). The group containing conventional cowpea (G) presented statistical similarity to the groups with biofortified cowpea Aracê (A), Xiquexique (X), and Tumucumaque (T) ($P < 0.05$).

Concerning gene expression, no statistical difference was observed between groups for hepatic ferritin levels. However, among transferrin, similarity was observed between the control group (FS) and the Xiquexique group (X) ($P < 0.05$), reaffirming the higher iron bioavailability of this cultivar. The other experimental groups did not differ ($P < 0.05$).

In contrast, groups A and X, both without the presence of β-carotene in the mixture, presented higher levels of ferritin. It is known that hepcidin, a central peptide in the systemic regulation of iron, has its mRNA levels modulated by vitamin A. Low concentrations of this hormone blocks ferroportin in the enterocytes, affecting the absorption of iron, and in macrophages and hepatocytes, inhibiting the iron mobilization of the latter's ferritin stocks (Cunha, 2013; Vyoral & Petrak, 2017).

The combination of biofortified foods with carotenoids and conventional and biofortified cowpea with iron and zinc was not sufficient to favor iron absorption, because the *in vitro* bioavailability of these mixtures was lower compared to the groups that did not receive sweet potato as source of β-carotene. The literature shows that vitamin A can bind to iron and form a complex that acts as a chelating agent, avoiding the inhibitory effect of phytate on iron absorption (Garcia-Casal & Larysse, 1998). However, to be converted into its active form, β-carotene must undergo the action of the zinc-dependent enzyme (Cozzolino, 2016). Thus, vitamin A may aid in the negative effect of phytate but be affected by zinc concentration.

It is believed that the combination of staple foods characteristic of Brazilian food habits, such as rice and beans, associated with biofortified vegetables with carotenoids, such as sweet potatoes, may increase the bioavailability of iron. The action of vitamin A on the mobilization of iron from hepatic stocks is known, favoring the availability of this mineral for hematopoiesis and hemoglobin synthesis (Semba & Bloem, 2002). In addition, vitamin A has been associated with the gene expression of hepcidin in the liver, a hormone that regulates the uptake and exportation of endogenous iron via the ferroportin receptor (Oates, 2007).

There is the premise that the mixing of these foods in the diet can increase the bioavailability of iron and absorption enhancers and minimize the negative effect of phytochemicals (Dias et al.,

Table 5–Iron and zinc content (mg/kg) and phytate:iron molar ratio of experimental diets.

Experimental diets	Fe (mg/kg)	Zn (mg/kg)	Phytate:iron
G	12.43	25.54	38.80
A	13.77	25.08	44.53
T	14.28	24.66	40.65
X	11.63	23.67	37.89
GB	11.58	25.33	43.79
AB	13.73	19.65	49.88
TB	12.15	18.83	45.77
XB	11.59	16.84	42.93
FS	1.87	15.41	–

Abbreviations: G, rice + BRS Guariba; A, rice + BRS Aracê; T, rice + BRS Tumucumaque; GB, rice + BRS Guariba + sweet potato; AB, rice + BRS Aracê + sweet potato; TB, rice + BRS Tumucumaque + sweet potato; XB, rice + BRS Xiquexique + sweet potato; FS, ferrous sulfate.

Table 6–Total food intake, total weight gain (total WG), and iron intake of Wistar (*in vivo* study).

	Food intake (g)[a]	Weight gain total (g)[a]	Iron intake (mg)
FS	229.01 ± 26.82	51.20 ± 15.87	0.43 ± 0.05[a]
G	244.28 ± 20.32	56.35 ± 9.61	3.04 ± 0.25[bcd]
A	237.56 ± 22.98	50.95 ± 8.99	3.27 ± 0.32[cd]
T	234.78 ± 19.22	53.38 ± 13.00	3.35 ± 0.27[d]
X	237.65 ± 32.07	49.18 ± 10.21	2.76 ± 0.37[b]
GB	241.04 ± 29.39	53.75 ± 7.69	2.79 ± 0.34[b]
AB	233.25 ± 24.85	45.70 ± 15.97	3.20 ± 0.34[bcd]
TB	246.60 ± 22.74	45.68 ± 32.44	3.00 ± 0.28[bcd]
XB	244.60 ± 9.16	56.85 ± 10.70	2.83 ± 0.11[bc]

Note. Data presented as mean and standard deviation. Averages in the columns followed by different lowercase letters differ by Tukey's test ($P \leq 0.05$). $N = 8$.
Abbreviations: FS, ferrous sulfate; GB, rice + BRS Guariba + sweet potatoes; AB, rice + BRS Aracê + sweet potato; TB, rice + BRS Tumucumaque + sweet potato; XB, rice + BRS Xiquexique + sweet potato; G, rice + BRS Guariba; A, rice + BRS Aracê; T, rice + BRS Tumucumaque; X, rice + BRS Xiquexique; BB, biofortified sweet potato.
[a]Not significant.

