Intra-amniotic administration and dietary inulin affect the iron status and intestinal functionality of iron-deficient broiler chickens

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ABSTRACT Inulin, a linear β-fructan, is present in a variety of plants, with relatively high levels of up to 20% in chicory root. It exhibits prebiotic properties and was shown to enhance mineral absorption. Our objectives were to assess the effect of intra-amniotic administration of inulin at 17 d of incubation on the iron status of broiler chicks (at hatch, 21 d) and to continue to monitor iron status with and without dietary inulin on these hatchlings for 42 d. The study included 3 prehatch treatment groups (n = 30): 1) inulin, inulin solution (4% inulin/0.85% saline); 2) control 1, untreated eggs; and 3) control 2, saline solution (0.85% saline). Solutions were injected into the naturally consumed amniotic fluid of 17-d-old chicken embryos (groups 1, 3). Upon hatch (93% hatchability), and from each group, 10 chicks were killed and their small intestine, liver, and cecum were removed for mRNA abundance of intestinal iron-related transporters, liver ferritin amounts, and bacterial analysis of cecal content, respectively. From the remaining chicks of each group, chicks were allocated to a standard corn-based diet (+/− 4% inulin, n = 10). During the trial, hemoglobin concentrations and body hemoglobin-Fe values were higher in the inulin group versus controls ($P < 0.05$). On d 42, birds were anesthetized and their duodenal loops were exposed. A nonocclusive catheter was inserted into the duodenal vein for blood sampling. A solution containing ^{58}Fe (0.1 mg of Fe/10 m*M* ascorbic acid) added to the digested diet sample was injected into the loop. Blood samples were collected every 5 min and for 90 min postinjection and analyzed by inductively coupled argon-plasma mass spectrometry for ^{58}Fe concentrations. At the end of the procedure, animals were killed and cecum contents and sections of the duodenum and liver were removed. Results showed that ^{58}Fe absorption rates were at times higher in the inulin group versus the other groups. Also, mRNA abundance of *DMT1* (an Fe transporter) and ferroportin in addition to liver ferritin amounts were higher ($P < 0.05$) in the inulin group versus controls. Results indicate that intra-amniotic administration and dietary inulin improved the iron status of iron-deficient broilers.

Key words: inulin, iron, bioavailability, intestine, chicken

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INTRODUCTION

Recently, efforts have been made to study how different nutrients might increase the dietary bioavailability of minerals. Prebiotics were shown to increase absorption of minerals, such as Ca, Mg, and possibly Fe (Ohta et al., 1998; Scholz-Ahrens et al., 2002; Scholz-Ahrens and Schrezenmeir, 2002; Bosscher et al., 2003; Yasuda et al., 2006; Tako et al., 2008). Of all the possible prebiotics, inulin and inulin-type fructans have been investigated the most.

Inulin, generally obtained from chicory roots (*Cichorium intybus* L.), is a mixture of lineal polymers and oligomers of fructose linked by a β (2-1)-glycosidic linkage, often with terminal glucose unit (Roberfroid, 1998). Because of its β-oxide bond configuration, inulin resists hydrolysis by digestive enzymes and selectively stimulates the growth and activity of beneficial bacteria in the intestine, mainly bifidobacteria and lactobacilli (Gibson et al., 1995; Roberfroid, 1998; Tako et al., 2008).

Studies conducted in mammal models, mainly rats, pigs, and in humans, have shown that inulin-type fructans stimulate mineral absorption, particularly Ca and Mg, and may have a beneficial effect on bone health (Delzenne et al., 1995; Lopez et al., 2000; Scholz-Ahrens et al., 2002; Zafar et al., 2004). Dietary inulin or fructo-oligosaccharides (**FOS**) increased intestinal Ca absorption in vitro (Bougle et al., 2002; Bosscher et al., 2003) and in humans (Scholz-Ahrens and Schrezenmeir, 2002). Also, in a study with gastrectomized rats, FOS enhanced intestinal Ca absorption and increased levels of calbindin-9 in the large intestine, indicating that dietary FOS may be useful for improving intestinal calcium absorption (Ohta et al., 1998).

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In regards to Fe absorption, it was previously shown that, 4% dietary inulin improved utilization of intrinsic iron in the corn-soybean meal diet by young anemic pigs, and this benefit was associated with increased soluble Fe in the digesta. Those piglets fed 4% inulin demonstrated a 28% improvement ($P < 0.01$) in hemoglobin repletion efficiency (Yasuda et al., 2006). It was also shown that transporters associated with Fe absorption, such as *DMT1*, *Dcytb*, ferroportin, ferritin, and *TfR* mRNA levels, in duodenal and colon samples were significantly higher in pigs given 4% dietary inulin (Tako et al., 2008). Also, the *Lactobacillus* and *Bifidobacterium* populations were significantly increased in the cecal content of the inulin-fed piglets (Tako et al., 2008; Patterson et al., 2010).

It was recently shown that a maize-soybean meal supplemented with 2% inulin did not affect growth performance and morphological measurements of the intestinal tract of broilers. However, inulin improved the relative apparent retention of Ca, Zn, and Cu. In addition authors suggested that inulin fed to broiler chickens may have a beneficial effect on bone quality (Ortiz et al., 2009). Also, broilers fed inulin-containing diets (2% or 4%) exhibited significantly improved BW gain. The dietary inulin had a positive and a significant effect on bifidobacteria and lactobacilli counts in both ileal and cecal contents, and, to an extent, also altered the fermentation patterns in the ceca (Rebolé et al., 2010).

