DESIRRÊ MORAIS DIAS

IN VITRO AND *IN VIVO* IRON BIOAVAILABILITY OF BIOFORTIFIED BEANS (*Phaseolus vulgaris* L.) AND ITS EFFECT ON INTESTINAL FUNCTION AND MICROBIOTA

Thesis submitted to the Universidade Federal de Viçosa, as part of the requirements of the Nutrition Science Graduate Program, to obtain the title of *Doctor Scientiae*.

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“*The fear of the Lord is the beginning of knowledge...*”

Proverbs 1:7

I dedicate this thesis to my beloved parents Nilza and Luiz Carlos, to my sisters Bianka and Franciely and to my grandparents, with much love and affection.

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BIOGRAPHY

Desirrê Morais Dias, daughter of Luiz Carlos Dias and Nilza da Silva Morais was born on June 10, 1990, in Cachoeiro de Itapemirim, Minas Gerais.

In March 2008, she joined the Nutrition Course of the Federal University of Espírito Santo (UFES), graduating Nutritionist in August 2012.

In March 2013, she began in the Graduate Program in Nutrition Science (UFV) in Master level, submitting to the defense of the dissertation in February 2015.

In March 2015, she began in the Graduate Program in Nutrition Science (UFV) in Doctorate level, submitting to the defense of the dissertation in February 2019.

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LIST OF ABBREVIATIONS

AOAC	Oficial Methods of Analysis
AP	Amino peptidase
BBM	Brush border membrane
BC	Fe-biofortified carioca bean based diet
cDNA	DNA complementary
Dcytb	Duodenal cytochrome B
DMT-1	Divalet metal transporter protein -1
DNAse	Deoxyribonuclease
EDTA	Acid etilen diamino tetraacetic acid
FAO	Food and Agriculture Organization
Fe	Iron
FPN	Ferroportin
Hb	Hemoglobin
HCP1	Haem Carrier Protein 1
HRE	Hemoglobin maintenance efficiency
IBGE	Instituto Brasileiro de Geografia e Estatística
mRNA	Messenger RNA
SC	Fe-standard carioca bean based diet
SCFA.	Short Chain Fat Acid
SI	Sucrose isomaltase
UPLC-M	Ultra performance liquid chromatography- tandem mass spectrometer
WHO	World Health Organization
ZnT-1	Zinc transporter protein-1

ABSTRACT

DIAS, Desirrê Morais, D.Sc., Universidade Federal de Viçosa, February, 2019**. *In vitro* and *in vivo* iron bioavailability of biofortified beans (*Phaseolus vulgaris* L.) and its effect on intestinal function and microbiota**. Adviser: Hércia Stampini Duarte Martino. Co-Advisers: Maria Eliza de Castro Moreira and Ceres Mattos Della Lucia.

Iron (Fe) deficiency is the most prevalent nutrient deficiency, affecting around 40% of the world population, particularly women and children in developing countries. Biofortified staple food crops have become an effective tool to reduce Fe deficiency. Common bean (*Phaseolus vulgaris*) is one of the crops target for biofortification program since it exhibits sufficient genetic variability in iron concentration. Thus, this study aimed to evaluate the *in vitro* and *in vivo* iron bioavailability of Fe-biofortified beans compared to the Fe-standard beans and its effect on the gut microbiota and intestinal function. For this study it was used different genotypes of Fe-biofortified beans and Fe-standard beans. The beans were cooked in a conventional pressure cooker for 40 minutes using distilled water, the beans were dried in an air oven for 17 hours at 60ºC, ground by stainless steel mill 090 CFT at 3000 rpm and stored at -12 °C. Firstly, it was evaluated the effect of the prebiotics extracted from the tested beans 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 (**Manuscript 2**). Eight treatment groups [(non-injected; 18MΩ H_2O; 40mg/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. The gene expression of the iron related proteins was performed by RT- qPCR and the abundance of bacteria was evaluated by PCR. Carioca bean groups presented an increased ($p<0.05$) relative abundance of bifidobacteria and lactobacilli in comparison to controls. The relative expression of zinc transprter 1 (ZnT1), ferroportin (FNP) and amino peptidase (AP) were up-regulated ($p<0/05$) in the BRS Cometa group (Fe-biofortified carioca beans). The second study (**Manuscript 3**), aimed to evaluate the *in vitro* iron bioavailability of different genotypes Fe-biofortified beans and Fe-standard beans by themselves and in a combination with others staple food crops. The bean genotypes used in this study were: BRS Perola; BRS Cometa; BRS Esteio and SMN39. The food proportions used were based on the Brazilian consumption. The polyphenol analysis was performed by UPLC-MS, the Fe concentration was evaluated by ICP-AES and the phytate concentration was evaluated using the Magazyme kit assay. *In vitro* iron bioavailability was evaluated using Caco-2 cells. Fe-biofortified beans (BRS Cometa ans SMN39) presented higher ($p<0.05$) iron concentration compared to Fe-standard beans (BRS Perola and BRS Esteio). Black beans (BRS Esteio and SMN39) showed higher ($p<0.05$) concentration of myricetin 3-glucoside and quercetin 3-glucoside compared to the carioca beans, whereas carioca beans (BRS Cometa and BRS Perola) presented higher ($p<0.05$) concentration of kaempferol 3-glucoside, epicatechin and catechin compared to the black beans. Also, both carioca beans presented higher ($p<0.05$) iron bioavailability compared to the both black beans and the combination of the beans and other staple food increased ($p< 0.05$) the iron bioavailability of both, carioca and black beans, except for the Fe-standard bean BRS Esteio. For the third study (**Manuscript 4**), it was performed a 6-week feeding trial in *Gallus gallus* ($n=15$), aimed to investigate the Fe status and the alterations in the gut microbiome following the administration of Fe-biofortified carioca bean based diet (BC) versus a Fe-standard carioca bean based diet (SC). The tested diets were designed based on the Brazilian food consumption survey. Two primary outcomes were observed: 1) a significant increase in total body Hb-Fe values in the group receiving the Fe-biofortified carioca bean based diet; and 2) changes in the gut microbiome composition and function were observed, specifically, significant changes in phylogenetic diversity between treatment groups, as there was increased abundance of bacteria linked to phenolic catabolism, and increased abundance of beneficial SCFA-producing bacteria. The BC group also presented a higher intestinal villi height compared to the SC group. Overall, our results demonstrate that Fe-biofortified beans (black and carioca) presented a higher iron concentration compared to the Fe-standard beans and the black beans presented higher content of polyphenols that can inhibit the iron absorption. Thus, carioca beans presented a higher iron bioavailability compared to the black beans (*in vitro*) and the combination of the beans with other staple foods increased the *in vitro* iron bioavailability. Our *in vivo* (*Gallus gallus*) results demonstrated that the Fe-biofortified carioca bean variety was able to moderately improve Fe status and to positively affect the intestinal functionality and bacterial populations. Overall, the carioca beans BRS Cometa presented the best results both *in vitro* and *in vivo*, improveing Fe status and to positively affecting the intestinal functionality and bacterial populations. Thus, it can be considered a promissor vehicle for the Fe biofortification.

RESUMO

DIAS, Desirrê Morais, D.Sc., Universidade Federal de Viçosa, fevereiro de 2019. **Biodisponibilidade *in vitro* e *in vivo* de ferro de feijões (*Phaseolus vulgaris* L.) biofortificados e seus efeitos na funcionalidade e na microbiota intestinal**. Orientadora: Hércia Stampini Duarte Martino. Coorientadoras: Maria Eliza de Castro Moreira e Ceres Mattos Della Lucia.

A deficiência de ferro (Fe) é a deficiência de nutrientes mais prevalente, afetando cerca de 40% da população mundial, particularmente mulheres e crianças em países em desenvolvimento. Culturas de alimentos básicos biofortificados tem se tornado uma ferramenta eficaz para reduzir a deficiência de Fe. O feijão comum (*Phaseolus vulgaris* L.) é uma das culturas alvo para o programa de biofortificação, uma vez que exibe variabilidade genética suficiente na concentração de ferro para o processo de biofortificação. Esta tese teve como objetivo avaliar a biodisponibilidade de ferro *in vitro* e *in vivo* de feijões biofortificados com Fe em comparação com feijões convencionais e seu efeito sobre a microbiota intestinal e a função intestinal *in vivo*. Foram utilizados diferentes genótipos de feijões biofortificados com Fe e feijões convencionais. Os feijões foram cozidos em panela de pressão convencional por 40 minutos utilizando água deionizada, secos em estufa de ar por 17 horas a 60ºC, e moídos em moinho de inox 090 CFT a 3000 rpm e armazenados a -12 °C. Inicialmente, avaliou-se o efeito da administração intra-amniótica de prebióticos extraídos dos feijões sobre a expressão gênica de proteínas relacionadas ao metabolismo de ferro e da membrana da borda em escova (BBM), e sobre a população bacteriana intestinal em modelo de aves (*Gallus gallus*) (**Manuscrito 2**). Foram testados oito grupos experimentais [(controle negativo não-injetado; 18MΩ $H2O$; 40mg/mL Inulina; 50 mg/mL BRS Pérola (convencional carioca); 50 mg/mL BRS Cometa (biofortificado carioca); 50 mg/mL BRS Esteio (convencional preto), 50 mg/mL de SMN 39 (biofortificado preto), 50 mg/mL de BRS Artico (convencional branco). A expressão gênica das proteínas relacionadas ao ferro foi realizada por RT-qPCR e a abundância das bactérias foi avaliada por PCR. Os grupos de feijão Carioca apresentaram aumento ($p<0,05$) da abundância relativa de bifidobactérias e lactobacilos em relação aos convencionais. A expressão gênica da proteína transportadora de zinco 1 (ZnT1), ferroportina (FNP) e amino peptidase (AP) apresentou-se aumentada ($p<0,05$) no grupo BRS Cometa (biofortificado carioca). O segundo estudo (**Manuscrito 3**) objetivou avaliar a biodisponibilidade de Fe *in vitro* de diferentes genótipos de feijão biofortificados com Fe e feijões convencionais, sozinhos e em combinação com outras culturas alimentares básicas. Os genótipos de feijão utilizados neste estudo foram: BRS Pérola, BRS Cometa, BRS Esteio e SMN39. As proporções alimentares utilizadas foram baseadas no consumo alimentar da população brasileira. A análise do polifenol foi realizada por UPLC-MS, a concentração de ferro foi analisada por ICP-AES e a concentração de fitato foi avaliada utilizando kit Magazyme. A biodisponibilidade de Fe *in vitro* foi avaliada usando células Caco-2. Os feijões biofortificados (BRS Cometa e SMN39) apresentaram maior (p<0,05) concentração de ferro em relação aos convencionais (BRS Pérola e BRS Esteio). Os feijões pretos (BRS Esteio e SMN39) apresentaram maior (p<0,05) concentração de miricetina 3-glicosídeo e quercetina 3-glicosídeo em relação aos feijões cariocas, enquanto feijão carioca (BRS Cometa e BRS Pérola) apresentou maior (p<0,05) concentração de 3-glucoside kaempferol, epicatequina e catequina em comparação com os feijões pretos. Além disso, ambos os feijões cariocas apresentaram maior biodisponibilidade de ferro (p <0,05) em relação aos feijões pretos e a combinação de feijão com outros alimentos básicos aumentou (p<0,05) a biodisponibilidade de ferro dos feijões carioca e preto, com exceção do feijão preto convencional BRS Esteio. No terceiro estudo (**Manuscrito 4**), foi realizado um experimento de 6 semanas em modelo de aves (*Gallus gallus*) (n = 15), com o objetivo de investigar o estado nutricional de Fe e as alterações no microbioma intestinal após a ingestão de feijão carioca biofortificado com Fe (BC) em comparação ao feijão carioca convencional (SC). As dietas testadas foram elaboradas com base no consumo alimentar da população brasileira. Dois resultados primários foram observados: 1) um aumento significativo nos valores de Hb-Fe total no organismo do grupo que recebeu a dieta à base de feijão carioca biofortificado; e 2) alterações na composição e função do microbioma intestinal, especificamente, mudanças significativas na diversidade filogenética entre os grupos de tratamento, pois houve aumento da abundância de bactérias relacionadas ao catabolismo de compostos fenólicos e aumento da abundância de bactérias benéficas produtoras de ácidos graxos de cadeia curta. O grupo BC também apresentou maior altura de vilosidades intestinais em relação ao grupo SC. De maneira geral, nossos resultados demonstram que os feijões biofortificados (preto e carioca) apresentaram maior concentração de ferro em relação ao feijão convencional e o feijão preto apresentou maior teor de polifenóis inibidores da absorção de ferro. Assim, o feijão carioca apresentou maior biodisponibilidade de ferro em relação ao feijão preto (*in vitro*) e a combinação do feijão com outros alimentos básicos aumentou a biodisponibilidade de ferro *in vitro*. Os resultados *in vivo* (Gallus gallus) demonstraram que a variedade feijão carioca biofortificado foi capaz de melhorar moderadamente o Fe e afetar positivamente a função intestinal e a composição da microbiota intestinal. De modo geral, o feijão BRS Cometa (carioca) apresentou os melhores resultados *in vitro* e *in vivo*, sendo capaz de melhorar o estado nutricional de ferro e afetando positivamente a funcionalidade intestinal e a população bacteriana. Assim, este feijão carioca biofortificado pode ser considerado um veículo promissor para a biofortificação com ferro.

1. GENERAL INTRODUCTION

Micronutrients deficiency affects approximately two billion people worldwide. Iron (Fe) deficiency is the most prevalent nutrient deficiency, affecting around 40% of the world population, particularly women and children in developing countries (WEGMÜLLER et al., 2016; WORLD HEALTH ORGANIZATION, 2011). It is estimated that around 46% of the population in Africa, 57% in South-East Asia and 19% in Latin America are anemic (DIAS et al., 2018; REED et al., 2017). Fe deficiency is highly prevalent in low-income countries (~30% in Brazil) due to a lack of meat consumption in addition to a notable dietary reliance on grains containing high amounts of Fe absorption inhibitors (e.g., phytic acid, polyphenolic compounds) (BOUIS; SALTZMAN, 2017; NESTEL et al., 2006; TAKO et al., 2015a; WHO, 2011). Major pathophysiological complications related to insufficient Fe intake may include stunted growth, impaired physical and cognitive development, and increased risk of morbidity and mortality in children (BHARGAVA; BOUIS; SCRIMSHAW, 2006; TAKO et al., 2015a). To alleviate Fe deficiency, an integral step involves the understanding of specific dietary patterns and components that contribute to Fe status in the particular population suffering from a deficiency.

Biofortified staple food crops have become an effective tool to address micronutrient deficiencies, especially that of Fe, in many at-risk populations (BOUIS; SALTZMAN, 2017; BROUGHTON et al., 2003; GLAHN et al., 2017; TAKO et al., 2015a). The common bean (*Phaseolus vulgaris*) is one of the crops target for biofortification program since it exhibits sufficient genetic variability in iron concentration, which is the basic requirement for biofortification (HARVESTPLUS, 2014; WHITE; BROADLEY, 2005). This crop is currently estimated to be one of the most important legume worldwide (PETRY et al., 2015), and is an important source of nutrients for more than 300 million people in parts of Eastern Africa and Latin America, representing 65% of total protein consumed, 32% of energy, and a major source of micronutrients (vitamins and minerals) (BLAIR et al., 2010; BROUGHTON et al., 2003; PETRY et al., 2015). For example, with a production of approximately 2.61 million tons per year (FAO, 2015), 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).

Previous studies using Fe biofortified beans (MOURA et al., 2014; HAAS et al., 2014; PETRY et al., 2014) have shown some improvement in Fe status in subjects consuming the biofortified beans versus a standard bean variety. However, a major challenge associated with biofortification of staple food crops, especially common beans, is that they contain factors such as polyphenols and phytic acid that can inhibit Fe bioavailability and absorption, hence limiting their nutritional benefit (PETRY et al., 2014; TAKO et al., 2014a). These inhibitory factors may increase with Fe concentration when these crops are biofortified via conventional breeding (HART; TAKO; GLAHN, 2017; PETRY et al., 2014; TAKO et al., 2014a). Therefore, it is necessary to measure the concentration of Fe, the amount of bioavailable Fe, and the concentration of potential inhibitors of Fe bioavailability in these biofortified crops (TAKO, REED, BUDIMAN, 2015; HART et al., 2015; TAKO et al., 2014a). It is also important to assess the other components of the diet in which these crops are consumed as the potential interactions can negate or even enhance the expected benefit of increased Fe content.

Despite containing inhibitory factors, legumes also carry other substances, referred to as promoters, which have the potential to counteract the effects of the inhibitory factors (HART; TAKO; GLAHN, 2017; PETRY et al., 2010; TAKO et al., 2014a; TAKO; GLAHN, 2010). One of the most notable promoters are prebiotic (TAKO et al., 2014a, 2014b). Prebiotics have been characterized as a group of carbohydrates that resist digestion and absorption in gastrointestinal tract (small intestine), such as raffinose and stachyose present in legumes, that beneficially affect gut health, by enhancing the growth and activities of probiotics (PACIFICI et al., 2017; TUOHY et al., 2005; WONG et al., 2006) and can improve mineral absorption (WELCH; GRAHAM, 2004). These compounds can survive the acidic and enzymatic digestion in the small intestine, and be fermented by probiotics that reside in the colon/cecum (DWIVEDI et al., 2014). The fermentation of prebiotics by probiotic bacteria leads to the production of short-chain fatty acids (SCFA), which may improve the intestinal function, increasing the absorption of minerals such as Fe (CHEN et al., 2017; TAKO et al., 2008, 2014b; ZIMMERMANN et al., 2010).

Biofortified crops have become an effective tool by which to address micronutrient deficiencies, especially that of Fe, in many at-risk populations (BLAIR, 2013; BLAIR et al., 2010; BOUIS et al., 2014). The combination of a Caco-2 cell bioassay and an *in vivo* (*Gallus gallus*) model that has been used extensively for nutritional research and shown to be an excellent model to assess dietary Fe bioavailability and the effect of the prebiotics present in the beans on the gut microbiota population (HOU et al., 2017; TAKO et al., 2013, 2015a; TAKO; BLAIR; GLAHN, 2011a).

Therefore, the evaluation of iron bioavailability of foods with higher iron content is necessary to the knowledge about the interaction of micronutrients consumed in the same diet, supporting the incentive of the using of these crops as a strategy to improve the nutritional status of populations at risk. In addition, since several varieties of beans (carioca and black) are being produced with higher concentrations of iron, the *in vitr*o evaluation of the iron bioavailability will contribute to the selection of promising varieties of beans that show better results to be further evaluated in the *in vivo* study with the poultry model (Gallus gallus).

Thus, the hypothesis of this study is that the high Fe beans will present higher *in vitro* and *in vivo* iron bioavailability compared to the standard beans and the consumption of these beans will affect the gut microbiota and intestinal function.

2. GENERAL OBJECTIVE

To evaluate the *in vitro* and *in vivo* iron bioavailability of Fe-biofortified beans compared to the Fe-standard beans and its effect on intestinal function and gut microbiota.

2.1 Specific objectives

- ✓ Identify the varieties of beans with higher iron concentration and iron bioavailability;
- ✓ To compare the concentrations of iron, phytate, phenolic compounds and dietary fiber of Fe-biofortified beans and Fe-standard beans;
- ✓ To evaluate the effect of intra-amniotic administration (*Gallus gallus*) of soluble dietary fiber extracted from beans on the intestinal microbiota and intestinal function of the animals;
- ✓ To evaluate the *in vitro* iron bioavailability of Fe-biofortified beans and Fe-standard beans, by themselves and in combination with staple foods of the Brazilian diet;
- ✓ To evaluate the *in vivo* bioavailability (*Gallus gallus*) Fe-biofortified beans and Fe-standard beans in a based Brazilian diet;

- ✓ To evaluate the effect of modulation of the intestinal bacteria population caused by the ingestion of Fe-biofortified beans and Fe-standard beans on the iron bioavailability in *Gallus gallus* model.

3. LITERATURE REVIEW

3.1 Micronutrient deficiency

It is estimated that more than 840 million people in the world do not consume enough food to meet their basic daily energy needs. And a larger number, more than 2 billion people, are affected by one or more micronutrient deficiencies (BAILEY; WEST; BLACK, 2015)

The most prevalent micronutrient deficiencies are iron, zinc, vitamin A, iodine and folate deficiency. Although these nutrients are required in very small amounts, they are essential for the maintenance of immune, cognitive and physical function, as well as for growth and body metabolism (ALLEN et al., 2006). In developing countries, multiple micronutrient deficiencies are frequently present in a single population. These nutritional deficiencies negatively impact the global health and the economic growth (ALLEN; PEERSON; OLNEY, 2009; MUTHAYYA et al., 2013).

In Brazil, the ingestion of some minerals, such as iron, calcium, zinc and selenium, is insufficient or they present a very low bioavailability in the diets (NUTTI; CARVALHO; WATANABE, 2006). The lack of these essential vitamins and minerals results in hidden hunger, which may hide the signs of malnutrition and hunger. Thus, people may have access to sufficient calories but lack adequate micronutrients (KENNEDY et al., 2007).

Micronutrient deficiency causes negative consequences on the human health and productivity, and may affect the development of a country (BURCHI; FANZO; FRISON, 2011). Among the most studied micronutrients, iron, zinc and vitamin A are identified as the ones with the greatest public health problem worldwide (GRAHAM et al., 2007; KENNEDY et al., 2007; NUTTI; CARVALHO; WATANABE, 2006).

3.1.1 Iron deficiency and anemia

Anemia is a public health problem that affect more than 1.6 billion people worldwide, corresponding to 24.8% of the world population (PASRICHA et al., 2013). The pre-school children (0 to 4.99 years) are the most affected group, with a prevalence around 47% (WHO, 2011). Iron deficiency is responsible for about 50% of the cases of anemia. According to WHO, in 2004 iron deficiency anemia (caused by iron deficiency) resulted in 273,000 deaths: 45% in Southeast Asia, 31% in Africa, 9% in the Eastern Mediterranean, 7% in the Americas, 4% in the Western Pacific, and 3% in Europe, about 97% of the deaths occur in low- and middle-income countries (WHO, 2011).

The diagnosis of iron deficiency anemia occurs when the hemoglobin levels are below 13 g/dL for men, 12 g/dL for women and children between 6 and 14 years of age, and 11 g/dL for pregnant women and children between 6 months and 6 years of age (BENOIST et al., 2008). In addition, ferritin can also be used to diagnose the iron deficiency anemia, considering values below 15 ng / mL for adults and 12 ng/mL for children younger than 5 years (30 ng / mL in the presence of inflammation) (WHO, 2011).

The symptoms of iron deficiency include deficiency of neural development in infants and children, impairment in language development, motor coordination and school performance, (BENOIST et al., 2008) abnormalities in the epithelium of the gastrointestinal tract and difficulty in healing. Also, the severe and moderate iron deficiency anemia is associated with increased mortality and susceptibility to infections (SHILS et al., 2003).

3.2 Iron metabolism and homeostasis

Iron is an essential element, which is involved in a large amount of biological reactions that are important to the cellular function. It is an essential component in the formation of the heme molecule, synthesized in all nucleated cells, which participates in the formation of several proteins, such as hemoproteins, which are responsible for the oxygen transport, cellular energy generation and detoxification (TANDARA; SALAMUNIC, 2012).

Iron is a transition metal that exists in two easily reversible redox states: ferrous (Fe^{2+}) and ferric (Fe^{3+}). At normal physiological oxygen concentrations iron form in most of its biological complexes is Fe^{3+}. Since the transmembrane transport of iron can only occur with Fe^{2+} form the reduction reactions of this mineral play a fundamental role in its metabolism (PUNTARULO, 2005; WATT; HILTON; GRAFF, 2010).

The amount of iron in the body of an adult is about 3-5 g (approximately 45 mg/kg in women and 55 mg/kg in men). Most of the iron in the body is incorporated into the hemoglobin (60-70%), ferritin (20 to 30%) and hemosiderin in hepatocytes and macrophages as an iron storage. A small amount of the residual iron is incorporated in the muscle myoglobin or into enzymes. In addition, around 3mg of the body iron is bound to the transferrin, an iron transporter protein. An adult man has about 0.2-0.5 g of stored iron, on the contrary, children, adolescents and women of childbearing age have almost no iron reserves (PAPANIKOLAOU; PANTOPOULOS, 2005; TANDARA; SALAMUNIC, 2012).

3.2.1 Intestinal absorption of iron

Iron absorption occurs in the small intestine and it is mediated by the enterocytes (epithelial cells). Dietary iron can be absorbed as inorganic iron (iron salts or chelates) or as part of the heme group, which is usually released after digestion of hemoglobin and myoglobin from meat (THEIL, 2011)

The intestinal iron uptake occurs in the apical membrane of the enterocyte, and it is mediated by the divalent metal transporter-1 (DMT-1), a nonheme iron transporter which also carries Mn^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+} (MAHLER; SHULER; GLAHN, 2009). However, the DMT-1 transports the ferrous iron (Fe^{2+}), thus the iron must be converted from Fe^{3+} to Fe^{2+}, this process is mediated by the duodenal cytochrome b ferrireductase (Dcytb) (GROTTO, 2010; SCHEERS, 2013).

The absorption of heme iron occurs by a different mechanism, which is not yet well understood. However, apparently a heme transporter protein (HCP-1) is responsible for the iron uptake in the apical membrane of the enterocytes (GROTTO, 2010; SHAYEGHI et al., 2005). Inside the cell, the heme iron is released from the protoporphyrin by heme oxygenase and it will be part of the same pool of nonheme iron, which can be stored as ferritin or released from the enterocyte into the blood (PIETRANGELO, 2004).

Iron is exported from the enterocyte to plasma by ferroportin (FPN) (PIETRANGELO, 2004). The ferroportin is an iron exporter that is located in the basolateral membrane of the cells. The iron in its ferrous form is re-oxidized by a ferroxidase, called hephaestin, to the ferric form, thus it can be transported in the bloodstream to the liver, spleen and bone marrow by the transferrin. Thus, mutations that inactivate FPN or hephaestin may lead to impairment in the absorption and accumulation of iron in the enterocyte and macrophages (GROTTO, 2010). Iron is stored as ferritin and hemosiderin in the liver, spleen and bone marrow. The amount of ferritin in the blood is strongly related to the body iron stores, which makes it an important tool for the clinical evaluation of the nutritional status of iron (GROTTO, 2008, 2010).

3.2.2 Iron homeostasis

Iron homeostasis occurs at two levels, the cellular and systemic level. At the cellular level it is regulated by the amount of iron inside the cell and it is modulated by transcriptional, translational and post-translational modifications. At the systemic level, iron homeostasis is modulated by the action of hepcidin (VASHCHENKO; MACGILLIVRAY, 2013)

Hepcidin is a small peptide hormone produced by the liver, which acts on the mucosal cell, inhibiting the iron uptake by the enterocytes and the iron release from macrophages. Hepcidin binds to ferroportin, resulting the degradation of this protein and consequent inhibit the iron exportation into the bloodstream. The amount of hepcidin produced by the liver is related to the amount of iron stored in the organism. Thus, hepcidin synthesis is induced by an iron overload (FRAZER et al., 2003; NEMETH et al., 2004).

The synthesis of proteins related to the iron metabolism may be affected by the modulation of transcription, mRNA stability, translation and post-translational modifications. These changes are directly related to the amount of intracellular iron. Hence, the regulation of the synthesis of these proteins is responsible for the iron homeostasis (HENTZE; MUCKENTHALER; ANDREWS, 2005). Thus, iron bioavailability studies have used the mRNA expression of these proteins, especially DMT-1, Dcytb and ferroportin, in order to evaluate the iron uptake by the intestine cells (TAKO et al., 2015; TAKO et al., 2013, 2015b).

