EFFECTS OF INTRA-AMNIOTIC ADMINISTRATION OF BEAN PREBIOTIC EXTRACTS ON THE GUT MICROBIOTA, BRUSH BORDER MEMBRANE FUNCTIONALITY AND IRON METABOLISM RELATED GENES IN THE *Gallus gallus* MODEL

A Project Paper

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Master of Professional Studies in Agriculture and Life Sciences

Field of Food Science

by

Naveena Lakshmanan

May 2018

ABSTRACT

Iron deficiency is a major micronutrient disorder affecting roughly 30% to 40% of the world's population. Common beans are a major source of food to a significant population in eastern Africa, Latin America and the Caribbean. Hence, beans are a good source to deliver iron to the population. This study used *Gallus gallus* as the animal model with intra-amniotic administration of bean prebiotic extracts to study the influence of the bean extracts on the intestinal microbial content, brush border membrane (BBM) functionality, and iron metabolism. Prebiotic extracts from seven different bean types grown in Brazil were the test solutions. Eggs(n=10/group) were injected with test solutions and control on the 17^{th} day. Upon hatching, chicks were euthanized and blood, liver, cecum and small intestine samples were collected for serum and liver iron concentrations, bacterial analyses of the cecum and for relative gene expression of proteins related to iron metabolism and BBM functionality. All the test groups had a significantly low relative abundance of potentially harmful bacteria in the gut relative to the positive control. The relative expression of ZnT1, ferroportin and AP were up-regulated in BRS Cometa group (iron biofortified carioca beans), but the study did not reveal any change in the iron status, possibly due to the short duration of the study. Hence, these results validate the need for a long-term study to elucidate the effects of the bean extracts on the iron status.

BIOGRAPHICAL SKETCH

Naveena Lakshmanan earned her bachelor's degree in Biotechnology from PSG College of Technology in India. She pursues her master's in food science and technology (M.P.S in Food Science) and graduates in May 2018. She is interested in food processing and in food safety and hopes to start her professional career in the food processing industry in India after graduation. Her project in Dr. Tako's lab involved investigating the effects of bean extracts on factors influencing the bioavailability of iron using a broiler chicken model.

ACKNOWLEDGMENTS

I express my heartfelt gratitude to Dr. Elad Tako, Research physiologist, USDA/ARS for his guidance and continuous support through the course of this project.

I would also like to extend my sincere gratitude to my colleagues in the lab, Mr. Nikolai Kolba, Ms. Desirre Morais Dias, Ms. Xuan Wang, Ms. Sybil T. Sha, Ms. Cathy K. Zhang, Ms. Michelle Ma and Ms. Jenny Kim for their support.

I am thankful to all the faculty and staff at the Department of Food Science, Cornell University who have been helpful through my period of study at Cornell University.

I am also extremely grateful to my family and friends who have stood by me at all times in every endeavor of mine.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1
INTRODUCTION

Iron deficiency is a major micronutrient deficiency in the world with two billion affected people and a million deaths a year[1,2]. Young children and women in reproductive age form a significant percentage of the at-risk population due to high iron demands during growth and pregnancy[1,2]. The incidence rate is high in low-income population as their diet comprises mainly of grains and legumes which are generally high in components that inhibit iron absorption and also due to a lack of meat consumption[3,4]. Hence, biofortification of staple food crops is one of the solutions to fight iron deficiency.

Common beans (*Phaseolus vulgaris*) are highly consumed in parts of eastern Africa and Latin America, and are the major source of proteins and micronutrients[5]. The global bean production in 2010 was roughly 23 million metric tons with 24% produced in Latin America and the Carribean[6]. Since beans form a major part of the staple diet, iron biofortification of beans is a sustainable solution to reduce iron deficiency in these populations. But the downside of biofortification of crops by conventional breeding is that, components like phytic acid and some inhibitory polyphenols hinder the absorption of iron, are also likely to be amplified in magnitude[7,8]. Studies have reported that prebiotics help in thwarting the negative effects of these inhibitory elements[9,10].

Prebiotics are non-digestible polysaccharides that aid in the promotion of gut health. These are fermented by probiotic bacteria to produce short chain fatty acids, which in turn, reduce the local pH, thus improving mineral solubility as well as inhibiting harmful bacteria[11]. They also increase the surface area of enterocytes for absorption, thus aiding in increasing iron bioavailability[12]. Reports indicate that beans have prebiotics like stachyose, raffinose, verbacose[13,14,15]. Hence these

prebiotics may play a role in improving the gut health and thereby have an indirect effect on mineral absorption.

Previous studies indicate that *Gallus gallus* can be a useful model to study the bioavailability of iron[16]. The rodent animal model has been the prevalent one to study nutrient bioavailability, but the rodent model is highly efficient in absorbing nutrients that exhibit low bioavailability in the human system[16]. Pigs have also been used for these studies. But the diets of pigs cannot be manipulated to the desired composition and the sample size when pig models tends to be small. In addition, the gut microbiota of *Gallus gallus* and humans at the phylum level is also considerably similar making chicken an intermediate test of in vivo iron bioavailability observations in preparation for human studies[17].

