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Daidzein is an isoflavone found primarily in soybean and various soy-based products such as tofu. In the intestines, daidzein is reductively transformed to its constituent metabolites equol and *O*-desmethylangolensin. Although the ingestion of daidzein has been associated with marked physiological improvements in various pathological states, namely cancer, metabolic syndrome, and cardiovascular disease, further understanding into the tissue-level effects of daidzein ingestion is needed. In this study, broiler chickens (*Gallus gallus,* n = 50,) were injected *in ovo* (day 17 of embryonic incubation) with varying concentrations of a 1.0mL pure daidzein in saline solution. Three treatment groups (0.5, 2.5, 5.0 mg/mL) and two controls (saline and non-injected) were utilized. Upon hatch, blood was taken for hemoglobin and total body iron hemoglobin determination, and chicks were then euthanized. Hepatic, duodenal, and caecal tissues were excised for ferritin analysis, mRNA gene expression of relevant brush border membrane and iron transporters/ proteins, and PCR quantification of three bacterial genera (*Bifidobacterium*, *Lactobacillus*, and *Clostridium*) and one bacterial species (*E. coli)*. Results revealed no significant differences in hemoglobin, total body hemoglobin iron, or ferritin between groups (p>0.05). Aminopeptidase and Na^+K^+ATPase were upregulated in daidzein-treated groups when compared to controls (p<0.05). Additionally, daidzein administration increased the expression of Dcyt B, an iron-specific cytochrome reductase (p<0.05). Further, daidzein injection resulted in the increased caecal abundance of *E. coli* in the 2.5 mg/mL group (p<0.05). Taken together, these results suggest a physiological role for daidzein administration in improving the functionality and development of the brush border membrane, as well for influencing the abundance of certain bacterial communities in the colon.

Abbreviations Daidzein; DE, Estrogen receptor-beta; ER-β, Equol; EQ, *O*-desmethylangolensin; *O*-DMA, Germ free; GF, Brush border membrane; BBM, Aminopeptidase; AP, Sucrase isomaltase; SI, Na^+K^+ATPase; Sodium Potassium ATPase, SGLT-1; Sodium glucose transporter-1, Iron; Fe, Hemoglobin; Hb, Total body iron hemoglobin; Hb-Fe, Hemoglobin; Hb, Hb-Fe; Total body hemoglobin Fe, Arbitrary Unit; AU, Operational taxonomic unit; OTU, Short chain fatty acids; SCFAs

1. Introduction

Daidzein (DE), an isoflavone especially concentrated in soybean, has shown to possess a variety of protective physiological properties. Such effects have been observed in a variety of *in vivo* models of cardiovascular diseases, metabolic syndrome and diabetes, osteoporosis, and certain cancers.[1,2] The pharmacological effects of isoflavone ingestion have been largely attributed to the structural similarity with steroid compounds, particularly the estrogens, as well as their potent binding to ER-β.[3]

Despite the well-studied physiological effects of DE and other isoflavones, characterization of the absorption and metabolism of DE in humans is still ongoing. Current research has shown that the resident intestinal microbiota possess glucosidase activity,[4] and appear to be responsible for reductively metabolizing DE to its metabolites equol (EQ) and *O*-desmethylangolensin (*O*-DMA).[5,6] Both metabolites possess greater biological activity than that of DE,[7] yet this activity is notably dissimilar between the two.[8] Further, after biotransformation, *O*-DMA production is greater than that of EQ.[9]

Studies in GF rats display a total lack of production of either EQ and *O*-DMA,[10,11] signifying a vital role for the gut microbiota in DE metabolism. In fact, *Eubacterim ramulus*, *Escherichia coli*, and certain species of *Clostridium*, *Bifidobacteria*, *Bacteroides*, and *Ruminococcus* have been implicated in DE metabolism.[12-15] However, specific microbes responsible for the metabolism of DE have yet to be elucidated.[8]

Despite the investigation of specific health benefits attributable to DE ingestion, and subsequent knowledge of DE administration on the gut microbiome composition, there is a paucity of understanding in regards to how DE affects the functionality of the small intestine, specifically the brush border membrane (BBM). The functional capacity (e.g., digestive enzyme production) of the BBM dictates the extent of food hydrolysis and nutrient uptake.[16,17] Hence, it remains a priority to investigate the interactions between bioactive compounds in the diet, such as DE, and the BBM.