Up to date, the underlying cellular/molecular mechanisms by which dietary FOS or inulin affect the absorption and transport of iron and other mineral cations are not fully understood. Therefore, the objective of this study was to determine the effect of intra-amniotic inulin administration followed by a dietary inulin inclusion on Fe uptake using the broiler chicken as an in vivo model. Hence, we documented how the Fe status of Fe-deficient broilers changes in response to intra-amniotic administration of inulin and dietary inulin (as part of corn-based diets with low Fe concentrations). This was done via measurement of blood hemoglobin, total body hemoglobin Fe, liver ferritin, duodenal Fe uptake, and the expression of Fe transport proteins, such as DMT1 (the Fe uptake transporter), duodenal cytochrome-B reductase (**DcytB**; reduces Fe at brush border membrane), and ferroportin (a protein involved in Fe transport across the enterocyte).

MATERIALS AND METHODS

Birds, Diets, and Study Design

One hundred twenty Cornish-cross fertile broiler eggs were obtained from a commercial hatchery (Moyer's chicks, Quakertown, PA), from a maternal flock 35 wk in lay. The eggs were incubated under optimal conditions at the Cornell University Animal Science poultry farm incubator.

Intra-Amniotic Administration

The intra-amniotic administration procedure was described previously (Tako et al., 2004, 2005; Uni et al., 2005; Smirnov et al., 2006). Briefly, at 17 d of embryonic incubation, eggs containing viable embryos (n = 112) were weighed and divided into 3 groups (n = 30), each with an average egg weight of 53 ± 1.25 g. All treatment groups were assigned eggs of similar weight-frequency distribution. A group of 60 eggs was then injected with the specified solution (1 mL per egg) with a 21-gauge needle into the amniotic fluid, which was identified by candling (Tako et al., 2004). The intra-amniotic treatment solution included the following: 40 g/L of inulin, 8.5 g/L of NaCl (n = 30). A control group (control 2, n = 30) was also injected with 8.5 g/L of NaCl and placed in hatching baskets. Control 1 (n = 30) was a noninjected group that paralleled routine procedures in commercial hatcheries. After all the eggs were injected, the injection holes were sealed with cellophane tape and the eggs placed in hatching baskets such that each treatment was equally represented at each incubator location. Hatchability was similar in all treatment groups and was approximately 93%.

Immediately after hatch and from each treatment group, 10 chicks were killed and their small intestine, liver, and cecum were removed for relative mRNA abundance of intestinal Fe-related transporters, relative liver ferritin amounts, and bacterial analysis of cecal content, respectively.

Posthatch Study Design

At hatch (hatchability rate was 93%), 2 chicks per 1-m^2 metal pen were randomly assigned to groups and housed in a total-confinement building. Hatchlings were divided into 3 treatment groups (5 pens per treatment group; n = 10): 1) inulin, prehatch treatment, intra-amniotic administration of 4% inulin (in 0.85% NaCl solution); posthatch treatment: standard corn-based diet supplemented with 4% inulin (no Fe added, 16.1 ± 0.6 ppm Fe); 2) control 1, prehatch treatment: noninjected; posthatch treatment: standard corn-based diet (no Fe or inulin added, 15.8 ± 0.5 ppm Fe); 3) control 2, prehatch treatment: intra-amniotic administration 0.85% NaCl solution; posthatch treatment: standard corn-based diet (no Fe or inulin added, 15.1 ± 0.7 ppm Fe). Diet compositions are shown in Table 1.

Birds were under indoor ambient temperatures and were provided 16 h of light. Each pen was equipped with an automatic nipple drinker and manual self feeder. All birds were given ad libitum access to water (Fe content was 0.379 ± 0.012 ppm).

Blood samples were collected from the wing vein (n = 10, ~100 μL) using microhematocrit heparinized capillary tubes (Fisher, Pittsburgh, PA). Samples were collected in the morning following an 8-h overnight fast. The samples were analyzed for hemoglobin (**Hb**) con-

Table 1. Composition of the experimental diets

Item	Inulin	Control 1	Control 2
Intra-amniotic administration (%, g/L)			
4% inulin[1] in 0.85% NaCl	+	−	−
0.85% NaCl only	−	−	+
Ingredient (g/kg)			
Corn	710	750	750
Dry skim milk	100	100	100
Corn oil	30	30	30
Corn starch	47	47	47
Vitamin/mineral premix (no Fe)[2]	70	70	70
Choline chloride	0.75	0.75	0.75
DL-Methionine	2.5	2.5	2.5
Ferric citrate	0	0	0
Inulin[1]	40	0	0
Total (g)	1,000	1,000	1,000
Determined content[3]			
Fe (μg Fe/g)	16.1 ± 0.6	15.8 ± 0.5	15.1 ± 0.7

[1]Synergy 1 (ORAFTI, Malvern, PA) was the source of inulin.