This study is aimed at looking into the effects of bean extracts on the intestinal microflora in vivo using the intra-amniotic administration[18] of bean extracts *Gallus gallus* model through an intra-amniotic mode of administration of the extracts. Three different bean varieties, carioca, black and white beans were used in this study. The aim was to study the effects of the bean prebiotic extracts on the intestinal microbial population. The study also investigated the effects of the prebiotic extracts on the expression of genes related to iron metabolism – DMT1, DCytB and Ferroportin. Brush border membrane functionality was evaluated by investigating the expression of aminopeptidase (AP), sucrose isomaltase (SI) and sodium glucose cotransporter1 (SGLT-1).

CHAPTER 2

MATERIALS AND METHODS

Bean samples

Three bean varieties grown in Brazil were used in the study. The beans were pressure cooked for 40 minutes, air dried in an oven at 60°C for 17 hours. The cooked beans were then milled in a stainless-steel mill 090 CFT at 3000 rpm and stored at -12° C. The bean samples were prepared by adding 5 ml of 50% v/v methanol/water solution to 1g of bean flour. This slurry was vortexed for 1 minute, placed in a water bath (24°C) for 20 minutes, vortexed again for 1 minute and centrifuged at 4000 xg for 15 minutes. The supernatant obtained was filtered using a 0.45µm Teflon syringe filter and stored at -20°C. The details of the beans used in the study are listed in Table 1.

Table 1: Characteristics of beans used in the study

Bean classes	Bean cultivars	Iron concentration in the bean flours (mg/g)	Source
White Carioca	BRS Artico BRS Perola BRS Cometa	65.59±5.66 (Fe standard) 77.39±3.01 (Fe standard) 94.95±0.74 (Fe biofortified)	Embrapa (Empresa Brasileira de Pesquisa Agropecuária, Goias, Brazil)
Black	BRS Esteio SMN 39	68.07±2.31 (Fe standard) 86.54±2.46 (Fe biofortified)	CIAT (International Center for Tropical Agriculture, Cali, Colombia)

The samples and standards were analyzed with a Waters Acquity UPLC followed by a Xevo G2 QTOF mass spectrometer (Waters Corp.) and ESI mass spectrometry was performed in negative ionization mode. Individual polyphenols in bean samples were tentatively determined by mass using MarkerLynx software, and their identities were confirmed by comparison of LC retention times with authentic standards.

Extraction of prebiotics from beans

The extraction of prebiotics was performed as described by Vidanarachchi et al (2009)[19], with some modifications[20,21]. Briefly, the bean flour samples were dissolved in distilled water (50 g/L) (60°C, 90 min) and then centrifuged at 3000 rpm for 20 min to remove particulate matter and then centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was collected and dialysed (MWCO 12–14 kDa) exhaustively against distilled water for 48 h. At last, the dialysate was collected and then lyophilized to yield a fine off-white powder.

Ethics statement

All animal protocols were approved by Cornell University Institutional Animal Care and Use committee (ethic approval code: 2007-0129).

Design of the study

Eggs from Cornish-cross broilers were obtained from a commercial hatchery (Moyer's chicks, Quakertown, PA, USA) and incubated under optimal conditions in the Cornell University Animal Science incubation facility. Prebiotics/polyphenolic extracts in powder form were separately diluted in 18 MΩ H2O to determine the concentrations necessary to maintain an osmolality value (Osm) of less than 320 Osm to ensure that the chicken embryos would not be dehydrated upon injection of the solution. Eggs were divided into eight different test groups with ten per group and on day 17, the eggs were injected with 1 ml of prebiotic solution/inulin/water/received no injection based on the group they were assigned to. The eight groups were: (1) non-injected; (2) 18 MWH2O; (3) 40mg/mL Inulin; (4) 50mg/mL Perola beans; (5) 50mg/mL Cometa beans; (6) 50mg/mL Esteio beans; (7) 50mg/mL SWN 39 beans; (8) 50mg/mL Artico beans. The eggs were candled to locate the amniotic fluid into which the solutions were injected. Post the injection, the pore created by the syringe was sealed and the eggs, separated

into groups, were restored to the incubator. The chicks emerged on day 21 and were euthanized with carbon-dioxide to collect liver, small intestine, blood and cecum samples.

Concentration of iron in the prebiotic extract and serum samples was measured using inductively-coupled argon-plasma/atomic emission spectrophotometry (Thermo iCAP 6500 series - Thermo Jarrell Ash Corp., Franklin, MA, USA).

. **Microbial analyses of the cecum**

The cecum were sterilely removed and treated as described previously [22]. The contents of the cecum were placed into a sterile 50 mL tube containing 9 mL of sterile PBS and homogenized by vortexing with glass beads (3 mm diameter) for 3 min. Debris was removed by centrifugation at 700 g for 1 min, and the supernatant was collected and centrifuged at 12,000 ×g for 5 min. The pellet was washed twice with PBS and stored at -20 ºC until DNA extraction. For DNA purification, the pellet was re-suspended in 50 mM EDTA and treated with lysozyme (Sigma Aldrich CO., St. Louis, MO, USA; final concentration of 10 mg/mL) for 45 min at 37ºC. The bacterial genomic DNA was isolated using a Wizard Genomic DNA purification kit (Promega Corp., Madison, WI, USA).