Therefore, a major aim of the study was to assess the effects of intra-amniotic daidzein administration on the BBM development and functionality in *Gallus gallus*, a novel animal system used to model the physiological effects of various nutritional solutions relevant to human nutrition.[20-22] To this end, gene expression of brush border digestive enzymes, such as AP, SI, ion antiporter ATPase, and glucose transporter SGLT-1, were measured.

A secondary aim was to further evaluate BBM functionality in the context of dietary Fe absorption. Fe deficiency is the most prevalent micronutrient deficiency affecting nearly 40% of the world's population.[18] A major strategy to combat Fe deficiency has been to increase the bioavailability of Fe in the diet, accomplished via increased Fe concentration, decreased Fe inhibitors (e.g., phytate, polyphenols), improved BBM function (e.g., increased enzyme output, activity, expression), and/or modulation of the gut microbiome to favor mineral uptake.[19] To accomplish this objective, we assessed Hb, Hb-Fe, liver ferritin concentrations, as well as the expression of DMT-1, the primary transporter of Fe^{2+} from the luminal side of the enterocyte, ferroportin, a basolateral exporter of dietary Fe^{2+}, and Dcyt B, a Fe-specific cytochrome reductase on the luminal side of the enterocyte. In addition, the immunoregulatory cytokines TNF-α and IL-6, as well as the transcription factor NF-κB, were measured to assess any potential modulation of systemic inflammation by DE. Relative genus-level changes (*Lactobacillus, Clostridium*, *Bifidobacterium*), as well as species-level changes (*Escherichia coli*) in the caecal content were also quantified to assess the effect of DE administration on the abundance of these microbes.

2. Methods and materials

2.1 Birds, diets, and study design

Cornish-cross fertile broiler eggs (n = 96) were obtained from a commercial hatchery from a maternal flock 35wk in lay. The eggs were incubated under optimal conditions at the Cornell University Animal Science Poultry Farm Incubator.

Analyte	Organ	Forward Primer (5'→3')	Reverse Primer (5'→3')	Length (base pairs)	GI Identifier
TNF-α	Liver	CATTTGGAAGCAGCGTTCGG	GAAGGGTAGGGGTGAGGAT	202	53854909
IL-6	Liver	AACAACCTCAACCTGCCCAA	AGGTCTGAAAGGCGAACAGG	112	302315692
NF-κB	Liver	GGATGGTCTGTTCCTGAAGA	ACCTCTGCCTGCTTTGTGAT	300	2130627
AP	Intestine	GAATGAGGGCTTTGCCTCCT	GAAGTTGCTGTTGGTGGCTG	610	45382360
SI	Intestine	CAGATCTCAGCCCGTCTTCC	CCAGAATGCCACCGGTAACT	282	2246388
ATPase	Intestine	CTGAGGGCAACGAAACAGTG	ATCCCTCGGGTTGACCTCC	74	14330321
SGLT-1	Intestine	GTGGGAATGCCTTGGAGGGTA	GCTTCCTCAGATACTCCGGCC	121	8346783
DMT-1	Intestine	TTCCTCCTCAACAACGTCGG	TCCCAATGCCATCCCAGTTC	154	206597489
Dcty B	Intestine	GGCCG GTTTGAGAACCACAATGTT	CGTTTGCAATCACGTTTCCAAAGAT	214	20380692
Ferroportin	Intestine	GATGCATTCTGAACAACCAAGGA	GGAGACTGGGTGGACAAGAACTC	250	61098365
18S rRNA	Liver, Intestine	CGATGCTCTTAACTGAGT	GGAGACTGGGTGGACAAGAACTC	300	7262899

Table 1 Measured genes (from *Gallus gallus*) and tissue-specific rRNA from mRNA.