3.3 Iron bioavailability

Bioavailability is the fraction of the nutrient that is available to use in metabolic processes or for storage (ARGYRI et al., 2011). The iron bioavailability depends on the type of food and the interaction between the iron and other nutrients present in the food. Meat contains heme iron derived from hemoglobin and myoglobin, therefore it presents a high iron bioavailability, whose absorption varies between 10 and 30% of iron ingested. However, plant foods (cereals, legumes and tubers) contain non-heme iron, presenting low bioavailability (SANTOS; AMANCIO; OLIVA, 2007)

There are a large number of compounds that may increase iron uptake by the body, such as citrate, malate and lactate, but the most effective is the ascorbic acid (ARGYRI et al., 2009; HURRELL et al., 1992). On the other hand, some intraluminal factors can negatively affect the amount of iron absorbed, such as oxalic acid, phytic acid and some kind of polyphenols, which can form insoluble chelates or macromolecules that decrease the iron absorption (MOURA; CANNIATTI-BRAZACA, 2006; TAKO et al., 2014a, 2015a). Thus, the increasing in the micronutrient contents in foods is not the only factor that should be considered to guarantee the adequate iron nutritional status.

Since the bioavailable iron in foods is an important factor to guarantee the adequate iron intake, many studies have been performed to evaluate the iron bioavailability from foods and diets. Some of these studies have showed higher iron bioavailability from biofortified beans (DIAS et al., 2015; GLAHN et al., 2017; HAAS et al., 2016; HAAS et al., 2014; TAKO et al., 2009, 2015a; TAKO; BLAIR; GLAHN, 2011b), biofortified pearl millet (TAKO et al., 2015), maize (TAKO et al., 2013) and rice (TRIJATMIKO et al., 2016). On the other hand, some of them showed similar iron bioavailability from biofortified and conventional beans (TAKO et al., 2014a; VAZ-TOSTES et al., 2016) and rice (HAAS et al., 2005).

The *Gallus gallus* model have been widely used for nutritional research and it is considered an excellent animal model to evaluate the iron bioavailability, since they can respond rapidly to the iron deficiency and its deficiency phenotype also includes growth retardation and hypertrophy (TAKO et al., 2015b; TAKO; RUTZKE; GLAHN, 2010a). In addition, this model has shown similar results compared to the *in vitro* model using Caco-2 cells. In fact, the combination of this animal model with the *in vitro* studies (Caco-2 cells) has shown to be an excellent model to assess dietary iron bioavailability (TAKO; BLAIR; GLAHN, 2011a; TAKO; RUTZKE; GLAHN, 2010a).

3.4 Intestinal microbiota and iron nutritional status

The intestinal microbiota is a complex microbial ecosystem, with many different species competing for nutrients. These organisms have a great impact on the nutrition and health of the human host, modifying nutrient supply, metabolism conversion and interactions with host cells (LOUIS et al., 2007). High bacterial density and occupation of ecological niches produce a barrier effect that helps to protect the host from colonization by environmental bacteria (GUARNER, 2006). Molecular approaches based on analysis of 16S rDNA have shown that the intestinal microbiota is composed mainly by Bacteroides phylum, Firmicutes (eg Clostridium, Roseburia, Ruminococcus, or Lactobacillus spp.), Actinobacteria for example, bifidobacteria) and Proteobacteria (enterobacteria) (ECKBURG et al., 2011; WU et al., 2010).

Iron is an essential trace element for most intestinal bacteria and many of them have active iron transport systems. Many members of the Enterobacteriaceae family have developed mechanisms, including siderophores, to acquire iron when there is a competition with other bacteria (ANDREWS; ROBINSON; RODRÍGUEZ-QUIÑONES, 2003). Despite the crucial role of iron for microorganisms, some animal and human studies have shown the effect of nutritional iron status on the intestinal microbiota composition, as well as the influence of the intestinal microbiota on the iron bioavailability (DOSTAL et al., 2012, 2013; JAEGGI et al., 2015; REED et al., 2017; ZIMMERMANN et al., 2010).

Some of these studies have shown an increasing in the amount of Bacteroides spp. and butyrate producing microorganisms, such as members of the Clostridium group, and a reduction of opportunistic pathogen microorganisms, such as Turicibacter spp. and Enterococcus spp. in animals feed with diets containing iron compared to iron-free diets (DOSTAL et al., 2013; WERNER et al., 2011).

In addition, iron deficiency may cause dysbiosis of the gut microbiota and reduction of the butyrate and propionate production (DOSTAL et al., 2012, DOSTAL et al., 2013). In contrast, the production of short chain fatty acids (SCFA) by the intestinal microbiota has been positively related to increased bioavailability of iron, since SCFAs reduce intestinal pH, which promote the iron releasing from protein complexes, thereby increasing iron absorption. SCFAs can also stimulate the proliferation of epithelial cells, increasing the absorption area, hence increasing the iron uptake (PATTERSON et al., 2008).

Human studies have observed that children supplemented with iron presented greater abundance of Enterobacteriaceae (ZIMMERMANN et al., 2010) and Bacteroides spp. (KREBS et al., 2013) and reduction of the number of lactobacilli (KREBS et al., 2013; ZIMMERMANN et al., 2010). On the other hand, it is known that lactobacilli and bifidobacteria are beneficial for humans with iron deficiency, since these microorganisms can reduce the abundance of pathogenic bacteria that use dietary iron by nutrient competition (PATTERSON; LEI; MILLER, 2008).

3.5 Strategies to reduce the micronutrient deficiency

There are many strategies for improving micronutrient intake in the human diet, such as diet diversification, mineral supplementation, post-harvest (industrial) fortification and biofortification of foods. However, the industrial fortification and mineral supplementation depend on a continuous investment, and current levels of iron fortification are frequently inadequate (BOUIS et al., 2014; BOUIS; SALTZMAN, 2017; NESTEL et al., 2006). Thus, biofortification is an alternative to fortification and mineral supplementation (TAKO; BLAIR; GLAHN, 2011).

3.5.1 Biofortification

Biofortification is the process of improving staple crops for micronutrient content as a way to address malnutrition in developing countries. The main goals of mineral biofortification have been to increase the concentration of iron, zinc and vitamin A in certain major cereals and legumes (ALLEN et al., 2006; BLAIR, 2013).

Currently, there are three common approaches for biofortification process: agronomic, conventional, and transgenic biofortification. Agronomic biofortification can provide temporary micronutrient increases through fertilizers, whereas the transgenic approaches can provide a nutrient that does not naturally exist in a crop (for example, provitamin A in rice), or when sufficient amounts of bioavailable micronutrients cannot be effectively bred into the crop. However, many countries lack legal frameworks to allow release and commercialization of these varieties (BOUIS et al., 2014; BOUIS; SALTZMAN, 2017). In addition, biofortification can also be achieved through conventional plant breeding, where parent lines with high micronutrient levels are crossed over several generations to produce plants that have higher nutrient content and agronomic traits. The advantage of this technique is that, once the initial research and development is completed, the benefits of this improvement in nutritional quality will be sustainable with little more future investment (SALTZMAN et al., 2013).

Biofortified staple foods cannot deliver as high a level of micronutrients per day as supplements or industrially fortified foods, but they can increase the daily adequacy of micronutrient intakes among individuals throughout the lifecycle (BOUIS at al., 2014). This strategy is not expected to treat micronutrient deficiencies or eliminate them, in fact no single intervention will solve this problem, but biofortification complements existing interventions to sustainably provide micronutrients to the most vulnerable people in a comparatively inexpensive and cost-effective way (NESTEL et al., 2006; SALTZMAN et al., 2013). Biofortification provides a feasible means of reaching malnourished rural populations who may have limited access to diverse diets, supplements, and commercially fortified foods (SALTZMAN et al., 2013).

Since 2003, HarvestPlus, an alliance of research institutions, has been developing seven products in three micronutrient combinations: cassava, corn and sweet potato enriched with pro-vitamin A; beans and millet enriched with iron and zinc-fortified rice and wheat (ALLEN et al., 2006). In Brazil, Embrapa coordinates the biofortification program, and it has been developed rice, beans, sweet potatoes, cassava, corn, wheat and pumpkin with higher concentration of micronutrients. Foods included in the Harvest-Plus program are already widely produced and consumed in Brazil, which means that farmers and consumers do not have to change their eating habits to benefit from biofortification (NUTTI; CARVALHO; WATANABE, 2006).

Several studies have demonstrated the positive impact of the consumption of biofortified foods, especially iron-biofortified millet (TAKO et al., 2015), beans (GLAHN et al., 2017; HAAS et al., 2016; TAKO et al., 2009, 2015b; TAKO; BLAIR; GLAHN, 2011a) and rice (TRIJATMIKO et al., 2016) on iron nutritional status.

Overall, the biofortification of staple crops with iron has been used as a public health strategy to address nutritional deficiencies of micronutrients, especially in vulnerable populations. However, increasing iron content in food may not mean an increasing of its bioavailability in the diet. In addition, the interaction of these micronutrients consumed in the same diet and the interaction of the intestinal microbiota with the iron nutritional status and absorption are factors that may affect the iron bioavailability. Thus, the evaluation of iron bioavailability of biofortified foods in a based diet could contribute to the knowledge about the interactions between iron and intestinal microbiota, as well as to support the use of these crops as a strategy to improve the nutritional status of populations at nutritional risk.

4. GENERAL METHODOLOGY

The bean flour preparation and the dietary fiber analysis were performed at the Experimental Nutrition laboratory-Departament of Nutrition and Health of the Federal University of Viçosa, MG, Brazil. The analysis of iron, polyphenolic compounds and phytate concentration, and the *in vitro* and *in vivo* iron bioavailability study in the bean flours were performed at the Trace Minerals and Nutrition Unit of the Center for Agriculture and Health, Cornell University, NY, USA.

4.1 Samples characterization and study design

Seven varieties of beans (*Phaseolus vulgaris* L.) target for biofortification with iron: BRS Pontal, BRS Cometa, BRS Esteio, BRS Supremo, SMN 39, SMR 80 and BIO 101 and three conventional beans (black BRS Estilo, carioca BRS Perola and white BRS Artico) were tested. The other dry staple foods (potato, rice, corn and pasta) were purchased at a local market.

The characterization and source of the beans used in this study are showed in the Table 1. Firstly, it was conducted an *in vitro* study, using Caco-2 cell model, to evaluate the iron bioavailability of the bean flours. This *in vitro* assay was used to select the beans with the best results to use in the further studies (the *in vitro* and *in vivo* iron bioavailability and the *in ovo* study). The scheme of the study design is showed in the Figure 1.

Table 1. Characterization and source of the beans

Source	Bean cultivars	Bean classes
Embrapa (Empresa Brasileira de Pesquisa Agropecuária, Goias, Brazil)	BRS Perola	Carioca
	BRS Cometa	Carioca
	BRS Estilo	Carioca
	BRS Pontal	Carioca
	BRS Esteio	Black
	BRS Supremo	Black
	BRS Esteio	Black
	BRS Supremo	Black
	BRS Artico	White
CIAT (International Center for Tropical Agriculture, Cali, Colombia)	SMN 39	Black
	SMR80	Carioca
	BIO 101	Carioca

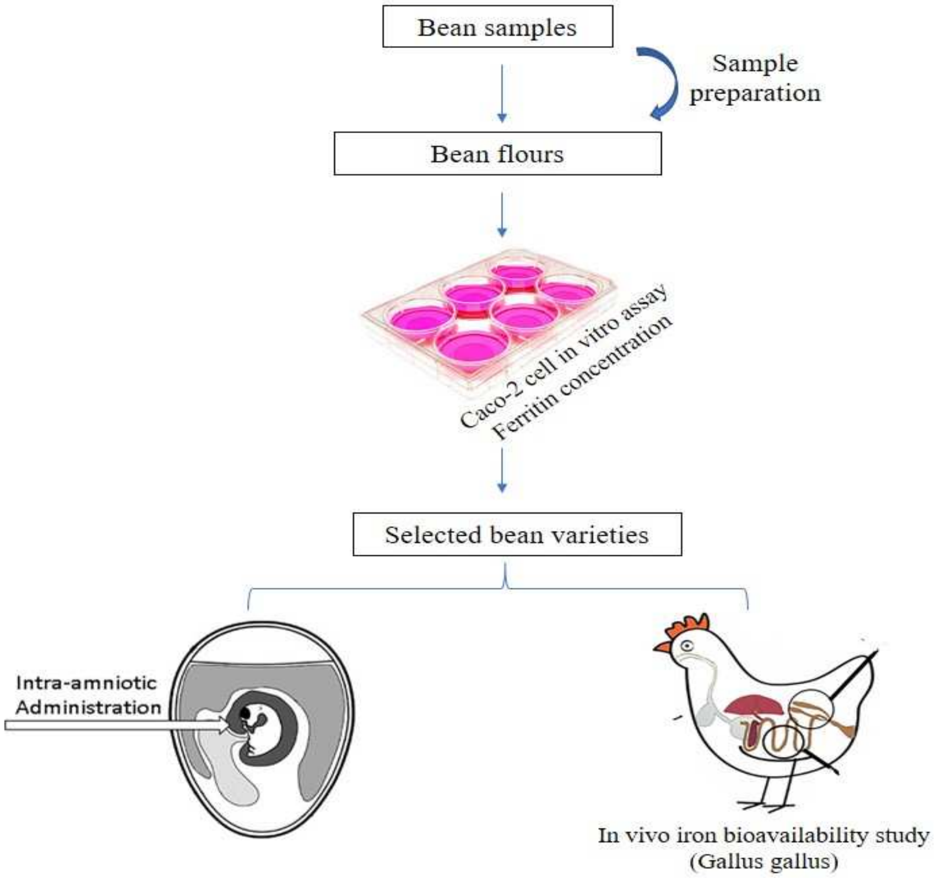

Figure 1. Technical sketch of the study design.

4.2 Sample preparation

The common beans were cooked in three replicates in a conventional pressure cooker for 40 minutes using a bean/distilled water ratio of 1:2.7 (w/v) for the carioca beans and 1:28 (w/v) for black beans. The beans were dried in an air oven for 17 hours at 60ºC. The other dry staple foods (potato, rice, corn and pasta) were purchased at a local market. All dried food were ground by stainless steel mill 090 CFT at 3000 rpm and stored at -12 °C (DIAS et al., 2015).

4.3 Iron content of food flours

All food flour samples (0.5 g) were digested with 3.0 mL nitric: perchloric mixture (60:40 HNO_3: $HClO_4$) into a Pyrex glass tube overnight. The mixture was then heated to 120 °C for two hours and 0.25 mL of 40 µg/g Yttrium (Sigma-Aldrich, St. Louis, MO, USA) 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 ten minutes 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, Thermo iCAP 6500 series (Thermo Jarrell Ash Corp., Franklin, MA, USA).

4.4 *In vitro* iron bioavailability assessment

An established *in vitro* digestion/Caco-2 cell culture model was used to assess Fe-bioavailability (GLAHN et al., 1998; TAKO; BAR; GLAHN, 2016; TAKO; RUTZKE; GLAHN, 2010a). Briefly, the cooked bean samples were subjected to simulated gastric and intestinal digestion. 0.5 g of the bean flours were utilized for each replication (n = 3). The intestinal digestion was carried out in cylindrical inserts closed on the bottom by a semipermeable membrane and placed in wells containing Caco-2 cell monolayers bathed in culture medium. The upper chamber was formed by fitting the bottom of Transwell insert ring (Corning) with a 15000 Da molecular weight cut off (MWCO) membrane (Spectra/Por 2.1. Spectrum Medical. Gardena. CA). The dialysis membrane was held in place using a silicone ring (Web Seal. Rochester. NY). Iron uptake by the Caco-2 cell monolayers was assessed by measuring ferritin concentrations in the cells. Six replicates of each Fe bioavailability measurement were performed. Caco-2 cells were obtained from the American Type Culture Collection (Rockville. MD) at passage 17 and used in experiments at passage 29. Cells were seeded at densities of 50.000 cells/cm^2 in collagen treated 6 well plates (Costar Corp. Cambridge. MA). The integrity of the monolayer was verified by optical microscopy. The cells were cultured at 37°C in an incubator with 5% CO_2 and 95% air atmosphere at constant humidity and the medium was changed every 48 h. The cells were maintained in Dulbecco's modified Eagle medium plus 1% antibiotic/antimycotic solution, 25 mmol/L HEPES, and 10% fetal bovine serum. 48 h prior the experiment the growth medium was removed from culture wells, the cell layer was washed and the growth medium was replaced with minimum essential media (MEM) at pH 7.0. The MEM was supplemented with 10 mmol/L PIPES, 1% antibiotic/antimycotic solution, 4 mg/ L hydrocortisone, 5 mg/L insulin, 5 µg/L selenium, 34 µg/L triiodothyronine, and 20 µg/L epidermal growth factor. This enriched MEM contained less than 80 µg Fe/L. All ingredients and supplements for cell culture media were obtained from GIBCO (Rockville. MD). The cells were used in the Fe uptake experiment at 13 days post seeding. In these conditions the amount of cell protein measured in each well was highly consistent between wells. On experiment day 1.5 mL of the digested sample was added to the insert's upper chamber and incubated for 2 h. Then, inserts were removed and 1 mL of MEM was added. Cell cultures were incubated for 22 h at 37°C. It was previously shown that intracellular ascorbic acid status might influence ferritin formation (i.e. cellular Fe uptake) and Fe related transporters and enzyme expression in Caco-2 cells (GLAHN et al., 1998; TAKO; RUTZKE; GLAHN, 2010b). Medium was removed from the culture well 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 at pH 7.0. The cells were harvested by adding an aliquot of deionized water and placing them in a sonicator (Lab- Line instruments. Melrose Park. IL). The ferritin and total protein concentrations were determined on an aliquot of the harvested cell suspension with a one-stage sandwich immunoradiometric assay (FERIRON II Ferritin assay. Ramco laboratories. Houston. TX) and a colorimetric assay (Bio-Rad DC Protein assay. Bio-Rad. Hercules. CA), 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 an index of the cellular Fe-uptake.

4.5 Selection of the beans

The beans were selected based on the iron concentration and iron bioavailability, which was determinated by the ferritin concentration (Table 2). Although the white bean (BRS Artico) presents low iron concentration, it presented a higher ($p<0.05$) iron bioavailability compared to the other colored beans, which was expect since the white beans presents lower polyphenols compared to the colored beans (HART; TAKO; GLAHN, 2017; TAKO et al., 2014a; TAKO; GLAHN, 2010). Among the colored beans it was selected the follow varieties to use in the further studies: BRS Cometa (carioca and Fe-biofortified bean); BRS Perola (carioca and Fe-standard bean); SMN39 (black and Fe-biofortified bean), BRS Esteio (black and Fe-standard bean).

It was considered as Fe-biofortified beans the beans target to the biofortification that presented, at least, a difference of 20 ppm in the iron concentration compared to the standard beans (carioca: BRS Perola, black: BRS Esteio).

Table 2. Iron concentration in the bean flours and ferritin formation in the Caco-2 cell exposed to the bean flours

Bean flours	**Iron (ppm)**	**Ferritin (ng/mg ptn)**
Carioca beans		
BRS Perola	$70.39^{de} \pm 0.84$	$3.3^{b} \pm 0.46$
BRS Pontal*	$73.24^{d} \pm 3.78$	$3.59^{b} \pm 0.24$
BRS Cometa*	$94.95^{a} \pm 0.74$	$3.64^{b} \pm 0.17$
BRS Estilo*	$66.87^{de} \pm 1.17$	$3.87^{b} \pm 0.30$
BRS SMR80*	$64.64^{e} \pm 0.5$	$3.63^{b} \pm 0.22$
BIO 101*	$70.28^{de} \pm 0.34$	$3.89^{b} \pm 0.36$
Black beans		
BRS Esteio	$68.08^{de} \pm 2.31$	$2.77^{b} \pm 0.22$
BRS Supremo*	$78.56^{c} \pm 2.15$	$2.7^{b} \pm 0.24$
BRS SMN 39*	$86.54^{b} \pm 2.47$	$2.85^{b} \pm 0.31$
White beans		
BRS Artico	$65.59^{e} \pm 5.67$	$22.31^{a} \pm 1.78$
#Baseline	-	$3.55^{b} \pm 1.11$

Values are mean ± SD, n = 3. Different letters indicate statistical differences at 5% probability by Tukey test. *Beans target to the biofortification with iron. #Baseline: Caco-2 cells without samples.

Figure 2 shows how the selected beans were used in the different experiments. Firstly, we tested five varieties in the *in ovo* experiment, in order to evaluate the effect of the soluble content extract from the three different classes of beans (carioca, black and white) on the gut microbiota and intestinal function. Then, we evaluated whether the addition of other staple crops (potato, rice, corn and pasta) would increase the *in vitro* iron bioavailability of standard and biofortified beans (carioca and black). Finally, we evaluated the *in vivo* iron bioavailability of the standard and biofortified carioca beans (BRS Perola and BRS Cometa, respectively), which presented the best results in the previous experiments.

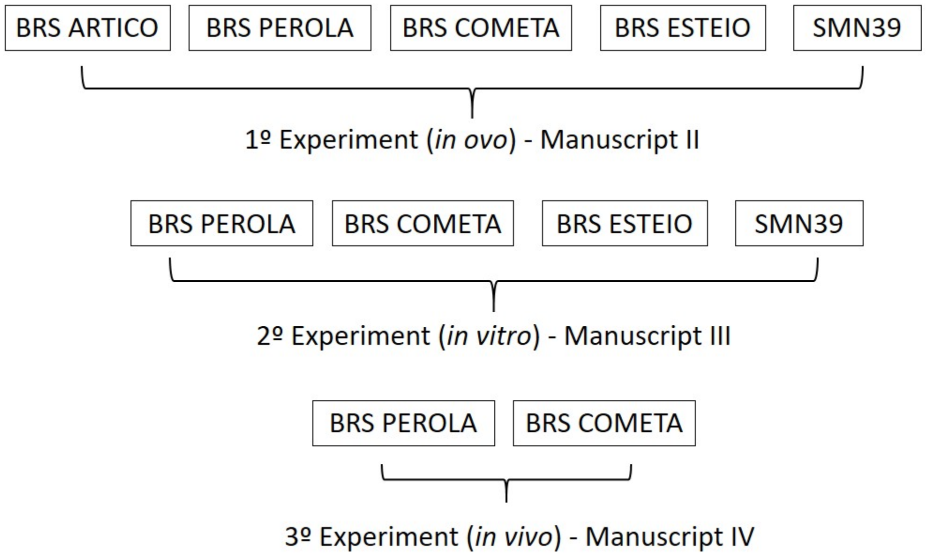

Figure 2. Scheme showing the selection of the beans and which beans were used in the different experiments. Carioca beans: BRS Cometa and BRS Perola; Black beans: BRS Esteio and SMN39; White beans: BRS Artico.

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6. MANUSCRIPT I

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CRITICAL REVIEWS IN FOOD SCIENCE AND NUTRITION
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Advantages and limitations of *in vitro* and *in vivo* methods of iron and zinc bioavailability evaluation in the assessment of biofortification program effectiveness

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ABSTRACT

Biofortification aims to improve the micronutrient concentration of staple food crops through the best practices of breeding and modern biotechnology. However, increased zinc and iron concentrations in food crops may not always translate into proportional increases in absorbed zinc (Zn) and iron (Fe). Therefore, assessing iron and zinc bioavailability in biofortified crops is imperative to evaluate the efficacy of breeding programs. This review aimed to investigate the advantages and limitations of *in vitro* and *in vivo* methods of iron and zinc bioavailability evaluation in the assessment of biofortification program effectiveness. *In vitro*, animal and isotopic human studies have shown high iron and zinc bioavailability in biofortified staple food crops. Human studies provide direct knowledge regarding the effectiveness of biofortification, however, human studies are time consuming and are more expensive than *in vitro* and animal studies. Moreover, *in vitro* studies may be a useful preliminary screening method to identify promising plant cultivars, however, these studies cannot provide data that are directly applicable to humans. None of these methods provides complete information regarding mineral bioavailability, thus, a combination of these methods should be the most appropriate strategy to investigate the effectiveness of zinc and iron biofortification programs.

KEYWORDS
Caco-2 cells; animal models; clinical trials; biofortification; mineral bioavailability

Advantages and limitations of *in vitro* and *in vivo* methods of iron and zinc bioavailabilityevaluationintheassessmentofbiofortificationprogram effectiveness

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Abstract

Biofortification aims to improve the micronutrient concentration of staple food crops through the best practices of breeding and modern biotechnology. However, increased zinc and iron concentrations in food crops may not always translate into proportional increases in absorbed zinc (Zn) and iron (Fe). Therefore, assessing iron and zinc bioavailability in biofortified crops is imperative to evaluate the efficacy of breeding programs. This review aimed to investigate the advantages and limitations of *in vitro* and *in vivo* methods of iron and zinc bioavailability evaluation in the assessment of biofortification program effectiveness. *In vitro*, animal and isotopic human studies have shown high iron and zinc bioavailability in biofortified staple food crops. Human studies provide direct knowledge regarding the effectiveness of biofortification, however, human studies are time consuming and are more expensive than *in vitro* and animal studies. Moreover, *in vitro* studies may be a useful preliminary screening method to identify promising plant cultivars, however, these studies cannot provide data that are directly applicable to humans. None of these methods provides complete information regarding mineral bioavailability, thus, a combination of these methods should be the most appropriate strategy to investigate the effectiveness of zinc and iron biofortification programs.

Keywords: Caco-2 cells; animal models; clinical trials; biofortification; mineral bioavailability

Introduction

Biofortification aims to improve the micronutrient concentration of staple food crops through the best practices of breeding and modern biotechnology. The most commonly targeted micronutrients include iron, zinc, and provitamin A carotenoids because of the high prevalence of deficiencies of these micronutrients in children and women of childbearing age (Bouis et al., 2011; La Frano et al., 2014). Biofortification may reach malnourished rural populations who may have limited access to supplements and commercially fortified foods (Saltzman et al., 2012).

However, the enhancement of zinc and iron concentrations in staple food crops may not translate into a proportional increase in absorbed zinc and iron since absorption inhibitors or enhancers may be present. Therefore, it is necessary to measure not only mineral concentration in enhanced crops but also bioavailability (Tako et al., 2011).

Several *in vitro* and *in vivo* models have been identified as appropriate for estimating human absorption and metabolism (La Frano et al., 2014). *In vivo* analyses of how food components are absorbed through the intestinal wall into the circulatory system to exert their biological effects are not easy to perform. Thus, *in vitro* studies represent useful tools to simulate the condition of the alimentary tract and enable more detailed research into cell metabolism under controlled conditions. Moreover, this approach is more ethical than research conducted on experimental animals (Olejinik et al., 2010). Finally, as advances in plant breeding are achieved, it is important to determine how effectively micronutrient content increments are utilized by humans, in addition to their public health benefits and sustainability of these benefits. Thus, this review aimed to identify the advantages and limitations of *in vitro* and *in vivo* methods of iron and zinc bioavailability evaluation to assess the effectiveness of biofortification programs.

Methods

A search was performed in the PubMed, Medline and Science Direct databases for articles published between 2000 and 2016 related to zinc and iron bioavailability in biofortified foods. Combinations of the following keywords were used: “iron”, “zinc”, “biofortified foods”, “biofortification”, and “bioavailability”. To identify studies not included in the initial search, a reverse search was conducted using the reference lists in the identified articles. *In vitro* (Caco-2 cells) and *in vivo* (human and animal models) studies regarding zinc and iron bioavailability in biofortified foods were included. Each article selected for inclusion in this review was critically analyzed.

Results

The search strategy resulted in the selection of 25 articles from 1556 articles researched (903 articles on iron and 653 articles on zinc) (Fig. 1). Eight references evaluated more than one mineral or evaluated one mineral by more than one method. Five references evaluated iron bioavailability using both *in vitro* and *in vivo* models (Tako et al., 2011; Tako et al., 2013; Tako et al., 2014; Tako et al., 2015a; Tako et al., 2015b). One reference evaluated zinc and iron bioavailability using human and Caco-2 cell models as well as iron bioavailability using a rat model (Vaz-Tostes et al., 2016). Furthermore, one reference assessed iron and zinc bioavailability in humans (Kodkany et al., 2013), and another study assessed iron and zinc bioavailability in Caco-2 cells (Jou et al., 2012). Thus, 13 articles were found that evaluated zinc bioavailability in biofortified crops (animal studies: 3; in vitro studies: 4; human studies: 6), and 23 articles were found that evaluated iron bioavailability in biofortified crops (animal studies: 8; in vitro studies: 7; human studies: 8), for a total of 36 articles on iron and/or zinc bioavailability.

