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Enriched sorghum cookies with biofortified sweet potato carotenoids have good acceptance and high iron bioavailability

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ABSTRACT

Aim was to evaluate acceptability and iron bioavailability of enriched sorghum cookies with biofortified sweet potato carotenoids. Acceptability and chemical composition were analyzed in four formulations with dry or extruded sorghum and its combination with high carotenoid sweet potato. Enriched cookies presented the highest acceptance as well as nutritional quality, and they were used to measure iron bioavailability by depletion/repletion. The animals fed with Fe-free diet during three weeks were divided in three groups for two weeks: ferrous sulphate control and two tests with the enriched cookies (DEC-D, EEC-D). Hemoglobin were similar among experimental groups and the enzymes expression related to iron metabolism increased in the duodenum of EEC-D. TAC in plasma was similar between test groups and higher when compared to the control. Therefore, biofortified sweet potato carotenoids increased nutritional and sensory quality of the cookies allowing potential action as functional food to reduce risk of iron deficiency anemia.

1. Introduction

Iron deficiency anemia is the most common nutritional disorder worldwide, affecting two billion people: over 30% of the world's population. This pathology is more prevalent in developing countries due to low iron intake and others nutritional deficiencies, as in cases of vitamin A insufficiency, frequently exacerbated by infectious diseases. It affects mainly preschool children and pregnant woman, contributing to 20% of all maternal mortality, being considered a public health condition of epidemic proportions. In this type of anemia, the number of red blood cells or their oxygen-carrying capacity is insufficient, causing fatigue, weakness, dizziness and drowsiness (WHO, 2017a).

Sorghum (*Sorghum bicolor* (L.) Moench) is a pseudo-cereal native to Africa which present nutritional and functional benefits, such as phenolic compounds and vitamin E. In addition, the sorghum is gluten-free and can protect celiac patients from food allergy to this protein combination (de Morais Cardoso, Pinheiro,

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Martino, & Pinheiro-Sant'Ana, 2017; de Morais Cardoso et al., 2015; Martino, Cardoso, Moraes, Sant'Ana, & Queiroz, 2014). The grain has also agronomic advantages due to its resistance to drought, high productivity, low nutritional requirement and low cost, but it is still mainly used as animal food (Martino et al., 2014; Moraes et al., 2012). Thus, it is necessary to develop new formulations with sorghum to ensure adequate sensory quality and increase human consumption.

Sweet potato (*Ipomoea batatas*) is a popular root native to Central and South America rich in carbohydrates with low glycemic index, which provides energy to daily activities and can help blood glucose control. This food shows high productivity in tropical climates of Brazil throughout the year, with high temperature, rainfall and fertile soil, requiring low or no technology, thus predominant in the familiar agricultural production (Henz, Silva, Lopes, & Magalhães, 2008). Vitamin A deficiency is the leading cause of preventable visual impairment, night blindness and reduction in the immunologic defense, leading to increased risk of death from opportunistic infections. This illness is a severe public health problem in more than half of all low-income countries, such as Brazil, mainly affecting children and pregnant women (WHO, 2017b). Due to the fast production, nutritional advantages and high micronutrient deficiency in Brazil (iron and Vitamin A), the Brazilian Company of Agricultural Research (EMBRAPA) is producing in its experimental fields the *Beauregard* sweet potato biofortified with provitamin A carotenoids (La Frano, de Moura, Boy, Lönnerdal, & Burri, 2014; Rodriguez-Amaya, Nutti, & Carvalho, 2011).

The vast majority of sorghum genotypes contain low iron levels and high antinutritional concentration, such as phytate, which binds itself to iron and becomes an insoluble molecule (de Morais Cardoso et al., 2017; Moraes et al., 2012). Furthermore, phenolic compounds are related to Fe^{+2} ion (ferrous) chelate which may reduce bioavailability, however, it blocks the oxidation in Fe^{+3} (ferric, insoluble form), which can increase the content of the iron available (Khalili, Ebrahimzadeh, & Kosaryan, 2015; Sheikh, Desai, & Tirgar, 2016; Wallace, 2016; Santos, Alvarenga Brizola, & Granato, 2017). Likewise, provitamin A carotenoids show antioxidant properties to free radical sequestration and protection against cell oxidative stress. Evidences shows that carotenoids can also improve iron absorption in the intestine, mobilization in the liver storage and the erythropoietic pathway (García-Casal & Leets, 2014; La Frano et al., 2014). Thus, the combination of sorghum with sweet potato rich in carotenoids could be a good option to increase iron bioavailability, antioxidant capacity and the options to prevent or control iron deficiency anemia.

We know that sensory quality is very important to establish a habit of food intake, assuring its benefits. Our hypothesis is that the combination of the sorghum flour with the sweet potato biofortified with carotenoids will increase cookies acceptance and iron bioavailability. The purpose of this study was to evaluate acceptability and iron bioavailability of enriched sorghum cookies with high level of carotenoids in the sweet potato.

2. Materials and methods

2.1. Raw material and preparation of the combined flours

To increase iron absorption potency and reduce the effect of the antinutritional compounds, a genotype with high levels of iron was selected through genetic improvement and two different heat treatment processing: dry heat and wet heat or extrusion. The sorghum genotype SC319 with high content of the antioxidant compounds was produced by Brazilian Company of Agricultural Research Maize and Sorghum (EMBRAPA Maize and Sorghum) in Nova Porteirinha, Minas Gerais, Brazil (de Morais Cardoso et al., 2015) and the *Beauregard* sweet potato biofortified with carotenoids was produced by EMBRAPA Middle North in Teresina, Piauí, Brazil (Dias et al., 2015). Both cultivars from the 2010 harvest were stored in polyethylene bags at −18 °C ± 1 °C until flour elaboration.

To prepare sorghum flours, the grains were selected, washed in deionized water due to iron bioavailability experiment and dried at room temperature to start the processing. Firstly, grains were subjected to dry heat treatment at 121 °C for 25 min in an oven with air circulation (Marconi®, 093 MA, São Paulo, Brazil) and ground in a rotor mill (Marconi®, 90/CFT MA, Piracicaba, São Paulo, Brazil) with a 30 mesh sieve. The dry sorghum flour resultant was stored in polyethylene bags at −18 °C ± 1 °C for later use in the development of cookies. Then, the extruded sorghum flour was elaborated in a co-rotating twin-screw (Clextral®, Evolum HT 25, Firminy, France) at constant screw speed and temperature profile according de Morais Cardoso et al. (2015). To compensate moisture differences in the samples and provide a final moisture content of 12%, distilled water was injected between the first and second feeding zones using a plunger metering pump (AILIPU Pump Co. Ltd.®, J-X 8/1, China) during 15–20 min. The extruded sorghum grains were so milled with a 30 mesh sieve (Marconi®, 90/CFT MA, Piracicaba, São Paulo, Brazil) and the flour resulting was stored in polyethylene bags covered with aluminum foil at −18 °C ± 1 °C until the development of cookies.

