FULL-LENGTH ARTICLE

Organic iron absorption and expression of related transporters in the small intestine of broilers

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ABSTRACT An experiment was conducted to investigate the effect of organic and inorganic Fe sources on Fe absorption and expression of related transporters in the small intestine of broilers. Iron-deficient intact broilers (7-day-old) were fed an Fe-unsupplemented corn-soybean meal basal diet or the basal diet supplemented with 60 mg Fe/kg as Fe sulfate (**$FeSO_4 \bullet 7H_2O$**), Fe-Met with weak chelation strength (**Fe-Met W**), Fe-proteinate with moderate chelation strength (**Fe-Prot M**) or Fe-proteinate with extremely strong chelation strength (**Fe-Prot ES**) for 14 d. The plasma Fe contents were enhanced ($P < 0.02$) by Fe addition, and greater ($P < 0.0002$) in Fe-Prot M and Fe-Prot ES groups than in Fe-Met W and $FeSO_4$ groups. Supplemental Fe decreased ($P < 0.03$) the divalent metal transporter 1 (**DMT1**) mRNA levels in the duodenum and jejunum, and ferroportin 1 (**FPN1**) mRNA levels in the duodenum on d 21, but no differences ($P > 0.20$) were detected among different Fe sources. Regardless of Fe source, the mRNA levels of *DMT1* and *FPN1* were higher ($P < 0.02$) in the duodenum than in the jejunum and ileum, and in the jejunum than in the ileum ($P < 0.05$). However, Fe addition did not affect ($P > 0.10$) the mRNA levels of amino acid transporters and protein levels of DMT1 and FPN1 in the small intestine of broilers. These results indicate that organic Fe sources with stronger chelation strength showed higher Fe absorption in broilers in vivo; the mRNA expression of Fe and amino acid transporters varied along with the extension of the small intestine; the absorption of Fe as organic Fe chelates was not mediated by the amino acid transporters in intact chicks in this study.

Key words: amino acid transporter, broiler, divalent metal transporter, ferriportin 1, iron absorption

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INTRODUCTION

Iron is an essential trace element, with Fe-containing proteins exerting a variety of vital functions in physiological and biochemical process of body (Evstatiev and Gasche, 2012). It was reported that Fe deficiency is one of the most common nutrition deficiencies worldwide and rapidly growing animals are vulnerable to Fe deficiency (Duque et al., 2014). Therefore, Fe is often added to animal diets in the form of supplements to meet animal growth and production. Traditionally, Fe as the inorganic iron sulfate is often added to diets of broilers (Zhang et al., 2016a). However, due to many disadvantages of the inorganic Fe supplement (e.g., low bioavailability, high hydroscopicity and oxidation, and high excretion; Ma et al., 2014), nowadays increasing attention has been paid to the development and use of organic Fe sources (Ma et al., 2014; Sun et al., 2015). According to the Association of American Feed Control Officials (AAFCO, 2018), organic chelated sources of Fe are available in the following 2 forms: Fe amino acid chelate and Fe proteinate. Fe amino acid chelate is the product resulting from the reaction of a Fe ion from a soluble Fe salt with amino acids with a mole ratio of one mole of Fe to 1 to 3 (preferably 2) moles of amino acids to form coordinate covalent bonds. Fe Proteinate is the product resulting from the chelation of a soluble Fe salt with

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amino acids and/or partially hydrolyzed protein. It was reported that chelated sources of Fe had 125 to 185% relative availabilities in comparison to ferrous sulfate (Henry and Miller, 1995). Previous studies from our laboratory have demonstrated that the relative bioavailabilities of organic Fe sources for broilers are closely correlated with their chelation strengths (quotient of formation [Q_f] values) between Fe and their ligands, and the bioavailability values vary greatly among Fe sources (Ma et al., 2014; Zhang et al., 2016a). Ma et al. (2013) found that Fe from Fe glycine chelate was easily absorbed than that from Fe sulfate in Caco-2 Cells. Recent studies from our laboratory have further indicated that organic Fe sources with greater Q_f values showed higher Fe absorption in situ ligated duodenum, jejunum, and ileum loops of broilers (Li et al., 2017; Zhang et al., 2017; Lu et al., 2018). However, these results need to be verified by in vivo studies of broilers.