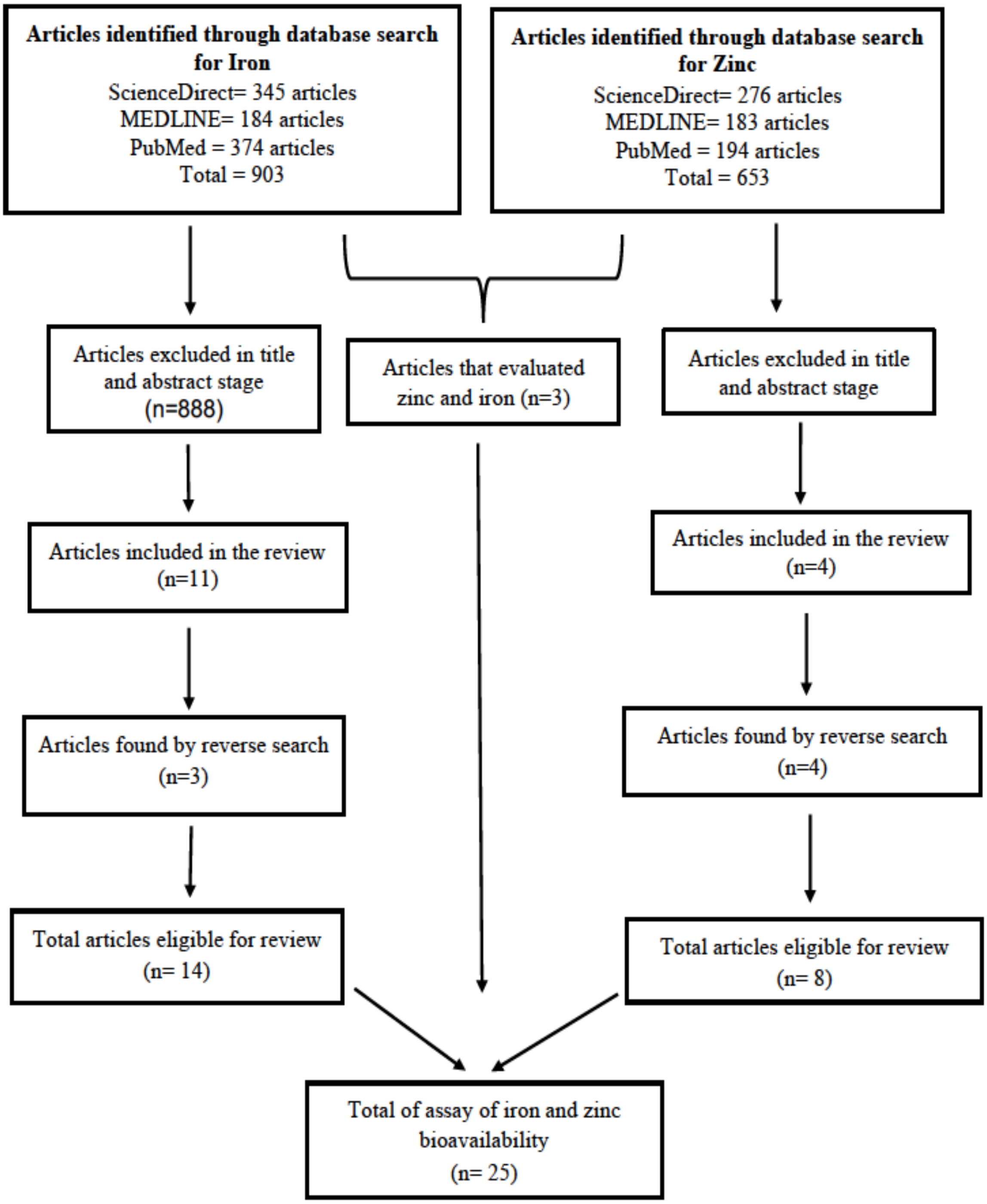

Figure 1. Search and selection of articles.

Methods of mineral bioavailability evaluation

In vitro model: Caco-2 cell culture

In vitro models of human tissues are gaining importance because of their relevance and wide applicability. The combination of cell culture and *in vitro* digestion models presents an alternative for studies that are frequently challenging to conduct in humans and animals because of ethical concerns (Payne et al., 2012).

Caco-2 cells are isolated from colon adenocarcinomas and mimic the typical characteristics of the human small intestinal epithelium (Wlodzimierz and Olejnik, 2004; Bermudez-Brito et al., 2014). This model can be used in *in vitro* Fe bioavailability studies combined with *in vitro* digestion methods and the high through put measurement of Caco-2 cell ferritin formation as a measure of Fe uptake (Yun et al., 2004). Caco-2 cell ferritin is a sensitive and clear marker of cell Fe uptake since cells produce ferritin proportionately in response to increases in intracellular iron (Ariza-Nieto et al., 2007).

Iron and zinc bioavailability may be assessed through the determination of nutrient uptake and transport by Caco-2 cells. Iron uptake may be estimated by ferritin formation or ^{59}Fe uptake (a radioisotope of iron). In contrast to ferritin formation, which is an indicator of iron uptake, there are no biomarkers of zinc uptake. Metallothionein, a cytoplasmic protein that stores zinc, has been used as an indicator of zinc uptake. However, this protein may also bind and store other metals, such as copper, selenium, cadmium, mercury, silver, and arsenic (Bell and Vallee, 2009). Thus, metallothionein is not specific for zinc, which makes its application as a biomarker for zinc bioavailability questionable (Etcheverry et al., 2012). Therefore, the combination of isotopic techniques with Caco-2 cells should be a better approach to estimate zinc absorption *in vitro* because similar results have been obtained from animal and human studies (Jou et al., 2012).

Iron uptake may be estimated by ^{59}Fe uptake (a radioisotope of iron). Whereas, zinc bioavailability studies have used an extrinsic labeling with ^{65}Zn. Extrinsic ^{65}Zn equilibrates with intrinsic Zn in complex food matrices of animal and plant origin after *in vitro* digestion (Jou et al., 2012). Thus, many studies have used this *in vitro* method to estimate the iron and zinc bioavailability from biofortified foods (Tables 1 and 2).

Animal models

Different approaches may be used to evaluate zinc- and iron biofortified foods as dietary mineral sources for humans. The most appropriate model is to directly evaluate different biofortified foods in human studies. However, these studies are expensive and time consuming. An alternative approach is to perform bioavailability studies on animals (Patterson et al., 2008; Carlson et al., 2014).

Iron bioavailability may be assessed in rats and pigs via the hemoglobin repletion assay reported by Perks and Miller (1996) to obtain a relative measure of iron absorption. Blood is sampled at the initiation of the feeding period to measure the initial hemoglobin concentrations. The animals are subsequently fed experimental diets for 2 to 5 weeks, after which blood samples are obtained for the determination of the final hemoglobin concentrations. The feed intake is measured throughout the period to calculate the iron intake. The blood volume is estimated from the body weight, and it is used to calculate the hemoglobin repletion efficiency (HRE), which is an indicator of iron bioavailability (Petterson et al., 2008).

Moreover, various techniques have been developed to assess mineral retention and absorption *in vivo*. One of the simplest methods for indirectly measuring the absorption of ingested iron and zinc is to assess fecal and urinary excretion levels and then differentially calculate the absorption/retention based on the ingested dose (Patterson et al., 2008). However, the main pathway of zinc and iron excretion is endogenous, which limits the utility of this method because it may underestimate the bioavailability of these micronutrients.

Another option involves the use of stable radioisotopes. Whole-body counting is performed to determine the retention level, and individual tissues may be assayed to determine the distribution patterns throughout the body. Isotope absorption is subsequently determined by measuring the changing isotopic ratios in tissue, blood, and/or urine against the more abundant, natural isotopic form (Griffin, 2002). Summaries of studies that have investigated zinc and iron bioavailability using these animal models are presented in Table 1 (iron studies) and Table 2 (zinc studies).

Human studies

The human studies that have evaluated iron and zinc bioavailability in biofortified foods have used radioactive and stable isotopes. This technique discriminates the amounts of the micronutrient provided by the diet from endogenous forms, which enables a more accurate measurement of bioavailability. Iron and zinc have been both intrinsically and extrinsically labeled. Intrinsic labeling is the biological incorporation of an isotope into a plant during its growth, whereas extrinsic labeling is the addition of an isotope to food prior to ingestion (Fairweather-Tait et al., 2005). Extrinsic labeling may be used or non-heme iron (present in plant foods) and zinc absorption studies in humans (Jou et al., 2012). These techniques are also useful to investigate the potential efficacy of different iron and zinc compounds for use in food fortification and mineral supplements (Hotz and Brown, 2004). Furthermore, they may be useful to assess the efficiency of mineral biofortification programs. Iron bioavailability studies have used isotopically labeled $^{58}FeSO_4$ and $^{57}FeSO_4$, and the amounts of ^{57}Fe and ^{58}Fe isotopic labels have been analyzed in blood samples (Petry et al., 2012; Cercamondi et al., 2013; Kodkany et al., 2013; Petry et al., 2014). For zinc bioavailability, human studies have utilized the stable isotopes ^{67}Zn, ^{68}Zn, and ^{70}Zn, and the amounts of the isotopic labels have been analyzed in urine (Rosado et al., 2009; Islam et al. 2013; Chomba et al., 2015; Brnic et al., 2016). These human studies are summarized in Table 1 (iron studies) and Table 2 (zinc studies).

Iron bioavailability evaluation

In vitro studies

Iron (Fe) deficiency is the most prevalent nutrient deficiency, affecting approximately 40% of the world's population, particularly women and children in developing countries (WHO, 2008; Muthaya et al., 2013). Strategies for reducing the prevalence of iron deficiency include the distribution of Fe supplements, food fortification and the diversification of diets. The common bean (*Phaseolus vulgaris,* L.), one of the staple food crops targeted for nutritional enhancement by HarvestPlus, is an attractive candidate for Fe biofortification because there is genetic variability in Fe concentration, and it is possible to increase the Fe concentrations in beans (Welch et al., 2000). Furthermore, the Fe concentrations in beans are high relative to those in other crops, therefore, beans may deliver substantial amounts of Fe (Tako et al., 2009). In this context, the CIAT (International Center for Tropical Agriculture, Cali, Colombia) has developed biofortified beans that contain up to 100 mg Fe/g bean, which represents a substantial increase over that in standard beans (Blair et al., 2010).

Two studies have used the *in vitro* method with Caco-2 cells to evaluate the iron bioavailability of biofortified red mottled beans (Tako et al., 2011) and carioca beans (Tako et al., 2015b) from the CIAT. In two studies, higher ferritin concentrations were found in cells exposed to the Fe-biofortified bean than in cells exposed to the standard Fe bean. These findings indicate increased amounts of bioavailable Fe in the Fe-biofortified beans. In contrast, Vaz-Tostes et al. (2016) did not identify differences in ferritin concentrations between common beans (PE) and the targeted bean for mineral biofortification (PO)

(PO: 13.1 ± 1.4 and PE: 13.6 ± 1.4 ng mg $^{-1}$protein). Maize (Zea mays L.) and pearl millet have also been used in a biofortification program as a strategy to increase iron intake in an at-risk population.

Maize is widely consumed in developing countries and provides energy, vitamins and minerals. Thus, maize is an attractive candidate for Fe biofortification (Cannon et al., 2011; Tako et al., 2013). An *in vitro* digestion/ Caco-2 cell culture model employed by Tako et al. (2013) showed higher amounts of bioavailable iron in biofortified maize than in common maize. Similarly, Tako et al. (2015a) identified increased amounts of bioavailable Fe in high-Fe pearl millet. Thus, maize and millet are promising vehicles to alleviate Fe deficiency in human populations where these foods are major dietary staples.

Biofortified rice has also been investigated for iron bioavailability in the Caco-2 cell model. Biofortified rice had more bioavailable iron than the control. Moreover, this result was even more pronounced in the presence of ascorbic acid, which is reported to be the most efficient promoter of iron absorption (Trijatmiko et al., 2016).

Animal studies

Despite the rat model presents differences from humans regarding Fe absorption, hemoglobin depletion/repletion studies are widely used to assess the relative bioavailability of Fe in foods (Tako et al., 2009). In this context, two studies have used this model to evaluate iron bioavailability in biofortified foods. In these studies, the authors identified increased iron bioavailability in beans targeted for iron biofortification (Dias et al., 2015; Vaz-Tostes et al., 2016). However, rats are more efficient than humans at iron absorption from plant foods since they produce phytase and vitamin C, which led these studies to overestimate the iron bioavailability in plant foods (Sant'ana et al., 2006).

Therefore, poultry may be a suitable model for the measurement of iron bioavailability because of their quick response to iron deficiency (Tako and Glahn, 2010; Tako et al., 2011). The modern broiler chicken is a fast-growing animal that is sensitive to dietary deficiencies of trace minerals such as Fe and they have limited ability to synthesize ascorbic acid (Tako et al., 2011; Tako et al., 2016). Physiological adaptations to iron deficiency may occur over time, thus, animals must be monitored for signs of anemia. In addition, this model has very good agreement with the results obtained from *in vitro* Caco-2 cells. Moreover, the combination of this animal model with the *in vitro* Caco-2 cell model has been effective for testing the bioavailability of iron in food crops (Tako et al, 2009; Tako and Glahn, 2011a; Tako et al, 2014).

Therefore, a group of researchers has investigated iron bioavailability by combining *in vitro* (Caco-2 cells) and *in vivo* (Gallus gallus) models. In this experimental model, two groups of animals are compared, including one group fed iron-free diets (which leads to iron deficiency) and a second group provided with the test foods as a dietary iron source. Blood variables (hemoglobin) and the gene expression of proteins related to iron metabolism were evaluated to determine the iron bioavailability. In this way, these authors have compared the iron bioavailability of biofortified red beans (Tako and Glahn, 2011b), biofortified black beans (Tako et al., 2014), biofortified maize (Tako et al., 2013) and pearl millet (Tako et al., 2015a) with those of their conventional counterparts. They identified an increase in iron bioavailability by increasing blood hemoglobin and liver ferritin and reducing the gene expression of divalent metal transporter 1 (DMT-1), duodenal cytochrome b (Dcytb) and ferroportin in the animal duodenum (Tako and Glahn, 2011b; Tako et al., 2013; Tako et al., 2014; Tako et al., 2015a).

Piglets have been used as a model for iron bioavailability studies because of similarities in gastrointestinal anatomy and physiology between pigs and humans. Pigs, similar to humans, are truly omnivorous, and the digestive and metabolic processes in pigs are similar to humans (Patterson et al., 2008). Moreover, pigs readily consume monotonous diets that may be formulated to simulate the human diets common in resource poor regions of the world. Furthermore, iron deficiency develops rapidly in young pigs (Tako et al., 2009). This model was used by Tako et al. (2009) to assess the iron bioavailability of iron-biofortified and standard black beans consumed in a maize-based diet in a 5-week feeding study. Hemoglobin regeneration efficiency, which represented a measure of iron bioavailability, did not differ between the groups, which indicates that although the biofortified beans contained increased the concentrations of iron, the bioavailable iron in these beans was equal to that in the standard beans.

Human studies

Current iron biofortification research programs have focused on increasing the iron concentration of staple crops, such as wheat, maize, rice, beans, and pearl millet (Nestel et al., 2006). Thus, these studies have evaluated the iron bioavailability in these biofortified foods (Table 1).

Petry et al. (2012) utilized an iron stable isotope to elucidate the potential of common beans as a biofortification vehicle for iron. This study was conducted in women with a low iron status. They observed that iron absorption from the high-iron bean was 40% lower than that from the normal-iron bean, which resulted in equal amounts of iron absorbed. In addition, when beans were combined with other meal components in multiple meals, high polyphenol concentrations had no negative impact on iron absorption. However, the quantity of iron absorbed from composite meals with high-iron beans was not different from that absorbed from meals with normal-iron beans, which indicates that efficacious iron biofortification may be difficult to achieve in beans rich in phytic acid (PA) and polyphenols.

Similarly, in an intervention study with high-iron beans, Vaz-Tostes et al. (2016) determined there were no changes in iron nutritional status in preschool children after high-iron bean intake (ferritin, 41.2 ± 23.2 and 28.9 ± 40.4 $\mu g\ L^{-1}$; hemoglobin, 13.7 ± 2.2 and 13.1 ± 3.2 $g\ dL^{-1}$, respectively). In contrast, Petry et al. (2014), using a stable isotope technique to assess the effect of PA on iron bioavailability in iron-biofortified beans (the beans were grown from certified seeds at CIAT), reported a higher quantity of absorbed iron from this variety than from the control bean.

Furthermore, they promoted bean dephytinization, and an increase in the quantity of iron absorbed from the biofortified bean was identified that was higher than that from the control bean. Thus, the authors concluded that the PA decreases iron bioavailability in iron-biofortified beans, and a high PA concentration is an important impediment to the optimal effectiveness of bean iron biofortification (Petry et al., 2014). However, PA is required for plant growth, thus, its reduction may lead to decreased productivity.

Because PA decreases iron bioavailability in beans, one study investigated whether low-PA beans provide more bioavailable iron than iron-biofortified beans. A multiple-meal crossover design with 25 young women was performed that utilized stable iron isotopes to assess iron absorption. The amount of bioavailable iron in low-PA beans did not differ from that available in the biofortified beans, however, the amount of bioavailable iron in the biofortified beans was >50% higher than that in the control beans (Petry et al., 2016).

Haas et al. (2016) conducted a randomized controlled trial to compare the efficacy of iron-biofortified beans (Fe-Beans) relative to standard unfortified beans (Control-Beans) in improving the iron status in iron-deficient women. Iron status was assessed via measurements of hemoglobin, serum ferritin, soluble transferrin receptor, and body iron. Fe-Beans were associated with significantly greater increases in hemoglobin, serum ferritin and body iron than Control-Beans. For every 1 g of Fe consumed from beans during the study, there was a significant 4.2-g/L increase in hemoglobin. Thus, the consumption of iron-biofortified beans significantly improved the iron status of iron-deficient women.

Another food targeted for iron biofortification is pearl millet, which has been reported to contain 7–8 mg/100 g of Fe (HarvestPlus, 2009; Hama et al., 2012), approximately double the iron content of other major cereal staples (Cercamondi et al., 2013). Thus, two studies have assessed the iron bioavailability in pearl millet (Cercamondi et al., 2013; Kodkany et al., 2013). Cercamondi et al. (2013) used stable iron isotopes to evaluate the potential of iron-biofortified millet to provide additional bioavailable iron compared with conventional millet and post-harvest iron-fortified millet in women with marginal iron status. It was reported that the total absorbed iron from biofortified millet was higher than that from conventional millet. Furthermore, the quantity of total absorbed iron from the post-harvest iron-fortified millet was higher than that from the conventional and iron-biofortified millet. Thus, although the fractional absorption of iron from biofortification was lower than that from fortification, iron-biofortified millet should be highly effective in combating iron deficiency in millet-consuming populations.

The same result was obtained by Kodkany et al. (2013), who used a stable isotope in iron-deficient children. They found a higher total amount of absorbed iron from biofortified pearl millet than from conventional pearl millet. In addition, the absorption of iron from the biofortified millet exceeded the physiological requirement (0.54 mg/d) for this age group (Kodkany et al., 2013).

Rice has been targeted for iron biofortification and was one of the first crops biofortified by HarvestPlus (Haas et al., 2005). The International Rice Research Institute (IRRI) recently developed a variety of rice for experimental use that has 400–500% more iron, after processing and cooking, than conventional varieties (Gregorio et al., 2000). Thus, one study (Haas et al., 2005) investigated the efficacy of consuming this biofortified rice in Filipino women at risk of iron deficiency. They used a randomized, controlled, double-blind and longitudinal (9 months) intervention trial. The study analyzed two groups (low-iron rice and high-iron rice) and two groups of subjects (anemic and non-anemic women). The biofortified and conventional rice produced no differences in blood hemoglobin or ferritin in the anemic women. However, in the non-anemic women, blood hemoglobin and ferritin were higher in the biofortified rice group than in the conventional rice group. Thus, the consumption of biofortified rice, without other changes in the diet, may be efficacious to improve the iron stores of women with iron-poor diets.

Zinc bioavailability evaluation

In vitro studies

Using a stable isotope, ^{65}Zn, in a Caco-2 model, Jou et al. (2012) compared zinc bioavailability in undermilled and polished biofortified rice and undermilled conventional rice. They showed that zinc absorption from biofortified rice, either undermilled or polished, was twofold higher than that from conventional rice. In addition, the molar ratio of phytate to zinc was lower in biofortified rice (19:1) than in the common varieties (35– 46:1), which may result in increased zinc bioavailability in biofortified rice. In addition to biofortification, foliar Zn fertilization produced significant increases in Zn retention, transport and uptake efficiency in Caco-2 cells (Wei et al., 2012). However, the latter study did not utilize stable isotopes, it analyzed zinc concentrations in the cells, which may overestimate zinc absorption.

It has been shown that both biofortification and rice genotype may influence zinc bioavailability in rice. Wei et al. (2012) compared *in vitro* Zn bioavailability between three genotypes of Zn-fortified germinated brown rice and normal germinated brown rice. They found higher percentages of Zn absorption by Caco-2 cells from the

Zn-fortified germinated brown rice than from the normal germinated brown rice, and there was bioavailability variation among the tested rice genotypes.

Beans were also assessed in the Caco-2 cell model in one study (Vaz-Tostes et al., 2016). In this study, two bean varieties were evaluated: the conventional BRS Perola (PE) (20.47 mg/kg Zn) and a variety targeted for mineral biofortification, BRS Pontal (PO) (26.1 mg/kg Zn). However, in contrast to rice, there was no difference in zinc uptake between the conventional bean and the bean targeted for mineral biofortification (PO: 15.9 ± 1.5 and PE: 15.5 ± 3.5 μmol mg $^{-1}$ protein).

Animal studies

Rodent models are suitable for the assessment of zinc bioavailability. For zinc, the rat pup model is the most appropriate model because young rats do not have intestinal phytase activity (La Frano et al., 2014). Jou et al. (2012) utilized this model to test Zn bioavailability in five varieties of rice. Four varieties represented several of the most highly produced varieties in Bangladesh (i.e., BR-28, BR-29, BR-11, and Paijam) (between 14 and 19 mg Zn/kg), and one variety represented a Zn-biofortified line (IR-68-1-44) (35 mg/kg of Zn in polished form) developed at the IRRI (Los Banños, Philippines).

Rats were fed a radiolabeled diet that contained ^{65}Zn. The radioactivity in the stomach, perfused intestine, perfusate, liver, carcass, and cecum-colon was measured via gamma counting. The absorbed Zn was expressed as fractional absorption and was calculated as radioactivity in the carcass, liver, kidney, and perfused small intestine as a percentage of the total recovery. The results indicated that the absorbed zinc from the biofortified rice was twice as high as that from the common rice. However, the phytate:zinc molar ratio was lower in biofortified rice (19:1) than in common varieties (35–46:1), which may result in increased zinc bioavailability in biofortified rice (Jou et al., 2012).

Similarly, Welch et al. (2000) used a rat model to determine the zinc bioavailability in the same genotype of the previously described zinc-biofortified rice (IR68144) and zinc biofortified beans from CIAT. Furthermore, they used the same methodology as Jou et al. (2012), which included the radioisotopes ^{65}Zn and ^{59}Fe. Bioavailability was calculated from the amount of radiolabeled zinc retained in the rats over a 10-day period, as determined each day via a whole body gamma spectrometry assay. The results demonstrated that increasing the amount of zinc in enriched rice grains and beans significantly increased the amount of bioavailable zinc. These findings also support the contention that the selection of traits in bean and rice genotypes that enrich the zinc concentration in their seeds and grains will provide more bioavailable zinc to target populations dependent on these foods as a major source of zinc in their diet.

Pigs have also been used to evaluate zinc bioavailability. This model is known to be a good model for humans. Carlson et al. (2012) fed two groups of pigs with zinc biofortified wheat and beans for seven days. They collected and weighed the urine and feces during the balance period. However, the authors were not able to calculate reliable zinc bioavailability values using this model because the animals had low zinc intake, which represents a limitation of this type of study. Moreover, the balance technique was not appropriate for estimating zinc bioavailability because the main pathway of zinc excretion is endogenous. Recently, two studies proposed a new biomarker of zinc status in a poultry model that can be used in human zinc status studies, the erythrocyte linoleic acid: dihomo-γ-linolenic acid (LA:DGLA) ratio. An elevation in the 18: 2ω6: 20: 3ω6 ratio may be a sensitive marker for zinc deficiency because this mineral is required by Δ^6-desaturase, which converts 18: 2ω6 to 20: 3ω6 (Reed et al., 2014; Knez et al., 2016).

Human studies

In a study of Bangladeshi children, Islam et al. (2013) used the dual-isotope tracer ratio technique to calculate the total absorbed zinc from high-zinc rice. The zinc intake from the conventional rice-based diet was 1 mg less than that from the high-zinc diet, however, the total absorbed zinc from these diets was not significantly different. This finding was a result of the lower fractional absorption (20.1%) from the high-zinc rice than from the conventional rice (25.1%) and the increased phytate content present in the high-zinc rice.

Using a double-isotope tracer ratio method in adults, Brnic et al. (2016) reported similar absorbed fractions of zinc from biofortified rice and fortified rice. Thus, rice biofortification is likely to be as good as post-harvest zinc fortification as a strategy to combat zinc deficiency.

Rosado et al. (2009) compared zinc absorption from high zinc- biofortified wheat and control wheat, which were extracted at high (95%) and moderate (80%) levels, respectively. To assess the fractional absorption of zinc, they used a dual-isotope tracer ratio technique in women. Both extraction rates resulted in similar reductions in the zinc and phytate contents, suggesting that the benefits of high-zinc wheat are not reduced by milling. Zn absorption was 31% (95% extraction group) and 33% (80% extraction group) higher from the Zn biofortified wheat than from the control. These findings suggest that Zn absorption from the same quantities of wheat flour is higher for Zn-biofortified wheat than for wheat with a more typical Zn concentration.

The bioavailability of zinc in biofortified pearl millet has been investigated in one human study with non-zinc-deficient children (Kodkany et al., 2013). Zinc consumption was 5.8 mg/d from biofortified pearl millet and 3.3 mg/d from regular pearl millet. Using stable isotope extrinsic labeling of zinc, the amount of absorbed zinc from biofortified millet was found to be higher than that from control millet, although the fractional absorption was less than expected.

Another food target for zinc biofortification is maize, particularly in countries with a higher consumption of this cereal. Therefore, Chomba et al. (2015) used the dual-isotope tracer technique in young children to compare the zinc absorption from control maize, zinc-biofortified maize and zinc-fortified maize. They found that the total daily absorption of zinc from the biofortified maize was higher than that from the control maize, however, it did not differ from that from the fortified maize. Thus, biofortified maize intake meets zinc requirements and provides an effective dietary alternative to regular maize for this vulnerable population.

These studies assessed zinc bioavailability in biofortified foods using isotopic techniques. However, in addition to these techniques, one study used plasma zinc and erythrocyte zinc determination to investigate the beneficial effects of beans targeted for biofortification on improving zinc nutritional status in preschool children (Vaz-Tostes et al., 2016). Thus, these researchers performed a nutritional intervention with the Pontal bean, which is a target variety for the mineral biofortification program of the Empresa Brasileira de Pesquisa Agropecuaria (EMBRAPA), Brazil. There were no differences in zinc nutritional status after Pontal bean consumption (plasma zinc: 119.2 ± 24.5 and 133.9 ± 57.7 $\mu g\ dL^{-1}$; erythrocyte zinc: 53.5 ± 13.8 and 59.4 ± 17.1 $\mu g\ g^{-1}$, respectively). Nonetheless, this type of study provides feedback for biofortification programs to produce beans with higher mineral bioavailability.