2015). Nevertheless, this study did not present favorable results to the combination of these foods. When discussing the bioavailability of certain micronutrients, one should consider the influence of the food matrix and the effects of dietary enhancers and inhibitors on the absorption process. Vitamin A comprises compounds having biological activity of retinol. Foods of animal origin provide the preformed source of vitamin A, whereas plant foods are made from the carotenoid forms of pro-vitamin A and need to be converted into vitamin A in the gut.

This conversion is influenced by factors that include the food matrix, pro-vitamin species, food preparation, and fat content of the meal as a whole (Casgrain et al., 2010). Thus, the mere presence of carotenoids in food is not a guarantee that these will be available for absorption and conversion into retinol.

3.3 Bioavailability *in vivo*

Rats have the intestinal phytase enzyme, but studies show that in young animals, such as those in this study, there is low activity of this enzyme (Frano et al., 2014). In this way, it is possible that the phytate–iron interaction has reduced the availability of this mineral for absorption and consequent bioavailability. The groups with higher phytate:iron molar ratio (A and T) had lower *in vitro* digestibility. The groups with lower molar ratio (G and X) had higher digestibility; however, when associated with sweet potatoes, they showed low *in vitro* bioavailability. This result is understandable when evaluating the phytate:iron molar ratio of the experimental diets (Table 5), in which high values were observed for the combinations that contained sweet potato.

There was no significant difference in total food intake, total weight gain, hemoglobin concentration (initial and final), and gain among groups ($P \geq 0.05$), as shown in Table 6.

Experimental groups of sweet potatoes presented iron intake similar to each other and when compared to their without sweet potato (Table 6). In addition, the hemoglobin gain of these groups was similar when compared to the control group (FS). However, the efficiency of hemoglobin regeneration was lower for the test groups than for ferrous sulfate, evidencing the lower amount of bioavailable iron in the experimental diets. The same was observed in the study by Gomes et al. (2017), in which there was no difference in the hemoglobin and HRE% gain in animals fed sorghum in the absence and presence of biofortified sweet potato.

Iron intake by the Xiquexique (X) group in the repletion phase was one of the lowest observed and may have led to an increase in the transferrin expression in the hepatic tissue as a way to compensate for the reduction of the supply of this mineral. The increase of this transporter may explain the increased transport of iron to the liver, increasing the ferritin gene expression (values close to the control) and reducing the availability of iron for hematopoiesis, a result observed in the levels of hemoglobin gain in the animals. Although not statistically significant, the animals that received sweet potato blends exhibited lower expression of ferritin compared to the groups without the addition of the carotenoid source, possibly due to the lower supply of bioavailable iron in these diets.

The hemoglobin gain was similar in all groups, even in the control group, which shows a greater efficiency of hemoglobin regeneration. A nutritional intervention in children, performed by Landim (2013), using cowpea flour cookies from the biofortified cultivar BRS Xiquexique, showed a significant increase in hemoglobin levels after a period of 60 days with the offer of 30 g, three times a week.

Conventional beans showed a higher percentage compared to all test groups and, among the biofortified cowpeas, Tumucumaque was highlighted, but still lower than its conventional counterpart. In spite of the similarity in iron intake and hemoglobin gain, the cowpeas mentioned differed in the *in vivo* bioavailability index (HRE%), and the other groups, although with similar results, presented values greater than 50%, mostly. This is in line with the findings of Cunha (2013) and Garcia et al. (2013), according to which, in the presence of anemia, physiological changes are responsible for altering some of the stimuli in the intestinal mechanism of Fe absorption, such as increased expression of DMT1 and TfR1, making dietary Fe more bioavailable and increasing its use for Hb synthesis.

With regard to HRE (Table 7), all combinations of sweet potato (GB, AB, XB, and TB) were similar to their sweet potato counterparts ($P < 0.05$), to the Xiquexique group (X), and to each other ($P < 0.05$). Groups AB and XB did not differ from the T and A groups, respectively ($P \geq 0.05$). On the groups without the addition of sweet potato (G, A, X, and T), the Aracê group was similar to the Tumucumaque and the Xiquexique to the Guariba and this one was statistically equal to all others (A, X, and T) ($P < 0.05$). Regarding the test groups, GB stood out in the percentage of HRE (59.65%).