Primers for *Lactobacillus*, *Bifidobacterium*, and E. coli were designed according to previously published data [23]. To evaluate the relative proportion of each examined bacteria, all products were expressed relative to the content of the universal primer product and proportions of each bacterial group are presented. PCR products were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide, and quantified using the Quantity One 1-D analysis software (Bio-Rad, Hercules, CA, USA).

Gene expression profiles in small intestine

Total RNA Extraction

Total RNA was extracted from 30 mg of the proximal duodenal tissue (n = 6) using Qiagen RNeasy Mini Kit (RNeasy Mini Kit, Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. RNA was quantified by absorbance at A 260/280. Integrity of the 28S and 18S ribosomal RNAs was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining.

RT-PCR

To create the cDNA, a 20 μL reverse transcriptase (RT) reaction was completed in a BioRad C1000 touch thermocycler using the Improm-II Reverse Transcriptase Kit (Catalog #A1250; Promega, Madison, WI, USA).

The primers used in the real-time PCR was designed based on 9 gene sequences from Genbank database, using Real-Time Primer Design Tool software (IDT DNA, Coralvilla, IA, USA). The sequences and the description of the primers used in this work are summarized in Table 2. The *Gallus gallus* primer 18S rRNA was designed as a reference gene. Results obtained from the qPCR system were used to normalize those obtained from the specific systems as described below.

RT-qPCR Design

As previously described [24], cDNA was used for each 10 μL reaction together with 2× BioRad SSO Advnaced Universal SYBR Green Supermix (Cat #1725274, Hercules, CA, USA) which included buffer, Taq DNA polymerase, dNTPs and SYBR green dye. Specific primers (forward and reverse (Table 2) and cDNA or water (for no template control) were added to each PCR reaction. The specific primers used can be seen in Table 2. For each gene, the optimal MgCl2 concentration produced the amplification plot with the lowest cycle product (Cp), the highest fluorescence intensity and the steepest amplification slope. Master

mix (8 µL) was pipetted into the 96-well plate and 2 µL cDNA was added as PCR template. Each run contained 7 standard curve points in duplicate. A no template control of nuclease-free water was included to exclude DNA contamination in the PCR mix. The double stranded DNA was amplified in the Bio-Rad CFX96 Touch (Hercules, CA, USA) using the following PCR conditions: initial denaturing at 95 ◦C for 30 s, 40 cycles of denaturing at 95 ◦C for 15 s, various annealing temperatures according to Integrated DNA Technologies (IDT) for 30 s and elongating at 60 ◦C for 30 s. The data on the expression levels of the genes were obtained as Cp values based on the "second derivative maximum" (=automated method) as computed by the software. For each of the 12 genes, the reactions were run in duplicate. All assays were quantified by including a standard curve in the real-time qPCR analysis. The next four points of the standard curve were prepared by a 1:10 dilution. Each point of the standard curve was included in duplicate. A graph of Cp vs. log 10 concentrations was produced by the software and the efficiencies were calculated as 10[1/slope]. The specificity of the amplified real-time RT-PCR products were verified by melting curve analysis (60–95 ◦C) after 40 cycles, which should result in a number of different specific products, each with a specific melting temperature. In addition, we electrophoresed the resulting PCR products on a 2%-agarose gel, stained the gel with ethidium bromide, and visualized it under UV light. PCR-positive products were purified of primer dimers and other non-specific amplification by-products using QIAquick Gel Kit (Qiagen Inc., Valencia, CA, USA) prior to sequencing. We sequenced the products using BigDye®Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) and ABI Automated 3430xl DNA Analyzer (Applied Biosystems) and analyzed them with Sequencing Analysis ver. 5.2 (Applied Biosystems). We aligned sequences of hepcidin with those from related organisms obtained from Gen Bank using a

basic alignment-search tool (BLAST; National Center for Biotechnology Information, Bethesda, MD, USA). Sequence alignments were performed for all samples. The ClustalW program was used for sequence alignment.

Table 2: Primers used in the study

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

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

Statistical analysis

The bean flours were analyzed in triplicates. Polyphenol and iron composition data was subjected to analysis of variance (ANOVA), and the post hoc Tukey test was used to compare the groups. Experimental treatments for the in ovo assay were arranged in a completely randomized design. The microbial and gene expression results were analyzed by ANOVA. For significant "F-value", post hoc Duncan test was used to compare test groups. Statistical analysis was carried out using GraphPad Prism version 5.0 software (GraphPad Software, California, CA, USA). Statistical significance was set at $p < 0.05$.

CHAPTER 3

RESULTS

Analysis of polyphenols in beans

The results of the polyphenol anaylsis are shown in Table 3. Carioca beans (BRS Perola and BRS Cometa) have a significantly higher concentration of kaempferol 3-glucoside, catechin and epicatechin, all of which have been shown to promote iron uptake[25]. On the other hand, black beans (BRS Esteio and SMN 39) seem to have a significantly higher concentration of myricetin 3-glucoside and quercetin 3-glucoside which are known inhibitors of iron absorption[12,13].

Body weight and cecum-to-body weight ratio

There is no significant difference in the body weight between the treatment groups (Figure 1) but there is a significant increase in cecum-to-body weight ratio between the groups treated with inulin, prebiotic extracts and the negative controls. The increase in cecum weight in the prebiotic treated groups indicates the increase in the microbial content in the gut.

