MARIANA JUSTE CONTIN GOMES

EFFECT OF DIFFERENTS CULTIVARS OF BRAZILIAN BEANS ON INTESTINAL HEALTH *IN VIVO*

Thesis submitted to the Science of Nutrition Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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Assent:

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I dedicate this achievement to God, my husband, Daniel, my parents, Marli and Everardo, my siblings, Cézar and Beatriz, and all my family and friends.

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ABSTRACT

GOMES, Mariana Juste Contin, D.Sc., Universidade Federal de Viçosa, September, 2022. **Effect of differents cultivars of Brazilian beans on intestinal health *in vivo*.** Adviser: Hércia Stampini Duarte Martino. Co-advisers: Desirrê Morais Dias, Natália Elizabeth Galdino Alves, Bárbara Pereira da Silva and Elad Tako.

Introduction: Beans are a legume widely consumed in Brazil and in the world, being especially rich in dietary fibers, proteins, and minerals. Carioca bean (*Phaseolus vulgaris* L.) has a promising chemical composition, with high content of proteins, rich in bioactive peptides, with functional potential in the health. BRSMG Madreperola is a cultivar of carioca bean used in this study due to its beneficial biological properties and low darkening along storage time. The intake of carioca bean and its protein hydrolyzate can modulate the intestinal microbiota and mitigating the effects of the high fat diet (HFD). In addition, beans are a promising crop for the biofortification of minerals such as zinc (Zn). Zn deficiency is one of the most prevalent nutritional deficiencies in Brazil and in the world, it impairs the quality of life of the population and generates an increase in the health expenditures. Cowpea (*Vigna unguiculata* L. Walp.) was target for Zn biofortification in Brazil. It has a large amount of dietary fibers, proteins, carbohydrates, starch, phenolic compounds, vitamins and minerals, including Zn. The high amount of dietary fibers (soluble and insoluble) present in cowpea beneficially affects intestinal health, favoring the growth of beneficial bacteria in the intestine, and improving the absorption of minerals. Furthermore, the use of Zn biofortified foods can be a good strategy to contribute to the reduction of Zn deficiency prevalence. However, the effects of the new cultivars of Zn biofortified cowpea in the intestinal functionality, gut microbiota, and mineral absorption, are not known. **Objectives:** (Review 1) To describe the effects of iron and Zn biofortified foods and gut microbiota *in vivo*. (Review 2) To analyze the effects of Zn biofortified foods on Zn status in humans. (Original Study 1) To evaluate the effect of the consumption of carioca bean flour and its protein hydrolyzate, associated with a HFD, on the modulation of the intestinal microbiota *in vivo.* (Original Study 2) To investigate the effects of soluble extracts of Zn biofortified cowpea on intestinal functionality and microbiota *in vivo*. **Methods:** Review articles were conducted according to PRISMA guidelines. Data search was performed at PubMed, Web of Science, Science Direct, and Scopus databases for experimental studies (Review 1), and PubMed, Cochrane, Scopus and Science Direct databases were searched for human studies (Review 2). For original study 1, 48 adult male BALB/c mice received a HFD associated or not with carioca bean flour or its

protein hydrolyzate for nine weeks. Four experimental groups were designed (n=12): (1) AIN 93M diet + 0.5 mL of deionized water by gavage; (2) high fat diet + 0.5 mL 6-propyl-2-thiouracil (PTU) (10 mg/kg body weight) by gavage; (3) high fat diet added with carioca bean flour + 0.5 mL of PTU by gavage; (4) high fat diet + 0.5 mL of carioca bean protein hydrolyzate (700mg/Kg) + PTU by gavage. Intestinal function markers were assessed at the end of the study, and the gut microbiota was assessed by 16S rRNA gene sequencing. In the original study 2, the biological assay with intra-amniotic administration (*Gallus gallus*) was designed in seven experimental groups (n=10): (1) non-injected; (2) 18MΩH_2O; (3) 50 mg/mL inulin; (4) 50 mg/mL BRS Pajeú bean (Zn standard); (5) 50 mg/mL BRS Aracê bean (Zn biofortified); (6) 50 mg/mL BRS Xiquexique bean (Zn biofortified) and (7) 50 mg/mL BRS Imponente bean (Zn biofortified). Evaluation of the intestinal morphology and intestinal functionality, as well as gut microbiota was performed at the end of the experiment. **Results:** Review 1 – Biofortified foods have positive effects on the composition and function of the gut microbiota, with an increase in *Lactobacillus* and *Ruminococcus*, producers of short chain fatty acids (SCFAs), and a decrease in pathogenic bacteria. Review 2 – Zn biofortified foods increase the Zn absorption in humans, favored by the lower phytate:Zn molar ratio in the studied food matrices. Advances in biofortification strategies to increase production, access, and consumption of Zn biofortified foods can contribute to improve the physiological state of Zn in vulnerable populations. Original study 1 – We observed an increase in Bacteroidetes and a reduction in the Firmicutes/Bacteroidetes ratio in the group that consumed a HFD associated with bean flour. In addition, this group showed an increase in cecum weight, moisture, and lipids content in the feces, compared to the controls. We did not observe changes in the gut microbiota of the group that consumed carioca bean protein hydrolyzate associated with the HFD, compared to the HFD control. Members of the *Muribaculaceae* family were more abundant in the group that consumed carioca bean flour, showing potential to improve the intestinal health. Functional analysis of the microbiota in this group showed a promising outcome of carioca beans in attenuating the effects of the HFD, without negatively altering its function. Original study 2 – There was a reduction in the abundance of *Clostridium* and *E. coli* in the groups treated with soluble extract of BRS Imponente and BRS Xiquexique beans (Zn biofortified). BRS Xiquexique increased the diameter and depth of the crypt, compared to the other groups. Gene expression of proteins involved with mineral absorption, brush border membrane (BBM) functionality and inflammation were similar to inulin and 18MΩH_2O controls. However, the promising effects of BRS Xiquexique and BRS Imponente on improving the Zn transport by BBM, and BRS Xiquexique on intestinal

morphology, indicate that these are promising cultivars to be considered by biofortification programs. **Conclusion:** Evidence suggests that the consumption of biofortified foods modifies the local microbial ecology, increasing the abundance of SCFA-producing bacteria and decreasing the abundance of potentially pathogenic bacteria. Carioca bean has dietary fibers which positively modulate the gut microbiota when associated with a HFD. Zn biofortified cowpea has the potential to improve the gut microbiota and increase the supply of Zn in the population.

Keywords: Prebiotic. Bioactive peptides. Mineral metabolism. *Gallus gallus*. Intra-amniotic administration. Microbiome.

RESUMO

GOMES, Mariana Juste Contin, D.Sc., Universidade Federal de Viçosa, setembro de 2022. **Efeitos de diferentes cultivares de feijão Brasileiro na saúde intestinal *in vivo*.** Orientadora: Hércia Stampini Duarte Martino. Coorientadores: Desirrê Morais Dias, Natália Elizabeth Galdino Alves, Bárbara Pereira da Silva e Elad Tako.

Introdução: O feijão é uma leguminosa amplamente consumida no Brasil e no mundo, sendo especialmente rico em fibras alimentares, proteínas, e minerais. O feijão carioca (*Phaseolus vulgaris* L.) possui uma composição química promissora, com elevado conteudo de proteínas, ricas em peptídeos bioativos, com potencial funcional na saúde. BRSMG Madreperola é uma cultivar de feijão carioca, que foi utilizada neste estudo devido às suas propriedades biológicas benéficas e baixo escurecimento ao longo do tempo de armazenamento. A ingestão de feijão carioca e de seu hidrolisado proteico pode modular a microbiota intestinal e amenizar os efeitos da dieta *high fat* (HFD). Além disso, o feijão é uma cultura promissora para a biofortificação de minerais como o zinco (Zn). A deficiência de Zn é uma das deficiências nutricionais mais prevalentes no Brasil e no mundo, prejudica a qualidade de vida da população e gera aumento dos gastos em saúde. O feijão caupi (*Vigna unguiculata* L. Walp.) foi alvo para biofortificação com Zn no Brasil. Ele possui grande quantidade de fibras alimentares, proteínas, carboidratos, amido, compostos fenólicos, vitaminas e minerais, dentre eles o Zn. A elevada quantidade de fibras alimentares (solúvel e insolúvel) presentes no feijão caupi afeta beneficamente a saúde intestinal, favorecendo o crescimento de bactérias benéficas no intestino, e melhorando a absorção de minerais. Além disso, o uso de alimentos biofortificados com Zn pode ser uma boa estratégia para contribuir para a redução da prevalência de deficiência de Zn. Entretanto, os efeitos de novos cultivares de feijão caupi biofortificados com Zn na funcionalidade intestinal, na microbiota intestinal, e na absorção de minerais não são conhecidos. **Objetivos:** (Revisão 1) Descrever os efeitos de alimentos biofortificados com ferro e Zn na microbiota intestinal *in vivo*. (Revisão 2) Analisar os efeitos de alimentos biofortificados com Zn sobre o status de Zn em humanos. (Estudo Original 1) Avaliar o efeito do consumo de farinha de feijão carioca e de seu hidrolisado proteico, associados à HFD, na modulação da microbiota intestinal *in vivo*. (Estudo Original 2) Investigar os efeitos de extratos solúveis de feijões caupi biofortificados com Zn na funcionalidade e microbiota intestinal *in vivo*. **Métodos:** Os estudos de revisão foram conduzidos de acordo com as diretrizes PRISMA. A busca foi realizada nas bases de dados PubMed, Web of Science, Science Direct e Scopus para os estudos experimentais (Revisão 1),

e as bases de dados PubMed, Cochrane, Scopus e Science Direct foram utilizadas para buscar estudos com humanos (Revisão 2). Para o estudo original 1, 48 camundongos BALB/c machos adultos receberam HFD associadas ou não à farinha de feijão carioca ou ao seu hidrolisado proteico, durante nove semanas. Foram delineados quatro grupos experimentais (n=12): (1) dieta AIN 93M + 0,5 mL de água deionizada por gavagem; (2) dieta *high fat* + 0,5 mL de 6-propil-2-thiouracil (PTU) (10 mg/kg de peso) por gavagem; (3) dieta *high fat* adicionada de farinha de feijão carioca + 0,5 mL de PTU por gavagem; (4) dieta *high fat* + 0,5 mL de hidrolisado proteico de feijão carioca (700mg/Kg) + PTU por gavagem. Marcadores da função intestinal foram analisados ao final do estudo, e a microbiota intestinal foi analisada por sequenciamento do gene rRNA 16S. No estudo original 2, o ensaio biológico com administração intra amniótica (*Gallus gallus*) foi delineado em sete grupos experimentais (n=10): (1) não-injetado; (2) 18MΩH_2O; (3) 50 mg/mL inulina; (4) 50 mg/mL feijão BRS Pajeú (convencional); (5) 50 mg/mL feijão BRS Aracê (biofortificado com Zn); (6) 50 mg/mL feijão BRS Xiquexique (biofortificado com Zn) e (7) 50 mg/mL feijão BRS Imponente (biofortificado com Zn). A avaliação da morfologia intestinal e funcionalidade intestinal, bem como a análise da microbiota intestinal foram realizadas ao final do experimento. **Resultados:** Revisão 1 – Os alimentos biofortificados exercem efeitos positivos na composição e função da microbiota intestinal, com aumento de *Lactobacillus* e *Ruminococcus*, produtores de ácidos graxos de cadeia curta (AGCC), e diminuição de bactérias patogênicas. Revisão 2 – Alimentos biofortificados com Zn aumentam a absorção de Zn em humanos, favorecido pela menor razão molar fitato:Zn nas matrizes alimentares estudadas. Avanços nas estratégias de biofortificação para aumentar a produção, acesso e consumo de alimentos biofortificados com Zn contribuem para melhorar o estado fisiológico de Zn em populações vulneráveis. Estudo Original 1 – Observamos aumento de Bacteroidetes e redução da razão Firmicutes/Bacteroidetes no grupo que consumiu HFD associada à farinha de feijão. Além disso, esse grupo mostrou aumento no peso do ceco, na umidade e conteúdo de lipídios nas fezes, comparado aos controles. Não observamos mudanças na microbiota intestinal no grupo que consumiu hidrolisado proteico de feijão carioca associado à HFD. Membros da família *Muribaculaceae* foram mais abundantes no grupo que consumiu farinha de feijão carioca, mostrando potencial para melhorar a saúde intestinal. A análise funcional da microbiota neste grupo mostrou desfecho promissor do feijão carioca em atenuar os efeitos da HFD, sem alterar negativamente a sua função. Estudo Original 2 – Houve redução da abundância de *Clostridium* e *E. coli* nos grupos tratados com extrado solúvel dos feijões BRS Imponente e BRS Xiquexique, biofortificados com Zn. BRS Xiquexique aumentou o diâmetro e a

profundidade da cripta, comparado com os demais grupos. A expressão gênica de proteínas envolvidas na absorção de minerais, funcionalidade da membrana da borda em escova (MBE) e inflamação foi semelhante aos controles inulina e 18MΩH_2O. Entretanto, os efeitos promissores do BRS Xiquexique e BRS Imponente na melhoria do transporte de Zn pela MBE e do BRS Xiquexique na morfologia intestinal, indicam que estes são as cultivares promissoras a serem consideradas por programas de biofortificação. **Conclusão:** As evidências sugerem que o consumo de alimentos biofortificados modifica a ecologia microbiana local, aumentando a abundância de bactérias produtoras de AGCC e diminuindo a abundância de bactérias potencialmente patogênicas. O feijão carioca possui fibras alimentares capazes de modular positivamente a microbiota intestinal frente ao consumo de HFD. O feijão caupi biofortificado com Zn apresenta potencial de melhorar a microbiota instestinal e aumentar o aporte de Zn na população.

Palavras-chave: Prebiótico. Peptídeos bioativos. Metabolismo de minerais. *Gallus gallus*. Administração intra-amniótica. Microbioma.

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LIST OF ACRONYMS AND ABBREVIATIONS

16S rRNA	16S ribosomal RNA
18S rRNA	18S ribosomal RNA
ANOVA	Analysis of variance
AP	Aminopeptidase
AA	Arachidonic acid
ARRIVE	Animal in research: reporting *in vivo* experiments
BBM	Brush border membrane
CCB	Cooked common bean
CD	Crypt depth
CML	Circular muscle layer
CO_2	Carbon dioxide
DCytb	Duodenal cytochrome b
DGLA	Dihomo-γ-linolenic acid
DMT-1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
EAR	Estimated average dietary requirements
Embrapa	*Empresa Brasileira de Pesquisa Agropecuária*
ESI	Electrospray ionization
FADS1	Fatty acid desaturase 1
FADS2	Fatty acid desaturase 2
FDR	False discovery rate
FPN	Ferroportin
Fe	Iron
FZA	Fractional zinc absorption
g	Gram
GRADE	Grading of recommendations, assessment, development, and evaluation
GLA	Gama-linolenic acid
HFBF	High fat diet + cooked common bean flour group
HFD	High fat diet
HFPH	High fat diet + cooked common bean protein hydrolysate group
HPLC	High performance liquid chromatography
HPLC-MS	Liquid chromatography-mass spectrometry
H_2O	Water
ICP-AES	Inductively coupled plasma-atomic emission spectroscopy
IDF	Insoluble dietary fiber
IL-8	Interleukin-8
KEGG	Kyoto encyclopedia of genes and genomes
kcal	Kilocalorie
kg	Kilogram
LA	Linoleic acid
LC-MS	Liquid chromatography coupled mass spectrometry
LDL	Low-density lipoprotein

LefSe	Linear discriminant analysis effect size
LML	Longitudinal muscle layer
MeSH	Medical subject headings
MUC-2	Mucin-secreting intestinal protein-2
NC	Normal control group
NF-κB	Nuclear factor kappa B
OSM	Osmolality
OTU	Operactional taxonomic unit
PCoA	Principal coordinate analysis
PICOS	Participants, interventions, comparisons, outcomes, and study design
PRISMA	Preferred reporting items for systematic review and meta-analysis
PTU	6-propyl-2-thiouracil
RNA	Ribosomal ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Real-time polymerase chain reaction
SCFA	Short chain fatty acid
SDF	Soluble dietary fiber
SGLT-1	Sodium-glucose transport protein 1
SI	Sucrase isomaltase
RS	Resistant starch
SYRCLE RoB	Systematic review centre for laboratory animal experimentation risk of bias
TDF	Total dietary fiber
TNF-α	Tumor necrosis factor alpha
TZA	Total zinc absorption
ZIP/ZnT	Zn transporters proteins
Zn	Zinc
ZnT1	Zinc transporter 1
μL	Microliter
μm	Micrometer
ω -3	Omega 3
ω -6	Omega 6

SUMMARY

1. GENERAL INTRODUCTION

Beans are a legume widely consumed in Brazil and in other countries, mainly in South America, Central America, and Africa (CASTRO GUERREIRO et al., 2016). In Brazil, beans together with rice constitute the basis of the Brazilian diet. The chemical composition of carioca bean (*Phaseolus vulgaris* L.) is formed by about 60 to 70% of carbohydrates, 15 to 30% of protein, 0.7 to 2% of lipids and 21 to 26% of total dietary fiber (MARTINO et al., 2012; DIAS et al., 2015). The high protein content of this kind of bean stands out and studies have shown that both the protein isolate and the protein hydrolyzate of carioca bean exert anti-inflammatory and antiatherogenic effects *in vitro* (ALVES et al., 2016a; ALVES et al., 2016b). In addition, carioca bean flour can reduce inflammation and risk factors for cardiovascular diseases, and its protein hydrolyzate has hypocholesterolemic activity and beneficial action on oxidative stress and vascular endothelium *in vivo* (LIMA et al., 2019; GOMES et al., 2020). The high content of total dietary fiber and its soluble and insoluble fractions (26.69 ± 0.45; 7.04 ± 1.27; 19.64 ± 0.92, respectively) (DIAS et al., 2015) contribute to intestinal health, as they undergo bacterial fermentation in the cecum and colon producing short chain fatty acids (SCFAs) that are fundamental for nutrition and maintenance of enterocytes (PACIFICI et al., 2017), reduce intestinal pH by inhibiting the growth of potentially pathogenic bacterial populations, and improve the absorption of minerals such as zinc (Zn) and iron (TAKO et al., 2008; TAKO et al., 2014; ZIMMERMANN et al., 2010).

Cowpea (*Vigna unguiculata* L.) is a crop of African origin, introduced in Brazil in the second half of the 16th century (EMBRAPA, 2019). This legume has about 33.7 to 57.8% of carbohydrates, 17.4 to 28.3% of protein, 1.0 to 1.6% of lipids and 19.5 to 35.6% of total dietary fiber (CARVALHO et al., 2012). Its promising chemical composition, combined with its undemanding agronomic characteristics, are favorable to low-income farmers, who have limited access to a balanced diet and are susceptible to higher rates of malnutrition, chronic hunger, and hidden hunger, which have been associated with prevalence of chronic diseases (GÖDECKE; STEIN; QAIM, 2018). In addition, the phenolic compounds found in cowpea have the potential to inhibit the production of reactive oxygen species, prevent LDL oxidation, protect against chronic diseases (OJWANG et al., 2015; HACHIBAMBA et al., 2013), and inhibit the expression of pro-inflammatory cytokines and cell adhesion molecules *in vitro* (OJWANG et al., 2015), providing evidence of the anti-inflammatory potential of the polyphenols present in this legume.

In recent years, research has focused on clarifying the role of the gut microbiota in health and disease and investigating the beneficial effect of foods and dietary ingredients, including dry beans, in modulating a healthy gut microbiota. Nowadays, it is known that the damage to the microbiota and intestinal function, caused by the consumption of ultraprocessed foods and a high fat diet (HFD), directly affects the absorption of nutrients, and can lead to nutritional deficiencies (VOLAND et al., 2022). The knowledge about gut microbiota has improved in the last few years due to the fast technological development, it has been possible to understand the functionality and role of microorganisms inhabit human gut, which constitutes a large set of microbial communities (SCHMIDT, RAES, BORK, 2018). The content of dietary fibers, proteins, and minerals in Brazilian beans benefically modulates the gut microbiota and can be a strategy to improve the intestinal health. In addition, the role of dietary fibers in modulating the intestinal function and microbiota has been investigated in animal models of mice and chicken (*Gallus gallus*) (GOMES et al., 2022a; GOMES et al., 2022b; HOU et al., 2017; DIAS et al., 2019).

Minerals have a dynamic interaction with the gut microbiota. Effects of minerals such as Zn on the intestinal microbiota and the effects of its deficiency on the intestinal barrier has been elucidated, and this interaction may be the key to clarify the physiology behind micronutrient deficiency (BIELIK, KOLISEK, 2021). Adequate supply of Zn in the diet has shown beneficial modulation of the microbiota *in vivo*, leading to favorable changes in their metabolic activity (SUN et al., 2019; LI et al., 2021; SKRYPNIK, SULIBURSKA, 2018; KOREN, TAKO, 2020). Also, an increase in SCFAs concentration and in species richness and diversity in the ileum were observed in animals fed a diet containing high levels of Zn oxide, reflecting the stability of the ecosystem in the presence of the mineral (PIEPER et al., 2012; SUN et al., 2019; PAJARILLO, LEE, KANG, 2021).

Zn deficiency affects about 17.3% of the world population (WESSELLS, BROWN, 2012), and an investigation in low- and middle-income countries assessed data of plasma Zn concentration and showed a prevalence > 20% Zn deficiency in the population (GUPTA, BRAZIER, LOWE, 2020). Low Zn intake associated with its low bioavailability in food is one of the factors that contribute to its nutritional deficiency (MARET, SANDSTEAD, 2006). Thus, the use of Zn biofortified foods can be a strategy to reduce the deficiency of this mineral and benefit the maintenance of a healthy intestinal microbiota (GUPTA, BRAZIER, LOWE, 2020). Food biofortification consists of improving the micronutrient content of basic cultivars produced in the field through conventional plant breeding, where plants of the same

species are selected and those with higher levels of desired nutrients and better agronomic characteristics are crossed with each other to obtain varieties with higher levels of iron, Zn, or pro-vitamin A (BOUIS et al., 2011; LA FRANO et al., 2014). There is evidence that using conventional breeding is the fastest way to get more nutritious crops into the hands of farmers and consumers (BOUIS, SALTZMAN, 2017). Cowpea was target for biofortification because it is a legume commonly cultivated and consumed in poorer regions of the Northeast and North of Brazil, where a high prevalence of Zn deficiency is observed, especially in children (PEDRAZA, SALES, 2017).

The present study aims to contribute to scientific advances for the use of functional foods and biofortified foods, as well as to provide a basis for the development of strategies to combat nutritional deficiencies in vulnerable populations. Initially, we systematically reviewed published data that evidence the effects of biofortified foods on gut microbiota and Zn status in humans. Subsequently, we investigated the effects of ingestion of carioca beans and their protein hydrolyzate, as well as Zn biofortified cowpea (included in the Embrapa biofortification program), on functionality, morphology, and intestinal microbiota. In the current literature, studies that showed the effects of the consumption of these foods and their bioactive compounds on the gut microbiota modulation, and on the maintenance of beneficial bacterial taxa are scarce. Thus, this study evaluated the effects of carioca bean flour and its protein hydrolyzate on intestinal function and microbiota *in vivo*, and the effects of soluble extracts of Zn biofortified cowpea on intestinal function and microbiota *in vivo*, using the intra-amniotic administration model (*Gallus gallus*).

The hypothesis of this study is based on the premise that (1) evidence from reviewed experimental (review 1) and clinical (review 2) studies will support the use of biofortified foods with micronutrients for the modulation of the healthy microbial rate of the host, and will exert beneficial effects on intestinal health, as well as for improve Zn status in the population; (2) the intake of carioca bean flour and its protein hydrolyzate will prevent deleterious effects of the HFD on the gut microbiome composition and functional prediction of the intestinal microbiota; (3) Intra-amniotic administration of soluble extracts of Zn biofortified cowpea will improve the expression of enterocyte brush border proteins involved in Zn absorption, in addition to maintaining healthy gut functionality, morphology and microbiota.

2. OBJECTIVES

2.1. General objective

To investigate the effects of consumption of differents cultivars of Brazilian beans, carioca bean and cowpea, on intestinal health *in vivo*.

2.2. Specific objectives

- ✓ To describe the effects of Fe and Zn biofortified foods on gut microbiota *in vivo*; and the potential of Zn biofortified foods to improve the Zn status in humans.
- ✓ To evaluate the effect of the consumption of carioca bean flour and its protein hydrolyzate in Balb/c mice fed a high fat diet on the modulation of the gut microbiota and intestinal function;
- ✓ To analyze the chemical and mineral composition (Fe and Zn) of Zn biofortified cowpea flour;
- ✓ To investigate the effect of intra-amniotic administration of Zn biofortified cowpea soluble extracts on gene expression of brush border membrane proteins, intestinal morphology and microbiota.

3. GENERAL METHODOLOGY

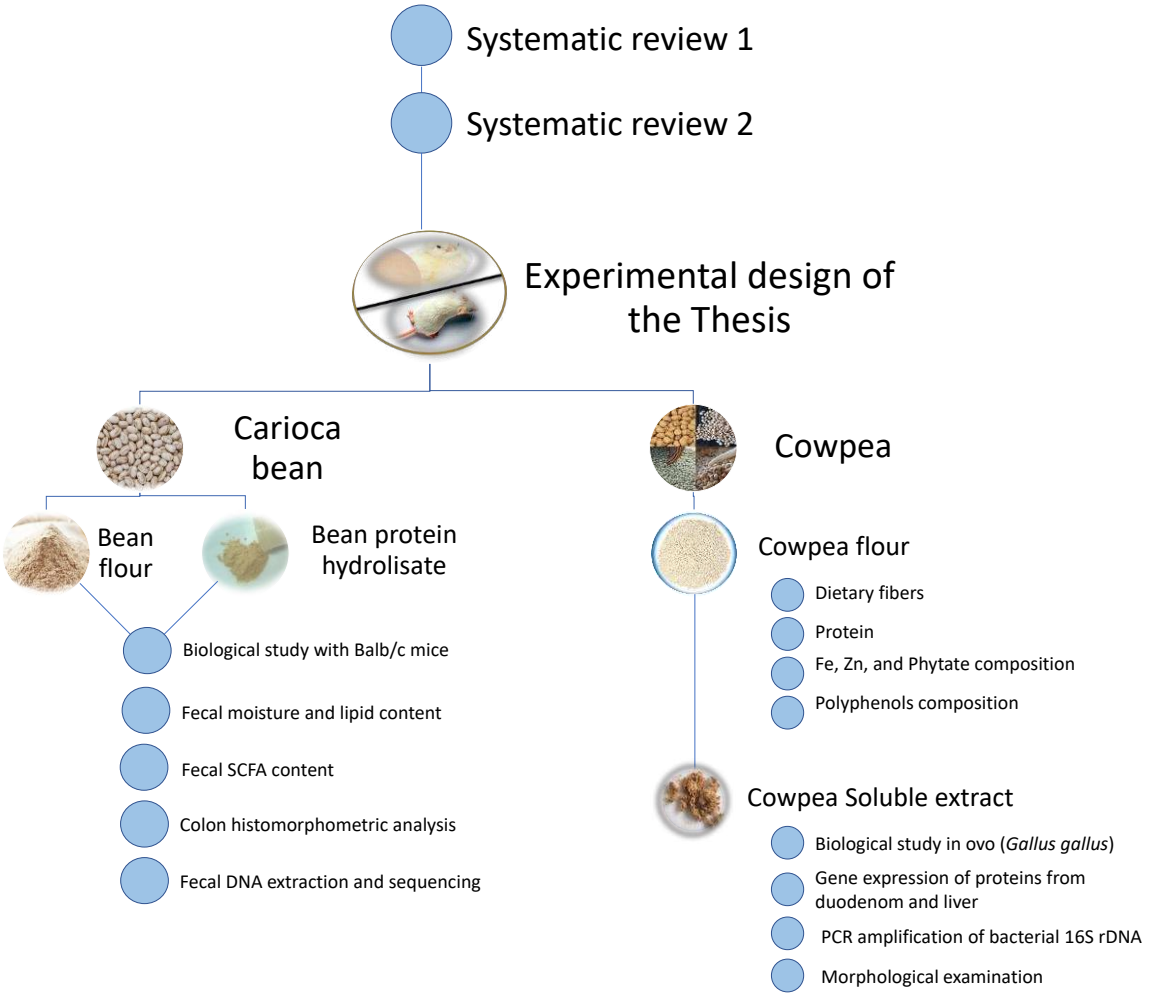

Figure 1. Representative scheme of the general methodology of the thesis.

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4. THESIS PUBLICATIONS

Paper	Paper title	Journal	Impact factor
Systematic Review 1	Effects of Iron and Zinc Biofortified Foods on Gut Microbiota *In Vivo* (*Gallus gallus*): A Systematic Review	Nutrients	6.706
Systematic Review 2	Zinc-biofortified staple food crops to improve zinc status in humans: a systematic review	Critical Reviews in Food Science and Nutrition	11.208
Original Paper 1	Cooked common bean flour, but not its protein hydrolysate, has the potential to improve gut microbiota composition and function in BALB/c mice fed a high-fat diet added with 6-propyl-2-thiouracil	Journal of Nutritional Biochemistry	6.117
Original Paper 2	Zinc Biofortified Cowpea (*Vigna unguiculata* L. Walp.) Soluble Extracts Modulate Assessed Cecal Bacterial Populations and Gut Morphology *In Vivo* (*Gallus gallus*)	Frontiers in Bioscience - Landmark	3.115

5. LITERATURE REVIEW

5.1. PAPER 1

Systematic Review published at Nutrients, Impact Factor: 6.706, Qualis CAPES: A1, DOI: 10.3390/nu13010189

Review

Effects of iron and zinc biofortified foods on gut microbiota *in vivo* (*Gallus gallus*): A systematic review

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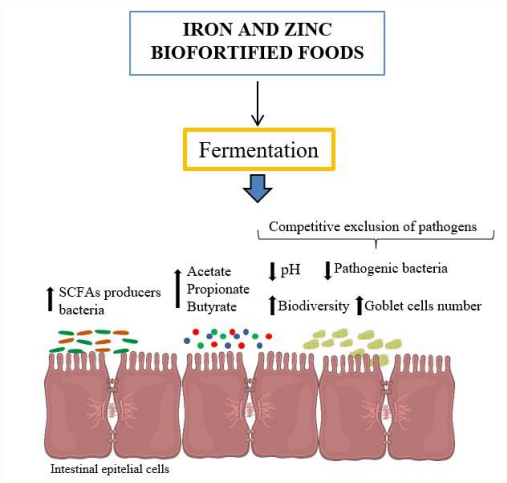
Abstract: Dietary iron and zinc deficiencies are a global health concern. Bacteria that colonize the gastrointestinal tract depend on minerals to maintain their activities; thus, recent evidence suggests that biofortified foods can modulate the host's beneficial bacterial taxa. The current review analyzes the research data that linked between iron and zinc biofortified foods and gut microbiota modulation. The data analysis was based on the PRISMA guidelines and the data search was performed at PubMed, Web of Science, Science Direct, and Scopus databases for experimental studies published from January 2010 until December 2020. The five selected studies were conducted in an experimental *in vivo* model (*Gallus gallus*). The identified and discussed research showed positive effects of biofortified foods on the composition and function of the gut microbiota. Further, an increase in short chain fatty acids producing bacterial populations as *Lactobacillus* and *Ruminococcus*, and a decrease in potentially pathogenic bacteria as *Streptococcus*, *Escherichia*, and *Enterobacter* was identified due to the consumption of biofortified foods. In conclusion, biofortified foods may contribute to improved gut health without increasing the colonization of pathogenic bacteria. The dietary inclusion of approximately 50% of iron/zinc biofortified foods has a significant beneficial effect on the gut microbiota. Additional studies in humans and animal models are warranted to further establish the suggested effects on the intestinal microbiome. PROSPERO (CRD42020184221).

Keywords: zinc; iron; minerals; short chain fatty acids; intestinal health; bacteria taxa; diversity analysis

Graphical abstract



1. Introduction

Dietary deficiencies of vitamins and minerals such as iron (Fe), zinc (Zn), vitamin A, iodine, and folic acid are common health concerns worldwide, with significant physiological and developmental consequences. Globally, Fe deficiency is the most widespread nutritional disorder that affects approximately two billion people [1]. Zinc is the second highest micronutrient deficiency, affecting approximately 17% of the global population [2]. As a part of the battle aimed at decreasing the dietary prevalence of micronutrient deficiencies, several governmental programs have been established, and strategies were developed in order to effectively produce Fe and Zn biofortified and fortified foods.

Biofortification is the process of conventionally breeding staple food crops that are rich in micronutrients, such as vitamin A, Zn, and Fe. Biofortification is a food-based approach that aims to improve the nutritional value of staple foods that are consumed by the relevant target populations that are most affected by a specific nutritional deficiency with potential or existing malnutrition [3]. Previous studies indicated that the consumption of biofortified foods such as common beans, rice, sweet potatoes, and pumpkins increased the dietary micronutrients delivery and intake, and therefore, improved the physiological status and overall health of the population [4,5,6,7]. Linked to the delivery of a greater amount of the target nutrient and as part of the biofortification process, the foods chosen by the biofortification programs are also rich in other compounds which may affect overall health. The food matrix of biofortified foods can have a differential effect on the gut microbiota and can modulate the bacterial taxa in the colon. In this review, we present the effects of biofortified foods with micronutrients or their fractions such as flour or soluble extracts on gut

microbiota *in vivo*, and only animal model studies that had an appropriate control group, composed by the test food or its conventional/non-biofortified fraction, were included. Common beans, for example, are targets for biofortification due to their multiple beneficial health effects, such as anti-inflammatory, antioxidative, and ability to reduce the risk of cardiovascular diseases *in vivo* [8,9], with further anti-inflammatory, antihyperlipidemic, and antihypertensive properties as demonstrated *in vitro* [10,11]. In addition, several governmental programs have focused on food fortification, which take place on an industrial scale and aimed to improve the amount of micronutrients consumed via basic foods and food products [12].