Table 1 Zinc (Zn) bioavailability from biofortified foods

REFERENCE	TESTED FOOD	STUDY DESIGN	RESULT
In vitro studies			
Jou et al. (2012)	Zn biofortified rice (undermilled :42,5mg/Kg; polished: 35,5mg/Kg) and convencional rice (undermilled: ~20,3mg/Kg; polished: ~17,5mg/kg Zn)	Caco-2 cell assay using stable isotope	Zn absorption was twice higher in the biofortified rice (undermilled or polished) than the common rice
Wei, Shohag and Yang (2012)	High-Zn polished rice (~27,02 mg/kg Zn) and control rice (22,92 mg/kg Zn)	Caco-2 cell assay	Higher Zn retention, transport and uptake efficiency.
Wei et al. (2012)	Zn fortified germinated brown rice (~59,9 mg/kg Zn) and normal germinated brown rice (~22,9 mg/kg Zn).	Caco-2 cell assay	Higher percentages of Zn bioavailability from Zn fortified germinated brown rice than the normal germinated brown rice.
Vaz-Tostes et al. (2015)	Common bean (20,47 mg/kg Zn) and Pontal bean (which is a targeted variety for mineral biofortification) (26,1 mg/kg Zn)	Caco-2 cell assay	The zinc uptake from common bean and from the Pontal bean was similar.
Animal models			

Jou et al. (2012)	Zn biofortified rice (undermilled :42,5mg/kg; polished: 35,5mg/kg) and convencional rice (undermilled: ~20,3mg/kg; polished: ~17,5mg/kg Zn)	Rat model by isotopic technique	The absorbed zinc was twice higher in the biofortified rice compared to common rice
Welch et al. (2000)	10 varieties of Zn biofortified rice (range from 35,09 to 60,5 mg/kg) and 24 genotypes of zinc biofortified beans from CIAT (range from 30,42 to 62,51 mg/kg)	Rat model by isotopic technique	The increasing the amount of zinc in enriched rice grain and beans significantly increases the amount of zinc bioavailable to rats.
Carlson et al. (2014)	Zn biofortified wheat (42,8 mg/kg Zn), control wheat (15,6 mg/kg/Zn) and Zn biofortified beans (41,4 mg/kg Zn) and common beans (29,9 mg/kg Zn)	Pig model by balance technique (urine and feces).	The authors were not able to calculate the reliable value for the zinc bioavailability in this model, since the animals had a very low zinc intake.
Human studies			
Islam et al. (2013)	High-zinc rice (26,0 mg/kg Zn) and conventional rice (13,5mg/kg Zn)	Dual-isotope ratio in Bangladeshi children	The total absorbed zinc from high-zinc rice and conventional rice was similar.
Brnic et al. (2016)	Zn biofortified rice (22 mg/kg	Double-isotope in adults	The fractional absorption of zinc from

	Zn) and control fortified rice (8mg/kg Zn)		biofortified rice similar to zinc fortified rice.
Rosado et al. (2009)	High-zinc biofortified wheat (41,3 mg/kg Zn) and control wheat (23,6 mg/kg Zn).	Dual isotope tracer ratio technique in women	Zn absorption was higher from the Zn biofortified wheat than the control wheat.
Kodkany et al., 2013	Biofortified pearl millet (84,1 mg/kg Zn) and control pearl millet (43,7 mg/kg Zn)	Stable isotope in no Zn deficient children	The amount of absorbed Zn was higher from the biofortified millet than the control millet.
Chomba et al. (2014)	Zn biofortified maize (43 mg/kg Zn), control maize (21mg/kg Zn) and fortified maize (60 mg/kg)	Dual isotope tracer ratio in young children.	The total absorption of Zn from the biofortified maize was higher than the control maize, but did not differ from the fortified maize
Vaz-Tostes et al. (2015)	Common bean (20,47 mg/kg Zn) and Pontal bean (which is a targeted variety for mineral biofortification) (26,1 mg/kg Zn)	Nutritional intervention in preschool children. Evaluation by plasma zinc and erythrocyte zinc determination	No differences in zinc nutritional status after the Pontal bean consumption

Table 2 Iron (Fe) bioavailability from biofortified foods

REFERENCE	TESTED FOOD	STUDY DESIGN	RESULT
In vitro studies			
Tako et al. (2011)	High-Fe red mottled (71 mg/kg Fe) beans and control red beans (49 mg/kg Fe)	Caco-2 cell assay	Higher amounts of bioavailable Fe in the Fe biofortified red mottled beans
Tako et al. (2015a)	High-Fe Pearl millet (84.9 μg/g Fe) and Low-Fe Pearl Millet (25.9 μg/g Fe)	Caco-2 cell assay	Higher amounts of bioavailable Fe in the High-Fe Pearl millet
Tako et al. (2015b)	Fe biofortified carioca beans (106 mg/kg Fe) and standard carioca beans (58 mg/kg Fe)	Caco-2 cell assay	Higher amounts of bioavailable Fe in the Fe biofortified carioca beans.
Vaz-Tostes et al. (2015)	Common bean (52,43 mg/kg Fe) and high-Fe bean (60,62 mg/kg Fe)	Caco-2 cell assay	No differences were observed in ferritin concentrations between common beans and a bean tagged for mineral biofortification.
Tako et al. (2013)	High-Fe bioavailability Maize (21 mg/kg Fe) and Low-Fe bioavailability maize (20 mg/kg Fe).	Caco-2 assay	Higher amount of bioavailable iron from the biofortified maize compared the common maize
Tako et al. (2014)	Fe biofortified (88 mg/kg Fe) and standard black beans (59 mg/kg Fe)	Caco-2 assay	Low Fe bioavailability from Fe biofortified beans and standard beans.
Trijatmiko et al.	Fe biofortifed polished rice	Caco-2 cells assay	Higher amount of bioavailable iron in

(2016)	(8,2 mg/kg Fe) and the wild type of rice (2-3 mg/kg Fe)		biofortified rice compared to the control
Animal models			
Dias et al. (2015)	High-Fe Pontal beans (75,2 mg/kg Fe)	In rats by hemoglobin depletion-repletion method	Higher iron bioavailability of beans target for iron biofortification.
Vaz-Tostes et al. (2015)	Common bean (52,43 mg/kg Fe) and high-Fe bean (60,62 mg/kg Fe)	In rats by hemoglobin depletion-repletion method	Higher iron bioavailability of beans target for iron biofortification compared to the control.
Tako et al. (2011)	High-Fe red mottled (71 mg/kg Fe) beans and control red beans (49 mg/kg Fe)	In poultry model by hemoglobin maintenance efficiency	Increased in iron bioavailability from High-Fe red mottled by increasing the blood hemoglobin and liver ferritin
Tako et al. (2014)	Fe biofortified (88 mg/kg Fe) and standard black beans (59 mg/kg Fe)	In poultry model by hemoglobin maintenance efficiency	The Fe bioavailability from Fe biofortified beans was lower than the standard beans.
Tako et al. (2013)	High-Fe bioavailability maize (21 mg/kg Fe) and Low-Fe bioavailability maize (20 mg/kg Fe).	In poultry model by hemoglobin maintenance efficiency (HME)	Increased in iron bioavailability from High-Fe bioavailability maize by increasing the blood hemoglobin and liver ferritin
Tako et al. (2015a)	High-Fe Pearl millet (84.9 µg/g Fe) and Low-Fe Pearl Millet (25.9 µg/g Fe)	In poultry model hemoglobin maintenance efficiency	Increased in iron bioavailability from High-Fe Pearl millet by increasing the blood hemoglobin and liver ferritin
Tako et al. (2009)	Fe-biofortified (106 mg/kg Fe) and standard black beans	In pig model by hemoglobin maintenance	The hemoglobin regeneration efficiency did not differ between biofortified beans and standard

	(71 mg/kg Fe)	efficiency	beans.
Tako et al. (2015b)	Fe biofortified carioca beans (106 mg/Kg Fe) and standard carioca beans (58 mg/Kg Fe)	In poultry model hemoglobin maintenance efficiency	Increased in iron bioavailability from Fe biofortified carioca beans by increasing the blood hemoglobin and liver ferritin
Human studies			
Petry et al. (2012)	High-Fe bean (9,1mg/kg Fe) and control bean (5,2mg/kg Fe)	Stable isotope in women with low iron status.	Fe absorption from the high iron bean was lower than from the normal iron bean, resulting in equal amounts of iron absorbed.
Vaz-Tostes et al. (2015)	High-Fe bean (60,62 mg/kg Fe)	Intervention study with in preschool children by ferritin and hemoglobin analysis.	No changes in Fe nutritional status in preschool children comparing before and after the consumption of high-iron bean
Petry et al. (2014)	Fe biofortified beans (88 mg/kg Fe), control beans (54 mg/kg Fe) and dephytinized biofortified beans	Stable isotope technique in women.	Higher quantity of absorbed iron from biofortified variety compared to the control bean. The dephytinized biofortified beans presented higher absorbed iron than the control bean.
Cercamondi et al (2013)	Fe biofortified pearl millet (88 mg/kg Fe), regular millet (25 mg/kg Fe) and post-harvest Fe-fortified millet (add 40 mg/kg Fe in the regular millet)	Stable iron isotopes in women with marginal iron status	The total Fe absorbed from biofortified millet was higher than the conventional millet. Also, the total Fe absorbed from the post-harvest Fe-fortified millet was higher than the conventional and biofortified millet.

Kodkany et al. (2013)	Fe biofortified from pearl millet (124 mg/kg Fe) and control millet (46,5 mg/kg Fe)	Stable isotope in iron-deficient children	The total amount of absorbed Fe was significantly higher for biofortified millet than the regular millet.
Haas et al. (2005)	Fe biofortified rice (3,2 mg/kg Fe) and control rice (0,57 mg/kg Fe)	Randomized, controlled, doubleblind, longitudinal, intervention trial in anemic and noanemic women.	No differences in blood hemoglobin and ferritin between biofortified and convectional rice in anemic group. In noanemic group, the hemoglobin and ferritin were higher in biofortifed rice group compered to convectional rice.
Haas et al. (2016)	Fe-biofortified beans (86 mg/kg Fe) and standard unfortified beans (50 mg/kg Fe)	Randomized controlled trial in Fe deficient women. Iron status was assessed by hemoglobin, serum ferritin and body iron.	The Fe-Beans group had significantly greater increases in hemoglobin, serum ferritin and iron body than standard unfortified beans
Petry et al. (2016)	Fe-biofortified beans (99 mg/kg Fe), Low-phytic acid bean (70 mg/kg Fe) and control bean (5,2 mg/kg Fe)	Multiple-meal crossover design with young women by stable iron isotopes	The amount of bioavailable iron from low phytic acid beans did not differ from the biofortified bean, however the biofortified bean was >50% higher than from control beans.

Correlation among *in vitro* (cell culture), animal and human studies: advantages and limitations

No single bioavailability method is ideal for all micronutrients, and all bioavailability methods present advantages and limitations (Table 3). The required equipment, costs of labor and animals, and funds available all play important roles in determining the method of choice. The selection of a method to use in determining micronutrient bioavailability in plant foods requires the consideration of several issues that may affect the results obtained. Several particularly important issues include intrinsic versus extrinsic labeling of the plant material, the bioavailability model to use (*in vitro* or animal species), the micronutrient status of the experimental subjects, and the levels of anti-nutrients and promoter substances in the test plant food and test meals (Welch et al., 2000).

Human studies provide the most applicable results because they are capable of considering host factors, disease states, and physiological changes during digestion. Therefore, these results may be interpreted more directly and used to assess the true absorption of nutrients (La Frano et al, 2014). However, human studies remain difficult to perform because of the social and ethical considerations that govern the invasive medical procedures necessary in accessing the human large intestine, thus, human studies are primarily limited to fecal sample analyses (Payne et al., 2012).

The use of radioactive and stable isotopes in human studies is an alternative to invasive methods and it enables the discrimination of the dose of micronutrients provided by the diet and endogenous forms, which provides a more accurate measurement of bioavailability. However, the use of isotopes has limitations because of the risk of radioactivity exposure, costs, complexity and labor-intense procedures required (Au and Reddy, 2000).

Therefore, animal models represent an alternative to human studies because they may provide useful information regarding *in vivo* bioavailability, particularly in the dissection and analysis of individual tissues to provide a whole-body assessment of absorption (La Frano et al., 2014).

Rats may be used to provide relative estimates of bioavailable iron and zinc in plant foods (Whelch et al., 2000). However, because of their quick response to micronutrient deficiencies, including low iron status, poultry may represent a suitable model to measure iron bioavailability (La Frano et al., 2014; Tako et al., 2016). The poultry model has found a useful niche as an intermediate test of *in vivo* iron bioavailability studies in preparation for subsequent human studies (Tako et al., 2015b). In addition, the *Gallus gallus* model has been used in numerous studies aimed at assessing iron bioavailability, absorption and status in vivo, specifically to assess the effectiveness of iron-biofortified crops to deliver more absorbable iron to maintain or improve iron status (Tako et al., 2014; Tako et al., 2015; Tako et al., 2016).

Piglets are also a good model for iron bioavailability studies because of their similarities to humans with respect to gastrointestinal anatomy and physiology. However, differences in body fat content may translate into differences in nutrient absorption because of the increased expression of hepcidin, which may inhibit iron absorption (Frazer and Anderson, 2005). The *in vitro* cell model has also been used to investigate the mineral bioavailability and it is especially important for investigating interactions between minerals and enhancers or inhibitors, such as the effect of polyphenolics and phytic acid. However, it lacks communication with other organs that are involved in the regulation of nutrient absorption *in vivo* (Pigeon et al., 2001; Roetto et al., 2003; Scheers et al., 2014).

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Table 3 Advantages and limitations of the methods for assessing the bioavailability of iron and zinc

METHODS	ADVANTAGES	LIMITATIONS
In vitro (Caco-2 cell)	✓ It is less cost, time and work intensive; ✓ It allows a large number of breeding lines to be compared in a single experiment; ✓ It is a better indicator of bioavailability than the solubility method; ✓ It can simulate gastric and intestinal digestion of food ✓ It allows to pinpoint genetic markers for iron bioavailability	✓ It cannot simulate all of the physiological and metabolic responses of the human body; ✓ Variability on the protocols; ✓ Changes in intestinal epithelial permeability because of modification in transporters and metabolic enzymes expression in carcinoma cell; ✓ There are no biomarkers of uptake for zinc; ✓ It lacks communication with other organs
In vivo		
Animal model	✓ It allows to analyze individual tissues to provide a whole-body assessment of absorption; ✓ Faster physiological responses than human; ✓ Poultry model has a quick response to micronutrient deficiency; ✓ Piglet has similarities to humans concerning gastrointestinal anatomy and physiology	✓ No animal model can exactly simulate the physiological response of the human; ✓ Different food intake, energy expenditure, body proportion, intestinal morphology and enteric microbiota from human; ✓ Rats endogenously synthesize ascorbic acid and phytase; ✓ Pigs have differences in body fat content from human; ✓ They practice coprophagy;

Relevance of *in vitro* and *in vivo* studies to evaluate the effectiveness of food biofortification programs

Biofortification involves the development of micronutrient enhanced staple crop varieties via traditional breeding practices or modern biotechnology (Nestel et al., 2006). Biofortification is potentially more sustainable and cost-effective than conventional fortification, and it implicitly targets low-income households in remote areas with a substantial daily consumption of a limited number of food staples and limited access to commercially marketed fortified foods (Meenakshi et al., 2010; Bouis et al., 2011). However, mineral biofortification improves zinc and iron status only if the additional amounts of these minerals provided by the biofortified crop are bioavailable (Cercamondi et al., 2013). Thus, prior to the implementation of an intervention and in addition to agronomic traits, environmental factors, and variability in micronutrient concentrations, bioavailability is a major concern (La Frano et al., 2014).

Bioavailability, which represents the amount of a nutrient that is accessible for utilization in normal physiological functions, metabolism and storage, may be enhanced or inhibited by the presence of food components and food-processing techniques. Therefore, the amount of a mineral that is present in food and available for absorption must be investigated to properly estimate the minimum amount of that mineral that breeders must achieve and to predict the success of these interventions. Therefore, the measurement of zinc and iron absorption from biofortified crops is an important first step prior to demonstrating the efficacy of these crops in improving the status of these minerals (Bouis et al., 2011; La Frano et al., 2014).

Since the nutrient absorption by the body is a prerequisite for the prevention of micronutrient deficiencies the change in the prevalence of mineral deficiencies with the long-term intake of biofortified staple foods must be directly measured. Therefore, human studies that demonstrate the impact of biofortified crops on the micronutrient status are required to provide evidence to support the release of biofortified crops (Bouis et al.,2011).

Zinc and iron bioavailability is an important factor that has been considered in biofortification programs. Thus, studies that evaluate the effectiveness of these programs have focused on this evaluation. *In vitro* studies have been used as a means to screen, rank, and categorize cultivars and foods, substantial numbers of genetic variants, food-processing effects, and influencers of absorption, as well as to direct attention to factors that may deserve further investigation *in vivo* (Etcheverry et al., 2012; La Frano et al., 2014).

Approximately 78% of the iron bioavailability assays reported higher iron absorption from biofortified foods than from conventional foods (Table 1). While, about 62% of the zinc bioavailability studies reported higher zinc absorption from bioforitified foods than from conventional foods (Table 2). These results indicate that zinc- and iron-biofortified foods may represent an effective strategy to combat nutritional deficiencies in populations at nutritional risk.

Conclusion

In vitro and *in vivo* studies have provided knowledge regarding zinc and iron bioavailability in biofortified foods and assessments of the effectiveness of mineral biofortification programs. Thus, these studies have reported higher amounts of absorbed zinc and iron from biofortified foods than from similar conventional foods. These results are likely due to the presence of higher amounts of Zn/Fe in these biofortified foods than in conventional foods.

The *in vitro* Caco-2 cell model cannot provide data directly applicable to humans because it cannot simulate all the physiological and metabolic responses of the organism. However, this model may be used as a useful preliminary screening method to identify promising plant cultivars to be tested in *in vivo* bioavailability assays. Furthermore, this method enables the investigation of interactions between zinc and iron and enhancers or inhibitors of their absorption at different concentrations in a shorter time and at a lower cost than the *in vivo* models.

The utilization of *in vivo* models is necessary to assess the physiological alterations caused by biofortified foods. Animal models enable the dissection and analysis of individual tissues to provide a whole-body assessment of absorption and an understanding of the gene expression alterations caused by biofortified food intake. Isotopic techniques in human studies have also been used to assess zinc and iron absorption rates, and they provide a more accurate measurement of bioavailability.

Human studies also provide direct knowledge regarding the effectiveness of biofortification because they enable the assessment of alterations in zinc and iron nutritional status. However, human studies are longer and more expensive than *in vitro* and animal studies. Thus, the combination of *in vitro* (Caco-2 cells), animal and human studies is most appropriate to investigate zinc and iron bioavailability and the effectiveness of biofortification programs. Therefore, it should be acceptable to perform screening in a cell culture assay as an initial step and subsequently investigate the best variety of foods identified in the cell culture study in animal studies. These biofortified varieties could then be tested in human studies.

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7. MANUSCRIPT II

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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; 18MΩ H_2O; 40mg/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.

Keywords: biofortification; dietary fiber; common beans; iron; brush border membrane; microbial populations

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, 2011). 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, 2011). 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; Dias et al., 2015; Bouis & Saltzman, 2017).

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 (White & Broadley, 2005; HarvestPlus, 2014). In addition, this crop is currently estimated to be one of the most important legumes worldwide, and is an important source of nutrients for more than 300 million people in parts of Eastern Africa and Latin America, representing 65% of total 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 182.9g 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; N. 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 Consortium, 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 minutes 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 hours at 60ºC, ground by stainless steel mill 090 CFT at 3000 rpm and stored at -12°C (Dias et al., 2015; Ramírez-Cárdenasi, Leonel, & Costa, 2008).

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)

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 (Tako, Glahn, et al., 2014; Hou et al., 2017). Briefly, the bean flour samples were dissolved in distilled water (50 g/L) (60ºC, 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 4ºC. 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 less than 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) 40mg/mL Inulin; (4) 50mg/mL Perola bean extract; (5) 50mg/mL Cometa bean extract; (6) 50mg/mL Esteio bean extract; (7) 50mg/mL SMN 39 bean extract; (8) 50mg/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 two hours 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 145ºC for 2 h. Then, the temperature of the heating block raised to 190 ◦C for ten minutes 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 minutes at 8,000 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 µMof 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 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 Advnaced 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.

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.

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 ºC 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 37ºC. 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 $p < 0.05$.

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 ($p<0.05$) compared to the other bean extracts (Figure 1).

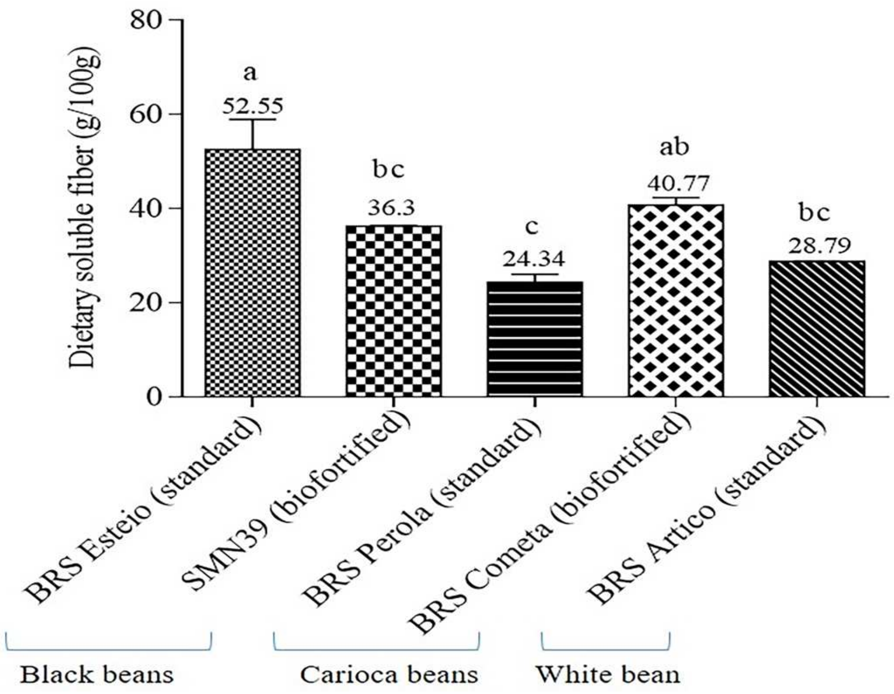
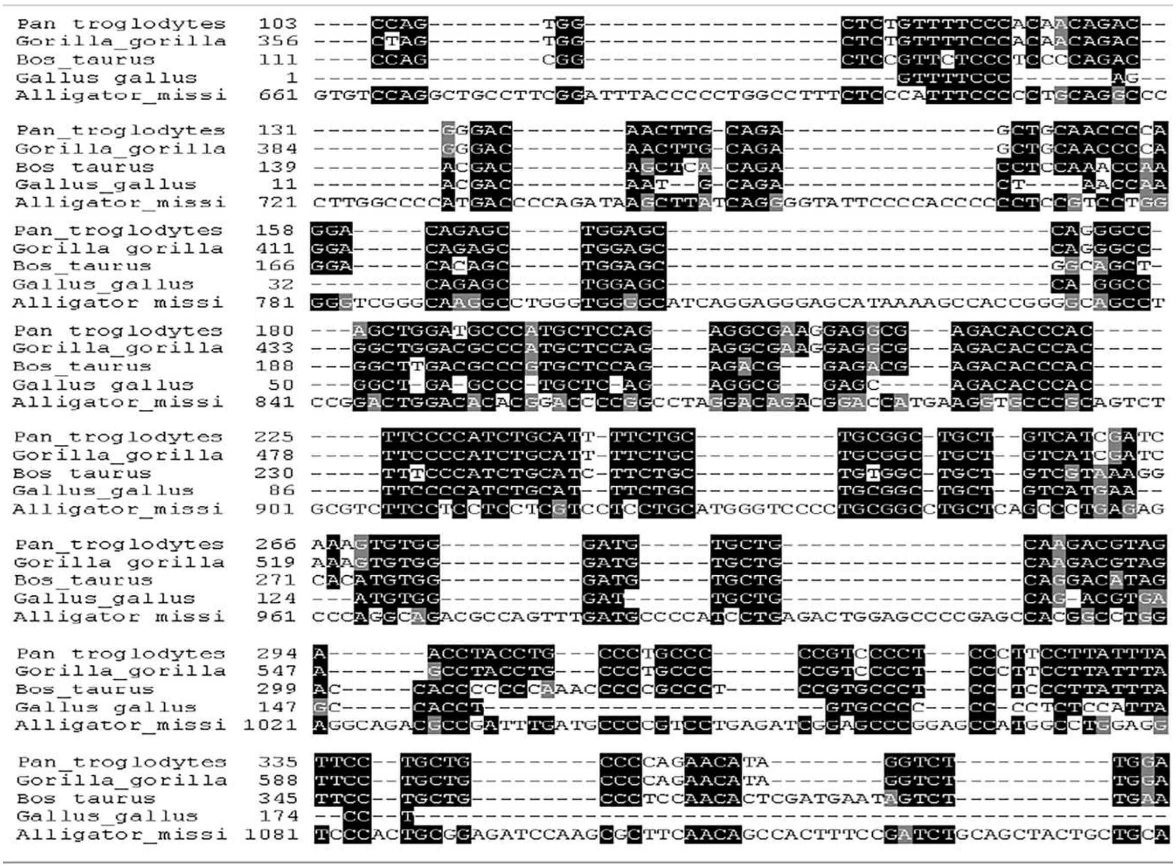


Figure 1. Dietary soluble fiber content in the bean extracts (g/100g of the extracts). Values are means ± SEM. Bean extracts not indicated by the same letter are significantly different ($p<0.05$) by Tukey test.

3.2 Isolation and sequencing of partial chicken hepatic hepcidin cDNA

As shown in Figure 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 miss* hepatic hepcidin genes. The cDNA sequence of the hepatic hepcidin was entered into the BioSample sequence database under accession number SAMN0805649.