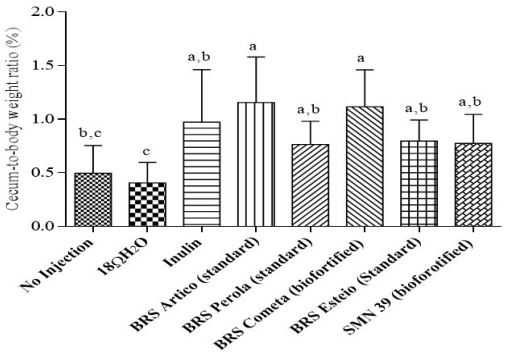

Figure 1:Cecum-to-body weight ratio (%). Values are means ± SEM, n = 12. a-c Per bacterial category, treatment groups not indicated by the same letter are significantly different ($p<0.05$).

Table 3: Characterization of polyphenols in beans

	Kaempferol 3-glucoside	**Catechin**	**Epicatechin**	**Procyanidin B1**	**Myricetin 3-glucoside**	**Quercetin 3-glucoside**	**Quercetin**
Carioca beans							
BRS Perola (standard)	17.3 ± 1^{a}	26.1 ± 1.3^{a}	12.8 ± 1.7^{a}	1.4 ± 0.2^{b}	-	0.2 ± 0.1^{c}	-
BRS Cometa (Biofortified)	16.2 ± 1.1^{b}	25.9 ± 4.6^{a}	11 ± 1.4^{b}	1.2 ± 0.2^{b}	-	-	-
Black beans							
BRS Esteio (standard)	2.4 ± 0.4^{c}	14.7 ± 2.7^{b}	6.9 ± 0.6^{c}	0.7 ± 0.2^{c}	3.9 ± 0.3^{a}	2.0 ± 0.2^{a}	-
SMN39 (biofortified)	1.5 ± 0.2^{d}	13 ± 1.4^{b}	2.7 ± 0.7^{d}	3.0 ± 0.2^{a}	1 ± 0.1^{a}	0.9 ± 0.1^{b}	0.07 ± 0.01
White bean							
BRS Artico	0.8 ± 0.3^{d}	-	-		-	-	-

Data presented as mean ± SD. Means with different letters in the same column present significant difference ($p < 0.05$).

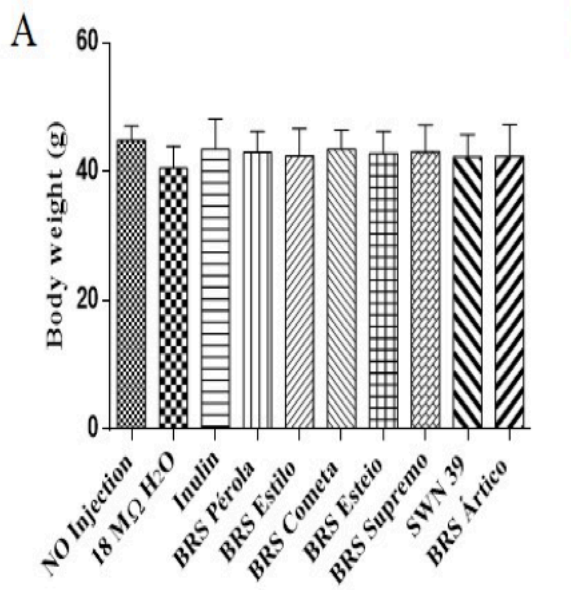

Figure 2: Body weights of chicken treated with prebiotic extracts of the bean varieties tested. The body weights were measured on the day of hatch before euthanizing the birds. There is no significant difference in the body weights.

Microbial analyses of the cecum

Inulin, the positive control, has increased the relative abundance of all the genera tested. There is no significant difference in the relative abundance of *Bifidobacterium* and *Lactobacillus* between the test groups and the negative controls (except BRS Esteio and BRS Artico). All the treated groups seem to lower the levels of *E.coli* and when compared to the positive control inulin (except SMN 39 whose *Clostridium* levels are comparable to that of inulin)(Figure 3).