The sweet potatoes were selected, washed in deionized water due to iron bioavailability experiment, peeled and sliced on a multiprocessor (Philips Walita®, Amsterdam, Netherland). The slices were submerged sodium bisulfite solution 0.5% for 10 min in order to prevent enzymatic browning and next subjected to dry heat treatment at 60 °C for 6 h in an oven with air circulation (New Ethics 400/6ND®, Vargem Grande Paulista, Sao Paulo, Brazil). Flour was ground in a rotor mill (Marconi®, 90/CFT MA, Piracicaba, São Paulo, Brazil) with a 60 mesh sieve and then stored in polyethylene bags at −22 °C for later use in the development of cookies.

2.2. Cookies development

Four cookies formulations were prepared with the sorghum and the sweet potato flours previously prepared: dry sorghum cookie, with 100% of dry sorghum flour (DSC); extruded sorghum cookie, with 100% of extruded sorghum flour (ESC); dry enriched cookie, with 50% of dry sorghum flour + 50% of sweet potato flour (DEC); extruded enriched cookie, with 50% of extruded sorghum flour + 50% of sweet potato flour (EEC) (Table S1). The ingredients were added slowly and manually mixed to provide a homogeneous dough. Chemical yeast was added last to assure great efficacy in baking. Subsequently, the cookies were molded into round 11 g portions and baked in a conventional gas oven at 280 °C for 10 min. Each cookie baked weight was 8 g, with a yield of 0.72.

The cookies were prepared twice and in three repetitions for analysis. Firstly, samples were prepared and stored in polyethylene bags at room temperature one day before the sensory tests. Next, were stored in polyethylene bags at −22 °C for chemical analysis.

2.3. Sensory analysis

Sensory evaluation was performed in laboratorial conditions after approved by the ethics committee of the Federal University of Minas Gerais (COEP/UFMG) in October 17, 2012, protocol number CAAE - 03591312.0.0000.5149.

Each sample was identified with a three-digit code and the four formulations of the cookies were presented to judge at once, according to the randomized block design. Participating of this study 100 untrained adult judge of both sexes who scored color, texture, flavor and overall impression using a 1–9 point hedonic

scale (from "like extremely" to "dislike extremely") (Gomes Natal, de Souza, Teixeira Ribeiro Vidigal, Ribeiro Silva, & Duarte Martino, 2013). Following, the two formulations with enriched flours were used to investigate if the iron bioavailability of sorghum improve with carotenoids addition.

2.4. Chemical composition

2.4.1. Proximate analyses and caloric value

The determination of water, protein, fat and ash of the three sorghum flours and of the experimental diets used in biological assay were performed according to methods of the Association of Official Analytical Chemists (AOAC, 2012). The soluble and insoluble dietary fibers were determined by enzymatic gravimetric method, according to the methodology proposed by AOAC (2012) method 985.29 and digestible carbohydrates content was calculated as the difference (Brasil, 2001). The caloric value was calculated by summing the calories supplied by proteins, carbohydrates and lipids using the conversion factors 4 kcal g^{-1}, 4 kcal g^{-1}, and 9 kcal g^{-1}, respectively (Frary & Johnson, 2005).

2.4.2. Iron content

The analysis of iron was performed according to Gomes and Oliveira (2011) protocol, in triplicate. All glassware used was previously demineralized in 10% nitric acid solution during 12 h and dry on air circulation oven. One gram of each cookie sample was weighed and transferred into a digestion tube, and added 10 mL of nitric acid. Subsequently, samples were heated in block digester with exhaustion at initial temperature of 80 °C. Temperature was increased progressively up to 160 °C, and submitted to this temperature for 4 h, until formation of clear solution. The tubes were cooled at room temperature and the content was transferred to a 50 mL volumetric flask previously washed with deionized water. This solution was agitated in vortex to avoid losses e then was used for reading of mineral contents using plasma emission spectrophotometry (Perkin Elmer-Optima® DV 3300, Norwalk, USA).

2.4.3. Resistant starch

This analysis was performed according to AOAC method 2009.01 & 2011.25 on the cookies by in vitro digestion (AOAC, 2012). Samples of 0.1 g were incubated in a shaking water bath with 4 mL of pancreatic α-amylase and amyloglucosidase at 37 °C for 16 h, during which time the starch was dissolved and hydrolyzed to D-glucose by the combined action of the two enzymes. The supernatant was removed and the resistant starch is recovered as a pellet on centrifugation by 1006g during 10 min. Pellets were dissolved in 2 M KOH (potassium hydroxide) by vigorously stirring in an ice-water bath over a magnetic stirrer. This solution was neutralized with sodium acetate buffer (pH3.8) and the resistant starch is quantitatively hydrolyzed to D-glucose by the action of the amyloglucosidase enzyme. D-Glucose was measured with glucose oxidase/peroxidase reagent (GOPOD) in an spectrophotometer (ThermoScientific®, Evolution 60S, EUA) at a 510 nm absorbance, in three repetitions.

2.5. Experimental design

The depletion-repletion hemoglobin method was applied to determine iron bioavailability according to Association of Official Analytical Chemists (AOAC, 2012), with a modification to the depletion phase to three weeks.

2.5.1. Preparation of diet

The experimental diets were based on the AIN-93G standard recommended to growing rats (Reeves, Nielsen, & Fahey, 1993). Firstly, we prepared a Fe-free depletion diet with mineral mixture without iron. On the second phase of the study, we prepared a control diet with 12 mg kg^{-1} ferrous sulphate as an iron source (AIN-93G) and two enriched cookies test diets, with sorghum and biofortified sweet potato carotenoids as iron sources: dry enriched cookie diet, with dry sorghum flour + sweet potato flour biofortified with carotenoids (DEC-D); and extruded enriched cookie diet, with extruded sorghum flour + sweet potato flour biofortified with carotenoids (EEC-D) (Table S2). Next, the iron analysis was performed by digestion in nitric acid followed by measurement with an atomic absorption and flame spectrophotometer according to the protocol previously described (Gomes & Oliveira, 2011). All the diet was calculated with 12 ppm of the mineral, however after the preparation there were a variation of 30%. In addition, since that protein can affect iron bioavailability, this nutrient was analyzed by the semi-micro Kjeldahl method and the final diets were also isoproteic (Kajarabille, Brown, Cucliciu, Thapaliya, & Latunde-Dada, 2017). For the biological experiment, the cookies were developed without canola oil in order to keep the experimental diets isocaloric.

2.5.2. Biological assay

Twenty-one male *Wistar* rats (Rattus norvegicus, *Wistar*, albinus variation) recently weaned, at 21 days of age and 75 g, were obtained from the Animal Center of Biological and Health Sciences, Federal University of Viçosa (UFV). Animals were distributed in individual stainless steel cages in controlled temperature environment (22 °C ± 3 °C) and automatically controlled 12 h light and dark cycles.

The animals received a Fe-free depletion diet and deionized water *ad libitum*, for three weeks to induce anemia. At the end of this phase, blood samples were collected from the rat-tails to determine hemoglobin (Hb) concentration.