Divalent metal transporter 1 (**DMT1**) is expressed in the apical transmembrane, and as one of key transporters of Fe, it actively transports Fe to intestinal enterocytes (Andrews, 1999). Once Fe is absorbed into the enterocyte, it can bind to ferroportin 1 (**FPN1**), which exists in the basal membrane of avian intestinal epithelial cells and is the only transmembrane transporter that transfers Fe from the cells to the blood circulation system (Tako et al., 2010). The results from a previous study in our laboratory indicated that both DMT1 and FPN1 were involved in the Fe absorption of ligated duodenal loops of broilers (Zhang et al., 2017). Besides, we found that L-type amino acid transporter 1 (**LAT1**) and B^0-type amino acid transporter 1 (**B^0AT1**) might participate in the absorption of Fe as Fe amino acid chelates in situ ligated jejunum and ileum loops of broilers (Lu et al., 2018). Nevertheless, to our knowledge, no in vivo study has been reported on the effect of Fe source on the Fe absorption and gene expression of the above-mentioned Fe and amino acid transporters in the small intestine of broilers.

We hypothesized that the organic Fe sources with greater Q_f values would have higher Fe absorption, and the greater absorption of Fe would be related to increased Fe and amino acid transporters in the small intestine of broilers. Therefore, the aim of this study was to determine the effect of organic and inorganic Fe sources on Fe absorption and gene expression of Fe and amino acid transporters in the small intestine using broilers in vivo to test the above hypothesis.

MATERIALS AND METHODS

Experimental Design and Treatments

A completely randomized design was used in the present study. A total of 5 dietary treatments were designed, including an Fe-unsupplemented corn-soybean meal basal diet (control, containing 69.86 mg Fe/kg by analysis) or the basal diet supplemented with 60 mg Fe/kg from one of the following four Fe sources, including Fe sulfate (**$FeSO_4 \cdot 7H_2O$** , reagent grade, 19.5% Fe by analysis; Beijing Chemical Co., Beijing, China), Fe-Met with weak chelation strength (**Fe-Met W**, feed grade, 14.7% Fe and $Q_f = 1.37$ by analysis; DeBon Agri-TECH Group, Shanghai, China), Fe proteinate with moderate chelation strength (**Fe-Prot M**, feed grade, 14.2 % Fe and $Q_f = 43.6$ by analysis; Alltech Inc., Nicholasville, KY), and Fe proteinate with extremely strong chelation strength (**Fe-Prot ES**, feed grade, 10.2% Fe and $Q_f = 8{,}590$ by analysis; Hebei Amino Acid Co., Baoding, China), respectively. The dietary added Fe level was based on the Fe requirement (a total dietary Fe level of about 136 mg/kg) of broilers from 1 to 21 d of age determined in our previous study (Ma et al., 2016).

Animals and Diets

All experimental procedures were approved by the Animal Management Committee (in charge of animal welfare issue) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (**IAS-CAAS**, Beijing, China) and performed in accordance with the ARRIVE guidelines for reporting animal research. Ethical approval on animal survival was given by the animal ethics committee of IAS-CAAS.

A total of 600 1-day-old Arbor Acres commercial male broilers were fed a Fe-deficient dextrose-casein diet (containing 3.26 mg Fe/kg of diet by analysis on an as-fed basis, Table 1) from d 1 to 7 to deplete the body Fe stores. At 8 d of age, after an overnight fast, a total of 480 broilers were weighed, selected and randomly allotted to 1 of 5 treatments with 8 replicate cages of 12 birds per cage. The broilers were maintained on a 24-h constant light schedule and handled in accordance with the Arbor Acres Broiler Management Guide (Aviagen, 2009), and allowed ad libitum access to tap water containing no detectable Fe. Mortality was recorded daily, and body weight and feed intake per cage were measured on d 21 to calculate the ADG, ADFI, gain: feed ratio, and mortality during d 8 to 21.

The dextrose-casein diet and corn-soybean meal basal diet were formulated to meet or exceed the nutrient requirements for broilers (NRC, 1994) except for Fe (Table 1). A single batch of basal diet was mixed and then divided into 5 aliquots according to the experimental treatments. The Fe sources were added to the basal diet according to the above experimental treatments. Variable small amounts of L-Lys monohydrochloride or DL-Met were added to the respective experimental diets according to the amounts of Lys and Met from supplemental organic Fe sources so as to balance Lys and Met in each experimental diet. The analyzed Fe concentrations in diets are listed in Table 2.

Sample Collections and Preparations

At both 14 and 21 d of age, 32 broilers (4 chickens from each replicate cage) from each treatment were selected based on the average body weight of the cage, and anesthetized by injections of sodium pentobarbital

Table 1. Ingredients and nutrient composition of the basal diets for broilers (as-fed basis).

