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Soluble extracts from carioca beans (*Phaseolus vulgaris* L.) affect the gut microbiota and iron related brush border membrane protein expression in vivo (*Gallus gallus*)

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ABSTRACT

The effect of soluble extracts with putative prebiotic ability extracted from various bean varieties on the intestinal brush border membrane (BBM) iron related proteins, and intestinal bacterial populations were evaluated using the *Gallus gallus* model and by the intra-amniotic administration procedure. Eight treatment groups [(non-injected; 18 MΩ H_2O; 40 mg/mL Inulin; 50 mg/mL BRS Perola (carioca standard); 50 mg/mL BRS Cometa (carioca, Fe biofortified); 50 mg/mL BRS Esteio (black, standard); 50 mg/mL SMN 39 (black, Fe biofortified); 50 mg/mL BRS Artico (white, standard)] were utilized. Tested groups reduced the relative abundance of *Clostridium* and *E. coli* compared to the Inulin group (positive control) and they did not affect the relative abundance of *Bifidobacterium* and *Lactobacillus* compared to the negative control (18MΩ H_2O). The relative expression of zinc transporter 1, ferroportin and amino peptidase were up-regulated in the BRS Cometa group (Fe-biofortified carioca beans). Results suggest that soluble extracts from carioca beans may improve the iron bioavailability by affecting intestinal bacterial populations, and BBM functionality.

1. Introduction

Consumption of diets with low bioavailable iron (Fe), that may lead to iron deficiency anemia, remains one of the most pervasive nutritional deficiencies worldwide (Wegmüller et al., 2016; WHO, 2011a, 2011b). In 2011 the World Health Organization (WHO) estimated that roughly 43% of children, 38% of pregnant women, and 29% of non-pregnant women are affected by anemia, and it is estimates that 50% of anemia cases worldwide are due to dietary iron deficiency (WHO, 2011a, 2011b). Global efforts to reduce the incidence of iron deficiency have been directed to increase the iron consumption through fortification of food products and biofortification of staple food crops (Blair, 2013; La Frano, de Moura, Boy, Lönnerdal, & Burri, 2014). The biofortification of staple crops is now considered an established plant breeding approach to alleviate Fe deficiency in under-developed countries. The biofortification process currently assumes that producing foods with higher Fe concentrations through the best practices of breeding and modern biotechnology will result in delivering more Fe for absorption and utilization by humans (Bouis, McClafferty, Meenakshi, & Pfeiffer, 2014; Bouis & Saltzman, 2017; Dias et al., 2015).

In this context, the common bean (*Phaseolus vulgaris* L.) is one of the crops that are targeted for biofortification. It has been established exhibits sufficient genetic variability and can be bred for enhanced iron concentration, which is a basic requirement for biofortification (Harvest Plus, 2014; White & Broadley, 2005). In addition, this crop is currently estimated to be one of the most important legumes worldwide, and is an important source of nutrients for > 300 million people in parts of Eastern Africa and Latin America, representing 65% of total

Abbreviations: Fe, Iron; BBM, Brush Border Membrane; DMT1, Divalent metal transporter 1; AP, Amino peptidase; SI, Sucrose isomaltase; FPN, Ferroportin; ZnT1, zinc transporter 1

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Table 1
Characterization and source of the beans.

Bean classes	Bean cultivars	Iron concentration in the bean flours (mg/g)	Source
White	BRS Artico	65.59 ± 5.66 (Fe standard)	Embrapa (Empresa Brasileira de Pesquisa Agropecuaria, Goias, Brazil)
Carioca	BRS Perola	70.39 ± 1.19 (Fe standard)	
	BRS Cometa	94.95 ± 0.74 (Fe biofortified)	
Black	BRS Esteio	68.08 ± 2.31 (Fe standard)	
	SMN 39	86.54 ± 2.46 (Fe biofortified)	CIAT (International Center for Tropical Agriculture, Cali, Colombia)

protein consumed, 32% of energy, and a major source of micronutrients (vitamins and minerals) (Blair, González, Kimani, & Butare, 2010; Broughton et al., 2003; Petry, Boy, Wirth, & Hurrell, 2015). For example, with a production of approximately 3.03 million tons per year (FAO, 2017), beans are a major dietary component in the Brazilian diet. The national survey (2009) data indicated that Brazilians consume on average 182.9 g per capita of cooked beans daily (IBGE, 2011), and 76.8% of inhabitants eat beans on a daily basis (IBGE, 2011).

A major challenge associated with biofortification of common beans, is that the seed coat can be high in polyphenols that inhibit Fe bioavailability (Hart, Tako, & Glahn, 2017; Tako, Beebe, Reed, Hart, & Glahn, 2014). Moreover, the cotyledon cell walls and phytic acid within the intracellular matrix have also been identified as major factors that can inhibit the iron absorption from beans (Glahn, Tako, Cichy, & Wiesinger, 2016). These inhibitory factors may increase with iron concentration when these crops are biofortified via conventional breeding (Hart et al., 2017; Petry et al., 2014).

Despite containing inhibitory factors, certain color classes of beans may also have significant concentrations of polyphenolics that can promote Fe bioavailability (Hart, Tako, Kochian, & Glahn, 2015). Alternatively, certain compounds such as arabinoxylans, stachyose and raffinose have demonstrated prebiotic effects that have been linked to improved gut functionality and Fe status (Pacifici et al., 2017; Tako, Glahn, Knez, & Stangoulis, 2014).