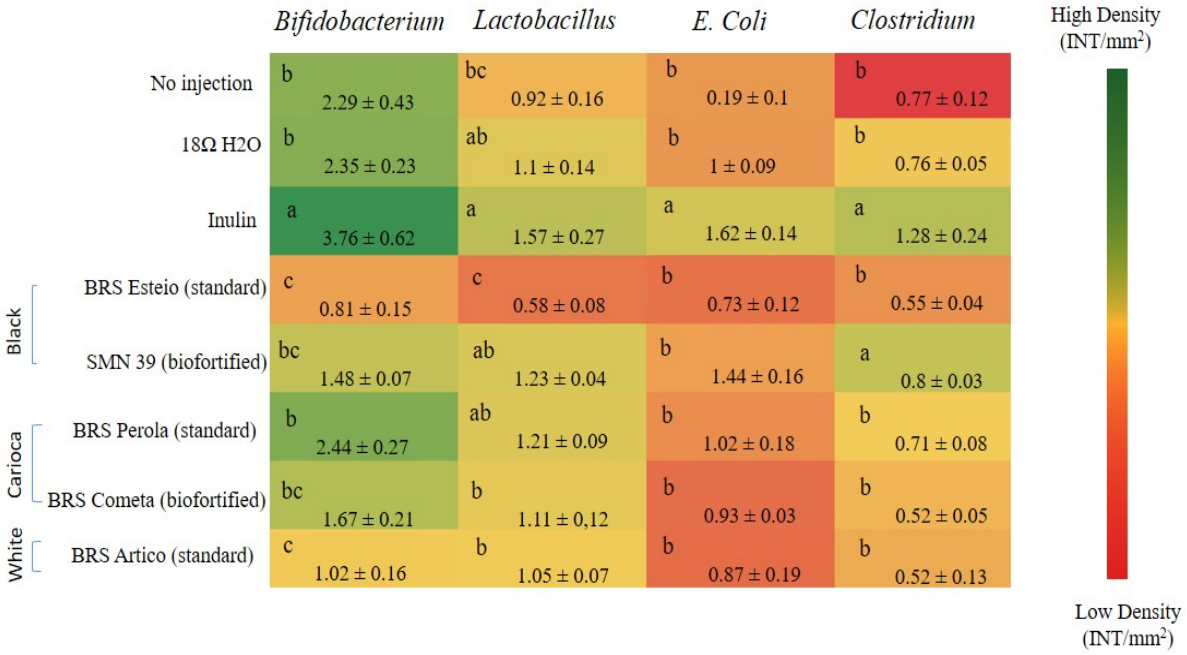

Figure 3:Genera and species-level bacterial populations (AU) from cecal contents measured on the day of hatch. Values are means ± SEM, n = 6. a-c Per bacterial category, treatment groups not indicated by the same letter are significantly different ($p<0.05$)

Influence of bean prebiotics on the iron status

While BRS Artico, had a significantly high iron concentration in the extract, it was not reflected in the serum or liver iron concentrations. There was no difference in the iron concentration in the serum or liver among the different treatment groups and the controls.

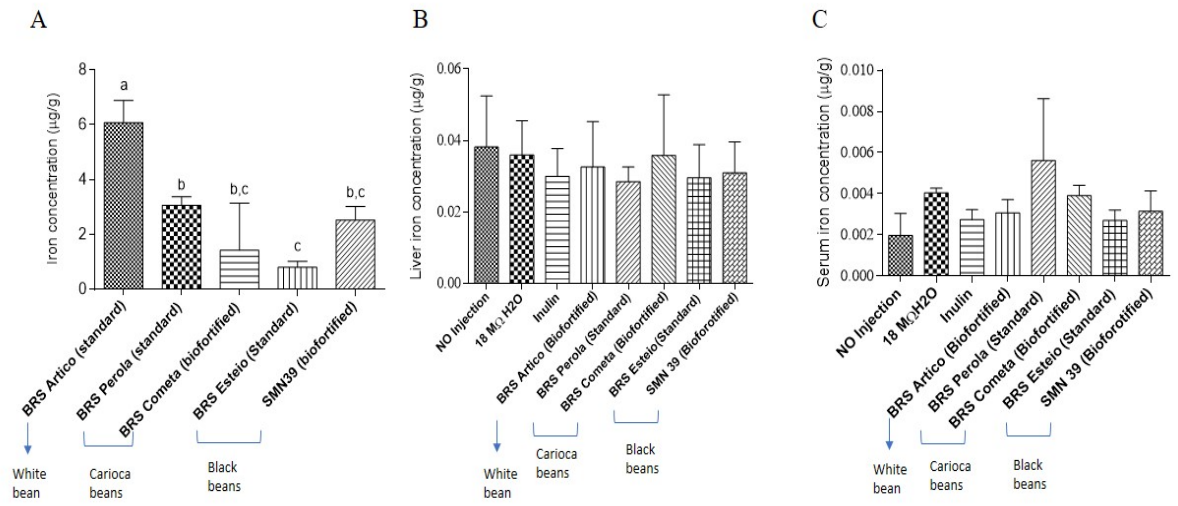

Figure 4:Figure 4. (A): Iron content in the bean prebiotics extracts; (B) Liver iron concentration (n=6); (C) Serum iron concentration (n=4). Values are means ± SEM. Different letters indicate statistical differences at $p<0.05$

Expression of genes related to iron metabolism in the small intestine

The relative expression of ZnT1, DCytB, Ferroportin and AP are upregulated in subjects that received BRS Cometa (a carioca bean) prebiotic extract when compared to the BRS Artico prebiotic treatment. The expression of ZnT1 and Ferroportin in the black bean SMN 39 is reduced when compared to the carioca bean BRS Cometa. Hepcidin is a peptide hormone that is capable of interfering with the iron absorption[26,27]. Hence, the expression of hepcidin in the liver was analysed. BRS Cometa had a significantly reduced expression of the gene whereas BRS Esteio, a black bean had upregulated the expression of this gene. There was no difference in the expression of DMT1, SI and SGLT1 among the treatment groups.