Next, the repletion phase was conducted. The animals were kept in individual steel cages and randomly divided into three groups (n = 7), where the hemoglobin (Hb) concentration was not statistically different among groups: AIN-93G, DMC-D and EMC-D. In this phase, ferrous sulphate was used in the AIN-93G control as the highest standard iron source and the two enriched cookies with sorghum and biofortified sweet potato carotenoids were tested for two weeks. Weight gain and food intake were monitored weekly, during the five weeks.

On the 36th day, after 12 h fasting, the animals were anesthetized with isoflurane (Isoforine, Cristália®) and submitted to euthanasia by cardiac puncture (Natal et al., 2017). Blood was centrifuged in test-tube with or without anticoagulant under 4 °C at 1006g for 10 min (Fanem®, 204, São Paulo, Brazil) to have plasma and serum, respectively. The liver and duodenum samples were immediately frozen in liquid nitrogen and stored at −80 °C before analysis.

All experimental procedures with animals were conducted in accordance with the ethical principles for animal experimentation and approved by the Ethics Committee of the Federal University of Viçosa (CEUA/UFV) in June 05, 2014, process N° 09/2014.

2.6. Hemoglobina (Hb) and iron bioavailability

To determine Hb concentration was used the cianometahemoglobin method (Labtest®, Sete Lagoas, Brazil) according to AOAC (2012). Blood samples 20 μL were collected by vein tails puncture at the start and end of repletion phase, and were added to 5 mL of Drabkin's color reagent. The hemoglobin levels were quantified by UV-visible spectrophotometry at 540 nm (Shimadzu®, Kyoto, Japan).

The iron bioavailability was calculated according to Hernández, Sousa, Moreno, Villapando, and López-Alarcón (2003). To obtain Hb gain (GHb), the value of Hb final was subtracted by Hb initial

and its relative biologic value was calculated (RBV GHb) by ration GHb test/GHb control. The iron concentration in hemoglobin was estimated by: [body weight (g) × Hb (g L^{-1}) × 0.335 × 6.7]/1000. This last variable was calculated assuming the total blood volume is equal to 6.7% of the rat's body weight and the body's iron content in hemoglobin being 0.335. Then, hemoglobin regeneration efficiency (HRE%) was calculated by the expression: HRE% = [(mg Fe final Hb − mg Fe initial Hb)/100]/mg Fe consumed. The usage of iron was calculated as: [HRE% × % dietary iron]/100 and, finally, its relative biologic value (RBV HRE) was quantified by ration HRE test/HRE control.

2.7. Real-time polymerase chain reaction (RT-PCR)

The total RNA extraction was performed in the liver and duodenum samples using Trizol Reagent (Invitrogen®, CA, USA) and MirvanaTM miRNA Isolation Kit (Ambion® by Life TechnologiesTM). Next, concentration and purity were evaluated by μDrop plate spectrophotometer Multiskan™ GO (Thermo Scientific®, DE, USA) and the integrity was confirmed by electrophoresis agarose gel. For cDNA synthesis it was used the M-MLV Reverse Transcriptase Kit (Invitrogen®, CA, USA). Relative quantification of gene expression was performed by RT-PCR using the AB Step One Real Time PCR System and 2X SYBR Green Master Mix (Applied Biosystems®, CA, USA). The initial parameters used in the run were 15 min at 95 °C and then 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C, followed by melting curve analysis. Real-time PCR data was analyzed with the ΔΔCT method (Hettinger et al., 2001) using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. In the liver were analyzed two primers sequences: ferritin and transferrin. Additionally, were analyzed four primers sequences in the duodenum: divalent metal transporter-1 (DMT-1), duodenal cytochrome *B* (DcytB), ferroportin and hephaestin (Table 1). The primers were designed with the Primer3 Plus Program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Noratto, Martino, Simbo, Byrne, & Mertens-Talcott, 2015).

2.8. Biochemical profile

The serum of the animals was utilized to determine fasting glucose, renal function (uric acid; creatinine) and hepatic function (ALT - alanine aminotransferase; AST - aspartate aminotransferase) in the biochemistry analyzer (Mindray®, BS-200, Shenzhen, China) using commercial kits (Bioclin®).

2.9. Total antioxidant capacity (TAC)

The total antioxidant capacity (TAC) was determined in the plasma for enzymatic immunoassay with a specific kit (Sigma®) according to da Silva et al. (2013). The reaction mixture contained ABTS (2,2′-azino-bis 3-etilbenzotiazolina-6-ácido sulfônico) radical cation, hidrogen peroxide (H_2O_2) and a peroxidase (metmyoglobin). This assay is based in the oxidation of the ABTS and resulted in the production of the blue-green solution. So, the antioxidants present in test plasma resulted in suppression of this reaction and the color production decrease proportionally to their concentration. The absorbance was measured in a spectrophotometer (Thermo Scientific®, model Multiskan GO) at 405 nm and the amount of antioxidants was standardized using Trolox, a water-soluble vitamin E analogue. From the calibration curve, the results obtained were expressed as Trolox equivalent [mM].

Table 1
Sequence of primers used in the RT-PCR analysis.

Genes	Oligonucleotide (5′-3′)	
	Forward	Reverse
GAPDH	AGGTTGTCTCCTGTCACTTC	CTGTTGCTGTAGCCATATTC
Ferritin	CAGCCGCCTTACAAGTCTCT	ATGGAGCTAACCGCGAAGAC
Transferrin	AGCTGCCACCTGAGAACATC	CGCACGCCCTTTATTCATGG
DMT-1	CTGATTTACAGTCTGGAGCAG	CACTTCAGCAAGGTGCAA
DcytB	TGCAGACGCAGAGTTAAGCA	CCGTGAAGTATACCGGCTCC
Ferroportin	TTCCGCACTTTTCGAGATGG	TACAGTCGAAGCCCAGGACCGT
Hephaestin	GGCACAGTTACAGGGCAGAT	AGTAACGTGGCAGTGCATCA

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; DMT-1: divalent metal transporter-1; DcytB: duodenal cytochrome B.

2.10. Statistical analysis

The sensory scores were assessed by Analysis of Variance (ANOVA) followed of the *post hoc* test Tukey at 5% probability and expressed as mean ± standard deviation. Then, the data were organized in a matrix of samples (in lines)/consumers (in columns) and the covariance matrix was evaluated by Principal Component Analysis (PCA) methodology (Alizadeh Behbahani, Tabatabaei Yazdi, Shahidi, Mortazavi, & Mohebbi, 2017; Ghosh & Chattopadhyay, 2012). The results were expressed as scatter plots of samples and individual consumers in relation to the first two principal components.

Chemical composition were arranged in completely randomized design and data analyzed by the ANOVA followed of the *post hoc* test, Tukey at 5% probability. The biological experiment results were also arranged in completely randomized design and data submitted by ANOVA followed by *post hoc* test Duncan at 5% probability. Results are showed as mean ± standard deviation.

All the analyses were performed in software SPSS Statistics, version 20.0, 2011 and graphics made in the system Sigma Plot, version 11.0, 2008.