[2]Vitamin and mineral premix provided per kilogram of diet: retinyl palmitate, 1,208 μg; ergocalciferol, 5.5 μg; DL-α-tocopheryl acetate, 10.72 mg; menadione, 0.5 mg; D-biotin, 0.05 mg; choline chloride, 0.5 g; folic acid, 0.3 mg; niacin, 15 mg; Ca-D-pantothenate, 10 mg; riboflavin, 3.5 mg; thiamine, 1 mg; pyridoxine, 1.5 mg; cyanocobalamin, 17.5 μg; $CuSO_4 \cdot 5H_2O$, 6 mg; $C_2H_8N_2 \cdot 2HI$ (ethylene diamine dihydroiodine), 0.14 mg; MnO, 4 mg; Na_2SeO_3, 0.3 mg; ZnO, 100 mg. The broiler chicken dietary Fe requirement is 80 mg/kg of diet (NRC, 1994).

[3]Data provided as mean ± SEM, n = 3 (by analysis). Iron concentrations in the diets were determined by an inductively coupled argon-plasma/atomic emission spectrophotometer (ICAP 61E Thermal Jarrell Ash Trace Analyzer, Jarrell Ash Co., Franklin, MA) following wet ashing.

centration. Body weights and hemoglobin concentrations were measured weekly. Iron bioavailability was calculated as total body hemoglobin Fe (Hb-Fe) that was calculated from hemoglobin concentrations and estimates of blood volume based on BW (a blood volume of 85 mL per kg of BW is assumed; Sturkie, 2000; Tako et al., 2009, 2010, 2011; Tako and Glahn, 2011):

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

Hemoglobin Measurements

Blood Hb concentrations were determined spectrophotometrically using the cyanmethemoglobin method (H7506-STD, Pointe Scientific Inc., Canton, MI) following the kit manufacturer's instructions.

Duodenal Loop Procedure

Exposure and surgical preparation of the duodenal loop of the birds was as described previously (Tako et al., 2010). Briefly, on d 42, birds from each treatment group (n = 4) were fasted overnight and anesthetized by intravenous injection of pentobarbital (30 mg/kg of BW; Henry Schein, Melville, NY). A small incision in the lower abdomen was made to expose the duodenal loop. A nonocclusive 22-gauge catheter was inserted into the duodenal vein. The wing vein was also exposed and a BPE-T50 polyethylene tubing (Solomon Scientific, San Antonio, TX) was inserted into the vein for maintenance of anesthesia and heparin administration to prevent coagulation in the duodenal cannula during the experiment. Initial baseline blood samples were taken from the duodenal vein. Then, each duodenal loop was injected with a 10 m*M* ascorbic acid solution with 0.1 mg of ^{58}Fe (total volume of 3 mL). The anesthetized birds were kept under lamps to maintain their body temperature, and wetted gauze pads with warm saline were placed over the loops to maintain their moisture. Blood samples were collected before the stable isotope injection (baseline) and then every 5 min until 90 min post solutions injection using a peristaltic pump (ALITEA VS-10R, Precision Instrumentation Ltd., Englewood, CO) that was set to draw blood at a rate of 0.13 mL/min (total blood volume collected, 11.7 mL/1.5 h per bird). Samples were analyzed by inductively coupled argon-plasma mass spectrometry (**ICP-MS**) for ^{58}Fe concentrations. At the end of procedure, an overdose of the pentobarbital served to euthanize the birds. Sections (5 cm) of the liver and midduodenum were immediately taken, and scrapings of the duodenal mucosa were isolated and stored in a −80°C freezer until gene expression analysis. In addition, colon contents were collected for microbial analyses. All animal protocols were approved by the Cornell University Institutional Animal Care and Use Committee.

Preparation of Fe Isotope Solutions for Intravenous Infusion

Enriched stable isotope of Fe (^{58}Fe 92.2%, enriched; Isoflex USA, San Francisco, CA) was used for this study. The ^{58}Fe (in the form of elemental iron powder) was dissolved in concentrated HCl (Fisher; 200 μL of HCl/mg of Fe). The solution was diluted with deionized water to a final concentration of 1 mg of ^{58}Fe/mL (Tako et al., 2009, 2010).

For each animal, a volume of 1 mL of the concentrated ^{58}Fe solution was transferred into a separate vial with 2 mL of the 10 m*M* ascorbic acid (pH = 4) solution. The stable isotope solutions were sterilized by filtration and aliquots were stored in sealed, sterile vials until use.

Measurement of Iron Absorption

Iron absorption was estimated from the concentrations of the stable isotope tracer (^{58}Fe) in blood plasma relative to ^{56}Fe natural abundance concentration. Blood plasma samples (0.1 mL) were wet-digested in concentrated HNO_3 followed by a 50/50 mixture of HNO_3 + $HClO_4$ and brought to near dryness in a heating block. The ash was dissolved in 15 mL of 2% (0.316 mol/L) nitric acid, and isotope ratios were determined via ICP-MS (Agilent 7500 CS; Patterson et al., 2009; Tako et al., 2009, 2010). The amounts of Fe stable isotope (^{58}Fe) in excess of the naturally occurring amounts in the total circulating hemoglobin of the animals were calculated from mass spectrometer isotope ratio analyses of whole blood, Hb concentration measurements, and estimates of blood volume (Frenkel et al., 1972).