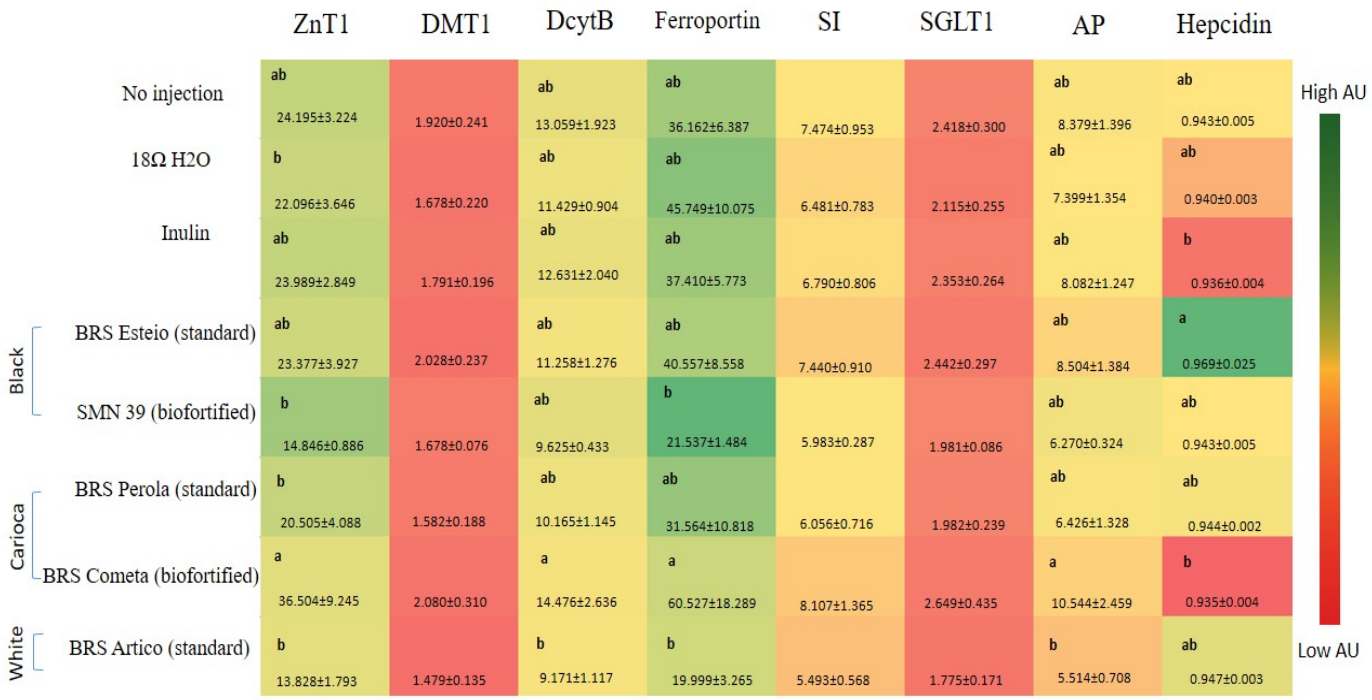

Figure 5: Effect of intra-amniotic administration of experimental solutions on the intestinal gene expression. Values are means ± SEM, n = 5. [a-c] Per gene, treatment groups not indicated by the same letter are significantly different ($p<0.05$). ZnT-1: Zinc transporter protein-1; DMT-1, Divalent Metal Transporter–1; DcytB, Duodenal cytochrome b; SI, Sucrose isomaltase; SGLT-1: Sodium-Glucose transport protein 1; AP: Amino peptidase.

CHAPTER 4

DISCUSSION

This study aimed at assessing the influence of prebiotic extracts from three different bean varieties – black, carioca and white beans with standard and biofortified lines, on the microbial content of the intestine and on the expression of genes related to iron absorption and BBM functionality.

This study tested for four different bacterial genera to assess the gut health. *Bifidobacterium* and *Lactobacilli* are known probiotics. *Clostridium* is pathogenic and *E.coli* can be beneficial or pathogenic depending on the strain[28,29]. Fig 1 suggests that the cecum to body weight ratio in all the treated groups have increased. This indicates that the prebiotic extracts played a role in increasing the microbial content of the cecum. This was followed by analysis of the 16S rDNA to analyze the relative abundance of the different genera. The treatment groups treated with the biofortified lines SMN 39 and BRS Cometa and the standard variety in BRS Perola showed a significantly high relative abundance of *Bifidobacterium* relative to the two standard lines in BRS Esteio (black bean) and BRS Artico (white bean). The relative abundance of *Lactobacilli* was also significantly high in the biofortified SMN 39 relative to the standard BRS Esteio. Also, all the treatment groups reduced the relative abundance of *Clostridium* and *E.coli* relative to the positive control inulin (Fig 3). These results show that prebiotic extracts from beans have the potential to improve gut health by increasing the relative abundance of beneficial bacteria over potentially harmful bacteria. This improved gut health can also aid in improving the absorption of iron, thus increasing the bioavailability of iron. *Bifidobacterium* and *Lactobacilli* produce short chain fatty acids which reduce the intestinal pH and this can improve iron absorption[30,34]. There have been studies that indicate that iron supplementation and fortification

can adversely affect the gut health by promoting pathogenic enteric bacteria[31,32]. But iron biofortification of beans, in addition to having the potential to combat iron deficiency, also seems to improve gut health.