2.2 Intra-amniotic administration

As previously described,[8, 20-22] on day 17 of embryonic incubation, eggs containing viable embryos (n = 50) were weighed and divided into 5 groups (n = 10). All treatment groups were assigned eggs of similar weight-frequency distribution. The intra-amniotic treatment solution (1mL per egg) was injected with a 21-gauge needle into the amniotic fluid,[20] and included selected concentrations of daidzein in saline at 0.5mg/mL, 2.5 mg/mL and 5mg/mL. Saline and non-injected (N-i) groups served as the controls. Eggs were placed in hatching baskets such that each treatment was equally represented at each incubator location. Hatchability was similar in all treatment groups and was approximately 85%.

Immediately after hatch (day 21), all chicks were euthanized by CO_2 exposure. The digestive tracts (duodenum and cecum) and liver were quickly removed from the carcass and separated into various sections for tissue analysis (~1-2 cm; 2-3 g was taken from small intestine and liver, respectively). The samples were immediately frozen in liquid nitrogen, and then stored in a −80°C freezer until analysis. All animal protocols were approved by Cornell University Institutional Animal Care and Use committee.

2.3 Blood analysis and Hb measurements

Blood was collected using micro-hematocrit heparinized capillary tubes (Fisher Scientific, Waltham, MA) immediately after hatch but before euthanization. Blood Hb concentrations were determined spectrophotometrically using the cyanomethomoglobin method (H7506-STD, Pointe Scientific Inc., Canton, MI,) following the kit manufacturer's instructions.

As a marker of Fe absorption, Hb-Fe was also calculated as previously described.[23]

2.3 Isolation of total RNA

Total RNA was extracted from 30 mg of duodenal (proximal duodenum, n = 10) and liver tissues (n = 10) using Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. All steps were carried out under RNase free conditions. RNA was quantified by absorbance at 260-280 nm. Integrity of the 28S and 18S rRNA was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining.

2.4 Gene expression analysis

As previously described,[16,23,24] RT-PCR was carried out with primers chosen from the

fragments of chicken duodenal and hepatic tissues. Highly conserved tissue-specific 18S rRNA was used as internal standard to normalize the results. All PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and quantified using the Quantity One 1-D analysis software (Bio-Rad, Hercules, CA). Table 1 represents the totality of genes assessed in this study.

2.5 Liver Ferritin

Liver samples were treated as described previously.[25,26] Briefly, the frozen tissue samples were thawed on ice for approximately 30 min. One gram of sample was diluted into 1 mL of 50 mM Hepes buffer, pH 7.4, and homogenized on ice using an Ultra-Turex homogenizer at maximum speed (5,000 × g) for 2 min. One milliliter of each homogenate was subjected to heat treatment for 10 min at 75°C to aid isolation of ferritin, since other proteins are not stable at that temperature.[25,27] After heat treatment, the samples were immediately cooled on ice for 30 min. Thereafter, samples were centrifuged at 13,000 × g for 30 min at 4°C until a clear supernatant was obtained and the pellet containing most of the insoluble denatured proteins was discarded. All tests were conducted in duplicates for each animal (n = 10).

2.6 Electrophoresis, staining, and measurement of the gels

Native PAGE was conducted using a 6% separating gel and a 5% stacking gel. Samples were run at a constant voltage of 100 V.[25] After electrophoresis, the gels were treated with either of the 2 stains as described by *Leong, et al.*[28]: Coomasie blue G-250 stain, specific for proteins, or potassium ferricyanide [$K_3Fe(CN)_6$] stain, specific for iron. The corresponding band found in the protein and iron-stained gel was considered to be ferritin.