3. Results

3.1. Chemical composition of the cookies

The water content was highest in cookies with extruded sorghum flour enriched with carotenoids (EEC) ($p \leq 0.05$) while the concentration of the lipids did not differ among the four formulations. Ashes concentration decreased in cookies with extruded sorghum flour (ESC) compared with cookies with dry sorghum flour (DSC) and the addition of the sweet potato flour also increased the concentration of this mineral in both enriched cookies (DEC, EEC). On the other hand, the protein content was lower in DEC and EEC compared to DSC and ESC. The highest content of total dietary fiber (TDF) and insoluble fraction were shown in the dry sorghum flour cookie, but there was not difference in soluble dietary fiber fraction among the formulations. Resistant starch (RS) is not broken down by human enzymes in the small intestine, and so it is generally considered one of the components that make up TDF. As 100% dry sorghum flour cookie showed the highest TDF content, this formulation also presented the highest content of RS in relation to the three others ($p \leq 0.05$). After the extrusion process, the iron concentration decreased in the ESC in relation to DSC and there were subsequent reduction in the two enriched cookies with biofortified sweet potato ($p \leq 0.05$). Similarly, the phytate concentration decreased considerably (2.7 times) after the extrusion process, resulting in a lower phytate:iron molar ratio ($p \leq 0.05$). The sweet potato flour increased phenolic compounds on enriched cookies, DEC and EEC. No difference was observed in the digestible carbohydrates concentration and in the caloric density among the four cookies formulations ($p > 0.05$; Table 2).

Table 2
Chemical composition of the four cookies formulations (g·100 g^{-1}).

Composition	DSC	ESC	DEC	EEC
Water	$7.06^{b} \pm 0.05$	$8.74^{b} \pm 0.50$	$8.26^{b} \pm 3.75$	$11.57^{a} \pm 1.19$
Lipids	$40.91^{a} \pm 1.65$	$46.51^{a} \pm 2.22$	$38.57^{a} \pm 7.16$	$37.12^{a} \pm 3.76$
Ashes	$2.74^{c} \pm 0.09$	$2.33^{d} \pm 0.05$	$3.40^{a} \pm 0.11$	$3.15^{b} \pm 0.03$
Proteins	$9.53^{a} \pm 0.01$	$10.60^{a} \pm 1.24$	$6.68^{b} \pm 0.24$	$7.14^{b} \pm 0.12$
Total dietary fiber	$8.36^{a} \pm 0.14$	$3.69^{c} \pm 0.07$	$5.75^{b} \pm 0.06$	$4.04^{c} \pm 0.20$
Soluble dietary fiber	$0.67^{a} \pm 0.19$	$0.57^{a} \pm 0.16$	$0.74^{a} \pm 0.05$	$0.95^{a} \pm 0.10$
Insoluble dietary fiber	$7.70^{a} \pm 0.33$	$3.11^{c} \pm 0.10$	$5.00^{b} \pm 0.07$	$3.02^{c} \pm 0.30$
Resistent starch	$5.09^{a} \pm 1.40$	$0.70^{b} \pm 0.09$	$1.17^{b} \pm 0.06$	$1.75^{b} \pm 0.23$
Iron (mg)	$1.95^{a} \pm 0.05$	$1.42^{b} \pm 0.10$	$1.36^{c} \pm 0.06$	$1.11^{d} \pm 0.07$
Phytate (mg)[**]	53.48	19.45	26.76	9.74
Molar ratio phytate:iron	27.43	13.70	19.68	8.77
Carotenoids (mg)[#]	–	–	28.09	28.09
Total phenolic (mgEGA)[1]	0.23	0.19	33.48	33.46
Digest carb	$31.40^{a} \pm 0.05$	$28.13^{a} \pm 1.61$	$34.12^{a} \pm 6.89$	$36.98^{a} \pm 2.80$
CD (kcal g^{-1})	5.30	5.78	5.10	5.11

*Means followed by the same letter in the same line did not differed by Tukey test at 5% of probability.
DSC: dry sorghum cookie, with 100% of dry sorghum flour; ESC: extruded sorghum cookie, with 100% of extruded sorghum flour; DEC: dry enriched cookie, with 50% of dry sorghum flour + 50% of sweet potato flour; EEC: extruded enriched cookie, with 50% of extruded sorghum flour + 50% of sweet potato flour; Digest carb: digestible carbohydrates; CD: caloric density.
** Phytate content of the sweet potato flour (Dias et al., 2015) and of the sorghum flour (Gomes et al., 2017) were used to calculated the phytate concentration in the cookies formulations.
# Carotenoids levels were calculated from the sweet potato flour (Dias et al., 2015).
1 Total phenolic concentration was estimated by sum of the sweet potato flour levels (Dias et al., 2015) plus of the sorghum flour content (de Morais Cardoso et al., 2015).

3.2. Sensory analysis

The frequency of the scores showed that all formulations were acceptable with the majority of scores around six on the hedonic scale by the Analysis of Variance (ANOVA). Additionally, there was greater acceptance of the enriched formulations, DEC and EEC, in relation to flavor and overall impression ($p \leq 0.05$), while the texture show the lowest acceptability in the extruded sorghum cookie ($p > 0.05$; Table 3).

The ANOVA showed that there were significant differences among the consumers. However, the average score values among the four formulations have shown little variation, indicating that there were a variety of similar responses to the same cookie. Therefore, the data were conducted to PCA analysis and shown by Internal Preference Mapping.

The Internal Preference Mapping of all consumer data showed that at least 77.0% of the variation in the preferences were explained by the two-first principal components, sufficiently high to discriminate difference among the cookies. Each little point represents the correlations between data from consumer acceptance and the two-first principal components. The samples were represented by the four shapes and are divided into the four quadrants according its acceptance. Consumers closest to the centre of the chart did not correlated with either of the two principal components and contributed little to the discrimination of the formulations (considered to have similar acceptance). The spatial arrangement of the samples suggests the formation of four distinct groups in the analysis of color, since each cookie was preferred for a different number of consumers (Fig. 1A). Otherwise, regarding flavor, two groups were formed: one preferring the dry and extruded sorghum cookies (DSC, ESC) and the favored the both enriched cookies with biofortified sweet potato carotenoids (DEC, EEC). For this sensory characteristic, the distance of the samples grouped in relation to the two-first principal components is approximately the same as the number of consumers (Fig. 1B). Regarding texture and overall impression, the formulations DSC and ESC were separated in two other groups, considering the high difference between the distance of the samples and the two-first principal components as well as the number of consumers correlated in each. The enriched cookies were kept in the same arrangement (Fig. 1C, D).

3.3. Effect of cookies in food intake, biometry and iron bioavailability in Wistar rats

The two formulations of the enriched cookies were used in the biological assay as iron source to make two experimental diets: dry enriched cookie diet, with dry sorghum flour + sweet potato flour biofortified with carotenoids (DEC-D); and extruded enriched cookie diet, with extruded sorghum flour + sweet potato flour biofortified with carotenoids (EEC-D). The body weight did not differ among the experimental groups, although the weight gain was lower in the group fed with DEC-D ($p \leq 0.05$). In addition, the group fed with the dry enriched cookie diet also had lower food intake ($p \leq 0.05$) and so the feed efficiency ratio (FER) did not differ among the three groups ($p > 0.05$). Independently of the difference in iron intake ($p \leq 0.05$), all bioavailability parameters, hemoglobin (Hb), hemoglobin gain (GHb), hemoglobin regeneration efficiency

Table 3
Acceptance scores of the cookies.