It was previously established that the human gastrointestinal tract inhabits a diverse and complex microbiota, composed of trillions of microorganisms that are distributed along the intestine in a symbiotic relationship with its host [13]. The abundance of this microbiome is modulated by several external and internal factors, amongst them, are the subject's dietary habits and composition [14]. Previously, the scientific literature defined a "healthy gut microbial profile" that is composed of short chain fatty acids (SCFA) producing bacteria that benefits the host by regulating the intestinal homeostasis, contributing to the absorption of minerals [15] via the efficient functionality of the duodenal brush border membrane (BBM). In addition, a beneficial bacterial taxa profile was suggested to reduce the incidence of preneoplastic lesions and tumors *in vivo*, and ability to delay the progression of cancer associated with inflammatory bowel disease [16].

Experimental studies that evaluated the effects of micronutrient fortified foods on the gut microbiota are scarce [17,18]. There are some clinical studies that evaluate these effects, however, these were conducted with toddler populations [19,20,21], that present a still forming eating patterns and in constant change. In addition, the frequent use of antibiotics may lead these populations to not have a well-established resident intestinal microbiota [22,23]. In contrast, the role of dietary biofortified foods and how it may affect the composition and function of the gut microbiome has been recently investigated. Despite the available knowledge on the effects of biofortified foods on dietary mineral bioavailability, there is no evidence that increased concentrations of dietary minerals and as part of a complete meal (containing dietary fibers, proteins, lipids) has a beneficial effect on gut microbiota *in vivo*.

Therefore, the objective of the current study was to systematically review the experimental studies that evaluated the effects of the consumption of Fe and Zn biofortified

foods or their derivatives, such as flour or soluble extracts, on the gut microbiota. Hence, if the current review provides evidence that Fe and Zn biofortified foods have a beneficial effect on the gut microbiota, we suggest further increased dietary consumption of Fe and Zn biofortified foods by populations with these micronutrient deficiencies.

2. Materials and Methods

2.1. Protocol and Registration

This systematic review was carried out in accordance with the Preferred Reporting Items for Systematic review and Meta-Analysis (PRISMA) protocols [24] and registered in PROSPERO (CRD42020184221). The research question to be reviewed was: "What are the effects of the consumption of biofortified foods with some micronutrient on the gut microbiota of *in vivo* models"? This is the first study to review the effects of biofortified foods on gut microbiota.

2.2. Literature Search

Two researchers independently searched for original articles. The search was carried out in PubMed, Web of Science, Science Direct, and Scopus databases for experimental studies conducted in animal models that evaluated the effects of biofortified foods on the gut microbiota. Filters were used to select articles published from January 2010 until December 2020. The last search date was 4 December 2020.

The descriptors were identified based on Medical Subject Headings (MeSH) and the following search strategy was designed and utilized: ("Microbial Profile" OR "Cecum Microbiome" OR "Gastrointestinal Microbiome" OR "Gastric Microbiome" OR "Gut Microbiome" OR "Gut Microbiomes" OR "Gut Microflora" OR "Gut Microbiota" OR "Gut Microbiotas" OR "Gastrointestinal Flora" OR "Gut Flora" OR "Gastrointestinal Microbiota" OR "Gastrointestinal Microbiotas" OR "Gastrointestinal Microbial Community" OR "Gastrointestinal Microflora" OR "Intestinal Microbiome" OR "Intestinal Microbiomes" OR "Intestinal Microbiota" OR "Intestinal Microbiotas" OR "Intestinal Microflora" OR "Intestinal Flora" OR "Microbial Populations" OR "Enteric Bacteria" AND Biofortification OR Biofortified OR "food biofortification" OR "foods biofortification" OR "Biofortified Foods" OR "Biofortified Food" OR "Biofortified Crops" OR "Biofortified Crop"). The logical operators "AND" or "OR" were used to combine the descriptors.

2.3. Screening and Eligibility of Records

The eligibility criteria were formulated with reference to participants, interventions, comparisons, outcomes, and study design (PICOS). Duplicate studies were excluded and, the search and screening for titles and abstracts were carried out independently by the authors according to the inclusion and exclusion criteria (Table 1). After screening, *in vitro* studies, reviews, consensus papers, letters to editor, books, book chapters, theses, dissertations, and non- animal studies were excluded, and studies with biofortified foods that evaluated the gut microbiota were selected.

Table 1. Participants, interventions, comparisons, outcomes, and study design (PICOS) criteria for inclusion and exclusion of studies.

Parameter	Inclusion criteria	Exclusion criteria
Population	*In vivo* animal studies	Human studies; *in vitro* studies; pregnancy and lactation; pathologies different from obesity and micronutrient deficiency
Intervention	Biofortified foods with some micronutrient or their fractions (e.g. flour, soluble extracts)	Do not correlate biofortified foods and gut microbiota; ultra-processed foods; biofortification with compounds different from vitamins and minerals; supplementation
Comparison	Standard foods, or their standard fractions; standard diet for rodents, with no biofortified foods	No control group
Outcomes	Modulation of the health gut microbiota and decrease of pathogenic bacteria	
Study design	Experimental placebo-controlled studies	*In vitro* studies; reviews; consensus papers; letters to editor; books; book chapters; theses and dissertations; non- animal studies; studies with more than 10 years from publication date

The potentially eligible research articles were read in full independently by authors and assessed for compliance with the established eligibility criteria. Discrepancies between reviewers were resolved through consensus with a third reviewer and the reference lists of the studies included were hand searched to identify other relevant trials. If the data were not reported or unclear, we directly contacted authors via e-mail.

2.4. Data Extraction

After reading and reviewing the selected research articles in full, the data were compared to ensure integrity and reliability. Divergent decisions were resolved by consensus.

For each experimental study included, we extracted relevant information related to the authors, publication year and experimental model features as species, sex and age. To access the research methods, we extracted specific information related to the experimental groups, number of animals per group, type of food intervention and method of consumption of the intervention. For the control of test food intake, we extracted information related to the type of biofortified food that was used in the intervention, the type of micronutrient incorporated in the food, the duration of the intervention, the methods of evaluation of the gut microbiota and the main results.

For this review, data from the eligible studies are expressed in tables and figures. We provided a narrative synthesis of the results according to the main characteristics and results related to the topic addressed.

2.5. Risk-Of-Bias Assessment

The methodological quality of the included studies was assessed, and the risk of bias was verified using the Systematic Review Centre for Laboratory Animal Experimentation Risk of Bias (SYRCLE RoB) tool [25], which is responsible for identifying the study quality and to measure the bias in research involving animal studies [26]. The SYRCLE RoB toll considers 10 entries that are related to six types of bias: selection bias, performance bias, detection bias, attrition bias, reporting bias, and other. For each included study, the six bias types were classified as “high” (+), “low” (–), or “unclear” (?).

To improve the quality evaluation of the included studies in this review, the criteria set forth in the Animal in Research: Reporting *in Vivo* Experiments (ARRIVE) guidelines [27] were used. A checklist of 20 items that evaluate essential descriptions about the experimental model, number of animals, study design, allocation of animals to experimental groups, methodological basis, statistical draw, and result measures were evaluated. For each criterion was filled out “0” for “not reported” or “1” for “reported”. The final score was displayed as a percentage for better visualization of the study quality.

3. Results

3.1. Selected Studies

The flow diagram with the number of selected or excluded articles in each selection step was built in accordance with PRISMA guidelines (Figure 1). Altogether, 592 articles were identified in the PubMed (n = 11), Web of Science (n = 18), Science Direct (n = 63), and

Scopus (n = 500). Of these, 587 articles were excluded: duplicate studies (n = 41), title, abstract and articles that were not suited to the topic (n = 336), review articles (n = 125), *in vitro* studies (n = 21), studies that could not be accessed (n = 3), and others scientific materials such as books, book chapters, or encyclopedia (n = 61). The remaining five articles were selected and after reading in full, all of them were eligible for this review. With the search in the reference lists we did not identify other relevant studies. All included studies were published from January 2010 and until December 2020.

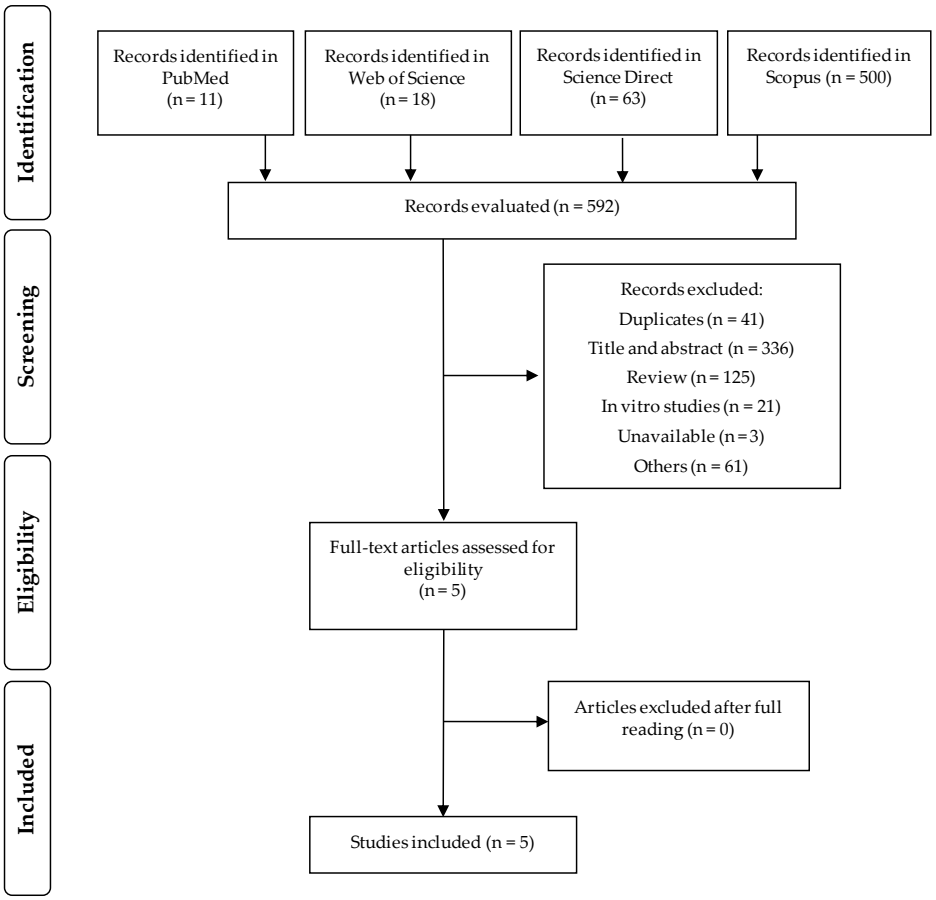


Figure 1. Flow diagram of the literature search process.

3.2. Characteristics of the Included Studies

The five original papers selected and included in the present systematic review were performed in the United States and the experimental model that was used was Cornish Cross broiler (*Gallus gallus*) [15,28,29,30,31]. Four studies used *Gallus gallus* hatchlings, male and female starting at day of hatch [15,28,30,31], and two studies performed the experiment at the embryonic stage (*Gallus gallus*) [28,29] (Table 2).

The studies were based on the consumption of biofortified foods with micronutrients. Three studies evaluated Fe biofortified carioca beans (*Phaseolus vulgaris* L.) [15,29,30], one

study evaluated Fe biofortified wheat (*Triticum aestivum*) [28], and one evaluated Zn biofortified wheat [31]. Other details of the characteristics of the eligible studies were included and described in Table 2.

Table 2. Characteristics of the eligible studies assessed for Fe and Zn biofortified foods in the gut microbiota modulation.

Reference	Animal model	Sex/age	Number of animals	Type of food to intervention	Method of administration	Duration of intervention (wk)
Reed et al., 2018 [31]	Cornish Cross broiler (*Gallus gallus*)	Male and female/ Hatchlings	30 (n = 15 per group)	Zinc biofortified wheat (*Triticum aestivum*)	Oral (in diet)	6
Reed et al., 2017 [30]	Cornish Cross broiler (*Gallus gallus*)	Male and female/ Hatchlings	28 (n = 14 per group)	Iron biofortified carioca bean (*Phaseolus vulgaris* L.)	Oral (in diet)	6
Dias et al., 2018 [15]	Cornish Cross broiler (*Gallus gallus*)	Male and female/ Hatchlings	28 (n = 14 per group)	Iron biofortified carioca bean (*Phaseolus vulgaris* L.)	Oral (in diet)	6
Dias et al., 2019 [29]	Chicken embryos (*Gallus gallus*)	Male and female/ Day 17th of embryonic incubation	80 eggs (n = 10 per group)	Iron biofortified carioca beans (*Phaseolus vulgaris* L.)	Intra-amniotic administration (1 mL per egg)	17th day to 21st day
Beasley et al., 2020 [28]	1st experiment: Chicken embryos (*Gallus gallus*) 2nd experiment: Cornish Cross broiler (*Gallus gallus*)	1st experiment: Male and female/ Day 17th of embryonic incubation 2nd experiment: Male and female/ Hatchlings	1st experiment: 40 eggs (n ≥ 5 per group) 2nd experiment: 30 (n = 15 per group)	Iron biofortified wheat (*Triticum aestivum* L.)	1st experiment: Intra-amniotic administration (1 mL per egg) 2nd experiment: Oral (in diet)	1st experiment: 17th day to 21st day 2nd experiment: 6

All included studies compared the biofortified food with the parallel standard food. The offered dosage varied according to the specific study and all reviewed studies were different. Reed et al. [31] tested 75% of Zn biofortified wheat in the diet, totaling 46.5 μg Zn/g; Reed et al. [30] tested 34.6% of Fe biofortified bean in the diet, totaling 48.7 μg Fe/g; Dias et al. [15] tested 42% of Fe biofortified bean in the diet, totaling 47.04 μg Fe/g; Dias et al. [29] performed utilized the intra amniotic administration (*in ovo* feeding) *in vivo* approach and assessed the effects of 50 mg/mL of Fe biofortified bean soluble extract per egg; and Beasley et al. [28] tested in the first study (via intra amniotic administration) 50 mg/mL of soluble extract from Fe biofortified wheat, and in a consecutive study evaluated 80% of Fe biofortified wheat in the diet, totaling 28.9 μg Fe/g.

3.3. Main Findings

The reviewed experimental studies demonstrated that Fe and Zn biofortified foods provide several health benefits to the host and improved the intestinal bacterial profile that leads to a healthier gut.

Of the five studies evaluated, three studies that performed the 16S rRNA gene sequencing reported significant differences in β-diversity between biofortified food vs. control treatment groups [15,28,31]. Two studies reported a significant increase in Firmicutes [15,30] and one study reported a reduction of the Firmicutes phyla [28]. Only one study reported a significant increase in the Bacteroidetes phyla after the consumption of the assessed biofortified food [31] (Table 3). Positive findings that are associated with SCFA producing bacteria were shown in the majority of the reviewed studies. These findings include an increased abundance of lactic acid bacteria [31], butyrate producing bacteria [30], and a general increased abundance of beneficial SCFA producing bacteria [15], that leads to an increase in SCFA production (acetic, propionic and valeric acids). Further, an increase in *Bifidobacterium* and *Lactobacillus* probiotic genera abundance in the Fe biofortified group was observed [28], and two studies reported an increase in bacteria linked to phenolic catabolism in the group that was fed the Fe biofortified material based diet [15,31].

Table 3. Methods and main findings in studies on the use of Fe and Zn biofortified foods in gut microbiota modulation.

Reference	Experimental groups	Method of evaluation of the gut microbiota	Microbial activity
Zn-biofortified food			
Reed et al., 2018 [31]	CZn: Standard wheat (75% wheat-based diet; 32.8 ± 0.17 µg Zn/g) BZn: Zn biofortified wheat (75% Zn wheat-based diet; 46.5 ± 0.99 µg Zn/g)	16S rRNA gene sequencing	Change in β-diversity between the CZn and BZn groups. ↔ no difference in abundance between *Firmicutes*, *Actinobacteria*, and *Proteobacteria* phyla according taxon-based analysis; ↔ no differences between groups at the genus level, according taxon-based analysis. LEfSe method: ↑ *Lactobacillus reuteri* and members of the *Dorea*, *Clostridiales*, *Ruminococcus* and *Lachnospiraceae* family in BZn group.
Fe-biofortified foods			
Reed et al., 2017 [30]	SFe: Fe standard, 34.6% cream seeded carioca bean based diet (33.7 ± 0.80 µg Fe/g) BFe: Fe biofortified bean, 34.6% cream seeded carioca bean based diet (48.7 ± 1.50 µg Fe/g)	16S rRNA gene sequencing	No change in β-diversity between the BFe and SFe groups; no difference in α-diversity between groups. ↑ *Elusimicrobioa* and *Euryarchaeota* phyla; ↑ *Dehalobacteriaceae* and *Enterococcaceae* family; ↑ unclassified *Dehalobacteriaceae* genus in the BFe group. ↓ *Elusimicrobiaceae*, *Methanobacteriaceae*, and *Methanomassiliicoccaceae* family; ↓ unclassified *Elusimicrobiaceae*, *Methanobrevibacter*, *vadinCA11*, and *Enterococcus* genus in the BFe group; LEfSe method: ↑ *Proteobacteria* and *Firmicutes*; ↓ *Elusimicrobiota* and *Euryarchaeota* at phylum level; ↑ *Campylobacterales*; ↓ *Enterobacteriales*, *Elusimicrobiales*, *Bacteroidales* and E2 at order level; ↑ *Helicobacteraceae*, *Dehalobacteriaceae*, and *Streptococcaceae*; ↓ *Enterobacteriaceae*, *Enterococcaceae*, *Elusimicrobiaceae*, *Coriobacteriaceae*, *Methanomassiliicoccaceae*, and *Methanobacteriaceae* at family level; ↑ *Helicobacter*, *Ruminococcus*, *Coprococcus*, and *Streptococcus;* ↓ *Lachnospira*, *Enterococcus*, *vadinCA11*, *Methanobacterium*, and *Methanobrevibacter* at genus level; ↑ OTUs enriched *Faecalibacterium prausnitzii*, *Barnesiella viscericola*, *Enterococcus cecorum*, and *vadinCA11* in the BFe group.
Dias et al., 2018 [15]	SC: Fe-standard carioca bean-based diet, 42% BRS Perola bean based diet (40.47 ± 1.84 µg Fe/g)	16S rRNA gene sequencing	Change in β-diversity between the BFe and SFe groups; no difference in α-diversity between groups; ↔ no significant differences between groups at the genus level; LEfSe method: Predominance of SCFA-producing Firmicutes in BC group;

	BC: Fe-biofortified carioca bean based diet, 42% BRS Cometa bean (47.04 ± 1.52 µg Fe/g)		↑ *Eggerthella lenta* and *Clostridium piliforme*; members of the *Coriobacteriaceae*, *Dehalobacteriaceae* and *Lachnospiraceae* in the BC group.
Dias et al., 2019 [29]	Non-injected 18 MΩH2O Inulin (40 mg/mL) Perola bean extract (Fe standard carioca bean) * Cometa bean extract (Fe biofortified carioca bean) * Esteio bean extract (Fe standard black bean) * SMN 39 bean extract (Fe biofortified black bean) * Artico bean extract (Fe standard white bean) * * 50 mg/mL	PCR amplification of bacterial 16S rDNA for *Lactobacillus, Bifidobacterium, Clostridium* and *E. coli*	↓ relative abundance of *Bifidobacterium* in biofortified carioca bean extract compared to standard; ↓ relative abundance of *E. coli* in biofortified carioca bean extract compared to standard; ↑ relative abundance of *Lactobacillus* in biofortified black bean extract compared to standard; ↑ relative abundance of *Clostridium* and *E. coli* in biofortified black bean extract compared to standard; ↔ relative abundance of *Lactobacillus* and *Clostridium* in biofortified carioca bean extract compared to standard; ↔ relative abundance of *Bifidobacterium* in biofortified black bean extract compared to standard.
Beasley et al., 2020 [28]	<u>1st experiment:</u> NI: non-injected H_2O: 18 MΩH_2O Fe: Fe solution (1 mg/mL) Fe-EDTA: Fe-EDTA solution (77 µM Fe) Fe-NA: Fe-Nicotinamine solution (1.6 mM) C WF: Control wheat flour extract* (0.91 µg Fe/g of extract) B WF: Fe biofortified wheat flour extract* (0.82 µg Fe/g of extract) * 50 mg/mL <u>2nd experiment:</u> Control: Fe-standard wheat, 80% wheat based diet (25.9 ± 0.12 µg Fe/g) Biofortified: Fe-biofortified wheat, 80% Fe wheat-based diet (28.9 ± 0.13 µg Fe/g)	<u>1st experiment:</u> PCR amplification of bacterial 16S rDNA for *Lactobacillus, Bifidobacterium,Escherichia* and *Clostridium* <u>2nd experiment:</u> 16S rRNA gene sequencing	<u>1st experiment:</u> ↔ relative abundance of *Bifidobacterium, Lactobacillus, Escherichia* and *Clostridium* in biofortified wheat flour extract compared to the Control. <u>2nd experiment:</u> Change in β-diversity and α-diversity between the Control and Biofortified groups; ↑ 1.9-fold the proportion of *Actinobacteria*; ↓ 1.2- and 2.0-fold, respectively, the proportion of *Firmicutes* and *Proteobacteria* in 'Biofortified' relative to 'Control' group at phyla level; ↑ 1.9- and 1.5-fold, respectively, the proportion of *Bifidobacterium* and *Lactobacillus*; ↑ abundance of *Enterococcus*; ↓ proportion of *Streptococcus* (1.7-fold), *Coprococcus* (1.4-fold), *Ruminococcus* (1.2-fold) *Faecalibacterium* (2-fold), and *Escherichia* (2-fold); ↓ *Dorea* abundance in 'Biofortified' relative to 'Control' group at genera level; ↓ 1.7-fold the proportion of *Lachnospiraceae* and ↑ abundance of *Enterococcaceae* families in 'Biofortified' relative to 'Control' group.

↔ no change; ↑ increased; ↓ reduced; LEfSe: linear discriminant analysis effect size.

In related to risk of bias, all studies included have described their titles and abstracts properly, presented the primary and secondary objectives in the manuscript introduction section, provided an ethical statement, included an adequate experimental protocol, and other relevant details in the manuscript methods section, and they all showed the dose of biofortified food offered to the animals. All studies described the route of consumption of biofortified food that was offered to the animals and all studies provided the information on how the biofortified foods were obtained and treated before use or provide the reference of a scientific article with the appropriate information and relevant methodologies.

Figure 2 summarizes the risk of bias that is related to each manuscript that was included in this review and the ARRIVE guideline was summarized in the Supplementary Material (Table S1). Due to the nature of studies included, the risk categorization is not specified. The random sequence generation, the random of the outcome assessment, and the blinding of the outcome assessor were the most uncertain points detected in the manuscripts included in this review. The randomness of outcomes improves the quality of the selection and measurement bias and it is advisable to follow a methodology of randomization and blind evaluation of the results [32].

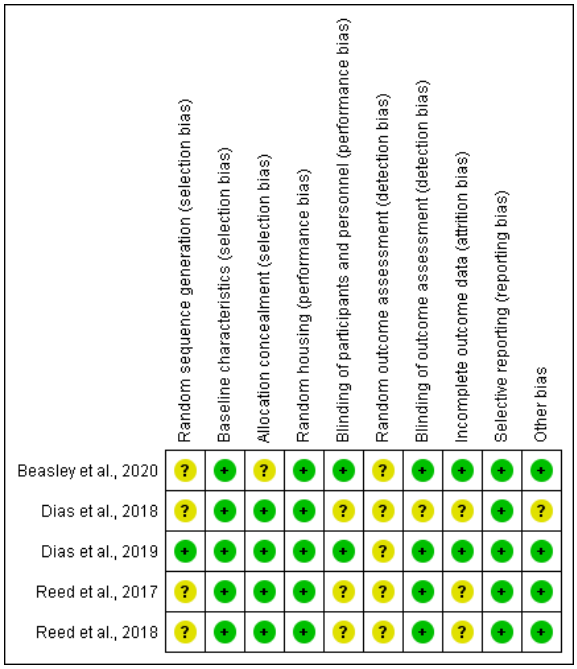


Figure 2. Risk of bias summary: review authors' judgments about each risk of bias item for each included study. +, low risk; ?, unclear.

4. Discussion

In recent years, there has been an increase in the overall consumption of biofortified foods and in the development of new strategies related to food biofortification, aiming to increase the supply of nutrients, minerals and vitamins to populations with higher risk of dietary deficiency. Hidden hunger is characterized by inadequate intake of one or more micronutrients such as pro-vitamin A, Fe, and Zn. These types of nutritional deficiencies install imperceptibly in the body and lead to serious complications, especially for most vulnerable groups, as children of preschool age, pregnant women, lactating women and the elderly [33]. Food fortification may be an effective strategy, but it may not be sustainable, and may not reach all relevant target populations. Biofortification, on the other hand, can be a more efficient, economical, and sustainable alternative to maintain long-term nutrient consumption and improve the health of relevant populations with poor access to balanced diet.

4.1. Impact of Fe and Zn Biofortification on the Gut Microbiota In Vivo

In recent years, several studies were designed and conducted to assess the efficacy of biofortified foods, also aiming to evaluate productivity, in an economic context, and the consumption to these foods by the population [34]. This includes assessing the dietary mineral bioavailability and gene expression of BBM proteins that are associated with mineral absorption [35], including the effects of the tested dietary composition on intestinal functionality and host's intestinal microbial taxa composition. However, there is still no consensus whether the increased micronutrients content in foods influences the gut microbiota. In this review, we provided evidence that the consumption of Fe and Zn biofortified foods may improve the host's gut microbiota composition and function.

All studies included in this review utilized the established *Gallus gallus* model, which has been used to assess the bioavailability of minerals, specifically Fe and Zn, as this model exhibits the appropriate responses to Fe and Zn deficiencies and can serve as a model for Fe and Zn dietary bioavailability and absorption [36,37,38]. The *Gallus gallus* model inhabits a dynamic and complex intestinal microbiota, similar to that of humans, with predominance of the Firmicutes, Proteobacteria and Actinobacteria phyla [15,30,39]. In addition, there is a great homology (>85%) between human and chicken intestinal genes responsible for the expression of BBM proteins involved with the Fe and Zn absorption, such as Divalent Metal Transporter 1 (DMT1), Duodenal cytochrome b (DcytB), Zinc Transporter 1 (ZnT1) and

Ferroportin (FPN) [40]. The 16S rRNA gene sequencing is a specific method for studying bacterial phylogeny and taxonomy and has been widely used in studies that evaluate human and animal microbiome. The 16S rRNA gene has been preserved for generations, it is present in most bacteria, allowing precise investigation in the field of the microbiome and it allows stratification at the genus and species level [41]. In this review, some studies used the linear discriminant analysis effect size (LEfSe) [42] to investigate significant bacterial biomarkers that could identify differences in the gut microbiota of treatment groups. The four studies that performed the 16S rRNA gene sequencing reported a change in β-diversity between treatment vs. control groups [15,28,31]. Changes in the β-diversity can occur in an experimental group after treatment, but do not necessarily indicate a beneficial variation in the bacterial taxa. Generally, the response of the microbial taxa to the consumption of Fe and Zn biofortified foods varied in terms of taxonomic abundance but show a similar pattern in qualitative terms.

Zinc is an essential mineral with catalytic, structural and regulatory functions that has a refined homeostatic control, making it difficult to identify its inadequate levels in the body [43]. Bacteria that colonize the gastrointestinal tract are dependent on minerals, and the bacterial activity contribute to minerals solubility, making Zn biofortified foods a promising strategy to improve intestinal health, and potentially via bacterial fermentation activities. Reed et al. [31] observed an increase in *Lactobacillus reuteri*, members of *Dorea*, *Clostridiales*, *Ruminococcus*, and *Lachnospiraceae* in the group that received Zn biofortified wheat-based diet. As Zn is essential for bacteria, the abundance of Zn-dependent microorganisms is up regulated in an environment with higher Zn bioavailability [44], this includes the *Ruminococcus* genus, that houses species of gram-positive bacteria that degrade cellulose or polysaccharides of the diet, especially resistant starch. The fermentation activity of SCFA-producing bacterial populations is recognized as an important contributor to the overall health of the gut ecosystem [45]. In addition, the abundance of *Lactobacillus reuteri* in the biofortified group suggests that the wheat-based diet provides prebiotic properties, with potential modulation and beneficial effect on the host's intestinal bacterial profile, since *L. reuteri* interacts with both epithelial and non-epithelial cells with potent anti-inflammatory effects [46,47]. Further, the *Clostridiales* order has also shown an increased abundance in the biofortified group. *Clostridiales* belongs to the Firmicutes phylum, and is represented by fermenting microorganisms and SCFA producers in the intestine, mainly butyrate, which may lead to improvement in the host's gut health [48,49]. The increase in SCFA-producing

bacteria, specifically butyrate producers in the Fe biofortified group was also documented [30].

The gut microbiota is shaped directly by the host's dietary habits and the presence of plant-origin dietary ingredients that modulate the colonization of selective bacterial populations. It was recently shown that in addition to the known plant primary metabolites, as soluble and insoluble dietary fibers, proteins and carbohydrates, the secondary metabolites, specifically phytochemicals, as phenolic compounds, terpenoids, and alkaloids, can also have antimicrobial properties, which can modify the composition and function of the intestinal microbiota [50]. The selective modulation of intestinal microorganisms that arises when consuming a certain food, occurs in conjunction with the process of metabolizing the components that are present in it. This allows the host to absorb and transform these phytochemicals and supply the required metabolites to the relevant bacterial populations. The presence of phenolic compounds and the highest content of Fe in biofortified beans [30] beneficially modulated the abundance of bacteria involved in phenolic catabolism, such as *Faecalibacterium prausnitzii*, *Enterococcus spp*., *Barnesiella spp*., and members of *Dehalobacteriaceae* and it does not seem to affect the composition nor genetic capacity of the gut microbiota [30].

This same qualitative pattern of the gut microbial taxa following the consumption of biofortified foods based diets was also shown in studies by Dias et al. [15,29]. An acute exposure study that evaluated the effects of Fe biofortified bean soluble extracts on the gut microbiota, observed a reduction in the relative abundance of pathogenic bacteria such as *Escherichia coli* and *Clostridium* [29]. In the long term *in vivo* feeding trial (*Gallus gallus*), the authors observed a predominance of SCFA-producing Firmicutes [15]. In addition, the authors found a greater abundance of *Eggerthella lenta* in the biofortified group. *E. lenta* is an intestinal bacterium belonging to the *Actinobacteria* phylum. These species have the ability to convert catechin and epicatechin into their metabolic derivatives, with potential to increase the bioavailability of catechin metabolites and improve intestinal health [51]. The greater abundance of *Coriobacteriaceae*, specifically *Eggerthella lenta* and *Lachnospiraceae*, butyrate producers [52], in the Fe biofortified bean group indicates on the protective potential of the biofortified foods to the host and via microbial activity, and without adverse changes in the microbiome's composition.

Wheat is also a target staple food crop for biofortification and fortification worldwide. The development of Fe biofortified wheat lines is a strategy to improve the bioavailable long

term dietary Fe supply to populations with higher risk of Fe dietary deficiency. The effects of Fe biofortified wheat flour on the gut microbiota were evaluated during a 6-week trial in a study conducted by Beasley et al. [28]. The authors observed an increase in the abundance of the Actinobacteria phylum and a reduction in the abundance of Firmicutes and Proteobacteria in the Fe biofortified wheat group when compared to the control group. Hence, since there is a positive relationship between the abundance of Actinobacteria and the consumption of dietary fiber from legumes (beans), fruits and vegetables [53], these observations suggest a potential beneficial effect of this phylum on overall intestinal health. Further, the observed reduction of Firmicutes, Proteobacteria, *Streptococcus*, and *Escherichia* genera confirms the hypothesis that food biofortification can improve the gut health and may reduce the abundance of pathogenic bacterial taxa.

Previously, the Proteobacteria phylum was associated with intestinal dysbiosis and inflammatory diseases, such as metabolic disorders and inflammatory bowel disease [54,55]. The Fe biofortified wheat flour contributed to the increased abundance of bacterial populations with recognized probiotic functions, specifically *Bifidobacterium* and *Lactobacillus*, that are able to maintain a symbiotic relationship with the host and to increase the production of SCFAs such as acetic, propionic and valeric acids, and by positively regulating the enzymes of glycolysis and gluconeogenesis [28]. In addition, improvements in dietary Fe bioavailability and physiological status were observed in the biofortified group relative to the control, indicating on a high potential of this food matrix to improve intestinal health.

The protective effects of Fe and Zn biofortified foods on gut microbiota were also evident by the intestinal morphometric evaluation. This association was performed in three of the research manuscripts that were evaluated. One study observed an increase in goblet cell density (per 10 villi) in the group that was fed the Zn biofortified food [31], one study observed an increase in goblet cell number in the group that was fed the Fe biofortified food [28], and one study observed an increase in villi height and diameter, and no difference in goblet cells number in the Fe biofortified group relative to control [15]. In addition, one manuscript reported a depletion in transcription-related proteins and mineral absorption according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in the Fe biofortified group compared to the control, suggesting that the luminal Fe was not used by the bacteria [30]. However, in four manuscripts the Fe and Zn biofortified foods affected the microbiome by leading towards increased abundance and capacity of intestinal resident

bacteria to provide beneficial SCFAs and therefore, favor mineral absorption by the host [15,28,29,31]. Both Fe and Zn are essential micronutrients that are required by beneficial bacteria that make up the gut microbiota. The goblet cells differentiation process is controlled by extrinsic and intrinsic factors, and a healthy gut microbiota increases goblet cells density, which synthesize and secrete the mucus that coats the intestinal lumen. The mucus layer is rich in polysaccharide/protein that protects epithelial cells from the growth of pathogenic bacteria and modifies the luminal environment to favor the absorption of micronutrients [56,57].

The body of evidence reported here indicates that biofortified foods act through beneficial modulation of bacterial taxa, with no adverse risk to the composition of gut microbiota.