Item[A], % unless noted	Dextrose-casein diet (d 1 to 7)	Corn−soybean meal diet (d 8 to 21)
Ingredient		
corn	-	54.57
Soybean meal	-	37.26
Dextrose[1]	61.78	-
Casein[1]	20.00	-
Cellulose[1]	3.00	-
Soybean oil	4.00	4.00
NaCl[1]	0.88	0.30
$CaHPO_4·H_2O$	-	2.01
$CaCO_3$[1]	2.66	1.10
KH_2PO_4[1]	1.61	-
$NaHCO_3$[1]	1.01	-
$MgSO_4·7H_2O$[1]	0.35	-
$KHCO_3$[1]	1.03	-
Gly[1]	2.01	-
DL-Met[1]	0.35	0.24
L-Arg[1]	1.01	-
Micronutrients[2,3]	0.31	0.32
Cornstarch + Fe[4]	-	0.20
Nutrient composition		
ME, MJ/kg	12.82	12.46
CP[5]	22.03	21.42
Lys	1.40	1.06
Met	0.86	0.58
Met + cys	0.94	0.92
Ca[5]	1.06	1.01
Total P	0.70	0.67
Nonphytate P	0.51	0.45
Fe[5], mg/kg	3.26	69.86

[1]Reagent grade.

[2]Provided per kilogram of diet (d 1 to 7): vitamin A (as retinyl acetate), 5,200 IU; cholecalciferol, 600 IU; vitamin E (as dl-α-tocopheryl acetate), 20 IU; vitamin K_3, 2 mg; vitamin B_1, 20 mg; vitamin B_2, 10 mg; vitamin B_6, 6 mg; vitamin B_{12}, 0.04 mg; calcium pantothenate, 30 mg; niacin, 50 mg; folic acid, 4 mg; biotin, 0.60 mg; ascorbic acid, 250 mg; choline chloride, 2,000 mg; Cu ($CuSO_4·5H_2O$), 8 mg, Zn ($ZnSO_4·7H_2O$), 40 mg; Mn ($MnSO_4·H_2O$), 80 mg; I (KI), 0.35 mg; Se (Na_2SeO_3), 0.15 mg; H_3BO_3, 9 mg; $NaMoO_4·2H_2O$, 9 mg.

[3]Provided per kilogram of diet (d 8 to 21): vitamin A (as retinyl acetate), 15,000 IU; cholecalciferol, 4,500 IU; vitamin E (as dl-α-tocopheryl acetate), 24 IU; vitamin K_3, 3 mg; vitamin B_1, 3 mg; vitamin B_2, 9.6 mg; vitamin B_6, 3 mg; vitamin B_{12}, 0.018 mg; calcium pantothenate, 15 mg; niacin, 39 mg; folic acid, 1.5 mg; biotin, 0.15 mg; choline chloride,700 mg; Cu ($CuSO_4·5H_2O$), 8 mg, Zn ($ZnSO_4·7H_2O$), 60 mg; Mn ($MnSO_4·H_2O$), 110 mg; I (KI), 0.35 mg; Se (Na_2SeO_3), 0.15 mg.

[4]Fe supplements added in place of equivalent weights of cornstarch.

[5]Determined by triplicate assays.

Table 2. Analyzed Fe concentrations in diets for broilers from 8 to 21 d of age.[1]

Fe source[2]	Added Fe, mg/kg	Analyzed Fe contents, mg/kg
Control	0	69.86
$FeSO_4·7H_2O$	60	127.89
Fe-Met W	60	133.42
Fe-Pro M	60	128.15
Fe-Pro ES	60	135.58

[1]Values of analyzed Fe contents are based on duplicate determinations.

[2]Fe-Met W = Fe-Met with a weak chelation strength ($Q_f = 1.37$); Fe-Prot M = Fe proteinate with moderate chelation strength ($Q_f = 43.6$); Fe-Prot ES = Fe proteinate with extremely strong chelation strength ($Q_f = 8.59 \times 10^3$).

(20 mg/kg body weight) via a wing vein (the birds were still alive and not euthanized). Then the blood was collected aseptically from the hepatic portal vein, and the plasma was separated and stored at -20°C for analysis of Fe content. Only at 21 d of age, after blood collection, the birds were killed by cervical dislocation, and the duodenum, jejunum, and ileum were separated and flushed with ice-cold saline solution, and slit lengthwise, and the intestinal mucosa was scraped from the underlying submucosa with an ice-cold microscope slide, immediately frozen in liquid nitrogen and then stored at −80°C until further analyses. The samples from 4 birds in each replicate cage were pooled into 1 sample in equal ratios before analysis.

Measurements of Fe, Ca, and CP Concentrations

The concentrations of Fe in the feed ingredients, diets, tap water, and plasma were measured by inductively coupled plasma emission spectroscope (model IRIS Intrepid II, Thermal Jarrell Ash, Waltham, MA) after wet digestions with HNO_3 and $HClO_4$ as described by Li et al. (2017). Validation of the Fe analysis was conducted using bovine liver powder (GBW (E) 080193, National Institute of Standards and Technology, Beijing, China) as a standard reference. Concentrations of Ca and CP in feed ingredient and diet samples were determined as described by AOAC (1990).