Formulations	Acceptance scores			
	Color	Flavor	Texture	Overall impression
DSC	$6.29^{ab} \pm 1.88$	$6.01^{b} \pm 1.84$	$6.25^{b} \pm 1.74$	$6.07^{b} \pm 1.71$
ESC	$6.01^{b} \pm 1.96$	$5.92^{b} \pm 1.77$	$5.10^{c} \pm 2.11$	$5.67^{b} \pm 1.81$
DEC	$6.75^{a} \pm 1.51$	$6.57^{a} \pm 1.74$	$6.97^{a} \pm 1.43$	$6.65^{a} \pm 1.60$
EEC	$6.50^{ab} \pm 1.72$	$6.71^{a} \pm 1.64$	$6.58^{ab} \pm 1.60$	$6.61^{a} \pm 1.60$

*Means followed by the same letter in the same column did not differed by Tukey test at 5% of probability.
DSC: dry sorghum cookie, with 100% of dry sorghum flour; ESC: extruded sorghum cookie, with 100% of extruded sorghum flour; DEC: dry enriched cookie, with 50% of dry sorghum flour + 50% of sweet potato flour; EEC: extruded enriched cookie, with 50% of extruded sorghum flour + 50% of sweet potato flour.

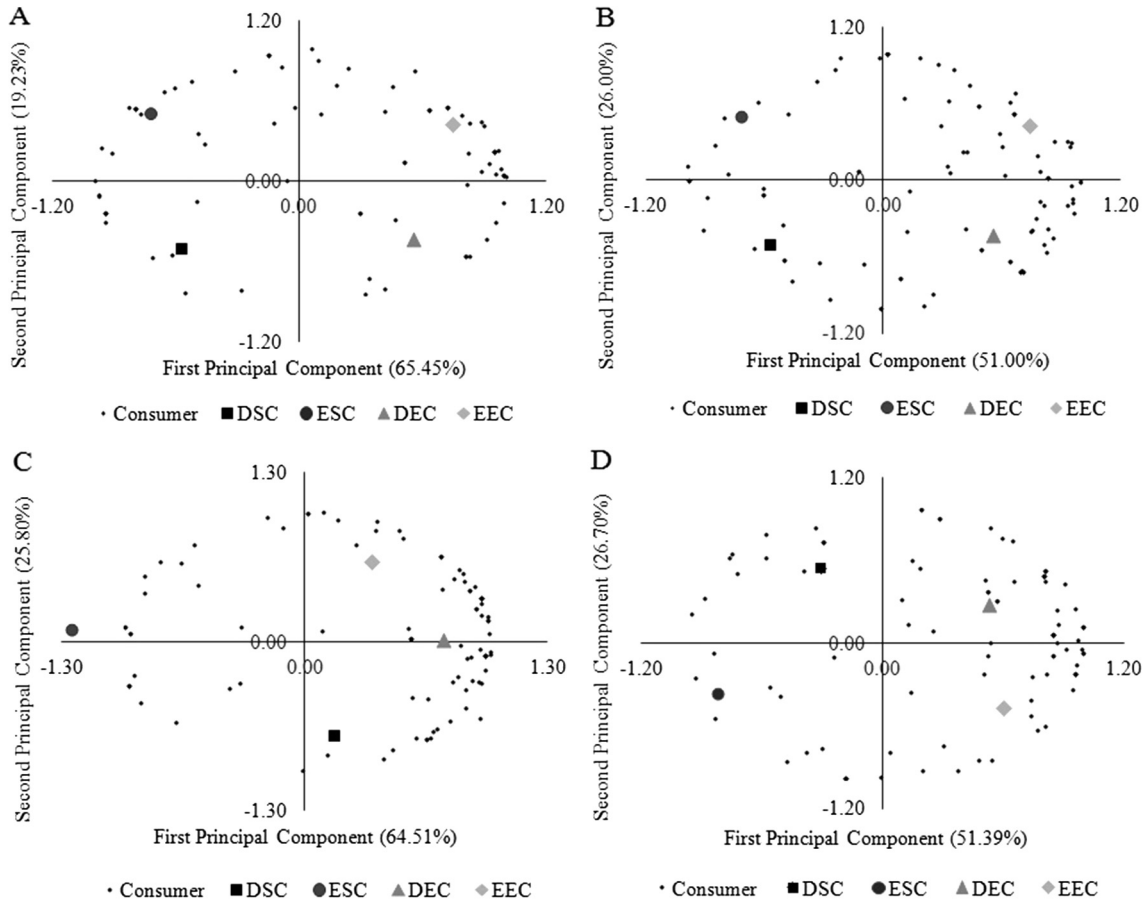

Fig. 1. Correlations between the data of each customer acceptance and the first two principal components in dispersion of cookies formulations in relation to acceptance for color (A), flavor (B), texture (C) and overall impression (D) attributes.
DSC: dry sorghum cookie, with 100% of dry sorghum flour; ESC: extruded sorghum cookie, with 100% of extruded sorghum flour; DEC: dry enriched cookie, with 50% of dry sorghum flour +50% of sweet potato flour; EEC: extruded enriched cookie, with 50% of extruded sorghum flour +50% of sweet potato flour.

(HRE), and its relative biologic value (RBV GHb and RBV HRE) were similar for the three experimental groups ($p > 0.05$; Table 4).

3.4. Modulation of the genes expression of the iron metabolism

The ferritin is the main protein to iron reservation found in all tissues, especially in those connected with iron metabolism such the liver. Otherwise, the transferrin is a plasmatic protein that carry the oxidized ion iron (Fe^{+3}) by blood releasing it to tissues of the body (Wallace, 2016). Both expressions were determined in the liver: ferritin decreased 25.0 and 100 fold in DEC-D and EEC-D test groups in relation to control ($p \leq 0.05$) and no difference was observed in transferrin among groups ($p > 0.05$; Fig. 2A, B). The intestinal absorption of the non-heme iron occur by the protein Dcytb and DMT-1 in the brush border. The only protein able to export some iron to enterocytes exterior is ferroportin, that also binds to hepcidin hormone liberating iron from enterocytes into the blood. Finally, the hephaestin is an enzyme responsible to oxide the Fe^{+2} to Fe^{+3} and so to allow its incorporation into serum transferrin (Wallace, 2016). Expression of these proteins above were investigated in the duodenum and the highest values showed by EEC-D test group were 36.8, 6.7, 155.2 and 66.1-fold, respectively, comparing to control ($p \leq 0.05$; Fig. 2C, F).

Table 4
Food intake, biometry and iron bioavailability parameters of the experimental rats.