4.2. Dosages and Reporting Quality

Despite the varied offered dosage, the *in vivo* studies are based on the analysis of personal food consumption and dietary patterns of relevant populations. Only one study [15] included the source of the database (country dietary survey) that was used to calculate the dietary content and level of the ingredients that were used, including the assessed biofortified food. Studies using Fe biofortified foods were used in a proportion of 34.6–80% from diet and provided between 26.9 and 48.7 µg Fe/g of diet [15,28,30], with no adverse effects on the composition nor genetic capacity of the gut microbiota. Only one study that evaluated zinc biofortified wheat used 75% wheat-based diet, and included 46.5 µg Zn/g of diet [31], with no adverse effects on the gut microbiota composition. Thus, the related microbiome results indicate a promising effect of biofortified foods, with beneficial modulation effect of the host's gut microbiota.

The current systematic review examines the effects of biofortified foods on gut microbiota. The selection of literature was performed on widely recommended and approved practices for systematic reviews. The risk of bias was verified using the SYRCLE RoB tool [25] and the ARRIVE guidelines [27], aimed to investigate and confirm all possible factors that influence the quality of the *in vivo* studies that are included in this review. Furthermore, the random sequence generation, the blinding of the investigators, the random of the outcome assessment, and the blinding of the outcome assessor may present potential limitations in some *in vivo* studies [58,59]. Also, according to ARRIVE guidelines and SYRCLE'S risk of

bias tool, to classify studies as “low risk of bias” must be considered and conducted carefully and appropriately.

The number of studies included in this review may be a limitation to directly demonstrate the link between the consumption of biofortified foods and the gut microbiota. Although the articles search was made in four of the most important databases, other databases may include more articles that were not selected. In regard to the *in vivo* studies with the experimental model of *Gallus gallus*, it is known and established that this model is suitable for the evaluation of dietary mineral bioavailability, mineral metabolism and the gut microbiota, however, no animal model provides physiological responses that can be completely extrapolated to the humans. In addition, in *in vivo* studies in general, animals consume the specific diet that is provided, based on the tested dietary ingredient and as part of controlled environment and specific study design, which is different from humans in qualitative and quantitative terms. The results found in this review serve as a guide for the development of future clinical trials that may clarify the role of biofortified foods in human health, and specifically the effects on the microbiome.

Hence, this paper provides new insights in this field, and highlights the necessity of more experimental studies and clinical trials to evaluate the gut microbiota due to consumption of biofortified foods. The selected studies allow us to observe and discuss the important associations between the consumption of biofortified foods and the gut microbiota, which is an emerging research area in the field of mineral nutrition.

5. Conclusions

The biofortification of foods is a strategy that aims to increase the supply of micronutrients, vitamins and minerals to diverse populations. However, the study of the effects of these foods on the intestinal microbiota is critical to further clarify the potential beneficial effects on host’ intestinal functionality and overall health. Despite the high interindividual variability in the microbiota composition, experimental results showed that the consumption of Fe and Zn biofortified foods modifies the local microbial ecology, increases the abundance of SCFAs producing bacteria and decreases the abundance of potentially pathogenic bacteria, such as *Streptococcus*, *Escherichia*, and *Enterobacter*. This review supports the prospective use of Fe and Zn biofortified foods to increase the colonization of the microbial taxa with beneficial bacteria and therefore to potentially improve the host’s intestinal health. A potential benefit on gut microbiota was verified with the consumption of

about 50% biofortified material-based diet. Further studies are needed to strengthen the evidence found in this systematic review, to confirm the amount of the biofortified foods which, in fact, presents a beneficial effect on the gut microbiota in animal model and in humans, and to develop public health strategies to strengthen biofortification programs and encourage the consumption of these foods worldwide.

Supplementary Materials

The following is available online at https://www.mdpi.com/2072-6643/13/1/189/s1,

Table S1. Risk of bias from experimental studies.

Reference	Reed et al., (2018)	Reed et al., (2017)	Dias et al., (2018)	Dias et al., (2019)	Beasley et al., (2020)	Percentage (%)
(1) Title	1	1	1	1	1	100
(2) Abstract	1	1	1	1	1	100
Introduction						
(3) Background information	1	0	1	1	1	80
(4) Primary and secondary objectives	1	1	1	1	1	100
Methods						
(5) Ethical statement	1	1	1	1	1	100
(6) Study design	1	1	1	1	1	100
(7) Experimental procedures	1	1	1	1	1	100
(8) Experimental animals detail	1	1	1	1	1	100
(9) Housing and husbandry conditions	1	1	1	1	1	100
(10) Sample size	0	0	0	0	0	0
(11) Allocating animals to experimental groups	1	1	1	1	1	100
(12) Experimental outcomes	1	1	1	1	1	100
(13) Statistical methods	1	1	1	1	1	100
Results						
(14) Baseline data	1	1	1	1	1	100
(15) Number of animals analyzed	0	0	0	0	1	20
(16) Outcomes and estimation	1	1	1	1	1	100
(17) Adverse events	1	1	1	0	1	80
Discussion						
(18) Interpretation/ scientific implications/study limitations	1	1	1	1	1	100
(19) Generalizability/translation/ relevance to human biology	1	1	1	0	1	80
(20) Funding	0	1	1	1	0	60

0: not reported; 1: reported.

Author Contributions

Conceptualization, M.J.C.G., H.S.D.M., and E.T.; methodology, M.J.C.G. and H.S.D.M.; writing—original draft preparation, M.J.C.G.; writing—review and editing, M.J.C.G., H.S.D.M., and E.T.; supervision, H.S.D.M. and E.T.; project administration, H.S.D.M. and E.T. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

All animal protocols related to studies reviewed in this manuscript were conducted according to the guidelines of the Declaration of Helsinki, and were approved by the Cornell University Institutional Animal Care and Use committee (ethic approval code: 2007–0129).

Data Availability Statement

The data analyzed in this study are openly available in reference numbers [15,28,29,30,31].

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5.2. PAPER 2

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Original Research

Zinc-biofortified staple food crops to improve zinc status in humans: a systematic review

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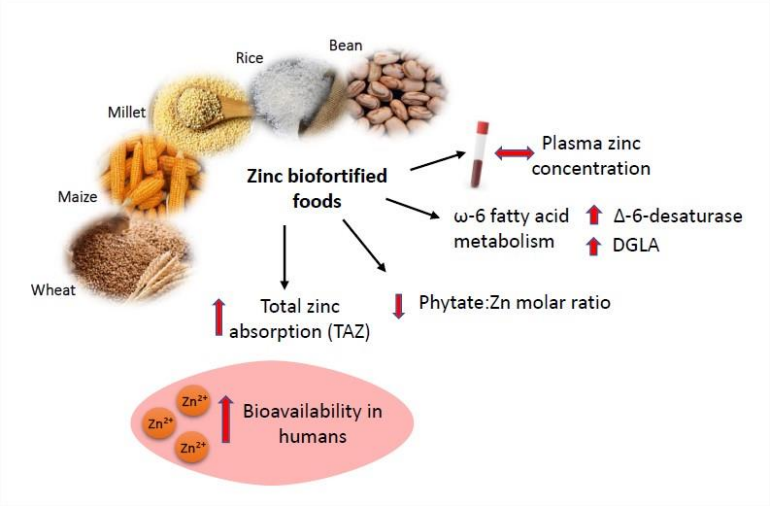
Abstract

Biofortified foods are a new approach to increase minerals in the diet, and evidence suggests that zinc (Zn) biofortification can improve Zn physiological status in humans. This systematic review aimed to answer the question: "What are the effects of the consumption of Zn biofortified foods on Zn status in humans?". This review was conducted according to PRISMA guidelines and registered in PROSPERO (CRD42021250566). PubMed, Cochrane, Scopus and Science Direct databases were searched for studies that evaluated the effects of Zn biofortified foods on Zn absorption. Of 4282 articles identified, nine remained after inclusion/exclusion criteria were applied. Limitations in study quality, external and internal validity (bias/confounding), and study power were evaluated. The Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) was used to assess the certainty of evidence. Of the nine articles included, five observed an increase in total Zn absorption, and one showed that Zn participated in the conversion of linoleic acid to dihomo-γ-linolenic acid. By increasing the amount of Zn in the food, Zn biofortification can reduce the phytate:Zn molar ratio and improve Zn absorption in humans. More studies are needed to clarify what portion of Zn biofortified foods/day is needed to achieve a significant effect on Zn status.

Keywords: biofortification; zinc metabolism; zinc bioavailability; zinc absorption; minerals

Graphical abstract



Introduction

Zinc (Zn) and Iron (Fe) deficiencies are the most prevalent worldwide and have a significant impact on public health, especially among women and children (Roba et al. 2018; Ramakrishnan 2002). Zn deficiency is concerning because Zn plays an essential role in numerous enzymatic reactions, it participates in the regulatory pathways of the immune system, and it is essential during the growth stage of children (Belay et al. 2021; Ackland and Michalczyk 2016). Zn intake through food may be relatively low in a non-balanced diet, and it is estimated that about 17% of the world population is affected by dietary Zn deficiency, which makes it the mineral with the second highest deficiency rate, second only to Fe (Wessells and Brown 2012). The low intake of Zn associated with its low bioavailability in food is one of the factors that contributes to its deficiency (Maret and Sandstead 2006). Thus, the use of Zn biofortified foods (Figure 1) can be a strategy to reduce dietary Zn deficiency rates, worldwide.

It was demonstrated that the prevalence of Zn deficiency is higher in Africa, Asia and Latin America, respectively, and mainly in certain countries as Bangladesh, Cameroon, Cambodia, Ecuador, Ethiopia, Kenya, Malawi and Vietnam (Gupta, Brazier, and Lowe 2020). A recent study indicated that in Ethiopia, the prevalence of Zn deficiency is alarming, specifically in rural populations (74.9%) and compared to the urban populations (55.3%), where these rates further demonstrate the need to establish strategies for the cultivation of biofortified foods with adequate supply in rural areas (Belay et al. 2021). Among the most produced biofortified crops, cereals (47%), legumes/pulses (25%), vegetables (19%), oilseed

(6%) and fruits (3%) have been shown to provide sufficient concentrations of minerals for target populations (Garg et al. 2018).

Biofortification by conventional breeding methods consists of increasing the micronutrient content of standard cultivars through conventional plant breeding, where plants of the same species are selected and those with higher content of nutrients, such as Zn, Fe, or provitamin A, are crossed with each other to obtain improved varieties (La Frano et al. 2014; Bouis et al. 2011). There is evidence that the use of conventional breeding is the fastest route for farmers and consumers to gain access for seeds of more nutritious crops (Bouis and Saltzman 2017). Other biofortification approaches are also widely used, as foliar biofortification, hydroponic cultivation and soil Zn application (Figure 1). In the foliar biofortification, solution rich in the mineral of interest are applied to the leaves. Foliar applications have shown several benefits in terms of quality of harvested crops, including, increase in the amount of minerals and plant nutrition (Alshaal and El-Ramady 2017). Similarly, hydroponic cultivation of biofortified foods aims to improve the mineral content in plants. This cultivation approach is made under controlled conditions, and has benefits in terms of plant growth time, it can be implemented in environments with limited physical space and low labour demand. However, there is still a demand for low cost hydroponic technologies, mainly in the context of method implementation and early stages of hydroponic food production (Sharma et al. 2018). Phytic acid (phytate) present in food, especially plant-based, reduces the bioavailability of minerals because its molecule is negatively charged, which leads to a strong potential to bind to bivalent cations, such as Zn and Fe, hence, reducing absorption. Biofortification of foods with Zn increases the content of this mineral and therefore can be a strategy to improve the phytate:zinc molar ratio (Sparvoli and Cominelli 2015).

Zinc found in cells throughout the body maintains a precise metabolic balance, which makes the assessment of Zn nutritional and physiological status a challenge for the scientific community. Stable isotope techniques have been used to evaluate Zn absorption in humans, although it is expensive and difficult to apply in large population studies. By using dual-isotope tracer ratio techniques, the fractional zinc absorption (FZA) and total zinc absorption (TZA) from dietary intervention can be assessed (Hambidge et al. 2006; Lowe, Fekete, and Decsi 2009). A promising biological indicator for Zn nutritional status that has been proposed *in vivo* (Knez et al. 2018; Reed et al. 2014) and in studies with humans (Knez et al. 2016; Knez et al. 2017) is the ratio between erythrocyte or plasma linoleic acid (LA): dihomo-γ-

linolenic acid (DGLA). Zn is an essential mineral used by Δ-6-desaturase enzyme to convert LA (C18:2n-6) to DGLA (C20:3n-6) and this metabolic pathway was demonstrated to reflect the Zn physiological status. In addition, serum and plasma Zn concentration is still used to evaluate Zn nutritional status, however, it was demonstrated to be a problematic predictor of Zn status in adult and children (Moran et al. 2012; Lowe et al. 2012; King 2018).

Despite the wide spectrum of benefits in maintaining an adequate Zn supply in the body, and efforts to increase the dietary Zn intake by vulnerable populations, limited information is available about the effects of consuming Zn biofortified foods on Zn absorption, in humans. Further, there is no evidence if the Zn content in these foods presents higher bioavailability in children and adults. The aim of this systematic review was to investigate if Zn physiological status can be improved by the consumption of Zn biofortified foods. This proposed goal aims to answer the question: "What are the effects of the consumption of Zn biofortified foods on Zn status in humans?" It is hypothesized that the higher amount of Zn provided by Zn biofortified foods increases the Zn absorption, and also reduces the phytate:Zn molar ratio, which can improve Zn bioavailability. This is the first systematic review that identifies evidence of how the consumption of Zn biofortified foods in humans affects Zn physiological status. Further, the results presented here should be considered by the relevant programs that focused in developing strategies aimed to increase the consumption of Zn biofortified foods by at risk populations.

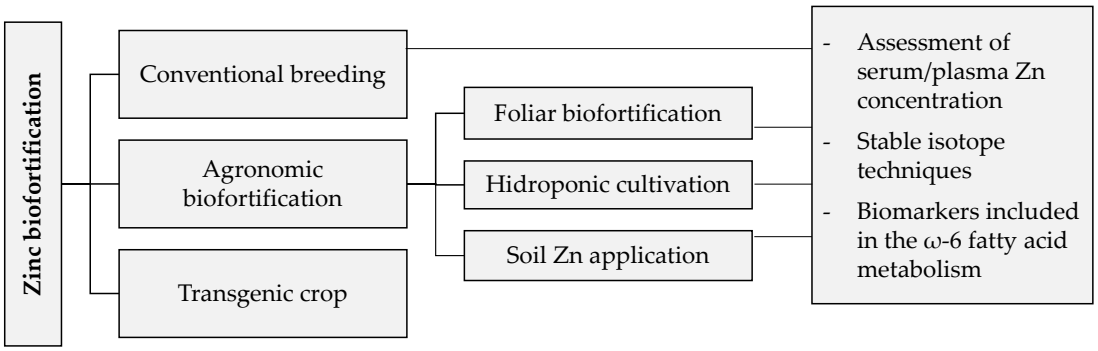


Figure 1. Schematic diagram of zinc biofortification methods and indicators of zinc absorption in humans.

Methods

Study identification and selection

The search was performed at the PubMed, Cochrane Library, Scopus and Science Direct databases, and no restrictions regarding the dates of the publications were added. English articles were selected by using descriptors from the Medical Subject Headings (MeSH), and

the search strategy was formed: (Biofortification OR "zinc bio-fortified") AND ("zinc deficiency" OR "zinc absorption" OR “zinc absorbed” OR "zinc bioavailability" OR "zinc requirement" OR "zinc status”). No filters were used. To combine the descriptors, the logical operators “AND” or “OR” were used in the search, whose last survey date was July 27, 2021.

As the topic addressed in this review is very new, this is the first study that reviewed the effects of Zn biofortified foods on Zn status in humans. The protocol for identification and selection of clinical trials was defined accordingly Figure 2. Two researchers (M.J.C.G. and H.S.D.M) independently analyzed the articles found with the search strategy and at the end of the selection, the reference list of the included studies was carefully analyzed to identify possible relevant articles that were not found in the search. The potentially eligible articles were read in full by the two authors and assessed for compliance with the established eligibility criteria. Discrepancies between reviewers were resolved through consensus with a third reviewer, and in case data is not reported or is unclear, we contacted the authors by email.

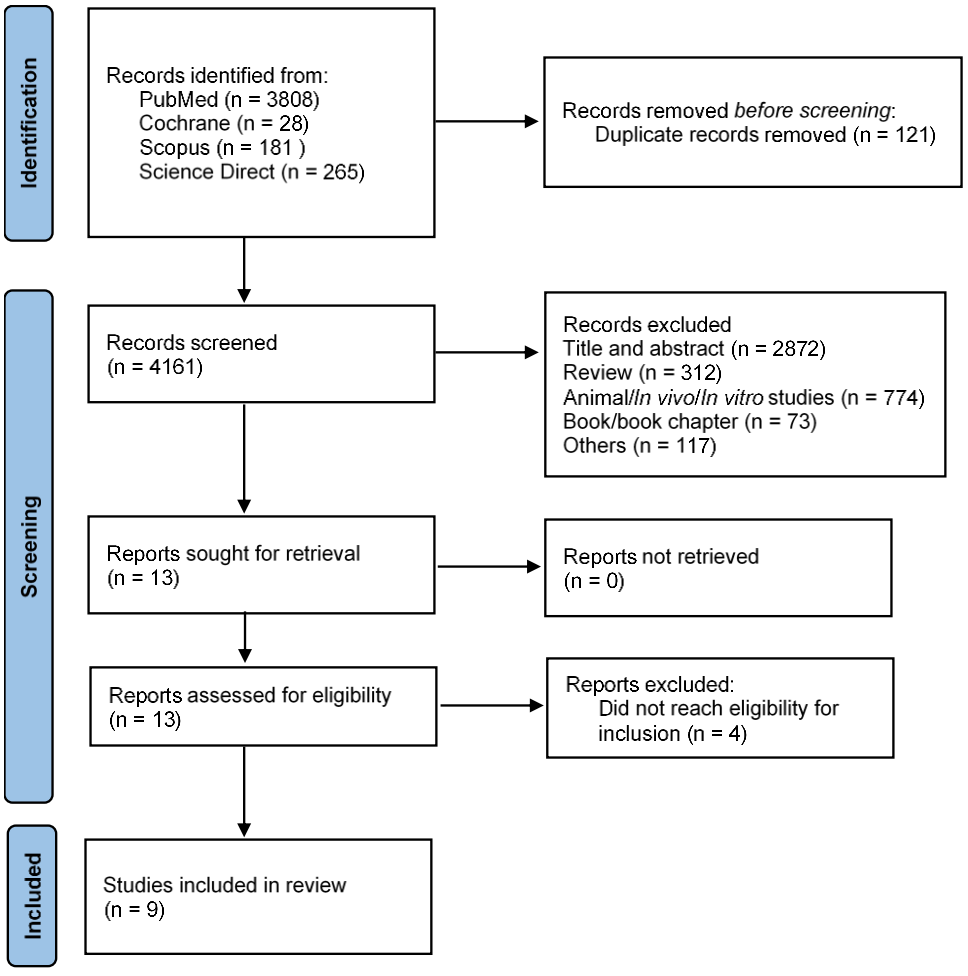


Figure 2. Flow diagram of the literature search included in the systematic review, according to PRISMA (2020).

Eligibility criteria

The eligibility criteria were elaborated with reference to participants, interventions, comparisons, outcomes, and study design (PICOS) (Table 1). Initially 4282 articles were identified, and only studies with Zn biofortified foods that evaluated the Zn absorption and Zn status in humans were selected.

Table 1. PICOS criteria for inclusion and exclusion of studies.

Parameter	Inclusion criteria	Exclusion criteria
Population	Human studies	*In vivo* or *in vitro* studies; pregnancy and lactation; pathologies different from obesity
Intervention	Zinc biofortified foods or their fractions (eg. flour, extracts)	Do not correlate Zn biofortified and Zn status; ultra-processed foods; biofortification with compounds different from zinc; fortification or supplementation
Comparison	Standard foods, or their standard fractions; standard meal, with no Zn biofortified or foods	No control group
Outcomes	Modulation of the Zn absorption increasing Zn status	
Study design	Randomized controlled study	*In vivo* or *in vitro* studies; reviews; consensus papers; letters to editor; theses and dissertations; non- human studies

Data extraction

For each study included, we extracted information about the authorship, publication year, country, study objective and study population. To access the research methods, we extracted information about the study design, the number of participants that completed the study, the type of food intervention, the Zn concentration in the food, duration of the intervention and the method used for biofortification. For the control of test food intake, information was extracted about the type of Zn biofortified food used in the intervention, the amount of Zn biofortified food offered and the experimental groups. To access the main results, we extracted information about the plasma Zn concentration, the fractional zinc absorption (FZA), total zinc absorption (TZA), Zn concentration in the food, phytate concentration and the phytate:Zn molar ratio.

Study quality assessment

The risk of bias assessment is a strategy to identify the quality of studies included in systematic reviews. A checklist based on the criteria proposed by Downs and Black (Downs and Black 1998) was used. Two authors (M.J.C.G and H.S.D.M) independently scored the overall quality of the papers based on 13 domains, assigning a score of 1 to each criterion satisfied and a score of 0 to each criterion not satisfied. The sum of the items evaluated is a predictor of the quality of the study, where ≤ 4 of 13 points indicates poor quality, 5–8 of 13 points indicates intermediate quality and ≥ 9 of 13 points indicates good quality.

Certainty of evidence and synthesis of results

The certainty of evidence was assessed using the Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) (GRADEpro, gradepro.org) (Balshem et al. 2011). The five domains analysed by GRADE (study design, risk of bias, inconsistency, indirectness, and imprecision) make possible to classify the certainty of evidence as high, moderate, low, or very low. The outcomes assessed were: "TZA", "FZA", "plasma Zn level after the intervention with Zn biofortified foods", "GLA/LA and AA/DGLA ratio", and "Phytate/Zn molar ratio".

A meta-analysis was not performed due to a high degree of study heterogeneity.

Results

Study selection

The flow diagram based on the selection process is presented in the Figure 2. PubMed (n = 3808), Cochrane Library (n = 28), Scopus (n = 181) and Science Direct (n = 265) were screened. Of the 4282 articles identified, 4148 were excluded: duplicate studies (n = 121), title, abstract and articles that were not suited to the topic (n = 2872), review articles (n = 312), animal studies, *in vivo* and *in vitro* studies (n = 774), book and book chapters (n = 73), and others as letters to editor, opinion, protocols, etc. (n = 117). After the selection, 13 original articles were assessed for eligibility with a full text screening, and four articles were excluded because they did not reach eligibility for inclusion. A total of nine articles were included in the present review. After the search in the reference lists, we did not identify others relevant studies (Figure 2).

Description of the included studies

Two articles were performed with children in India (Kodkany et al. 2013; Sazawal et al. 2018), one with adults from Pakistan (Ahsin et al. 2020), one with children from Zambia (Chomba et al. 2015), one with children from Bangladesh (Islam et al. 2013), one with men and women from Switzerland (Signorell et al. 2019), two articles with solely woman in fertile age from Mexico (Rosado et al. 2009) and United States (Donangelo et al. 2003), and one with adult men (Liong et al. 2021).

All of these were cross-sectional and randomized studies. One article (Sazawal et al. 2018) assessed long-term (6 months) consumption of Zn biofortified food in 5102 participants, one evaluated the 3 months-consumption of Zn biofortified wheat produced by the farmers themselves who were selected to participate in the study (156 participants) (Ahsin et al. 2020), and one was a 8 weeks Zn-controlled feeding trial with 36 participants (Liong et al. 2021). However, most articles evaluated the acute Zn biofortified food intake, and sample sizes varied from 18 to 55 individuals. The Zn biofortified food tested in the selected articles varied between wheat (Liong et al. 2021; Sazawal et al. 2018; Signorell et al. 2019; Rosado et al. 2009; Ahsin et al. 2020), maize (Chomba et al. 2015), rice (Islam et al. 2013), millet (Kodkany et al. 2013) and bean (Donangelo et al. 2003). The Zn concentration consumed varied between 3.6 to 13.6 mg/day for the biofortified groups, and 1.01 to 9.3 mg/day for control groups (Table 2).

Table 2. Characteristics of clinical studies on the use of zinc biofortified foods in the zinc absorption and metabolism

Reference	Study objective	Country and Study population	Study design and methodology	Sample	Zinc concentration	Duration
Ahsin et al. (2020)	To measure the effects of biofortified wheat on human Zn status	Pakistan Adult women and men (19-80 y), n = 156	Randomized controlled study; Analysis: Plasma Zn dosage	Zn biofortified wheat consumed as chapati (24 µg Zn/g)	↑ Biofortified: 9.0 mg/d Control: 7.0 mg/d	3 months
Signorell et al. (2019)	*Study 1:* To compare Zn absorption from intrinsic and extrinsic labels in biofortified wheat and control wheat *Study 2 and 3:* To compare Zn absorption using extrinsic labels at 80% and 100%, extraction rates	Switzerland *Study 1:* Men and women (18-45 y), n = 18 *Study 2 and 3:* women (18-45 y), n = 41	Randomized, single-blind, crossover studies; Analysis: Plasma Zn dosage, FZA, TZA	*Study 1:* Hydroponically Zn biofortified wheat (21.1% ^{67}Zn enrichment) *Study 2:* (100% Ext): Zn biofortified wheat (43.5 µg/g) *Study 3:* (80% Ext): Zn biofortified wheat (31 µg/g)	*Study 1:* Biofortified: 4.0 mg/d*; Control: 1.01 mg/d* *Study 2:* (100% Ext): Biofortified: ↑ 10.06 mg/d; Control: 6.54 mg/d *Study 3:* (80% Ext): Biofortified: ↑ 7.54 mg/d; Control: 4.96 mg/d	Acute (2 meals/3 days with 4-wk wash-out period)
Sazawal et al. (2018)	To evaluate the efficacy of Zn biofortified wheat flour on Zn status and its impact on morbidity among children and woman	India Children (4-6 y) and women (15-49 y), n = 5102)	Double-masked randomized controlled study; Analysis: Plasma Zn dosage	Zn biofortified wheat (30 µg Zn/g)	Zn biofortified wheat: 10.8 mg/d for adults and 3.6 mg/d for children Control wheat: 7.2 mg/d for adults and 2.4 mg/d for children	6 months
Liong et al. (2021)	To evaluate the effect of consuming zinc-biofortified wheat on plasma zinc concentrations and biomarkers of zinc-dependent functions	United States Adult men (18-51 y), n = 36	Nonblinded, randomized controlled study; Analysis: Plasma Zn dosage, GLA/LA, AA/DGLA ratios	Zn biofortified wheat (amount NS)	↑ Biofortified: 10.9 mg/d Control: 9.3 mg/d	8 weeks

Chomba et al. (2015)	To determine whether substitution of biofortified maize for control maize was adequate to meet zinc physiologic requirements	Zambia Children (1-5 y), n = 38	Randomized, cross-sectional observational study; Analysis: Plasma Zn dosage, FZA, TZA	Zn biofortified maize (34 mg Zn/g)	↑ Biofortified: 5.0 mg/d Control: 2.3 mg/d	Acute (3 meals/1 day)
Islam et al. (2013)	To examine the bioavailability of zinc from a mixed diet containing a relatively high-zinc rice cultivar	Bangladesh Children: men and women (36–59 mo), n = 42	Randomized, cross-sectional observational study; Analysis: Plasma Zn dosage, FZA, TZA	Zn biofortified rice (2.60 mg Zn/g dry weight)	Biofortified: 4.81 mg/d Control: 3.81 mg/d	Acute (3 meals/1 day)
Kodkany et al. (2013)	To determine the absorption of Fe and Zn from pearl millet biofortified	India Children: men and women (22–35 mo), n = 40	Double-blinded, randomized, controlled study; Analysis: Plasma Zn dosage, FZA, TZA	Zn biofortified millet (84.1 µg/g)	↑ Biofortified: 5.8 mg/d Control: 3.3 mg/d	Acute (3 meals/1 day)
Rosado et al. (2009)	To determine the increase in quantity of Zn absorbed achieved by Zn biofortified wheat	Mexico Adult women (18-42 y), n = 26	Short-term, cross-sectional study; Analysis: FZA, TZA	Zn biofortified wheat: 95% Ext (40.5 µg/g); 80% Ext (23.8 µg/g)	↑ Biofortified 95% Ext (13.6 mg/d) compared to Control (7.9 mg/d); ↑ Biofortified 80% Ext (6.6 mg/d) compared to Control (3.9 mg/d)	Acute (3 meals/2 days)
Donangelo et al. (2003)	To compare Fe and Zn absorptions of Zn and Fe biofortified beans	United States Adult women (20-28 y), n = 23	Randomized controlled study; Analysis: Plasma Zn dosage, FZA, TZA	Zn biofortified bean (55.4 µg/g)	Estimate value (intrinsic + extrinsic): Biofortified: 8.08 mg/d Control: 3.21 mg/d	Acute (1 meal/1 day)

↑ increased ($p<0.05$); NS: not specified; Ext: extraction rate; FZA: fractional Zn absorption; TZA: total Zn absorption; AA: arachidonic acid; DGLA: dihomo-γ -linolenic acid; GLA: γ -linolenic acid; LA: linoleic acid. * Wheat porridge made of 50 g of wheat flour of hydroponically biofortified or control with both intrinsic (^{67}Zn) and extrinsic (^{70}Zn) labels.

Main findings

We observed that biofortified crops varieties contain a higher concentration of Zn relative the conventional crops varieties. This higher amount in Zn concentration translates into an increase in total Zn absorbed from foods prepared from Zn biofortified crops, relative to conventional crops. Studies show that despite an increase in some inhibitors of Zn absorption, such as phytate, the phytate:Zn molar ratio was lower in most of the Zn biofortified foods compared to their conventional one. This indicates that a high amount of dietary Zn from Zn biofortified crop can be absorbed compared to the standard food.

Five studies assessed Zn biofortified foods by conventional breeding methods (Rosado et al. 2009; Islam et al. 2013; Chomba et al. 2015; Kodkany et al. 2013; Liong et al. 2021), one study used soil Zn application (Ahsin et al. 2020), one study used foliar biofortification (Sazawal et al. 2018), one study used both foliar and hydroponically biofortification (Signorell et al. 2019), and in one study the beans were grown in nutrient solutions (Donangelo et al. 2003) (Table 3).

Using a foliar biofortification approach, Sazawal et al. (2018) observed an increase in plasma Zn levels after the consumption of both Zn biofortified wheat and control wheat, with no difference between the groups. Liong et al. (2021) used a biofortified sample by conventional breeding methods, and Ahsin et al. (2020) a soil Zn application approach. They did not observe changes in the plasma Zn levels after the consumption of Zn biofortified wheat. Four studies performed this evaluation only at baseline (Islam et al. 2013; Kodkany et al. 2013; Donangelo et al. 2003; Signorell et al. 2019), two studies assessed plasma Zn levels only at endpoint (Chomba et al. 2015; Ahsin et al. 2020) and one article did not evaluated this parameter (Rosado et al. 2009) (Table 3).

Most of the studies presented positive findings relative to dietary Zn absorption from Zn biofortified foods. Four studies observed an increase in the TZA after the consumption of Zn biofortified foods compared to control group, with no difference in the FZA (Donangelo et al. 2003; Chomba et al. 2015; Kodkany et al. 2013; Rosado et al. 2009), and only one study found no difference in the TZA between Zn biofortified and control groups (Islam et al. 2013) (Table 3). Signorell et al. (2019) showed that both hydroponic and foliar biofortification were effective in increasing the Zn content in wheat, which led to increased TZA values. Authors performed three experiments: study 1 with hydroponically Zn biofortified wheat, and studies 2 and 3 with foliar Zn biofortified wheat at 100% and 80% milling extraction rate, respectively. In the study 1, they observed a decrease in the FZA, but a 76% increase in TZA

compared to the control group. In the studies 2 and 3, the TZA increased by 48% and 40%, respectively, compared to the control group (Table 3).

Liong et al. (2021) showed an improvement in the Zn status of the studied population by observing an increased activity of FADS2 enzyme which participates in the conversion of LA to DGLA. Further, the concentration of gamma-linolenic acid (GLA) and DGLA increased in the group that received the Zn biofortified wheat flour, and as mentioned by the authors, this change was identified with an increase of just 1.6 mg of Zn/day, in comparison to the control (Liong et al. 2021).