Determinations of mRNA Expression Levels by Real-Time Quantitative PCR

The primer information of *DMT1, FPN1*, related to $b^{0,+}$-type amino acid transporter (**rBAT**), excitatory amino acid transporter 3 (**EAAT3**), *LAT1, B^0AT1*, y^+ L-type amino acid transporter 1 (**y^+LAT1**), y^+ L-type amino acid transporter 2 (**y^+LAT2**), β-actin (housekeeping gene) and glyceraldehyde 3-phosphate dehydrogenase (**GAPDH**, housekeeping gene) genes (housekeeping genes were chosen with Primer Express Software [Applied Biosystems Inc., Foster, CA]) was the same as shown in our previous studies (Lu et al., 2018; Liao et al., 2019). The RNA isolation, reverse transcription, and real-time qPCR were performed as described previously (Lu et al., 2018).

Western Blotting

The intestinal mucosa samples were homogenized in ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) containing protease inhibitors (Roche, Penz-berg, Germany). Then they were centrifuged for 4 min (12000 × *g*, 4°C) and the supernatants were collected for Western-blot analysis (Zhang et al., 2017). The primary antibodies and dilution rates were as follows: DMT1, bs-3577R, 1:500 (Bioss, Beijing, China); FPN1, bs-4906R, 1:500 (Bioss); and GAPDH, ab22555, 1:5,000 (Abcam, Cambridge, MA). The GAPDH protein was used to normalize the expression levels of the target protein (Qin et al., 2017).

Statistical Analyses

All data were analyzed by one-way ANOVA using the general linear model procedures of SAS (version 9.2; SAS Inst. Inc., Cary, NC). Each replicate cage served as the experimental unit. Differences among means were tested by the LSD method, and statistical significance was detected at $P \leq 0.05$.

RESULTS

Growth Performance and Mortality

Fe source did not affect ($P > 0.31$) the ADG, ADFI, gain: feed ratio, and mortality during d 8 to 21 (data not shown).

Iron Contents in Plasma From the Hepatic Portal Vein

Iron source did not affect ($P > 0.97$) plasma Fe contents from the hepatic portal vein of broilers at 14 d of age, but influenced ($P < 0.001$) them at 21 d of age (Table 3). The plasma Fe contents were higher ($P < 0.02$) in Fe-supplemented groups (0.98−1.38 μg/mL) than in the control group (0.80 μg/mL), and in Fe-Prot M (1.38 μg/mL) and Fe-Prot ES (1.30 μg/mL) groups than in Fe-Met W (0.98 μg/mL) and $FeSO_4 \cdot 7H_2O$ (1.00 μg/mL) groups on d 21 ($P < 0.0002$).

mRNA Levels of Fe and Amino Acid Transporters

The mRNA levels of *DMT1* and *FPN1* were greater ($P < 0.02$) in the duodenum than in the jejunum and ileum, and in the jejunum than in the ileum ($P < 0.05$) (Table 4). Compared with the control group, the addition of Fe decreased ($P < 0.02$) the *DMT1* mRNA levels in the duodenum and jejunum and *FPN1* mRNA levels in the duodenum, and no differences ($P > 0.20$) were observed among different Fe sources.

The *rBAT* and y^+LAT2 mRNA levels of broilers were greater ($P < 0.03$) in the duodenum and jejunum than in the ileum (Tables 5 and 7). And the B^0AT1, *LAT1* or y^+LAT1 mRNA expression also tended to decrease along the intestinal tract (Tables 5−7). However, the *EAAT3* mRNA levels of broilers were lower ($P < 0.05$) in the duodenum than in the jejunum and ileum (Table 6). However, Fe source did not affect ($P > 0.10$) all of the abovementioned parameters in all of small intestinal segments of broilers.

Table 3. Effect of dietary Fe source on Fe contents in plasma from the hepatic portal vein of 14 and 21-day-old chicks.[1]

Fe Source[2]	Fe contents in plasma[2], μg/mL	
	d 14	d 21
Control	0.81	0.80[c]
$FeSO_4 \cdot 7H_2O$	0.80	1.00[b]
Fe-Met W	0.74	0.98[b]
Fe-Pro M	0.80	1.38[a]
Fe-Pro ES	0.73	1.30[a]
Pooled SE	0.073	0.065
P-value	0.978	<0.001

[a,b,c]Means within the same column lacking a common superscript differ ($P < 0.03$).