Isolation of Total RNA

Total RNA was extracted from 30 mg of the distal duodenal tissue using Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Briefly, tissues were disrupted and homogenized with a rotor-stator homogenizer in buffer RNA lysis tissue (for lysis of cells/tissues before RNA isolation), containing β-mercaptoethanol. The tissue lysate was centrifuged for 3 min at 8,000 × *g* at 4°C in a microcentrifuge. An aliquot of the supernatant was transferred to another tube, combined with 1 volume of 70% ethanol, and mixed immediately. Each sample (700 μL) was applied to an RNeasy mini column, centrifuged for 15 s at 8,000 × *g*, and the flow-through material was discarded. Next, the RNeasy columns were transferred to new 2-mL collection tubes, and 500 μL of buffer RNA buffer ethanol was pipetted onto the RNeasy column, followed by centrifugation for 15 s at 8,000 × *g*. An additional 500 μL of buffer RPE was pipetted onto the RNeasy column and centrifuged for 2 min at 8,000 × *g*. Total RNA was eluted in 50 μL of RNase-free water. All steps were carried out under RNase-free conditions. The RNA was quantified by absorbance at $A_{260/280}$. Integrity of the 28S and 18S ribosomal RNA was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining. The DNA contamination was removed using TURBO DNase treatment and removal kit from Ambion (Austin, TX).

DMT-1, DcytB, and Ferroportin Gene Expression Analysis

As described previously (Tako et al., 2005, 2010, 2011), first strand cDNA were synthesized from 5 μg of total RNA from each bird using oligo $(dT)_{18}$ as primers in the presence of MLV reverse transcriptase (Fermentase), for 1 h at 42°C. The PCR was carried out with primers chosen from the fragment of the chicken duodenal *DMT1* gene (GI 206597489; forward: 5′-AGC CGT TCA CCA CTT ATT TCG-3′; reverse: 5′-GGT CCA AAT AGG CGA TGC TC-3′), *DcytB* gene (GI 219943161; forward: 5′-GGC CGT GTT TGA GAA CCA CAA TGT T-3′; reverse: 5′-CGT TTG CAA TCA CGT TTC CAA AGA T-3′), and ferroportin gene (GI 61098365; forward: 5′-GAT GCA TTC TGA ACA ACC AAG GA-3′; reverse: 5′-GGA GAC TGG GTG GAC AAG AAC TC-3′). Ribosomal 18S was used to normalize the results, with primers from the *Gallus gallus* 18S ribosomal RNA (GI 7262899; forward: 5′-CGA TGC TCT TAA CTG AGT-3′; reverse: 5′- CAG CTT TGC AAC CAT ACT C-3′). Determination of the linear phase of the PCR amplification was performed with Tfi-DNA polymerase (Access RT-PCR system, Promega) with pooled aliquots removed at 15, 20, 25, 30, 35, 40, 45, 50, and 55 cycles. Amplification of the chicken duodenal *DMT1*, *DcytB*, and ferroportin genes were performed for 32, 33, and 30 cycles, respectively, which consisted of denaturation (95°C, 30 s), annealing (48°C, 1 min), and extension (72°C, 1 min); ribosomal 18S was amplified at 32 cycles under identical conditions in a different tube. Ribosomal 18S (426 bp) and chicken duodenal *DMT1* (300 bp), *DcytB* (214 bp), and ferroportin (300 bp) PCR products were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide, and quantified using the Quantity-One 1-D analysis program (Bio-Rad, Hercules, CA).

Liver Ferritin

Liver samples were treated as described previously (Mete et al., 2005; Tako and Glahn, 2010, 2011). Briefly, the frozen tissue samples were thawed on ice for approximately 30 min. One gram of sample was diluted into 1 mL of 50 m*M* 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, becasue other proteins are not stable at that temperature (Passaniti and Roth, 1989; Mete et al., 2005). After heat treatment, the samples were immediately cooled down 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 denaturated proteins was discarded. All tests were conducted in duplicates for each bird.

Electrophoresis and Staining 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 (Mete et al., 2005). After electrophoresis, the gels were treated with either of the

2 stains as described earlier (Leong et al., 1992): 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.

Gel Measurements

The gels were scanned with a Bio-Rad densitometer. Measurements of the bands were conducted using the Quantity-One 1-D analysis program (Bio-Rad). 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 (Passaniti and Roth, 1989; Pietrangelo et al., 1995; Mete et al., 2005; Tako et al., 2010, 2011).

Collection of Microbial Samples and DNA Isolation

The cecum was removed and treated as described previously (Zhu et al., 2002; Tako et al., 2008). The contents of the cecum were squeezed out 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 $\times$ *g* for 1 min, and the supernatant was collected and centrifuged at 12,000 $\times$ *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 resuspended in 50 m*M* 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). The DNA concentration was determined spectrophotometrically.