Table 3. Biofortification methods and main results of studies that evaluated the Zn absorption from the consumption of Zn biofortified foods

Reference	Biofortification method	Experimental groups	Plasma Zn levels (baseline and endpoint)	Fractional Zn absorption (FZA), %	Total Zn absorption (TZA), mg/day
Ahsin et al. (2020)	Soil Zn application	Zn biofortified wheat (24 µg/g) Control wheat (18 µg/g)	Zn Biofortified: Baseline: NP; ↔ Endpoint: 75 µg/dL Control: Baseline: NP; Endpoint: 71 µg/dL	NP	NP
Signorell et al. (2019)	*Study 1:* Hydroponically biofortified wheat with ^{67}Zn *Study 2 and 3:* Foliar biofortified wheat were sprayed 3 times with 0.5% $ZnSO_4 7H_2O$	*Study 1:* Zn biofortified wheat: (80 µg/g); Control wheat: (20.2 µg/g); *Study 2:* (100% extraction): Zn biofortified wheat (43.5 µg/g); Control wheat (25.9 µg/g) *Study 3:* (80% extraction): Zn biofortified wheat (31 µg/g); Control wheat (18.1 µg/g)	*Study 1:* Baseline: 87.0 µg/dL Endpoint: NP *Study 2:* Baseline: 78.9 µg/dL Endpoint: NP *Study 3:* Baseline: 76.6 µg/dL Endpoint: NP	*Study 1:* ↓ Biofortified (5.68) compared to Control (8.93) *Study 2:* (100% extraction): ↔ Biofortified (8.37) compared to Control (8.08) *Study 3:* (80% extraction): ↓17% Biofortified (12.3) compared to Control (14.8)	*Study 1:* ↑ 76% Biofortified (0.30 mg) compared to Control (0.17) *Study 2:* (100% extraction): ↑ 48% Biofortified (0.71) compared to Control (0.48) *Study 3:* (80% extraction): ↑ 40% Biofortified (0.83) compared to Control (0.59)
Sazawal et al. (2018)	Foliar biofortified wheat were sprayed with 0.5% zinc sulphate fertilizer	Zn biofortified wheat (30 µg/g) Control wheat (20 µg/g)	<u>Zn Biofortified</u>: *Children*: Baseline: 55.9 µg/dL ↑ 12.7% Endpoint: 63.0 µg/dL *Women*: Baseline: 55.4 µg/dL ↑ 10% Endpoint: 60.9 µg/dL <u>Control</u>: *Children*: Baseline: 56.9 µg/dL ↑ 11% Endpoint: 63.1 µg/dL *Women*: Baseline: 54.9 µg/dL ↑ 10.5% Endpoint: 60.7 µg/dL ↔ between Zn biofortified and control	NP	NP

Liong et al. (2021)	Conventional breeding methods	Zn biofortified wheat (amount NS) Control wheat (amount NS)	Zn Biofortified: Baseline: 79.4 µg/dL; Endpoint: 77.9 µg/dL Control: Baseline: 79.4 µg/dL; Endpoint: 76.0 µg/dL ↔ between Zn biofortified and control	GLA/LA and AA/DGLA ratio ↑ GLA/LA Biofortified (0.025) compared to Control (0.020) ↓ AA/DGLA Biofortified (5.53) compared to Control (6.37)	
Chomba et al. (2015)	Conventional breeding methods	Zn biofortified maize (34 µg/g) Control maize (21 µg/g)	Zn Biofortified: Baseline: NP; ↔ Endpoint: 59 µg/dL Control: Baseline: NP; Endpoint: 58 µg/dL	↔ Biofortified (0.22) compared to Control (0.28)	↑ Biofortified (1.1) compared to Control (0.6)
Islam et al. (2013)	Conventional breeding methods	HZnR: high-zinc rice (26 µg/g) CR: conventional rice (13.5 µg/g)	Zn Biofortified: Baseline: 12.1 µmol/L; Endpoint: NP Control: Baseline: 12.1 µmol/L; Endpoint: NP	↓ Biofortified (20.7) compared to Control (25.1)	↔ Biofortified (1.0) compared to Control (0.96)
Kodkany et al. (2013)	Conventional breeding methods	Zn biofortified millet (84.1 µg/g) Control millet (43.7 µg/g)	Zn Biofortified: ↔ Baseline: 78.5 µg/dL; Endpoint: NP Control: Baseline: 75.6 µg/dL; Endpoint: NP	↔ Biofortified (0.17) compared to Control (0.20)	↑ Biofortified (1.0) compared to Control (0.7)

Rosado et al. (2009)	Conventional breeding methods	Zn biofortified wheat: 95% extraction (40.5 µg/g); 80% extraction (23.8 µg/g) Control wheat: 95% extraction (23 µg/g); 80% extraction (14.4 µg/g)	NP	↔ Biofortified 95% extraction (0.15) compared to Control (0.20) ↔ Biofortified 80% extraction (0.31) compared to Control (0.38)	↑ Biofortified 95% extraction (2.1) compared to Control (1.6) ↑ Biofortified 80% extraction (2.0) compared to Control (1.5)
Donangelo et al. (2003)	Cultivation in nutrient solution	Zn biofortified bean (55.4 µg/g) Control bean (28.0 µg/g)	Zn Biofortified: Baseline: 10.26 µmol/L; Endpoint: NP Control: Baseline: 10.24 µmol/L; Endpoint: NP	↔ Biofortified (Extrinsic) (13.4) compared to Control (16.1)	↑ Biofortified (Extrinsic) (0.68) compared to Control (0.36)

↑ increased ($p<0.05$); ↓ reduced ($p<0.05$); ↔ no change ($p>0.05$); NP: not performed; NS: not specified. GLA/LA (FADS2 activity) and AA/DGLA (FADS1 activity) ratio. AA: arachidonic acid; DGLA: dihomo-γ -linolenic acid; GLA: γ -linolenic acid; LA: linoleic acid; FADS1: fatty acid desaturase 1; FADS2: fatty acid desaturase 2.

The amount of Zn biofortified foods consumed, the Zn and phytate concentration consumed from Zn biofortified foods, and the phytate:Zn molar ratio are shown in the Table 4.

Four studies showed an increased phytate concentration in the Zn biofortified dietary groups (Chomba et al. 2015; Sazawal et al. 2018; Islam et al. 2013; Rosado et al. 2009), however, due to an even greater increases in dietary Zn content in the biofortified treatments, the phytate:Zn molar ratio was lower in most of the biofortified groups relative to the control groups (Signorell et al. 2019; Sazawal et al. 2018; Chomba et al. 2015; Kodkany et al. 2013; Liong et al. 2021; Donangelo et al. 2003) (Table 4). Of the six articles that showed lower phytate:Zn molar ratio, five showed an improvement in Zn absorption in the Zn biofortified dietary group, compared to the control group (Signorell et al. 2019; Chomba et al. 2015; Kodkany et al. 2013; Liong et al. 2021; Donangelo et al. 2003). In general, the amount of Zn biofortified food consumed varied from 40 to 360 g/day (Table 4).

Table 4. Zn and phytate concentration, and phytate to Zn molar ratio of Zn biofortified foods

Reference	Amount of biofortified food consumed	Zn concentration	Phytate concentration	Phytate:Zn molar ratio
Ahsin et al. (2020)	~ 357 g/day	↑ Biofortified: 9.0 mg/d Control: 7.0 mg/d	↓ Biofortified: 2000 mg/d Control: 2400 mg/d	NP
Signorell et al. (2019)	*Study 1:* 50 g/day *Study 2 and 3:* 200 g/day	*Study 1:* Biofortified: 4.0 mg/d*; Control: 1.01 mg/d* *Study 2:* (100% extraction): Biofortified: ↑ 10.06 mg/d; Control: 6.54 mg/d *Study 3:* (80% extraction): Biofortified: ↑ 7.54 mg/d; Control: 4.96 mg/d	*Study 1:* NP *Study 2:* (100% extraction): ↔ Biofortified: 1610 mg/d; Control: 1660 mg/d *Study 3:* (80% extraction): ↔ Biofortified: 1000 mg/d; Control: 1000 mg/d	*Study 1:* Biofortified: 4.7:1; Control: 20.2:1 *Study 2:* (100% extraction): Biofortified: 16:1; Control: 25:1 *Study 3:* (80% extraction): Biofortified: 13:1; Control: 20:1
Sazawal et al. (2018)	Children: 120 g/day Women: 360 g/day	Zn biofortified wheat: 10.8 mg/d for adults and 3.6 mg/d for children Control wheat: 7.2 mg/d for adults and 2.4 mg/d for children	↑ Biofortified: 3.87 mg/g † Control: 3.34 mg/g †	↓ Biofortified: 12.5:1 † Control: 15.8:1 †
Liong et al. (2021)	NS	↑ Biofortified: 10.9 mg/d Control: 9.3 mg/d	Biofortified: 596 mg/d Control: 2096 mg/d	Biofortified: 5.42:1 Control: 22.32:1
Chomba et al. (2015)	~ 100 g/day	↑ Biofortified: 5.0 mg/d Control: 2.3 mg/d	↑ Biofortified: 1569 mg/d Control: 848 mg/d	↓ Biofortified: 34:1 Control: 38:1
Islam et al. (2013)	150 g/day	Biofortified: 4.81 mg/d Control: 3.81 mg/d	Biofortified: 771 mg/100 g (1083 mg/d) Control: 544 mg/100 g (767 mg/d)	Biofortified: 22:1 Control: 20:1

Kodkany et al. (2013)	~ 70 g/day	↑ Biofortified: 5.8 mg/d Control: 3.3 mg/d	↓ Biofortified: 7.5 mg/g Control: 10.3 mg/g	(In the millet grain) Biofortified: 9:1 Control: 24:1
Rosado et al. (2009)	~ 300 g/day of 95 or 80% extracted wheat flour	↑ Biofortified 95% extraction (13.6 mg/d) compared to Control (7.9 mg/d); ↑ Biofortified 80% extraction (6.6 mg/d) compared to Control (3.9 mg/d)	↑ Biofortified 95% extraction (2400 mg/d) compared to Control (2200 mg/d) ↑ Biofortified 80% extraction (770 mg/d) compared to Control (650 mg/d)	NP
Donangelo et al. (2003)	40 g/day	Estimate value (intrinsic + extrinsic): Biofortified: 8.08 mg/d Control: 3.21 mg/d	↔ Biofortified: 1.97 mg/g Control: 1.84 mg/g	↓ Biofortified: 35.6:1 Control: 65.8:1

↑ increased ($p<0.05$); ↓ reduced ($p<0.05$); ↔ no change ($p>0.05$); NP: not performed; * Wheat porridge made of 50 g of wheat flour of hydroponically biofortified or control with both intrinsic (^{67}Zn) and extrinsic (^{70}Zn) labels. † The value was calculated by authors.

Risk of bias

The risk of bias was based on the analysis of 13 domains, and a score of 1 and 0 was marked for each criterion satisfied and not satisfied, respectively (Downs and Black 1998). All studies included in this review were classified as being of good quality (sum of the items evaluated ≥ 9 points). The assessed manuscripts described the hypothesis and objectives, the main outcomes to be assessed, presented the characteristics of the population included in the study, the interventions of interest, and the main findings. However, the representativeness of the population, the presence of statistical power correctly described, and the blinding of the study subjects were the most uncertain points detected (Figure 3).

Only three studies correctly described the presence of statistical power (Kodkany et al. 2013; Sazawal et al. 2018; Liong et al. 2021), and one study reported representativeness of the evaluated population (Sazawal et al. 2018) (Figure 3).

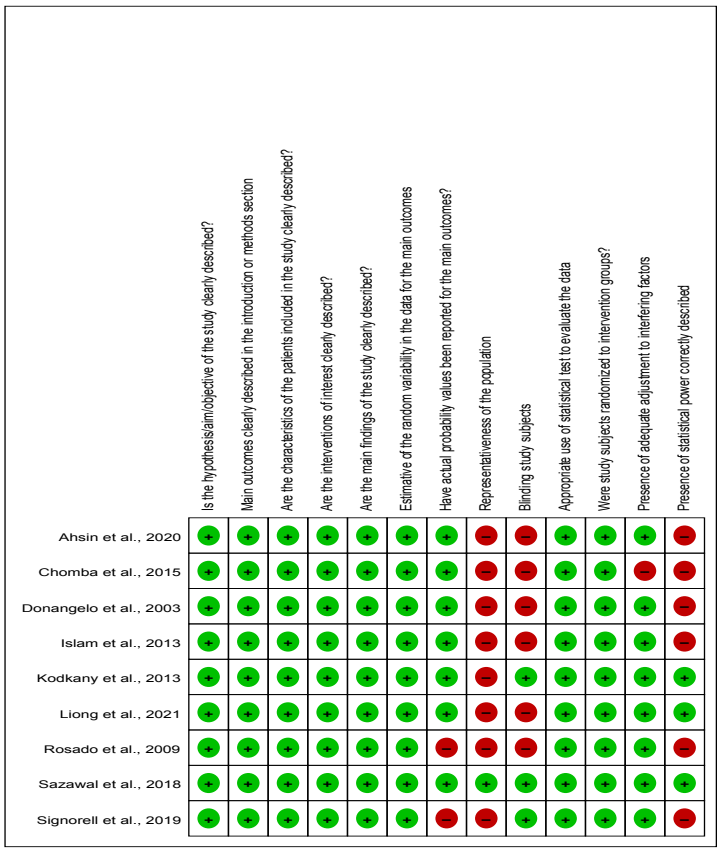


Figure 3. Risk of bias analysis of the included studies.

Quality of evidence

The GRADE evaluation showed a moderate certainty of evidence for FZA, TZA and GLA/LA and AA/DGLA ratio, and low certainty of evidence for plasma Zn level, and Phytate/Zn molar ratio (Table 5). These results can be associated with the risk of bias of included studies and the indirectness of the results assessed.

Table 5. GRADE evidence profile table

Certainty assessment							№ of patients			
№ of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	Zn biofortified foods	Standard foods	Certainty	Importance
TZA (follow up: mean 1.5 days)										
6	Randomized trials	Serious [a,b,c]	Not serious	Not serious	Not serious	None	123/245 (50.2%)	122/245 (49.8%)	⊕⊕⊕◯ MODERATE	Critical
FZA (follow up: mean 1.5 days)										
6	Randomized trials	Serious [a,b,c]	Not serious	Not serious	Not serious	None	123/245 (50.2%)	122/245 (49.8%)	⊕⊕⊕◯ MODERATE	Critical
Plasma Zn level after intervention (mean 11 weeks)										
4	Randomized trials	Serious [a,b,c]	Not serious	Serious [d]	Not serious	None	2645/5332 (49.6%)	2687/5332 (50.4%)	⊕⊕◯◯ LOW	Important
GLA/LA and AA/DGLA ratio (8 weeks)										
1	Randomized trials	Serious [a,b]	Not serious	Not serious	Not serious	None	36/36 (100.0%)	36/36 (100.0%)	⊕⊕⊕◯ MODERATE	Critical
Phytate:Zn molar ratio (mean 4.7 weeks)										
7	Randomized trials	Serious [a,b,c]	Not serious	Serious [e]	Not serious	None	2727/5340 (51.1%)	2613/5340 (48.9%)	⊕⊕◯◯ LOW	Important

[a] Poor representativeness of the population; [b] Deficiencies in the blinding study subjects; [c] Low statistical power; [d] This is not an accurate marker to assess Zn status; [e] This is an indirect evidence to estimate the Zn bioavailability. TZA: total Zn absorption; FZA: fractional Zn absorption; GLA: γ -linolenic acid; LA: linoleic acid; AA: arachidonic acid; DGLA: dihomo-γ -linolenic acid; ⊕ indicates positive certainty of evidence; ◯ indicates a lack of certainty of evidence.

Discussion

The production of biofortified foods has increased worldwide with the aim of reducing dietary deficiencies rates (HarvestPlus 2021; HarvestPlus 2019). Despite accelerated technological and industrial development in the food production sector, a large portion of the global population is at risk of hidden hunger, which is characterized by inadequate dietary intake of minerals (Eggersdorfer et al. 2018; Lowe 2021; HarvestPlus 2021). In addition to the low Zn intake by the at risk populations, studies revealed that the phytate:Zn molar ratio higher than 15:1 can affect Zn bioavailability (Norhaizan and Nor Faizadatul Ain 2009; Morris and Ellis 1989; Bel-Serrat et al. 2014), and it can reduce the fractional Zn absorption by 45% of control values (Bel-Serrat et al. 2014). In recent years, *in vivo* studies have evaluated the bioavailability of minerals from biofortified foods, and changes in gene expression of key brush border membrane proteins, responsible for mineral digestion and absorption (Dias et al. 2019; Dias et al. 2018; Gomes, Martino, and Tako 2021; Tako et al. 2015). However, there is still no consensus if the increase in the Zn concentration in biofortified foods is effective in reducing the phytate:Zn molar ratio and increasing the Zn absorption in humans.

In this systematic review, we observed that four of the nine manuscripts showed a higher phytate concentration in the Zn biofortified dietary group, compared to the control group (Sazawal et al. 2018; Chomba et al. 2015; Islam et al. 2013; Rosado et al. 2009). Phytic acid is a natural substance in plants, and is involved in the biosynthesis of cell wall polysaccharides and plant growth regulators (Sparvoli and Cominelli 2015). However, phytic acid has high binding affinity to positively charged minerals, such as Zinc, Iron, Calcium and Phosphorous. This binding results in an insoluble complex (phytate), which is difficult to be hydrolysed by phytases (Sparvoli and Cominelli 2015; Pramitha et al. 2021). Previous study showed that phytic acid has a higher ability to bind Zn, compared to other di- and trivalent cations, as iron, manganese, calcium, and magnesium (Maenz et al. 1999). This may explain the fact that many Zn biofortified foods presented a higher phytate content, compared to conventional ones. However, it is important to note, that in the assessed Zn biofortified foods, the increase in Zn content was significantly higher, relative to increase in phytate content, which therefore, reduced the phytate:Zn molar ratio, compared to the control foods (Signorell et al. 2019; Sazawal et al. 2018; Chomba et al. 2015; Kodkany et al. 2013; Liong et al. 2021; Donangelo et al. 2003). Among the six manuscripts that presented a lower phytate:Zn molar ratio, in the Zn biofortified dietary group, five have observed an increased Zn absorption,

compared to the control (Signorell et al. 2019; Chomba et al. 2015; Kodkany et al. 2013; Liong et al. 2021; Donangelo et al. 2003). The indicators that were used to evaluate Zn absorption are summarized in Figure 4.

Of the nine studies assessed, five showed that the consumption of Zn biofortified foods increased the TZA rate (Donangelo et al. 2003; Chomba et al. 2015; Kodkany et al. 2013; Rosado et al. 2009; Signorell et al. 2019), and one study showed that FADS2 activity in the ω-6 metabolic pathway significantly increased during the period of which participants consumed the Zn biofortified food (Liong et al. 2021). According to the International Zinc Nutrition Consultative Group (IZiNCG) (Gibson, King, and Lowe 2016), the minimal amount of absorbed Zn required to replenish total endogenous losses, are 2.69 mg Zn/day, for men, and 1.86 mg/day, for women. In this review, we observed that the mean amount of absorbed Zn, after the consumption of Zn biofortified foods, was 1.08 mg/day. This is equivalent to 58% of the amount required to replenish total endogenous losses in women, and 40% of the amount required in men. The Estimated Average Dietary Requirements (EAR) for Zn varies according to age, gender, and dietary phytate:Zn molar ratio. For children aged 4-6 years, the EAR is 3.0 mg/day, when the individual consumes a diet with phytate:Zn molar ratio of 4-18:1, and the EAR is 4.0 mg/day, when the individual consumes a diet with phytate:Zn molar ratio higher than 18:1. For young adults, ages 15-18 years, the EAR is 8.0 mg/day, for male, and 7.0 mg/day, for female, when the individual consumes a diet with phytate:Zn molar ratio of 4-18:1. However, this amount increases to 11.0 mg/day, for male, and 9.0 mg/day for female, when the individual consumes a diet with phytate:Zn molar ratio higher than 18:1 (Gibson, King, and Lowe 2016).

As observed in this review, the amount of dietary Zn from Zn biofortified foods supplied the EAR for most studies. Three studies observed a phytate:Zn molar ratio higher than 18:1 (Chomba et al. 2015; Islam et al. 2013; Donangelo et al. 2003), and of these, only one study did not reach the EAR for Zn intake, from the consumption of Zn biofortified bean (Donangelo et al. 2003). However, the authors still observed an increase in TZA, suggesting a promising potential of Zn biofortified foods to improve the Zn physiological status in humans.

Four studies assessed the plasma Zn concentration at the endpoint of the experiment (Ahsin et al. 2020; Sazawal et al. 2018; Liong et al. 2021; Chomba et al. 2015), however, no studies observed a significant difference in this Zn physiological status marker (Figure 4). Plasma Zn metabolism, specifically pathways that are related to the cellular metabolism and the transient transfer of zinc from plasma/serum to cells or tissues, is a dynamic process. The

plasma/serum Zn pool turns over about 150 times/day, in order to provide sufficient Zn for Zn dependent pathways throughout the body, such as chemical reactions and enzymatic activity.

Because of that, the Zn turnover does not permit a stable Zn concentration in the plasma (King 2018). Previous study showed that the plasma concentration is not a sensitive biomarker to assess Zn status in adults, indicating that despite doubling the amount of Zn ingested via food, plasma Zn concentration only increased by 6% (Lowe et al. 2012). In children, a meta-analysis of 18 randomized controlled trials (median duration of 24 weeks) showed that by doubling Zn intake, plasma Zn concentration may increase by 9%. Furthermore, the authors suggested that the Zn concentrations in serum or plasma are not accurate in assessing the Zn physiological status (Moran et al. 2012).

Sazawal et al. (2018) evaluated the efficacy of Zn biofortified wheat, by foliar approach, on Zn physiological status of children and women. The authors did not observe changes in plasma Zn concentration in the groups that received the Zn biofortified food compared to controls. However, it was observed that 21% of women in the Zn biofortified group, and 22.4% of women in the control group, who were Zn deficient at baseline, presented an adequate plasma Zn concentration after the six-month of dietary intervention. In addition, the study demonstrated that the consumption of Zn biofortified wheat can reduce the morbidity rate among children, compared to standard wheat, specifically rates of pneumonia (17% lower), vomit (39% lower) and ear discharge (17% lower) (Sazawal et al. 2018). These results agree with another study that showed a decrease in morbidity and mortality rates due to gastrointestinal and respiratory diseases in children post Zn dietary supplementation (Aggarwal, Sentz, and Miller 2007).

The studies included in this review differ mainly in relation to the biofortification methods that were used (Figure 4). Despite variations in nutrient concentration, crop genetic background, and differences in the soil in which biofortified foods are grown (Zaman et al. 2018), the data presented in this review suggest that the biofortification process increases the total amount of Zn in the plant. The conventional breeding method was applied in five of the assessed studies, and four of them showed positive findings in the Zn absorption rates (Chomba et al. 2015; Kodkany et al. 2013; Rosado et al. 2009; Liong et al. 2021). Further, a three-day acute study assessed the hydroponic and foliar Zn biofortification, and observed that both treatments increased Zn absorption rate, compared to control (Signorell et al. 2019). However, other studies that assessed foliar Zn biofortification (Sazawal et al. 2018) and soil Zn application (Ahsin et al. 2020), did not show differences in Zn absorption biomarkers.

Unlike foliar biofortification, which requires greater financial investment for the application of Zn fertilizers to plants (Cakmak and Kutman 2018), and according to this review, the conventional breeding method is more common, and more studies have evaluated its efficacy in providing absorbable Zn. This method has an advantage of low cost between planting and harvesting, and it allows small farmers and vulnerable populations to have better access, and potential ability to grow biofortified foods (Birol et al. 2014; Lividini et al. 2018). However, due to the heterogeneity of the methods applied for biofortification, and the limited number of studies, it is not possible to conclude which is the most effective method to increase the Zn concentration in the plant, and additional studies are needed.

Despite the multiple methods that were used to assess Zn physiological status, it is possible to evaluate the effectiveness of Zn biofortified foods by grouping the studies that utilized similar biofortification approaches, as was done in this review. Therefore, this methodology is valuable in providing the basis for further advancement of various biofortification strategies.

Dosages and reporting quality

In this systematic review, we assessed the Zn absorption from Zn biofortified foods consumed by children (about 60 and 150 g/day), and by adults (about 40 and 360 g/day). These portions provided between 26 and 84.1 μg Zn/g of biofortified food, and did not show adverse effects in the studied populations. However, due to the high heterogeneity among the assessed studies, it was not yet possible to define the recommended dietary portion of Zn biofortified foods, which may allow to achieve a significant effect on Zn physiological status in humans.

The factors that could influence the quality of the reviewed human efficacy trials were evaluated according to Downs and Black (1998), and all of the reviewed studies had met the adequate quality standards. However, the studies included in this review presented high heterogeneity related to the type of Zn biofortified foods assessed and the duration of the intervention. According to the GRADE, the level of evidence defined was not high. We suggest that future trials include details related to study's subjects blinding, to reduce potential risk of bias, and present clear information about the representativeness of the population/subjects, and study's statistical power.

Conclusions

The development of Zn biofortified foods is related not only to the increase in the supply of specific essential minerals to target populations, but also to reduce the incidence of several metabolic disorders that are related to hidden hunger. Of the nine studies included in this review, six studies showed a positive increase in Zn absorption rates after the consumption of approximately 34-84.1 µg Zn/g of Zn biofortified wheat, maize, millet, or bean. Therefore, Zn biofortified foods improve dietary Zn absorption and can provide lower phytate:Zn molar ratio, resulting in improved Zn physiological status (Figure 4).

In this review, we presented data that justify the development of public strategies to increase the production, access, and consumption of Zn biofortified foods by vulnerable populations, primarily infants and children. However, additional long-term efficacy trials are required to further demonstrate and confirm the Zn bioavailability ratio from Zn biofortified foods, and the recommended dietary portions that may effectively provide the potential positive effects that are reported in this systematic review.

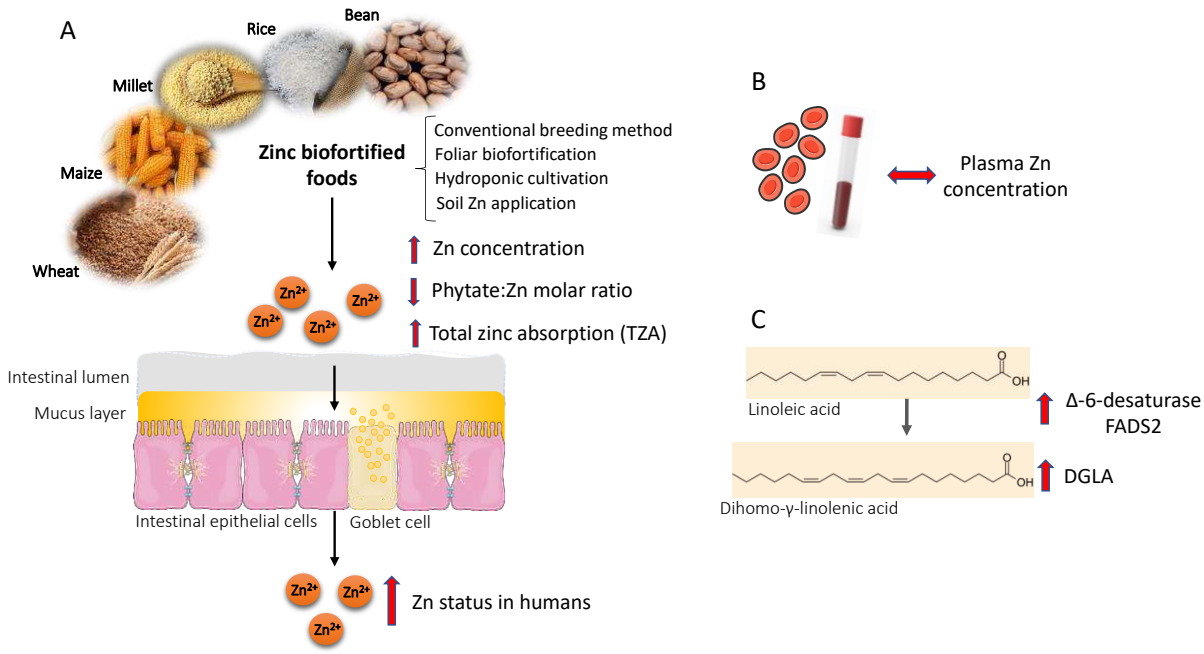


Figure 4. Indicators of absorption and bioavailability of zinc biofortified foods. A: Zinc absorption by enterocytes. B: Changes in plasma zinc concentration reported by studies after the consumption of zinc biofortified foods. C: Changes in biological marker of zinc bioavailability by ω-6 fatty acid metabolism. Zn: zinc; FADS2: fatty acid desaturase 2; DGLA: dihomo-γ-linolenic acid.

Registration and Protocol

This systematic review was designed to evaluate and answer the following research question: "What are the effects of the consumption of Zn biofortified foods on Zn absorption in humans?". This study was carried out in accordance with the Preferred Reporting Items for Systematic review and Meta-Analysis (PRISMA) guidelines 2020 (Page et al. 2021) and registered in PROSPERO (CRD42021250566).

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the following articles: Ahsin et al. (2020), Signorell et al. (2019), Sazawal et al. (2018), Liong et al. (2021), Chomba et al. (2015), Islam et al. (2013), Kodkany et al. (2013), Rosado et al. (2009), and Donangelo et al. (2003).

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6. ORIGINAL RESEARCH RESULTS

6.1. PAPER 3

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Research paper

Cooked common bean flour, but not its protein hydrolysate, has the potential to improve gut microbiota composition and function in BALB/c mice fed a high-fat diet added with 6-propyl-2-thiouracil

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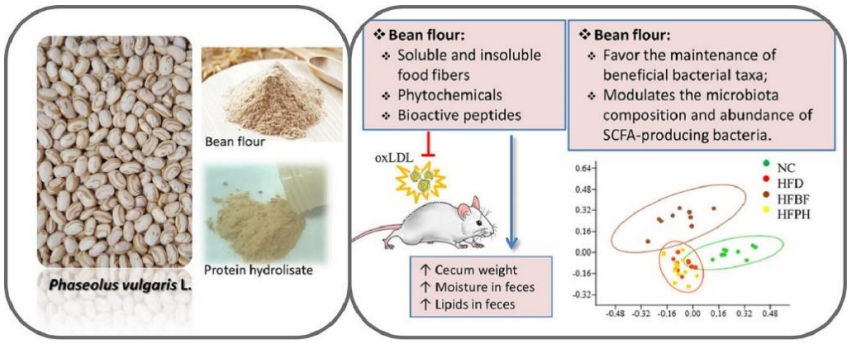
Abstract

Common bean has the potential to improve gut microbiota function due to its chemical composition and content of dietary fiber. This study evaluated the effect of cooked common bean (CCB) flour and its protein hydrolysate as part of a high-fat diet (HFD) added with 6-propyl-2-thiouracil (10 mg/kg/d), an inhibitor of thyroid hormone synthesis, on gut health of BALB/c mice. Forty-eight adult mice were divided into four groups: normal control; HFD; HFD plus CCB flour (346.6 g/kg of diet) (HFBF group) and HFD plus CCB protein hydrolysate (700 mg/Kg/d) (HFPH group). HFBF, but not HFPH, increased cecum weight, and the moisture, and lipids in the excreted feces, compared to control groups. Sequencing of the 16S rRNA gene of the cecal microbiota indicated changes in the beta-diversity between the HFBF and HFPH groups, compared to the normal control. The abundance of Bacteroidetes increased and the Firmicutes/Bacteroidetes ratio decreased in the HFBF compared to control groups. However, HFPH was not able to prevent the damage caused by a HFD to the gut bacterial communities. The OTUs enriched by HFBF were mainly assigned to members of the *Muribaculaceae* family, which shows potential to improve gut health. The intake of CCB flour improved intestinal health and modulated the composition and function

of the cecal microbiota, attenuating the effects of the HFD, added wit 6-propyl-2-thiouracil, when fed to BALB/c mice.

Keywords: *Phaseolus vulgaris*; Bioactive peptides; Gut microbiome; Diversity analysis.

Graphical Abstract



1. Introduction

Excessive consumption of saturated fat in the diet is dramatically increasing the prevalence of overweight and obesity in the world population [1]. Saturated fat influences the general state of health, increases intestinal permeability and has a deleterious effect on the gut microbial ecosystem, leading to an increase in gram-negative and a reduction in gram-positive bacteria [2]. Although fatty acids have different effects on health, it is known that saturated fat changes the composition and the function of microorganisms that colonize the gastrointestinal tract [2,3], besides increasing the pro-inflammatory immune response and susceptibility to colitis [4].

To evaluate the effects of food compounds or diet associated with a high-fat diet (HFD), experimental studies have used the thyroid hormone inhibitors, known as 6-propyl-2-thiouracil (PTU), to generate a hypothyroidism model and induce metabolic changes that are common in obese individuals or those with metabolic syndrome [5], [6], [7]. PTU inhibits the thyroperoxidase enzyme, leading to a reduction in the circulating levels of thyroxine and triiodothyronine [7,8]. Therefore, this drug can be used to promote weight gain, and increase total cholesterol, LDL-c and triglycerides in animal models [5], [6], [7].

Inadequate eating habit is a relevant factor for the development of chronic noncommunicable diseases, and regular consumption of legumes has shown to be beneficial for reducing inflammatory processes and increasing the body's antioxidant defense,

contributing to the improvement of the population's health status [9]. In this sense, the identification of specific foods that may improve beneficial bacterial populations can help to prevent associated diseases.

Common bean (*Phaseolus vulgaris* L.) is a widely consumed legume in Brazil and other countries [10]. The world production of common bean has increased, and in 2018 it was 30.43 thousand tons [11]. Cooked common bean (CCB) is a source of protein, carbohydrates, dietary fiber, starch, phenolic compounds, vitamins, and minerals [12,13]. Studies on common bean have intensified in the last years due to its rich chemical composition and high content of dietary fiber, about 26.7% (7.0% of soluble fiber and 19.6% of insoluble fiber) [14,15]. Moreover, the so called carioca bean flour, used in this study, presents a high content of soluble and insoluble dietary fibers, as well as phytochemicals such as catechin, kaempferol [16], phytosterols, and saponins [17]. These compounds may inhibit DNA damage and prevent LDL-c oxidation induced by a HFD [18]. Therefore, the consumption of beans associated with a HFD has been shown to reduce obesity and insulin resistance [19], hyperlipidemia [20], and disorders related to serum glucose and lipid metabolism, in addition to modulate the gut microbiota [21], [22], [23].

Moreover, bioactive peptides that are encoded in the primary structure of animal [24], [25], [26], [27] and plant [27], [28], [29], [30], [31] proteins, may be released by *in vivo* and/or *in vitro* proteolysis [27,32]. Among the proteolysis methods, the simulated gastrointestinal digestion provides a more realistic enzymatic hydrolysis of the complete food matrix [32]. In this sense, considering the worldwide consumption and relatively low cost of common beans, their proteins have been studied regarding the generation of biologically active hydrolysates and peptides, which have important physiological functions [32], [33], [34], [35], [36]. It has been shown that whole bean flour and bean protein hydrolysates reduce inflammation and the risk factors for cardiovascular diseases [16,[37], [38], [39]. Common bean protein hydrolysate possesses hypocholesterolemic activity and can prevent inflammation and dysfunction of vascular endothelium, decreasing oxidative stress *in vivo* [16,39]. Moreover, bean protein hydrolysates have demonstrated antihyperlipidemic, anti-inflammatory, and antihypertensive properties *in vitro* [37,38]. Regarding the effect on gut microbiota, *Gallus gallus* animal model fed with iron biofortified carioca bean flour-based diet, improved the gut microbiome composition and function, with a higher abundance of bacteria linked to phenolic catabolism, and beneficial short-chain fatty acids (SCFA) producing bacteria [40].