	AIN-93G	DEC-D	EEC-D
Food intake (g)	$199.96^{a} \pm 8.06$	$171.92^{b} \pm 19.63$	$194.11^{a} \pm 21.78$
Body weight (g)	$197.75^{a} \pm 20.44$	$179.52^{a} \pm 16.59$	$195.89^{a} \pm 33.05$
Weight gain (g)	$48.92^{a} \pm 11.18$	$29.06^{b} \pm 7.54$	$44.68^{a} \pm 7.03$
FER	$0.24^{a} \pm 0.06$	$0.17^{a} \pm 0.03$	$0.22^{a} \pm 0.03$
Iron intake (mg)	$3.70^{b} \pm 0.15$	$3.13^{c} \pm 0.36$	$4.60^{a} \pm 0.52$
Hb initial (mg)	$6.18^{a} \pm 1.46$	$6.23^{a} \pm 1.46$	$6.27^{a} \pm 1.41$
Hb final (mg)	$10.21^{a} \pm 1.89$	$9.11^{a} \pm 1.70$	$10.16^{a} \pm 2.03$
GHb (mg)	$4.38^{a} \pm 0.56$	$3.36^{a} \pm 1.08$	$3.89^{a} \pm 0.85$
RBV GHb**	–	0.91 ± 0.29	1.02 ± 0.23
HRE (%)	$37.95^{a} \pm 11.07$	$36.68^{a} \pm 12.50$	$30.19^{a} \pm 7.83$
RBV HRE**	–	0.97 ± 0.33	0.79 ± 0.21

*Means followed by the same letter in the same line did not differed by Duncan test at 5% of probability.
AIN-93G: control diet; DEC-D: dry enriched cookie diet; EEC-D: extruded enriched cookie diet; FER: feed efficiency ratio; Hb: hemoglobin; GHb: hemoglobin gain; RBV GHb: relative biological value of the hemoglobin gain; HRE: hemoglobin regeneration efficiency; RBV HRE: relative biological value of the hemoglobin regeneration efficiency.
** Means following by star differed by ANOVA at 5% of probability.

3.5. Changes in the biochemical parameters after intervention with enriched cookie diets

No difference was observed on fasting glucose, uric acid, creatinine levels and on the hepatic enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels among the control and the two test groups fed with enriched cookie diets, DEC-D and EEC-D ($p > 0.05$; Fig. 3).

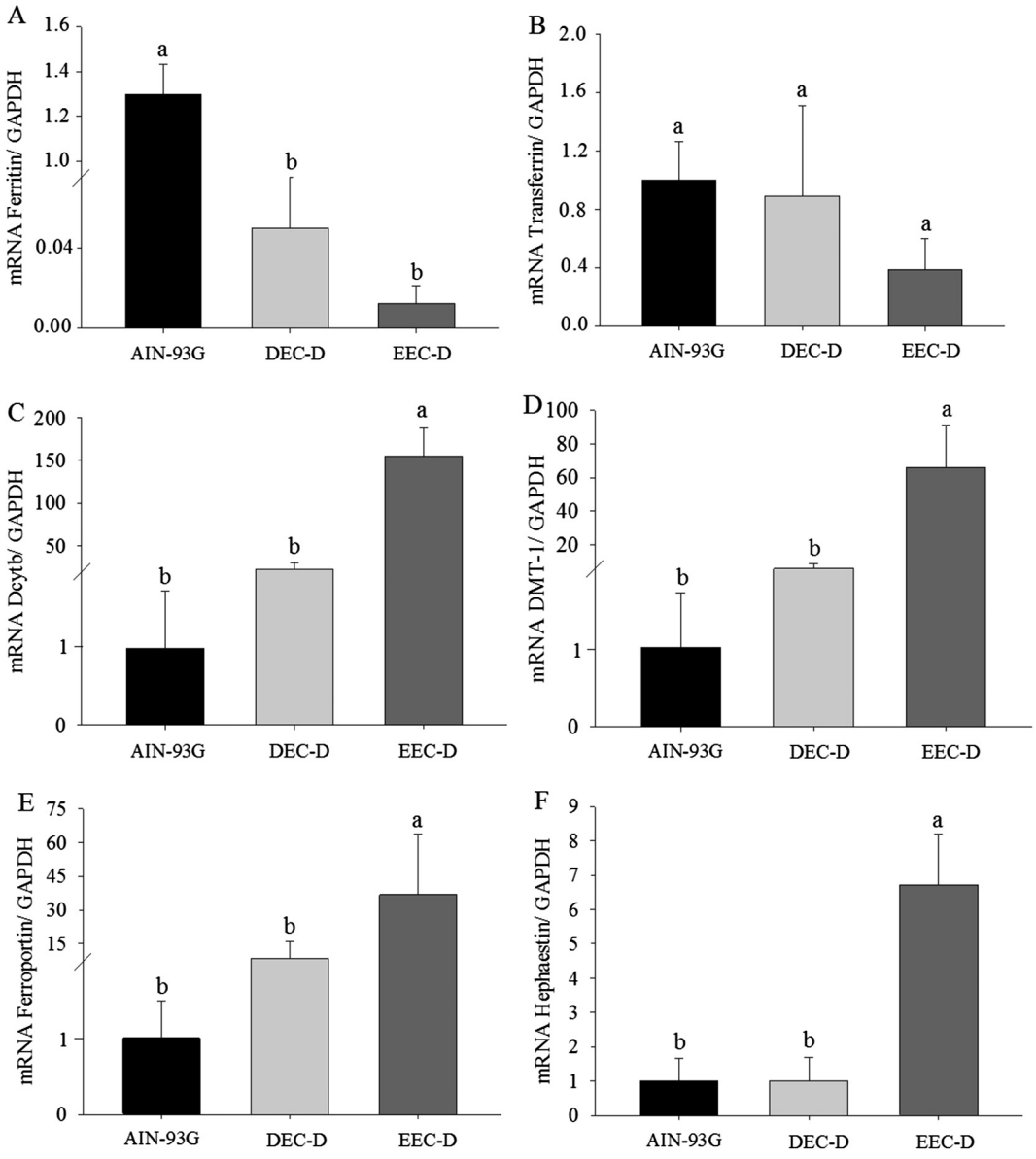

Fig. 2. Blood biochemistry values of the experimental animals the treatment with the cookies diets (mg dL^{-1}).*Means followed by the same letter in the same graphic did not differed by Duncan test at 5% of probability. AIN-93G: control diet; DEC-D: dry enriched cookie diet; EEC-D: extruded enriched cookie diet; ALT - alanine aminotransferase; AST - aspartate aminotransferase.

3.6. Effect of the cookies intake on total antioxidant capacity (TAC) in plasma

Free radical sequestration is one of the mechanisms by which antioxidants contribute to protect against cell oxidative damage (Tóth et al., 2014; Zengin et al., 2017). The total antioxidant capacity was similar ($p > 0.05$) between the groups fed with enriched cookie diets (DEC-D, EEC-D) and higher ($p \leq 0.05$) when compared with the control group (AIN-93G) (Fig. 4A). According with the total phenolic compounds and carotenoids concentration in the two cookies, it was calculated the antioxidant intake by the animals: both groups showed high intake of the phenolic compounds, but the rats in the EEC-D presented the highest carotenoids intake ($p \leq 0.05$; Fig. 4B). Therefore, the pairs TAC/total phenolic compounds and TAC/carotenoids were positively correlated, in other words, the variables tend to increase together ($p < 0.01$; Fig. 4C, D).