[1]Data represent the means of 8 replicate cages (4 birds per cage; $n = 8$).

[2]Fe-Met W = Fe-Met with a weak chelation strength ($Q_f = 1.37$), Fe-pro M = Fe proteinate with a moderate chelation strength ($Q_f = 43.6$), Fe-pro ES = Fe proteinate with an extremely strong chelation strength ($Q_f = 8.59 \times 10^3$).

DMT1 and FPN1 Protein Levels

Iron source did not affect ($P > 0.25$) protein expression levels of both DMT1 and FPN1 in the duodenum, jejunum, and ileum of broilers (Table 8).

DISCUSSION

In the present study, the birds were depleted of Fe from d 0-7 post-hatch to increase their sensitivity to Fe addition. Similar approach was used in our previous studies on Fe absorption in broilers (Zhang et al., 2016b, 2017; Lu et al., 2018). The first part of our hypothesis that the organic Fe sources with greater Q_f values would have higher Fe absorption has been supported by the results of the present study. However, the second part of our hypothesis that the greater absorption of Fe would be related to increased Fe and amino acid transporters in the small intestine of broilers has not been supported by the present results. Iron absorbed from intestinal-mucosal cells is transported to the portal blood of liver, and thus Fe content in plasma from the hepatic portal vein can accurately reflect the Fe transport and absorption from the intestinal lumen. There was no effect on plasma Fe on d 14 amongst treatments compared with on d 21 in the present study. This might be because the Fe accumulation in the body on 14 was less than that on d 21, and the time of d 14 was not long enough to reflect the differences in plasma Fe among treatments of different Fe sources. The results on the plasma Fe contents from the hepatic portal vein on d 21 indicated that the absorption of Fe from the Fe-Prot M and Fe-Prot ES was higher than that of Fe from Fe-Met W and $FeSO_4$, indicating that organic Fe sources with greater Q_f values had higher Fe absorption in the small intestine of broilers. This might be due to that the organic Fe with greater Q_f values could better resist to the chelating effect of interference factors such as dietary Ca, rendering higher Fe absorption. These findings are in agreement with our previous results obtained from in situ ligated small intestinal loops of broilers (Zhang et al., 2017; Lu et al., 2018). These results are also similar to our previous findings on Mn or Zn contents in plasma from the hepatic portal vein of broilers, which indicated that organic Mn or Zn absorption increased with increasing chelation strengths (Ji et al., 2006; Bai et al., 2012; Yu et al., 2017; Liao et al., 2019).

The DMT1 and FPN1 are important transporters in Fe absorption. The DMT1 is located in the apical membrane of intestinal mucosal cells, and free Fe^{2+}

Table 4. Effect of dietary Fe source on *DMT1* and *FPN1* mRNA levels in small intestinal segments of broilers at 21 d of age.[1]

Fe source	*DMT1*, RQ[2,3]					*FPN1*, RQ[2,3]				
	Duodenum[3]	Jejunum[3]	Ileum[3]	Pooled SE	*P* value	Duodenum[3]	Jejunum[3]	Ileum[3]	Pooled SE	*P* value
Control	2.54^{Aa}	0.60^{Ba}	0.054^{C}	0.362	<0.001	1.36^{Aa}	0.65^{B}	0.047^{C}	0.129	<0.001
$FeSO_4 \cdot 7H_2O$	1.19^{Ab}	0.25^{Bb}	0.052^{C}	0.133	<0.001	0.98^{Ab}	0.32^{B}	0.044^{C}	0.062	<0.001
Fe-Met W	0.72^{Ab}	0.31^{Bb}	0.052^{C}	0.013	<0.001	0.89^{Ab}	0.41^{B}	0.044^{C}	0.063	<0.001
Fe-Pro M	0.84^{Ab}	0.23^{Bb}	0.055^{C}	0.106	<0.001	0.79^{Ab}	0.48^{B}	0.042^{C}	0.105	<0.001
Fe-Pro ES	0.79^{Ab}	0.33^{Bb}	0.051^{C}	0.083	<0.001	0.75^{Ab}	0.38^{B}	0.041^{C}	0.066	<0.001
Pooled SE	0.262	0.23	0.006			0.104	0.107	0.005		
P-value	<0.001	0.015	0.979			<0.001	0.253	0.929		

[a,b]Means within the same column lacking a common superscript differ ($P < 0.02$).
[A,B,C]Means within the same row lacking a common superscript differ ($P < 0.03$).
[1]Fe-Met W = Fe-Met with a weak chelation strength ($Q_f = 1.37$), Fe-pro M = Fe proteinate with a moderate chelation strength ($Q_f = 43.6$), Fe-pro ES = Fe proteinate with a extremely strong chelation strength ($Q_f = 8.59 \times 10^3$); *DMT1* = divalent metal transporter 1; *FPN1* = ferroportin 1; RQ = relative quantity.
[2]The mRNA levels were calculated as the ratio of target gene mRNA to the geometric mean of GAPDH and β-action mRNA, and $RQ = 2^{-\Delta\Delta CT}$ (CT = threshold cycle).
[3]Data represent the means of 8 replicate cages (4 birds per cage; $n = 8$).

