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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) 18 MΩ 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

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 com-

pounds 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 [22–24].

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 [26,28–30]. 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 [23,31–33]. 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 [34–37].

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 ultrapure 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 (HPLC-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 (CMS; 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 LCMS, 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) 18 MΩ 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.

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-κB1	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, Aminopeptidase; SI, Sucrose 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.

2.7 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 [44–46].

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.

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 $\times$ 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 MgCl2 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

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

	BRS Pajeú	BRS Aracê	BRS Imponente	BRS Xiquexique
TDF (g/100 g)	19.02 ± 0.24^{a}	13.82 ± 0.08^{c}	11.65 ± 0.23^{d}	15.10 ± 0.04^{b}
SDF (g/100 g)	1.59 ± 0.19^{a}	1.07 ± 0.21^{a}	1.16 ± 0.44^{a}	0.91 ± 0.06^{a}
IDF (g/100 g)	17.43 ± 0.05^{a}	12.75 ± 0.29^{c}	10.50 ± 0.67^{d}	14.19 ± 0.02^{b}
Protein (g/100 g)	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/100 g)	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.

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 cow-

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 $\pm$ SEM (n = 5). Means sharing different letters 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.

pea 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).

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).

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, 18 MΩ 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, 18 MΩ 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 18 MΩ 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, 18 MΩ 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).

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), sucrose 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 18 MΩ 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 18 MΩ 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.

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 admin-

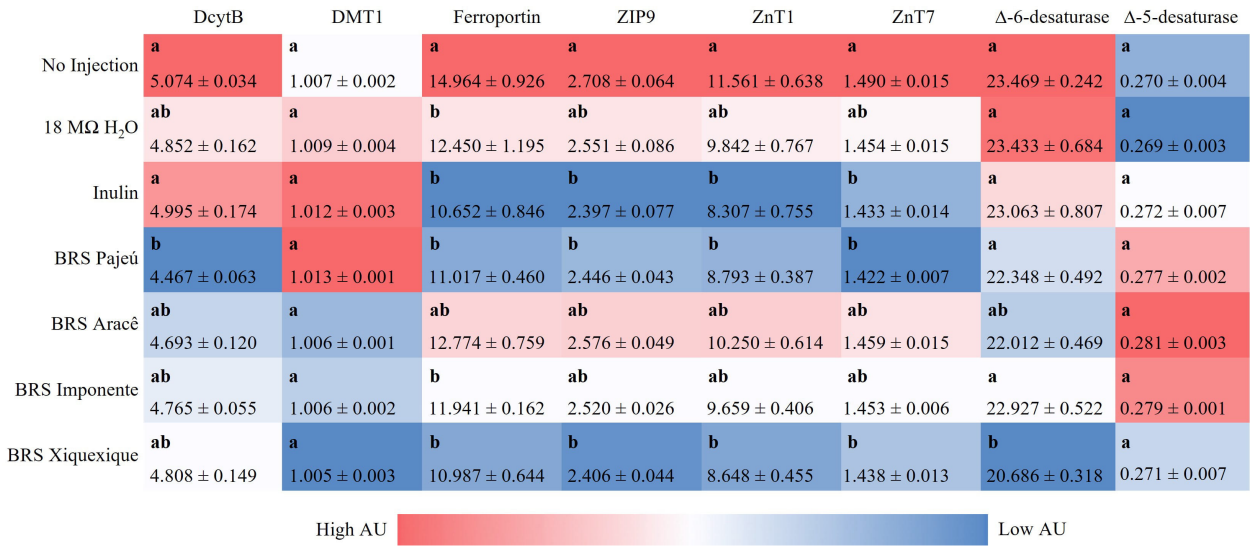

Fig. 1. Effect of the intra-amniotic administration of cowpea soluble extracts on gene expression of proteins involved with Zn and Fe metabolism. Values are the means ± SEM, n = 5. [a-b] Per gene, treatments groups not indicated by the same letter are significantly different ($p < 0.05$). Dcytb, Duodenal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