Similar to other legumes as lentil and chickpeas, beans contain prebiotic compounds (Dwivedi, Sahrawat, Puppala, & Ortiz, 2014; Hou, Kolba, Glahn, & Tako, 2017; Johnson, Thavarajah, Combs, & Thavarajah, 2013; Nilsson, Johansson, Ekström, & Björck, 2013; Pacifici et al., 2017), which have been characterized as a group of carbohydrates that resist the initial digestion in the upper gastrointestinal tract (small intestine). These compounds may beneficially affect gut health, by the enhancing the growth and activities of probiotics (Pacifici et al., 2017); Tuohy, Rouzaud, Brück, & Gibson, 2005; Wong, de Souza, Kendall, Emam, & Jenkins, 2006) and may indirectly improve mineral absorption (Welch & Graham, 2004). Prebiotics can resist the acidic and enzymatic digestion in the small intestine, and thus can be fermented by probiotics that reside in the colon/cecum (Dwivedi et al., 2014). The fermentation of prebiotics by probiotics leads to the production of short-chain fatty acids (SCFA), which reduce the intestinal pH, inhibiting the growth of potentially pathogenic bacterial populations and potentially improving the absorption of minerals such as iron and zinc (by increasing their solubility) (Tako et al., 2008; Tako, Glahn, et al., 2014; Zimmermann et al., 2010).

The *Gallus gallus* model (broiler chicken) has been established as a model for human iron and zinc bioavailability (Knez et al., 2016; Reed et al., 2014; Tako, Bar, & Glahn, 2016). Additionally, there is > 85% homology between gene sequences of human and chicken intestinal genes such as DMT1, DcytB, ZnT1, and FPN (International Chicken Genome Sequencing Consrotium, 2004). Moreover, *Gallus gallus* harbors a complex and dynamic gut microbiota, strongly influenced by host genetics, environment and diet (Yegani & Korver, 2008). There is considerable similarity at the phylum level between the gut microbiota of broilers (*Gallus gallus*) and humans, with *Bacteroidetes*, *Firmicutes*, Proteobacteria, and Actinobacteria representing the four dominant bacterial phyla in both (Backhed, 2005; Dias et al., 2018; Hou et al., 2017; Reed, Neuman, Glahn, Koren, & Tako, 2017).

In the current study, the effect of soluble extracts with putative prebiotic ability (extracted from common beans) on the promotion of Fe uptake was studied in vivo and by utilizing the intra-amniotic administration model (*Gallus gallus*) (Hou et al., 2017; Pacifici et al., 2017). It was previously demonstrated that soluble extracts from wheat, lentil and chickpea affected the expression of BBM Fe related proteins, and intestinal bacterial populations (Hou et al., 2017; Tako, Glahn, et al., 2014). Hence, the first objective of this study was to assess the effects of the tested bean soluble extracts on Fe related BBM proteins, specifically, the expression of Fe metabolism-related genes (DMT1, the major iron intestinal transporter; DcytB, Fe reductase; and FPN, the major intestinal enterocyte Fe exporter). In addition, the intestinal (BBM) functionality was evaluated by assessing the expression of biomarkers proteins of BBM digestive and absorptive ability (AP- aminopeptidase, SI- sucrase isomaltase, and SGLT1- sodium glucose cotransporter-1). The second objective was to evaluate the effects of the intra-amniotic administration of the tested beans soluble extracts on the intestinal bacterial populations; this was done by measuring the relative abundance of probiotic health-promoting populations bacteria such as *Bifidobacterium* and *Lactobacillus* versus those of potentially pathogenic bacteria such as *E. coli* and *Clostridium*.

2. Materials and methods

2.1. Sample preparation

The bean cultivars and classes are listed in Table 1. The beans were cooked in three replicates in a conventional pressure cooker for 40 min using a bean/distilled water ratio of 1:2.7 (*w/v*) for the carioca beans, 1:28 (w/v) for black beans and 1:3 (w/v) for the white beans. The beans were dried in an air oven for 17 h at 60 °C, ground by stainless steel mill 090 CFT at 3000 rpm and stored at −12 °C (Dias et al., 2015; Ramírez-Cárdenasi, Leonel, & Costa, 2008).

2.2. Extraction of soluble content from tested beans

The extraction of soluble content of the beans was performed as described by Vidanarachchi et al. (Vidanarachchi, Iji, Mikkelsen, Sims, & Choct, 2009), with some modifications (Hou et al., 2017; Tako, Glahn, et al., 2014). Briefly, the bean flour samples were dissolved in distilled water (50 g/L) (60 °C, 90 min) and then centrifuged at 3000 rpm for 20 min to remove particulate matter and then centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was collected and dialysed (MWCO 12–14 kDa) exhaustively against distilled water for 48 h. At last, the dialysate was collected and then lyophilized to yield a fine off-white powder.

2.3. Dietary soluble fiber content in the bean extracts

Dietary soluble fiber concentration was performed by the enzymatic-gravimetric method (AOAC, 2012), using the enzymatic hydrolysis for a heat-resistant amylase, protease and amyloglucosidase (Total dietary fiber assay Kiyonaga, Sigma®, Kawasaki, Japan).

2.4. Animals and design

Cornish-cross fertile broiler eggs ($n = 110$) were obtained from a commercial hatchery (Moyer's chicks, Quakertown, PA, USA). The eggs were incubated under optimal conditions at the Cornell University Animal Science poultry farm incubator. All animal protocols were approved by Cornell University Institutional Animal Care and Use committee (ethic approval code: 2007–0129). Soluble extracts in powder form were separately diluted in 18 MΩ H_2O to determine the concentrations necessary to maintain an osmolality value (OSM) of < 320 OSM to ensure that the chicken embryos would not be dehydrated upon injection of the solution. At day 17th of embryonic incubation, eggs containing viable embryos were weighed and divided into 8 groups ($n = 10$). All treatment groups were assigned eggs of similar weight frequency distribution. Each group was then injected with the specified solution (1 mL per egg) with a 21-gauge needle into the amniotic fluid, which was identified by candling. The 8 groups were assigned as follows: (1) non-injected; (2) 18 MΩH_2O; (3) 40 mg/mL Inulin; (4) 50 mg/mL Perola bean extract; (5) 50 mg/mL Cometa bean extract; (6) 50 mg/mL Esteio bean extract; (7) 50 mg/mL SMN 39 bean extract; (8) 50 mg/mL Artico bean extract. After all the eggs were injected, the injection holes were sealed with cellophane tape and the eggs placed in hatching baskets such that each treatment was equally represented at each incubator location. Immediately after hatch (21 days) and from each treatment group, chicks were euthanized by CO_2 exposure and their small intestine, blood, cecum and liver were collected. Blood sample was collected using micro-hematocrit heparinized capillary tubes (Fisher Scientific, Waltham, MA) immediately after hatch but before euthanization.