Primer Design and PCR Amplification of Bacterial 16S rDNA

Primers for *Lactobacillus* and *Bifidobacterium* were designed according to previously published data (Langendijk et al., 1995; Zhu et al., 2002; Amit Romach et al., 2004; Tako et al., 2008). The primers used in this study are shown in Table 2. Universal primers identifying all known bacteria were designed using the invariant region in the 16s rDNA of the bacteria. The universal primer set was used for determining the total microflora population. For PCR amplification of the bacterial targets from cecal contents, 5 μL of DNA extract was added to 45 μL of PCR mixture containing 27.5 μL of nuclease-free water, 5 μL of each primer (10 μg/mL), 1.5 μL of nucleotide (dNTP) mix, 5 μL of PCR buffer, and 1 μL of *Taq* polymerase (Go-*Taq*, Promega). The PCR thermal conditions were as follows: 1 cycle of 94°C for 3 min, 35 cycles of 94°C for 30 s, 60°C for 1 min, 68°C for 2 min, and finally 1 cycle of 68°C for 7 min. The PCR reaction was run with different numbers of cycles (25, 30, 35, 40, 45, or 50) for each primer set, and 35 cycles was in the center of the exponential increase in PCR products. The PCR products were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide, and quantified using a Gel-Pro analyzer version 3.0 (Media Cybernetics, Bethesda, MD). 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, where the total of the examined bacteria was set at 100%. Results are presented as means ± SEM.

Statistical Analysis

Results were analyzed by one-way ANOVA using the GLM procedure of SAS software (SAS Institute Inc., Cary, NC). 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).

RESULTS

Growth Rates, Hb, and Total Body Hb Fe

At d 0, no significant differences in BW or Hb were measured. During the study, BW of all birds were low, as previously shown; this was probably due to the Fe-deficient diets that were given to the birds (Tako et al., 2010). The Hb concentrations were at times significantly higher in the inulin group and in comparison with the control 1 group ($P < 0.05$; Table 3). As for total body Hb-Fe, higher values in the inulin group versus other groups were measured at hatch and during the study ($P < 0.05$; Table 3).

Table 2. Microbial polymerase chain reaction primers used

Bacterial group	Primer[1]	Sequence (5′–3′)	Length (bp)	Reference
Lactobacillus	LAA-f	CATCCAGTGCAAACCTAAGAG	286	Wang et al., 1996
	LAA-r	GATCCGGTGCAAACCTAAGAG		
Bifidobacterium	Bif164-f	GGGTGGTAATGCCGGATG	510	Langendijk et al., 1995
	Bif662-r	CCACCGTTACACCGGGAA		
Universal	Unibac-f	CGTGCCAGCCGCGGTAATACG	611	
	Unibac-r	CGTGCCAGCCGCGGTAATACG		

[1]For sequences, f = forward and r = reverse.

Table 3. Body weights, blood hemoglobin (Hb) concentrations, and total body hemoglobin iron in experimental treatment groups from d 0 to 42[1]

Item	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42
BW (g)							
Inulin	47.4 ± 3.5	182.0 ± 10.2	355.5 ± 25.8	435.2 ± 31.5	520.5 ± 48	610.9 ± 53	706.8 ± 61
Control 1	46.3 ± 3.9	159.3 ± 12.1	332.7 ± 23.6	411.6 ± 32.9	495.9 ± 51	564.1 ± 45	642.7 ± 49
Control 2	46.5 ± 3.3	174.1 ± 11.5	340.1 ± 21.3	418.1 ± 35.2	506.4 ± 38	578.8 ± 48	678.5 ± 52
Hb concentration (g/L)							
Inulin	93.1 ± 5.0^{a}	92.6 ± 4.1^{a}	88.9 ± 6.2^{a}	90.5 ± 4.5^{a}	89.5 ± 5.5^{a}	89.9 ± 4.2^{a}	88.2 ± 6.3^{a}
Control 1	82.1 ± 4.5^{a}	81.5 ± 4.0^{b}	79.5 ± 3.6^{a}	78.5 ± 8.0^{a}	78.0 ± 3.8^{a}	76.5 ± 3.9^{b}	77.5 ± 4.9^{a}
Control 2	82.5 ± 5.5^{a}	82.6 ± 3.0^{ab}	81.5 ± 3.5^{a}	81.0 ± 3.8^{a}	80.5 ± 3.3^{a}	79.5 ± 3.5^{ab}	79.2 ± 5.5^{a}
Total body Hb Fe[2](mg)							
Inulin	1.25 ± 0.07^{a}	4.80 ± 0.2^{a}	8.98 ± 0.5^{a}	11.2 ± 0.6^{a}	13.2 ± 0.7^{a}	15.6 ± 0.8^{a}	17.7 ± 0.8^{a}
Control 1	1.08 ± 0.05^{b}	3.69 ± 0.2^{a}	7.51 ± 0.4^{b}	9.18 ± 0.5^{b}	10.9 ± 0.6^{b}	12.2 ± 0.6^{b}	14.1 ± 0.7^{b}
Control 2	1.09 ± 0.05^{b}	4.09 ± 0.2^{a}	7.89 ± 0.4^{ab}	9.64 ± 0.4^{ab}	11.5 ± 0.5^{ab}	13.0 ± 0.6^{ab}	15.2 ± 0.7^{ab}

[a,b]Within a column and for each variable tested, means without a common superscript are significantly different ($P < 0.05$).

[1]Values are means ± SEM; n = 10.