Although studies have shown the effect of the bean consumption on the host health and gut microbiota associated with both low-fat [41,42] and high-fat diet consumption [22,23,43], there are no studies that have assessed the effects of a CCB flour diet of slow-darkening (named Madreperola cultivar) [44], and its protein hydrolysate in preventing the deleterious effects caused by a HFD on the intestinal microbiota. Therefore, the aim of this study was to evaluate the effect of CCB flour and its protein hydrolysate, as part of a HFD on colon histomorphometry, and gut bacteriome composition and function. PTU was added to the diets in order to exacerbate the deleterious effect of the HFD in adult BALB/c mice.

2. Material and methods

2.1. Sample material

Common bean (*Phaseolus vulgaris* L.), cultivar BRSMG Madreperola, is a slow-darkening genotype during storage [44]. The sample was provided by EMBRAPA Rice and Bean (Santo Antônio de Goiás, GO, Brazil). First, the beans were washed under running water and cooked under pressure (1:2 beans/water) for 50 min at 120°C. Beans were then oven-dried for 8 h at 60°C and crushed in an automatic mill (sieve of 600 μm aperture size, 30 mesh; Grinder Vertical Rotor MA 090 CFT, Marconi Equipment, Brazil). The protein hydrolysate was obtained by a simulated gastrointestinal digestion process, as described by Alves et al. [38] and Megías et al. [45]. Briefly, pepsin (pepsin/bean flour 1:20, pH 2.0) and pancreatin (pancreatin/bean flour 1:20, pH 7.5) were used for sequential enzyme digestion for 2 h at 35°C, in triplicate. The hydrolysate was centrifuged at 20,000 × *g* for 15 min at 4°C, dialyzed (500 Da molecular weight cut-off membrane, Sigma Aldrich, San Louis, MO, USA) and freeze-dried (LabConco FreeZone, Kansas, MO, USA). Samples were kept at –20°C until analysis. The bean flour and bean protein hydrolysate have been previously characterized by de Lima et al. [16] and Alves et al. [38], respectively.

2.2. Animals and diets

Forty-eight male BALB/c mice (*Mus musculus*, class *Rodentia*), 60 d of age, were obtained from the Central Animal Facility of the Center for Life Sciences and Health at Federal University of Viçosa (Viçosa, MG, Brazil). Adult mice were chosen to evaluate the preventive effects of the treatment compared to control and to eliminate possible hormonal variations due to the animals' growth phase. Animals were randomly allocated into four groups (n=12 per group) in individual stainless-steel cages under a controlled temperature

environment (22 ± 2°C) and a 12 h light/dark cycle. The groups received deionized water ad libitum, and its experimental diets based on AIN-93M [46] and high-fat high-cholesterol diet [47,48] weekly and for 9 weeks (Table 1).

Table 1. Composition of experimental diets (g/kg of diet).

Ingredients (g/Kg)	NC	HFD	HFBF	HFPH
Casein*	170.73	218.19	124.47	218.19
Whole bean flour	0.00	0.00	346.60	0.00
Dextrinized starch	155.00	105.50	0.00	105.50
Sucrose	100.00	300.00	218.63	300.00
Lard	0.00	200.00	200.00	200.00
Cellulose	62.01	62.01	0.00	62.01
Soy oil	40.00	40.00	36.00	40.00
Mineral mix	35.00	35.00	35.00	35.00
Vitamin mix	10.00	10.00	10.00	10.00
Cholesterol	0.00	20.00	20.00	20.00
Choline bitartrate	2.50	2.50	2.50	2.50
L-cystine	1.80	1.80	1.80	1.80
Colic Acid	0.00	5.00	5.00	5.00
Corn starch	422.96	0.00	0.00	0.00
Carbohydrate (%)	76.29	45.89	44.35	45.89
Protein (%)	19.21	24.69	24.85	24.69
Lipids (%)	4.50	29.42	30,80	29.42
Energy (kcal/kg)	3754.76	4834.76	4677.97	4834.76
CD (kcal/g^{-1})	3.75	4.83	4.67	4.83
Bean protein hydrolysate (mg/Kg body weight)	-	-	-	700.00

*Purity of 82%. NC: normal control; HFD: high-fat diet; HFBF: high-fat diet added with cooked common bean flour; HFPH: high-fat diet added with cooked common bean protein hydrolysate; CD: caloric density.

The experimental groups received the following diets: normal control (NC); high-fat diet (HFD), HFD added with CCB flour (HFBF), and HFD added with CCB protein hydrolysate (HFPH). As CCB flour is a good source of dietary fibers and protein [14,16], it was added to the diet of the HFBF group to supply 100% of dietary fibers (Table 1). The total amount of 346.6g of whole CCB flour/kg of diet was also adequate to supply 50% of the dietary protein requirements for the adult mice. The protein content was equivalent for all HFD groups (HFD, HFBF, and HFPH). The diet of the three HFD groups was isocaloric, isolipidic, and isoglycidic.

The CCB protein hydrolysate was formulated according to Mojica et al. [49] and offered by oral gavage in the amount of 700 mg/kg/d. All the experimental groups received a daily treatment by gavage for 9 weeks. The HFD and HFBF groups received the PTU (10 mg/kg/d) diluted in deionized water, by gavage, as a suppressor of thyroid hormones [50,51],

and the HFPH group was administered by oral gavage with CCB protein hydrolysate (700 mg/kg/d) plus PTU (10 mg/kg/d), diluted in deionized water. The NC group received deionized water by oral gavage to mitigate the stress level of the animals.

Body weight and feed intake were monitored weekly. On the 62nd day, excreted feces were collected for moisture, lipid, and SCFA quantification. On the 63rd day, after 12h fasting, animals were anesthetized with isoflurane (Isoforine, Cristália, Brazil) and euthanized by cardiac puncture. The colon segment was collected, flushed with phosphate buffer saline solution, and fixed in Karnovsky solution (glyceraldehyde 1:1 vol/vol and formaldehyde 4%) for 24 h and kept in ethanol 70% for histological analysis. Cecum weight was measured, and the cecum content was collected in a sterile microtube, immediately frozen in liquid nitrogen, and stored at –80°C for analysis. All experimental procedures using animals were performed in accordance with the ethical principles for animal experimentation, and the study protocol was approved by the Ethics Committee of the Federal University of Viçosa (Protocol No. 97/2015) (Appendix I).

2.3. Fecal moisture and lipids content

The moisture content in the feces was determined by the gravimetric method. For this, the samples were oven-dried at 105°C for 24 h [52]. Lipids quantification was performed by extraction in Soxhlet apparatus, using ethyl ether as the extractor, for 8 h, under reflux [52]. Briefly, a 10 g dry sample was added inside the extraction thimble. 250 mL balloons with known weight were used in the extraction system. After 8 h of extraction under heating, the balloons were kept in a fume hood to allow the ether to evaporate and then were transferred to an oven at 105°C for 12 h. The balloon's final weight was measured to quantify the total oily extracted from each sample.

2.4. Fecal short-chain fatty acids content

The SCFA analysis followed the methodology proposed by Smiricky-Tjardes et al. [53] with modification. The samples were kept at low temperatures throughout the analysis. Briefly, 50 mg of feces were homogenized in MiliQ water following a Vortex shaking protocol/rest of the samples for 30 min to extract the SCFA. After this step, samples were centrifuged at 19,350 × *g* for 30 min at 4°C (Hitachi Koki Co., Ltd, Tokyo, Japan) and the supernatant was collected and filtered at 0.45 μm. The quantification of SCFA was performed by high-performance liquid chromatography (HPLC).

The SCFA were determined in a Dionex Ultimate 3000 Dual detector HPLC apparatus (Dionex Corporation, Sunnyvale, CA, USA) equipped with a refractive index detector Shodex RI-101 maintained at 40°C. The SCFA were separated on a Phenomenex Rezex ROA ion exclusion column (300 × 7.8 mm) (Phenomenex Inc. Torrance, CA, USA) maintained at 45°C. Analyses were performed isocratically under the following conditions: mobile phase sulfuric acid 5 mmol L^{-1}, flow rate 0.7 mL min^{-1}, column temperature 40°C, injection volume 20 µL. Stock solutions of the standards were prepared using acetic, propionic, and butyric acid. All SCFA were prepared with a final concentration of 10 mmol/L. Stock solutions were diluted 2-, 4-, 8-, 16-fold in 5 mmol L^{-1} sulfuric acid (0.08 to 10 mM) to be used as standards in the HPLC analysis.

2.5. Colon histomorphometric analysis

Semi-serialized histological proximal colon fragments of 3 µm thickness were obtained on a semi-automated rotating microtome (Leica, Brazil) and stained using the hematoxylin/eosin technique. Slides were examined under a CX31 photomicroscope (Olympus, Japan). To measure crypt depth and thickness of the circular and longitudinal muscle layers (CML and LML, respectively), twenty random fields per animal were selected. Only crypts with definite and visible connective epithelium were used [54] and the images were processed using the ImagePro-Plus software, version 4.5 (Media Cybernetics, Rockville USA).

2.6. DNA extraction and sequencing

Total genomic DNA was extracted from cecum content, following a mechanical disruption and phenol/chloroform extraction protocol [55]. Polymerase chain reaction amplicon libraries targeting the hypervariable V4-region of the 16S rRNA gene were produced using the primers 515F (5′GTGYCAGCMGCCGCGGTAA3′) and 806R (GGACTACNVGGGTWTCTAAT3′) and a barcoded primer set adapted for the Illumina MiSeq platform (Illumina, San Diego, California, USA) [56,57]. Samples were loaded onto an Illumina flow cell for paired-end sequencing reactions using the Illumina MiSeq platform in the Environmental Sample Preparation and Sequencing Facility at Argonne National Laboratory (Lemont, Illinois, USA).

Amplicons were sequenced on a 151bp x 12bp x 151bp MiSeq run using customized sequencing primers and procedures [56]. The sequences obtained for all samples in the

present study were submitted to Sequence Read Archive on the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sra) under the accession number PRJNA658699.

Data processing and analysis were performed using the software Mothur v.1.40.0 [58]. In summary, the R1 and R2 paired-end reads were joined, and sequences that were smaller than 150 or greater than 300 bp were removed. Sequences that had homopolymers with at least eight nucleotides or containing ambiguous base pairs were also eliminated. Chimera sequences were detected and removed using UCHIME [59]. After cleaning the sequences, they were aligned with the 16S rRNA gene using SILVA database v.132 [60].

Taxonomic classification was performed using SILVA database v.132 and the Operational Taxonomic Units (OTUs) were grouped with a 97% sequence similarity cutoff. The coverage of all samples was assessed by Good's coverage estimator (Bacteria>97%). To correct for sampling bias due to unequal amplicon library sizes, the samples were normalized for the lowest number of sequences produced from any sample (Supplementary Table S1). The standardized data table was used for calculating alpha- and beta-diversity, as well as for calculating the relative abundance of OTUs. The indices, Chao1, Shannon, and Simpson were used for estimates of alpha-diversity. Beta-diversity between dietary groups was assessed by Principal Coordinate Analysis based on the Bray-Curtis dissimilarity index and between sample-diversity, using unweighted UniFrac [61]. Metagenome functional predictive analysis was carried out using PICRUSt2 software [62]. Normalized OTU abundance was identified, and the assigned functional traits were predicted based on reference genomes using the Kyoto Encyclopedia of Genes and Genomes (KEGG). The most abundant metabolic processes and significant fold-change differences in functional pathways between experimental groups were plotted. The KEGG less abundant metabolic routes that showed a statistical difference, compared to the control group, are shown in Supplementary Table S3.

2.7. Statistical analysis

Data for food consumption, body weight gain, colonic histomorphometric characteristics, and concentrations of SCFA, were initially submitted to a Kolmogorov-Smirnov normality test, and *one-way* analysis of variance followed by the *post-hoc* test of Newman-Keuls. Experimental treatments were arranged in a completely randomized design

with twelve repetitions. Data are presented as means ± standard deviation and statistical significance were established at $P<.05$.

The Chao1, Shannon and Simpson indices were used to estimate alpha diversity. To evaluate the clustering of the samples according to treatment, a Principal Coordinate Analysis plot, based on Bray-Curtis dissimilarity metrics, was performed to show the distance in the bacterial communities of mice allocated in different dietary groups. Nonparametric analysis of similarities (ANOSIM, number of permutation=10,000) was performed to evaluate the OTU composition of the gut microbiota across experimental groups using the Past software [63].

Datasets were tested for homogeneity of variance by the Kolmogorov-Smirnov test and nonparametric and independent samples were submitted to Kruskal-Wallis test with a Dunn's multiple comparison test. Data were corrected for multiple comparisons using false discovery rate (FDR) in the Statistical Analyses of Metagenomic Profiles software. Statistical analysis was performed using SPSS software version 20.0 with Bonferroni correction. The level of significance was established at $P<.05$.

3. Results

3.*1. Mice fed a HFD and PTU added with CCB protein hydrolysate experienced lower food consumption and low body weight gain after 9 weeks*

In the present study, the HFPH group presented the lowest ($P<.05$) dietary consumption in the first three weeks of treatment, compared to the NC and HFD control groups. In weeks four to nine, there was no difference ($P>.05$) between the HFBF and HFPH treatment groups compared to controls (Fig. 1A). Despite this variation, the HFPH group showed a lower ($P<.05$) daily and total dietary consumption, compared to the other groups, and the weekly calorie intake followed this same pattern.

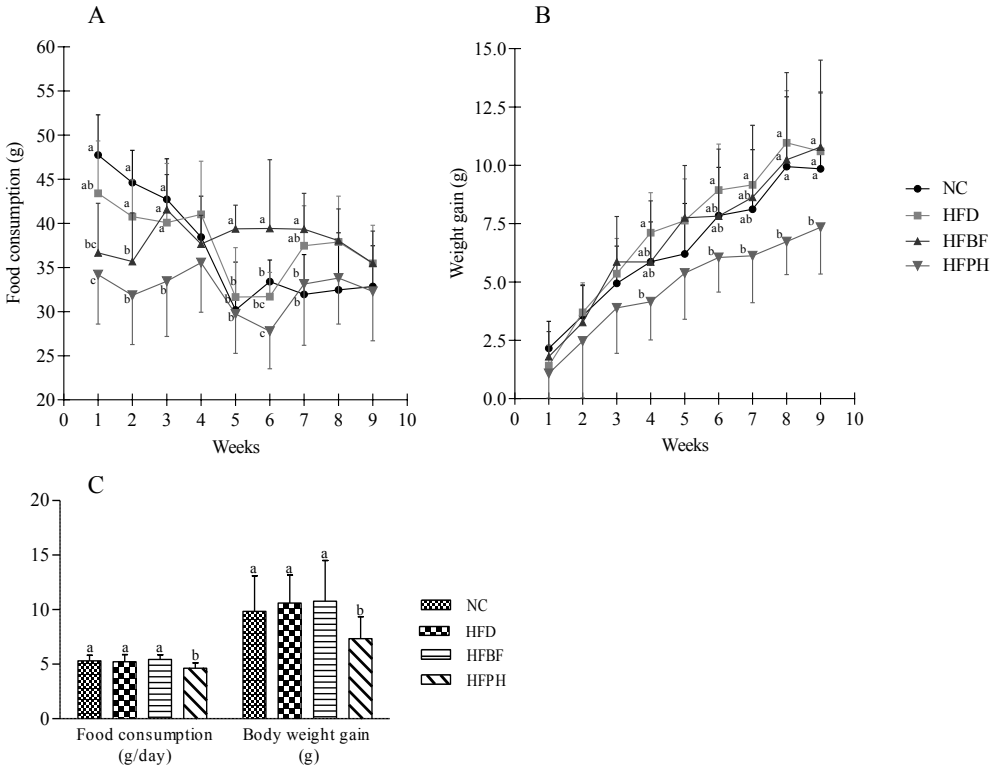


Fig. 1. Food consumption and body weight gain of adult BALB/c mice after 9 weeks of treatment (n=12). A) Weekly food consumption; (B) Weekly weight gain; (C) Daily food consumption and total body weight gain. Mean followed by different letters in the column indicate difference by Newman-Keuls test (P<.05). NC, normal control; HFD, high-fat diet; HFBF, high-fat diet plus CCB flour; HFPH, high-fat diet plus CCB protein hydrolysate.

In addition, in week four, the HFPH group reduced (P<.05) weight gain, compared to the HFD control, and in the weeks six to nine, the HFPH treatment group decreased the weight gain compared to the HFD group (Fig. 1B). However, the model HFD plus PTU did not induce the weight gain on adult BALB/c mice, and there was no difference in the total body weight gain between the HFD and HFBF treatment groups, compared to the NC group (P>.05) (Fig. 1C). The total protein intake was 0.95±0.07 g/wk (0.14±0.01 g/d) in the HFBF group, and 0.80±0.08 g/wk (0.11±0.01 g/d) for the HFPH group. The CCB protein hydrolysate consumption was, on average, 26.12 mg/d. This dosage was calculated weekly using the updated weight gain of the animals, aiming to provide 700 mg/Kg body weight during the 9 weeks of experimentation. During the experiment, the protein intake from HFBF and HFPH groups was equivalent.

3.2. Mice fed a HFD and PTU added with CCB flour showed high cecum weight, and increased moisture and lipids in the feces after 9 weeks

The consumption of whole CCB flour (HFBF group) increased (P<.05) the cecum weight, the moisture concentration in the feces, and increased the lipid excretion, compared with the other experimental groups (Table 2). The crypt depth was higher (P<.05) in the HFD, HFBF, and HFPH groups compared to the NC group. The circular muscle layer did not differ among the experimental groups (P>.05), and the thickness of the longitudinal muscle layer was similar (P>.05) in the HFBF group compared to the HFD group, but increased (P<.05) in the HFPH compared to the HFD group, with no difference compared to the NC group (Table 2).

Table 2. Colonic histomorphometric characteristics of adult BALB/c mice after 9 wk of treatment

	NC	**HFD**	**HFBF**	**HFPH**
Cecum weight (g)	0.26 ± 0.06^{b}	0.29 ± 0.06^{b}	0.49 ± 0.14^{a}	0.33 ± 0.06^{b}
Moisture in feces (%)	11.00 ± 2.70^{b}	7.77 ± 0.62^{b}	15.90 ± 5.36^{a}	9.04 ± 4.02^{b}
Lipids in feces (%)	0.77 ± 0.55^{c}	19.19 ± 1.81^{b}	21.40 ± 1.77^{a}	19.16 ± 2.18^{b}
Crypt height (μm)	103.81 ± 21.08^{b}	$123{,}54 \pm 13.32^{a}$	131.69 ± 8.73^{a}	128.75 ± 7.24^{a}
CML (μm)	61.40 ± 12.18^{a}	47.08 ± 13.94^{a}	53.68 ± 13.12^{a}	54.16 ± 9.95^{a}
LML (μm)	24.21 ± 3.81^{a}	19.51 ± 2.57^{c}	20.71 ± 1.96^{bc}	23.42 ± 2.50^{ab}

Values represent means ± SD, n = 12/group (cecum weight, moisture, and lipids in feces) and n = 8/group (histological analysis). NC: normal control; HFD: high-fat diet; HFBF: high-fat diet added with cooked common bean flour; HFPH: high-fat diet added with cooked common bean protein hydrolysate; CML: circular muscle layer; LML: longitudinal muscle layer. Data were analyzed by one-way ANOVA. Means followed by different letters differ by Newman-Keuls *post-hoc* test ($p < 0.05$). Representative images are shown in Supplementary Fig. 1 and 2.

3.3. Mice fed a HFD and PTU added with CCB flour showed lower acetic acid concentration but no changes in propionic and butyric acids in the feces after 9 weeks

Among organic acids analyzed, acetic acid decreased (P<.05) in the feces of HFBF group compared to NC and HFD groups, and no difference (P>.05) was observed between HFBF and HFPH groups (figure 2A). However, propionic acid and butyric acid in the feces did not differ (P>.05) among experimental groups (Fig. 2B and C).

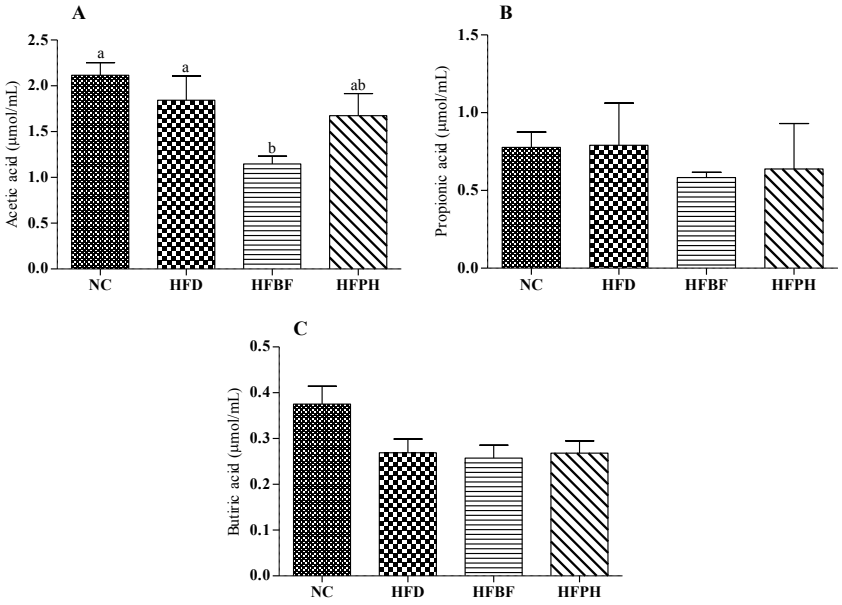


Fig. 2. Short-chain fatty acids (SCFA) concentration in the feces of adult BALB/c mice after 9 weeks of treatment (n=12). Different letters indicate differences by the Newman-Keuls test (P<.05). NC, normal control; HFD, high-fat diet; HFBF, high-fat diet plus CCB flour; HFPH, high-fat diet plus CCB protein hydrolysate.

3.4. Mice fed a HFD and PTU added with CCB flour showed changes in the gut microbiota after 9 weeks

The 16S rRNA gene sequencing of the cecal content generated 1.656.308 raw sequences. After filtering and cleaning the sequencing data, were obtained 1.173.011 good quality sequences. The Good's coverage estimator was always >99% across samples, indicating that the current sequencing depth could represent most of the bacterial community in the experimental groups (Supplementary Table S1).

The Chao1 richness index and Shannon and Simpson diversity indexes were used to evaluate the alpha-diversity and did not indicate a difference (P>.05) among the experimental groups (Supplementary Table S2). Data spatial ordination and analysis of similarities (ANOSIM) showed statistical differences in the distance metrics among treatments (ANOSIM, P=.00009, F=0.6639). The clustering of the bacterial community in the HFBF group differed from the NC and HFD control groups (ANOSIM, P<.0001, F=0.8042, and F=0.8016, respectively), while the HFPH group did not show differences in the distance metrics compared to the HFD group (ANOSIM, P=.164, F=0.0624) (Fig. 3A). A similar result was observed when these treatments were compared using a distance metric (unweighted UniFrac) based on the phylogeny of their collections of sequences (Fig. 3B).

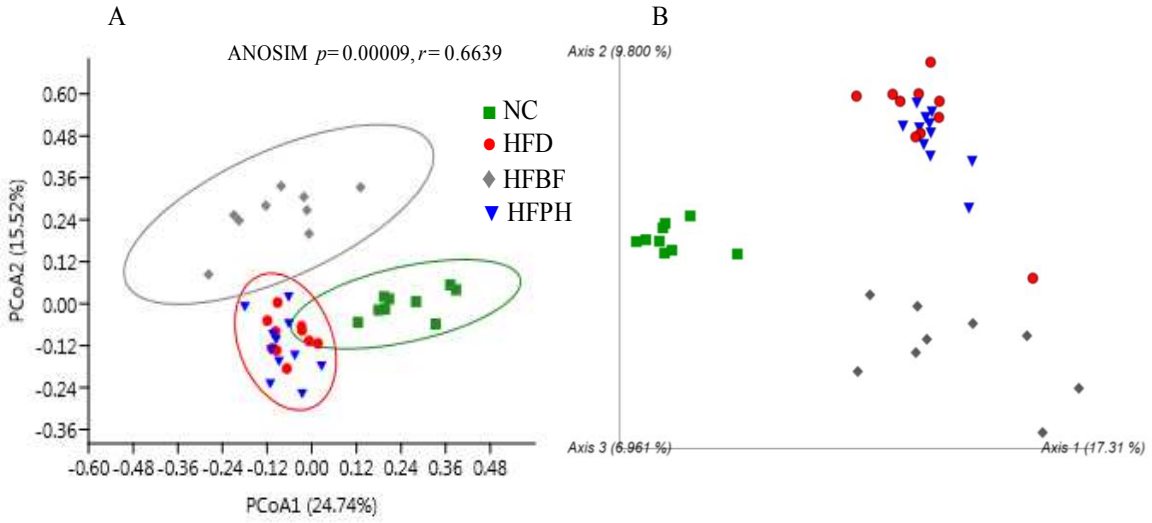


Fig. 3. Microbial diversity of the cecal content of adult BALB/c mice after 9 weeks of treatment. (A) Principal coordinate analysis (PCoA) performed from the Bray-Curtis dissimilarity index; (B) Measure of beta-diversity using unweighted UniFrac distances separated by the first three principal components (PCoA). Individual points represent each animal within its respective experimental group, n=9/group (NC, HFBF groups), n=10/group (HFD group) and n=11/group (HFPH group). NC, normal control; HFD, high-fat diet; HFBF, high-fat diet plus CCB flour; HFPH, high-fat diet plus CCB protein hydrolysate.

The taxonomic classification of samples showed 16 phyla, 25 classes, 48 orders, 88 families and 249 genera, and the stratification of the phyla that comprised more than 0.2% of relative abundance, after FDR correction, is shown in Fig. 4. The HFBF group showed increased levels of Bacteroidetes (P=.015) compared to the other experimental groups, and the abundance of Firmicutes reduced in this group (50.19±8.71) relative to HFPH (61.29±4.99) (P=.025) (Fig. 4A). In addition, the Firmicutes/Bacteroidetes ratio was lower (P<.05) in the HFBF group compared to the other experimental groups (Fig. 4B).

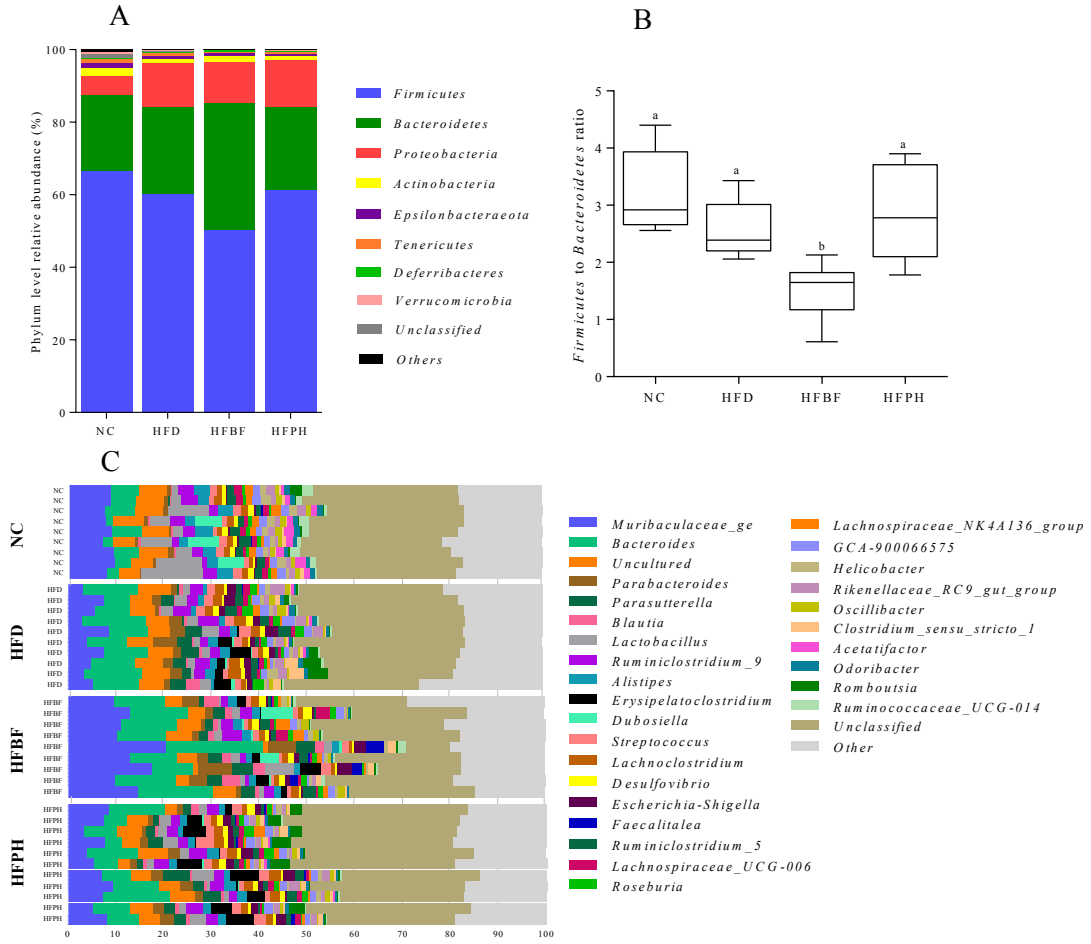


Fig. 4. Relative abundances of bacterial microbiota composition at phylum and genera level of adult BALB/c mice after 9 weeks of treatment. (A) Relative abundance of each identified phylum; (B) Firmicutes/Bacteroidetes ratio; (C) Genera samples displayed according to each experimental group (NC, HFD, HFBF, and HFPH). n=9/group (NC, HFBF groups), n=10/group (HFD group) and n=11/group (HFPH group). Only phyla with abundance >0.2% and genera with abundance >1% in at least one group were displayed. Data were analyzed by Dunn's test with FDR and Bonferroni corrections. NC, normal control; HFD, high-fat diet; HFBF, high-fat diet plus CCB flour; HFPH, high-fat diet plus CCB protein hydrolysate.

The Proteobacteria phylum showed no difference (P>.05) among experimental groups (HFD, HFBF, and HFPH), however, the abundance was higher (P<.05) than NC group. The relative abundance of the phylum Actinobacteria had no difference relative to HFD group (P>.05) (Fig. 4A).

HFBF group showed increased levels of the family *Muribaculaceae* (13.35% vs. 5.39%, P<.0001) and the genus *Blautia* (2.01% vs. 0.67%, P=.015) in their gut microbiota, while displaying reduced levels of *Lachnoclostridium* (1.13% vs. 1.81%, P=.042), *Rikenellaceae RC9 gut* group (0.19% vs. 1.59%, P<.0001) and *Odoribacter* (0.17% vs. 0.68%, P=.015) compared to HFD group. In the HFPH group, the genera did not differ (P>.05) compared to the HFD group (Fig. 4C).

The genera displayed in the HFBF group, compared to HFPH group, decreased the levels of *Lachnoclostridium* (1.13% vs. 1.77%, respectively; P=.016) and *Rikenellaceae RC9 gut* group (0.19% vs. 1.18%, respectively; P=.015), while increased the levels of *Muribaculaceae* (13.35% vs. 6.76%, respectively; P<.0001) and *Blautia* (2.01% vs. 0.60%, respectively; P<.018) (Fig. 4C).

The similarity between the sequences was assessed and classified into OTUs. We assessed 1559 OTUs among the four experimental groups, 14.7% were exclusive to the NC group, 9.8% to HFD, 14.8% to HFBF, and 8.6% to HFPH. The HFBF group shared 420 OTUs (26.9%) with HFD, and displayed 356 OTUs (22.8%) that were shared with the NC group. In turn, the HFPH group shared 552 OTUs (35.4%) with HFD, and 374 OTUs (24.0%) were shared with the NC group (Supplementary Fig. S3).

The key phylotypes were identified among the four groups using Statistical Analyses of Metagenomic Profiles software. The OTUs with abundance >1% shared by all experimental groups were assigned according to the taxonomy. The OTU00008 (assigned *Bacteroides*) was the most abundant within this genus, followed by the Otu00020 (assigned *Muribaculaceae*) and Otu00014 (unclassified *Lachnospiraceae*). In the HFBF group, the relative abundance increased (P<.05) for 11 OTUs and decreased (P<.05) for four OTUs compared to the HFD group. The most abundant OTUs were partly assigned to *Muribaculaceae* (8 OTUs), *Prevotellaceae UCG-001* (2.25% relative abundance in the HFBF group), *Blautia* (2 OTUs, 1.71 and 2.12% relative abundance), and *Alloprevotella* (2.00% relative abundance) (P<.05). The OTUs *Rikenella*, *Rikenellaceae RC9 gut* group, and unclassified *Ruminococcaceae* and *Peptostreptococcaceae* were decreased in the HFBF group, compared to the HFD group (P<.05). The HFPH group had no change in the relative abundance of OTUs compared with the HFD group (P<.05) (Table 3).

Table 3. Most abundant bacterial OTUs identified in fecal samples of adult BALB/c mice after nine weeks of treatment.