2.5. Iron content in the bean extracts, liver and serum

The bean extract samples (0.5 g) and serum (100 μL) were digested with 3.0 mL of nitric:perchloric mixture (60,40 HNO_3,$HClO_4$) into a Pyrex glass tube and left for overnight to destroy organic matter. The mixture was then heated to 120 °C for 2 h and 0.25 mL of 40 μg/g Yttrium added as an internal standard to compensate for any drift during the subsequent inductively coupled plasma atomic emission spectrometer (ICP-AES) analysis. The temperature of the heating block was then raised to 145 °C for 2 h. Then, the temperature of the heating block raised to 190 °C for 10 min and turned off. The cooled samples in the tubes were then diluted to 20 mL, vortexed and transferred into auto sample tubes to analyze via ICP-AES. The model of the ICP used was a Thermo iCAP 6500 series (Thermo Jarrell Ash Corp., Franklin, MA, USA).

2.6. Isolation of total RNA from chicken duodenum

Total RNA was extracted from 30 mg of the proximal duodenal tissue ($n = 6$) using Qiagen RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. Briefly, tissues were disrupted and homogenized with a rotor-stator homogenizer in buffer RLT®, containing β-mercaptoethanol. The tissue lysate was centrifuged for 3 min at 8000 *g* in a micro centrifuge. An aliquot of the supernatant was transferred to another tube, combined with 1 volume of 70% ethanol and mixed immediately. Each sample (700 μL) was applied to a RNeasy mini column, centrifuged for 15 s at 8000 *g*, and the flow through material was discarded. Next, the RN easy columns were transferred to new 2-mL collection tubes, and 500 μL of buffer RPE® was pipetted onto the RNeasy column followed by centrifugation for 15 s at 8000 *g*. An additional 500 μL of buffer RPE were pipetted onto the RNeasy column and centrifuged for 2 min at 8000 *g*. Total RNA was eluted in 50 μL of RNase free water.

All steps were carried out under RNase free conditions. RNA was quantified by absorbance at A 260/280. Integrity of the 28S and 18S ribosomal RNAs was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. DNA contamination was removed using TURBO DNase treatment and removal kit from AMBION (Austin, TX, USA).

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

To create the cDNA, a 20 μL reverse transcriptase (RT) reaction was completed in a BioRad C1000 touch thermocycler using the Improm-II Reverse Transcriptase Kit (Catalog #A1250; Promega, Madison, WI, USA). The first step consisted of 1 μg of total RNA template, 10 μM of random hexamer primers, and 2 mM of oligo-dT primers. The RT protocol was to anneal primers to RNA at 94 °C for 5 min, copy the first strand for 60 min at 42 °C (optimum temperature for the enzyme), then heat inactivate at 70 °C for 15 min and hold at 4 °C until ready to analyze by Nanodrop (Waltham, MA, USA). The concentration of cDNA obtained was determined by measuring the absorbance at 260 nm and 280 nm using an extinction coefficient of 33 (for single stranded DNA). Genomic DNA contamination was assessed by a real-time RT-PCR assay for the reference genes samples.

2.6.2. Primer design

The primers used in the real-time PCR was designed based on 9 gene sequences from Genbank database, using Real-Time Primer Design Tool software (IDT DNA, Coralvilla, IA, USA). The sequences and the description of the primers used in this work are summarized in Table 2. We analyzed the follow protein genes: DMT-1 (Divalent Metal Transporter–1), DcytB (Duodenal cytochrome *b*), Znt-1 (Zinc transporter protein-1), SI (Sucrose isomaltase), SGLT-1 (Sodium-Glucose transport protein 1) and AP (Amino peptidase). These proteins are present in the

Table 2
DNA sequences of the primers used in this study.

Analyte	Forward primer (5′-3′) (nucleotide position)	Reverse primer (5′-3′)	Base pairs length	GI identifier
Iron metabolism				
DMT-1	TTGATTCAGAGCCTCCCATTAG	GCGAGGAGTAGGCTTGTATTT	101	206597489
Ferroportin	CTCAGCAATCACTGGCATCA	ACTGGGCAACTCCAGAAATAAG	98	61098365
DcytB	CATGTGCATTCTCTTCCAAAGTC	CTCCTTGGTGACCGCATTAT	103	20380692
Hepcidin	AGACGACAATGCAGACTAACC	CTGCAGCAATCCCACATTTC	132	
Zinc metabolism				
Znt-1	GGTAACAGAGCTGCCTTAACT	GGTAACAGAGCTGCCTTAACT	105	54109718
BBM functionality				
SI	CCAGCAATGCCAGCATATTG	CGGTTTCTCCTTACCACTTCTT	95	2246388
SGLT-1	GCATCCTTACTCTGTGGTACTG	TATCCGCACATCACACATCC	106	8346783
AP	CGTCAGCCAGTTTGACTATGTA	CTCTCAAAGAAGCTGAGGATGG	138	45382360
18S rRNA	GCAAGACGAACTAAAGCGAAAG	TCGGAACTACGACGGTATCT	100	7262899

DMT-1, Divalent Metal Transporter – 1; DcytB, Duodenal cytochrome *b*; Znt-1: Zinc transporter protein-1; 18S rRNA, 18S Ribosomal subunit; SI, Sucrose isomaltase; SGLT-1: Sodium-Glucose transport protein 1; AP, Amino peptidase.

brush border membrane in the small intestine and they participate in the nutrient absorption, thus their increase suggests an improvement of the intestinal functionality (Hou et al., 2017; Pacifici et al., 2017).