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Pan_troglodytes     103  ----CCAG--------TGG------------------CTCTGTTTCCCACAACAGAC--
Gorilla_gorilla     356  ----CTAG--------TGG------------------CTCTGTTTCCCACAACAGAC--
Bos_taurus          111  ----CCAG--------CGG------------------CTCCGTTCTCCCTCCCAGAC--
Gallus_gallus         1  -----------------------------------------GTTTTCCC--------AG--
Alligator_missi     661  GTGTCCAGGCTGCCTTCGGATTTACCCCCTGGCCTTTCTCCCATTTCCCCCTGCAGGCCC

Pan_troglodytes     131  ---------GGGAC--------AACTTG-CAGA---------------GCTGCAACCCCA
Gorilla_gorilla     384  ---------GGGAC--------AACTTG-CAGA---------------GCTGCAACCCCA
Bos_taurus          139  ---------ACGAC--------AGCTCA-CAGA---------------CCTCCAAACCAA
Gallus_gallus        11  ---------ACGAC--------AAT--G-CAGA---------------CT----AACCAA
Alligator_missi     721  CTTGGCCCCATGACCCCAGATAAGCTTATCAGGGGTATTCCCCACCCCCCTCCGTCCTGG

Pan_troglodytes     158  GGA-----CAGAGC----TGGAGC----------------------------CAGGGCC-
Gorilla_gorilla     411  GGA-----CAGAGC----TGGAGC----------------------------CAGGGCC-
Bos_taurus          166  GGA-----CACAGC----TGGAGC----------------------------GGCAGCT-
Gallus_gallus        32  --------CAGAGC----TGGAGC----------------------------CA-GGCC-
Alligator_missi     781  GGGTCGGGCAAGGCCTGGGTGGGGCATCAGGAGGGAGCATAAAGCCACCGGGGCAGCCT

Pan_troglodytes     180  ---AGCTGGATGCCCATGCTCCAG----AGGCGAAGGAGGCG---AGACACCCAC-----
Gorilla_gorilla     433  ---GGCTGGACGCCCATGCTCCAG----AGGCGAAGGAGGCG---AGACACCCAC-----
Bos_taurus          188  ---GGCTTGACGCCCGTGCTCCAG----AGACG---GAGACG---AGACACCCAC-----
Gallus_gallus        50  ---GGCT-GA-GCCC-TGCTC-AG----AGGCG---GAGC-----AGACACCCAC-----
Alligator_missi     841  CCGGACTGGACACACGGACCCCGGCCTAGGACAGACGGACCATGAAGGTGCCCGCAGTCT

Pan_troglodytes     225  -----TTCCCATCTGCATT-TTCTGC-----------TGCGGC-TGCT--GTCATCGATC
Gorilla_gorilla     478  -----TTCCCATCTGCATT-TTCTGC-----------TGCGGC-TGCT--GTCATCGATC
Bos_taurus          230  -----TTTCCCATCTGCATC-TTCTGC-----------TGTGGC-TGCT--GTCGTAAAGG
Gallus_gallus        86  -----TTCCCATCTGCAT--TTCTGC-----------TGCGGC-TGCT--GTCATGAA--
Alligator_missi     901  GCGTCTTCCTCCTCCTCGTCCTCCTGCATGGGTCCCCTGCGGCCTGCTCAGCCCTGAGAG

Pan_troglodytes     266  AAAGTGTGG----------GATG-----TGCTG-----------------CAAGACGTAG
Gorilla_gorilla     519  AAAGTGTGG----------GATG-----TGCTG-----------------CAAGACGTAG
Bos_taurus          271  CACATGTGG----------GATG-----TGCTG-----------------CAGGACATAG
Gallus_gallus       124  ---ATGTGG----------GAT------TGCTG-----------------CAG-ACGTGA
Alligator_missi     961  CCCAGGCAGACGCCAGTTTGATGCCCCATCCTGAGACTGGAGCCCCGAGCCACGGCCTGG

Pan_troglodytes     294  A-------ACCTACCTG---CCCTGCCC------CCGTCCCCT---CCCTTCCTTATTTA
Gorilla_gorilla     547  A-------GCCTACCTG---CCCTGCCC------CCGTCCCCT---CCCTTCCTTATTTA
Bos_taurus          299  AC-----CACCCCCCCAAACCCCGCCCT------CCGTGCCCT---CC-TCCCTTATTTA
Gallus_gallus       147  GC-----CACCT------------------------GTGCCCC---CC-CCTCTCCATTA
Alligator_missi    1021  AGGCAGACGCCGATTTGATGCCCCGTCCTGAGATCGGAGCCCGGAGCCATGGCCTGGAGG

Pan_troglodytes     335  TTCC--TGCTG---------CCCCAGAACATA--------GGTCT-----------TGGA
Gorilla_gorilla     588  TTCC--TGCTG---------CCCCAGAACATA--------GGTCT-----------TGGA
Bos_taurus          345  TTCC--TGCTG---------CCCTCCAACACTCGATGAATAGTCT-----------TGAA
Gallus_gallus       174  --CC--T-----------------------------------------------------
Alligator_missi    1081  TCCCACTGCGGAGATCCAAGCGCTTCAACAGCCACTTTCCGATCTGCAGCTACTGCTGCA
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Figure 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.

3.3 Body weight and cecum-to-bodyweight

There was no significant difference in body weight between treatment (data not shown) ($p > 0.05$). The cecum-to-body weight ratios were ($p<0.05$) higher in the treatment groups and inulin group compared to the negative control (18MΩ H_2O) (Figure 3). The cecum of animals that received bean soluble extracts increased, suggesting an increase in their content of bacterial populations (Figure 4).

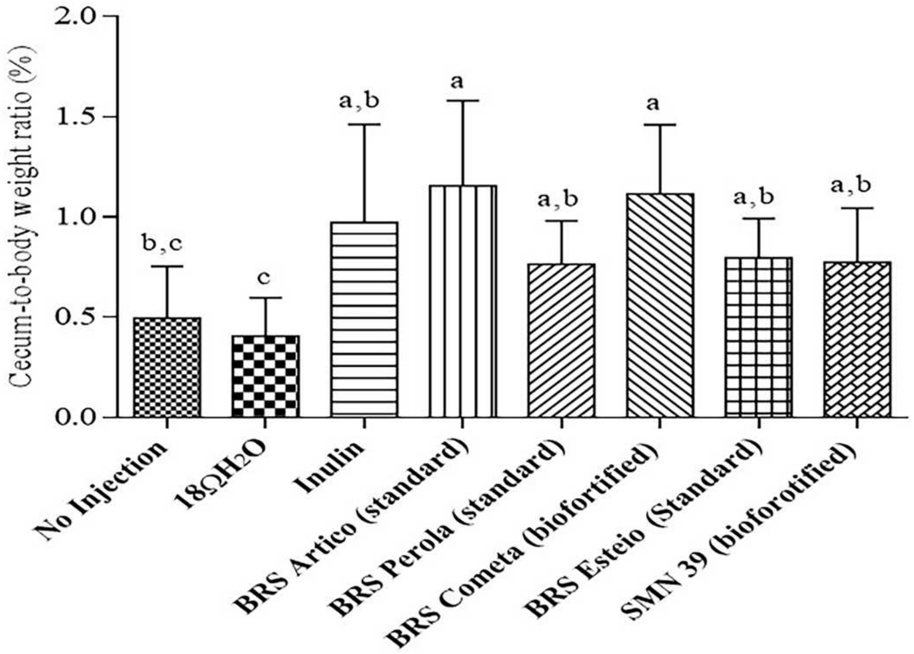
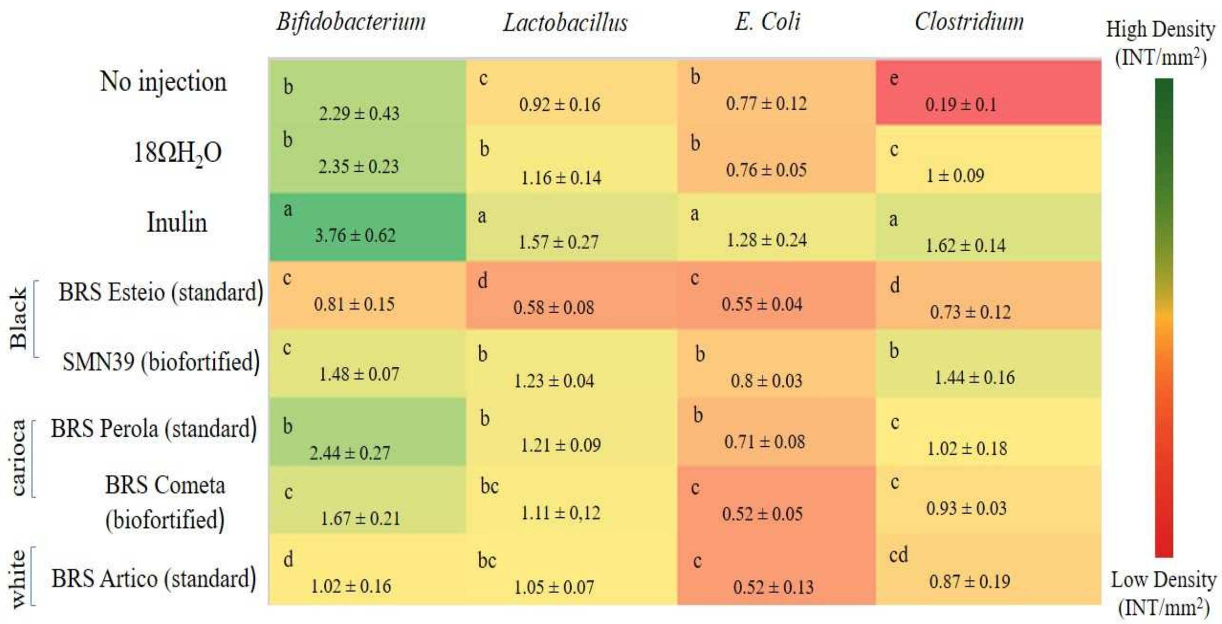


Figure 3. Cecum-to-body weight ratio (%). Values are means ± SEM, n = 12. Treatment groups not indicated by the same letter are significantly different ($p<0.05$) by Newman-Keuls test.

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 ($p< 0.05$) relative abundance of all bacteria genera compared to the negative (non-injected and 18MΩ H_2O) and test groups (Figure 2). The relative abundance of *Bifidobacterium* in the BRS Perola group was higher ($p< 0.05$) compared to the other tested groups. All groups presented lower ($p<0.05$) relative abundance of *E. coli* and *Clostridium* compared to the Inulin group. In general, tested groups reduced ($p< 0.05$) 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) (Figure 4).



		Bifidobacterium	*Lactobacillus*	*E. Coli*	*Clostridium*
	No injection	b 2.29 ± 0.43	c 0.92 ± 0.16	b 0.77 ± 0.12	e 0.19 ± 0.1
	$18\Omega H_2O$	b 2.35 ± 0.23	b 1.16 ± 0.14	b 0.76 ± 0.05	c 1 ± 0.09
	Inulin	a 3.76 ± 0.62	a 1.57 ± 0.27	a 1.28 ± 0.24	a 1.62 ± 0.14
Black	BRS Esteio (standard)	c 0.81 ± 0.15	d 0.58 ± 0.08	c 0.55 ± 0.04	d 0.73 ± 0.12
	SMN39 (biofortified)	c 1.48 ± 0.07	b 1.23 ± 0.04	b 0.8 ± 0.03	b 1.44 ± 0.16
carioca	BRS Perola (standard)	b 2.44 ± 0.27	b 1.21 ± 0.09	b 0.71 ± 0.08	c 1.02 ± 0.18
	BRS Cometa (biofortified)	c 1.67 ± 0.21	bc 1.11 ± 0,12	c 0.52 ± 0.05	c 0.93 ± 0.03
white	BRS Artico (standard)	d 1.02 ± 0.16	bc 1.05 ± 0.07	c 0.52 ± 0.13	cd 0.87 ± 0.19

Figure 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 ($p<0.05$) 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 ($p< 0.05$) Fe concentration compared to the other bean extracts (Figure 5A). However, the higher iron concentration in the extracts did not affect ($p>0.05$) the serum Fe (Figure 5C) and the Fe storage in the liver (Figure 5B).

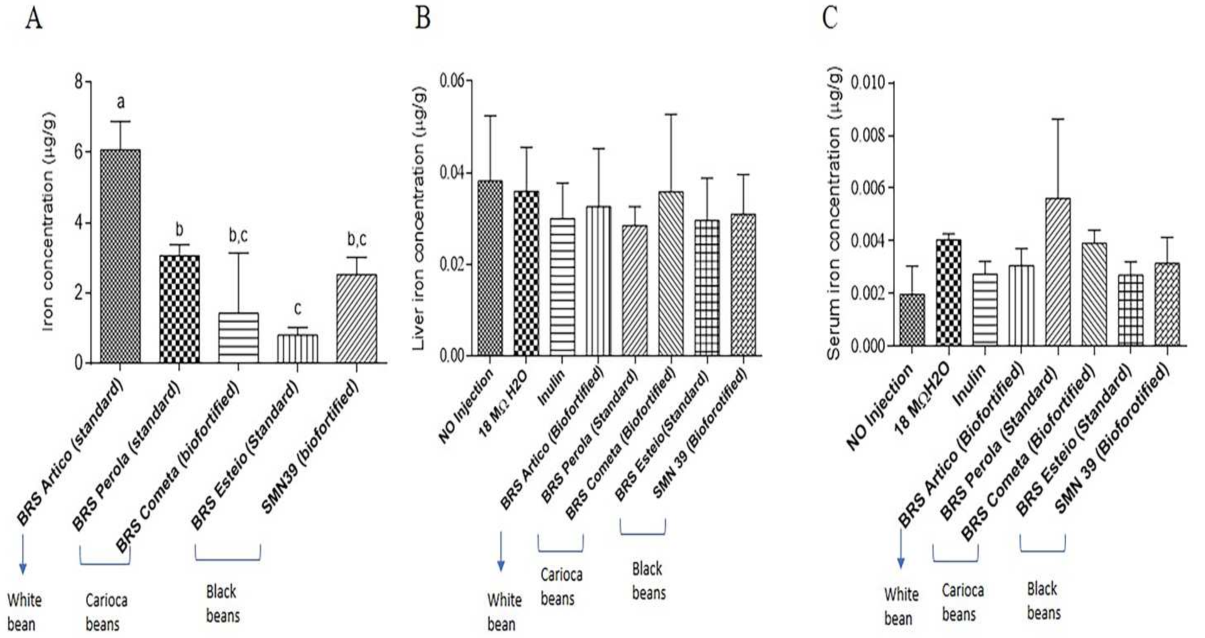
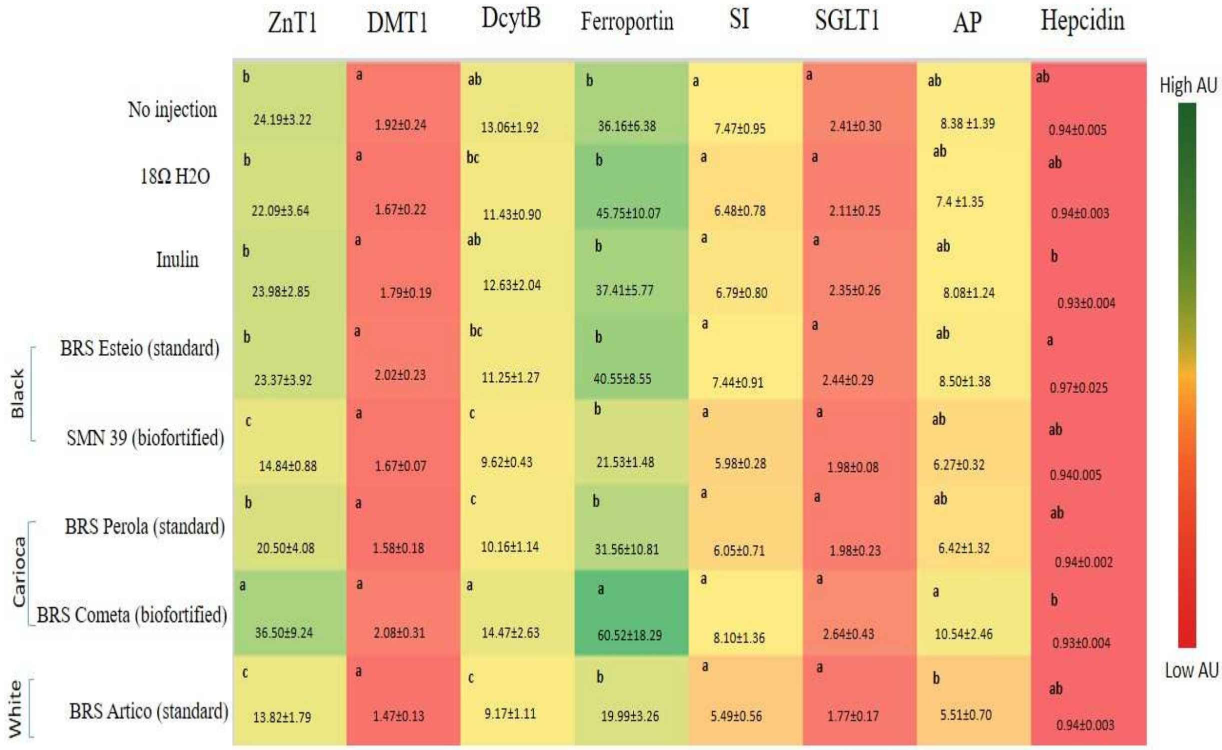


Figure 5. (A): Iron content in the bean prebiotics extracts; (B) Liver iron concentration (n=6); (C) Serum iron concentration (n=4). Values are means ± SEM. Different letters indicate statistical differences at 5% by Newman-Keuls test.

3.5.2 Gene expression of BBM proteins

Figure 6 shows the gene expression of proteins involved in the Fe metabolism and BBM functionality. There was no difference ($p>0.05$) 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 ($p<0.05$) in the presence of soluble extracts from BRS Cometa (carioca bean). Moreover, the relative expression of ZnT1 was down-regulated ($p<0.05$) by SMN39 (black bean) and BRS Artico (white bean). The heat map (figure 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.

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 ($p<0.05$) in the BRS Esteio group and lower ($p<0.05$) in the BRS Cometa group and Inulin group.



		ZnT1	DMT1	DcytB	Ferroportin	SI	SGLT1	AP	Hepcidin
	No injection	b 24.19±3.22	a 1.92±0.24	ab 13.06±1.92	b 36.16±6.38	a 7.47±0.95	a 2.41±0.30	ab 8.38 ±1.39	ab 0.94±0.005
	18Ω H2O	b 22.09±3.64	a 1.67±0.22	bc 11.43±0.90	b 45.75±10.07	a 6.48±0.78	a 2.11±0.25	ab 7.4 ±1.35	ab 0.94±0.003
	Inulin	b 23.98±2.85	a 1.79±0.19	ab 12.63±2.04	b 37.41±5.77	a 6.79±0.80	a 2.35±0.26	ab 8.08±1.24	b 0.93±0.004
Black	BRS Esteio (standard)	b 23.37±3.92	a 2.02±0.23	bc 11.25±1.27	b 40.55±8.55	a 7.44±0.91	a 2.44±0.29	ab 8.50±1.38	a 0.97±0.025
	SMN 39 (biofortified)	c 14.84±0.88	a 1.67±0.07	c 9.62±0.43	b 21.53±1.48	a 5.98±0.28	a 1.98±0.08	ab 6.27±0.32	ab 0.940.005
Carioca	BRS Perola (standard)	b 20.50±4.08	a 1.58±0.18	c 10.16±1.14	b 31.56±10.81	a 6.05±0.71	a 1.98±0.23	ab 6.42±1.32	ab 0.94±0.002
	BRS Cometa (biofortified)	a 36.50±9.24	a 2.08±0.31	a 14.47±2.63	a 60.52±18.29	a 8.10±1.36	a 2.64±0.43	a 10.54±2.46	b 0.93±0.004
White	BRS Artico (standard)	c 13.82±1.79	a 1.47±0.13	c 9.17±1.11	b 19.99±3.26	a 5.49±0.56	a 1.77±0.17	b 5.51±0.70	ab 0.94±0.003

Figure 6. Effect of intra-amniotic administration of experimental solutions on the intestinal gene expression. Values are means ± SEM, n = 5. $^{a\text{-}c}$ Per gene, treatment groups not indicated by the same letter are significantly different (p<0.05) 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.

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-pe 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<0.05) compared to the standard BRS Perola (carioca) (Figure 1). However, both lines, biofortifed BRS Cometa and standard BRS Perola, presented similar ($p > 0.05$) relative abundance of *Lactobacillus*. Also, the relative abundance of these bacterial populations was higher ($p < 0.05$) in the biofortified black beans SMN39 group compared to its corresponding standard BRS Esteio (Figure 4), even though the soluble fiber content in the soluble extracts from BRS Esteio was higher ($p < 0.05$) than in the SMN39 (Figure 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 ($p < 0.05$) 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 (Pacifici et al., 2017; Hou & Tako, 2018).

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 (Eung, Ymond, Ahn, & El, 2005; Welch & Graham, 2004).

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 (Figure 3) in all treatment groups was higher ($p<0.05$) 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 & Glahn, 2012; Tako, Ferket, & Uni, 2005). 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 ($p<0.05$) (Figure 5A), however, this did not affect ($p>0.05$) the Fe storage in the liver nor iron serum concentrations (Figure 5B and 5C). 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 ($p<0.05$) of the gene expression of ZnT1 and AP compared to other groups, whereas the soluble extract from BRS Artico promoted a down-regulation ($p<0.05$) of these proteins (Figure 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 ($p<0.05$) in the BRS Esteio group and lower ($p<0.05$) in the BRS Cometa and BRS Supremo groups (Figure 6). However, this difference did not affect FPN expression and serum iron concentration (Figure 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.

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Conflicts of Interest

The authors declare no conflict of interest.

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8. MANUSCRIPT III

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Fe-biofortified carioca and black beans (*Phaseolus vulgaris* L.)-based Brazilian diet deliver more absorbable iron *in vitro*

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Abstract

Biofortification aims to improve the micronutrient concentration and bioavailability in staple food crops. Common bean (*Phaseolus vulgaris* L.) is one of the crops target for iron (Fe) biofortification program. In this study, we used an *in vitro* (Caco-2 cell) approach to assess the Fe bioavailability from two Fe-standard and Fe-biofortified bean varieties, by themselves and in a combination with other staple food crops. The bean genotypes used in this study were: BRS Perola (carioca, Fe-standard); BRS Cometa (carioca, Fe-biofortified); BRS Esteio (black, Fe-standard) and SMN39 (black, Fe-biofortified). The food proportions used were based on the Brazilian consumption. Both Fe-biofortified beans presented higher ($p<0.05$) Fe and protein concentration compared to the Fe-standard beans. Black beans (BRS Esteio and SMN39) presented higher ($p<0.05$) concentration of myricetin 3-glucoside and quercetin 3-glucoside compared to the carioca beans, whereas carioca beans (BRS Cometa and BRS Perola) presented higher ($p<0.05$) concentration of kaempferol 3-glucoside, epicatechin and catechin. Also, both carioca beans presented higher ($p<0.05$) Fe bioavailability compared to the both black beans and the combination of the beans and other staple food crops increased ($p< 0.05$) the Fe bioavailability of both, carioca and black beans, except for the Fe-standard bean BRS Esteio. Overall, the carioca beans presented a higher iron bioavailability compared to the black beans and the combination of Brazilian staple

food crops with Fe-biofortified carioca and black beans increased the iron bioavailability.

Key-words: biofortification; phytate; polyphenol compounds; Caco-2 cell; iron; staple food crops

1. Introduction

Iron (Fe) deficiency is the most common and widespread nutritional disorder in the world, affecting around 40% of the world population, particularly women and children in developing countries [1,2]. Fe deficiency is highly prevalent in low-income countries (~30% in Brazil) due to a lack of meat consumption in addition to a notable dietary reliance on grains containing high amounts of Fe absorption inhibitors (e.g., phytic acid, polyphenolic compounds) [3–5].

Biofortified staple food crops have become an effective tool by which to address micronutrient deficiencies, especially that of Fe, in many at-risk populations [6–8]. The common bean (*Phaseolus vulgaris*) is one of the crops target for biofortification program since it exhibits sufficient genetic variability in iron concentration, which is the basic requirement for biofortification [9,10]. This crop is currently estimated to be one of the most important legumes worldwide [8,10]. However, a major challenge associated with biofortification of staple food crops, especially common beans, is that they contain factors such as polyphenols and phytic acid that can inhibit Fe bioavailability and absorption, hence limit their nutritional benefit [5,11]. These inhibitory factors may increase with Fe concentration when these crops are biofortified via conventional breeding [5,12]. Hence, it is necessary to measure the Fe concentration, the amount of bioavailable Fe, and the concentration of potential inhibitors of Fe bioavailability in these biofortified crops [12–14]. It is also important to assess the other components of the diet in which these crops are consumed, since the potential interactions can negate or even enhance the expected benefit of increased Fe content.

Studies on iron (Fe) biofortification of the common bean were published as early as 2000, approximately a year or two before the term "biofortification" was coined [6,15]. Before that, experiments were primarily performed in rodent models using intrinsically labelled crops, or in humans with extrinsically labelled foods and meals [6,16]. The cost and limitations of such *in vivo* studies often prevented the experimental approach from addressing important aspects of bean Fe bioavailability, such as the effects of polyphenols, phytate, and the influence of other foods consumed with beans. The coupling of the *in vitro* digestion techniques with Caco-2 cell monolayers was a significant advance that enabled direct *in vitro* examination of factors that influence Fe bioavailability [6,17]. When this *in vitro* approach is further coupled with an established animal model of Fe bioavailability it represents an effective approach to refine the experimental approach for human studies [18–20].

Thus, this study used an *in vitro* (Caco-2 cell) approach to assess the Fe bioavailability from two Fe-standard and Fe-biofortified bean varieties, by themselves and in a combination with other staple food crops. The second objective of this study was to compare the chemical composition of the carioca standard and biofortified beans and black standard and biofortified beans, as well as, to evaluate the Fe concentration in the bean seed coat and cotyledon.

2. Materials and Methods

2.1 Sample characterization and preparation

The characterization and source of the beans used in this study are showed in the Table 1. The common beans were cooked in three replicates in a conventional pressure cooker for 40 minutes using a bean/distilled water ratio of 1:2.7 (w/v) for the carioca beans and 1:28 (w/v) for black beans. The beans were dried in an air oven for 17 hours at 60ºC. The other dry staple foods were purchased at a local market. All dried food were ground by stainless steel mill 090 CFT at 3000 rpm and stored at -12 °C[21].

In order to separate the seed coat from the cotyledon the whole seed was soaked in $18\Omega H_2O$ and the separated parts were freeze-dried and ground by stainless steel mill.

Table 1. Characterization and source of the beans

Source	Bean cultivars	Bean classes
Embrapa (Empresa Brasileira de Pesquisa Agropecuária, Goias, Brazil)	BRS Perola (Fe-standard)	Carioca
	BRS Cometa (Fe-biofortified)	Carioca
	BRS Esteio (Fe-standard)	Black
CIAT (International Center for Tropical Agriculture, Cali, Colombia)	SMN 39 (Fe-biofortified)	Black

2.2 Protein and dietary fiber analysis

Protein concentration was determined by micro-Kjeldahl method according to the AOAC procedure[22]. The determination of total dietary fiber and soluble and insoluble fractions was performed by the enzymatic-gravimetric method. according to AOAC[22], using the enzymatic hydrolysis for a heat-resistant amylase, protease and amyloglucosidase (Total dietary fiber assay Kiyonaga. Sigma®).

2.3 Iron content in the samples

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 ºC for two hours and 0.25 mL of 40 µg/g Yttrium (Sigma-Aldrich, St. Louis, MO, USA) 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 ºC for 2 h. Then, the temperature of the heating block raised to 190 ºC for ten minutes 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).

2.4 Polyphenols analysis

2.4.1 Bean sample preparation

To 1 g of flour beans was added 5 mL of methanol/water (50:50 v/v). The slurry was vortexed for 1 min, placed in a 24 °C sonication water bath for 20 min, vortexed again for 1 min, and centrifuged at 4000g for 15 min. The supernatant was filtered with a 0.45 µm Teflon syringe filter and stored for later use in a −20 °C freezer.

2.4.2 LC-MS Analysis

Extracts and standards were analyzed with a Waters Acquity UPLC. Five microliter samples were injected and passed through an Acquity UPLC BEH Shield RP18. 1.7 µm, 2.1 × 100 mm. column (Waters) at 0.5 mL/min. The column was temperature-controlled at 40 °C. The mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Polyphenols were eluted using linear gradients of 86.7−84.4% A in 1.5 min. 84.4−81.5% A in 0.2 min. 81.5−77% A in 2.8 min. 77−55% A in 0.5 min. 55−46% A in 1 min. and 46−86.7% A in 0.2 min and a 0.8 min hold at 86.7% A for a total 7 min run time.

From the column, flow was directed into a Waters Acquity photodiode array detector set at 300−400 nm and a sampling rate of 20/s. Flow was then directed into the source of a Xevo G2 QTOF mass spectrometer (Waters Corp.), and ESI mass spectrometry was performed in negative ionization mode with a scan speed of 5/s in the mass range from 50 to 1200 Da. Capillary and cone gas voltages were set at 2.3 kV and 30 V, respectively. Desolvation gas flow was 800 L/h. and desolvation gas temperature was 400 °C. Source temperature was 140 °C. Lock-mass correction was used with leucine encephalin as the lock-mass standard and a scan frequency of 25 s. Instrumentation and data acquisition were controlled by MassLynx software. Individual polyphenols in bean samples were tentatively determined by mass using MarkerLynx software. and their identities were confirmed by comparison of LC retention times with authentic standards. Polyphenol standard curves for flavonoids were derived from integrated areas under UV absorption peaks from 10 replications. Standard curves for catechin and 3.4-dihydroxybenzoic acid were constructed from MS ion intensities using 10 replications.