Table 5. Effect of dietary Fe source on B^0AT1 and *rBAT* mRNA levels in small intestinal segments of broilers at 21 d of age.[1]

Fe Source	B^0AT1, RQ[2,3]					*rBAT*, RQ[2,3]				
	Duodenum[3]	Jejunum[3]	Ileum[3]	Pooled SE	P-value	Duodenum[3]	Jejunum[3]	Ileum[3]	Pooled SE	*P* value
Control	1.31^{A}	1.29^{A}	0.69^{B}	0.14	0.012	1.09^{A}	0.82^{B}	0.36^{C}	0.082	<0.001
$FeSO_4 \cdot 7H_2O$	0.85	0.81	0.57	0.10	0.113	0.99^{A}	0.73^{B}	0.40^{C}	0.056	<0.001
Fe-Met W	0.99	0.93	0.58	0.13	0.075	0.94^{A}	0.77^{A}	0.34^{B}	0.063	<0.001
Fe-Pro M	1.12	1.01	0.85	0.13	0.382	0.93^{A}	0.72^{B}	0.44^{C}	0.064	<0.001
Fe-Pro ES	0.99	0.91	0.73	0.12	0.252	1.04^{A}	0.71^{B}	0.34^{C}	0.073	<0.001
Pooled SE	0.14	0.13	0.084			0.065	0.09	0.03		
P-value	0.235	0.123	0.154			0.372	0.923	0.112		

[A,B,C]Means within the same row lacking a common superscript differ ($P < 0.05$).
[1]Fe-Met W = Fe-Met with a weak chelation strength ($Q_f = 1.37$), Fe-pro M =Fe proteinate with a moderate chelation strength ($Q_f = 43.6$), Fe-pro ES = Fe proteinate with a extremely strong chelation strength ($Q_f = 8.59 \times 10^3$); B^0AT1 = $b^{0,+}$-type amino acid transporter 1; *rBAT* = related to $b^{0,+}$-type amino acid transporter; RQ = relative quantity.
[2]The mRNA levels were calculated as the ratio of target gene mRNA to the geometric mean of GAPDH and β-action mRNA, and $RQ = 2^{-\Delta\Delta CT}$ (CT = threshold cycle).
[3]Data represent the means of 8 replicate cages (4 birds per cage; $n = 8$).

Table 6. Effect of dietary Fe source on *EAAT3* and *LAT1* mRNA levels in small intestinal segments of broilers at 21 d of age[1.]

Fe Source	*EAAT3*, RQ[2,3]					*LAT1*, RQ[2,3]				
	Duodenum[3]	Jejunum[3]	Ileum[3]	Pooled SE	*P* value	Duodenum[3]	Jejunum[3]	Ileum[3]	Pooled SE	*P*value
Control	1.18	1.77	1.56	0.21	0.162	0.89^{A}	0.72^{A}	0.26^{B}	0.11	0.001
$FeSO_4 \cdot 7H_2O$	0.87^{B}	1.43^{A}	1.58^{A}	0.14	0.003	0.85^{A}	0.71^{A}	0.23^{B}	0.097	<0.001
Fe-Met W	0.94^{B}	1.53^{A}	1.59^{A}	0.13	0.004	1.05^{A}	0.62^{B}	0.23^{C}	0.12	<0.001
Fe-Pro M	0.97^{C}	1.37^{B}	1.82^{A}	0.12	<0.001	1.17^{A}	0.52^{B}	0.28^{B}	0.086	<0.001
Fe-Pro ES	1.11	1.35	1.56	0.18	0.243	1.08^{A}	0.51^{B}	0.22^{B}	0.14	0.001
Pooled SE	0.12	0.2	0.16			0.14	0.13	0.03		
P-value	0.341	0.562	0.803			0.483	0.692	0.561		