The amplicon length was limited to 90 to 150 bp. The length of the primers was 17–25-mer and the GC content was between 41% and 55%. The specificity of the primers was tested by performing a BLAST search against the genomic National Center for Biotechnology Information (NCBI) database. The Gallus gallus primer 18S rRNA was designed as a reference gene. Results obtained from the qPCR system were used to normalize those obtained from the specific systems as described below.

2.6.3. Real-time qPCR design

As was previously described (Pacifici et al., 2017), cDNA was used for each 10 µL reaction together with 2× BioRad SSO Advanced Universal SYBR Green Supermix (Cat #1725274, Hercules, CA, USA) which included buffer, Taq DNA polymerase, dNTPs and SYBR green dye. Specific primers (forward and reverse (Table 2) and cDNA or water (for no template control) were added to each PCR reaction. The specific primers used can be seen in Table 2. For each gene, the optimal MgCl2 concentration produced the amplification plot with the lowest cycle product (Cp), the highest fluorescence intensity and the steepest amplification slope. Master mix (8 µL) was pipetted into the 96-well plate and 2 µL cDNA was added as PCR template. Each run contained 7 standard curve points in duplicate. A no template control of nuclease-free water was included to exclude DNA contamination in the PCR mix. The double stranded DNA was amplified in the Bio-Rad CFX96 Touch (Hercules, CA, USA) using the following PCR conditions: initial denaturing at 95 °C for 30 s, 40 cycles of denaturing at 95 °C for 15 s, various annealing temperatures according to Integrated DNA Technologies (IDT) for 30 s and elongating at 60 °C for 30 s. The data on the expression levels of the genes were obtained as Cp values based on the "second derivative maximum" (= automated method) as computed by the software. For each of the 12 genes, the reactions were run in duplicate. All assays were quantified by including a standard curve in the real-time qPCR analysis. The next four points of the standard curve were prepared by a 1:10 dilution. Each point of the standard curve was included in duplicate. A graph of Cp vs. log 10 concentrations was produced by the software and the efficiencies were calculated as 10[1/slope]. The specificity of the amplified real-time RT-PCR products were verified by melting curve analysis (60–95 °C) after 40 cycles, which should result in a number of different specific products, each with a specific melting temperature. In addition, we electrophoresed the resulting PCR products on a 2%-agarose gel, stained the gel with ethidium bromide, and visualized it under UV light. PCR-positive products were purified of primer dimers and other non-specific amplification by-products using QIAquick Gel Kit (Qiagen Inc., Valencia, CA, USA) prior to sequencing. We sequenced the products using BigDye®Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) and ABI Automated 3430xl DNA Analyzer (Applied Biosystems) and analyzed them with Sequencing Analysis ver. 5.2 (Applied Biosystems). We aligned sequences of hepcidin with those from related organisms obtained from Gen Bank using a basic alignment-search tool (BLAST; National Center for Biotechnology Information, Bethesda, MD, USA). Sequence alignments were performed for all samples. We used the ClustalW program for sequence alignment.

2.7. Collection of microbial samples and intestinal contents DNA isolation

The cecum were sterilely removed and treated as described previously (Hartono, Reed, Ankrah, Glahn, & Tako, 2015). The contents of the cecum were placed into a sterile 50 mL tube containing 9 mL of sterile PBS and homogenized by vortexing with glass beads (3 mm diameter) for 3 min. Debris was removed by centrifugation at 700 *g* for 1 min, and the supernatant was collected and centrifuged at 12,000 ×*g* for 5 min. The pellet was washed twice with PBS and stored at −20 °C until DNA extraction. For DNA purification, the pellet was re-suspended in 50 mM EDTA and treated with lysozyme (Sigma Aldrich CO., St. Louis, MO, USA; final concentration of 10 mg/mL) for 45 min at 37 °C. The bacterial genomic DNA was isolated using a Wizard Genomic DNA purification kit (Promega Corp., Madison, WI, USA).

2.7.1. Primers design and PCR amplification of bacterial 16S rDNA

Primers for *Lactobacillus*, *Bifidobacterium*, *Clostridium* and *E. coli* were designed according to previously published data (Zhu, Zhong, Pandya, & Joerger, 2002). To evaluate the relative proportion of each examined bacteria, all products were expressed relative to the content of the universal primer product and proportions of each bacterial group are presented. PCR products were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide, and quantified using the Quantity One 1-D analysis software (Bio-Rad, Hercules, CA, USA).

2.8. Statistical analysis

All values are expressed as means and standard deviation. The bean flours were analyzed in triplicates. Iron content data was subjected to analysis of variance (ANOVA), and the post hoc Tukey test was used to compare the groups. Experimental treatments for the *in ovo* assay were arranged in a completely randomized design. The serum and hepatic iron concentration and the microbial and gene expression results were analyzed by ANOVA. For significant "F-value", post hoc Newman-Keuls test was used to compare test groups. Statistical analysis was carried out using GraphPad Prism version 5.0 software (GraphPad Software, California, CA, USA). The level of significance was established at $p < .05$.

3. Results

3.1. Dietary fiber content in the bean extracts

The extracts from BRS Esteio (black bean) and BRS Cometa (carioca bean) presented the higher dietary soluble fiber content ($p < .05$) compared to the other bean extracts (Fig. 1).