2.5 Phytate analysis in the bean flours

Dietary phytic acid (phytate)/total phosphorus was measured as phosphorus released by phytase and alkaline phosphatase following the kit manufacturer's instructions (K-PHYT 12/12. Magazyme International. Ireland).

2.6 In vitro iron bioavailability assessment

An *in vitro* digestion/Caco-2 cell culture model[17,23] was used to assess Fe-bioavailability. The Fe-biofortified and Fe-standard beans were analyzed by themselves and in combination with staple food crops (rice, potato, corn and pasta). The specific Brazilian dietary formulation used in the study was based on the Brazilian food consumption survey with modifications[24]. In this experiment we reduced the proportion of rice from 39% (165g/day [24]) to 5.5% (22.3g/day) and it was increased the potato proportion from 3% (15.3g/day[24]) to 37% (156g/day). This modification was based on a study that showed that the addition of rice to beans reduced the iron bioavailability. Thus, the food proportion used in this study was: 44% of beans (186.9g/day); 37% of potato (156g/day); 5.8% of corn (20.3g/day); 9.1% of pasta (38.6g/day) and 5.5% of rice (22.3g/day).

The beans by themselves and the food combination were subjected to simulated gastric and intestinal digestion. Briefly, the intestinal digestion is carried out in cylindrical inserts closed on the bottom by a semipermeable membrane and placed in wells containing Caco-2 cell monolayers bathed in culture medium. The upper chamber was formed by fitting the bottom of Transwell insert ring (Corning) with a 15000 Da molecular weight cut off (MWCO) membrane (Spectra/Por 2.1, Spectrum Medical Gardena, CA). The dialysis membrane was held in place using a silicone ring (Web Seal Rochester, NY). Iron uptake by the Caco-2 cell monolayers was assessed by measuring ferritin concentrations in the cells. Six replicates of each Fe bioavailability measurement were performed. In terms of materials for the study, Caco-2 cells were obtained from the American Type Culture Collection (Rockville. MD) at passage 17 and used in experiments at passage 29. Cells were seeded at densities of 50.000 cells/cm2 in collagen treated 6 well plates (Costar Corp Cambridge. MA). The integrity of the monolayer was verified by optical microscopy. The cells were cultured at 37°C in an incubator with 5% CO2 and 95% air atmosphere at constant humidity and the medium was changed every 48 h. The cells were maintained in Dulbecco's modified Eagle medium plus 1% antibiotic/antimycotic solution 25 mmol/L HEPES and 10% fetal bovine serum, 48 h prior the experiment the growth medium was removed from culture wells, the cell layer was washed and the growth medium was replaced with minimum essential media (MEM) at pH 7.0. The MEM was supplemented with 10 mmol/L PIPES 1% antibiotic/antimycotic solution 4 mg/L hydrocortisone, 5 mg/L insulin, 5 μg/L selenium, 34 μg/L triiodothyronine and 20 μg/L epidermal growth factor. This enriched MEM contained less than 80 μg Fe/L. All ingredients and supplements for cell culture media were obtained from GIBCO (Rockville. MD). The cells were used in the Fe uptake experiment at 13 days post seeding. In these conditions, the amount of cell protein measured in each well was highly consistent between wells. On experiment day, 1.5 mL of the digested sample was added to the insert's upper chamber and incubated for 2 h. Then, inserts were removed and 1 mL of MEM was added. Cell cultures were incubated for 22h at 37°C. It was previously shown that intracellular ascorbic acid status might influence ferritin formation (i.e. cellular Fe uptake) in Caco-2 cells [17,25]. Medium was removed from the culture well 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 at pH 7.0. The cells were harvested by adding an aliquot of deionized water and placing them in a sonicator (Lab- Line instruments. Melrose Park. IL). The ferritin and total protein concentrations were determined on an aliquot of the harvested cell suspension with a one-stage sandwich immunoradiometric assay (FERIRON II Ferritin assay, Ramco laboratories Houston TX) and a colorimetric assay (Bio-Rad DC Protein assay, Bio-Rad, Hercules, CA) respectively Caco-2 cells synthesize ferritin in response to increases in intracellular Fe concentration. Therefore, the ratio of ferritin/total protein (expressed as ng ferritin/mg protein) was used as an index of the cellular Fe-uptake.

2.7 Statistical analysis

All values are expressed as means and standard deviation. The bean flours were analyzed in triplicates. Polyphenol results and iron concentration from the bean flours and from seed fractions (cotyledon, seed coat and whole flour) was subjected to analysis of variance (ANOVA), and the post hoc Tukey test was used to compare the groups. The concentration of iron, phytate, dietary fiber and protein were subject to t-test in order to compare the Fe-biofortified bean and Fe-standard bean. The *in vitro* assay was performed in triplicate and the results were analyzed by ANOVA. For significant “F-value”, post hoc test was used to compare test groups. In order to compare the iron bioavailability from the beans by themselves and the beans combined with other foods the results were subjected to the t-test. Statistical analysis was carried out using GraphPad Prism version 5.0 software (GraphPad Software, California, CA, USA). The level of significance was established at $p < 0.05$.

3. Results

3.1 Chemical composition of bean flours

The Fe-biofortified carioca bean (BRS Cometa) presented higher concentration of iron ($p<0.05$) and lower concentration of phytate ($p<0.05$) compared to the Fe-standard carioca bean (BRS Perola), hence BRS Cometa presented lower phytate/Fe molar ratio ($p<0.05$) compared to the Fe-standard bean (Table 2).

Similarly, the Fe-biofortified black bean (SMN39) presented higher concentration of iron ($p<0.05$) compared to the Fe-standard black bean (BRS Esteio). However, the Fe-biofortified bean also presented a higher content of phytate ($p<0.05$), which led to an increase in the phytate/Fe molar ratio ($p<0.05$) in this bean compared to the Fe-standard bean (Table 2).

Table 2. Phytate and iron content and molar ratios of phytate to iron of staple food flours.

Food flours	**Phytate (g/100g)**	**Iron (mg/kg)**	**Phytate/Fe molar ratio**
Carioca beans			
BRS Perola (Fe-standard)	$1.15^{*} \pm 0.018$	$70.39^{*} \pm 1.19$	$13.85^{*} \pm 0.01$
BRS Cometa (Fe-biofortified)	0.9 ± 0.019	94.95 ± 1.29	8.1 ± 0.24
Black beans			
BRS Esteio (Fe-standard)	$0.79^{*} \pm 0.002$	$68.08^{*} \pm 2.31$	$9.84^{*} \pm 0.58$
SMN39 (Fe-biofortified)	1.68 ± 0.01	86.54 ± 2.46	16.45 ± 0.79

Data presented as mean ±SD. * Means significant difference between Fe-standard beans and Fe-biofortified beans by t-test ($p<0.05$).

Interestingly, both Fe-biofortified beans (BRS Cometa and SMN39) presented a higher protein concentration ($p<0.05$) compared to the Fe-standard beans (BRS Perola and BRS Esteio). Moreover, the BRS Cometa (Fe-biofortified) presented higher

($p<0.05$) concentration of total dietary fiber compared to the BRS Perola (Fe-standard) (Table 3).

The polyphenol profile analysis showed that the both black beans presented higher ($p<0.05$) amount of myricetin 3-glucoside and quercetin 3-glucoside compared to the carioca beans, whereas both carioca beans presented higher ($p<0.05$) concentration of kaempferol 3-glucoside, catechin and epicatechin (Table 4). Moreover, the black beans SMN39 (biofortified) presented a higher ($p<0.05$) amount of the procyanidin B1 compared to the other beans.

Table 3: Chemical composition of bean flours (dry basis) (g/100g).

Food flours	Protein (g)	Dietary fiber (g/100g)		
		Soluble	*Insoluble*	*Total*
Carioca beans				
BRS Perola (Fe-standard)	23.98 ± 0.23	3.02 ± 3.39	25.56 ± 0.05	28.58 ± 3.34
BRS Cometa (Fe-biofortified)	26.45* ± 0.31	5.60 ± 2.11	29.75 ± 2.23	35.34* ± 0.12
Black beans				
BRS Esteio (Fe-standard)	22.67 ± 0.02	4.05 ± 2.7	28.47 ± 0.35	32.52 ± 3.05
SMN39 (Fe-biofortified)	24.75* ± 0.81	6.7 ± 4.33	26.91 ± 4.47	33.60 ± 0.14

Data presented as mean ±SD. * Means significant difference between Fe-standard beans and Fe-biofortified beans by t-test ($p<0.05$).

Table 4. Concentration of polyphenol present in common bean flours (μM)

Food flours	Kaempferol 3-glucoside	Catechin	Epicatechin	Procyanidin B1	Myricetin 3-glucoside	Quercetin 3-glucoside	Quercetin
Carioca beans							
*BRS Perola (Fe-standard)	17.3 ± 1^{a}	26.1 ± 1.3^{a}	12.8 ± 1.7^{a}	1.4 ± 0.2^{b}	-	0.2 ± 0.1^{c}	-
*BRS Cometa (Fe-biofortified)	16.2 ± 1.1^{a}	25.9 ± 4.6^{a}	11 ± 1.4^{b}	1.2 ± 0.2^{bc}	-	-	-
Black beans							
BRS Esteio (Fe-standard)	2.4 ± 0.4^{b}	14.7 ± 2.7^{b}	6.9 ± 0.6^{c}	0.7 ± 0.2^{c}	3.9 ± 0.3^{a}	2 ± 0.2^{a}	-
SMN39 (Fe-biofortified)	1.5 ± 0.2^{c}	13.0 ± 1.4^{b}	2.7 ± 0.7^{d}	3.0 ± 0.2^{a}	1 ± 0.1^{b}	0.9 ± 0.1^{b}	0.07 ± 0.01

Data presented as mean ± SD. Means with different letters in the same column present significant difference ($p<0.05$) by Tukey test. * Polyphenol data already published[2].

3.1.1 Iron content in the seed coat, cotyledon and whole flour

In general, the highest iron concentration was found in the seed coat of all beans (carioca and black) (Figure 1, p<0.05). This finding is a contrast with some studies that show a higher iron concentration in the seed cotyledon[26,27].

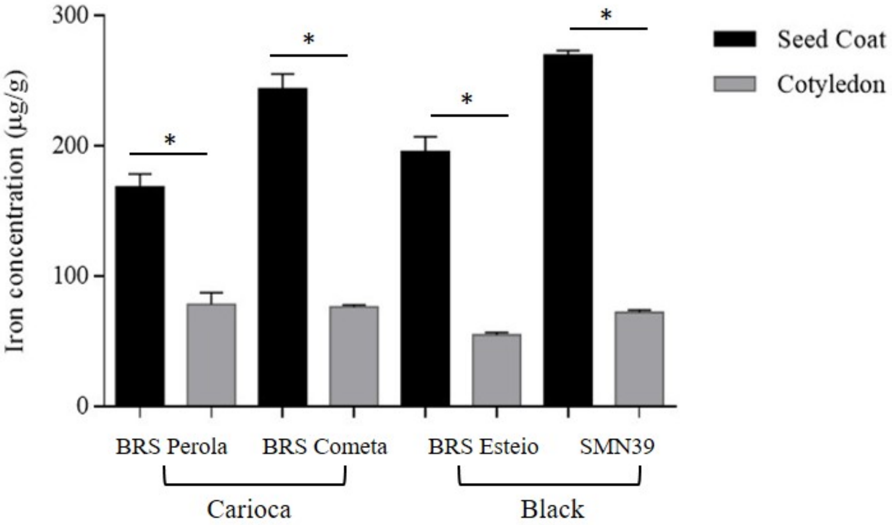

Figure 1. Iron concentration in the seed coat and cotyledon of carioca and black, biofortified and standard beans. BRS Perola (satandard); BRS Cometa (biofortified); BRS Esteio (standard); SMN39 (biofortified). * Means statistical differences at 5% of probability by Tukey test.

3.2 In vitro iron bioavailability

There was no difference (p>0.05) in the iron bioavailability between the Fe-biofortified beans and Fe-standard beans (Figure 2A). However, the carioca beans (BRS Perola and BRS Cometa) by themselves presented higher (p<0.05) iron bioavailability compared to the black beans (BRS Esteio and SMN39) (Figure 2A).

When it was combined other food crops to the beans (carioca and black) it was observed an increase (p<0.05) in the iron bioavailability (Figure 2B, 2B and 2E), except for the BRS Esteio (black, Fe-standard) (Figure 2D).

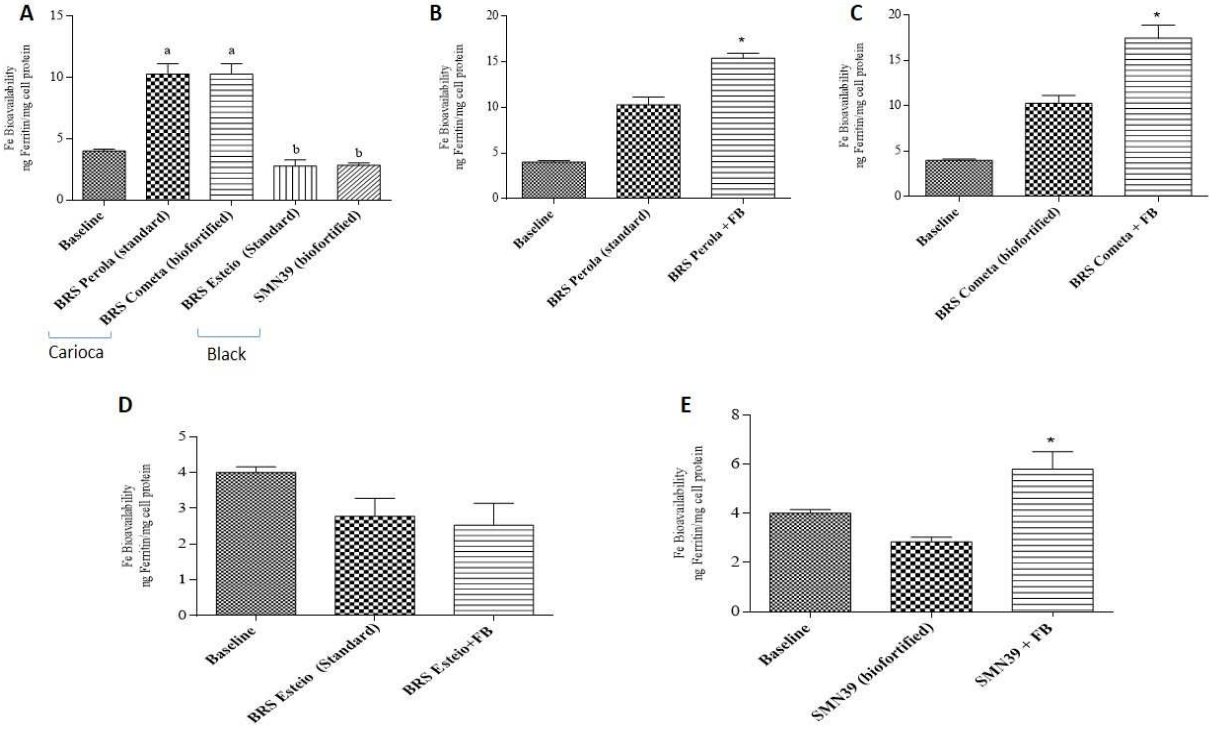

Figure 2. *In vitro* Fe bioavailability comparison between (A) beans by themselves and beans + Food basket (FB). B-C: carioca beans; D-E: Black beans. Iron content in the food basket: 18.4 µg/g ± 5.18. * Means significant difference by t-test ($p<0.05$). ^{a,b} Means statistical difference by Tukey test ($p<0,05$).

4. Discussion

In Fe biofortification studies, there is a clear need and advantage to have in place screening tools capable to evaluate the biofortified lines of staple food crops, both individually and in the context of the diet for which they are consumed[3,5,28]. Such screening tools are also useful in identifying processing steps that can affect Fe content and bioavailability and perhaps negate or enhance the effectiveness of the biofortified crop[29,30]. Thus, we used an *in vitro* (Caco-2 cell) approach to assess the Fe bioavailability from two Fe-standard and Fe-biofortified bean varieties, by themselves and in a combination with other staple food crops.

In this study, as it was expected, both Fe-biofortified beans (BRS Cometa and SMN39) presented higher ($p<0.05$) iron concentration compared to the Fe-standard beans (Table 2). However, unexpectedly, they also presented higher concentration of protein and the BRS Cometa (carioca beans) presented higher dietary fiber content compared to the standard beans (Table 3). These results are especially interesting since a higher protein contented has been show to increase the villi height and intestinal cell proliferation in animals[31]. In addition, undigested dietary proteins and dietary fibers are fermented in the intestine and this fermentation process produces SCFAs (mainly composed by acetate, propionate and butyrate). Functionally, SCFAs affect the metabolism, gut health, and intestinal function, which may increase the mineral absorption[2,32]. Therefore, besides the high iron content, the higher concentration of protein and dietary fiber in these Fe-biofortified beans may contribute to the iron absorption, as it was showed in a recent *in vivo* iron bioavailability study [2].

Contrary to studies that showed that the phytate concentration simultaneously increases with the iron concentration, in this study [11,33,34], the Fe-biofortified carioca bean presented lower ($p<0.05$) concentration of phytate compared to the Fe-standard beans (Table 2). This is a positive finding since the phytate is considerate the most potent inhibitor of iron absorption [11,35,36], which can limit the success of Fe biofortification of beans. Although the Fe-biofortified bean presented higher iron concentration and lower phytate content compared to the Fe-standard bean, the *in vitro* iron bioavailability from both carioca beans was similarly low ($p>0.05$) (Figure 2A). One possible explanation for this effect could be that the higher iron in this bean variety was in the seed coat (Figure 1) outside the cotyledon cells, which is more available[27]. This finding is in a contrast with other studies that show a highest iron concentration is in the seed cotyledon [26,27].

Moreover, the carioca beans (BRS Perola and BRS Cometa) presented higher iron bioavailability compared to the black beans (BRS Esteio and SMN39) by themselves (Figure 2A, $p<0.05$). This is probably due to the fact that the black beans presented higher concentration of polyphenols that can inhibit the iron absorption (quercetin 3-glucoside and myricertin 3-glucoside), whereas the carioca beans presented higher concentration of polyphenols that may increase the iron uptake (kaempferol 3-glucoside, catechin and epicatechin)[5,12,36] (Table 4, $p<0.05$).

Another objective of this study was to investigate if the combination of the beans with other staple food crops which are widely consumed by the Brazilian population could affect the iron bioavailability. In this experiment we reduced the proportion of rice from 39% (165g/day [24]) to 5.5% (22.3g/day) and increased the potato proportion from 3% (15.3g/day[24]) to 37% (156g/day). This modification was based on a study that showed that the addition of rice to beans reduced the iron bioavailability, eliminating the increase in iron uptake from the high iron bean. On the other hand, the addition of potato increases the overall iron uptake from the meal. This is likely due to the fact that potato does contribute more iron to the food matrix relative to rice[6].

Thus, using this proportion, the addition of the staple foods significantly increased ($p<0.05$) the iron bioavailability for the BRS Perola (Figure 2B), BRS Cometa (Figure 2C) and SMN39 (Figure 2E). The association of beans with the other staple foods may have led to a reduction in the phytate and polyphenols concentration in the meal, which led to an increase in the soluble iron available to the iron uptake. Also, the higher amount of potato added to the meal contributed to increase the iron content to the food combination. All these facts contributed to increase the iron bioavailability. On the other hand, this effect was not observed for the BRS Esteio combination (Figure 2D, $p>0.05$). This result may be due to lower iron concentration and the higher concentration of myricertin 3-glucoside and quercetin 3-glucoside in this bean (Table 4, $p<0.05$), which are strong inhibitor of the iron uptake[5,12].

The observations of the present study and others Fe-biofortified bean studies[2,6,11,33] clearly suggest that several factors should be considered when evaluating and advancing Fe-biofortified beans for either iron bioavailability studies or release to farmers. First, it is important to consider how other foods commonly consumed with beans can affect the iron bioavailability. For example, in this study, we observed that, specially the consumption of carioca beans with other staple foods (with high proportion of potato) increased the iron uptake from the meal.

The present study also demonstrates that the high Fe black beans (SMN39) also contained higher levels of uptake-inhibiting compounds, such as phytic acid (Table 3) and polyphenols (Table 4). Increased inhibitory polyphenols were also demonstrated in other lines of high Fe black beans and high Fe pearl millet [14,28]. This consistent association of enhanced iron content with greater polyphenol levels reinforces the need to perform bioavailability assessments of lines targeted for biofortification. Moreover, the finding of increased inhibitory polyphenols and phytic acid with increased iron content is an example of how breeding solely for enhanced iron content could result in a misdirection of breeding. Such a misdirection could result in an end product with no nutritional benefit, despite enhanced content, or one that achieves a less than maximal benefit.

It is important to note that the compounds listed in the present study represent the ones that are currently known to be among the list of polyphenols found in bean seed coats and are known to influence iron bioavailability. There may be others, but this list is simply based upon what has been identified and characterized from previous work that utilized the Caco-2 cell bioassay and LC-MS technology[5,6,12].

Our findings are also in agreement with recent *in vivo* study that demonstrated that the carioca bean-based Brazilian diet delivered more absorbable iron[2]. Therefore, we suggest to further utilize the *in vitro* and *in vivo* screening tools to guide future studies aimed to assess biofortified staple food crops, especially for the carioca and black beans, as this approach will allow proceeding to human efficacy studies more effectively. In addition, these screening tools also have the capacity to cost-effectively monitor Fe biofortified crops once they are released to farmers and dispersed into the food system. Such monitoring will likely be needed to ensure the biofortification effect.

5. Conclusions

Overall, carioca beans presented higher iron bioavailability compared to the black beans, which is likely due to the greater amount of uptake-inhibiting polyphenols, such as myricertin 3-glucoside and quercetin 3-glucoside in these beans. Moreover, the addition of staple food crops, which are widely consumed by the Brazilian population, to the Fe-biofortified beans increased the iron uptake. This effect was not observed for the Fe-standard black bean (BRS Esteio), which also presented lower iron concentration and a higher uptake-inhibitor polyphenol concentration.

Further and similar to previous data, the current research suggests that increased iron content may not necessarily result in an increased absorbable Fe, and a key factor is the measurement of dietary iron bioavailability in Fe biofortified crop variety-based diets, and as part of the breeding process.

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Conflicts of Interest: The authors declare no conflict of interest.

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9. MANUSCRIPT IV

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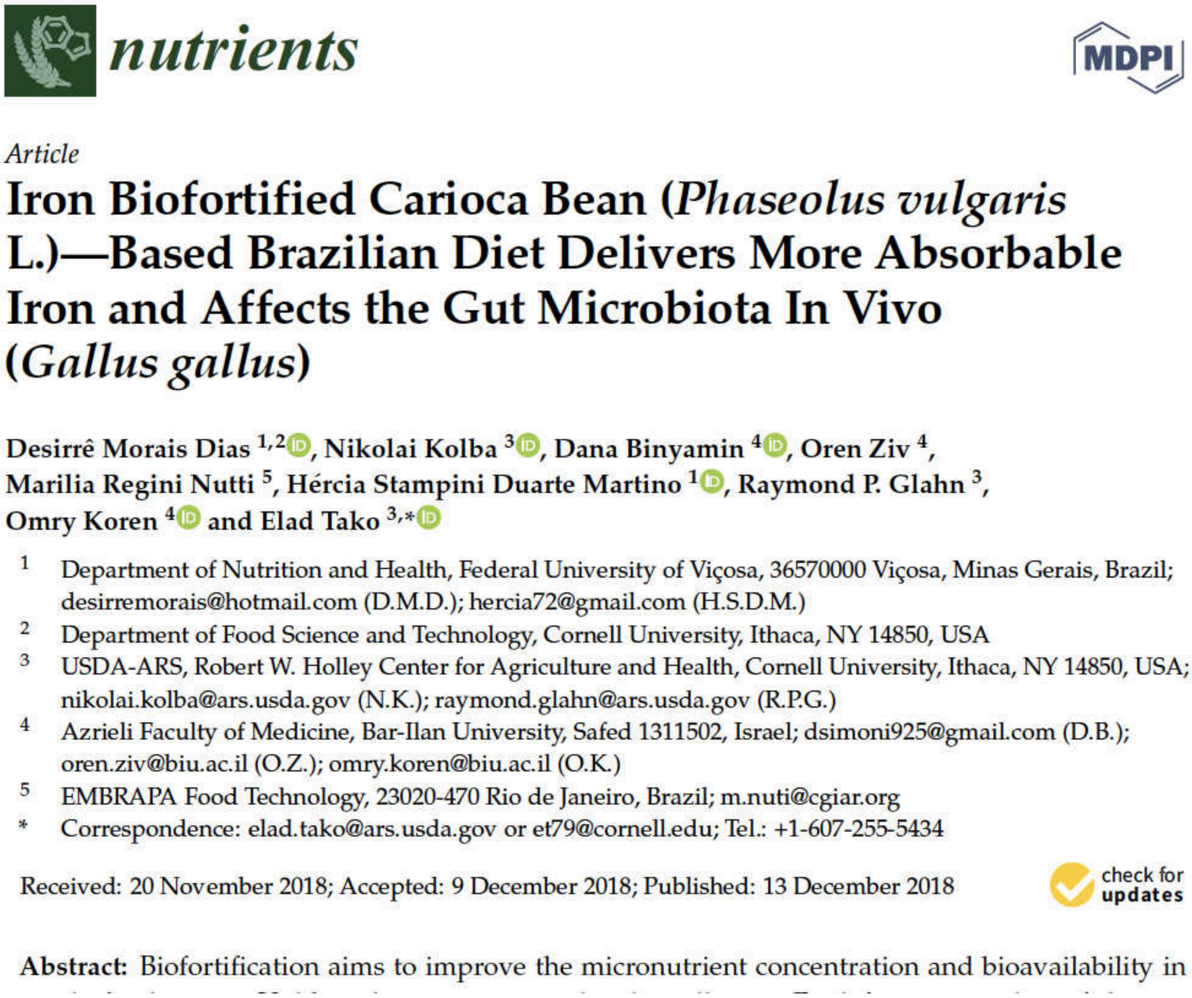

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Article

Iron Biofortified Carioca Bean (*Phaseolus vulgaris* L.)—Based Brazilian Diet Delivers More Absorbable Iron and Affects the Gut Microbiota In Vivo (*Gallus gallus*)

Desirrê Morais Dias [1,2], Nikolai Kolba [3], Dana Binyamin [4], Oren Ziv [4], Marilia Regini Nutti [5], Hércia Stampini Duarte Martino [1], Raymond P. Glahn [3], Omry Koren [4] and Elad Tako [3,*]

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Abstract: Biofortification aims to improve the micronutrient concentration and bioavailability in staple food crops. Unlike other strategies utilized to alleviate Fe deficiency, studies of the gut microbiota in the context of Fe biofortification are scarce. In this study, we performed a 6-week feeding trial in *Gallus gallus* (*n* = 15), aimed to investigate the Fe status and the alterations in the gut microbiome following the administration of Fe-biofortified carioca bean based diet (BC) versus a Fe-standard carioca bean based diet (SC). The tested diets were designed based on the Brazilian food consumption survey. Two primary outcomes were observed: (1) a significant increase in total body Hb-Fe values in the group receiving the Fe-biofortified carioca bean based diet; and (2) changes in the gut microbiome composition and function were observed, specifically, significant changes in phylogenetic diversity between treatment groups, as there was increased abundance of bacteria linked to phenolic catabolism, and increased abundance of beneficial SCFA-producing bacteria in the BC group. The BC group also presented a higher intestinal villi height compared to the SC group. Our results demonstrate that the Fe-biofortified carioca bean variety was able to moderately improve Fe status and to positively affect the intestinal functionality and bacterial populations.