4. Discussion

This study focus on the potential of the carotenoids present in the biofortified sweet potato to show good acceptance and high iron bioavailability of sorghum cookies in *Wistar* rats, since the acceptability of the product is essential to ensure its intake and healthy eating habits.

The formulations presented approximately the same caloric value as the commercial products. However, they were prepared from functional and whole ingredients with phytochemical (phenolic compounds), vitamins (provitamin A carotenoids), minerals (iron), and can help maintain good health. All cookies presented high concentrations of lipids: dry and extruded sorghum cookies (DSC, ESC) and the two enriched cookies with sweet potato (DEC, EEC). But the canola oil added in the cookies increased the density of the mono and polyunsaturated fatty acids, such as omega-3 and omega-9, being beneficial factors to protect against

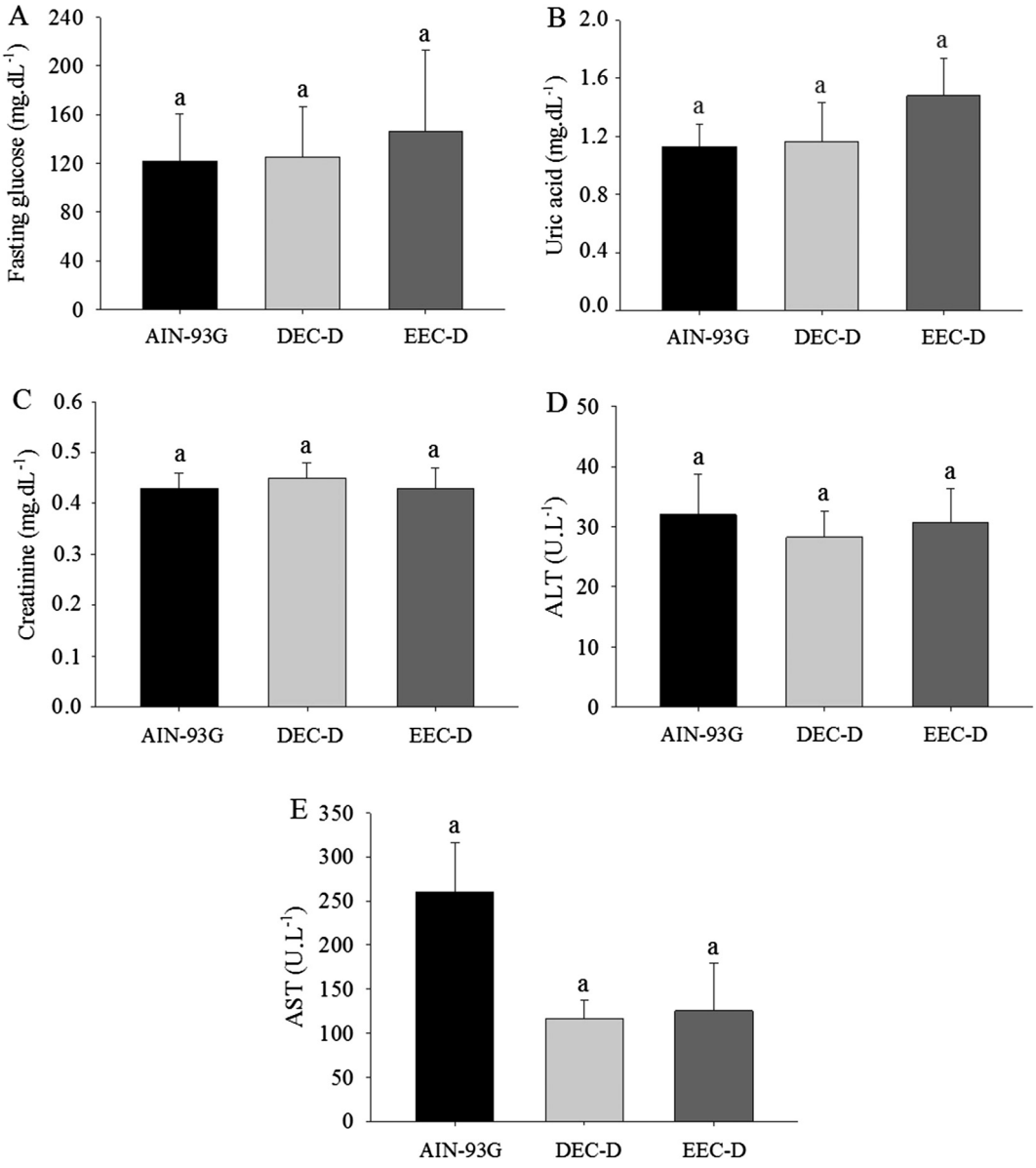

Fig. 3. Changes in genes expression of the liver proteins, ferritin (A), transferrin (B), and duodenum proteins, DcytB (C), DMT-1 (D), ferroportin (E) and hephaestin (F) after the iron bioavailability test in *Wistar* rats.
*Means followed by the same letter in the same graphic did not differed by Duncan test at 5% of probability. AIN-93G: control diet; DEC-D: dry enriched cookie diet; EEC-D: extruded enriched cookie diet; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; DMT-1: divalent metal transporter 1; DcytB: duodenal cytochrome B.

cardiovascular diseases (Guijas, Meana, Astudillo, Balboa, & Balsinde, 2016).

There was not any sorghum cookie development at this moment in the scientific researches, even considering the high nutritional value of this food product. According to Brazilian legislation to complementary nutritional information, the cookies were classified as a source of protein and dietary fiber (6% and 3% minimum, respectively) (Brasil, 2012). The sorghum cookies, enriched or not with biofortified sweet potato carotenoids, are gluten-free products; thus, they are a good option to celiac patients allergic to this protein combination. Although the extrusion process decreased the dietary fiber, all products are considered functional foods that promote healthy intestinal function (Han et al., 2017). In addition, the extrusion process also decrease the phytate concentration and so the phytate:iron molar ratio in the cookies, promoting a better use of this mineral by the organism (Gomes et al., 2017). The sweet potato added to cookies increased the phenolic compounds concentration and provided carotenoids to the products developed. Both compounds have antioxidant characteristic to bind free radical, helping people to protect themselves against cell oxidative damage and increase the iron absorption in the intestine (de Morais Cardoso et al., 2017; Protti et al., 2017).