3.2. Isolation and sequencing of partial chicken hepatic hepcidin cDNA

As shown in Fig. 2, a 174-bp fragment of the chicken hepatic hepcidin gene was isolated by reverse transcriptase-PCR and subjected to sequence analysis. It exhibited 14.8% homology to *Gorilla gorilla*, 24% homology to *Pan troglodytes*, 17.5% to *Bos taurus* and 6.2% to *Alligator*

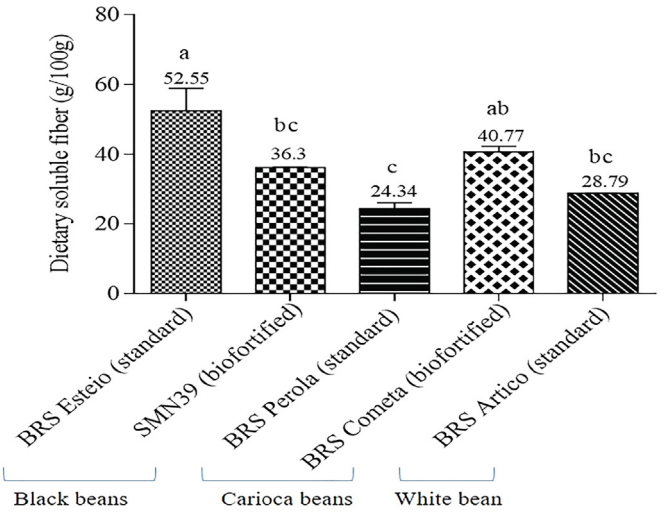

Fig. 1. Dietary soluble fiber content in the bean extracts (g/100 g of the extracts). Values are means ± SEM. Bean extracts not indicated by the same letter are significantly different ($p < .05$) by Tukey test.

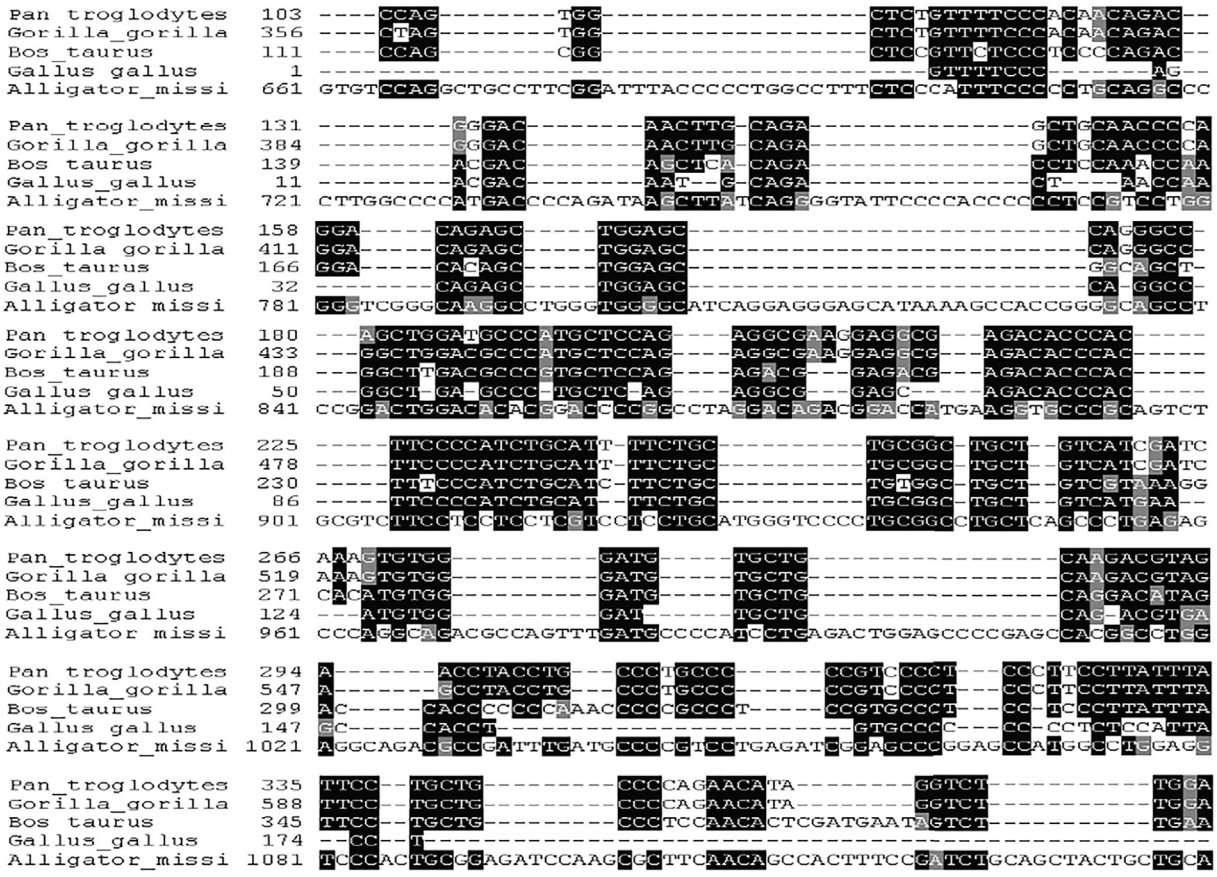

Fig. 2. Predicted partial amino acid sequences of the chicken hepatic hepcidin. The alignment of predicted amino acid sequences of chicken hepatic hepcidin with chimpanzee hepcidin (NM001109693.1), gorilla hepcidin (XM_004060516.2), Cow hepcidin (NM_001114508.2), alligator (XM_014600736.2) is shown. Homologous residues are shaded.

miss hepatic hepcidin genes. The cDNA sequence of the hepatic hepcidin was entered into the BioSample sequence database under accession number SAMN0805649.

3.3. Body weight and cecum-to-bodyweight

There was no significant difference in body weight between treatment (data not shown) ($p > .05$). The cecum-to-body weight ratios were ($p < .05$) higher in the treatment groups and inulin group compared to the negative control (18MΩ H_2O) (Fig. 3). The cecum of animals that received bean soluble extracts increased, suggesting an increase in their content of bacterial populations (Fig. 4).

3.4. Effect of bean extracts on the abundance of intestinal bacterial populations

Inulin group was used as a positive control and as was previously demonstrated (Tako et al., 2008), this group presented the higher ($p < .05$) relative abundance of all bacteria genera compared to the negative (non-injected and 18MΩ H_2O) and test groups (Fig. 2). The relative abundance of *Bifidobacterium* in the BRS Perola group was higher ($p < .05$) compared to the other tested groups. All groups presented lower ($p < .05$) relative abundance of *E. coli* and *Clostridium* compared to the Inulin group. In general, tested groups reduced ($p < .05$) the relative abundance of *Clostridium* and *E. coli* compared to the Inulin group and they did not affect the relative abundance of *Bifidobacterium* and *Lactobacillus* compared to the negative controls (non-injected and 18MΩ H_2O) (Fig. 4).