Tako et al. (2011) showed that biofortified color beans exhibited greater bioavailability of iron compared to conventional samples in the *in vivo* and *in vitro* assay. Different results were observed in the present study; the *in vivo* results using rats showed bioavailability of Fe similar to ferrous sulfate and *in vitro* this was not observed. In the present study, the *in vivo* results do not support the

Table 7–Hemoglobin concentration, hemoglobin regeneration efficiency (HRE), and relative biological value of HRE (RBV-HRE).

	Initial Hb[a] (g/dL)	Final Hb[a] (g/dL)	Hb Gain[a] (g/dL)	HRE (%)	RBV-HRE
G	6.97 ± 0.80	8.45 ± 0.92	1.48 ± 0.84	51.00 ± 8.49[ab]	0.25 ± 0.15
A	6.98 ± 0.79	8.50 ± 0.92	1.52 ± 1.28	46.58 ± 8.06[ab]	0.22 ± 0.09
T	6.98 ± 0.81	8.69 ± 0.87	1.71 ± 0.91	40.50 ± 11.38[a]	0.21 ± 0.16
X	6.99 ± 0.76	8.68 ± 0.64	1.69 ± 0.93	58.50 ± 8.01[ab]	0.30 ± 0.19
GB	6.97 ± 0.85	8.76 ± 0.58	1.79 ± 0.63	59.65 ± 13.93[b]	0.31 ± 0.22
AB	6.95 ± 0.82	9.00 ± 0.94	2.05 ± 0.87	49.04 ± 13.13[ab]	0.25 ± 0.16
TB	6.96 ± 0.80	9.21 ± 0.93	2.26 ± 0.84	54.92 ± 15.18[ab]	0.28 ± 0.18
XB	6.97 ± 0.80	8.00 ± 0.56	1.62 ± 0.35	55.17 ± 14.72[ab]	0.29 ± 0.23

Note. Data presented as mean and standard deviation. Averages in the columns followed by different lowercase letters differ by Tukey's test ($P \leq 0.05$). $N = 8$.
Abbreviations: FS, ferrous sulfate; GB, rice + BRS Guariba + sweet potatoes; AB, rice + BRS Aracê + sweet potato; TB, rice + BRS Tumucumaque + sweet potato; XB, rice + BRS Xiquexique + sweet potato; G, rice + BRS Guariba; A, rice + BRS Aracê; T, rice + BRS Tumucumaque; X, rice + BRS Xiquexique; BB, biofortified sweet potato.
[a]Not significant.

in vitro analyzes of bioavailability. Furthermore, all the biofortified cowpeas evaluated did not differ from the conventional counterparts in the bioavailability indices, that is, in the bioavailability and *in vitro* digestibility and HRE results.

Pachón, Ortiz, Araujo, Blair, and Restrepo (2009) did not observe differences for dialysable iron in biofortified beans and their conventional counterpart; on the other hand, both presented high bioavailability in the animal model. In this study, the group that received BRS Aracê (A) showed lower *in vitro* digestibility and gene expression of ferritin and transferrin in the presence of sweet potato. It is important to emphasize a possible competition between iron and zinc for the same site in foods biofortified with these minerals, due to its chemical similarity, affecting the availability of iron for absorption together with the lower conversion of β-carotene to vitamin A as a result of the lower presence of enzymatic cofactor for retinal reductase (Pedrosa & Cozzolino, 1993). Modulation of vitamin A in gene expression at the post-transcriptional level of iron metabolism proteins is known.

4. CONCLUSION

The combination of iron and zinc biofortified foods together with foods rich in carotenoids pro-vitamin A, contrary to expected, did not increase the gene expression of proteins involved in iron metabolism, neither favored the bioavailability of this mineral. It was not possible to observe differences between the treatments with regard to the *in vivo* study, nevertheless, there was a greater expression of mRNA for ferritin in the Xiquexique group.

BRS Xiquexique cowpea showed to be the most promising compared to other cultivars, exhibiting higher iron content in the digestible fraction, better *in vitro* bioavailability of iron, and ferritin gene expression. Nevertheless, it is interesting to evaluate the interaction of inhibitory and potentiating factors of the bioavailability of iron present in foods as a preliminary step to the possible mixtures of the same, because the high phytate content found in the biofortified sweet potato may have influenced negatively or counteracted the possible beneficial effect of beta-carotene on iron bioavailability of biofortified cowpeas. The effect of other food components of, such as vitamin C, on iron bioavailability is also needed in future studies in humans and different animal models.

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AUTHOR CONTRIBUTIONS

S. R. Corrêa performed the test and collected the data. P. Brigide drafted the manuscript and corrected the manuscript. M. d. G. Vas-Tostes helped in the interpretation of the results. N. M. B. Costa developed the study concept.

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