We know that a food matrix or food product with high nutritional value is important, but it is not enough, since the color, texture, flavor and global impression are fundamental attributes to ensure sensory quality (Laranjo et al., 2017). Enriched cookies with sweet potato showed a better acceptance for flavor and overall impression, by ANOVA. The Internal Preference Mapping shows the Principal Component Analysis (PCA) and clarifies the better appreciation in relation to texture: the highest grouping of consumers positively correlated with the first principal component indicates a greater acceptance of the enriched cookies, DEC and EEC, in relation to texture, flavor and overall impression. Thus,

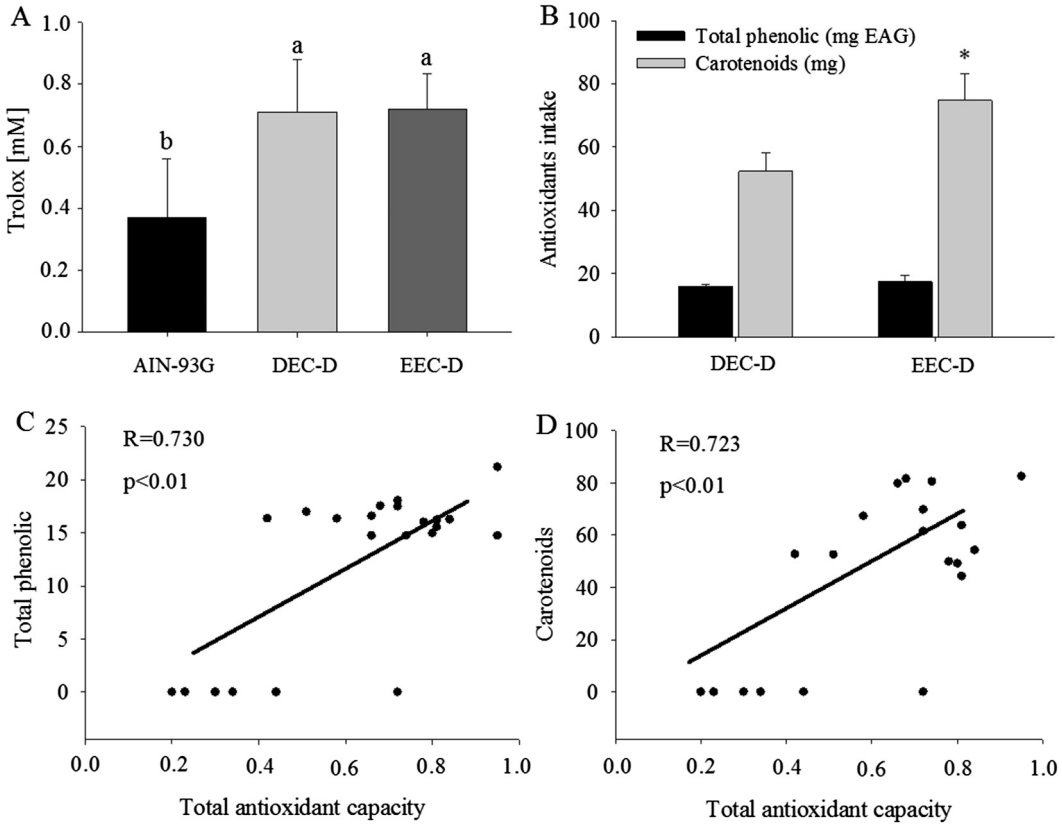

Fig. 4. Total antioxidant capacity (A), total antioxidants intake (B) and correlation between its variables (C, D) of the experimental animals.
*Means followed by the same letter in the graphic A did not differed by Duncan test at 5% of probability. **Means in the graphic B did not differed by ANOVA at 5% of probability. AIN-93G: control diet; DEC-D: dry enriched cookie diet; EEC-D: extruded enriched cookie diet; EAG: equivalent of the gallic acid.

the enriched cookies could improve the eating habits and the health of the consumers.

The lowest food intake was observed in the group fed with the dry enriched cookie (DEC-D), which could be due to higher insoluble dietary fiber (IDF) intake by the animals. This nutrient promoted satiety and decreased weight gain in relation to others two experimental groups (Sapkota, Marchant-Forde, Richert, & Lay, 2016). However, all the diets resulted proportionality in a same weight gain for each gram intake, since the feed efficiency ratio (FER) related food intake with weight gain and was similar among the groups. Although, the iron intake was higher in the extruded enriched diet group (EEC-D) compared to the control (AIN-93G) and the dry enriched cookie diet group, this measure did not indicated the intestinal absorption and utilization by the organism. The hemoglobin gain, hemoglobin regeneration efficiency and its relative biological value were similar among the test groups and the control (ferrous sulphate), indicating good biovailability of iron from enriched sorghum cookies with biofortified sweet potato carotenoids.

In previous research in our laboratory, the dry sorghum flour was unable to keep the iron bioavailability similar to ferrous sulphate. However, the extrusion process decreased phytate concentration in the extruded sorghum flour, increasing the iron bioavailability (Gomes et al., 2017). The addition of sweet potato with high phenolic compounds content in the cookies did not promote insoluble complex formation. On this account, iron bioavailability parameters of the both test groups were similar to control group ferrous sulphate. Furthermore, the carotenoids in the cookies probably formed soluble complex with the Fe^{+2} and, in such a way, increased iron absorption (Dias et al., 2015). Therefore, we can say that the enriched sorghum cookies can be a potential food product to prevent or control the iron deficiency anemia.

High ferritin levels are related to tissues toxicity in the brain, kidney and its reduction promote better iron absorption in the intestine (Meyron-Holtz et al., 2014; Wallace, 2016). Both enriched sorghum cookies reduced ferritin expression but only the extruded enriched cookie (EEC) increased the expression of the protein related with iron intestinal absorption: Dcytb, DMT-1, ferroportin and hephaestin. Similarly to this present work, a previous study in the current laboratory also verified that the extrusion process reduced phytate concentration, improved iron bioavailability parameters and increased Dcytb (Gomes et al., 2017). We know that the changes in the protein expression are the first to occur and indicate a future probability. So, the extrusion process may contribute to a long-term increase in iron absorption and bioavailability.

The majority of the blood biochemical parameters showed values into the standard range to male *Wistar* rats and only the AST presented high concentration in the control group in relation to standard (fasting glucose: 79–144; uric acid: 0.9–2.0; creatinine: 0.44–0.64; ALT: 36–58; AST: 81–180) (Melo, Dória, Serafini, & Araújo, 2013). Control and test groups with both enriched cookies were similar, which indicated that the cookies are adequate to maintain a normal physiological function of the β-cell in the pancreas, kidney and liver. Additionally, the DEC-D and EEC-D test groups showed a higher total antioxidant capacity (TAC) of the plasma in relation to AIN-93G control group, since sorghum and sweet potato ensure good intake of antioxidant compounds (de Morais Cardoso et al., 2017; Dias et al., 2015). In this work, the total phenolic compounds and carotenoids contents were calculated in cookie formulations, but the sorghum have other antioxidant com-

pounds such as anthocyanins and vitamin E (de Morais Cardoso et al., 2015; Martino et al., 2014).

5. Conclusion

The addition of biofortified sweet potato carotenoids to sorghum flours improved the nutritional and sensory quality of the cookies formulations. Both enriched sorghum cookies showed iron bioavailability similar to ferrous sulphate control, high total antioxidant capacity and adequate expression of the intestinal proteins related to absorption of the iron. Therefore, biofortified sweet potato carotenoids increased nutritional and sensory quality of the sorghum cookies allowing potential act as functional food to prevent or control iron deficiency anemia.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.08.044.

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