		Relative abundance (%)				
OTU	Taxonomy*	**NC**	**HFD**	**HFBF**	**HFPH**	***p*-value**
Otu00008	*Bacteroides*	2.47	1.36	5.39	1.57	0.115
Otu00016	*Bacteroides*	1.58	1.73	0.98	2.10	0.570
Otu00010	*Bacteroides*	0.20^{b}	2.61^{a}	4.20^{a}	2.33^{ab}	0.013
Otu00009	*Bacteroides*	0.62^{b}	6.09^{a}	1.40^{ab}	2.39^{ab}	0.028
Otu00020	*Muribaculaceae_ge*	0.05^{b}	0.10^{b}	4.58^{a}	0.07^{b}	0.002
Otu00026	*Muribaculaceae_ge*	0.04^{b}	0.09^{b}	2.18^{a}	0.28^{ab}	0.002
Otu00044	*Muribaculaceae_ge*	0.00^{b}	0.00^{b}	1.76^{a}	0.03^{ab}	0.020
Otu00032	*Muribaculaceae_ge*	0.75^{b}	0.02^{c}	1.72^{ab}	0.05^{c}	0.000
Otu00028	*Muribaculaceae_ge*	0.88^{ab}	0.28^{b}	1.28^{a}	0.45^{b}	0.010
Otu00058	*Muribaculaceae_ge*	0.00^{b}	0.00^{b}	1.23^{a}	0.05^{b}	0.032
Otu00060	*Muribaculaceae_ge*	0.00^{b}	0.00^{b}	1.14^{a}	0.03^{b}	0.000
Otu00079	*Muribaculaceae_ge*	0.00	0.00	1.10	0.03	0.323
Otu00061	*Muribaculaceae_ge*	0.06^{ab}	0.00^{c}	1.10^{a}	0.04^{bc}	0.000
Otu00001	*Escherichia-Shigella*	0.03^{b}	10.36^{a}	5.44^{ab}	14.66^{a}	0.003
Otu00006	*Dubosiella*	11.11	0.00	4.61	0.04	0.591
Otu00005	*Parasutterella*	0.00^{b}	4.24^{a}	3.87^{a}	4.84^{a}	0.009
Otu00002	*uncultured*	5.30	8.52	2.92	5.39	0.114
Otu00038	*Prevotellaceae_UCG-001*	0.00^{b}	0.00^{b}	2.25^{a}	0.02^{b}	0.000
Otu00035	*Blautia*	0.00^{b}	0.22^{b}	1.71^{a}	0.08^{b}	0.004
Otu00022	*Blautia*	0.00	0.87	2.12	0.36	0.184
Otu00034	*Alloprevotella*	0.14^{b}	0.02^{b}	2.00^{a}	0.04^{ab}	0.032
Otu00007	*Erysipelatoclostridium*	0.00^{b}	1.96^{ab}	1.90^{ab}	6.37^{a}	0.026
Otu00045	*Faecalitalea*	0.00	0.00	1.46	0.19	0.144
Otu00014	*Lachnospiraceae_unclassified*	0.00	1.08	4.49	0.51	0.066
Otu00036	*Lachnospiraceae_unclassified*	0.13	0.06	1.22	0.47	0.067
Otu00067	*Lachnospiraceae_unclassified*	0.00	0.00	0.99	0.02	0.568
Otu00015	*Lachnospiraceae_unclassified*	0.45	1.66	0.11	3.67	0.191
Otu00021	*Lachnoclostridium*	0.08^{c}	1.80^{ab}	0.70^{bc}	2.02^{a}	0.014
Otu00030	*Helicobacter*	2.12^{a}	0.41^{ab}	0.56^{ab}	0.09^{b}	0.006
Otu00017	*Roseburia*	0.03^{b}	4.20^{a}	0.44^{ab}	1.36^{ab}	0.028
Otu00029	*Muribaculaceae_ge*	0.07	0.57	0.43	1.28	0.615
Otu00003	*Lactobacillus*	15.71^{a}	2.87^{ab}	0.57^{b}	3.64^{ab}	0.002
Otu00023	*Lactobacillus*	3.27	0.02	0.27	0.49	0.470
Otu00013	*Lactobacillus*	9.69^{a}	0.04^{b}	0.01^{b}	0.04^{b}	0.034
Otu00041	*Rikenella*	0.44^{ab}	1.10^{a}	0.03^{b}	0.45^{ab}	0.032
Otu00019	*Clostridium_sensu_stricto_1*	0.00^{b}	1.83^{ab}	0.02^{b}	2.86^{a}	0.010
Otu00065	*Clostridium_sensu_stricto_1*	0.00	1.42	0.00	0.02	0.762
Otu00011	*Rikenellaceae_RC9_gut_group*	2.45^{a}	4.37^{a}	0.02^{b}	1.45^{a}	0.003
Otu00031	*Ruminococcaceae_unclassified*	0.98^{a}	1.76^{a}	0.02^{b}	0.24^{ab}	0.010
Otu00025	*Enterococcus*	0.86	0.92	0.01	1.65	0.113
Otu00012	*Romboutsia*	1.15^{b}	1.43^{ab}	0.00^{b}	4.26^{a}	0.011
Otu00027	*Paeniclostridium*	0.00	2.96	0.00	0.00	0.473

Otu00004	*Peptostreptococcaceae unclassified*	0.00^{bc}	4.19^{ab}	0.00^{c}	8.37^{a}	0.001
Otu00018	*Erysipelatoclostridium*	0.00	1.05	0.00	3.48	0.171

*Taxonomy of each OUT is given at the highest classifiable level. n = 9/group (NC, HFBF groups), n = 10/group (HFD group) and n = 11/group (HFPH group). OUT: operational taxonomic unit; NC: normal control; HFD: high-fat diet; HFBF: high-fat diet added with cooked common bean flour; HFPH: high-fat diet added with cooked common bean protein hydrolysate. Data were analyzed by Dunn's test with FDR correction and different letters in line differ ($p < 0.05$). Only OTUs with abundance > 1% were displayed.

3.5. Mice fed a HFD and PTU added with CCB flour altered the functional capacity of the gut microbiota after 9 weeks

We investigated whether the treatments with CCB flour or its protein hydrolysate associated with a high-fat diet influenced the genetic repertoire of the microbiota and explored the possible functional alterations.

Using PICRUSt2 [62], metagenome functional predictive analysis revealed that 208 of the 300 (~69%) KEGG metabolic routes analyzed among the four experimental groups had differences (P<.05). After FDR correction, 204 of the 300 (68%) metabolic processes analyzed were differently enriched between experimental groups. We performed a peer comparison test to identify differences between the treatments (HFBF or HFPH) and the HFD control group. The result indicated that 41 (20%) metabolic processes analyzed were differentially enriched in the HFBF group compared to HFD (P<.05). The KEGG most abundant metabolic processes (e.g., Top 5: “DNA repair and recombination proteins,” “Starch and sucrose metabolism,” “Pentose phosphate pathway,” “Galactose metabolism,” “Bacterial motility proteins”) are shown in Figure 5, and the less abundant metabolic processes were presented in the Supplementary Table S3. In addition, two metabolic processes (“L-isoleucine biosynthesis II” and “Flavin biosynthesis I (bacteria and plants)”) were differentially enriched in the HFPH group compared to HFD (P<.05), and the relative abundance is shown in the Supplementary Table S3.

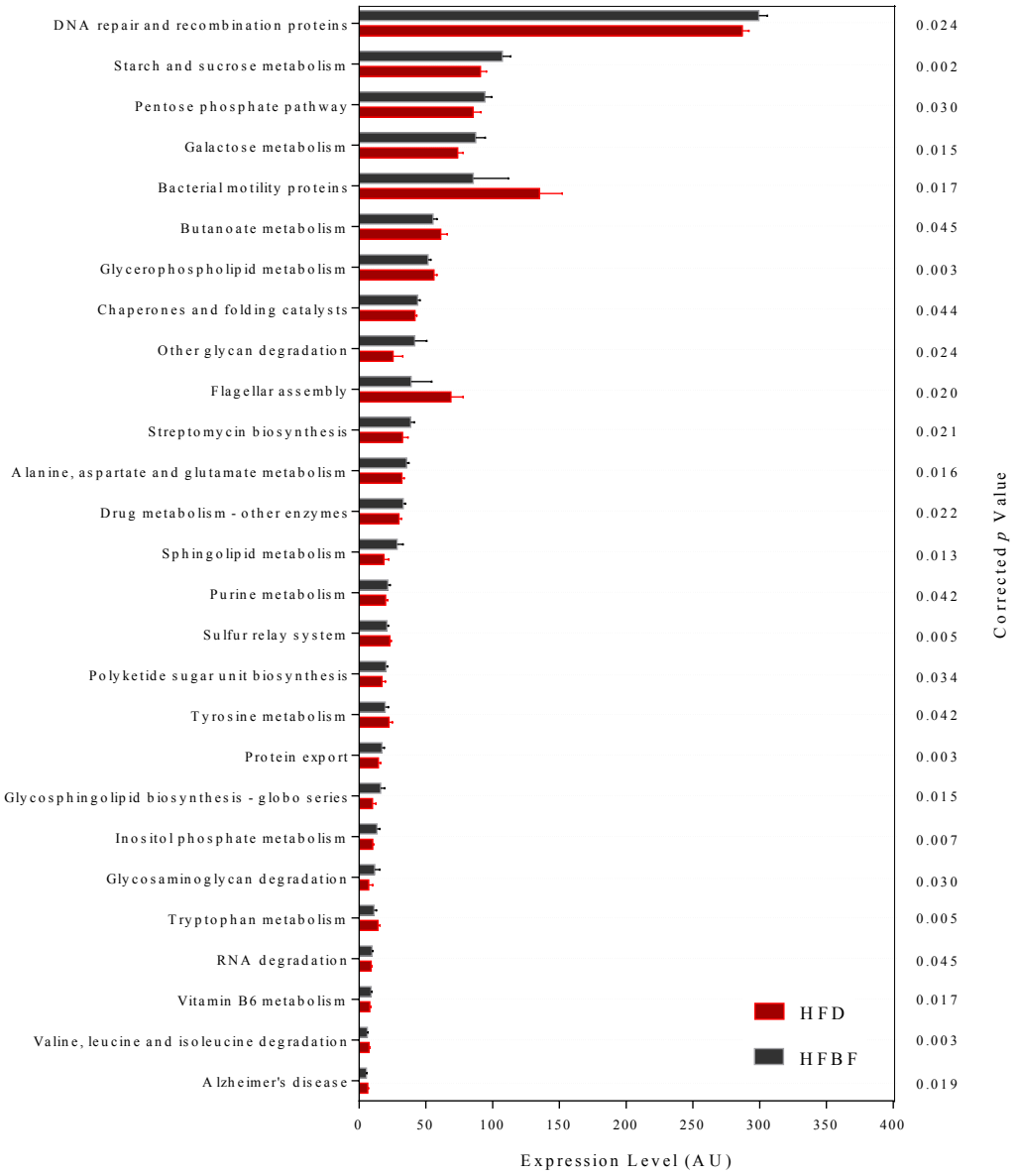


Fig. 5. Functional capacity of the gut microbiota is altered following a high-fat diet plus CCB flour. Relative abundance of differentially enriched KEGG microbial metabolic pathways in the microbiota. Treatment groups are indicated by the different colors, and P-values are displayed on the y-axis. HFD, high-fat diet; HFBF, high-fat diet plus CCB flour.

4. Discussion

Several studies point out the beneficial effects of CCB consumption on the health and gut microbiome of humans and animals [64], [65], [66], [67]. The beneficial effects of the CCB protein hydrolysate are due to the content of phenolic compounds and bioactive peptides with antihyperlipidemic, anti-inflammatory, and antihypertensive effects [16,38,39,68,69]. Some of these nutritional and bioactive properties were previously demonstrated for BRSMG Madreperola CCB flour and its protein hydrolysates [16,38,39]. This CCB flour contained a high concentration of protein (24.63±0.36 g/100 g) [38], which was able to generate bioactive

peptides after gastrointestinal digestion, with a yield of 51.2±4% and degree of hydrolysis of 56.1±14.1% [38].

Using a HFD plus PTU diet model in BALB/c mice, this study showed that the consumption of BRSMG Madreperola CCB flour, a slow-darkening cultivar [44], and its protein hydrolysate, rich in bioactive properties with promising health benefits [16],[37], [38], [39], promote distinct compositional changes in the gut microbiota. In addition, we highlight that this difference may be due to the distinct compositional properties of the two treatments.

The mice fed the HFBF diet received about 1.8 g of whole CCB flour per day (346.60 g CCB flour/Kg of diet) and the HFPH group received a HFD associated with 700 mg/kg of body weight per day [49] of CCB protein hydrolysate by intragastric gavage. In the present study, PTU was used to increase the weight gain in the groups that received a HFD, since previous studies showed the potential of this drug to induce hypothyroidism [5,7,51,70]. Despite the potential of PTU for increasing body weight, it was not observed in the present study, and there was no difference in the weight gain between the control groups and the HFBF treatment group (Fig. 1). A possible explanation may be the short experimentation time, which was not long enough to induce weight gain in the experimental model used. However, the HFPH group showed lower food consumption and weight gain after 9 weeks, compared to the other experimental groups. This result was expected since a previous study showed the potential of CCB protein hydrolysate on act in the hunger and satiety center [39].

On the other hand, mice in the HFBF group showed improved gastrointestinal tract characteristics, such as higher cecum weight and higher moisture and lipids in the feces, which could be due to the higher consumption of soluble and insoluble dietary fibers present in CCB flour. The dietary fibers present in the bean husk are oligosaccharides that function as prebiotics and exert effects on the solubility and viscosity of the fecal content as can stimulate or inhibit its distension, weight and motility in the intestine [71]. Beta-diversity analysis revealed differences in the gut bacterial community of mice allocated in the HFBF group, compared to animals from other treatments, and these results indicate that CCB dietary fibers differentially affected microbial diversity in the colon.

In this study, an increase in crypt depth was observed in the gut of animals fed with a high-fat diet (HFD, HFBF, and HFPH), compared to the mice fed the NC diet. There is a relationship between the consumption of a high-fat diet and an increased crypt depth in the large intestine [72,73], but the small intestine can have a different response. In a *Gallus gallus* feeding model, the digestive and absorptive capabilities of the brush border membrane can

have a direct relationship with the morphometric parameters, such as villi height, crypt depth, and the ratio between villi height and crypt depth [74]. We did not observe expressive changes in the thickness of the circular and longitudinal muscle layers in the colon. Similar results were observed by Moraes et al. [75], who evaluated the intake of a HFD and sorghum. However, in general, the high amount of soluble and insoluble dietary fiber increases intestinal motility and fecal volume, tending to increase the thickness of muscle layers.

Further, we found no relationship between the consumption of CCB and its protein hydrolysate in the production of propionic and butyric acids (Fig. 2). These results are in accordance with the histomorphometric data assessed, since we did not observe differences in the crypt height, circular and longitudinal muscle layers (Table 2) in the large intestine of animals from HFBF group compared to the HFD group. It was shown by others [76,77] that an increase in the production of SCFA could increase the cellular activity in the gut, reflecting on the thickness of the muscle layers. In the present study, the absence of this effect could be related to some metabolic processes of reabsorption of these SCFAs, which is an interesting result that merits future investigation.

The HFBF group presented the lowest concentration of acetic acid in the feces. An inverse correlation between the amount of resistant starch (RS) in the diet and the acetic acid concentration has been reported [78,79]. The cooked common brown beans present about 2.7±1.6 g/100 g of RS and this content is increased by the storage time [78], indicating that beans used in the present study possibly contained high RS content, and this could result in a lower production of this SCFA. In addition, a positive covariance between members of the order *Clostridiales* and the acetate production has been reported, and the high RS content in the diet may reduce the presence of genera belonging to the *Clostridiales*, thus decreasing acetate production [80]. The HFBF group presented a lower relative abundance of the genus *Lachnoclostridium* belonging to the order *Clostridiales*, and this microbial modulation possibly affected the production of acetic acid in this group. Ferrario et al. [80] also reported a negative correlation between increased populations of *Alloprevotella spp*. in rats fed a RS supplemented diet and acetate production.

We did not find significant effects of the dietary treatments on species richness and diversity of the BALB/c mice gut microbiota after 9 weeks, indicating that intra-species diversity remained stable between groups. On the other hand, the beta-diversity analysis showed that the gut microbiota composition of the NC group was different from the other groups (Fig. 3A). Bacterial communities of the HFPH group were similar to the HFD group,

however distant from the NC and HFBF groups. This observation was confirmed when a phylogenetic distance metric, unweighted UniFrac, was used (Fig. 3B) and indicated that diet has distinct effects on the bacterial communities among experimental groups. As observed by the Venn diagram, the HFD and HFPH groups showed a high number of shared OTUs (552), which are probably part of the core microbiome, and represent OTUs present in most of the samples (Supplementary Fig. 3). We also observed that the number of unique OTUs in the HFPH and HFD groups was less than in the NC group. However, the HFBF group showed a high number of unique OTUs when compared to the control groups. This observation suggests that the CCB flour helped to maintain rare species in the gut.

The HFBF group showed a higher abundance of Bacteroidetes and lower Firmicutes to Bacteroidetes ratio compared to the HFD group. Although the abundance of Firmicutes does not differ between these groups, we can infer that the consumption of CCB flour improved the composition of the bacterial community at the phylum level, with potential benefits to intestinal health, and a similar result was recently showed [81]. Further, the benefits of common bean designed based on the Brazilian food consumption survey has shown promising potential for modulating the gut microbiota [40,41,43]. The lowest Firmicutes to Bacteroidetes ratio is considered beneficial and has been reported for lean and healthy individuals [82]. Possibly, the modulatory effects of CCB flour are related to its chemical composition, the presence of phenolic compounds, such as catechin, epicatechin, kaempferol, quercetin 3-glucoside, and the presence of resistant starch that can help to improve the gut microbiota composition of the animals feed a high-fat diet [16,39,40,81].

It is known that the chemical structure of the RS molecule can modulate the accessibility by groups of colonic bacteria and favor specific bacterial populations [83]. However, this mechanism remains uncertain due to the range of factors that can modify the gut microbiota in the short and long term.

We did not observe a significant increase in the *Actinobacteria* in the HFBF and HFPH groups compared with the HFD group, and the HFPH group showed only subtle changes in the abundance of this phylum compared with the NC group. However, Dominianni et al. [66] showed a correlation between the abundance of *Actinobacteria* and the consumption of dietary fiber from beans, fruits and vegetables shaping the intestinal microbiome. The high abundance of *Proteobacteria* in the groups that consumed high-fat diets may be caused by harmful effects of saturated fats on the gut microbiota. *Proteobacteria* has been associated

with intestinal dysbiosis and inflammatory diseases, such as metabolic disorders and inflammatory bowel disease [84], [85], [86].

Stratification of the microbiota at the genus level showed an increase in *Muribaculaceae* and *Blautia* in the HFBF group compared to the HFD group. The genus *Blautia* has been linked to a healthy microbiota. Some members are SCFA producers [81] and the abundance of *Blautia spp*. in the intestine is directly related to the body composition of lean mass *in vivo* [87]. An interesting observation from our study was the high abundance of members of *Muribaculaceae* family in the HFBF group and the high relative abundance of OTUs classified to derived genus (Table 3). It is remarkable how the consumption of CCB flour increases the *Muribaculaceae* in the microbial community of mice, compared with both HFD and NC control groups. It is also interesting that OTUs 20, 26, 44, 32, 28, 58, 60, and 61, assigned as *Muribaculaceae*, were differentially abundant in the HFBF group, while most remain unchanged in the other experimental groups. In agreement with other studies that showed similar results [22,64], we suggest that some components of the beans can modulate the bacterial community composition *in vivo*.

Studies have investigated the effects of *Muribaculaceae* on intestinal health [22,64,88], and the existing studies suggest that members of the *Muribaculaceae* family, historically named *S24-7* [89], have a functional potential in the gut, being able to ferment polysaccharides into SCFAs [90]. The findings of this study suggest that the whole CCB flour does not seem to adversely affect the composition nor genetic repertoire of the gut microbiota.

The HFBF group reduced the abundance of genera *Lachnoclostridium*, *Rikenellaceae* and *Odoribacter* compared to HFD group. *Lachnoclostridium* belongs to the *Lachnospiraceae* family and both have been correlated with increased body weight and diet-induced obesity [91,92]. The genus *Rikenellaceae* belongs to the *Rikenellaceae* family, which was correlated with inflammatory process in the murine microbiota [93], and the effects of *Odoribacter* genus to intestinal/host health are less clear.

Among the most abundant bacterial taxa, *Prevotellaceae* and *Alloprevotella* showed higher abundance in the HFBF group compared to the HFD group, probably due to the fact that these are fiber-degradation bacteria commonly enriched in the presence of high-fiber diets [94]. Prevotella was also related with the increased glucose metabolism and liver glycogen content after a diet rich in complex polysaccharides, due its potential to ferment fibers and increases the glycogen storage [95]. Notably, mice supplemented with CCB flour consumed more soluble dietary fiber, suggesting an effect on the microbiome even when

associated with high saturated fat content. However, we did not observe an increase in the SCFA concentration in the feces, suggesting that the time of experimentation or the experimental model used in this study could also be a limitation. As proposed [96], members of the *Prevotella* genera potentially serve as effective biomarkers of plant-based diets rich in polysaccharides and dietary fiber. In addition, there was a trend towards a reduction in an OTU identified as *Escherichia-Shigella* in the HFBF group, which indicates the beneficial potential found in this group.

According to the functional analysis of the microbiota, we observed beneficial changes in the host genetic capacity, especially in the metabolic pathways involved with glucose metabolism. As shown in Figure 5, the KEGG metabolic pathways involved with starch and sucrose metabolism, as well as the galactose metabolism were enriched in the HFBF group compared to the HFD control group. Nutrigenomics refers to the study of how the food compounds act in the genetic expression modulation, and explains how the diet can initiate different responses among individuals due to genetic variability or polymorphisms [97]. In the present study, the relative abundance of bacteria of the genus *Prevotella* was enriched by the consumption of CCB, and these results indicate that the chemical composition of soluble dietary fibers, proteins and bioactive compounds present in common bean-based nutrition, even associated with a high-fat diet, has a significant impact on the gut microbiota and host metabolism.

The HFPH group did not show significant difference in the composition/abundance of bacterial genera when compared to the HFD group, indicating that although the protein hydrolysate has a promising phytochemical composition and bioactive peptides, it was not able to prevent the deleterious effects caused by HFD. In this sense, more studies are necessary to assess the effects of CCB protein hydrolysate associated with other dietary patterns, to investigate the gaps that exist when we combine food and the gut microbiota. In addition, when we compare both treatments (HFBF x HFPH), we observe a reduced abundance of *Lachnoclostridium* and *Rikenellaceae RC9 gut* group and increased abundance of *Muribaculaceae* and *Blautia* in the HFBF group. The significant increase in the abundance of beneficial microorganisms in the gut microbiota of mice that received CCB flour reinforces the positive impact of dietary fibers, phytochemicals and bioactive compounds present in this food matrix in the gut microbiota. Thus, as shown in the present study, the consumption of common bean should be encouraged as a potential strategy to modulate the intestinal microbiota against the high-fat diet, especially among the poor population.

5. Conclusions

This study shows that cooked BRSMG Madreperola bean flour, a slow-darkening cultivar, improves the composition of gut microbiota of mice fed a HFD associated with PTU, without negatively altering its function. The CCB flour preserved the bacterial taxa that is beneficial to intestinal health, and increased the cecum weight, moisture and lipids in the feces. However, the CCB protein hydrolysate was not able to prevent damage to the bacterial communities caused by a the HFD added with PTU.

Collectively, these findings show that consumption of common beans can prevent the deleterious effect of a HFD in experimental animals and that it could be a promising strategy to improve gastrointestinal health and the abundance of SCFA-producing bacteria. This is particularly important for people leaving in low- and middle-income countries, where common bean is a low-cost legume easily accessible to the population. However, further clinical trials are needed to confirm the present results.

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Supplementary Material

Table S1. Summary of sequencing data for adult BALB/c mice after nine weeks of treatment.

Treatment	Good's coverage	Raw sequences	After filtering and clean-up		After normalization	
		Reads	Reads	OTUs	Reads	OTUs
NC	0.998 ± 0.001	35866 ± 9339	26196 ± 6100	406 ± 74	15700 ± 24	402 ± 69
HFD	0.998 ± 0.000	40529 ± 10737	27679 ± 8148	340 ± 51	15679 ± 28	321 ± 37
HFBF	0.999 ± 0.000	47942 ± 12464	35772 ± 9118	364 ± 103	15697 ± 17	338 ± 90
HFPH	0.998 ± 0.000	45159 ± 10204	30774 ± 7183	355 ± 61	15682 ± 15	327 ± 42

Values presented as mean ± SD, n = 9/group (NC, HFBF groups), n = 10/group (HFD group) and n = 11/group (HFPH group). NC: normal control; HFD: high-fat diet; HFBF: high-fat diet added with cooked common bean flour; HFPH: high-fat diet added with cooked common bean protein hydrolysate.

Table S2. Alpha-diversity metrics of bacterial communities in the cecum content of adult BALB/c mice after nine weeks of treatment.

	Chao	Shannon	Simpson
NC	602.00 ± 141.43	3.51 ± 0.93	0.12 ± 0.11
HFD	570.97 ± 194.65	3.42 ± 0.23	0.07 ± 0.03
HFBF	619.58 ± 204.49	3.65 ± 0.52	0.06 ± 0.04
HFPH	559.47 ± 153.90	3.21 ± 0.27	0.09 ± 0.04
p-value	0.559	0.119	0.214

Values presented as mean ± SD, n = 9/group (NC, HFBF groups), n = 10/group (HFD group) and n = 11/group (HFPH group). NC: normal control; HFD: high-fat diet; HFBF: high-fat diet added with cooked common bean flour; HFPH: high-fat diet added with cooked common bean protein hydrolysate. Data were analyzed by Kruskal-Wallis with a Dunn's test for peer comparison.

Table S3. KEGG less abundant metabolic routes that showed a statistical difference between the treatment groups compared to the control group.

	Relative abundance (%)		
Description	**HFBF**	**HFD**	***p*-value**
Starch degradation V	0.82 ± 0.07	0.65 ± 0.09	0.043
D-fructuronate degradation	0.57 ± 0.07	0.40 ± 0.08	0.037
Thiazole biosynthesis I	0.50 ± 0.04	0.41 ± 0.06	0.048
UDP-N-acetyl-D-glucosamine biosynthesis I	0.40 ± 0.04	0.50 ± 0.07	0.050
Pyrimidine Deoxyribonucleotides de novo biosynthesis II	0.39 ± 0.02	0.43 ± 0.03	0.046
Superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis (E. coli)	0.39 ± 0.02	0.43 ± 0.03	0.031
Superpathway of hexuronide and hexuronate degradation	0.29 ± 0.06	0.17 ± 0.04	0.025
Taxadiene biosynthesis	0.29 ± 0.05	0.44 ± 0.04	0.020
Superpathway of D-glucuronide and D-glucuronate degradation	0.28 ± 0.06	0.16 ± 0.05	0.028
Thiazole biosynthesis II (Bacillus)	0.20 ± 0.04	0.28 ± 0.04	0.037
Reductive TCA cycle I	0.12 ± 0.09	0.29 ± 0.11	0.049
Superpathway of *Clostridium acetobutylicum* acidogenic fermentation	0.08 ± 0.05	0.20 ± 0.07	0.041
1,4-dihydroxy-6-naphthoate biosynthesis I	0.04 ± 0.03	0.09 ± 0.03	0.039
Glutaryl-CoA degradation	0.02 ± 0.01	0.07 ± 0.03	0.022
	HFPH	**HFD**	***p*-value**
L-isoleucine biosynthesis II	0.82 ± 0.06	0.88 ± 0.10	0.050
Flavin biosynthesis I (bacteria and plants)	0.46 ± 0.07	0.54 ± 0.06	0.036

Values presented as mean relative abundance ± SD. n = 9/group (HFBF groups), n = 10/group (HFD group) and n = 11/group (HFPH group). HFD: high-fat diet; HFBF: high-fat diet added with cooked common bean flour; HFPH: high-fat diet added with cooked common bean protein hydrolysate. Data were analyzed by the Mann-Whitney test.

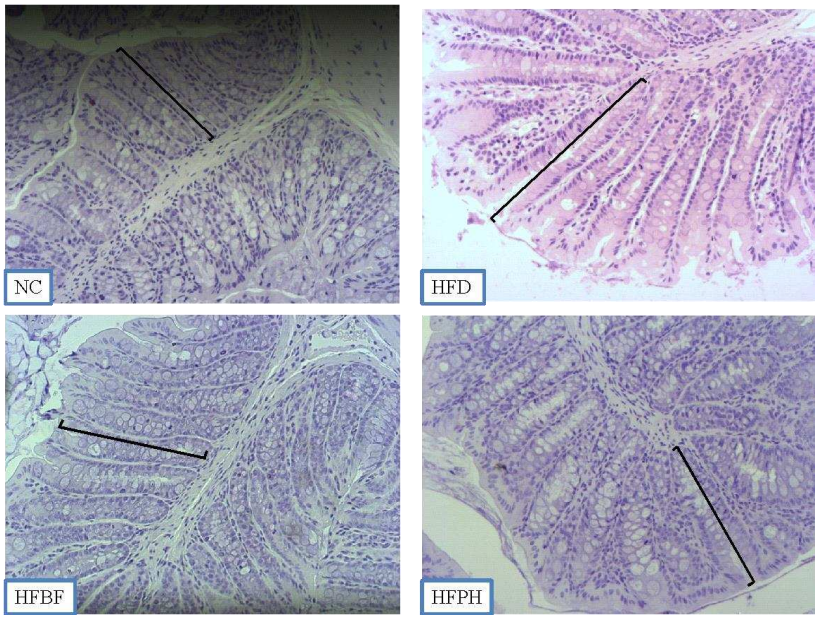

Fig. S1. Colonic histomorphometric characteristics of adult BALB/c mice after nine weeks of treatment (n = 8). Effect of bean flour and bean protein hydrolysate intake on crypt height. NC: normal control; HFD: high-fat diet; HFBF: high-fat diet added with cooked common bean flour; HFPH: high-fat diet added with cooked common bean protein hydrolysate.

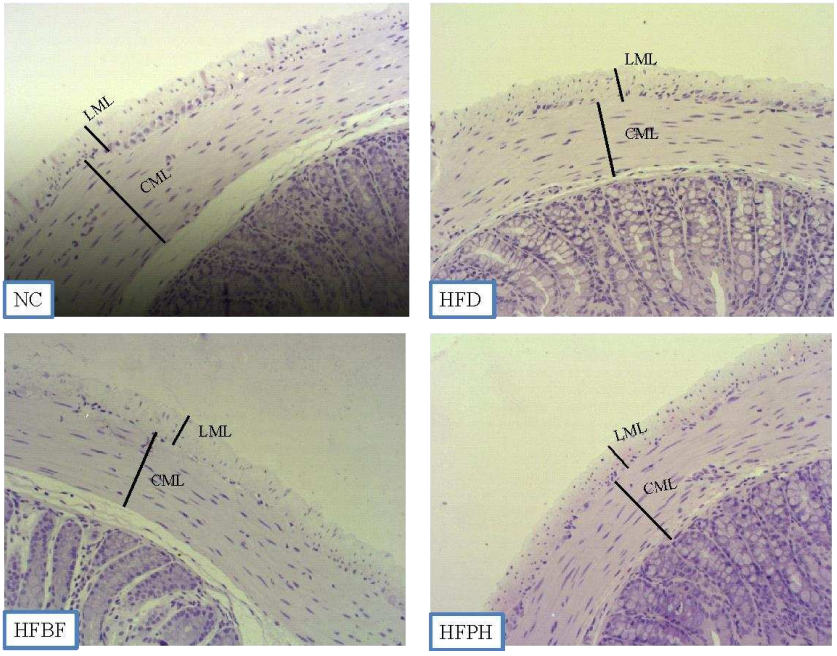

Fig. S2. Colonic histomorphometric characteristics of adult BALB/c mice after nine weeks of treatment (n = 8). Effect of bean flour and bean protein hydrolysate intake on circular and longitudinal muscle layers. NC: normal control; HFD: high-fat diet; HFBF: high-fat diet added with cooked common bean flour; HFPH: high-fat diet added with cooked common bean protein hydrolysate; CML: circular muscle layer; LML: longitudinal muscle layer.

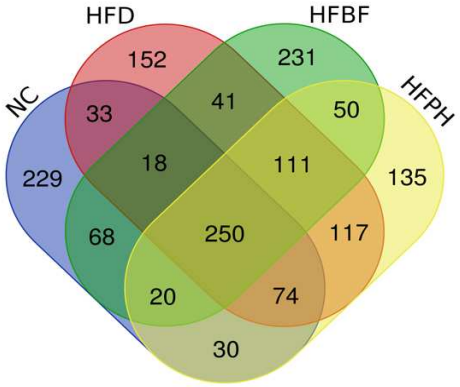

Fig. S3. Venn diagram showing the number of bacterial OTUs shared between adult BALB/c mice after nine weeks of treatment. n = 9/group (NC, HFBF groups), n = 10/group (HFD group) and n = 11/group (HFPH group). NC: normal control; HFD: high-fat diet; HFBF: high-fat diet added with bean flour; HFPH: high-fat diet added with bean protein hydrolysate. Only OTUs with abundance > 1% were represented.

6.2. PAPER 4

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Original Research

Zinc biofortified cowpea (*Vigna unguiculata* L. Walp.) soluble extracts modulate assessed cecal bacterial populations and gut morphology *in vivo* (*Gallus gallus*)

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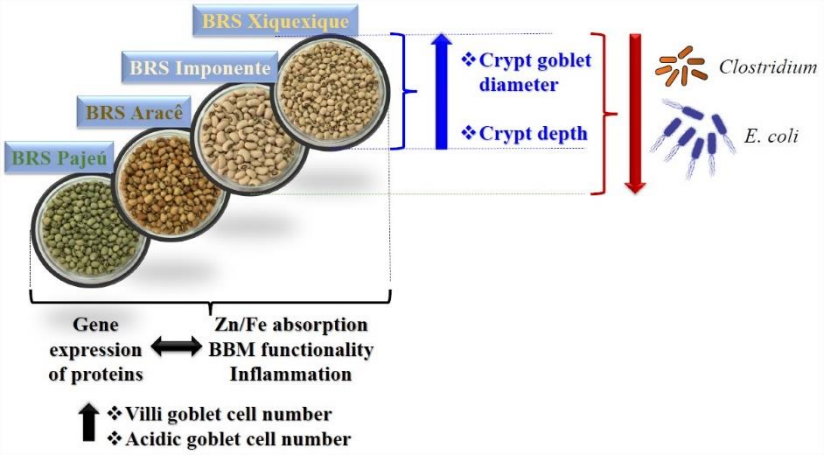
Abstract

Background: Biofortification is a method that improves the nutritional value of food crops through conventional plant breeding. The aim of this study was to evaluate the effects of intra-amniotic administration of soluble extracts from zinc (Zn) biofortified and Zn standard cowpea (*Vigna unguiculata* L. Walp.) flour on intestinal functionality and morphology, inflammation, and gut microbiota, *in vivo*. **Methods**: Seven treatment groups were utilized: (1) No Injection; (2) 18MΩ H_2O; (3) 50 mg/mL Inulin; (4) 50 mg/mL BRS Pajeú soluble extract (Zn standard); (5) 50 mg/mL BRS Aracê soluble extract (Zn biofortified); (6) 50 mg/mL BRS Imponente soluble extract (Zn biofortified); (7) 50 mg/mL BRS Xiquexique soluble extract (Zn biofortified). **Results**: Treatment groups with BRS Imponente and BRS Xiquexique reduced the abundance of *Clostridium* and *E. coli* when compared with all other experimental groups. All cowpea soluble extracts increased villi goblet cell number (total), specifically acidic goblet cell type number per villi relative to inulin and 18MΩ H_2O groups. Moreover, BRS Xiquexique increased the crypt goblet diameter and the crypt depth compared to all treatments and controls. The Zn content in the Zn biofortified cowpea flours was higher when compared to the Zn standard flour (BRS Pajeú), and the phytate:Zn molar ratio was lower in the Zn biofortified flours compared to the Zn standard flour. In general, all cowpea soluble extracts maintained the gene expression of proteins involved with Zn and iron absorption, brush border membrane (BBM) functionality and inflammation compared to inulin and 18MΩ H_2O. **Conclusions**: This study demonstrates the potential nutritional benefit of standard and biofortified cowpea treatment groups to improve intestinal morphology, BBM

functionality, inflammation, and gut microbiota, with the highest effect of BRS Xiquexique soluble extracts to improve assessed cecal microflora populations and intestinal morphology.