Keywords: iron deficiency; Biofortification; intestinal morphometry; gut microbiome; metagenome; polyphenols

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1. Introduction

Micronutrients deficiency affects approximately two billion people worldwide. Iron (Fe) deficiency is the most prevalent nutrient deficiency, affecting around 40% of the world population, particularly women and children in developing countries [1,2]. It is estimated that around 46% of the population in Africa, 57% in South-East Asia and 19% in America are anemic [3]. Fe deficiency is highly prevalent in low-income countries (~30% in Brazil) due to a lack of meat consumption in addition to a notable dietary reliance on grains containing high amounts of Fe absorption inhibitors (e.g., phytic acid, polyphenolic compounds) [4–7]. Major pathophysiological complications related to insufficient Fe intake may include stunted growth, impaired physical and cognitive development, and increased risk of morbidity and mortality in children [4,8,9]. To alleviate Fe deficiency, an integral step involves the understanding of specific dietary patterns and components that contribute to Fe status in the particular population suffering from a deficiency.

Biofortified staple food crops have become an effective tool by which to address micronutrient deficiencies, especially that of Fe, in many at-risk populations [4,6,10,11]. The common bean (*Phaseolus vulgaris*) is one of the crops target for biofortification program since it exhibits sufficient genetic variability in iron concentration, which is the basic requirement for biofortification [12,13]. This crop is currently estimated to be one of the most important legumes worldwide [13,14], and is an important source of nutrients for more than 300 million people in parts of Eastern Africa and Latin America, representing 65% of total protein consumed, 32% of energy, and a major source of micronutrients (vitamins and minerals) [10,14,15].

Previous studies using Fe biofortified beans in Mexico [16], and Rwanda [17,18] have shown some improvement in Fe status in subjects consuming the biofortified beans versus a standard bean variety. However, a major challenge associated with biofortification of staple food crops, especially common beans, is that they contain factors such as polyphenols and phytic acid that can inhibit Fe bioavailability and absorption, hence limit their nutritional benefit [17,19]. These inhibitory factors may increase with Fe concentration when these crops are biofortified via conventional breeding [17,19,20]. Hence, as was previously suggested, it is necessary to measure the concentration of Fe, the amount of bioavailable Fe, and the concentration of potential inhibitors of Fe bioavailability in these biofortified crops [19,21,22]. It is also important to factor in and assess the other components of the diet in which these crops are consumed as the potential interactions can negate or even enhance the expected benefit of increased Fe content.

Despite containing inhibitory factors, legumes also carry other substances, referred to as promoters, which have the potential to counteract the effects of the inhibitory factors [19,20,23,24]. One of the most notable promoters are prebiotic [19,25,26]. Prebiotics have been characterized as a group of carbohydrates that resist digestion and absorption in the gastrointestinal tract (small intestine), that beneficially affect gut health, by enhancing the growth and activities of probiotics [26–28] and can improve mineral absorption [29]. These compounds can survive the acidic and enzymatic digestion in the small intestine, and thus can be fermented by probiotics that reside in the colon/cecum [30]. The fermentation of prebiotics by probiotics leads to the production of short-chain fatty acids (SCFA), which may improve the intestinal function, increasing the absorption of minerals such as Fe [25,31–33]. At the same time, some polyphenols present in the common beans can stimulate the growth of commensal and beneficial microbiota while pathogenic strains are inhibited or unaffected [34].

Biofortified crops have become an effective tool by which to address micronutrient deficiencies, especially that of Fe, in many at-risk populations [15,35,36]. By using the combination of a Caco-2 cell bioassay and an in vivo (*Gallus gallus*) model that has been used extensively for nutritional research and shown to be an excellent model to assess dietary Fe (and Zinc) bioavailability [4,37–39], the objective of the current study was to evaluate the ability of the Fe biofortified carioca bean line to deliver more Fe for hemoglobin (Hb) synthesis. Also, we aimed to evaluate the effect of the Fe biofortified carioca bean intake on the intestinal microbiota composition and function. If this in vivo assessment indicates that nutritional benefit exists, we suggest to further employ these screening tools to guide future studies aimed to assess biofortified staple food crops, as this approach will allow proceeding to human efficacy studies with greater confidence and success.

2. Materials and Methods

2.1. Sample Preparation

The two carioca bean lines: BRS Perola (Fe Standard) and BRS Cometa (Fe Biofortified) that were used in this study were obtained from Embrapa (Empresa Brasileira de Pesquisa Agropecuaria, Goias, Brazil), and were shipped to Ithaca, New York in sealed containers imported as flours. 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) and dried in an air oven for 17 h at 60 ◦C. The dried beans were ground by stainless steel mill 090 CFT at 3000 rpm and stored at −12 ◦C [40].

2.2. Polyphenols Analysis

2.2.1. Polyphenol Extraction

1 g of bean flour was added with 5 mL of methanol/water (50:50 v/v). The slurry was vortexed for 1 min, placed in a 24 ◦C sonication water bath for 20 min, vortexed again for 1 min and centrifuged at 4000× g for 15 min. The supernatant was filtered with a 0.45 µm Teflon syringe, filtered, and stored for later use at −20 ◦C. 2.2.2. Ultra Performance Liquid Chromatography—Mass Spectrometry (UPLC—MS) Analysis of Polyphenols Extracts and standards were analyzed with a Waters Acquity UPLC (Waters, Milford, MA, USA). Five microliter samples were injected and passed through an Acquity UPLC BEH Shield RP18, 1.7 µm. 2.1 × 100 mm column (Waters, Milford, MA, USA) at 0.5 mL/min. The column was temperature-controlled at 40 ◦C. The mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Polyphenols were eluted using linear gradients of 86.7–84.4% A in 1.5 min, 84.4–81.5% A in 0.2 min, 81.5–77% A in 2.8 min, 77–55% A in 0.5 min, 55–46% A in 1 min and 46–86.7% A in 0.2 min and a 0.8 min hold at 86.7% A for a total 7 min run time. From the column flow was directed into a Waters Acquity photodiode array detector set at 300–400 nm and a sampling rate of 20/s. Flow was then directed into the source of a Xevo G2 QTOF mass spectrometer (Waters, Milford, MA, USA) and ESI mass spectrometry was performed in negative ionization mode with a scan speed of 5/s in the mass range from 50 to 1200 Da. Capillary and cone gas voltages were set at 2.3 kV and 30 V respectively. Desolvation gas flow was 800 L/h. and desolvation gas temperature was 400 ◦C. Source temperature was 140 ◦C. Lock-mass correction was used with leucine encephalin as the lock-mass standard and a scan frequency of 25s. Instrumentation and data acquisition were controlled by MassLynx software (version 4.2, Waters, Milford, MA, USA). Individual polyphenols in bean

samples were tentatively determined by mass using MarkerLynx software (Waters, Milford, MA, USA), and their identities were confirmed by comparison of LC retention times with authentic standards. Polyphenol standard curves for flavonoids were derived from integrated areas under UV absorption peaks from 10 replications. Standard curves for catechin and 3.4-dihydroxybenzoic acid were constructed from MS ion intensities using 10 replications.

2.3. Phytate Analysis

Dietary phytic acid (phytate)/total phosphorus was measured as phosphorus released by phytase and alkaline phosphatase, following the kit manufacturer's instructions (n = 5) (K-PHYT 12/12. Megazyme International. Bray, Ireland).

2.4. Iron Content of Bean Flour, Serum and Liver

The bean flour samples and liver samples (0.5 g) and serum (100 μL) were treated with 3.0 mL of 60:40 HNO3 and HClO4 mixture into a Pyrex glass tube and left for overnight to destroy organic matter. The mixture was then heated to 120 ◦C for two hours and 0.25 mL of 40 μg/g Yttrium (Sigma-Aldrich, Nutrients 2018, 10, 1970 4 of 20 St. Louis, MO, USA) 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 ten minutes 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).

2.5. Protein and Dietary Fiber Analysis in the Bean Flour

Protein concentration was determined by micro-Kjeldahl method according to the Official Methods of Analysis (AOAC International, Rockville, MD, USA) procedure [41]. The determination of total fiber and soluble and insoluble fractions was performed by the enzymatic-gravimetric method according to AOAC [41], using the enzymatic hydrolysis for a heat-resistant amylase, protease and amyloglucosidase (Total dietary fiber assay Kiyonaga, Sigma®, Kawasaki, Japan).

2.6. In Vitro Iron Bioavailability Assessment

An established in vitro digestion/Caco-2 cell culture model was used to assess Fe-bioavailability [37,42]. The staple food flour samples (biofortified and standard beans, rice, potato) were analyzed by themselves and in a food combination ("food basket"). With this method, the cooked bean samples, additional meal plan components and the formulated diets were subjected to simulated gastric and intestinal digestion. 0.5 g of the freeze dried cooked beans and diet samples were utilized for each replication (n=6) of the in vitro digestion [11,21,43].

2.7. Harvesting of Caco-2 Cells for Ferritin Analysis

The protocols used in the ferritin and the total protein contents analyses of Caco-2 cells were similar to those previously described [19,22,23,37,39,44]. 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 an indicator of cellular Fe uptake. All glassware used in the sample preparation and analyses was acid washed.

2.8. Animals, Diets and Study Design

Cornish cross—fertile broiler eggs (n=60) 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. Upon hatching (hatchability rate = 50%), chicks were allocated into 2 treatment groups on the basis of body weight and blood hemoglobin concentration (aimed to ensure equal concentration between groups), (1) Fe-standard carioca bean based diet (SC): 42% carioca bean (BRS Perola) based diet (n = 14), and (2) Fe-biofortified carioca bean based diet (BC): 42% carioca bean (BRS Cometa) based diet (n=14). Experimental diets (Table 1) had no supplemental Fe. The specific Brazilian dietary formulation used in the study (Table 1) was based on the Brazilian food consumption survey [45]. Chicks were housed in a total confinement building (4 chicks per 1 m2 metal cage). The birds were under indoor controlled temperatures and were provided 16 h of light. Each cage was equipped with an automatic nipple drinker and a manual self-feeder. All birds were given ad libitum access to water. Feed intakes were measured daily (as from day 1), and Fe intakes were calculated from feed intakes and Fe concentration in the diets. The body weight and the hemoglobin concentration in the blood were measured weekly.

Table 1. Composition of the experimental bean based diets$^{1–3}$

Ingredient	Fe content (μg Fe/g sample)	Fe-standard carioca based diet (SC) (g/kg by formulation)	Fe-biofortified carioca bean based diet (BC) (g/kg by formulation)
BRS Perola (Fe-standard bean)	64.3 ± 0.54	420	-
BRS Cometa (Fe-biofortified bean)	84.97 ± 2	-	420
Potato	12.89 ± 0.43	320	320
Corn	31.36 ± 4.74	70	70
Pasta (non-enriched)	13.82 ± 1.04	70	70
Rice	4.21 ± 0.8	50	50
Vitamin/mineral premix (no Fe)	0.0	70	70
DL-Methionine	0.0	2.5	2.5
Vegetable oil	0.0	30	30
Choline chloride Total (g)	0.0	0.75	0.75
Total (g)			
Selected components			
Dietary Fe concentration (μg/g)	-	40.47 ± 1.84	47.04 ± 1.52*
Phytic acid (μg/g)	-	1.71 ± 0.16*	1.15 ± 0.053
Phytate:Fe molar ratio	-	35.76	20.84

[1] Vitamin and mineral premix provided/kg diet (330002 Chick vitamin mixture; 235001 Salt mix for chick diet; Dyets Inc. Bethlehem, PA, USA). 2 Iron concentrations in the dietswere determined by an inductively-coupled argon-plasma/atomic emission spectrophotometer. 3 Method for determining phytate is described in the materials and method s section. * Statistical difference by t-test at 5% of probability (Comparison between Standard diet and Biofortified diet).

2.9. Blood Analysis, Hemoglobin (Hb) Determination, and Tissue Collection

Blood samples were collected weekly from the wing vein (100 μL) using micro-hematocrit heparinized capillary tubes (Fisher, Pittsburgh, PA, USA). Weekly blood Hb concentrations were determined spectrophotometrically using the Triton/NaOH method following the kit manufacturer's instructions. Fe bioavailability was calculated as hemoglobin maintenance efficiency (HME):

$$\text{HME} = \frac{\text{Hb-Fe, mg (final)–Hb-Fe, mg (initial)}}{\text{Total Fe intake, mg}} \times 100$$

where Hb-Fe (index of Fe absorption) = total body hemoglobin Fe. Hb-Fe was calculated from hemoglobin concentrations and estimates of blood volume based on body weight (a blood volume of 85 mL per kg body weight is assumed):

$$\text{Hb-Fe (mg)} = \text{BW (kg)} \times 0.085 \text{ blood/Kg} \times \text{Hb (g/L)} \times 3.35 \text{ mg Fe/g Hb}$$

At the end of the experiment (day 42), birds were euthanized by CO_2 exposure. The digestive tracts (small intestine and cecum) and livers were quickly removed from the carcass. The samples were immediately frozen in liquid nitrogen, and then stored in a −80 °C freezer until further analysis.

All animal protocols were approved by the Cornell University Institutional Animal Care and Use Committee (protocol name: Intestinal uptake of Fe and Zn in the duodenum of broiler chicken: extent, frequency, and nutritional implications; approved: 15 December 2016; protocol number: 2007–0129).

2.10. Isolation of Total RNA from Chicken Duodenum and Liver

Total RNA was extracted from 30 mg of the proximal duodenal tissue ($n = 8$) and liver ($n = 8$) 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 an 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).

2.11. Real Time Polymerase Chain Reaction (RT-PCR)

As was previously described [46], cDNA was used for each 10 µL reaction together with 2× BioRad SSO Advnaced Universal SYBR Green Supermix (BioRad, 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 seven 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 (Bio-Rad Laboratories, Hercules, CA, USA) using the following PCR conditions: initial denaturing at 95 ºC for 30 s, 40 cycles of denaturing at 95 ºC for 15 s, various annealing temperatures according to Integrated DNA Technologies (IDT) for 30 s and elongating at 60 ºC 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 ºC) 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.

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	
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; 18S rRNA, 18S Ribosomal subunit; SI, Sucrose isomaltase; SGLT-1: Sodium-Glucose transport protein 1; AP, Amino peptidase; TRCP1: Transferrin Receptor Protein 1; BBM, Brush border membrane.

2.12. 16S rRNA Gene Amplification and Sequencing

Microbial genomic DNA was extracted from cecal samples using the PowerSoil DNA isolation kit, as described by the manufacturer (MoBio Laboratories Ltd., Carlsbad, CA, USA). Bacterial 16S rRNA gene sequences were PCR-amplified from each sample using the 515F-806R primers for the V4 hypervariable region of the 16S rRNA gene, including 12-base barcodes, as previously published [47]. PCR procedure reactions consisted of 25 μL Primestar max PCR mix (Takara Kusatsu, Shiga, Japan), 2 μM of each primer, 17 μL of ultra-pure water, and 4 μL DNA template. Reaction conditions consisted of an initial denaturing step for 3 min at 95 ºC followed by 30 cycles of 10 s at 98 ªC, 5 s at 55 ªC, 20 s at 72 ªC, and final elongation at 72 ºC for 1 min. PCR products were then purified with Ampure magnetic purification beads (Beckman Coulter, Atlanta, GA, USA) and quantified using a Quant-iT PicoGreen dsDNA quantitation kit (Invitrogen, Carlsbad, CA, USA). Equimolar ratios of total samples were pooled and sequenced at the Faculty of Medicine of the Bar Ilan University (Safed, Israel) using an Illumina MiSeq Sequencer (Illumina, Inc., Madison, WI, USA).

2.13. 16S rRNA Gene Sequence Analysis

Data analysis was performed using QIIME2 [48]. Sequence reads were demultiplexed by per-sample barcodes and Illumina-sequenced amplicon reads errors were corrected by Divisive Amplicon Denoising Algorithm (DADA2) [49]. A phylogenetic tree was generated and sequences were classified taxonomically using the Greengenes [50] reference database at a confidence threshold of 99%. The Greengenes taxonomies were used to generate summaries of the taxonomic distributions of features across different levels (phylum, order, family, and genus). Alpha and beta diversity analysis were calculated based on a feature table with samples containing at least 7026 sequences. Richness and evenness, alpha diversity parameters, were calculated using the Faith's Phylogenetic Diversity and Pielou's Evenness measures [51]. Beta diversity was analyzed using weighted and unweighted UniFrac distances [52]. Linear discriminant analysis Effect Size (LEfSe) [53] was used to determine the features significantly differ between samples according to relative abundances. Metagenome functional predictive analysis was carried out using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) [54] software (version 1.1.3). Briefly, feature abundance was normalized by 16S rRNA gene copy number, identified and compared to a phylogenetic reference tree using the Greengenes database, and was assigned functional traits and abundance based on known genomes and prediction using the Kyoto Encyclopedia of Genes and Genomes (KEGG). Data representing significant fold-change differences in functional pathways between experimental groups was plotted.

2.14. Morphological Examination

As was previously described [26,46], intestinal samples (duodenal region as the main intestinal Fe absorption site) were collected at the conclusion of the study and from each treatment group. Samples were fixed in fresh 4% (v/v) buffered formaldehyde, dehydrated, cleared, and embedded in paraffin. Serial sections were cut at 5 µm and placed on glass slides. Sections were deparaffinized in xylene, rehydrated in a graded alcohol series, stained with hematoxylin and eosin, and examined by light microscopy. Morphometric measurements of villus height, width and goblet cell diameter were performed with a light microscope using EPIX XCAP software (Standard version, Olympus, Waltham, MA, USA).

2.15. Statistical Analyses

The in vivo and in vitro results were analyzed by ANOVA using the general linear models procedure of SAS software (SAS Institute Inc., Cary, NC, USA), and differences between treatment groups were compared by using the Student's *t*-test and values were considered statistically different at $p < 0.05$ (values in the text are means ± SEM). For the microbiome results, the Faith's Phylogenetic Diversity and Pielou's Evenness measures difference between groups were analyzed by Kruskal–Wallis (pairwise) test. Differences between Weighted/Unweighted UniFrac distances were analyzed by Pairwise permanova test. Analysis of composition of microbiomes (ANCOM) is a bioinformatics method to identify features that are differentially abundant (i.e., present in difference abundances) across sample groups. Significant *p*-values ($p < 0.05$) associated with microbial clades and functions identified by LEfSe were corrected for multiple comparisons using the Benjamini Hochberg false discovery rate (FDR) correction. Statistical analysis was performed using SAS version 9.3 (SAS Institute, Cary, NC, USA). The level of significance was established at $p < 0.05$.

3. Results

3.1. Phytate Concentration and Polyphenol Profile in the Bean Flours

The concentration of the five most prevalent polyphenolic compounds found in the bean seed coats is presented in Table 3. The Fe-standard beans (BRS Perola) presented higher ($p < 0.05$) concentration of epicatechin and quercetin 3-glucoside compared to the Fe-biofortified beans (BRS Cometa). There was no difference ($p > 0.05$) in the phytate ($n = 5$) concentration between the Fe-biofortified and Fe-standard carioca bean flour.

Table 3. Phytate concentration and polyphenol profile (µM) present in common bean flours

Food flours	Phytate (g/100g)	Polyphenol profile				
		Kaempferol 3-glucoside	**Catechin**	**Epicatechin**	**Procyanidin B1**	**Quercetin 3-glucoside**
BRS Perola (Fe-standard)	1.05 ± 0.03	17.3 ± 1	26.1 ± 1.3	12.8 ± 1.7*	1.4 ± 0.2	0.2 ± 0.1
BRS Cometa (Fe-biofortified)	1.08 ± 0.00	16.2 ± 1.1	25.9 ± 4.6	11 ± 1.4	1.2 ± 0.2	-

Values are means ± SEM. *Statistical difference by t-test at 5% of probability.

3.2. Dietary Fiber and Protein Concentration in the Bean Flours

There was no difference ($p > 0.05$) in the insoluble, soluble and total dietary fiber. However, the protein concentration is higher ($p < 0.05$) in the Fe-biofortified bean (BRS Cometa) compared to the Fe-standard bean (BRS Perola) (Table 4).

Table 4. Dietary fiber and protein concentration in the beans (g/100 g).

Beans	Insoluble Fiber	Soluble Fiber	Total Fiber	Total Protein
BRS Perola (Fe-standard)	20.80 ± 0.02	3.77 ± 1.03	24.56 ± 1.05	24.15 ± 0.44
BRS Cometa (Fe-biofortified)	18.71 ± 0.94	4.85 ± 0.33	23.55 ± 1.27	29.01 * ± 0.29

* Statistical difference by *t*-test ($p = 0.0001$).

3.3. In Vitro Assay (Caco-2 Cell Ferritin Formation)

Ferritin, the cellular Fe storage protein was used as an indicator of Fe bioavailability [42,43]. Ferritin concentrations were significantly higher in cells exposed to the Fe-biofortified (BC) bean based diet versus the Fe-standard (SC) bean based diet ($p < 0.05$, $n = 6$, Table 5). These results indicate greater amounts of bioavailable Fe in the Fe-biofortified bean based diet.

Table 5. Ferritin concentration in Caco-2 cells exposed to samples of bean based diets, and additional meal plan ingredients [1–2].

Tested Sample	Ferritin (ng/mg of Protein)
Tested Diets	
Standard Fe diet (SC) (40.4 ± 1.8 µg/g diet)	5.04 ± 0.37
Biofortified Fe diet (BC) (47.0 ± 1.5 µg/g diet)	6.10 ± 0.29 *
Ingredients	
BRS Perola (Fe-standard bean) (64.3 ± 0.5 µg/g bean)	7.87 ± 1.15 [d]
BRS Cometa (Fe-biofortified bean) (84.9 ± 2 µg/g bean)	5.74 ± 0.34 [d]
Potato flour (12.8 ± 0.4 µg/g flour)	21.74 ± 0.83 [a]
Pasta flour (13.8 ± 1.0 µg/g flour)	12.79 ± 0.60 [b]
Corn flour (31.3 ± 4.7 µg/g flour)	10.38 ± 0.94 [c]
Rice flour (4.2 ± 0.8 µg/g flour)	6.12 ± 1.02 [d]

[1] Caco-2 bioassay procedures and preparation of the digested samples are described in the materials and methods sections. [2] Cells were exposed to only MEM (minimal essential media) without added food digests and Fe (n = 6). All samples were run in the same experiment. [a–d] Values are means ± SEM. Different letters indicate statistical differences at 5% by Newman–Keuls test. * Indicates statistical differences at 5% by t-test between the experimental diets.

3.4. In Vivo Assay (Gallus Gallus Model)

3.4.1. Growth Rates, Hb, Hb-Fe, and HME

The feed intake and the Fe intake were higher ($p < 0.0001$) in the BC group (average consumption of 56.49 g diet/day ± 0.8) and cumulative (day 42) Fe intake of 111.3 mg Fe ± 1.5, compared to the SC group (average consumption of 51.06 g diet/day ± 2.1), and cumulative (day 42) Fe intake of 86.9 mg Fe ± 3.3. In addition, as from day 14 of the study, body weights were consistently higher ($p < 0.05$) in the group receiving the Fe-biofortified bean diet versus the group receiving the standard bean diet (Figure 1A). There were no significant differences ($p > 0.05$) in the hemoglobin concentrations between the treatments at any time point (Figure 1B). As from day 21, the total body Hb-Fe was significantly greater in the group receiving the Fe-biofortified carioca bean ($p < 0.05$, Figure 1C). However, no differences in HME values were measured between the groups ($p > 0.05$, Figure 1D).

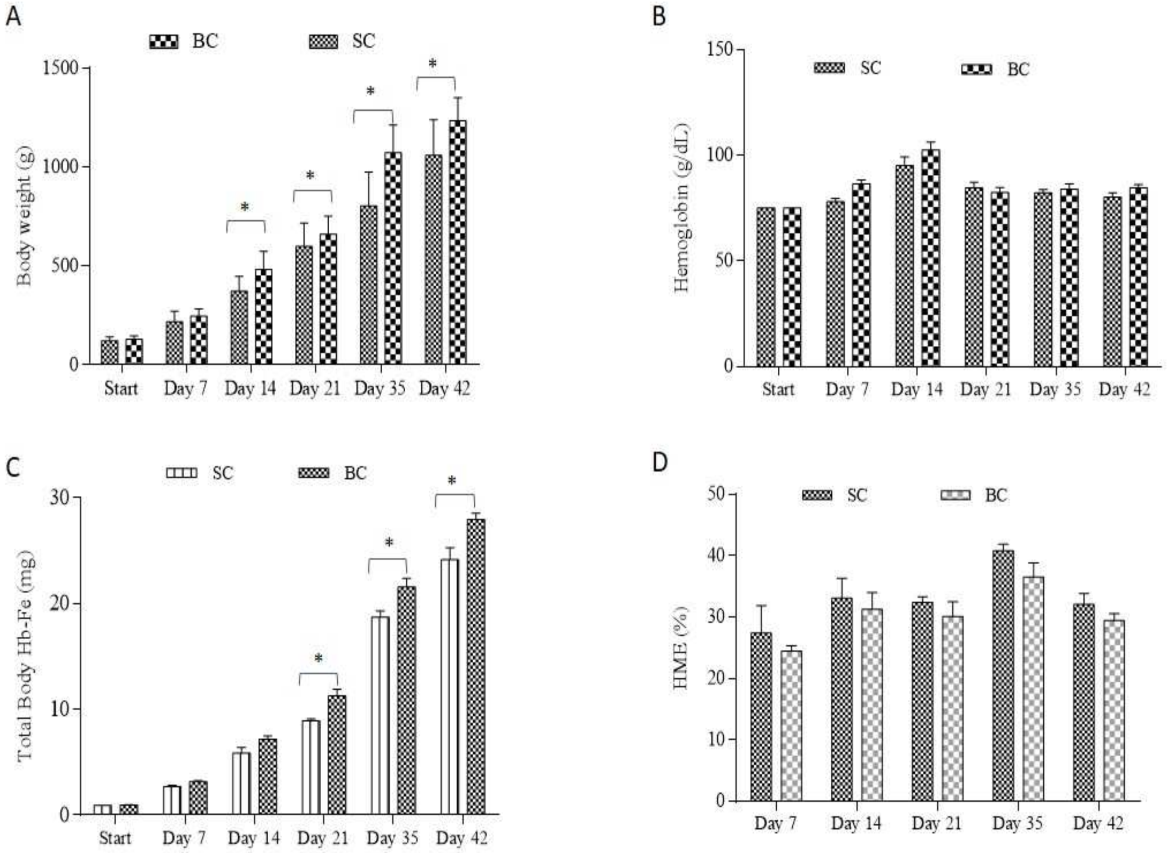

Figure 1. Fe-related parameters assessed during the study. (**A**): Body weight (g); (**B**) Blood hemoglobin concentration (g/L); (**C**): Total body Hb-Fe (mg); (**D**): Hemoglobin maintenance efficiency (%). Values are means ± SEM. * Statistical difference by *t*-test at 5% of probability. SC: Fe-standard carioca bean diet; BC: Fe-biofortified carioca bean diet.

3.4.2. Gene Expression of Fe—Related and BBM Functional Proteins

Relative to 18S rRNA, duodenal gene expression of ferroportin was significantly elevated ($p < 0.05$) in the group receiving the Fe-biofortified carioca bean based diet (BC) (Figure 2). However, no significant differences ($p > 0.05$) in the expression of the other Fe-related proteins were observed between treatment groups (Figure 2).