The expression of genes associated with iron metabolism and BBM functionality in the small intestine were also studied. The groups treated with prebiotic extracts from BRS Cometa (carioca) had a significant up-regulation of ZnT1 and AP relative to other groups, however, those that received the prebiotic extract from BRS Artico had a downregulation of these genes (Fig 5). This suggests that BRS Cometa prebiotic extract increased BBM functionality. This is consistent with previous study that suggests that dietary prebiotics can have a positive influence on BBM functionality and intestinal cell proliferation[33].

Iron consumed orally enters the bloodstream where DCytB reduces Fe^{3+} to Fe^{2+}. This facilitates DMT-1 to transport the resulting iron from the lumen of the intestine to the intestinal cells and ferroportin is responsible for transporting iron from the enterocytes into the hepatic portal vein[34,35]. In this study, BRS Cometa (carioca) upregulated the expression of Ferroportin and DCytB. This suggests that more iron can be absorbed and transported into the bloodstream. But there was no significant difference in the iron content in the liver or in the serum. This may be due to the short time period of this study and validates the need for a long-term feeding trial to study the effects on the iron status.

Polyphenol analysis of the beans (Table 3) shows that carioca beans are rich in compounds like kaempferol 3-glucoside, catechin and epicatechin which are known to promote the absorption of iron. However, black beans are rich in compounds like myrcetin 3-glucoside and quercetin 3-glucoside which inhibit iron absorption[36]. These findings further suggest that carioca beans are better vehicles for mineral biofortification.

Iron uptake is also regulated by the peptide hormone, hepcidin. Hepcidin negatively regulates iron uptake as it is capable of binding to ferroportin and initiating the degradation of ferroportin, which is the only known iron exporter, thereby limiting the entry of iron into the plasma[27,37]. Hence, the expression of hepcidin in liver was also analysed. The expression of hepcidin was significantly higher in groups treated with BRS Esteio (black bean) relative to those treated with BRS Cometa (carioca bean) (Fig 5). However, the effect of this difference was not seen in ferroportin expression or in the serum iron concentration (Fig 4).

CHAPTER 5

CONCLUSION

The data suggest that carioca beans can inhibit *E.coli* and *Clostridium*, while promoting the beneficial bacteria, *Bifidobacterium* and *Lactobacillus*, thereby improving iron bioavailability. Also, carioca beans upregulated ZnT1, AP, ferroportin and DcytB, which in turn can improve iron bioavailability. This in addition to the fact that carioca beans have significantly less iron inhibitory polyphenols (myrcetin 3-glucoside, quercetin 3-glucoside) and significantly high iron enriching polyphenols (kaempferol 3-glucoside, catechin, epicatechin) than black beans make carioca better varieties for iron biofortification. While this short-term study provides no evidence of improvement in iron status, the microbial analyses and gene expression studies are promising and validate the need for a long term feeding study.

REFERENCES

1. WHO | Overview. *WHO* Available at: http://www.who.int/whr/2002/overview/en/. (Accessed: 11th January 2018)
2. WHO. The global prevalence of anemia in 2011. Geneva: World Health Organization; 2015.
3. Bouis, H. E. Plant Breeding: A New Tool for Fighting Micronutrient Malnutrition. *J. Nutr.* **132,** 491S-494S (2002).
4. Nestel, P., Bouis, H. E., Meenakshi, J. V. & Pfeiffer, W. Biofortification of Staple Food Crops. *J. Nutr.* **136,** 1064–1067 (2006).
5. Petry, N., Boy, E., Wirth, J. P. & Hurrell, R. F. Review: The Potential of the Common Bean (Phaseolus vulgaris) as a Vehicle for Iron Biofortification. *Nutrients* **7,** 1144–1173 (2015).
6. Luque, R. D., José, J. & Creamer, B. Major constraints and trends for common bean production and commercialization; establishing priorities for future research = Principales restricciones y tendencias en la producción y comercialización de fríjol común; estableciendo prioridades de investigación. *Agron. Colomb.* (2014). doi:https://dx.doi.org/10.15446/agron.colomb.v32n3.46052
7. Tako, E., Beebe, S. E., Reed, S., Hart, J. J. & Glahn, R. P. Polyphenolic compounds appear to limit the nutritional benefit of biofortified higher iron black bean (Phaseolus vulgarisL.). *Nutr. J.* **13,** 28 (2014).
8. Petry, N. *et al.* Phytic Acid Concentration Influences Iron Bioavailability from Biofortified Beans in Rwandese Women with Low Iron Status,2. *J. Nutr.* **144,** 1681–1687 (2014).
9. Pacifici, S. *et al.* Intra Amniotic Administration of Raffinose and Stachyose Affects the Intestinal Brush Border Functionality and Alters Gut Microflora Populations. *Nutrients* **9,** (2017).