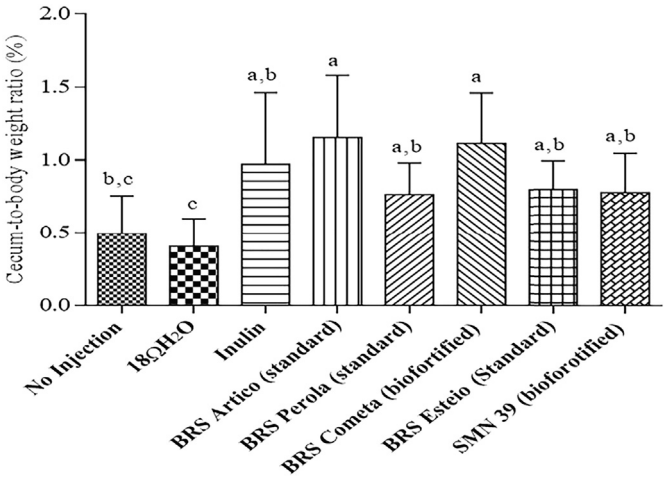

Fig. 3. Cecum-to-body weight ratio (%). Values are means ± SEM, $n = 12$. Treatment groups not indicated by the same letter are significantly different ($p < .05$) by Newman-Keuls test.

3.5. Effect of bean extracts on iron status

3.5.1. Iron concentration in the soluble extract, liver and blood samples

The soluble extract from BRS Artico presented higher ($p < .05$) Fe concentration compared to the other bean extracts (Fig. 5A). However, the higher iron concentration in the extracts did not affect ($p > .05$) the serum Fe (Fig. 5C) and the Fe storage in the liver (Fig. 5B).

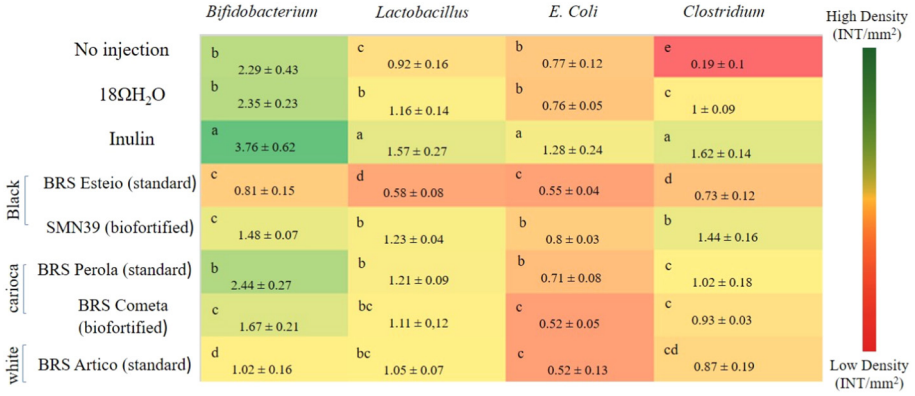

Fig. 4. Genera and species-level bacterial populations (AU) from cecal contents measured on the day of hatch. Values are means ± SEM, $n = 6$. [a–c] Per bacterial category, treatment groups not indicated by the same letter are significantly different ($p < .05$) by Newman-Keuls test.

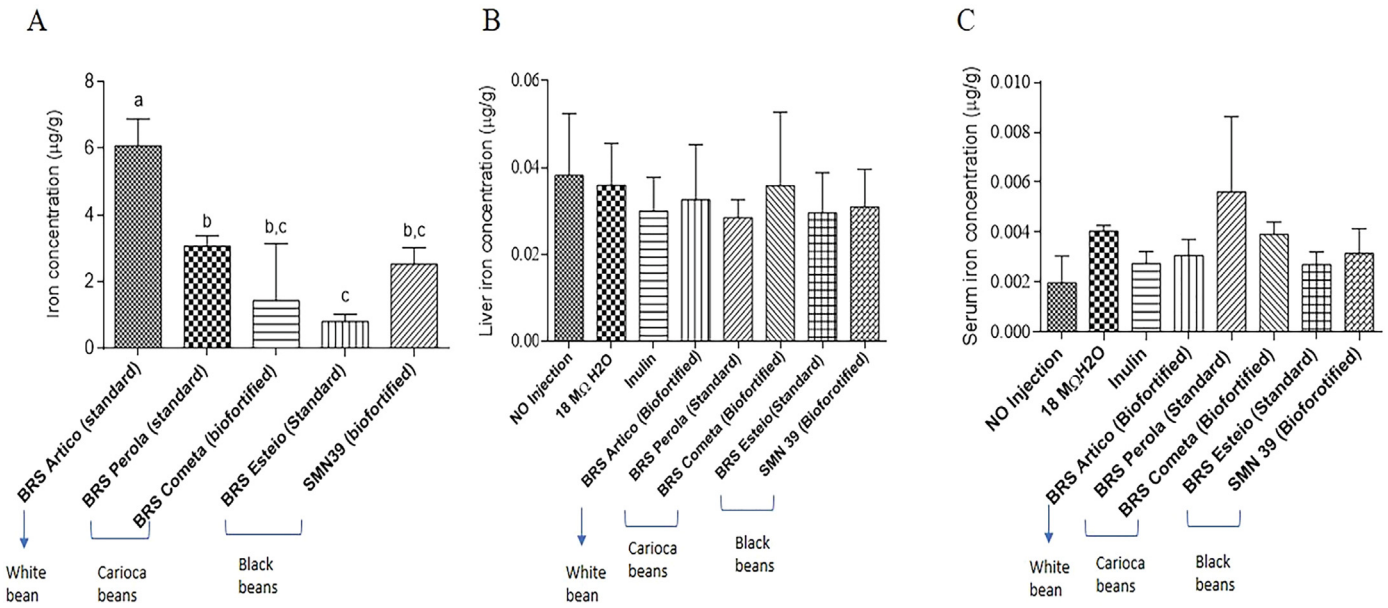

Fig. 5. (A): Iron content in the bean prebiotics extracts; (B) Liver iron concentration (n = 6); (C) Serum iron concentration ($n = 4$). Values are means ± SEM. Different letters indicate statistical differences at 5% by Newman-Keuls test.