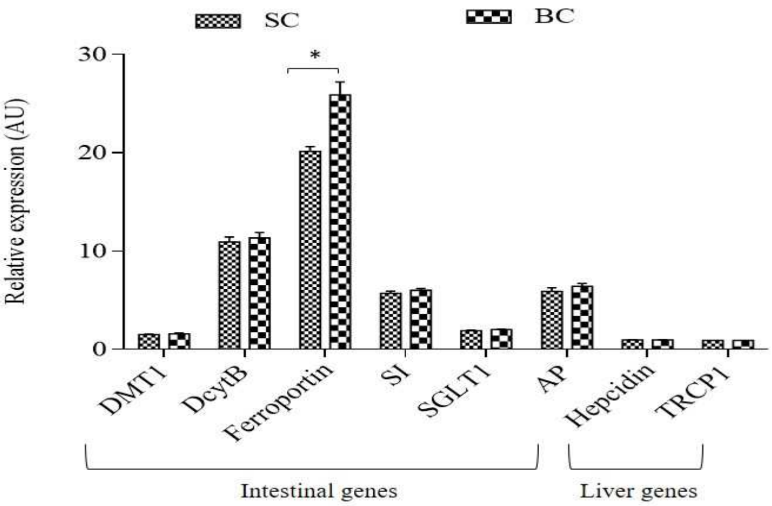

Figure 2. Duodenal and liver mRNAgene expression of Fe-related proteins collected on day 42. Changes in mRNA expression are shown relative to expression of 18S rRNA in arbitrary units (AU, * $p < 0.05$). SC: Fe-standard carioca bean diet; BC: Fe-biofortified carioca bean diet; DMT1: Divalent Metal Transporter 1; DcytB: Duodenal cytochrome b; SI: Sucrose isomaltase; SGLT1: Sucrose isomaltase 1; AP: Amino peptidase; TRCP1: Transferrin Receptor Protein 1.

3.4.3. Morphometric Measurements

The BC group presented higher ($p < 0.0001$) villi height (Figure 3A) and diameter (Figure 3B) compared to the SC group. This serves as a mechanical measurement of brush border membrane absorptive ability and improvement in brush border membrane functionality and overall gut health [56]. It indicates that the consumption of Fe biofortified carioca beans could lead to a proliferation of enterocytes.

There were no significant differences ($p > 0.05$) in goblet cells (mucus producing and secreting cells) number per intestinal villi. However, the goblet cell diameter was slightly higher ($p < 0.05$) in the SC group (4.74 µM ± 2.03) compared to the BC group (4.56 µM ± 1.84).

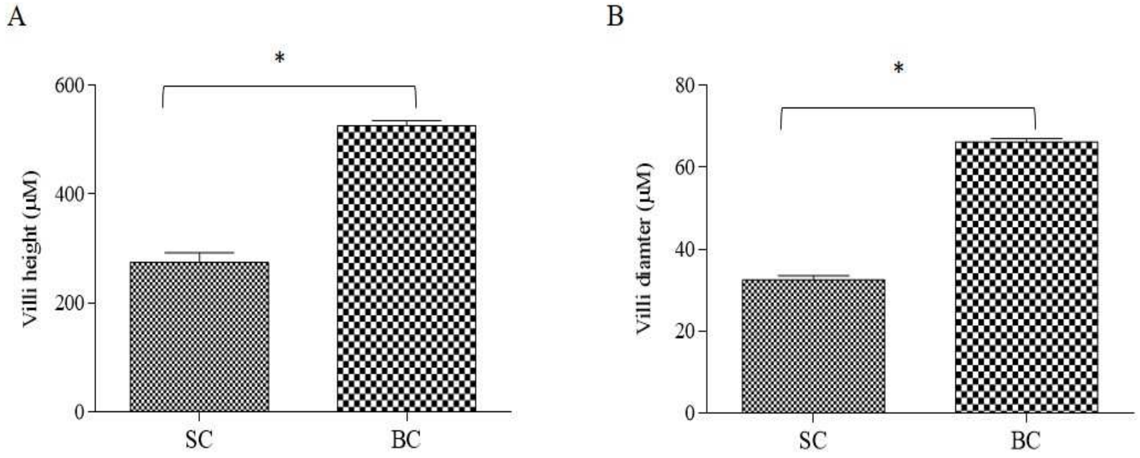

Figure 3. Effect of Standard and Biofortified diets on the duodenal small intestinal parameters: (**A**) Intestinal villi height (μM); (**B**) Intestinal villi diameter. A representation of intestinal morphology from two experimental groups is shown (**C**). SC: Fe-standard carioca bean diet; BC: Fe-biofortified carioca bean diet. Values are means ± SEM, $n = 5$. * Statistical difference by *t*-test ($p < 0.0001$).

3.4.4. Microbial Analysis

Comparisons were made between Fe-biofortified carioca bean diet (BC) and Fe-standard carioca bean diet (SC) beans groups. Cecal contents samples from the standard and biofortified varieties were collected and used for bacterial DNA extraction and sequencing of the V4 hypervariable region in the 16S rRNA gene. The contents of the cecum highly diverse and abundant microbiota and represent the primary site of bacterial fermentation [57].

The diversity of the cecal microbiota between the standard carioca bean (SC) and biofortified carioca bean (BC) was assessed initially through measures of α and β-diversity. Faith's phylogenetic diversity, used to assess α-diversity (Figure 4A), was not significant between SC and BC groups ($p > 0.05$). We utilized unweighted UniFrac distances as a measure of β-diversity to assess the effect of BC diet on between-individual variation in bacterial community (Figure 4B). Principal coordinate analysis showed statistically significant difference in clustering between the BC and SC groups, suggesting that individual samples were more similar to other samples within the same group, as opposed to samples of the other group ($p > 0.05$). Furthermore, individual samples of the BC group clustered significantly closer to each other than did members of the SC group ($p < 0.05$).

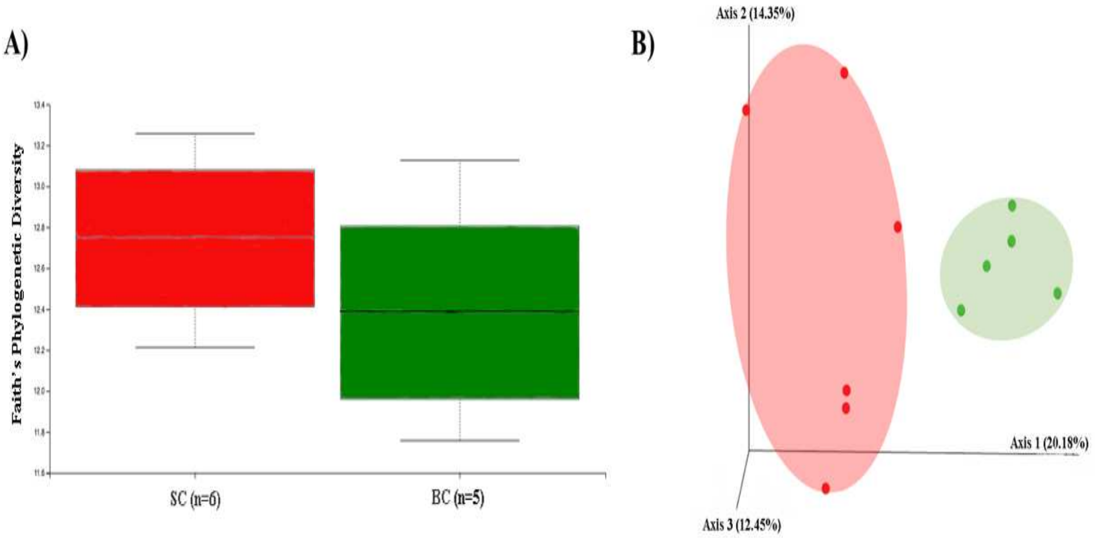

Figure 4. Microbial diversity of the cecal microbiome in Carioca diet. SC: Fe-standard carioca bean diet; BC: Fe-biofortified carioca bean diet. (A) Measure of α-diversity using the Faith's Phylogenetic Diversity; and (B) Measure of β-diversity using unweighted UniFrac distances separated by the first three principal components (PCoA). Each dot represents one animal, and the colors represent the different treatment groups within Carioca beans (red=SC; green=BC).

Following α and β-diversity, we conducted a taxon-based analysis of the cecal microbiota. 16S rRNA gene sequence revealed that >98% of all bacterial sequences in both treatment groups of the carioca variety. Both of the treatment groups were dominated by two major phyla: Firmicutes and Proteobacteria, whereas sequences of Tenericutes and Verrucomicrobia were also identified, but in much lower abundance. After FDR correction, there were no significant differences between groups at the genus level for the carioca variety (Figure 5A,B). As in the human gut [56], the Firmicutes phyla vastly predominated in the Gallus gallus cecum [57].

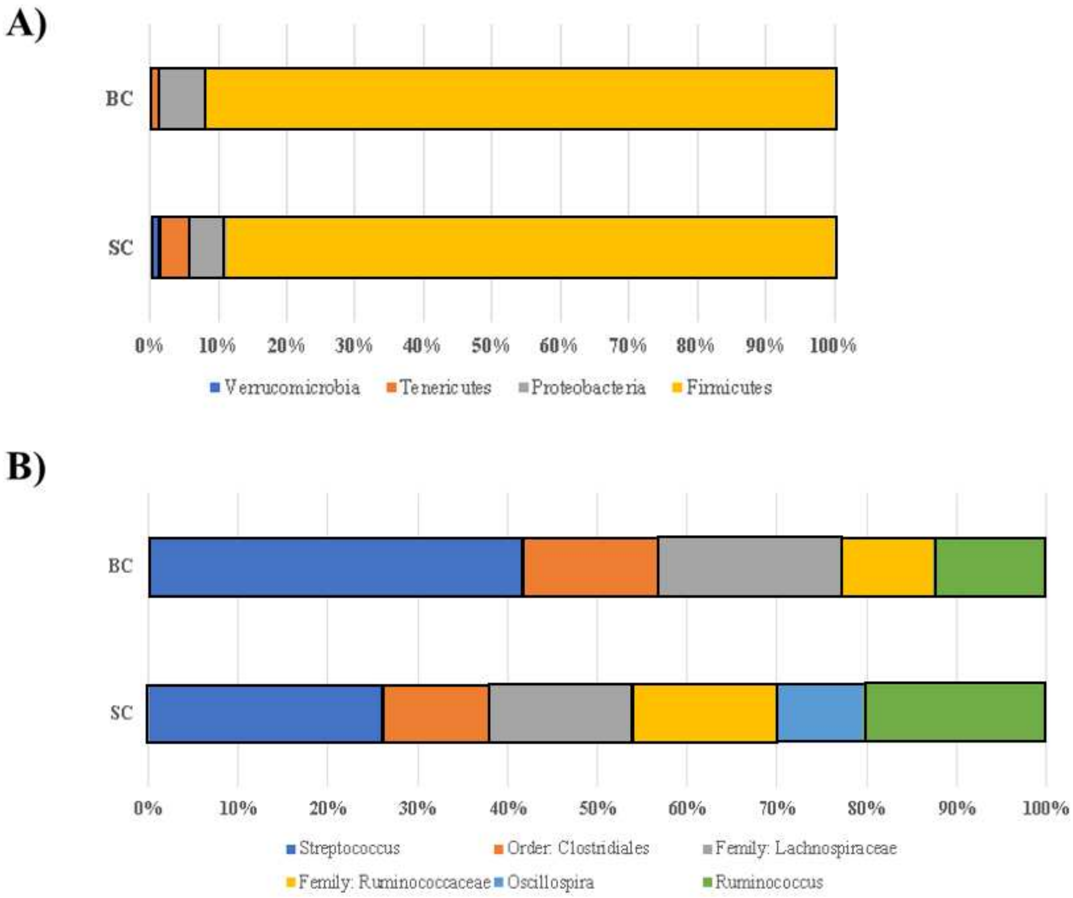

Figure 5. Compositional changes of gut microbiota in response to a Carioca standard versus biofortified diet. SC: Fe-standard carioca bean diet; BC: Fe-biofortified carioca bean diet. (A) Phylum level changes in the BC and SC groups as measured at the end of the study (day 42). Only phyla with abundance ≥1% are displayed; (B) Genus level changes in the BC and SC groups as measured at the end of the study (day 42). Only genera with abundance ≥5% are displayed.

The final analysis investigation of relative abundances at all taxonomic levels with carioca beans was carried out using the linear discriminant analysis effect size (LEfSe) method to investigate significant bacterial biomarkers that could identify differences in the gut microbiota of SC and BC groups [53]. Figure 6 A, B present the differences in abundance between groups at the various taxonomic levels, with their respective LDA (Linear discriminant analysis) scores. We observed a general taxonomic delineation between the SC and BC groups, whereby the SCFA-producing Firmicutes predominated in the BC groups. Specifically, Eggerthella lenta (LDA score = 3.65, p = 0.011) and Clostridium piliforme (LDA = 3.90, p = 0.006); there were members of the Coriobacteriaceae (LDA = 3.65, p = 0.011), Dehalobacteriaceae (LDA = 3.52, p = 0.044), Lachnospiraceae (LDA = 3.90, p = 0.006) were significantly enriched in the BC group. In the SC group, however, members of the Firmicutes, Tenericutes and Proteobacteria were the predominantly-enriched phyla. Specifically, Ruminococcus albus (LDA score = 3.72, p = 0.017), and members of the Oscillospira (LDA score = 4.41, p = 0.044) and Clostridium (LDA score = 3.75, p = 0.006) genera were significantly enriched in the SC group.

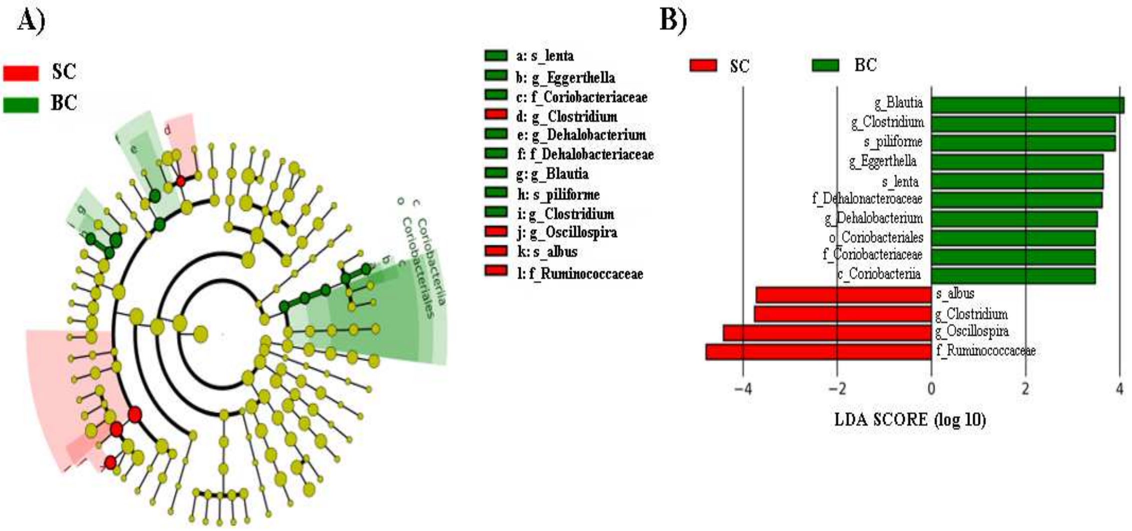

Figure 6. LEfSe method identifying the most differentially enriched taxa in the Standard and Biofortified Carioca diet groups. SC: Fe-standard carioca bean diet; BC: Fe-biofortified carioca bean diet. (A) Taxonomic cladogram obtained using LEfSe analysis of the 16S rRNA sequences. Treatment groups are indicated by the different colors, with the brightness of each dot proportional to its effect size; (B) Computed LDA (Linear discriminant analysis) scores of the relative abundance difference between the standard Carioca bean diet and the biofortified Carioca bean diet. Negative LDA scores (red) are enriched in standard Carioca bean diet while positive LDA scores (green) are enriched in biofortified Carioca beans.

4. Discussion

In studies of Fe biofortification, there is a clear need and advantage to have in place screening tools capable of evaluating biofortified lines of staple food crops, both individually and in the context of the diet for which they are consumed [4,7,36]. The present study, therefore evolved as an opportunity to demonstrate how the in vitro digestion/Caco-2 cell model and the *Gallus gallus* in vivo model of Fe bioavailability could be applied in the design of an Fe bioavailability study aimed at assessing the Fe bioavailability of Fe biofortified versus standard carioca beans. The diets that were used were specifically formulated according to the Brazilian dietary survey [47] (Table 1). Overall, the data presented in this manuscript are in agreement with previously published research [4,19,22], indicating that this dual in vitro/in vivo screening approach is effective in the assessment of Fe bioavailability of Fe biofortified beans.

The in vivo results showed that although Hb levels were not significantly increased in the Fe biofortified carioca bean group, significant differences in total body Hb-Fe, a sensitive biomarker of dietary Fe bioavailability and status [60], were observed starting on week four of the study (Figure 1), indicating on an improvement in Fe status in the Fe biofortified group. In addition, the animals receiving the standard bean variety had a higher HME at each time point when compared to the group receiving the Fe biofortified carioca beans, indicating an adaptive response (e.g., a relative up-regulation of absorption) to less absorbable dietary Fe [4,22,23,39]. The Fe-biofortified carioca bean diet presented higher Fe content and lower PA: Fe ratio compared to the Fe-standard carioca bean diet (Table 1), which could contribute to the higher dietary Fe bioavailability of this group [61–63].

Additionally, the in vitro assay (Table 5) further supported the in vivo findings. Ferritin values in cells exposed to the Fe-biofortified bean variety only, were low and similar to ferritin values in cells exposed to the standard bean variety only. In contrast, once the Fe biofortified bean variety was included in the experimental bean based diet, an increase in ferritin formation was observed relative to cells exposed to the standard bean based diet. This could be due to the higher Fe content and the lower PA: Fe ratio presented in the Fe-biofortified bean based diet, but can also be attributed to the other dietary ingredients, and their potential effect on dietary Fe bioavailability.

These results are in agreement with previous studies aimed at assessing the Fe promoting effects of Fe-biofortified black beans [19], red mottled beans [45] and pearl millet [22]. Thus, since a number of intrinsic factors, including polyphenol compounds and phytates, may influence the bioavailability of Fe from these beans and other crops [4,17,20,64], and limit their nutritional benefit. This suggests that increased bean Fe concentration alone may not be sufficient to yield significant physiological improvements in Fe status. In this context, it is important to note that in addition to increased Fe content, the Fe-biofortified bean variety had a higher protein content ($p < 0.05$, Table 4), this may further affect the nutritional benefit of this bean variety. Current results are in agreement with recent research indicating that dietary ingredients as potato may enhance the Fe absorption when consumed with beans, whereas other foods consumed with beans, as rice, might negatively affect Fe bioavailability (in vitro) [11].

Previous studies have shown a higher concentration of polyphenolic compounds (PP) and phytate in the Fe-biofortified beans compared to the Fe-standard beans [11,19,65]. However, in the current study the Fe-biofortified carioca bean presented lower concentrations of some PPs and no difference in the phytate concentration compared to the Fe-standard carioca bean (Table 3). This is an interesting finding since the PPs and phytate are known as strong inhibitors of Fe bioavailability [19,23,24,66]. Thus, this Fe-biofortified variety could be a more effective vehicle for the Fe biofortification program. This point was demonstrated, as the totality of the results indicated that the Fe biofortified carioca bean based diet was moderately effective at increasing the bioavailable and therefore absorbable dietary Fe both in vitro and in vivo.

Further, the duodenal gene expression of ferroportin (FPN) was significantly elevated in the group receiving the Fe-biofortified bean diet ($p < 0.05$, Figure 2). However, no significant differences in the expression of the other Fe-related and brush border membrane functional proteins were observed between treatment groups. In contrast, some studies have shown a down-regulation of the gene expression of these proteins (DMT-1, ferroportin and Dcytb) in Fe-biofortified diets compared to the Fe-standard diets [22,37,67,68]. Ferroportin is an Fe exporter protein that transfer the Fe across the basolateral membrane of the enterocyte [34]. Thus, since the BC group presented a higher expression of FPN, more Fe can be released from the enterocyte into the blood circulation, therefore, this mechanism suggests increased amounts of absorbable Fe, hence, the total body Hb-Fe increased in the Fe biofortified group compared to the standard.

As is the case in humans and the vast majority of animals, the *Gallus gallus* model harbor a complex and dynamic gut microbiota [69], heavily influenced by host genetics, environment and diet [70]. There is considerable similarity at the phylum level between the gut microbiota of *Gallus gallus* and humans, with Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria representing the four dominant bacterial phyla in both [71]. In the current study, a general taxonomic delineation between the SC and BC group was observed, whereby the SCFA-producing Firmicutes predominated in the BC group. Specifically, *Eggerthella lenta* and *Clostridium piliforme* (Figure 6B). The increase in the SCFA-producing bacteria could lead to an increased SCFA concentration in the intestinal lumen, which in return can promote intestinal cell proliferation [72], as was observed in the BC group that presented an increase in duodenal villi height (Figure 3). This observation is in agreement with previous research indicating that duodenal villi height was significantly increased due to dietary fiber (as xylooligosaccharides) that have led to increased SCFA bacterial production in vivo [73]. Also, the Fe-biofortified bean presented a higher, although not significant, soluble fiber content compared to the Fe-standard bean (Table 4). Soluble fiber can increase the villi height by increasing the intestinal cell proliferation [74] (Figure 3).

In addition, and as was mentioned above, the Fe-biofortified bean presented higher ($p < 0.05$) protein content compared to the Fe-standard bean (Table 4), a higher protein contented in a diet was shown increase villi height and intestinal cell proliferation [75]. Undigested dietary proteins and fibers are fermented in the intestine and this fermentation process produces SCFAs (mainly composed by acetate, propionate, and butyrate). Functionally, SCFAs affect the metabolism and gut health [76]. Acetate and propionate are energy substrates for peripheral tissues and butyrate is preferentially used as an energy source by colonic epithelial cells [77,78].

In this study, the abundance of members of the *Coriobacteriaceae*, specially *Eggerthella lenta* and *Lachnospiraceae* were enriched in the BC group (Figure 5B). These results demonstrate a potential beneficial effect of the Fe-biofortified bean diet on the intestinal microbial composition, since these microorganisms can improve the host health [79,80]. *Lachnospiraceae* is a butyrate producer family [79]. This short chain fatty acid (SCFA) is an energy source of colonocytes and it stimulates the immunogenicity of cancer cells [80]. *Coriobacteriacea* acts on the conversion of bile salts and steroid hormones*,* and the *Eggerthella lenta* was recently found to reductively cleave the heterocyclic C-ring of the epicatechin and catechin [81], and the breakdown product (3-(3,4-dihydroxyphenyl) propionic acid) presents anti-inflammatory effects [82]. This result is especially important since in general, carioca beans present these flavonoids (Table 3), thus they can be metabolized by the bacteria from the BC group.

Further, one of the aims of this study was to determine whether ingestion of an Fe biofortified diet would lead to an increased pathogenic bacterial load in the gut microbiota. Dietary Fe supplementation has been associated with an inflammatory-promoting gut microbiota, most likely due to the increased presence of luminal Fe [83], subsequent generation of free radicals, and ensuing epithelial stress and microbial dysbiosis [84]. Many of the nutritional methods used to combat Fe deficiency, such as Fe supplementation and Fe fortification, induce dysbiotic conditions and an expansion of pathogenic bacteria in the gut microbiota of subjects receiving Fe replete diets [83,85]. In contrast to these findings, we did not observe significant increase in pathogenic taxa in the BC group that have been previously associated with dietary Fe intake (e.g., Salmonella and other Enterobacteria) [85]. Therefore, this finding suggests that the use of biofortified beans instead of Fe fortification or Fe supplementation can be an effective and potentially sustainable strategy to reduce the Fe deficiency, with additional improvement in the gut bacterial populations.

Overall, we demonstrate in vitro that the potential consumption of the Fe-biofortified bean in a food basket context may increase the Fe uptake. The in vivo analyses demonstrated a significant remodeling of the gut microbiota in animals receiving a Fe-biofortified diet, which also presented higher amount protein. This microbiota remodeling increased the SCFA-producing bacteria abundance, improving the morphometric parameters (villi height), and increasing the intestinal absorptive surface area, these findings can potentially lead to increased Fe bioavailability and uptake. Therefore, under these experimentalal conditions, the results suggest that the consumption of the Fe biofortified carioca bean with other staple foods (i.e., food basket), increased Fe bioavailability, improved Fe status, and improved the composition and function of the gut microbiota. Understanding the effect of Fe biofortification on the gut microbiota may help to further biofortification efforts by improving the safety and efficacy profile of the food crop, as we understand more about the relationship between biofortified diets and the resident gut microbiota.

5. Conclusions

Nutritional methods aimed to alleviate global Fe deficiency, such as Fe supplementation or Fe fortification, have been moderately efficacious at attaining optimal Fe status. However, any improvement in serum Fe levels comes at the expense of decreased gut health in the form of dysbiosis and infection. This study showed how Fe-biofortification affects the composition and metagenome of the gut microbiota and intestinal function. Animals (*Gallus gallus*) that consumed the Fe biofortified carioca bean-based diet had less abundance of pathogenic bacteria, with concomitant increases in SCFA-producing bacteria that have known phenolic catabolic capacity, which have led to an improvement in intestinal morphology. In addition, and for the first time, the Fe-biofortified carioca bean presented similar concentration of phytate and polyphenols, yet, a higher protein content, in comparison to the Fe-standard bean, which potentially can increase the Fe bioavailability, and intestinal functionality, respectively.

Further and similar to previous data, the current research suggests that increased Fe content may not necessarily result in an increased absorbable Fe, and a key factor is the measurement of dietary Fe bioavailability in Fe biofortified crop varieties based diets, and as part of the breeding process.

Collectively, the findings presented here provide evidence that, unlike other nutritional methods of increasing Fe status, the Fe biofortification appear to improve the gut microbiota, and they raise the possibility that this strategy can further improve the efficacy and safety of the crop Fe biofortification approach. We suggest the utilization of the discussed in vitro and in vivo screening tools to guide studies aimed to develop and evaluate Fe biofortified staple food crops, and their potential nutritional benefit.

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10. GENERAL CONCLUSION

In this study, we demonstrated how Fe-biofortification affects iron *in vitro* and *in vivo* bioavailability, as weel as the composition and metagenome of gut microbiota and intestinal function. Our results demonstrated that the Fe-biofortified beans (black and carioca) presented higher iron concentration compared to the Fe-standard beans. Also, the black beans presented higher concentration of certain polyphenol compounds that can strongly inhibit the iron absorption compared to the carioca beans. Hence, carioca beans (BRS Cometa and BRS Perola) presented higher *in vitro* iron bioavailability compared to the black beans (BRS Esteio and SMN39). Moreover, iron bioavailability incresead when other staple foods (rice, potato, corn and pasta) were added to the bean-based diet.

Also, animals (*Gallus gallus*) that consumed the Fe biofortified carioca bean-based diet had less abundance of pathogenic bacteria, with concomitant increases in SCFA-producing bacteria that have known phenolic catabolic capacity, which have led to an improvement in intestinal morphology. This modification in the gut microbiota was also showed in the *in ovo* study, wherein the intra-amniotic administration of prebiotics extracted from the carioca beans incresead the abundance of beneficial bacteria (*Bifidobacterium* and *Lactobacillus*) and reduced the pathogenic bacteria (*E. coli* and *Clostridium*).

In addition, and for the first time, the Fe-biofortified carioca bean presented similar concentration of phytate and polyphenols, yet, a higher protein content, in comparison to the Fe-standard bean, which potentially can increase the Fe bioavailability, and intestinal functionality, respectively.

Collectively, the findings presented here provide evidence that, unlike other nutritional methods of increasing Fe status, the Fe biofortification appear to improve the gut microbiota, and they raise the possibility that this strategy can further improve the efficacy and safety of the crop Fe biofortification approach.

The carioca beans BRS Cometa presented the best results both *in vitro* and *in vivo*, improveing Fe status and to positively affecting the intestinal functionality and bacterial populations. Thus, it can be considered a promissor vehicle for the Fe biofortification.

11. FINAL CONSIDERATIONS

Overall, our findings showed that the Fe-biofortified carioca bean BRS Cometa improved the iron status and the gut microbiota *in vivo*. So, we suggest further studies evaluating the effect of the consumption of this Fe-biofortified carioca bean on the nutritional status of iron and the composition of gut microbiota in humans. Therefore, all these results (*in vitro*, in animals and in humans) can contribute to the knowledge how this Fe-biofortified carioca beans can affect the iron nutritional status and gut health, contrinuting to the efforts to reduce the iron deficiency. Also, all these studies build a scientific basis for the release of biofortified common beans to the famers and population.