The gels were scanned with a Bio-Rad densitometer. Measurement of the bands was conducted using the Quantity-One 1-D analysis program (Bio-Rad Inc., Hercules, CA). The local background was subtracted from each sample. Horse spleen ferritin (Sigma Aldrich Co., St. Louis, MO) was used as a standard for calibrating ferritin protein and iron concentrations of the samples. [25-27]

2.8 Collection of microbial samples and intestinal contents DNA isolation

The ceca were sterilely removed and treated as described previously.[29,30] The contents of the ceca were placed into a sterile 50mL tube containing 9mL of sterile PBS and homogenized by vortexing with glass beads (3mm diameter) for 3 min. Debris was removed by centrifugation at 700 × g for 1 min, and the supernatant was collected and centrifuged at 12,000 × g for 5 min. The pellet was washed twice with PBS and stored at −20°C until DNA extraction. For DNA purification, the pellet was re-suspended in 50 mM EDTA and treated with lysozyme (Sigma Aldrich Co., St. Louis, MO; final concentration of 10 mg/mL) for 45 min at 37°C. The bacterial genomic DNA was isolated using a Wizard Genomic DNA purification kit (Promega Corp., Madison, WI).

2.9 Primer design and PCR amplification of bacterial 16S rDNA

Primers for *Lactobacillus*, *Bifidobacterium, Clostridium, and E. coli* were designed according to previously published data.[29-32] 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).

2.10 Statistical analysis

Results were analyzed by a one-way multiple analysis of variance (MANOVA) using the

JMP software (SAS Institute Inc., Cary, NC, USA). Differences between treatments were compared by Tukey's test, and values were considered statistically different at $p<0.05$ (values in the text are means ± SEM).

3. Results and discussion

3.1 Body weight, Hb, Hb-Fe, and Liver Ferritin

There were no differences in body weight, Hb, Hb-Fe, or liver ferritin levels between treatment groups ($p>0.05$). We have observed similar findings using this model to explore intra-amniotic administration of prebiotics on various parameters of Fe status.[23] Although both the *in vivo* and *in ovo Gallus gallus* models are sensitive to dietary micronutrient deficiencies, namely Fe,[26,33,34] we suggest that the lack of significant differences in these parameters between groups could be a function of time, since our study was a relatively short-term intervention. Further, as we are limited by the volumetric amount of DE injected into the amniotic fluid (i.e., the osmolality of the solution must not cause embryonic dehydration), perhaps the concentration of DE within the system was not significant enough to affect these Fe status biomarkers. The choice of concentration for the three treatment groups was based on previous studies showing biological activity of DE at or above 2.5 mg/mL.[35]

3.2 Gene expression

As shown in Figure 1, significant differences were observed in the relative expression of a number of genes between different treatment groups and controls. AP was significantly elevated ($p<0.05$) in the 0.5 mg/mL group, compared to both DE treatments and both controls. ATPase was significantly elevated among all three treatment groups ($p<0.05$), but not significantly different from one another. AP, a peptidase, and ATPase, a sodium-potassium exchanger, are both proteins located on the BBM. Thus, the relative increase in the mRNA expression of these two enzymes via DE injection into the amniotic fluid resulted in increased BBM enzymatic expression, and possibly its functionality. Further, *Wilkinson et al.*[36] recently demonstrated that hydrolysis of DE is accomplished through the stimulation of certain enzymes on the BBM (mainly lactase), representing a major step of its biotransformation. Our preliminary results using this model may confirm the BBM-stimulating properties of intra-amniotic DE administration. Further study should elucidate additional routes by which DE is transported