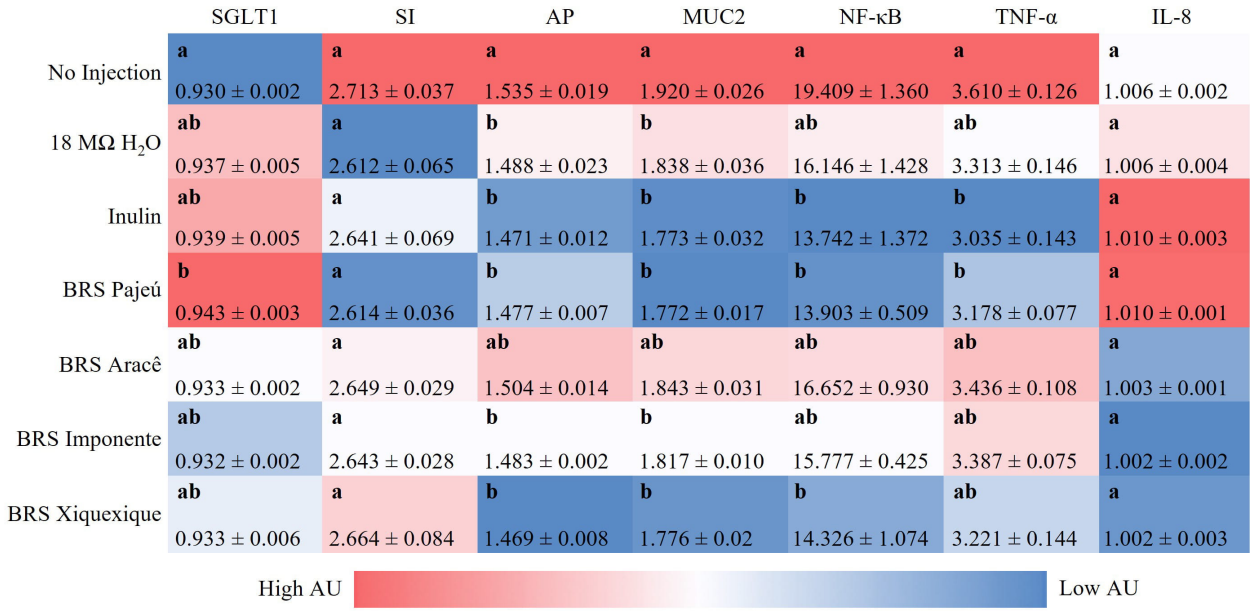

Fig. 2. Effect of the intra-amniotic administration of cowpea soluble extracts on gene expression of proteins involved with the BBM functionality and inflammation. Values are the means ± SEM, n = 5. [a-b] Per gene, treatments groups not indicated by the same letter are significantly different ($p < 0.05$).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

istration 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).

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 letters in each column are significantly different ($p \leq 0.05$) by *post-hoc* of Duncan test.

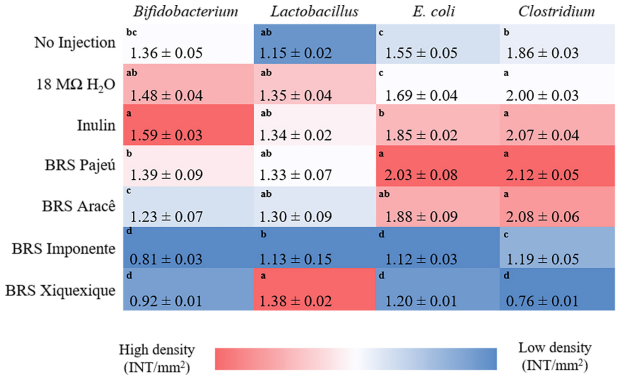

Fig. 3. Effect of the intra-amniotic administration of Zn biofortified cowpea soluble extract on genera- and species-level bacterial populations from cecal contents measured on the day of hatch. Values are the means ± SEM, n = 8. Means sharing different letters in each column are significantly different ($p \leq 0.05$) 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 18 MΩ 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 18 MΩ 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 18 MΩ 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 18 MΩ 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).

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 18 MΩ 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).

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

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}
18 MΩ 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 ^{c}	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 letters in each column are significantly different ($p \leq 0.05$) by *post-hoc* of Duncan test.

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 18 MΩ H_2O control groups. The Xiquexique group was also associated with increased villus surface area and crypt acidic goblet cell number compared to the 18 MΩ 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 18 MΩ 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, 18 MΩ 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 18 MΩ 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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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2705140.

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