10. Tako, E., Glahn, R. P., Knez, M. & Stangoulis, J. C. The effect of wheat prebiotics on the gut bacterial population and iron status of iron deficient broiler chickens. *Nutr. J.* **13,** 58 (2014).
11. Hou, T. & Tako, E. The In Ovo Feeding Administration (Gallus Gallus)—An Emerging In Vivo Approach to Assess Bioactive Compounds with Potential Nutritional Benefits. *Nutrients* **10,** 418 (2018).
12. Scholz-Ahrens, K. E. *et al.* Prebiotics, Probiotics, and Synbiotics Affect Mineral Absorption, Bone Mineral Content, and Bone Structure. *J. Nutr.* **137,** 838S-846S (2007).
13. Su, P., Henriksson, A. & Mitchell, H. Selected prebiotics support the growth of probiotic mono-cultures in vitro. *Anaerobe* **13,** 134–139 (2007).
14. Macfarlane, G. T. & Cummings, J. H. Probiotics and prebiotics: can regulating the activities of intestinal bacteria benefit health? *BMJ* **318,** 999–1003 (1999).
15. Prebiotics: Metabolism and Symbiotic Synergy with Probiotics in Promoting Health | IntechOpen. Available at: /books/probiotics-and-prebiotics-in-human-nutrition-and-health/prebiotics-metabolism-and-symbiotic-synergy-with-probiotics-in-promoting-health. (Accessed: 26th April 2018)
16. Tako, E., Rutzke, M. A. & Glahn, R. P. Using the domestic chicken (Gallus gallus) as an in vivo model for iron bioavailability. *Poult. Sci.* **89,** 514–521 (2010).
17. Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A. & Gordon, J. I. Host-Bacterial Mutualism in the Human Intestine. *Science* **307,** 1915–1920 (2005).
18. Tako, E. & Glahn, R. P. Intra-amniotic administration and dietary inulin affect the iron status and intestinal functionality of iron-deficient broiler chickens. *Poult. Sci.* **91,** 1361–1370 (2012).

19. Vidanarachchi, J. K., Iji, P. A., Mikkelsen, L. L., Sims, I. & Choct, M. Isolation and characterization of water-soluble prebiotic compounds from Australian and New Zealand plants. *Carbohydr. Polym.* **77,** 670–676 (2009).
20. Tako, E., Glahn, R. P., Knez, M. & Stangoulis, J. C. The effect of wheat prebiotics on the gut bacterial population and iron status of iron deficient broiler chickens. *Nutr. J.* **13,** 58 (2014).
21. Hou, T., Kolba, N., Glahn, R. & Tako, E. Intra-Amniotic Administration (Gallus gallus) of Cicer arietinum and Lens culinaris Prebiotics Extracts and Duck Egg White Peptides Affects Calcium Status and Intestinal Functionality. *Nutrients* **9,** 785 (2017).
22. Hartono, K., Reed, S., Ankrah, N. A., Glahn, R. P. & Tako, E. Alterations in gut microflora populations and brush border functionality following intra-amniotic daidzein administration. *RSC Adv* **5,** 6407–6412 (2015).
23. Zhu, X. Y., Zhong, T., Pandya, Y. & Joerger, R. D. 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. *Appl. Environ. Microbiol.* **68,** 124–137 (2002).
24. Pacifici, S. *et al.* Intra amniotic administration of raffinose and stachyose affects the intestinal brush border functionality and alters gut microflora populations. *Nutrients* **9,** (2017).
25. Identification of Black Bean (Phaseolus vulgaris L.) Polyphenols That.pdf.
26. Przybyszewska, J. & Żekanowska, E. The role of hepcidin, ferroportin, HCP1, and DMT1 protein in iron absorption in the human digestive tract. *Przegląd Gastroenterol.* **9,** 208–213 (2014).
27. Nemeth, E. & Ganz, T. The Role of Hepcidin in Iron Metabolism. *Acta Haematol.* **122,** 78–86 (2009).

28. Gibson, G. R., Beatty, E. R., Wang, X. & Cummings, J. H. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. *Gastroenterology* **108,** 975–982 (1995).

29. Roberfroid, M. B., Van Loo, J. A. & Gibson, G. R. The bifidogenic nature of chicory inulin and its hydrolysis products. *J. Nutr.* **128,** 11–19 (1998).

30. Welch, R. M. & Graham, R. D. Breeding for micronutrients in staple food crops from a human nutrition perspective. *J. Exp. Bot.* **55,** 353–364 (2004).

31. Kortman, G. A. M., Boleij, A., Swinkels, D. W. & Tjalsma, H. Iron Availability Increases the Pathogenic Potential of Salmonella Typhimurium and Other Enteric Pathogens at the Intestinal Epithelial Interface. *PLoS ONE* **7,** (2012).

32. Jaeggi, T. *et al.* Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants. *Gut* **64,** 731–742 (2015).

33. Hou, T., Kolba, N., Glahn, R. P. & Tako, E. Intra-Amniotic Administration (Gallus gallus) of Cicer arietinum and Lens culinaris Prebiotics Extracts and Duck Egg White Peptides Affects Calcium Status and Intestinal Functionality. *Nutrients* **9,** (2017).

34. Eisenstein, R. S. Iron regulatory proteins and the molecular control of mammalian iron metabolism. *Annu. Rev. Nutr.* **20,** 627–662 (2000).

35. Ganz, T. & Nemeth, E. Iron homeostasis in host defence and inflammation. *Nat. Rev. Immunol.* **15,** 500–510 (2015).

36. Hart, J. J. & Glahn, R. Identification of bean polyphenols that inhibit and enhance iron uptake by Caco-2 cells. *FASEB J.* **27,** 634.13-634.13 (2013).

37. Blanchette, N. L., Manz, D. H., Torti, F. M. & Torti, S. V. Modulation of hepcidin to treat iron deregulation: potential clinical applications. *Expert Rev. Hematol.* **9,** 169–186 (2016).