[A,B,C]Means within the same row lacking a common superscript differ ($P < 0.05$).
[1]Fe-Met W = Fe-Met with a weak chelation strength ($Q_f = 1.37$), Fe-pro M =Fe proteinate with a moderate chelation strength ($Q_f = 43.6$), Fe-pro ES = Fe proteinate with a extremely strong chelation strength ($Q_f = 8.59 \times 10^3$); *EAAT3* = Excitatory amino acid transporter 3; *LAT1* = L-type amino transporter 1; RQ = relative quantity.
[2]The mRNA levels were calculated as the ratio of target gene mRNA to the geometric mean of GAPDH and β-action mRNA, and $RQ = 2^{-\Delta\Delta CT}$ (CT = threshold cycle).
[3]Data represent the means of 8 replicate cages (4 birds per cage; $n = 8$).

discharged from the stomach can only be absorbed into the epithelial cells of the small intestine through DMT1 (Tako et al., 2010). The FPN1 exists in the basement membrane of mammalian intestinal epithelial cells, which is the sole Fe exporter that transfers Fe from enterocytes to bloodstream (Donovan et al., 2005). Some studies in bull calves and pigs have demonstrated that the addition of Fe resulted in a decrease of *DMT1* and *FPN1* expressions in the duodenum (Hansen et al., 2009, 2010). Jia et al. (2015) reported that supplemental Fe as $FeSO_4$ or Fe-Gly chelate decreased the mRNA expression of *DMT1* in the ligated duodenal loops of broilers. Our previous studies also confirmed that regardless of Fe source, the addition of Fe decreased the mRNA expression of *DMT1* in situ ligated duodenal, jejunal, and ileal loops of broilers (Zhang et al., 2016b; Lu et al., 2018). Similarly, in the present study, broilers fed diets supplemented with Fe had lower duodenal

Table 7. Effect of dietary Fe source on y^+LAT1 and y^+LAT2 mRNA levels in small intestinal segments of broilers at 21 d of age.[1]

Fe Source	y^+LAT1, RQ[2,3]					y^+LAT2, RQ[2,3]				
	Duodenum[3]	Jejunum[3]	Ileum[3]	Pooled SE	*P* value	Duodenum[3]	Jejunum[3]	Ileum[3]	Pooled SE	*P* value
Control	1.07^{A}	0.73^{B}	0.34^{C}	0.10	<0.001	1.17^{A}	0.65^{B}	0.19^{C}	0.13	<0.001
$FeSO_4$·$7H_2O$	1.19^{A}	0.72^{B}	0.40^{C}	0.14	0.002	1.05^{A}	0.71^{A}	0.20^{B}	0.14	0.001
Fe-Met W	1.03^{A}	0.73^{AB}	0.41^{B}	0.13	0.015	0.95^{A}	0.54^{B}	0.14^{C}	0.095	<0.001
Fe-Pro M	1.12^{A}	0.75^{B}	0.49^{B}	0.12	0.007	1.04^{A}	0.67^{B}	0.21^{C}	0.11	<0.001
Fe-Pro ES	1.05^{A}	0.79^{A}	0.38^{B}	0.12	0.002	1.08^{A}	0.54^{B}	0.16^{C}	0.10	<0.001
Pooled SE	0.15	0.14	0.052			0.14	0.14	0.02		
P-value	0.953	0.992	0.403			0.843	0.882	0.134		

[A,B,C]Means within the same row lacking a common superscript differ ($P < 0.05$).

[1]Fe-Met W = Fe-Met with a weak chelation strength ($Q_f = 1.37$), Fe-pro M =Fe proteinate with a moderate chelation strength ($Q_f = 43.6$), Fe-pro ES = Fe proteinate with a extremely strong chelation strength ($Q_f = 8.59 \times 10^3$); y^+LAT1 = y^+ L-type amino acid transporter 1; y^+LAT2 = y^+ L-type amino transporter 2; RQ = relative quantity.

[2]The mRNA levels were calculated as the ratio of target gene mRNA to the geometric mean of β-action and glyceraldehyde-3-phosphate dehydrogenase mRNA, and $RQ = 2^{-\Delta\Delta CT}$ (CT = threshold cycle).

[3]Data represent the means of 8 replicate cages (4 birds per cage; $n = 8$).

Table 8. Effect of dietary Fe source on DMT1 and FPN1 protein levels in small intestinal segments of broilers at 21 d of age.[1]

Fe Source	DMT1, RQ[2,3]			FPN1, RQ[2,3]		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
Control	1.00	1.00	1.00	1.00	1.00	1.00
FeSO4·$7H_2O$	1.20	1.10	1.09	1.00	1.02	0.91
Fe-Met W	1.30	1.05	0.92	0.86	0.95	0.87
Fe-Pro M	1.28	0.97	1.04	0.83	1.06	0.97
Fe-Pro ES	1.16	0.97	0.97	0.89	0.90	0.99
Pooled SEM	0.13	0.05	0.08	0.08	0.11	0.08
P-value	0.513	0.262	0.621	0.453	0.893	0.712

[1]Fe-Met W = Fe-Met with a weak chelation strength ($Q_f = 1.37$), Fe-pro M = Fe proteinate with a moderate chelation strength ($Q_f = 43.6$), Fe-pro ES = Fe proteinate with a extremely strong chelation strength ($Q_f = 8.59 \times 10^3$). DMT1 = divalent metal transporter 1; FPN1 = ferroportin 1.