[2]Hemoglobin Fe was calculated from hemoglobin concentrations and estimates of blood volume based on BW (a blood volume of 85 mL per kg of BW is assumed; Sturkie, 2000; Tan et al., 2008; Tako et al., 2009, 2010, 2011): Hb Fe (mg) = BW (kg) × 0.085 L of blood/kg × Hb (g/L blood) × 3.35 mg of Fe/g of Hb.

Gene Expression of Iron Transporters (DMT-1 and Ferroportin) and Enzyme (DcytB) in the Duodenum

Semiquantitative reverse-transcription (RT)-PCR analysis of duodenal samples, with results reported relative to 18S rRNA, showed no significant differences in mRNA abundance of *DMT1*, ferroportin, and *DcytB* between groups at d 0. However, on d 42, *DMT1* and ferroportin mRNA abundance was significantly higher ($P < 0.05$) in the inulin group versus the control 1 group (Figure 1).

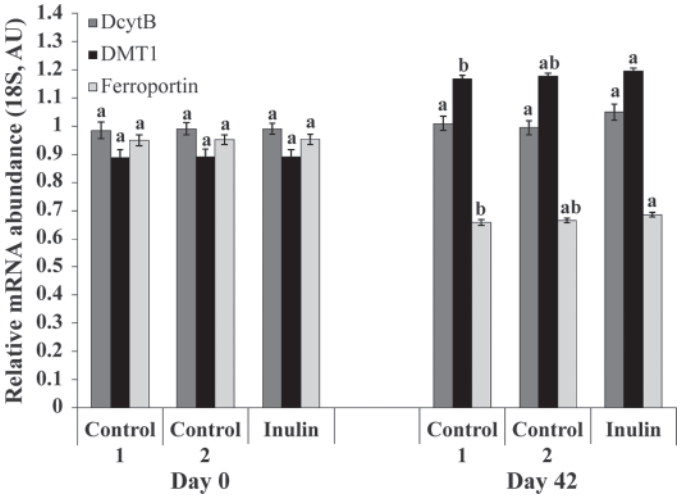

Figure 1. Duodenum mRNA abundance of *DMT1*, divalent metal transporter 1; *DcytB*, duodenal cytochrome b reductase; and ferroportin in broiler chickens at the age 6 of wk. Changes in mRNA abundance were measured by semiquantitative reverse-transcription PCR and expressed relative to the expression of 18S rRNA in arbitrary units (AU). Values are means (n = 10), with their standard errors represented by vertical bars. [a,b]Mean values within genes tested with unlike letters were significantly different ($P < 0.05$).

Liver Ferritin

Ferritin relative amounts were significantly higher on both d 0 and on d 42 of the study in the inulin group versus the control 1 treatment group ($P < 0.05$; Figure 2).

Bacterial Populations in the Chicken Cecum Contents

Analysis of cecal contents on d 0 and on d 42 of the experiment showed that bifidobacterium and lactobacilli genera proportions were higher ($P < 0.05$) in intestinal contents of the inulin group versus the control 1 group (Figure 3).

Fe Absorption

Fe absorption rates of ^{58}Fe were at times significantly higher in the inulin group versus other treatment groups. Average absorption rates were 5.15 ± 0.34%, 5.10 ± 0.32%, and 5.67 ± 0.38% for ^{58}Fe absorption in the control 1, control 2, and inulin birds, respectively (values are mean ± SEM; n = 4; Figure 4).

DISCUSSION

A combination of an innovative approach for presenting exogenous nutrients to developing chick and a long-term dietary feeding trial was used in this study. The intra-amniotic administration into the late-term embryo has been shown to be useful in investigating the effects of specific nutrients at particular stages of intestinal development (Tako et al., 2004, 2005; Uni et al., 2005; Smirnov et al., 2006; Foye et al., 2006, 2007). The long-term feeding trial approach was shown to be effective when testing nutrient affect in general and inulin in particular on Fe-deficient subjects (Haas et al., 2005; Yasuda et al., 2006; Tako et al., 2008, 2009, 2010, 2011; Tan et al., 2008; Patterson et al., 2009).

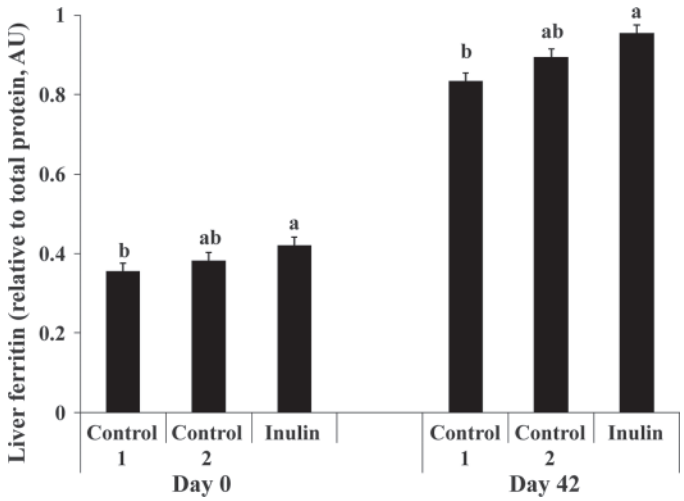

Figure 2. Liver ferritin protein amounts in arbitrary units (AU) in broiler chickens at the age of 6 wk. Values are means (n = 10), with their standard errors represented by vertical bars. [a,b]Mean values within genes tested with unlike letters were significantly different ($P < 0.05$).