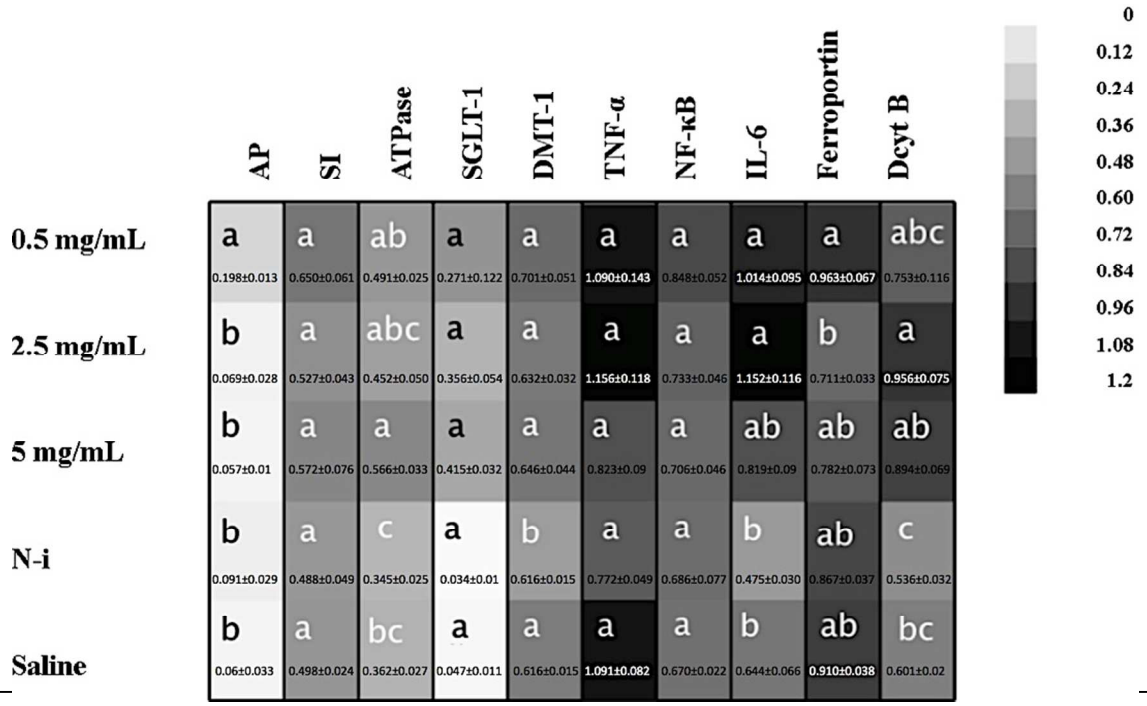

Figure 1. Duodenal mRNA expression (in AU) of measured genes on the day of hatch. Per gene, treatment groups not indicated by the same letter are significantly different ($p<0.05$).

from the enterocyte lumen to the systemic circulation.

An additional gene upregulated by DE administration included Dcyt B (p<0.05). Dcyt B is a cytochrome reductase which, in part, modulates intracellular Fe concentrations by regulating the conversion of Fe^{3+} to Fe^{2+} on the luminal side of the intestinal enterocyte.[37,38] The upregulation of Dcyt B in the 2.5 mg/mL treatment group compared to both controls suggests that DE administration has the potential to improve dietary Fe bioavailability (which may not be dose-dependent, Figure 1). Our previous work using this model has shown upregulated gene expression of this Fe homeostasis protein indicative of a relative Fe deficiency or an increased Fe load from the diet.[23,26,34] To increase dietary Fe absorption/ bioavailability, tissue expression of this, and other factors will increase to meet the Fe demands of the organism. It is interesting to note that ferroportin, a Fe transporter which shuttles Fe across the basolateral membrane, was not significantly different between groups (p>0.05). This, coupled with the lack of significant differences in Hb, Hb-Fe, ferritin concentration, and DMT-1 may generally indicate that Fe status was not altered by DE administration. Future work is needed to clarify whether the effects of DE on Dcyt B and Fe bioavailability/ status extend to Hb, Hb-Fe, and ferritin during a longer-term study.

Differences between SI, SGLT-1, TNF-α, and NF-κB were not observed (p>0.05).

3.3 Bacterial Populations in Cecum Contents

As shown in Figure 2, although the 2.5 mg/mL treatment groups had enriched abundance of *Bifidobacterum* and *Lactobacillus*, these differences were not significant when compared to controls (p>0.05). Similarly, although the 2.5 mg/mL group had the greatest abundance of *Clostridium,* the differences were not significant between the treatment and control groups (p>0.05).