3.5.2. Gene expression of BBM proteins

Fig. 6 shows the gene expression of proteins involved in the Fe metabolism and BBM functionality. There was no difference ($p > .05$) in the gene expression of DMT-1, SI and SGLT-1 between the groups. The relative expression of ZnT1, FNP, Dcytb and AP were up-regulated ($p < .05$) in the presence of soluble extracts from BRS Cometa (carioca bean). Moreover, the relative expression of ZnT1 was down-regulated ($p < .05$) by SMN39 (black bean) and BRS Artico (white bean). The heat map (Fig. 6) shows that soluble extracts from BRS Cometa (carioca beans) up-regulated the gene expression of almost all proteins (except hepcidin), even if for some of them (DMT1, SI and SGLT1) the increasing was not statistically significant.

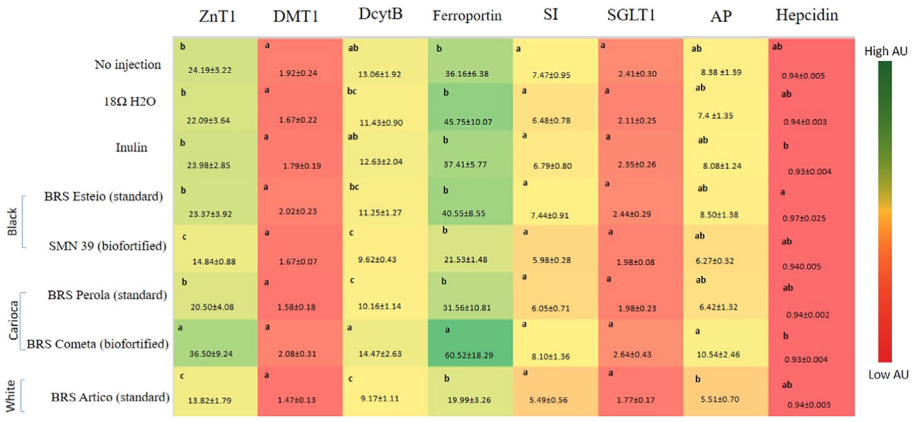

Fig. 6. Effect of intra-amniotic administration of experimental solutions on the intestinal gene expression. Values are means ± SEM, $n = 5$. [a–c] Per gene, treatment groups not indicated by the same letter are significantly different ($p < .05$) by Newman-Keuls test. ZnT-1: Zinc transporter protein-1; DMT-1, Divalent Metal Transporter–1; DcytB, Duodenal cytochrome *b*; SI, Sucrose isomaltase; SGLT-1: Sodium-Glucose transport protein 1; AP: Amino peptidase.

It was also evaluated the expression of hepcidin in the animal liver. Hepcidin is the iron regulatory hormone that controls iron absorption and distribution (Pasricha et al., 2014), by binding to FPN which causes the endocytosis of FPN and diminishes iron export to the plasma from all of its major sources, trapping iron in duodenal enterocytes (Ganz & Nemeth, 2015). The expression of hepcidin was higher ($p < .05$) in the BRS Esteio group and lower ($p < .05$) in the BRS Cometa group and Inulin group.

4. Discussion

In this study, the intra-amniotic administration procedure was used to assess the potential effects of soluble extracts with putative prebiotic ability derived from black, carioca and white beans varieties (standard vs. Fe biofortified), on the intestinal bacterial populations and the expression of BBM Fe related and tissue functionality proteins. This procedure has been shown to be useful in investigating the effects of specific nutrients at particular stages of intestinal development (Hou & Tako, 2018; Tako, Ferket, & Uni, 2004; Tako & Glahn, 2012). It is also used to demonstrate the potential effect of plant origin prebiotics on iron bioavailability and gut functionality (Pacifici et al., 2017; Tako & Glahn, 2012; Tako, Glahn, et al., 2014).

It was previously demonstrated that beans (as other legumes) contain prebiotic compounds, hence these compounds may affect the intestinal bacterial populations composition and function (Feregrino-Pérez et al., 2008; Hou et al., 2017; Johnson et al., 2013; Laparra, Glahn, & Miller, 2009). In this study, the soluble extracts from biofortified BRS Cometa (carioca) presented higher soluble fiber content ($p < .05$) compared to the standard BRS Perola (carioca) (Fig. 1). However, both lines, biofortifed BRS Cometa and standard BRS Perola, presented similar ($p > .05$) relative abundance of *Lactobacillus*. Also, the relative abundance of these bacterial populations was higher ($p < .05$) in the biofortified black beans SMN39 group compared to its corresponding standard BRS Esteio (Fig. 4), even though the soluble fiber content in the soluble extracts from BRS Esteio was higher ($p < .05$) than in the SMN39 (Fig. 1). These results can be due to the different type of soluble fiber presented in the bean extracts. We did not identify the different types of soluble fiber present in the extracts, but our findings suggest that the type of fiber may be more relevant to the gut microbiota composition than the total soluble fiber amount.

In general, tested groups reduced ($p < .05$) the relative abundance of *Clostridium* and *E. coli* compared to the Inulin group and they did not affect significantly the relative abundance of *Bifidobacterium* and *Lactobacillus* compared to the negative controls. This result may be due to the presence of non-digestible oligosaccharides present in beans, such as raffinose and stachyose, which can be metabolized by the beneficial gut bacteria increasing their abundance in the intestine and reducing the pathogenic bacteria (Hou & Tako, 2018; Pacifici et al., 2017).