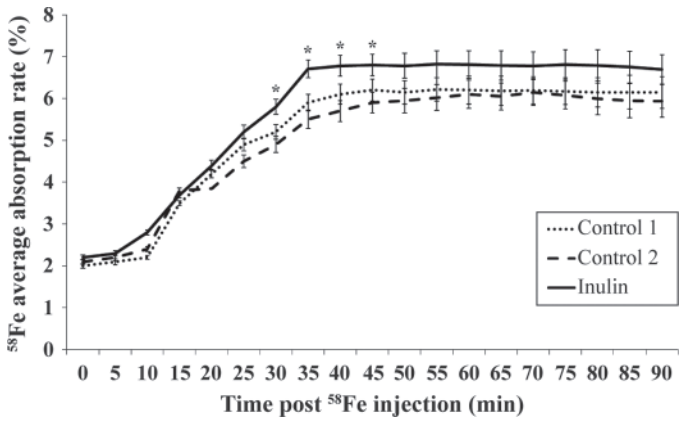

Figure 4. Duodenal loop iron absorption rate. Blood samples were collected prior to stable isotope injection and then every 5 min and for 90 min post solutions injection. Samples were analyzed by inductively coupled argon-plasma mass spectrometry for ^{58}Fe concentrations [absorption of ^{58}Fe (%); values are treatment means ± SEM; n = 4]. *Indicates significantly different, $P < 0.05$.

The current study showed that inulin administration into the amniotic fluid of the developing broiler embryo followed by dietary inulin regimen had improved the Fe status of Fe-deficient boiler chickens, as indicated by the increased Hb values (d 35), total body Hb-Fe (as from d 14), and liver ferritin concentrations. Also, bacterial populations were affected as beneficial cecal bifidobacteria and lactobacilli population proportions were elevated in the birds receiving inulin. Results also suggested that dietary inulin increased the dietary Fe availability (from corn-based diet) and to Fe-deficient broilers (by using a stable isotope tracer). These observations confirm previous studies showing a possible systemic affect of inulin in Fe-deficient piglets (Yasuda et al., 2006; Tako et al., 2008; Patterson et al., 2009, 2010).

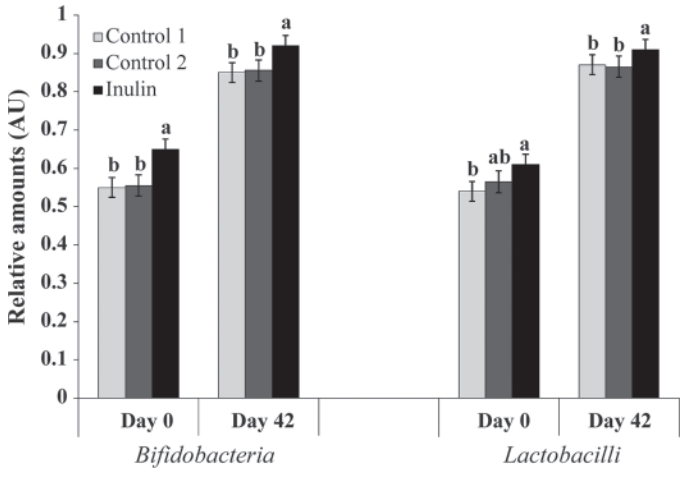

Figure 3. The effect of intra-amniotic inulin administration (4% inulin) compared with control 1 (noninjected) and control 2 (0.85% NaCl) on the proportion of lactobacilli and bifidobacteria generas in intestinal contents expressed in arbitrary units (AU). Values are means (n = 10), with their standard errors represented by vertical bars. [a,b]Mean values within each bacterial species tested with unlike letters were significantly different ($P < 0.05$). Changes in microflora populations were measured by PCR analysis of 16S rDNA of *Lactobacillus* and *Bifidobacterium* species and 16S rDNA of invariant sequences of all known intestinal bacterial species (Universal) in the intestinal contents.

Previously, Biggs et al. (2007) reported that feeding 4 or 8 g of inulin/kg of diet had no effect on the growth performance of young chicks. Similarly, Zduńczyk et al. (2007) did not find any positive response in BW gain with an inulin dietary inclusion of 4 g/kg given to turkeys. In contrast, Yusrizal and Chen (2003) showed that inulin improved the growth performance of female birds but not of male birds, and Verdonk et al. (2005) observed a positive effect of dietary inulin on broiler male performance. It was suggested that this variability in the effectiveness of inulin may be due to the effect of different factors: inclusion level, type of diet, animal characteristics, and degree of hygiene in husbandry conditions (Verdonk et al., 2005).

Recently, it was shown that 1% dietary inulin given to broilers increased intestinal villus height and deeper crypts (Rehman et al., 2007). Deeper crypts indicate fast cellular turnover to permit renewal of the villus as needed in response to normal sloughing or inflammation from pathogens or their toxins and high demands for tissue (Yason et al., 1987). A shortening of the villi and deeper crypts may lead to poor nutrient absorption, increased secretion in the gastrointestinal tract, and lower performance (Xu et al., 2003). On the other hand, increased villus length means that more energy and nutrients would be required for faster turnover of the gut mucosa (Rehman et al., 2007).