[2]The protein levels were calculated as the RQ of the target gene protein to the glyceraldehyde-3-phosphate dehydrogenase protein, and the average expression level of protein in the control treatment was used as a calibrator.

[3]Data represent the means of 8 replicate cages (4 birds per cage; $n = 8$).

DMT1 and *FPN1* mRNA levels than those fed the control diet, indicating that Fe supplementation downregulates Fe transporters and this gives more evidence that Fe transporter expression is related to supplemental Fe level in diets.

In addition, in the present study, there were no differences in the *DMT1* and *FPN1* mRNA levels among different Fe sources, which is consistent with our previous results (Zhang et al., 2017; Lu et al., 2018). However, Zhuo et al. (2014) found that the rats perfused with Fe-Gly chelate had lower *DMT1* and *FPN1* mRNA levels in the duodenum than those perfused with $FeSO_4$ at 2 or 4 h after gavage. The above disparities might be caused by different experimental animals, methods of Fe administration or Fe sources used in these studies. The present study demonstrated that portal vein Fe was increased as Fe was added to the diet, but this increase was not mediated by changes in Fe transporters in the intestinal tract. Thus, Fe-Prot M and Fe-Prot ES sources are more bioavailable compared with Fe-Met W and $FeSO_4$ when fed to broilers.

Proteins are degraded in the stomach and small intestine into small oligopeptides and amino acids. And then, amino acids are transported into the enterocyte via different amino acid transporters that vary in substrate specificity (Kanai and Hediger, 2004; Palacin and Kanai, 2004; Verrey et al., 2004). In the current study, the *rBAT, LAT1, y^+LAT1* and *y^+LAT2* mRNA levels were greater in the duodenum than in the ileum, whereas the *EAAT3* mRNA levels were lower in the duodenum than in the ileum. However, Liao et al. (2019) found that the mRNA expression of *rBAT* and *LAT1* increased gradually from proximal to distal small intestine. The above disparities might be caused by different experimental diets used in the 2 studies. Gilbert et al. (2007) confirmed that the mRNA expression of *EAAT3* was the greatest in the ileum, the lowest in the duodenum, and intermediate in the jejunum of broilers, which is similar to our findings, implying increased capacity for glutamate absorption in the distal small intestine.

It is hypothesized that organic mineral complex or chelate might be absorbed in their intact form (Ashmead, 2001). A previous study in rats demonstrated that the Zn−L-His complex could be absorbed via its intact form in the perfused rat intestine (Wapnir et al., 1983). Some earlier studies stated that amino acids facilitated the absorption of copper, but the mechanism is not well understood (Kies and Fox, 1989; Aoyagi and Baker, 1994). Gao et al. (2014) revealed that the absorption of copper in amino acid complex appeared to be mediated by amino acid transporters in caco-2 cells. A previous study from our laboratory demonstrated that LAT1, and B^0AT1 might participate in the absorption of Fe as Fe amino acid chelates in situ ligated jejunum or ileum loops of broilers (Lu et al., 2018). However, in the present study, there were no differences in the

mRNA expression levels of amino acid transporters among different Fe sources, indicating that these amino acid transporters are not involved in the absorption of Fe from the organic Fe sources. The different methods of Fe administration (intestinal perfusion *v.* dietary supplementation) in the 2 studies might partially explain the inconsistency. Therefore, in the present study, it could not be elucidated why the organic Fe sources with stronger chelation strengths had a higher Fe absorption in the small intestine. Further efforts are needed to address the mechanisms of the absorption of Fe as these organic Fe sources in the small intestine of broilers.

In conclusion, the results from the present study indicate that our hypothesis is partially correct. The organic Fe sources with stronger chelation strengths exhibited higher Fe absorption in the small intestine of intact broilers. The mRNA expression of Fe and amino acid transporters varied from proximal to distal small intestine. Fe and amino acid transporters were not different among broilers fed different sources of Fe and this indicates that the greater Fe content in in portal plasma of broilers fed Fe-Prot M and Fe-Prot ES was not due to increased Fe and amino acid transporters and that further research is necessary to determine why Fe content in portal plasma was greater in broilers fed Fe-Prot M and Fe-Prot ES.

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DISCLOSURES

The authors declare that no conflicts of interest exist.

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