All these results indicate a potential beneficial effect of soluble extracts from beans on gut health. Lactobacilli and bifidobacteria are known as probiotics, whereas *Clostridium* is a potentially pathogenic genera and *E. coli* can be either pathogenic or beneficial, depending on the strain (Tako et al., 2008); Gibson, Beatty, Wang, & Cummings, 1995; Roberfroid, Van Loo, & Gibson, 1998). Also, bifidobacteria and lactobacilli produce short chain fatty acids (SCFA) (Gibson et al., 1995; Wong et al., 2006), which reduce the intestinal pH, improving the absorption of minerals such as iron, potentially increasing Fe solubility, and there increase Fe bioavailability (Welch & Graham, 2004; Yeung, Glahn, Welch, & Miller, 2005).

Therefore, the use of biofortified beans instead of iron fortification or Fe supplementation can be an effective and potentially sustainable strategy to reduce the iron deficiency, since they can also improve the gut microbiota. It has been observed that iron fortification and supplementation can increase the abundance of enterobacteria, such as *E. coli* and reduce the lactobacilli genera (Lee et al., 2008; Zimmermann et al., 2010). Most enteric gram-negative pathogens, including *E. coli* (Naikare, Palyada, Panciera, Marlow, & Stintzi, 2006), take up iron siderophore complexes via specific outer membrane receptors. In vitro, enteric bacteria display increased virulence in situations of increased Fe availability (Bullen, Griffiths, Rogers, & Ward, 2000). Thus, it is possible that more soluble forms of iron, such as ferrous sulfate, could have a greater effect on enteropathogen growth (Zimmermann et al., 2010).

Moreover, the cecum-body weight ratio (Fig. 3) in all treatment groups was higher ($p < .05$) than the non-injected and 18MΩ H2O groups. This indicated that the cecal content in treatment groups that received the intra-amniotic soluble extracts was greater than those that did not. This observation supported the hypothesis that the cecum-body weight ratio could be used as an indicator for a potential increase in cecal bacterial populations and activity (Pacifici et al., 2017).

The *Gallus gallus* model is a fast growing animal with relatively high mineral requirements, and hence can develop deficiency considerably quickly (Tako, Rutzke, & Glahn, 2010). Previous studies have shown that intra-amniotic administration is a useful approach for investigating the effects of specific nutrients at particular stages of intestinal development (Hou et al., 2017; Pacifici et al., 2017; Tako, Ferket, & Uni, 2005; Tako & Glahn, 2012). Hence, this study also investigated the effect of the intra-amniotic administration of soluble content extracted from biofortified and standard beans on the iron status of chickens. First, it was observed that extracts from BRS Perola and BRS Cometa (carioca beans) and BRS Artico (white) presented the higher Fe concentration ($p < .05$) (Fig. 5A), however, this did not affect ($p > .05$) the Fe storage in the liver nor iron serum concentrations (Fig. 5B and C). Although, the soluble extracts form beans had positively affected on the microbial populations, it did not affect the hatchlings Fe status. However, this may be due to a short exposure time, as in a long-term feeding trial, the BRS Cometa promoted affected the intestinal bacterial populations composition and function, which have led to an improvement in Fe status in vivo (Dias et al., 2018).

The soluble extracts from BRS Cometa promoted an up-regulation ($p < .05$) of the gene expression of ZnT1 and AP compared to other groups, whereas the soluble extract from BRS Artico promoted a down-regulation ($p < .05$) of these proteins (Fig. 6). This result indicates that intra-amniotic administration of soluble extracts of carioca bean improved BBM functionality. Thus, it suggests that dietary prebiotics might lead to enterocyte proliferation and BBM functionality (Hou et al., 2017).

Moreover, the BRS Cometa group up-regulated the FPN and DcytB expressions. DcytB reduces the Fe ions to Fe^{2+}, which is then transported into the enterocyte via DMT-1, whereas FPN exports Fe from the enterocyte into portal blood (Knutson, 2017; Ludwiczek, Theurl, Artner-Dworzak, Chorney, & Weiss, 2004). Thus, since the BRS Cometa group presented an increased expression of DcytB and FPN, more Fe can potentially be transported by DMT-1 into the enterocyte, and then released from the to the blood circulation by FPN. Similarly, a long-term feed trial using the *Gallus gallus* model and the same bean varieties (biofortified BRS Cometa vs standard BRS Perola) demonstrated an up-regulation of the FPN, which leaded to an increasing in the total body Hb-Fe (sensitive biomarker of dietary Fe bioavailability and status) in the biofortified line BRS Cometa (Dias et al., 2018).

Since FPN expression is directly affected by hepcidin concentration (Lopez, Cacoub, Macdougall, & Peyrin-Biroulet, 2016; Pasricha et al., 2014; Sangokoya, Doss, & Chi, 2013), we also investigated the hepatic hepcidin expression. The hepcidin expression was higher ($p < .05$) in the BRS Esteio group and lower ($p < .05$) in the BRS Cometa and BRS Supremo groups (Fig. 6). However, this difference did not affect FPN expression and serum iron concentration (Fig. 5C). Some studies have shown lower hepcidin concentration in Fe- deficient children (Pasricha et al., 2014) and pregnant woman (Bah et al., 2017), and it appears to be a useful diagnostic marker for Fe deficiency (Bah et al., 2017; Wegmüller et al., 2016).

5. Conclusion

Overall, our data suggests that the intra-amniotic administration of soluble extracts with prebiotic ability extracted from carioca beans may improve the intestinal luminal Fe solubility and therefore bioavailability, by limiting the abundance of potentially pathogenic bacterial populations (*Clostridium* and *E. coli*) and increase the activity of *Lactobacillus* and *Bifidobacterium*.

In addition, the soluble extracts from BRS Cometa up-regulated the gene expression of Znt1, AP, FPN and DcytB, which can also contribute to the Fe (and zinc) BBM transport. Thus, the results presented here suggest that carioca beans could be an effective vehicle for mineral biofortification, since they might improve the gut microbial populations, and therefore, potentially increase iron bioavailability.

Conflicts of interest

The authors declare no conflict of interest.

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