However, the relative concentration of *E. coli* was significantly elevated in 2.5 mg/mL and 5 mg/mL treatment groups compared to both controls (p<0.05). This finding is

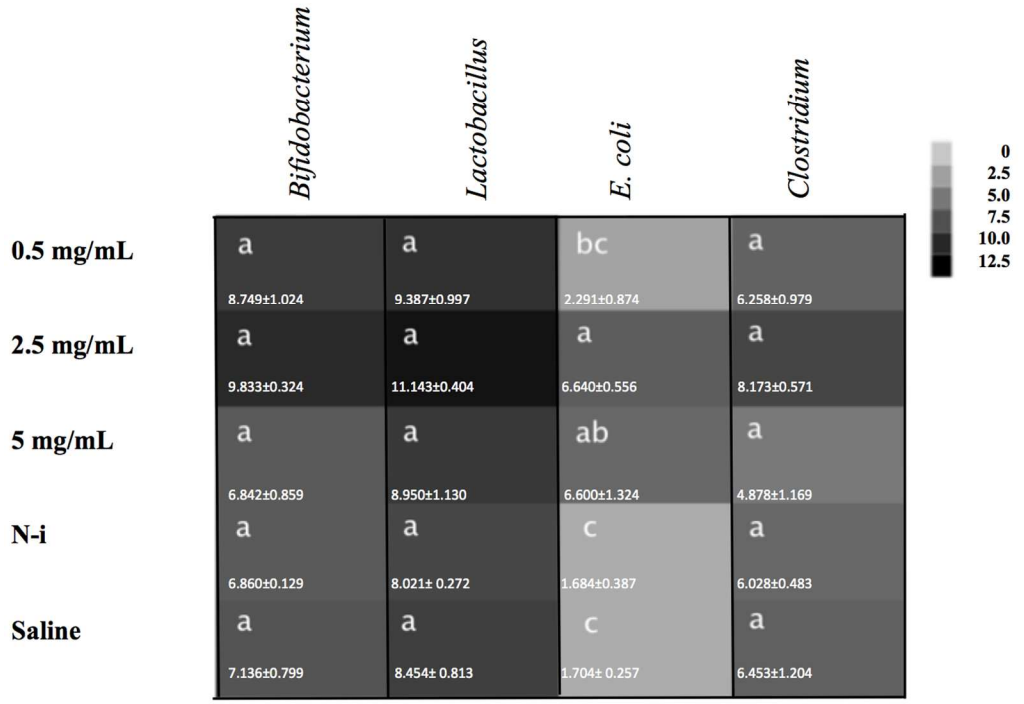

Figure 2. Genera and species-level bacterial populations (AU) from caecal contents measured on the day of hatch. Per bacterial category, treatment groups not connected by the same letter are significantly different (p<0.05).

consistent with numerous others who have found increased abundance of *E. coli* following administration of DE.[8,12] Therefore, in this model, *E. coli* may represent a candidate bacterial species directly involved in the biotransformation of DE to its bioactive metabolites, EQ and *O*-DMA. The increase in *E. coli* abundance among the DE injected groups may explain why increased relative mRNA expression of Dcyt B was observed, since SCFA products such as butyric and/or acetic acid produced via fermentation may increase the solubility of Fe^{3+} and, thus, increase Dcyt B expression.[16]

Future studies, especially those using next generation sequencing of caecal contents, will aid in determining *i*) OTU-level changes in caecal microbial ecology following DE administration, and *ii*) additional EQ and *O*-DMA- producing microbes.

4. Conclusions

In this preliminary study, we have demonstrated that intra-amniotic administration of DE affects BBM functionality by upregulating the expression of the brush-border enzymes AP and ATPase. We have further shown that DE administration increases the Fe-related homeostatic protein Dcyt B, signifying the role DE, and its metabolites, may possess in increasing dietary Fe bioavailability. Additionally, increases in the relative abundance of *E. coli* following DE administration signifies the potential role this microbe has in the biotransformation of DE to its metabolites. In our study, the biological effects of DE do not appear to be dose-dependent. Future studies which assess the effects of DE on the development, the functionality, and the interaction between the host and its gut microbiome are warranted.

Notes and references

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