Keywords: biofortification; dietary fiber; cowpea beans; mineral deficiency; intestinal functionality; microbial populations

Graphical Abstract



1. Introduction

Zinc (Zn) is essential for human health due to its key role as a required cofactor in numerous enzymatic reactions in the body. Zn holds a vital role during infants' growth and development phase, and contributes to immune system maintenance [1, 2]. Zn deficiency has been correlated with stunted growth, immune system depletion, and adverse pregnancy outcomes [3, 4]. An estimative of World Health Organization (WHO) showed that one-third of the global population is at risk for Zn deficiency, data calculated considering those individuals with intake lower than the daily requirements of Zn [5], thus improving Zn status through an increase of dietary Zn absorption is considered a critical challenge to public health [6, 7]. Worldwide, Zn deficiency is the second most prevalent mineral deficiency, just behind iron (Fe) deficiency, and is estimated to affect 17% of the global population. This is mainly attributed to the low Zn bioavailability in food [4, 8].

Cowpea is a nutritious crop and widely consumed in West Africa and North and Northeast Brazil [9], and its high tolerance to heat and drought makes it a relevant target crop for Zn biofortification. Biofortified cowpea cultivars present equal to or above 40 and 60 mg Kg^{-1} of Zn and Fe in the grain, respectively [10]. Cowpea cultivars biofortified in these minerals have been released in Brazil by Embrapa's cowpea breeding program, these include

BRS Xiquexique (Zn and Fe), BRS Aracê (Zn and Fe), BRS Tumucumaque (Zn and Fe), and BRS Imponente (Zn). BRS Xiquexique and BRS Aracê are more recommended for family farmers, while BRS Tumucumaque and BRS Imponente are more suitable for business farmers [11].

The promising chemical and polyphenolic composition [12] of the grain, combined with its undemanding agronomic characteristics, make cowpea favourable to low-income farmers, who have limited access to nutritionally-balanced diets and are highly susceptible to micronutrient malnutrition [13]. Polyphenols are a class of compounds naturally present in beans; some coloured beans have a higher content of phenolic compounds, which can potentially inhibit Zn bioavailability [10, 14, 15]. However, phenolic compounds have also been associated with beneficial health effects, such as anti-inflammatory and antioxidant properties [16, 17] and improvement of intestinal health [18, 19].

Cowpea flour also contains soluble compounds, such as soluble dietary fiber, which can act as prebiotics. Prebiotics are non-digestible complex carbohydrates that resist digestion in the gastrointestinal tract and are fermented in the colon [20]. Metabolites produced by gut microbiota fermentation of prebiotics can confer benefits to host health [21]. Gut microbiota fermentation of prebiotics can lead to the production of short-chain fatty acids (SCFA) and a decrease in intestinal lumen pH, beneficially affecting the gut microbiome and intestinal health [22, 23, 24].

Previous studies have shown the role of Zn to support ω-6 fatty acid metabolism and an association between low dietary Zn intake and fatty acid desaturase 1 (FADS1) and fatty acid desaturase 2 (FADS2) activities [25, 26]. FADS2 is a Δ-6-desaturase, and FADS1 is a Δ-5-desaturase. Δ-5- and Δ-6-desaturases are essential for metabolizing linoleic acid (LA) to arachidonic acid (AA) and can be used as a marker of FADS1 and FADS2 activities [27]. In addition, *in vivo* studies have shown the influence of Zn physiological status on intestinal microbiota composition and function [26, 28, 29, 30]. The consumption of different types of cowpea has been shown to increase cecal *Lactobacillus* populations, decrease the cecal pH, and increase the weight of the cecum, indicative of an overall beneficial effect on intestinal function *in vivo* [22]. Moreover, we have previously shown that Zn or Fe biofortified foods can improve gut microbiota composition and function in vivo (Gallus gallus) [23]. The *Gallus gallus* model is well-established in evaluating the effects of mineral status on brush border membrane (BBM) functionality, intestinal morphology, and gut microbiome [23, 31, 32, 33]. The gut microbiome of the *Gallus gallus* has significant resemblance at the phyla level

compared to humans, with Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria being the most dominant phyla [34, 35, 36, 37].

Studies evaluating the effects of food intake as part of biofortification programs on intestinal functionality, morphology and microbiota are limited. This is the first study with Zn biofortified cowpea in this line of investigation; as the effects of intra-amniotic administration of soluble extracts from Zn biofortified cowpea cultivars on intestinal health are unknown. Hence, the objectives of this study were to investigate the effects of the Zn biofortified and standard cowpea soluble extracts on Zn and Fe related BBM proteins and BBM functionality and inflammation, as well as to assess the effects of cowpea cultivars on the cecal microbiota and intestinal morphology *in vivo* (*Gallus gallus*). In addition, this study aimed to contribute to scientific advances and the utilization of Zn biofortified foods, and provide the basis for developing dietary strategies aimed to combat micronutrient deficiencies in vulnerable populations.

2. Materials and Methods

2.1 Sample Preparation

Grains of four cowpea cultivars were used to conduct this experiment: Zn standard BRS Pajeú, and Zn biofortified BRS Aracê, BRS Imponente and BRS Xiquexique. All cultivars were obtained from Embrapa Meio-Norte, Teresina, PI, Brazil. The cultivars' grains were shipped to the Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Brazil, and were cooked in three replicates in a conventional pressure cooker for 25 min using a bean/distilled H_2O ratio of 1:1.3 (w/v). Cowpeas were dried in an air oven for 16 h at 60 °C, ground by stainless steel mill 090 CFT at 3000 rpm, and stored at –12 °C until analysis [38]. Cowpeas flours were shipped to Ithaca, NY, in sealed containers, where the *in vivo* experiment was conducted.

2.2 Extraction of Soluble Compounds from Cowpeas

As previously described [33, 39], the cowpeas flour samples were homogenized in distilled H_2O (50 g/L) for 90 min, at 60 °C, and centrifuged at 3000 rpm for 30 min at 4 °C to remove suspended particles. The collected supernatant was dialyzed (MWCO 12–14 kDa) exhaustively against distilled H_2O for 48 h. Finally, the dialysate was collected and lyophilized to yield a light brown powder.

2.3 Dietary Fiber, Protein, Iron, Zinc and Phytate Composition Analysis of the Cowpea Flour

The dietary fiber and protein content were determined according to the methodology proposed by the Association of Official Analytical Chemistry (AOAC) [40], in duplicate. For dietary fiber assessment, samples were enzymatically hydrolyzed using heat-resistant amylase, protease and amyloglucosidase enzymes from total dietary fiber assay (Kiyonaga, Sigma®, Kawasaki, Japan). Dietary phytic acid (phytate)/total phosphorous assay was used to determine phytate content following specific kit instructions (K-PHYT 12/12, Megazyme International, Bray, Ireland).

Determination of Fe and Zn concentration in cowpeas flour was performed as previously described [33, 35]. For analysis, 500 mg samples of each respective cowpea flour were pre-processed at room temperature for 16 h, in borosilicate glass tubes added with 3 mL concentrated nitric acid and perchloric acid (60:40 v/v). After, samples were maintained for 4 h in a heated (120 °C) digestion block (Martin Machine, Ivesdale, IL, USA). After incubation, an ultra-pure nitric acid (2 mL) was added to the samples, and the digestion block temperature was adjusted to 145 °C for 2 h. After, the digestion block temperature was adjusted to 190 °C for ten minutes. Digested samples were re-suspended in 20 mL of ultrapure water and then analyzed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Thermo iCAP 6500 Series, Thermo Scientific, Cambridge, UK), with quality control standards (High Purity Standards, Charleston, SC, USA). As an internal standard, it was used Yttrium (High Purity Standards, 10M67-1). All samples were digested and measured with 0.5 µg/mL of Yttrium (final concentration) to ensure batch-to-batch accuracy and correct matrix inference during digestion.

2.4 Polyphenols Composition Analysis of the Cowpea Flour

2.4.1 Polyphenol Extraction

1 g of each respective cowpea flour was added with 5 mL of methanol/H_2O (50:50 v/v). Samples were vortexed for 1 min, followed by sonication in water bath for 20 min (24 °C), vortexed again for 1 min and finally centrifuged at 4000 × *g* for 15 min. The supernatant was filtered with a 0.20 µm Teflon syringe and stored at –20 °C.

2.4.2 Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis of Polyphenols

Extracts and standards were assessed using an Agilent 1220 Infinity Liquid Chromatograph (LC; Agilent Technologies, Inc., Santa Clara, CA, USA) combined with an

Advion expression LC mass spectrometer (LC-MS; Advion Inc., Ithaca, NY, USA). 10 µL cowpea extracts were inserted into an XBridge Shield RP18 3.5µm; 2.1 × 100 mm column (Waters, Milford, MA, USA) at 0.6 mL/minute. The temperature of the column was adjusted to 40 °C. The mobile phase consisted of ultra-pure H_2O with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). To elute polyphenols, linear gradients of 94.0 to 84.4% A in 1.50 min, 84.4 to 81.5% A in 2.25 min, 81.5 to 77.0% A in 6.25 min, 77.0 to 55.0% in 1.25 min, 55.0 to 46.0% in 2.25 min, 46.0 to 94.0% in 2.25 min and hold at 94.0% A for 2.25 min were used, with a complete run time of 18 minutes. The flow of the column was led into a variable wavelength UV detector set at 265–278 nm. After, flow was led into LC-MS, and an environment with negative ionization mode was used by ESI mass spectrometry (scan time of 200 msec). Capillary temperature and voltages were 250 °C and 180 volts, respectively, the desolvation gas flow was 240 L/h, and the ESI source voltage and gas temperature were 2.5 kilovolts and 250 °C, respectively. Data were extracted from Advion Mass Express™ software. Polyphenols in the samples were identified and confirmed after comparing the retention time of standards, and the standard curves were created from integrating areas under UV absorption peaks from 5 replications.

2.5 Intra-Amniotic Administration (Gallus Gallus Model)

Cornish-cross fertile broiler eggs (n = 63), acquired from a commercial hatchery (Moyer's Chicks, Quakertown, PA, USA), were properly incubated [41] at Cornell University Animal Science Poultry Farm incubator. Lyophilized soluble extracts were separately diluted in deionized H_2O to verify the final concentrations corresponding to an osmolality (OSM) <320 OSM. Eggs with viable embryos were weighed and divided into seven groups (n = 9) with approximately equal weight distribution. The seven treatment groups were assigned as follows: (1) No injection; (2) 18MΩ H_2O; (3) Inulin, 50 mg/mL; (4) BRS Pajeú extract, 50 mg/mL; (5) BRS Aracê extract, 50 mg/mL; (6) BRS Imponente extract, 50 mg/mL; (7) BRS Xiquexique extract, 50 mg/mL. 1 mL solution was injected intra-amniotically utilizing a 21-gauge needle into amniotic fluid following candling. Immediately following the injection, the injection site was sterilized with 70% ethanol and sealed with cellophane tape. The eggs were then placed into hatching baskets according to their treatment groups, with each treatment groups equally represented at each location within the same incubator.

Immediately after hatch (21 days), chicks were weighed and then euthanized by CO_2 exposure. Ceca were weighed before storage, and the cecum, duodenum (proximal small

intestine), and liver were collected in separate sterile cryovials (Simport, Beloeil, QC, Canada) and stored at –80 °C until analysis. All animal protocols were approved by Cornell University Institutional Animal Care and Use Committee (IACUC #2020-0077).

2.6 Extraction of Total RNA from Duodenum and Liver

30 mg of the liver tissue or proximal duodenal tissue (n = 5) were weighed for the total RNA extraction. Qiagen RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Inc., Valencia, CA, USA) was applied according to the kit manufacturer's protocol. All stages were executed under RNase-free conditions. Briefly, with a rotor–stator homogenizer and containing β-mercaptoethanol, tissues in buffer RLT®, were disrupted and homogenized. Next, in a microcentrifuge (C2400-R, Labnet International Inc, Edison, NJ, USA), the lysate was centrifuged for 3 min at 8000 × *g*. The supernatant was transferred to a new tube, blended with 70% ethanol, and slightly mixed.

Each sample (700 µL) was put in RNeasy mini-columns, centrifuged for 15 s at 8000 × *g*, and the flow-through material was removed. Following to new 2 mL collection tubes, the RNeasy columns were transferred, and 500 µL of buffer RPE® was pipetted onto the RNeasy column followed by centrifugation for 15 s at 8000 × *g*. Again, 500 µL of buffer RPE was added onto the RNeasy column and centrifuged for 2 min at 8000 × *g*. The total RNA was eluted in 50 µL of free RNase water, and the sample containing the RNA solution was analyzed and quantified by absorbance at 260/280 nm. Integrity test of the 18S ribosomal RNA was confirmed by 1.5% agarose gel electrophoresis with ethidium bromide staining. TURBO DNase treatment and removal kit from AMBION (Austin, TX, USA) was applied to remove the DNA contamination.

2.7 Real-time Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed as previously published [39, 42, 43]. Briefly, 20 µL reverse transcriptase (RT) reaction was completed in a BioRad C1000 Touch Thermocycler applying the Improm-II Reverse Transcriptase Kit (Catalog #A1250; Promega, Madison, WI, USA) to form the cDNA. cDNA concentration was quantified by the absorbance at 260/280 nm using an extinction coefficient of 33 (for single-stranded DNA). Genomic DNA contamination was measured by a real-time RT-PCR assay for the reference gene samples [44, 45, 46].

The primers used in the real-time PCR were designed. This procedure was based on gene sequences from the GenBank database, using Real-Time Primer Design Tool software

(IDT DNA, Coralville, IA, USA), as previously described [39, 42, 43]. Primers sequences used in this study were summarized in Table 1. Through performing a BLAST search against the genomic National Center for Biotechnology Information (NCBI) database, the specificity of the primers was tested. The reference gene used was the 18S rRNA specific for the *Gallus gallus* model.

Table 1. The sequences of the primers used in this study.

Analyte	Forward Primer (5'-3')	Reverse Primer (5'-3')	Base Pairs Length	GI Identifier
Zinc and Iron Metabolism				
DMT-1	TTGATTCAGAGCCTCCCATTAG	GCGAGGAGTAGGCTTGTATTT	101	206597489
Ferroportin	CTCAGCAATCACTGGCATCA	ACTGGGCAACTCCAGAAATAAG	98	61098365
DcytB	CATGTGCATTCTCTTCCAAAGTC	CTCCTTGGTGACCGCATTAT	103	20380692
ZnT-1	GGTAACAGAGCTGCCTTAACT	GGTAACAGAGCTGCCTTAACT	105	54109718
ZnT-7	GGAAGATGTCAGGATGGTTCA	CGAAGGACAAATTGAGGCAAAG	87	56555152
ZIP-9	CTAAGCAAGAGCAGCAAAGAAG	CATGAACTGTGGCAACGTAAAG	100	237874618
Δ-6-desaturase*	GGCGAAAGTCAGCCTATTGA	AGGTGGGAAGATGAGGAAGA	93	261865208
Δ-5-desaturase*	GTACTTCTTCATCATTGGTCCC	CCCAGGATACCCTTCACAC	171	423120
BBM Functionality				
AP	CGTCAGCCAGTTTGACTATGTA	CTCTCAAAGAAGCTGAGGATGG	138	45382360
SI	CCAGCAATGCCAGCATATTG	CGGTTTCTCCTTACCACTTCTT	95	2246388
SGLT-1	GCATCCTTACTCTGTGGTACTG	TATCCGCACATCACACATCC	106	8346783
MUC-2	CTGCTGCAAGGAAGTAGAA	GGAAGATCAGAGTGGTGCATAG	272	423101
Inflammation				
NF-κB	CACAGCTGGAGGGAAGTAAAT	TTGAGTAAGGAAGTGAGGTTGAG	100	2130627
TNF-α	GACAGCCTATGCCAACAAGTA	TTACAGGAAGGGCAACTCATC	109	53854909
IL-8	TCATCCATCCCAAGTTCATTCA	GACACACTTCTCTGCCATCTT	105	395872
18s rRNA	GCAAGACGAACTAAAGCGAAAG	TCGGAACTACGACGGTATCT	100	7262899

DMT-1: Divalent metal transporter-1; DcytB: Duodenal cytochrome B; Znt and ZIP: Zinc transporter proteins; BBM: Brush border membrane; AP: Amino peptidase; SI: Sucrase isomaltase; SGLT-1: Sodium-glucose transport protein 1; MUC-2: Mucin-secreting intestinal protein-2; NF-κB: Nuclear factor kappa B; TNF-α: Tumor necrosis factor Alpha; IL-8: Interleukin-8; 18s rRNA: 18s Ribosomal subunit. * Liver analysis.

2.8 RT-qPCR Design

For the RT-qPCR design, all procedures were conducted as previously described [35, 39, 42, 43]. Each 10 µL reaction consisted of 2 × BioRad SSO Advanced Universal SYBR Green Supermix (Cat #1725274, Hercules, CA, USA), cDNA, buffer, Taq DNA polymerase, dNTPs and SYBR green dye. Specific primers (forward and reverse) (Table 1), and cDNA or water, were added to each PCR reaction. The optimal $MgCl_2$ concentration provided the amplification plot with the lowest cycle product (Cp), the highest fluorescence intensity, and

the steepest amplification slope for each gene. Master mix (8 μL) was pipetted into the 96-well plate, and 2 μL cDNA was added as a PCR template. Each run contained, in duplicate, seven standard curve points. No template control of nuclease-free water was included to exclude DNA contamination in the PCR mix. The Bio-Rad CFX96 Touch (Hercules, CA, USA) was used to provide the amplification of the double-stranded DNA utilizing 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.

Gene expressions were quantified as Cp values based on the "second derivative maximum" (automated method) as computed by Bio-Rad CFX Maestro 1.1 (Version 4.1.2433.1219, Hercules, CA, USA). All tests were measured by including a standard curve in the real-time qPCR analysis. The standard curve was prepared using 1:10 serial dilution, in duplicate. Software generated a graph with the concentrations of Cp vs. log10, and the efficiencies were calculated as 10[1/slope]. The specificity of the amplified real-time RT-PCR products was verified by melting curve analysis (60–95 °C) after 40 cycles, in which several different specific products should be obtained, with a specific melting temperature for each one.

2.9 Collection of Microbial Samples and DNA Isolation

As was previously described, the cecum was sterilely removed and treated [24, 34]. To collect microbial samples, the cecum content was placed into a sterile 15 mL tube, containing 9 mL of sterile PBS, and homogenized with glass beads (3 mm diameter) for 3 min. Through centrifugation, debris was removed, at 1000 × *g* for 5 min, and the supernatant was collected and centrifuged at 4000 × *g* for 10 min. The pellet was washed with PBS and stored at –20 °C until DNA extraction. For DNA purification step, the pellet was re-suspended in 50 mM EDTA and treated with lysozyme (Sigma Aldrich CO., St. Louis, MO, USA) for 60 min at 37°C. Employing a Wizard Genomic DNA purification kit (Promega Corp., Madison, WI, USA), the bacterial genomic DNA was isolated.

2.10 Primer Design and PCR Amplification of Bacterial 16S rDNA

As previously described, primers for *Lactobacillus*, *Bifidobacterium*, *Clostridium* and *E. coli* were utilized [33, 39]. To estimate the relative proportion of each studied bacteria, each product was expressed relative to the content of the universal primer product, and proportions

of each bacterial group are presented. PCR products were separated using electrophoresis on 2% agarose gel, stained with ethidium bromide, and quantified using the Quantity One 1-D analysis software (Bio-Rad, Hercules, CA, USA).

2.11 Morphological Examination

Analysis of the intestinal morphology was conducted as previously described [39, 42]. Briefly, samples from the duodenum were fixed in fresh 4% (v/v) buffered formaldehyde, dehydrated, cleared and implanted 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, and stained with Alcian Blue/Periodic acid-Schiff. Morphometric measurements in the crypt and villi were performed with a light microscope equipped with EPIX XCAP software (Standard version, Olympus, Waltham, MA, USA), applying five biological samples per treatment group (n = 5) and four segments for each biological sample. The morphometric measurements are indicated by a representative duodenal histological cross-section image (Supplementary Figs. 1,2).

2.12 Statistical Analysis

The data were expressed as means and standard deviation. Experimental groups for the intra-amniotic administration procedure were arranged in a completely randomized design. Determined parameters were noticed to have a normal distribution and equal variance through a Shapiro-Wilk test and were, therefore, acceptable for one-way analysis of variance (ANOVA). For significant "p-value", test groups were compared using Duncan *post-hoc* test, with the significance level established at $p < 0.05$. The IBM SPSS Statistics 26 (IBM Analytics, Armonk, NY, USA) was executed for each statistical analysis.

3. Results

3.1 Concentration of Dietary Fiber, Protein, Iron, Zinc, Phytate, Phytate: Iron and Phytate: Zinc Molar Ratio in Cowpea Flour

The total dietary fiber and insoluble dietary fiber concentrations were higher ($p < 0.05$) in the BRS Pajeú flour (Zn standard) compared to the other Zn biofortified cowpea bean flours. The dietary fiber content in the cowpea flour soluble extracts did not change ($p > 0.05$) between the Zn standard and Zn biofortified cultivars. BRS Aracê (Zn biofortified) flour had the highest ($p < 0.05$) protein content compared to BRS Pajeú flour (Zn standard). The Zn

concentration was higher ($p < 0.05$) in the Zn biofortified cowpea flours compared to the Zn standard BRS Pajeú flour, and the Fe content in the Zn biofortified cowpea flours was similar ($p > 0.05$) to the Zn standard flour (Table 2).

Table 2. Chemical composition of Zn biofortified cowpea flour, on dry basis.

	BRS Pajeú	BRS Aracê	BRS Imponente	BRS Xiquexique
TDF (g/100g)	19.02 ± 0.24^{a}	13.82 ± 0.08^{c}	11.65 ± 0.23^{d}	15.10 ± 0.04^{b}
SDF (g/100g)	1.59 ± 0.19^{a}	1.07 ± 0.21^{a}	1.16 ± 0.44^{a}	0.91 ± 0.06^{a}
IDF (g/100g)	17.43 ± 0.05^{a}	12.75 ± 0.29^{c}	10.50 ± 0.67^{d}	14.19 ± 0.02^{b}
Protein (g/100g)	22.28 ± 0.20^{c}	26.08 ± 0.70^{a}	25.03 ± 0.16^{b}	23.04 ± 0.39^{c}
Fe (µg/g)	55.27 ± 1.29^{ab}	54.54 ± 3.13^{ab}	49.47 ± 1.12^{b}	61.25 ± 0.41^{a}
Zn (µg/g)	31.09 ± 0.09^{c}	36.34 ± 0.98^{b}	40.91 ± 0.20^{a}	37.19 ± 0.17^{b}
Phytate (g/100g)	0.78 ± 0.00^{c}	0.76 ± 0.00^{c}	0.90 ± 0.01^{a}	0.81 ± 0.01^{b}
Phytate:Fe molar ratio	11.91 ± 0.09^{b}	11.81 ± 0.06^{b}	15.49 ± 0.22^{a}	11.18 ± 0.11^{c}
Phytate:Zn molar ratio	24.78 ± 0.18^{a}	20.74 ± 0.11^{c}	21.92 ± 0.31^{b}	21.55 ± 0.20^{bc}

Values are means ± SD. Means sharing the same letter in each row are not significantly different ($p \leq 0.05$) by *post-hoc* of Duncan test. BRS Pajeú: Zn-standard; BRS Aracê, BRS Imponente and BRS Xiquexique: Zn-biofortified; TDF: Total dietary fiber; SDF: Soluble dietary fiber; IDF: Insoluble dietary fiber; Fe: Iron; Zn: Zinc.

3.2 Polyphenol Profile in the Cowpea Flour

The concentration of the eight most prevalent polyphenolic compounds found in the Zn biofortified and Zn standard cowpea flours is shown in Table 3. BRS Aracê flour showed the highest ($p < 0.05$) concentration of epicatechin, kaempferol 3-sambubioside, myricetin, and quercetin 3-rutinoside, compared to the other cowpea flours, and BRS Xiquexique flour showed higher ($p < 0.05$) content of epicatechin compared to BRS Pajeú. In addition, BRS Pajeú showed a higher ($p < 0.05$) content of myricetin 3-glucoside, protocatechuic acid, and quercetin 3-glucoside compared to the other flour samples (Table 3).

Table 3. Polyphenol profile (μM) present in the Zn biofortified cowpea flours.

	BRS Pajeú	BRS Aracê	BRS Imponente	BRS Xiquexique
Epicatechin	37.23 ± 0.05^{c}	39.00 ± 0.01^{a}	-	38.94 ± 0.02^{b}
Kaempferol 3-sambubioside	0.19 ± 0.01^{b}	0.24 ± 0.01^{a}	0.13 ± 0.03^{c}	0.19 ± 0.01^{b}
Myricetin	22.45 ± 0.13^{b}	22.95 ± 0.07^{a}	22.51 ± 0.21^{b}	22.68 ± 0.12^{b}
Myricetin 3-glucoside	5.08 ± 0.05^{a}	2.47 ± 0.02^{b}	1.87 ± 0.20^{d}	2.33 ± 0.06^{c}
Protocatechuic acid	12.19 ± 0.05^{a}	0.21 ± 0.02^{d}	0.70 ± 0.03^{b}	0.26 ± 0.01^{c}
Quercetin	1.68 ± 0.02^{a}	1.51 ± 0.07^{a}	-	-
Quercetin 3-glucoside	1.62 ± 0.03^{a}	0.36 ± 0.04^{bc}	0.32 ± 0.05^{c}	0.42 ± 0.02^{b}
Quercetin 3-rutinoside	0.24 ± 0.03^{b}	0.35 ± 0.03^{a}	0.25 ± 0.02^{b}	0.30 ± 0.04^{ab}

Values are means ± SEM (n = 5). Means sharing different letter in each row are significantly different ($p \leq 0.05$) by *post-hoc* of Duncan test. BRS Pajeú: Zn-standard; BRS Aracê, BRS Imponente and BRS Xiquexique: Zn-biofortified.

3.3 In Vivo Assay (Gallus Gallus Model)

3.3.1 Effect of the Cowpea Soluble Extracts on Biometric Parameters

There was no significant difference ($p > 0.05$) in the body weight, cecum weight, and cecum to bodyweight ratio between the Zn biofortified and Zn standard cowpea treatment groups when compared to the control groups (No injection, 18MΩ H_2O, and inulin).

3.3.2 Effect of the Cowpea Soluble Extracts in the Gene Expression of Proteins Involved with Zn and Fe Metabolism

The gene expression of duodenal cytochrome b (DcytB), divalent metal transporter 1 (DMT1) and ferroportin in the three Zn biofortified cowpea soluble extracts were similar ($p > 0.05$) to the Zn standard BRS Pajeú, 18MΩ H_2O and inulin groups. The relative expression of Zn transporters and importers (ZnT1, ZnT7, and ZIP9) did not differ ($p > 0.05$) between cowpea soluble extracts treatment groups compared to 18MΩ H_2O and inulin control groups, however, these proteins were downregulated ($p < 0.05$) in the BRS Xiquexique group, compared to no injection control. Δ-6- and Δ-5-desaturase are involved with the fatty acid biosynthesis, and the gene expression of these proteins has been demonstrated to be a sensitive and specific indicator of Zn status [27]. Δ-6-desaturase was significantly downregulated ($p < 0.05$) in the BRS Xiquexique treatment group compared to no injection, 18MΩ H_2O, and inulin control groups compared to the Zn standard BRS Pajeú. There was no difference ($p > 0.05$) in the expression of Δ-6-desaturase between the other Zn biofortified soluble extracts compared to the controls, and the treatments did not affect ($p > 0.05$) the expression of Δ-5-desaturase (Fig. 1).

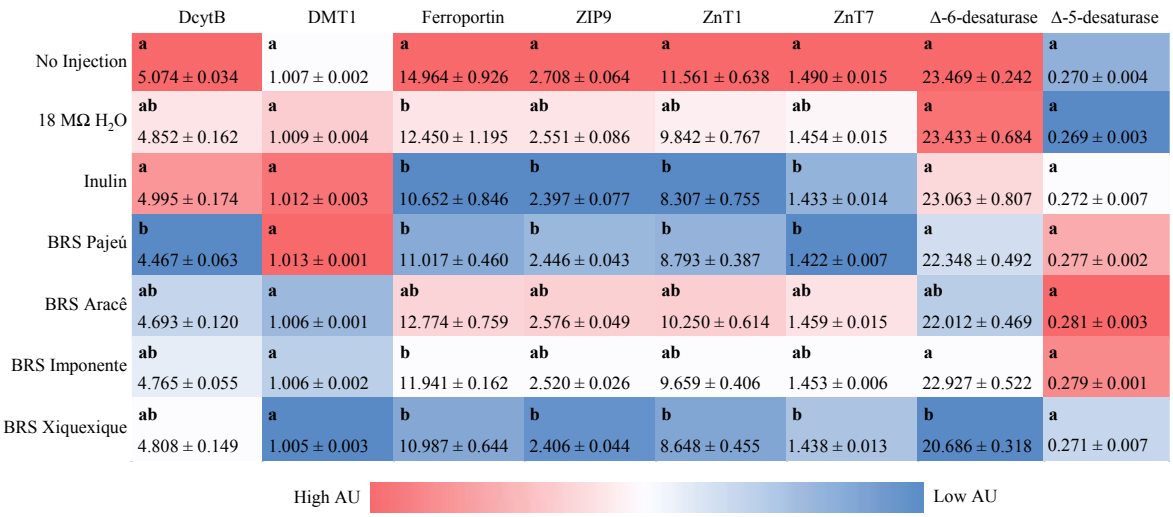


Fig. 1. Effect of the intra-amniotic administration of cowpea soluble extracts on gene expression of proteins involved with Zn and Fe metabolism. Values are the means ± SEM, n = 5. [a–b] Per gene, treatments groups not indicated by the same letter are significantly different ($p < 0.05$). Dcytb: Duodenal cytochrome b; DMT1: Divalent metal transporter 1; ZnT and ZIP: Znic transporter proteins.

3.3.3 Effect of the Cowpea Soluble Extracts in the Gene Expression of Proteins Involved with the BBM Functionality and Inflammation

The gene expression of sodium-glucose transport protein 1 (SGLT1), sucrase isomaltase (SI), aminopeptidase (AP), and mucin-secreting intestinal protein 2 (MUC2) are commonly used as biomarkers of BBM digestive and absorptive functions. In the present study, the treatment with soluble extracts of Zn biofortified cowpea did not alter ($p > 0.05$) the SGLT1, SI, AP and MUC2 expression compared to the 18MΩ H_2O and inulin control groups. However, the expression of SGLT1, AP and MUC2 was downregulated ($p < 0.05$) in the BRS Pajeú compared to the no injection control (Fig. 2).

The expression of markers related to inflammatory mechanisms is presented in Fig. 2. The expression of NF-κB, TNF-α, and IL-8 did not change ($p > 0.05$) after the intra-amniotic administration of Zn biofortified and Zn standard cowpea soluble extracts to the 18MΩ H_2O and inulin controls. In addition, BRS Pajeú soluble extract and inulin downregulated ($p < 0.05$) the expression of NF-κB and TNF-α, and BRS Xiquexique, downregulated ($p < 0.05$) the expression of NF-κB, compared to the no injection group.

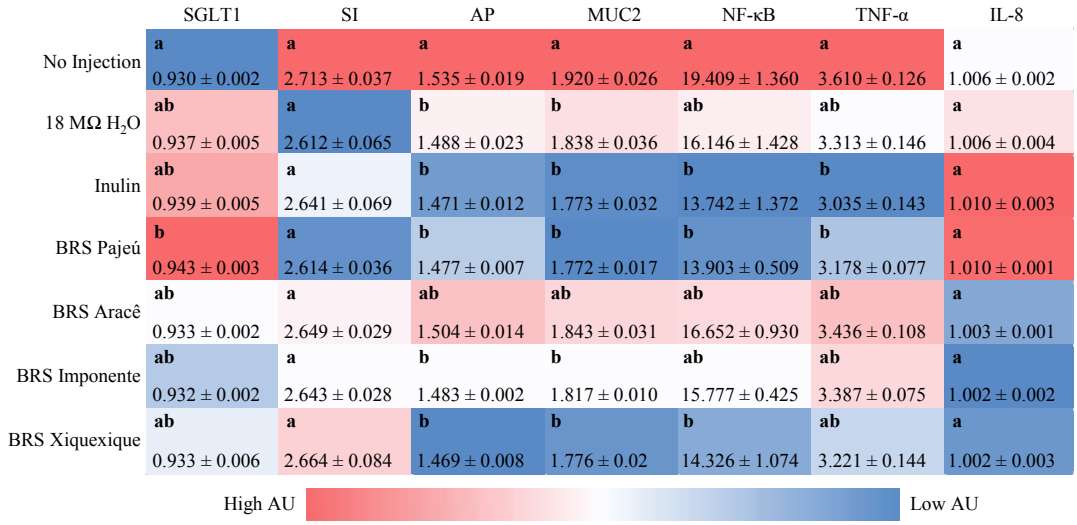


Fig. 2. Effect of the intra-amniotic administration of cowpea soluble extracts on gene expression of proteins involved with the BBM functionality and inflammation. Values are the means ± SEM, n = 5. [a–b] Per gene, treatments groups not indicated by the same letter are significantly different ($p < 0.05$). SGLT-1: Sodium-glucose transport protein 1; SI: Sucrose isomaltase; AP: Amino peptidase; MUC2: Mucin-secreting intestinal protein-2; NF-κB: Nuclear factor kappa B1; TNF-α: Tumor necrosis factor alpha; IL-8: Interleukin-8.