Our hypothesis in the present study was that intra-amniotic administration of inulin followed by inulin dietary inclusion will improve the Fe status of the late-term Fe-deficient broiler embryo and hatchling. Hence, to gain insight into the mechanism of action, we adopted a different approach aimed to test the additive

affect of inulin on the intestinal tissue functionality and specifically on Fe metabolism by introducing it to the developing intestine of the broiler's embryo followed by incorporating it into a low-Fe diet and at a higher level of 4%.

Recently, we have demonstrated that the broiler chicken is a suitable model for long-term dietary Fe availability feeding trials, and we have also shown the use of the duodenal loop model to measure Fe uptake (Tako et al., 2010). We also documented the late-term broiler embryo and hatchling Fe status, which indicated an Fe deficiency as day of hatch approaches (Tako and Glahn, 2011). This makes the broiler chicken embryo and hatchling a relevant in vivo model to test our hypothesis.

It was previously shown that Fe absorption is regulated, in part, by intracellular Fe concentrations in the intestinal enterocytes (Ludwiczek et al., 2004). In general, ferric Fe (Fe^{3+}) reaches the duodenal brush border membrane and is then reduced by Dcytb to Fe^{2+}, which is then transported into the enterocyte by DMT1. Within the cell, Fe is either stored as ferritin or trafficked to the basolateral membrane and exported into the circulation. Transport across the basolateral membrane is accomplished by the coordinated action of ferroportin, an Fe transporter, and hephaestin, an oxidase that oxidizes Fe^{2+} to Fe^{3+}. The Fe^{3+} then binds to transferrin for distribution throughout the body via the plasma circulation (Collins et al., 2005).

In the current study, liver ferritin amounts were higher when tested in both time points (d 0 and 42 of the experiment) in the inulin group versus the control 1 group. This indicates that these animals had higher Fe stores and thus better Fe status. In addition, on d 42, duodenal Fe-related transporter gene expression was elevated in birds receiving the inulin diet versus control 1. These observations are similar to a previous study that demonstrated that 4% dietary inulin increased the expression of *DcytB*, *DMT1*, ferroportin, and other related Fe transporters/proteins in the duodenum and colon (Tako et al., 2008) along with increasing Fe intestinal solubility and hemoglobin repletion efficiency (Yasuda et al., 2006) in Fe-deficient piglets. Coinciding with the differences in Fe transport proteins was the presence of enhanced populations of both lactobacilli ($P > 0.05$) and bifidobacteria ($P < 0.05$) in the cecal contents of the inulin birds. Previously, inulin was shown to enhance the proliferation of selected colonic microflora and alter the composition toward a more beneficial community (Gibson et al., 1995; Tzortzis et al., 2005).

It was reported that inulin-type fructans serve as a substrate for short-chain fatty acid synthesis by the intestinal bacteria and therefore may enhance the colon absorption of Ca, Zn, and Fe (Høverstad and Bjorneklett, 1984; Høverstad et al., 1984a,b; Cummings et al., 1987). In vitro, inulin was found to stimulate the growth of bifidobacteria and lactobacilli, genera considered beneficial to health (Gibson et al., 1995; Mentschel and Claus, 2003). It has been reported that bifidobacteria have a nutritional advantage compared with other intestinal microorganisms due to their β-1,2-glycosidase activity, which results in greater short-chain fatty acid production (Cummings et al., 1987; Conly and Stein, 1992; Kolida et al., 2002; Hopkins and Macfarlane, 2003; Bouhnik et al., 2004a,b; de Wiele et al., 2004). Short-chain fatty acid production in the intestinal lumen has been linked to improved intestinal functionality and overall health of the intestine (Mentschel and Claus, 2003; Daly et al., 2005). It is therefore reasonable to suspect that the improved Fe status of the birds receiving inulin was due to more efficient Fe uptake and transfer by the intestine.

With the above possibility in mind, we used the intestinal duodenal loop model for a direct measurement of Fe uptake using a stable isotope tracer as a marker (Tako et al., 2010). As in humans and other animals, the duodenal loop of poultry is the major intestinal site of Fe absorption (Sáiz et al., 1993; Aoyagi and Baker, 1995). In the present study, the plots of Fe absorption percentage against time post intraluminal injection of the stable Fe isotope showed that there were asymptotic time-dependent increases of Fe absorption. Iron absorption rates of ^{58}Fe were at times significantly higher in the inulin group versus other treatment groups ($P < 0.05$; Figure 4). This observation supports the hypothesis that dietary inulin improved the Fe uptake efficiency of the intestine via the change in bacterial population and overall health of the intestine.

In conclusion, in this study we demonstrate a positive additive affect of intra-amniotic administration and dietary inulin on the Fe status of broilers at the day of hatch and at the age of 6 wk. A nutritional solution contained 4% inulin administrated into the amniotic fluid that is naturally consumed by the developing broiler embryo, followed by 4% inulin dietary inclusion to low-Fe corn-based diet, increased dietary Fe uptake, increased intestinal beneficial bacterial populations, and improved the Fe status of Fe-deficient broilers.

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