3.3.4 Effect of the Cowpea Soluble Extracts in the Genera- and Species-Level Bacterial Populations

There was no difference ($p > 0.05$) in the relative abundance of *Lactobacillus* after the intra-amniotic administration of Zn biofortified and Zn standard cowpea soluble extracts compared to the controls. BRS Xiquexique (Zn biofortified) increased ($p < 0.05$) the relative abundance of *Lactobacillus* compared to BRS Imponente (Zn biofortified), and despite the relative abundance of *Bifidobacterium* has decreased ($p < 0.05$) in the BRS Imponente and BRS Xiquexique, compared to the controls, *E. coli* and *Clostridium* showed a decreased ($p < 0.05$) relative abundance in these treatment groups, compared to the other experimental groups (Fig. 3).

In addition, the standard BRS Pajeú (Zn standard) soluble extract increased the ($p < 0.05$) the relative abundance of *Bifidobacterium*, compared to the Zn biofortified soluble extracts, and BRS Xiquexique, BRS Pajeú, and BRS Aracê showed an abundance of *Lactobacillus* similar ($p > 0.05$) to inulin (positive control) (Fig. 3).

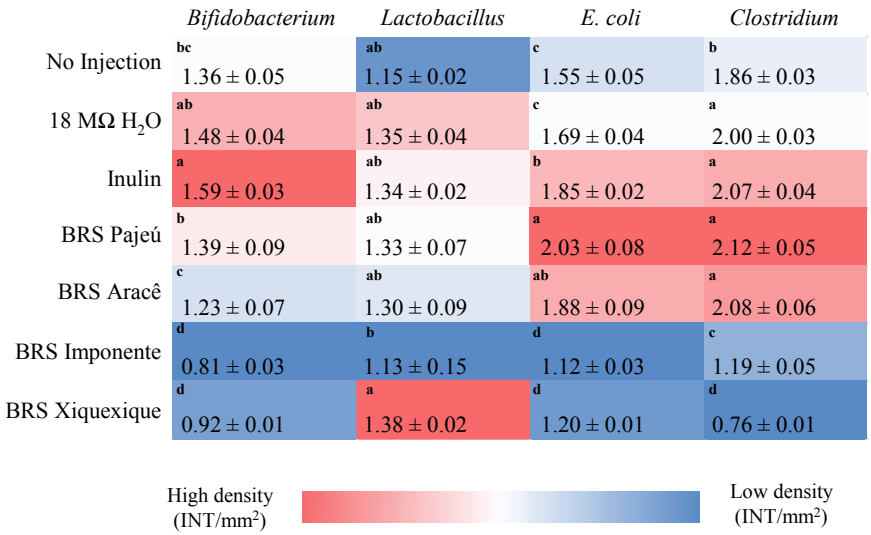


Fig. 3. Effect of the intra-amniotic administration of Zn biofortified cowpea soluble extract on genera- and species-level bacterial populations from cecal contents measured on the day of hatch. Values are the means ± SEM, n = 8. Means sharing different letter in each column are significantly different ($p \leq 0.05$) by post-hoc of Duncan test.

3.3.5 Effect of the Cowpea Soluble Extracts on Duodenal Morphometric Parameters

The villus surface area was higher ($p < 0.05$) in the BRS Xiquexique treatment group compared to the BRS Pajeú (Zn standard) and 18MΩ H_2O control group; however, it was lower ($p < 0.05$) in all treatment groups compared to inulin control. Related to the goblet cells, a mucus producer cell, the BRS Xiquexique group increased ($p < 0.05$) the villi goblet cell number compared to the 18MΩ H_2O (ultrapure water) and inulin control groups. However, there was no difference ($p > 0.05$) in the villi goblet diameter between the treatment groups with Zn biofortified cowpeas and Zn standard compared to the controls (Table 4). Representative images are shown in **Supplementary Fig. 1**.

In relation to the types of goblet cells in the crypt epithelium, we observed an increase ($p < 0.05$) in the number of acid goblet cells in the villus in all treatment groups with cowpea soluble extracts, compared to the 18MΩ H_2O and inulin control groups. Further, a decrease ($p < 0.05$) in the neutral goblet cell in the groups injected with cowpea soluble extracts compared to the 18MΩ H_2O and no injection groups was observed. In addition, the administration of BRS Xiquexique and BRS Aracê soluble extracts decreased ($p < 0.05$) the number of mixed goblet cells relative to the other experimental groups, except inulin control (Table 4).

Table 4. Effect of the intra-amniotic administration of Zn biofortified cowpea soluble extract on the duodenal small intestinal villus.

Treatment group	Villus surface area (mm^2)	Villi goblet cell number (Unit)	Villi goblet diameter (μm)	Villus goblet cell number (Unit)		
				Acid	Neutral	Mixed
No Injection	353.39 ± 8.14 ^{bc}	39.63 ± 0.93 ^{a}	3.45 ± 0.07 ^{a}	31.89 ± 0.89 ^{ab}	1.85 ± 0.19 ^{a}	5.89 ± 0.28 ^{a}
18 MΩ H_2O	261.12 ± 7.36 ^{e}	28.94 ± 0.76 ^{c}	3.43 ± 0.06 ^{a}	16.27 ± 0.67 ^{d}	1.42 ± 0.17 ^{b}	5.29 ± 0.72 ^{a}
Inulin	384.57 ± 12.58 ^{a}	25.50 ± 0.75 ^{d}	3.20 ± 0.06 ^{b}	23.96 ± 0.68 ^{c}	0.08 ± 0.02 ^{e}	1.46 ± 0.14 ^{c}
BRS Pajeú	326.62 ± 10.12 ^{cd}	40.56 ± 0.82 ^{a}	3.28 ± 0.07 ^{ab}	33.82 ± 0.83 ^{a}	0.88 ± 0.12 ^{c}	5.86 ± 0.55 ^{a}
BRS Aracê	318.97 ± 8.12 ^{d}	34.14 ± 0.90 ^{b}	3.31 ± 0.06 ^{ab}	30.63 ± 0.88 ^{b}	0.44 ± 0.07 ^{d}	3.20 ± 0.16 ^{b}
BRS Imponente	327.45 ± 9.59 ^{bcd}	36.47 ± 0.86 ^{a}	3.30 ± 0.06 ^{ab}	31.58 ± 0.83 ^{ab}	0.65 ± 0.10 ^{c}	4.30 ± 0.30 ^{a}
BRS Xiquexique	351.28 ± 9.12 ^{b}	36.05 ± 1.00 ^{b}	3.30 ± 0.08 ^{ab}	32.31 ± 1.00 ^{ab}	0.33 ± 0.06 ^{de}	3.41 ± 0.24 ^{b}

Values are the means ± SEM, n = 5. Means sharing different letter in each column are significantly different ($p \leq 0.05$) by *post-hoc* of Duncan test.

In the crypt, we observed an increase ($p < 0.05$) in the goblet diameter, crypt goblet cell number, and Paneth cell diameter in the BRS Xiquexique treatment group, compared to the BRS Pajeú (Zn standard). The crypt depth was increased ($p < 0.05$) in the BRS Xiquexique in relation to all the other experimental groups, and the Paneth cell per crypt was increased ($p < 0.05$) in the BRS Xiquexique group compared to the no injection and BRS Pajeú groups. However, the Paneth cell per crypt did not differ ($p > 0.05$) from the BRS Aracê and BRS Imponente compared to the standard BRS Pajeú (Table 5). Representative images are shown in **Supplementary Fig. 2**.

In relation to the types of goblet cells in the crypt, the BRS Xiquexique presented the highest and BRS Imponente presented the lowest ($p < 0.05$) acid goblet cell number compared to the other experimental groups. The treatments with Zn biofortified and Zn standard cowpea soluble extracts decreased ($p < 0.05$) the neutral goblet cell number related to the 18MΩ H_2O control, and the mixed goblet cell number was lower ($p < 0.05$) in the BRS Xiquexique and BRS Imponente groups compared to the inulin group (Table 5).

Table 5. Effect of the intra-amniotic administration of Zn biofortified cowpea soluble extract on the duodenal small intestinal crypt and Paneth cell.

Treatment group	Crypt goblet diameter (μm)	Crypt goblet cell number (Unit)	Crypt depth (μm)	Paneth cell/crypt (Unit)	Paneth cell diameter (μm)	Crypt goblet cell number (Unit)		
						Acid	Neutral	Mixed
No Injection	3.24 ± 0.04 ^{a}	10.15 ± 0.41 ^{a}	22.04 ± 0.66 ^{b}	1.81 ± 0.07 ^{c}	2.88 ± 0.10 ^{a}	7.74 ± 0.24 ^{bc}	1.56 ± 0.24 ^{bc}	0.86 ± 0.11 ^{c}
18MΩ H_2O	2.74 ± 0.04 ^{b}	11.14 ± 0.35 ^{a}	17.8 ± 0.54 ^{c}	2.32 ± 0.08 ^{a}	1.70 ± 0.04 ^{bcd}	7.66 ± 0.22 ^{bc}	2.62 ± 0.21 ^{a}	0.86 ± 0.08 ^{c}
Inulin	2.18 ± 0.04 ^{d}	10.78 ± 0.43 ^{a}	21.49 ± 0.64 ^{b}	2.29 ± 0.09 ^{a}	1.64 ± 0.04 ^{cd}	8.45 ± 0.36 ^{ab}	0.63 ± 0.11 ^{de}	1.70 ± 0.15 ^{a}
BRS Pajeú	2.80 ± 0.04 ^{b}	8.35 ± 0.33 ^{b}	22.31 ± 0.77 ^{b}	1.81 ± 0.06 ^{c}	1.58 ± 0.02 ^{d}	7.40 ± 0.30 ^{c}	0.29 ± 0.06 ^{e}	0.66 ± 0.08 ^{c}
BRS Aracê	2.02 ± 0.04 ^{e}	11.01 ± 0.46 ^{a}	21.7 ± 0.73 ^{b}	1.89 ± 0.07 ^{bc}	1.80 ± 0.03 ^{b}	7.49 ± 0.29 ^{c}	2.04 ± 0.27 ^{b}	1.48 ± 0.13 ^{ab}
BRS Imponente	2.22 ± 0.06 ^{c}	9.87 ± 0.29 ^{b}	20.66 ± 0.47 ^{c}	1.81 ± 0.06 ^{c}	1.81 ± 0.03 ^{b}	6.89 ± 0.24 ^{d}	1.60 ± 0.10 ^{cd}	1.38 ± 0.10 ^{b}
BRS Xiquexique	3.27 ± 0.05 ^{a}	10.26 ± 0.43 ^{a}	25.22 ± 0.82 ^{a}	2.04 ± 0.08 ^{b}	1.77 ± 0.03 ^{bc}	8.96 ± 0.39 ^{a}	0.66 ± 0.12 ^{de}	0.65 ± 0.08 ^{c}

Values are the means ± SEM, n = 5. Means sharing different letter in each column are significantly different ($p \leq 0.05$) by *post-hoc* of Duncan test.

4. Discussion

In the present study, four cowpea cultivars (*Vigna unguiculata* L. Walp.) were assessed following intra-amniotic administration (*Gallus gallus*) of its soluble extracts, with the aim to investigate the potential of standard (BRS Pajeú) and Zn biofortified cowpeas (BRS Aracê, BRS Imponente and BRS Xiquexique) in improving intestinal bacterial composition and morphology, brush border membrane (BBM) functionality and inflammation. Cowpeas are a nutritious crop and a widely consumed legume in West Africa and North and Northeast Brazil with a high tolerance to heat and drought, making cowpeas a great target crop for Zn biofortification [9, 11]. The cowpea flour used in this study showed a significant concentration of protein, dietary fiber, Zn and Fe, and polyphenols, specifically, epicatechin, myricetin and quercetin (Tables 2 and 3). Studies have shown the potential of soluble fiber, phenolic compounds and minerals from biofortified foods to improve mineral bioavailability and gut functionality [23, 33, 35, 37, 47, 48].

In this study, we observed that BRS Imponente and BRS Xiquexique soluble extracts decreased the populations of *Clostridium* and *E. coli* in comparison to all the other experimental groups. Further, despite the reduction in *Bifidobacterium*, the BRS Xiquexique treatment group demonstrated an increased relative abundance of *Lactobacillus* compared to BRS Imponente (Fig. 3). These observations are also associated with improved intestinal morphology, as indicated by increased crypt depth, crypt goblet diameter, villi goblet number and villi acidic goblet cell number, in the BRS Xiquexique group, compared to the inulin and 18MΩ H_2O control groups. The Xiquexique group was also associated with increased villus surface area and crypt acidic goblet cell number compared to the 18MΩ H_2O control group (Tables 4 and 5). These promising results can be explained by the chemical and polyphenolic composition of this newly developed cultivar (Tables 2 and 3). Recent literature has shown that the gut microbiome is directly affected by the compositional profile of the foods, mainly its dietary fibers and polyphenols. The intra amniotic administration of quinoa fiber and quercetin showed potential to modulate the microbiome and improve intestinal morphology [49]. In addition, cowpea showed its prebiotic properties by modulating the gut microbiota *in vitro*, with a significant increase in *Bifidobacterium* and *Lactobacillus* [50].

The BRS Xiquexique flour showed the lowest phytate:Fe molar ratio compared to the other tested Zn biofortified cowpea flour, and a lower phytate:Zn molar ratio than the standard BRS Pajeú. This may indicate higher mineral bioavailability in the intestinal lumen, where minerals could be utilized by bacteria that colonize the gastrointestinal tract [48, 51]. The

chemical composition of the food matrix of a bean cultivar can determine its effects on intestinal functionality and health [33, 35, 47]. BRS Xiquexique showed improved results, compared to the other varieties with increased levels of Zn, possibly due to its higher content of dietary fiber the lower phytate:Fe molar ratio, which increases the mineral bioavailability and is associated with its polyphenolic profile. Further, BRS Xiquexique showed high levels of gallic and ferulic acids, supporting an antioxidant and functional potential demonstrated in the present study [52]. Bacteria that inhabit the gut lumen are mineral dependent, therefore, an increased supply of Zn and Fe can increase the abundance of beneficial phyla and genera [31, 43]. Several bacterial species have the ability to ferment dietary soluble fibers and produce short-chain fatty acids (SCFA), which is a valuable metabolite used by enterocytes as a source of energy and nutrition [53]. *Lactobacillus* is a probiotic genus generally regarded as safe (GRAS); this genus harbors SCFA producing species, where SCFA production has been associated with anti-inflammatory properties [54, 55]. Further, the reduction in potentially pathogenic *Clostridium* and *E. coli* is associated with two treatment groups, Zn biofortified BRS Imponente and BRS Xiquexique, and suggests an improvement in the gut health [24, 33].

Among treatment groups with Zn biofortified cowpea beans soluble extracts, BRS Aracê showed an increase total goblet cell number and acidic type goblet cell number per villi, compared to the inulin and 18MΩ H_2O control groups. Despite no difference in the *Lactobacillus*, *E. coli* and *Clostridium* in the cecum of this treatment group, compared to the controls, the morphology assessment may indicate an improvement in duodenal functionality and health. The predominance of goblet cells with acidic characteristics can indicate increased SCFA production by bacterial populations, mainly acetate, propionate and butyrate, which decreased the intraluminal pH, turning it into a more acidic environment and reflecting in the cell hyperplasia [56]. Therefore, not only the production of SCFAs, but also the composition of polyphenols may have contributed to this result, and within the microbial ecosystem, different substrates affect the gut microbiota composition and modulate SCFA production [56].

Paneth cells play a key role in intestinal immunity and host defense, secreting anti-microbial compounds and other substances that contribute to maintaining the intestinal barrier [57]. Paneth cell number was increased in the Zn biofortified-BRS Xiquexique treatment group, compared to the standard Zn standard-BRS Pajeú. Further, an increased Paneth cell diameter was measured in the three Zn biofortified soluble extracts treated groups compared

to the Zn standard (Table 5). Paneth cells number and size can reflect the early stage of intestinal inflammation since Paneth cell-produced lysozyme regulates intestinal anti- and pro-inflammatory responses [58, 59]. Current data agrees with the gene expression of NF-κB1, TNF-α, and IL-8, which showed no difference between treatment groups versus control groups (Fig. 2).

BRS Xiquexique and BRS Aracê flour presented higher epicatechin contents than BRS Pajeú, which may explain the improved barrier function in these treatment groups. Compared with cultivars of Fe biofortified and Fe standard common bean [35], the cowpea flour polyphenolic profile assessed in the present study had a higher concentration of epicatechin and quercetin 3-glucoside. As previously demonstrated [60], derivates of myricetin and quercetin constitute the most abundant flavonoids in the cowpea, and this flavonoid profile has a major impact on the bioactive properties of this legume. Flavonoids, such as epicatechin, are metabolized by the gut microbiota, generating metabolites that are more potent than the primary compound, such as epicatechin-3′-O-glucuronide 3′-O-methyl-epicatechin-5-sulfate, and epicatechin-3′-sulfate [61]. In addition, some gut microbial enzymes are involved in metabolic reactions of flavonoids, such reactions may lead to improved flavonoid absorption in the gastrointestinal tract [62]. The gut microbiota can biotransform flavonoids, such as quercetin, kaempferol, naringenin, apigenin, and luteolin, into phenolic metabolites [63]. However, this transformation may not be necessary for flavonoid absorption. Recently, it was demonstrated the role of the microbiome in metabolizing kaempferol and quercetin *in vivo*, and it was suggested a potential flavonoid bioavailability modulation by gut microbiota [64].

The current study also assessed the potential effects of Zn biofortified and Zn standard cowpea soluble extracts on the gene expression of key Fe and Zn metabolism associated BBM proteins BBM functional and inflammation proteins. In general, there were no significant differences in the gene expression of proteins related to Zn and Fe absorption (DcytB, DMT1, ferroportin, ZIP9, ZnT1, ZnT7, and Δ-5-desaturase) between treatment groups of Zn biofortified soluble extract, relative to the inulin, 18MΩ H_2O, and no injection control groups (Fig. 1). This indicates that despite the slight difference between the cowpea cultivars, in terms of color, polyphenolic profile, mineral and proximal composition of the flour (Table 2), they presented a similar nutritional value. Another interesting result was observed in the BRS Xiquexique group, in which there was a downregulation of Δ-6-desaturase, a new proposed sensitive biomarker for Zn status assessment [65], compared to the control groups. In addition

to the higher Zn content in the BRS Xiquexique flour, compared to Zn standard-BRS Pajeú, the lower expression of Δ-6-desaturase may indicate that increased Zn absorptive efficiency might occur [66], since BRS Xiquexique cultivar demonstrated the lowest phytate:Fe molar ratio, and a lower phytate:Zn molar ratio, compared to Zn standard BRS Pajeú (Table 2).

Studies with biofortified foods show that the increased amounts of Zn and Fe in food matrices have the potential to improve the absorption of these minerals by improving BBM functionality [33, 35, 48, 67]. In the present study, we did not observe differences in the gene expression of proteins associated with BBM functionality (SGLT1, SI, AP, and MUC2) in the Zn biofortified and standard treatment groups in comparison to the inulin and 18MΩ H_2O controls. Considering that the embryonic *Gallus gallus* model has limited capacity to digest and absorb nutrients before hatch [68], these data indicate that the soluble extracts from cowpea maintained and supported BBM functionality and did not cause inflammation. Similar results were observed after an intra-amniotic administration (*Gallus gallus*) of Fe biofortified common bean soluble extract [33], and post a long-term feeding trial (*Gallus gallus*), aimed to assess Fe biofortified common bean flour [35].

Thus, our results demonstrate the potential benefit of biofortified cowpea extracts to improve intestinal morphology, BBM functionality, inflammation, and gut microbiota. These observations were significant specifically in the BRS Xiquexique group, with clear improvements in intestinal bacterial populations and intestinal morphological biomarkers.

5. Conclusions

The intra-amniotic administration of Zn biofortified cowpea soluble extracts demonstrated potential nutritional benefit, as was demonstrated by the improved intestinal morphology, BBM functionality, and cecal microbial composition. The promising effects shown by BRS Xiquexique and BRS Imponente in improving Zn BBM transport and by BRS Xiquexique in improving intestinal morphology indicate these are the most promising cultivars to be considered by biofortification programs.

In addition, we underlined the need for continuous studies on the benefits of new Zn biofortified cowpea cultivars, and we emphasize that the consumption of these beans' cultivars should be encouraged in other regions of the world besides West Africa and Northern Brazil. Based on the results of our preliminary study, the new cowpea cultivars have the potential to improve human health, although further studies are necessary to support these findings.

Author Contributions

ET led the research, conceived and supervised the project; MJCG, HSDM and ET developed and designed the experiment; MJCG, NK and ET conducted the experiment; MJCG, NK, JC and NA analyzed the data; MJCG drafted the manuscript; NK, JC, NA and MMR participated in data analysis and manuscript editing.

Ethics Approval and Consent to Participate

All animal care and experimental procedures complied with the Cornell University Institutional Animal Care and Use Committee (approval number: IACUC #2020-0077).

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Supplementary Material

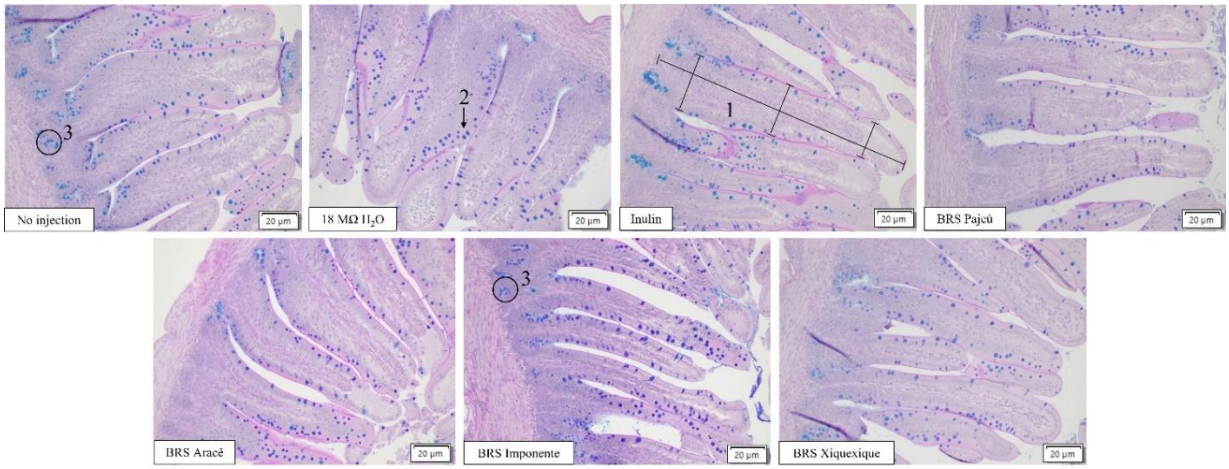


Fig. S1. Representation of the intestinal morphology for each experimental treatment group, with each parameter investigated indicated by numbers. (1) villus length and measurements applied to quantify the villus surface area (mm^2); (2) goblet cells; (3) crypt. BRS Pajeú is a Zn-standard; BRS Aracê, BRS Imponente and BRS Xiquexique are Zn-biofortified.

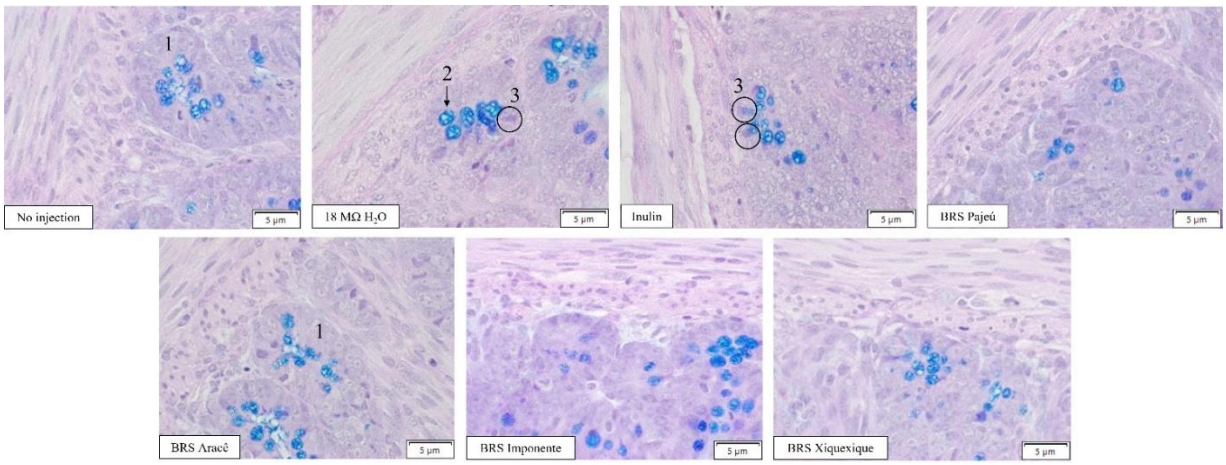


Fig. S2. Representation of the duodenal small intestinal crypt and Paneth cells for each experimental treatment group, with each parameter investigated indicated by numbers. (1) crypt; (2) goblet cells inside the crypt; (3) Paneth cells. BRS Pajeú is a Zn-standard; BRS Aracê, BRS Imponente and BRS Xiquexique are Zn-biofortified.

7. GENERAL CONCLUSIONS

The results and discussions presented in the manuscripts that make up this study contribute to understand the effects of carioca beans and cowpea on intestinal health *in vivo*. Further, it contributes with public health strategies to strengthen biofortification programs and encourages the production and consumption of biofortified foods (such as biofortified beans) worldwide.

We described the beneficial effects of the consumption of Fe and Zn biofortified foods in modifying the local microbial ecology, increasing the abundance of SCFAs producing bacteria, and decreasing the abundance of potentially pathogenic bacteria, such as *Streptococcus*, *Escherichia* and *Enterobacter*. We showed that biofortified foods provide substrates for fermentation in the large intestine, which favor the exclusion of pathogens by competition mechanisms. This is a natural selection, that favors the local biodiversity, increases the hyperplasia and/or hypertrophy of BBM cells, and the mucus production. In addition, we showed that Zn biofortified foods can provide lower phytate:Zn molar ratio, and has potential to improve the dietary Zn absorption in humans, specifically children. Also, we provided evidence that the consumption of about 34-84.1 μg Zn/g of Zn biofortified foods can increase Zn absorption rates in humans, and we strongly believe that this research is vital for contributing to nutritional science strategies and policies seeking to eradicate global micronutrient deficiencies, via the elucidation of factors that may contribute to digestion and absorption of micronutrients.

Carioca beans are daily consumed by most of Brazilian people. We showed that the consumption of carioca bean, but not its protein hydrolyzate, can attenuate the negative effects of a high fat diet, and positively modulate the gut microbiota composition and function *in vivo.* This kind of bean flour improved the colonic histomorphometric characteristics, and the gut microbiota composition *in vivo*, and contributed to beneficially change metabolic pathways of the host. It indicates that this legume should be encouraged as a potential strategy to modulate the gut microbiota against the high fat diet, especially among the poor population, as it is a cheap food, easily accessible and of high production yield worldwide.

Finally, we showed by an innovative study, that new cultivars of Zn biofortified cowpea soluble extracts, specially BRS Xiquexique and BRS Imponente soluble extracts, demonstrated potential nutritional benefit to improve the intestinal morphology, brush border membrane (BBM) functionality, and cecal microbial composition *in vivo*. In addition, cultivars of Zn biofortified beans presented higher Zn content and lower phytate:Zn molar

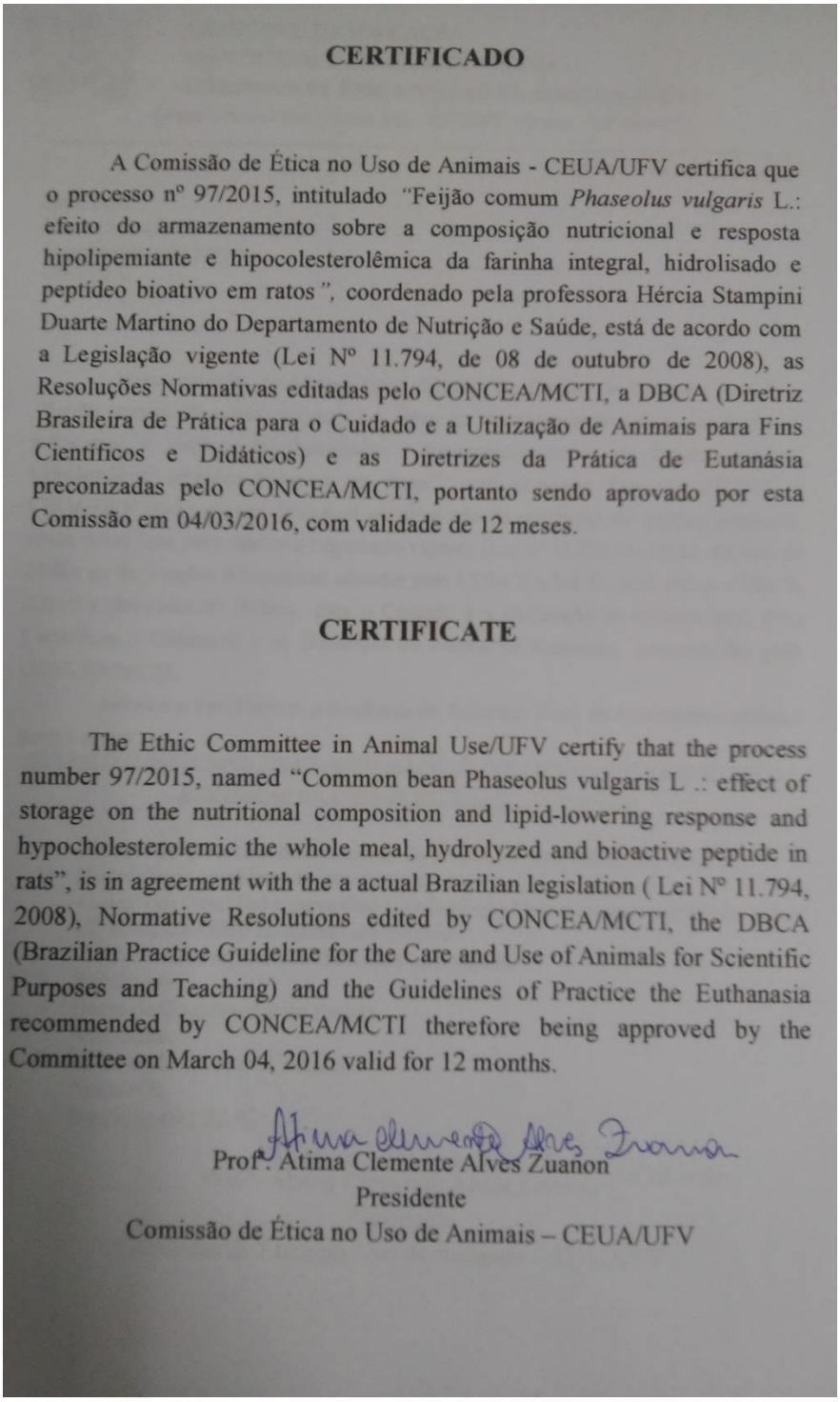
ratio, compared to the Zn standard. Our results showed that all cowpea soluble extracts improved the intestinal morphology compared to the control groups, but BRS Xiquexique presented the best results. Hence, these findings are significant and relevant to the field of micronutrients dietary deficiencies, mineral bioavailability, and intestinal functionality.

8. FINAL CONSIDERATIONS

We suggest that long-term feeding trials are performed to evaluate the effects of Zn biofortified cowpea, especially the cultivars BRS Xiquexique and BRS Imponente, on gut health, microbiome, and minerals absorption. These kinds of beans showed the highest potential to improve intestinal health, when assessed by the intra amniotic trial. In addition, this study highlights the importance of rescuing our culture of consuming not only beans, but foods in their entirety, instead of supplements containing extracts rich in bioactive compounds, for example.

We showed in our study that the effects of carioca bean flour consumption on the intestinal microbiota were different from the group that received the bean protein hydrolyzate, which reinforces the certainty that the complex food matrix generates systemic beneficial effects, different from isolated compounds. It is known that, in recent years, the consumption of beans *per capita* by Brazilian population has decreased, which, once again, reinforces the need to expand strategies for access to cultivars, especially by family farming, and the use of beans as a vehicle for biofortification.

APPENDIX I



CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 97/2015, intitulado "Feijão comum *Phaseolus vulgaris* L.: efeito do armazenamento sobre a composição nutricional e resposta hipolipemiante e hipocolesterolêmica da farinha integral, hidrolisado e peptídeo bioativo em ratos", coordenado pela professora Hércia Stampini Duarte Martino do Departamento de Nutrição e Saúde, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 04/03/2016, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 97/2015, named "Common bean Phaseolus vulgaris L .: effect of storage on the nutritional composition and lipid-lowering response and hypocholesterolemic the whole meal, hydrolyzed and bioactive peptide in rats", is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on March 04, 2016 valid for 12 months.

Profª. Atima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV