AMERICAN SOCIETY OF ANIMAL SCIENCE

Journal of Animal Science, 2021, Vol. 99, No. 1, 1–11

doi:10.1093/jas/skab002
Advance Access publication January 9, 2021
Received: 21 September 2020 and **Accepted:** 7 January 2021
Non Ruminant Nutrition

NON RUMINANT NUTRITION

Effects of dietary iron level on growth performance, hematological status, and intestinal function in growing-finishing pigs

Qingqing Deng,[†,1] Yancan Wang,[†,1] Xin Wang,[†,1] Qiye Wang,[†] Zhenfeng Yi,[†] Jun Xia,[†] Yuyao Hu,[†] Yiming Zhang,[†] Jingjing Wang,[‡] Lei Wang,[†] Shuzhong Jiang,[||] Rong Li,[$] Dan Wan,[‡] and Huansheng Yang,[†,‡,2] and Yulong Yin[‡]

[†]Hunan Provincial Key Laboratory of Animal Intestinal Function and Regulation, Hunan International Joint Laboratory of Animal Intestinal Ecology and Health, Laboratory of Animal Nutrition and Human Health, College of Life Sciences, Hunan Normal University, Changsha, Hunan 410081, China, [‡]Laboratory of Animal Nutritional Physiology and Metabolic Process, Key Laboratory of Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, Hunan 410125, China, [||]Hunan Jiuding Technology (Group) Co., Ltd. Yueyang, Hunan 414000, China, [$]Hunan Longhua Agriculture and Animal Husbandry Development Co., Ltd., TRS Group, Zhuzhou, Hunan 412000, China

[1]These authors contributed equally to this work.
[2]Corresponding author: yhs2121@126.com

ORCiD number: 0000-0003-1164-5771 (H. Yang).

Abstract

This study investigated the different addition levels of iron (Fe) in growing-finishing pigs and the effect of different Fe levels on growth performance, hematological status, intestinal barrier function, and intestinal digestion. A total of 1,200 barrows and gilts ([Large White × Landrace] × Duroc) with average initial body weight (**BW**; 27.74 ± 0.28 kg) were housed in 40 pens of 30 pigs per pen (gilts and barrows in half), blocked by BW and gender, and fed five experimental diets (eight replicate pens per diet). The five experimental diets were control diet (basal diet with no $FeSO_4$ supplementation), and the basal diet being supplemented with 150, 300, 450, or 600 mg/kg Fe as $FeSO_4$ diets. The trial lasted for 100 d and was divided into the growing phase (27 to 60 kg of BW) for the first 50 d and the finishing phase (61 to 100 kg of BW) for the last 50 d. The basal diet was formulated with an Fe-free trace mineral premix and contained 203.36 mg/kg total dietary Fe in the growing phase and 216.71 mg/kg in the finishing phase based on ingredient contributions. And at the end of the experiment, eight pigs (four barrows and four gilts) were randomly selected from each treatment (selected one pig per pen) for digesta, blood, and intestinal samples collection. The results showed that the average daily feed intake ($P = 0.025$), average daily gain ($P = 0.020$), and BW ($P = 0.019$) increased linearly in the finishing phase of pigs fed with the diets containing Fe. On the other hand, supplementation with different Fe levels in the diet significantly increased serum iron and transferrin saturation concentrations ($P < 0.05$), goblet cell numbers of duodenal villous ($P < 0.001$), and *MUC4* mRNA expression ($P < 0.05$). The apparent ileal digestibility (**AID**) of amino acids (**AA**) for pigs in the 450 and 600 mg/kg Fe groups was greater ($P < 0.05$) than for pigs in the control group. In conclusion, dietary supplementation with 450 to 600 mg/kg Fe improved the growth performance of pigs by changing hematological status and by enhancing intestinal goblet cell differentiation and AID of AA.

Key words: apparent ileal digestibility, growth performance, intestinal development, iron

© The Author(s) 2021. Published by Oxford University Press on behalf of the American Society of Animal Science.
All rights reserved. For permissions, please e-mail: journals.permissions@oup.com.

Abbreviations

AA	amino acids
ADFI	average daily feed intake
ADG	average daily gain
AID	apparent ileal digestibility
BW	body weight
DM	dry matter
F:G	feed to gain ratio
HCT	hematocrit
HGB	hemoglobin
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MPV	mean platelet volume
PCR	polymerase chain reaction
RBC	red blood cell
SI	serum iron
TAST	transferrin saturation
TIBC	total iron-binding capacity
UIBCI	unsaturated iron-binding capacity
WBC	white blood cell

Introduction

Essential trace element iron (Fe) is an important component of many enzymes and is widely involved in oxygen transport, electron transport, tricarboxylic acid cycle, production of hemoglobin (**HGB**) and myoglobin, DNA synthesis, cell differentiation, gene expression regulation, and other life processes in the body (David and Boldt, 1999; Lieu et al., 2001; Hentze et al., 2010; Martina et al., 2017). The Fe deficiency may lead to animal malnutrition, and severe Fe deficiency may cause Fe deficiency anemia or related dysfunction (Rincker et al., 2004; Bhattarai and Nielsen, 2015; Neto et al., 2019). In addition, Fe deficiency significantly inhibits the proliferation of various types of cells (Ranasinghe et al., 1987; Dayani et al., 2004; Hohnholt et al., 2010). It should be noted that Fe affects the function and differentiation of adaptive immune cells, such as T lymphocytes (Minamiyama et al., 2010), and the development of the immune system also depends on the availability of Fe (Beard, 2001; Bonaccorsi-Riani et al., 2015). Thus, given the significance of Fe, the NRC (2012) recommended that the appropriate Fe level in the diet for growing-finishing pigs is approximately 50 mg/kg.

However, grains that are commonly used in pigs' diets generally have substantial levels of native Fe (organic non-heme source), which means that the total Fe levels (native plus supplemental levels) in the diet may higher than the pigs' needs (Dalto and Matte, 2020). Moreover, supplementation of Fe in most of the commercial diets of pig is approximately 6 to 10 times that of the NRC requirement estimates. A survey conducted by Flohr et al. (2016) on current vitamin and trace mineral feeding programs in the U.S. pig industry also found that the Fe concentration in approximately 2.3 million pigs' diets was higher than the estimated Fe requirements reported by the NRC.

Excess Fe generates immoderate hydroxyl radicals through the Fenton reaction, triggering some death pathways inside and outside the mitochondria, such as apoptosis and necrosis (Qi et al., 2019). Furthermore, excessive Fe intake in animals may cause lipid peroxidation in intestinal mucosal epithelial cells, enhance membrane permeability, and impair intestinal barrier function (Knutson et al., 2000; Lu et al., 2011; Tan et al., 2013). The main function of the intestinal epithelium is the digestion and absorption of nutrients, and it also acts as a barrier against noxious antigens and pathogens (Wijtten et al., 2011). Impaired intestinal barrier function or an increased intestinal permeability may cause a decrease in transcellular absorption, which in turn leads to a decrease in the digestibility of nutrients (Pluske et al., 1997; Wijtten et al., 2011). Ferrous Fe and ferric Fe are two major Fe sources for bacteria. Besides, many bacteria, especially pathogens, have outer membrane receptors that bind or acquire Fe-containing organics such as transferrin, lactoferrin, and heme (Barton and Acton, 2019). Thus, Fe is not only important to the host but also important to microorganisms (Drakesmith and Prentice, 2012). Inappropriate dietary Fe may promote the growth of pathogenic bacteria. Furthermore, mammalian Fe homeostasis is maintained through the complex regulation of tissues that transport, store, and utilize Fe (Donovan et al., 2005). Therefore, maintaining Fe homeostasis that avoids both Fe deficiency and toxicity is essential for optimal development and function (Rao and Georgieff, 2007).

We hypothesized that greater dietary Fe than the NRC (2012) recommendation in pigs would promote growth performance via modulating intestinal barrier function and nutrient digestibility. The main objective of this research was to investigate the effects of supplementation with different levels of Fe on Fe status, growth performance, intestinal development, and immune function, as well as nutrient digestibility in growth-finishing pigs.

Materials and Methods

The care and handling of the weaned piglets used in this study followed the standards of the Animal Care and Use Committee of Hunan Normal University, Changsha City, Hunan, China. This study was conducted at a commercial farm in the Hunan Province of Southern China. The experimental period was from May to August 2019.

Animals and experimental design

A total of 1,200 barrows and gilts ([Large White × Landrace] × Duroc) with average initial body weight (**BW**; 27.74 ± 0.28 kg, gilts and barrows in half) were randomly allocated to five treatments with eight replicate pens per treatment and 30 pigs per pen (gilts and barrows in half). The dietary treatments were as follows: control group (basal diet with no $FeSO_4$ supplementation, which is formulated with a Fe-free trace mineral premix, and other nutrients in the formula met the pigs' recommended requirements estimated by the NRC (2012; Table 1) and the basal diet supplemented with 150, 300, 450, or 600 mg/kg Fe as $FeSO_4$ groups. All pigs were divided into heavy, medium, and light pens according to their weight and then adjusted to the 40 pens for the experiment according to their weight and sex. Finally, 30 pigs from each pen were randomly assigned to 8 pens of each experimental treatment. The pigs were housed in three replicate fattening rooms, with each room containing 16 pens, each pen size being 30 m^2 (1 m^2/pig), each room had wet curtains, ventilation fans were automatically controlled, and the indoor temperature was controlled at 25 to 30 °C. The pigs had ad libitum access to water and were on an experimental diet throughout the experiment. The trial lasted for 100 d and was divided into the growing phase (27 to 60 kg of BW) for the first 50 d and the finishing phase (61 to 100 kg of BW) for the last 50 d.

Sample treatment and collection

One day before the end of the experiment, eight pigs (four barrows and four gilts) were randomly selected from each treatment (selected one pig per pen), they were labeled with ear tags, isolated, and fasted overnight, and then blood samples were collected by 5-mL heparin tubes and non-heparinized tubes following jugular vein puncture. The serum was obtained by non-heparinized tube centrifugation at 3,000 × *g* for 10 min at 4 °C and then stored at −80 °C for subsequent biochemical analysis. Whole blood samples were obtained by heparin tubes and sent to the laboratory for hematological analysis.

At the end of the experiment, all the pigs were weighed at 6:00 a.m., and then 40 pigs with ear tags were transported to the commercial abattoir for sample collection. The pigs were slaughtered by electrical stunning (1.3 A for at least 3 s to apply 240 V stunning voltage), and then they were exsanguinated, scalded, skinned, eviscerated, and split down from the midline according to standard commercial procedures. The boundaries between the jejunum, cecum, and colon were ligatured to prevent chyme flow into other parts of the intestine. Then, ileal digesta samples were collected in 50-mL centrifuge tubes and immediately frozen in liquid nitrogen and stored at −80 °C until required for amino acids (**AA**) digestibility analysis. The weight and length of the small intestine were recorded. Intestinal index (ratio of the small intestine weight to BW) was calculated based on the finishing BW of the pigs. Intestinal tissues from the middle part of the duodenum, ileum, and colon were collected (approximately, 6 cm of each tissue) after being washed with phosphate-buffered saline (pH = 7.2 to 7.4). One segment (approximately 3 cm each tissue) was fixed in 10% phosphate-buffered formalin (pH = 7 to 7.4) for paraffin embedding, and the remaining segment was immediately frozen in liquid nitrogen and stored at −80 °C until the use for mRNA analysis.

Table 1. Experimental diets' ingredient and chemical composition (as-fed basis)

	Growing stage	Finishing stage
Items	27 to 60 kg	61~100 kg
Ingredients, %		
Corn	57.50	58.07
Wheat flour	10.00	7.00
Rice bran meal	7.70	12.80
Soybean meal, 43% CP	22.00	19.60
Limestone	0.90	1.00
Dicalcium phosphate	0.79	0.51
Vitamin and mineral premix[1]	0.50	0.50
Antifungal agent	0.05	0.05
Antioxidants	0.03	0.03
Choline chloride (50%)	0.04	0.03
L-lysine.H_2SO_4 (70%)	0.43	0.39
DL-Methionine	0.03	0.01
L-Threonine	0.02	0.01
L-Tryptophan	0.01	–
Total	100.00	100.00
Calculated composition		
Crude protein, %	17.06	16.42
Metabolic energy, MJ/kg	11.83	11.22
Calcium, %	0.56	0.55
Phosphorus, %	0.32	0.28
Lys[2], %	0.98	0.91
Met[2], %	0.28	0.25
Met + Cys[2], %	0.56	0.52
Thr[2], %	0.56	0.53
Trp[2], %	0.18	0.17
Analyzed composition		
Fe, mg/kg	203.36	216.71

[1]Vitamin–mineral premix supplied per kilogram of feed in growing phase: 6,500 IU of vitamin A, 225 IU of vitamin D3, 7 IU of vitamin E, 0.77 mg of vitamin K3, 1.06 mg of thiamine, 3.08 mg of riboflavin, 5.39 mg of D-pantothenic acid, 1.26 mg of pyridoxine, 0.008 mg of vitamin B12, 80 mg of Zn ($ZnSO_4$), 10 mg of Mn ($MnSO_4$), 5 mg of Cu ($CuSO_4$), 0.1 mg of Se (Na_2SeO_3); in finishing phase: 6,500 IU of vitamin A, 225 IU of vitamin D3, 7 IU of vitamin E, 0.78 mg of vitamin K3, 1.07 mg of thiamine, 3.09 mg of riboflavin, 5.34 mg of D-pantothenic acid, 1.26 mg of pyridoxine, 0.008 mg of vitamin B12, 80 mg of Zn ($ZnSO_4$), 10 mg of Mn ($MnSO_4$), 5 mg of Cu ($CuSO_4$), 0.1 mg of Se (Na_2SeO_3).
[2]Standardized ileal digestible.

Growth performance measurement

The total BW of the pigs in each pen was recorded at the beginning of the experiment and the end of each phase, and then the feed consumption per pen and the number and weight of the dead pigs were recorded daily. These data were used to calculate the average daily feed intake (**ADFI**), average daily gain (**ADG**), feed to gain ratio (**F:G**), and mortality rate (%, total numbers of dead pigs per pen × 100/total numbers of pigs per pen).

Dietary Fe concentration analysis

The microelement Fe in feed was analyzed by inductively coupled plasma optical emission spectrometry (**ICP**-OES; ICP720 ES; Agilent, USA). The detection and analysis were conducted according to our previous method (Zhang et al., 2017). The feed samples were dried in a constant temperature drying oven at 65 °C and then ground into dry powder. Following this, the feed sample was weighed at 5.0 ± 0.20 g in triplicate in a conical flask, and 15 mL of an acid mixture (nitric acid: perchloric acid = 4:1) was added for heating digestion on an electric heating plate. The digestion procedure was as follows: 80 °C for 60 min, 120 °C for 30 min, 180 °C for 30 min, and drying by evaporation at 260 °C. Subsequently, 5 mL of 1% HNO_3 was added to dissolve the residue and that was transferred to a 25-mL colorimetric tube and diluted with 1% HNO_3. Finally, the samples were filtered and subjected to ICP analyses for confirmation with standard reference. The Fe concentrations in the feed of five treatments are as follows: growing phase (203.36, 308.84, 461.62, 536.93, and 739.88 mg/kg) and finishing phase (216.71, 336.52, 483.97, 584.60, and 788.90 mg/kg).

Digestibility study and chemical analysis

Before analyses, ileal digesta samples were lyophilized using a freeze drier (Labconco Freezone 2.5L Freeze Dry System; Marshall Scientific, Hampton, NH), and then, the test diets and freeze-dried ileal digesta samples were ground through a 1-mm screen. The dry matter (**DM**) content of the experimental diets was analyzed by drying the samples at 135 °C for 2 h (method 930.15; AOAC Int., 2007). The acid-insoluble ash analysis method was based on those described by van Keulen and Young (1977). Briefly, feed and ileal digesta samples were weighed at 2.0 ± 0.001 g in triplicate in a conical flask, and then 100 mL of 4 N HCl was added and boiled on an electric heating plate for 30 min. A condenser was attached to the conical flask to prevent the loss of HCl. The thermal hydrolysate was then filtered with an ash-free filter paper (Whatman No. 541), and the residue was washed with acid-free hot double-distilled water at 85 to 100 °C. Subsequently, the filter paper and residue were transferred to a tared crucible and dried for 48 h at 103 °C and ashed for 4 h in a high-temperature furnace at 600 °C. After ashing, the

crucible was cooled to room temperature in a desiccator and weighed. The acid-insoluble ash content was calculated using the following equation:

$$\text{Acid-insoluble ash, }\% = (\text{Wf} - \text{We})/\text{Ws} \times 100$$

where Wf represents the weight of crucible with ash, We represents the weight of empty crucible, and Ws represents the weight of the sample DM.

Feed and ileal digesta AA concentrations were analyzed using Hitachi Amino Acid Analyzer (L-8900, Hitachi High-Tech Science Corporation, Tokyo, Japan). Prior to analysis, the samples were weighted at 1.0 ± 0.001 g (in triplicate) in a hydrolysis tube, and then 10 mL of 6 N HCl was added, sealed by an alcohol blast burner, and hydrolyzed at 110 °C for 24 h. After that, the hydrolysis tube was cooled to room temperature, then the tube was opened, and the hydrolysate was filtered into a 50-mL colorimetric tube via Whatman No. 1 paper that was rinsed with H_2O for three times, and then H_2O was used to constant volume. After mixing, a 1-mL solution was transferred to a 10-mL colorimetric tube and dried in a water bath at 65 °C. The dry hydrolysate was dissolved in 2-mL 0.02 N HCl and filtered through a 0.22-μm membrane. Then, the AA contents were determined using an automatic AA analyzer.

The apparent ileal digestibility (**AID**) of AA was calculated based on internal acid-insoluble ash as a marker.

$$\text{AID, }\% = \left[1 - \left(\text{ash}_{\text{diets}}/\text{ash}_{\text{digesta}}\right) \times \left(\text{AA}_{\text{digesta}}/\text{AA}_{\text{diets}}\right)\right] \times 100$$

where $\text{ash}_{\text{diets}}$ and $\text{ash}_{\text{digesta}}$ represent the internal acid-insoluble ash concentrations in the diets and ileal digesta from pigs, respectively (g per kg DM), and AA_{diets} and $\text{AA}_{\text{digesta}}$ represent the AA concentrations in the diets and ileal digesta from pigs, respectively (g per kg DM).

Hematological parameters and Fe metabolism analysis

Concentrations of serum unsaturated iron-binding capacity (**UIBCI**) and serum iron (**SI**) were determined by Cobas C311 analyzer (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer's instructions. The data of the UIBCI and SI were used to calculate the total iron-binding capacity (**TIBC**, the sum of UIBCI and SI). This was followed by the calculation of the transferrin saturation percentage (**TAST%**, SI concentration divided by TIBC per100).

The whole blood of white blood cell (**WBC**), total red blood cell number (**RBC**), HGB, hematocrit (**HCT**), mean corpuscular volume (**MCV**), mean corpuscular hemoglobin (**MCH**), mean corpuscular hemoglobin concentration (**MCHC**), red blood cell volume distribution width-CV (**RDW-CV**), red blood cell volume distribution width-SD (**RDW-SD**), total platelet number (**PLT**), mean platelet volume (**MPV**), platelet distribution width (**PDW**), and plateletcrit (**PCT**) were determined by the BC-5000VET automatic vet hematology analyzer for animals (Mindray, Guangdong, China).

Histological analysis

Paraffin-embedded intestinal segments were prepared according to the method described by Xie et al. (2018). Briefly, the fixed intestinal segments were flushed with flowing water for 12 h, dehydrated in 70%, 75%, 80%, 85%, 90%, and 95% ethanol for 1 h, and dehydrated twice in 100% ethanol for 30 min each. Finally, all intestinal segments were cleared in xylene for 10 min and embedded in paraffin wax. Three cross-sections of 5-μm thickness were then stained with hematoxylin and eosin (H&E). Images were obtained at 40× magnification and captured by a DFC450C CCD camera (Leica, Switzerland) coupled with a Leica DM 3000 microscope. The Image-Pro software (Media Cybernetics, Rockville, MD) was used to measure the villus height and crypt depth as well as the counts of goblet cells for each segment. The villus height-to-crypt depth ratio was calculated as villus height divided by crypt depth. For intestinal morphology, no fewer than 25 intact villi and their associated crypts were measured for each piglet. Goblet cells were counted in 15 villi and 15 crypts of each section.

Extraction of RNA and real-time quantitative polymerase chain reaction

Total RNA was isolated from the duodenum samples frozen in liquid nitrogen using the TRIZOL reagent (TaKaRa, Dalian, China) and then treated with DNase I (TaKaRa, Dalian, China) to remove traces of DNA. The RNA of 1 μg was reverse-transcribed (**RT**) to cDNA using an RT reagent kit (TaKaRa, Dalian, China) and then diluted five times with RNase-free water for real-time polymerase chain reaction (**PCR**). Primers were designed with Primer 5.0 (Table 2). Each PCR reaction consisted of 5 μL of SYBR Green mix (Luminaris Color HiGreen High POX qPCR Master Mix, Thermo Scientific), 3.4 μL of sterile double-distilled H_2O, 1 μL of cDNA, and 0.3 μL each of forward and reverse primers (Deng et al., 2020). Each gene was performed in triplicate, and the relative mRNA expression of the target genes was calculated as follows:

$$2^{-\Delta\Delta\text{Ct (sample}-\text{control)}}, \text{ where } -\Delta\Delta\text{Ct}$$
$$(\text{sample} - \text{control}) = (\text{Ct of target gene} - \text{Ct of }\beta - \text{actin})_{\text{sample}} - (\text{Ct of target gene} - \text{Ct of }\beta - \text{actin})_{\text{control}}.$$

Statistical analysis

The data were analyzed using the SPSS software (version 17.0; IBM Corp., Chicago, IL, USA). The histograms and the Shapiro–Wilk test were used to check the normality of data distribution and any value that departed more than 3 SD from the standardized mean was examined. If the means follow a normal distribution, use one-way analysis of variance (ANOVA); otherwise, use chi-square test (mortality rate in finishing phase). Linear and quadratic orthogonal contrasts were used to determine the effects of inclusion of Fe in the diets. Differences among the treatments were estimated using Duncan's multiple comparisons, and values were presented as means ± SEM. The differences were considered significant at $P < 0.05$, and P-values between 0.05 and 0.10 were considered as trends.

Results

Growth performance

No significant difference in the BW of start and growing and ADFI, ADG, F:G, and mortality rate was observed between the treatments in the growing phase as well as overall ($P > 0.05$; Table 3). However, increasing the dietary Fe concentration significantly increased BW (linear, $P = 0.019$), ADFI (linear,

Table 2. Primers used for real-time PCR analysis

Gene[1]	Sequence(5′-3′)[2]	Product size, bp	Accession No.
IL-10	F: GGGCTATTTGTCCTGACTGC R: GGGCTCCCTAGTTTCTCTTCC	105	NM_214041.1
IFN-γ	F: CCATTCAAAGGAGCATGGAT R: GAGTTCACTGATGGCTTTGC	146	NM_213948.1
IL-1β	F: CCTGGACCTTGGTTCTCT R: GGATTCTTCATCGGCTTCT	123	XM_021085847.1
TNF-α	F: ACAGGCCAGCTCCCTCTTAT R: CCTCGCCCTCCTGAATAAAT	102	NM_214022.1
ZO-1	F: TTGATAGTGGCGTTGACA R: CCTCATCTTCATCATCTTCTAC	126	XM_021098896.1
Occludin	F: GAGTGATTCGGATTCTGTCT R: TAGCCATAACCATAGCCATAG	181	XM_005672525.3
Claudin-1	F: CTAGTGATGAGGCAGATGAA R: AGATAGGTCCGAAGCAGAT	250	XM_005670262.3
SOD1	F: GAGACCTGGGCAATGTGACT R: CCAAACGACTTCCAGCATTT	189	NM_001190422.1
GPX1	F: AGCCCAACTTCATGCTCTTC R: CATTGCGACACACTGGAGAC	159	NM_214201.1
Nrf2	F: GAAAGCCCAGTCTTCATTGC R: TTGGAACCGTGCTAGTCTCA	190	XM_021075133.1
MUC2	F: GGTCATGCTGGAGCTGGACAGT R: TGCCTCCTCGGGGTCGTCAC	181	XM_021082584.1
MUC4	F: GATGCCCTGGCCACAGAA R:TGATTCAAGGTAGCATTCATTTGC	89	XM_021068274.1
β- actin	F:AGTTGAAGGTGGTCTCGTGG R: TGCGGGACATCAAGGAGAAG	216	XM_003357928.4

[1]*IL-10, interleukin-10; IFN-γ, interferon-γ; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; IFN-α, interferon-α; ZO-1, zona occludens 1; SOD1, superoxide-dimutase-1; GPX1, glutathione peroxidase 1; Nrf2, nuclear factor-erythroid 2-related factor-2; MUC2, mucin2; MUC4, mucin4.*
[2]F, forward primer; R, reverse primer.

P = 0.025), and ADG (linear, P = 0.020) in the finishing phase but did not affect F:G. The mortality rate (P = 0.070) decreased when the diet was supplemented with 300 mg/kg Fe.

Hematological parameters and Fe metabolism-related parameters

There were no effects of dietary treatments on the count of WBC, RBC, HGB, HCT, PLT, and PCT (P > 0.05; Table 4). However, the addition of Fe in diets significantly increased RDW-SD (quadratic, P = 0.004) and MPV (quadratic, P < 0.001). Supplementation with Fe in the diets increased MCV (linear, P < 0.001; quadratic, P < 0.001), MCH (linear, P < 0.001; quadratic, P < 0.001), MCHC (linear, P < 0.001; quadratic, P = 0.003), and PDW (linear, P < 0.001; quadratic, P < 0.001) and decreased RDW-CV (linear, P < 0.001; quadratic, P = 0.005). The diet supplemented with Fe decreased UIBCI (linear, P < 0.001; quadratic, P = 0.002) and TIBC (linear, P < 0.001; quadratic, P = 0.010) and increased SI (linear, P = 0.013) and TAST (linear, P < 0.001; quadratic, P = 0.008). The concentrations of UIBCI and TIBC significantly increased (P < 0.001) in the control group; however, the concentration of SI and % of TAST decreased (P < 0.050).

Intestinal morphology, intestinal index, and goblet cells

The duodenal villus height (P = 0.024) and the colonic crypt depth (P = 0.087) increased when the diet was supplemented with 300 mg/kg Fe; however, the ileal villus height significantly reduced with a supplement of 150 mg/kg Fe (quadratic, P =0.073; Table 5). The intestinal weight (P = 0.027) and the intestinal index (P = 0.047) showed a linear decrease in the pigs on the diet supplemented with Fe. However, increasing the Fe content in diet did not affect the duodenal and ileal crypt depth, villus width, villus height:crypt depth, and intestinal length (P > 0.05).

No significant difference in the numbers of goblet cells in both villus and crypt of ileum as well as in the crypt of the duodenum was observed between the treatments (P > 0.05; Table 6). Increasing the dietary Fe concentration linearly increased the goblet cell numbers in the villus of the duodenum (P < 0.001).

Cytokines, tight junction proteins, antioxidant-related genes, and mucin mRNA expression

The mRNA expression of *mucin4* (***MUC4***; linear, P = 0.019; quadratic, P = 0.029) significantly increased in 600 mg/kg Fe treatment compared with other treatments (Table 7). *Claudin-1* (P = 0.062) mRNA expression showed a quadratic increase in trend as Fe content increased in the diets. However, the mRNA expression of interleukin-10 (*IL-10*), interferon-γ (*IFN-γ*), interleukin-1β (*IL-1β*), tumor necrosis factor-α (*TNF-α*), zona occludens-1 (*ZO-1*), *occluding*, superoxide-dismutase-1 (*SOD1*), glutathione peroxidase 1 (*GPX1*), nuclear factor erythroid 2-related factor-2 (*Nrf2*), and mucin2 (*MUC2*) were not affected by the Fe included in the diets (P > 0.05).

The AID of AA

The AID of histidine, leucine, lysine, phenylalanine, threonine, valine, and all dispensable AA for pigs in the 450 and 600 mg/kg Fe diets was greater (P < 0.05) than for pigs fed the control diet, and there was linear increase in the AID of all indispensable AA and all dispensable AA as increasing concentrations of Fe were fed (P < 0.05; Table 8). In addition, the AID of arginine was greater than for pigs fed the 600 mg/kg Fe diet compared with

Table 3. Effect of Fe on growth performance in growing-finishing pigs[1]

Items	Fe, mg/kg[2]					SEM	P-value		
	Control	150	300	450	600		ANOVA	Linear	Quadratic
BW									
At start	27.74	27.72	27.82	27.70	27.73	0.28	1.000	0.988	0.967
Growing	61.57	60.64	60.83	60.52	60.47	0.55	0.978	0.600	0.813
Finishing	104.14	106.30	104.49	108.48	109.48	0.74	0.100	0.019	0.556
Growing phase									
ADFI	1.53	1.51	1.50	1.49	1.53	0.02	0.933	0.770	0.444
ADG	0.69	0.67	0.67	0.66	0.67	0.01	0.913	0.450	0.660
F:G	2.24	2.27	2.24	2.25	2.29	0.01	0.517	0.294	0.526
Mortality rate, %	1.67	1.25	0.83	0.83	1.25	0.31	0.914	0.583	0.439
Finishing phase									
ADFI	2.40[b]	2.55[ab]	2.53[ab]	2.53[ab]	2.59[a]	0.02	0.095	0.025	0.404
ADG	0.82	0.86	0.86	0.87	0.88	0.01	0.155	0.020	0.446
F:G	2.94	2.96	2.93	2.90	2.92	0.02	0.883	0.518	0.974
Mortality rate, %	0.83	0.42	0.00	1.25	0.83	0.21	0.070	0.585	0.357
Overall									
ADFI	1.97	2.03	2.01	2.01	2.05	0.02	0.466	0.151	0.826
ADG	0.75	0.77	0.77	0.77	0.77	0.01	0.801	0.263	0.777
F:G	2.62	2.66	2.63	2.62	2.65	0.01	0.776	0.720	0.944
Mortality rate[3], %	2.50	1.67	0.83	2.08	2.08	0.38	0.182	0.880	0.253

[1]Data are means of eight replicate pens with 30 pigs per treatment.
[2]The control group (basal diet with no $FeSO_4$ supplementation, which is formulated with an Fe-free trace mineral premix) and the basal diet supplemented with 150, 300, 450, or 600 mg/kg Fe as $FeSO_4$ groups.
[3]Mortality rate was calculated using the following equation: mortality rate (%) = (total numbers of dead pigs per pen × 100)/total numbers of pigs per pen.
[a,b]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

Table 4. Effect of Fe on hematological parameters and SI-related parameters in growing-finishing pigs[1]

Items[2]	Fe, mg/kg[3]					SEM	P-value		
	Control	150	300	450	600		ANOVA	Linear	Quadratic
WBC, 10^9/L	24.45	28.51	28.13	25.40	27.63	0.97	0.624	0.643	0.472
RBC, 10^12/L	5.53	4.06	4.50	5.23	4.67	0.30	0.556	0.797	0.405
HGB, g/L	68.38	72.63	85.50	95.38	85.88	4.86	0.414	0.102	0.458
HCT, %	22.20	22.25	26.04	29.01	25.91	1.50	0.582	0.195	0.579
MCV, fL	40.66[b]	55.73[a]	57.61[a]	55.74[a]	55.73[a]	1.13	<0.001	<0.001	<0.001
MCH, pg	12.51[b]	18.31[a]	19.00[a]	18.33[a]	18.51[a]	0.42	<0.001	<0.001	<0.001
MCHC, g/L	306.63[b]	329.13[a]	329.63[a]	329.13[a]	332.50[a]	2.07	<0.001	<0.001	0.003
RDW-CV, %	25.91[a]	23.25[b]	21.29[c]	20.65[c]	20.91[c]	0.42	<0.001	<0.001	0.005
RDW-SD, fL	39.13[c]	47.93[a]	45.71[ab]	42.83[bc]	43.24[bc]	0.80	0.003	0.517	0.004
PLT, 10^9/L	397.00	460.63	400.13	345.88	420.50	17.66	0.361	0.589	0.849
MPV, fL	8.29[c]	9.70[a]	9.31[ab]	9.53[a]	8.59[bc]	0.15	0.003	0.634	<0.001
PDW, %	14.34[b]	15.14[a]	15.10[a]	15.06[a]	15.09[a]	0.06	<0.001	<0.001	<0.001
PCT, %	0.33	0.45	0.37	0.34	0.36	0.02	0.173	0.714	0.319
UIBCI, μmol/L	113.43[a]	86.64[b]	75.75[b]	70.18[b]	73.91[b]	3.42	<0.001	<0.001	0.002
SI, μmol/L	22.18[b]	23.65[ab]	27.66[a]	28.65[a]	26.80[ab]	0.82	0.048	0.013	0.140
TIBC, μmol/L	135.61[a]	110.29[b]	103.41[b]	98.83[b]	100.71[b]	3.21	<0.001	<0.001	0.010
TAST, %	16.56[c]	21.83[b]	27.11[a]	29.04[a]	26.88[a]	1.03	<0.001	<0.001	0.008

[1]Data are means of eight piglets (four barrows and four gilts were randomly selected from each treatment) per treatment.
[2]WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW-CV, red blood cell volume distribution width CV; RDW-SD, red blood cell volume distribution width-SD; PLT, total platelet number; MPV, mean plasma volume; PDW, platelet distribution width; PCT, plateletcrit; UIBCI, unsaturated iron binding capacity; SI, serum iron; TIBC, total iron binding capacity; TAST, transferrin saturation.
[3]The control group (basal diet with no $FeSO_4$ supplementation, which is formulated with an Fe-free trace mineral premix) and the basal diet supplemented with 150, 300, 450, or 600 mg/kg Fe as $FeSO_4$ groups.
[a–c]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

Table 5. Effect of Fe on intestinal morphology and intestinal index in growing-finishing pigs[1]

	Fe, mg/kg[2]						P-value		
Items	Control	150	300	450	600	SEM	ANOVA	Linear	Quadratic
Duodenum									
Villus height, µm	448.23[ab]	381.87[b]	547.30[a]	406.47[b]	423.12[b]	17.1	0.024	0.813	0.287
Crypt depth, µm	429.81	398.97	421.19	429.55	402.00	13.20	0.912	0.796	0.950
Villus width, µm	155.97	168.12	176.36	164.50	166.51	3.35	0.376	0.458	0.160
Villus height:crypt depth	1.17	1.03	1.37	1.01	1.12	0.05	0.201	0.720	0.675
Ileum									
Villus height, µm	433.34	358.04	392.36	385.74	411.69	10.56	0.191	0.832	0.073
Crypt depth, µm	273.80	224.25	217.17	229.57	240.12	9.93	0.413	0.386	0.107
Villus width, µm	153.74	140.18	142.52	138.87	142.36	2.82	0.476	0.241	0.252
Villus height:crypt depth	1.80	1.76	2.11	1.94	2.08	0.08	0.541	0.200	0.806
Colon									
Crypt depth, µm	481.65[ab]	443.77[b]	511.94[a]	452.36[ab]	432.23[b]	10.27	0.087	0.196	0.263
Small intestine									
Intestinal weight, kg	1.86	1.83	1.67	1.66	1.67	0.04	0.179	0.027	0.454
Intestinal length, m	18.55	16.86	18.44	17.88	17.20	0.27	0.206	0.378	0.955
Intestinal index[3], %	1.94	1.76	1.72	1.69	1.71	0.04	0.203	0.047	0.173

[1]Data are means of eight piglets (four barrows and four gilts were randomly selected from each treatment) per treatment.
[2]The control group (basal diet with no $FeSO_4$ supplementation, which is formulated with an Fe-free trace mineral premix) and the basal diet supplemented with 150, 300, 450, or 600 mg/kg Fe as $FeSO_4$ groups.
[3]Intestinal index: ratio of intestinal weight: BW, g/kg.
[a,b]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

Table 6. Effect of Fe on the number of goblet cells of the small intestine in growing-finishing pigs[1]

	Fe, mg/kg[2]						P-value		
Items	Control	150	300	450	600	SEM	ANOVA	Linear	Quadratic
Duodenum									
Villus	17.99[c]	18.02[c]	25.01[b]	26.94[ab]	30.08[a]	1.05	<0.001	<0.001	0.852
Crypt	30.73	32.51	32.07	31.84	35.45	0.87	0.528	0.166	0.602
Ileum									
Villus	24.39	25.50	25.98	25.98	24.75	0.73	0.948	0.824	0.426
Crypt	36.98	32.26	38.10	35.59	38.50	1.29	0.570	0.495	0.531

[1]Data are means of eight piglets (four barrows and four gilts were randomly selected from each treatment) per treatment.
[2]The control group (basal diet with no $FeSO_4$ supplementation, which is formulated with an Fe-free trace mineral premix) and the basal diet supplemented with 150, 300, 450, or 600 mg/kg Fe as $FeSO_4$ groups.
[a–c]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

pigs fed control and 450 mg/kg Fe diets ($P = 0.042$). Pigs fed 450 and 600 mg/kg Fe diets had an increasing trend ($P = 0.062$) in the AID of isoleucine than those fed the control diet.

Discussion

Iron is an essential trace element in pigs, which plays an important role in growth performance and immunity. Most of the dietary Fe sources for the pigs were from the feed and additional Fe supplements. However, excessive Fe supplementation may impair the growth performance or intestinal function in pigs (Furugouri, 1972; Hansen et al., 2009; Qi et al., 2019). In this study, the concentration of Fe increased in the diet, and the BW, ADFI, and ADG of pigs in the finishing phase increased linearly. Many previous studies have shown that adding Fe to diets can improve the growth performance of pigs; for instance, Feng et al. (2007) found that increasing Fe-glycine chelate from 0 to 120 mg/kg Fe in the diets linearly increased ADG of weanling piglets. Similarly, Luiggi et al. (2014) showed that as the Fe supplement level increased from 50 to 150 mg/kg, there was a linear increase in ADG and a linear reduction in the F:G ratio in weaned piglets. However, excess Fe (500 mg/kg Fe) also has harmful effects on the growth performance in weaned piglets (Hansen et al., 2009). Our results suggest that the maximum dietary Fe supplementation in growth-finishing pigs is 600 mg/kg, and if a high dose of Fe is continued, it may have adverse effects on the growth performance of pigs.

Iron absorption primarily occurs in the duodenum. For the absorption of dietary non-heme Fe, firstly, Fe^{3+} is reduced to Fe^{2+} by duodenal cytochrome b (McKie, 2008) and then Fe^{2+} is transported by divalent metal transporter 1 into the absorption cells of intestinal mucosa epithelium (Chua et al., 2007; Hentze et al., 2010). It is then released from the intestinal absorption cells to extracellular by recombinant ferroportin, oxidizes Fe^{2+} to Fe^{3+} by hephaestin to blood circulation, and binds to transferrin that is transported to various tissues of the body (Abboud and Haile, 2000). The Fe status is commonly evaluated by several indicators such as HGB, RBC, HCT, MCV, MCH, MCHC, SI, TAST, and TIBC. The Fe-deficient and anemic pigs have fewer MCHC, HCT, HGB, and MCV compared with normal pigs (Dong et al.,

Table 7. Effect of Fe on the mRNA abundance of cytokines, tight junction proteins, antioxidant-related genes, and mucin of the duodenum in growing-finishing pigs[1]

	Fe, mg/kg[3]						*P*-value		
Items[2]	Control	150	300	450	600	SEM	ANOVA	Linear	Quadratic
IL-10	1.18	1.55	2.28	1.59	0.85	0.29	0.626	0.764	0.150
IFN-γ	1.27	1.45	1.48	2.25	0.73	0.29	0.620	0.895	0.295
IL-1β	1.16	1.39	1.40	1.87	1.16	0.15	0.587	0.649	0.276
TNF-α	1.08	1.89	2.30	2.56	1.35	0.33	0.594	0.609	0.140
IFN-α	1.10	2.12	2.36	1.51	1.71	0.32	0.765	0.794	0.329
ZO-1	1.09	0.97	1.23	0.82	0.85	0.10	0.651	0.361	0.638
Occludin	1.04	0.84	0.92	0.69	1.01	0.08	0.656	0.697	0.283
Claudin-1	1.05	1.16	1.47	1.54	1.00	0.10	0.275	0.674	0.062
SOD1	1.04	1.40	1.80	2.49	0.97	0.26	0.330	0.600	0.115
GPX1	1.03	0.99	1.59	1.79	0.90	0.19	0.479	0.685	0.195
Nrf2	1.10	1.15	1.43	1.25	1.16	0.10	0.845	0.761	0.381
MUC2	1.08	0.92	1.10	1.23	1.30	0.09	0.709	0.244	0.580
MUC4	1.15[b]	0.69[b]	1.00[b]	1.11[b]	3.00[a]	0.26	0.030	0.019	0.029

[1]Data are means of eight piglets (four barrows and four gilts were randomly selected from each treatment) per treatment.
[2]*IL-10, interleukin-10; IFN-γ, interferon-γ; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; IFN-α, interferon-α; ZO-1, zona occludens 1; SOD1, superoxide-dimutase-1; GPX1, glutathione peroxidase 1; Nrf2, nuclear factor-erythroid 2-related factor-2; MUC2, mucin2; MUC4, mucin4.*
[3]The control group (basal diet with no $FeSO_4$ supplementation, which is formulated with an Fe-free trace mineral premix) and the basal diet supplemented with 150, 300, 450, or 600 mg/kg Fe as $FeSO_4$ groups.
[a,b]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

Table 8. Effect of Fe on the AID[1] of AA in growing-finishing pigs[2]

	Fe, mg/kg[3]						*P*-value		
Items	Control	150	300	450	600	SEM	ANOVA	Linear	Quadratic
Indispensable AA, %									
Arginine	85.08[b]	88.87[ab]	89.50[ab]	89.87[b]	90.53[a]	0.653	0.042	0.011	0.212
Histidine	83.40[c]	88.54[ab]	85.78[bc]	92.81[ab]	90.34[a]	0.701	<0.001	<0.001	0.313
Isoleucine	80.87	84.55	81.61	87.36	86.57	0.825	0.062	0.018	0.970
Leucine	84.14[c]	88.49[abc]	86.02[bc]	91.20[a]	90.55[ab]	0.654	0.002	0.001	0.643
Lysine	80.60[c]	89.33[ab]	84.18[bc]	91.81[a]	89.05[ab]	0.832	0.002	0.001	0.109
Phenylalanine	84.78[c]	90.56[ab]	87.76[bc]	93.64[ab]	90.98[a]	0.607	0.001	<0.001	0.079
Threonine	71.96[c]	79.11[bc]	76.07[bc]	88.72[a]	83.33[ab]	1.091	<0.001	<0.001	0.254
Valine	75.32[c]	83.94[ab]	79.94[bc]	89.10[a]	87.16[a]	0.967	<0.001	<0.001	0.264
Dispensable AA, %									
Alanine	78.58[c]	85.11[ab]	80.60[bc]	88.98[a]	86.81[a]	0.868	0.001	0.001	0.498
Aspartic acid	87.03[c]	90.00[abc]	88.12[bc]	93.29[a]	91.58[ab]	0.554	0.005	0.002	0.593
Glutamic acid	86.11[c]	90.60[ab]	87.39[bc]	92.74[a]	91.61[a]	0.574	0.001	0.001	0.542
Glycine	67.83[b]	74.59[ab]	74.29[ab]	79.40[a]	80.42[a]	1.34	0.017	0.002	0.570
Proline	83.27[d]	88.22[bc]	84.65[cd]	92.70[a]	90.80[ab]	0.651	<0.001	<0.001	0.658
Serine	75.80[d]	82.42[bc]	80.12[cd]	91.82[a]	86.59[ab]	0.931	<0.001	<0.001	0.156
Tyrosine	75.68[c]	81.36[abc]	79.03[bc]	87.34[a]	84.56[ab]	1.012	0.008	0.001	0.430

[1]The AID of AA was calculated based on internal acid-insoluble ash as a marker.
[2]Data are means of eight piglets (four barrows and four gilts were randomly selected from each treatment) per treatment.
[3]The control group (basal diet with no $FeSO_4$ supplementation, which is formulated with an Fe-free trace mineral premix) and the basal diet supplemented with 150, 300, 450, or 600 mg/kg Fe as $FeSO_4$ groups.
[a–d]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

2020). In the current study, with the increase of dietary Fe concentration, there is a linear increase in MCV, MCH, MCHC, RDW-SD, MPV, PDW, SI, and TAST, whereas a linear decrease in RDW-CV, UIBCI, and TIBC compared with the control, indicating that supplementing different doses of Fe in the diet increased the Fe status in pigs. This result is similar to the findings of Luiggi et al. (2014) who found that weaned piglets supplemented with increasing Fe levels showed a linear increase in the values of HGB, HCT, SI, and TAST as well as a linear decrease in the values of Tf, latent Fe-binding capacity, and TIBC. Yu et al. (2000) described the increasing levels of dietary Fe, HGB, PCV, and Fe concentrations, and the linear decrease in the TIBC value in the blood. Feng et al. (2007) observed that the HGB and Fe concentrations and PCV increased with the increasing levels of Fe in weanling pigs. It should be noted that low TAST and high TIBC levels are associated with anemia (Donker et al., 2014; Zhang et al., 2015). Therefore, low levels of TAST and high levels of UIBCI and TIBC in the control group predicted that pigs might be in the Fe-deficient state, which was confirmed by the HGB concentration of 68.38 g/L in the blood in this study, as the

HGB concentration of 70 g/L indicates that the animals were suffering from anemia (McDowell, 1992).

Intestinal morphology is closely related to the digestion and absorption of nutrients (Pluske et al., 1996a, 1996b). Also, the intestinal villi affected the feed intake of pigs after weaning (Pluske et al., 1996a; van Beers-Schreurs et al., 1998; Lallès et al., 2004). Furthermore, claudins are important tight junction proteins that regulate paracellular permeability and barrier functions of epithelial cells (Garcia-Hernandez et al., 2017). In the present study, the villus height in the duodenum and the crypt depth in the colon as well as mRNA expression of *claudin-1* increased in the diet supplemented with 300 mg/kg Fe. However, the villus height in the ileum showed a decreasing trend in the diet supplemented with 150 mg/kg Fe. This suggested that adding 300 mg/kg Fe to the diets of growing-finishing pigs could promote the development of intestinal morphology and maintain the function of cell polarity and tight junction barrier, which may increase the effective absorption area and ultimately Fe absorption. Similarly, Lee et al. (2019) found that dietary supplementation with 100 mg/kg Fe significantly increased villus height in the duodenum and jejunum of weanling pigs compared with 50 mg/kg Fe. Zhuo et al. (2018) pointed out that the addition of Fe to the diets of weaned piglets significantly increased the villus height of the duodenum and jejunum. Both Fe deficiency and excess Fe would impair intestinal barrier function (Qi et al., 2019), hence, adding an adequate dose of Fe to pig diets may maintain intestinal health and function. Interestingly, in the current study, the control group increased the small intestine weight and intestinal index, which may be related to the decreased the AID of AA in that group. Similarly, the reduced supply of exogenous nutrients (energy) leads to an increase in stem cell activity per crypt and promotes intestinal regeneration (Yilmaz et al., 2012). Nilaweera and Speakman (2018) found that energy deficit promotes the growth of the intestine via a signaling mechanism involving the hypothalamus. Mitchell et al. (2017) indicated that calorie restriction also increased the gut size in the C57BL/6 mouse.

AA digestibility is one of the important indexes to evaluate the digestibility and utilization of AA in animals. In this study, the AID of AA was significantly increased by increasing the supplemental level of Fe in the diet, indicating that a high level of Fe was beneficial to the digestion and absorption of AA. This may be due to the fact that Fe affects the digestibility of nutrients by affecting enzymes related to digestion. It is well known that more than half of the enzymes and factors in the tricarboxylic acid cycle perform their biochemical functions in the presence of Fe. Previous reports have indicated that AA can significantly improve animal production performance (Thompson et al., 2019; Aymerich et al., 2020). Therefore, we found that the growth performance of pigs increased with the increase of AA digestibility. In addition, the improvement of AA digestibility may promote the development of intestinal villi and maintain intestinal barrier function. In intrauterine growth retardation piglets, methionine effectively enhanced the intestinal tight junction protein expression, villus height, and oxidative status (Su et al., 2018). Gut mucosal proteins and mucins, which contribute to intestinal integrity, are rich in cysteine (Badaloo et al., 2012; Yin et al., 2016) . Arginine stimulates intestinal cell migration (Rhoads et al., 2004), enhances intestinal mucosal immune barrier function, and maintains intestinal integrity in weaned pigs after the Escherichia *coli* LPS challenge (Zhu et al., 2012). Thus, the upregulating mRNA expression of *MUC4* and increased the goblet cell numbers in villus of the duodenum in the 600 mg/kg Fe group may be responsible for the increased AID of AA. In turn, the larger number of goblet cells in the high Fe group may have promoted the intestinal secretion of more mucus, which promoted intestinal health, and then improved the digestibility of nutrients, and ultimately improved the growth performance of pigs. Since, goblet cells are specialized secretory cells that help synthesize and secrete mucus, including proteins, trefoil factors, and mucins in the human and animal intestines, and play an important role in maintaining tissue homeostasis (McCauley and Guasch, 2015).

In conclusion, dietary supplementation with 450 to 600 mg/kg Fe improved the growth performance of pigs by changing hematological status and by enhancing intestinal goblet cell differentiation and AID of AA.

Acknowledgments

This work was supported by the National Key R & D Program (2016YFD0501201), Key Programs of frontier scientific research of the Chinese Academy of Sciences (QYZDY-SSWSMC008), and Changsha Zhuzhou Xiangtan High-level Talent Concentration Project (2017XK2022).

Conflict of interest statement

The authors declare that they have no competing interests.

Literature Cited

Abboud, S., and D. J. Haile. 2000. A novel mammalian iron-regulated protein involved in intracellular iron metabolism. *J. Biol. Chem.* **275**:19906–19912. doi:10.1074/jbc.M000713200

AOAC Int. 2007. *Official methods of analysis of AOAC Int*. 18th ed. 2nd rev. ed. Gaithersburg (MD): AOAC International.

Aymerich, P., C. Soldevila, J. Bonet, J. Gasa, J. Coma, and D. Solà-Oriol. 2020. Increasing dietary lysine impacts differently growth performance of growing pigs sorted by body weight. *Animals (Basel)*. **10**:1032–1048. doi:10.3390/ani10061032

Badaloo, A., J. W. Hsu, C. Taylor-Bryan, C. Green, M. Reid, T. Forrester, and F. Jahoor. 2012. Dietary cysteine is used more efficiently by children with severe acute malnutrition with edema compared with those without edema. *Am. J. Clin. Nutr.* **95**:84–90. doi:10.3945/ajcn.111.024323

Barton, J. C., and R. T. Acton. 2019. Hepcidin, iron, and bacterial infection. *Vitam. Horm.* **110**:223–242. doi:10.1016/bs.vh.2019.01.011

Beard, J. L. 2001. Iron biology in immune function, muscle metabolism and neuronal functioning. *J. Nutr.* **131**(2S-2):568S–579S; discussion 580S. doi:10.1093/jn/131.2.568S

van Beers-Schreurs, H. M. G., M. J. A. Nabuurs, L. Vellenga, H. J. K. der. Valk, T. Wensing, and H. J. Breukink. 1998. Weaning and the weanling diet influence the villous height and crypt depth in the small intestine of pigs and alter the concentrations of short-chain fatty acids in the large intestine and blood. *J. Nutr.* **128**:947–953. doi:10.1093/jn/128.6.947

Bhattarai, S., and J. P. Nielsen. 2015. Early indicators of iron deficiency in large piglets at weaning. *J. Swine Health. Prod.* **23**:10–17.

Bonaccorsi-Riani, E., R. Danger, J. J. Lozano, M. Martinez-Picola, E. Kodela, R. Mas-Malavila, M. Bruguera, H. L. Collins, R. C. Hider, M. Martinez-Llordella, *et al.* 2015. Iron deficiency impairs intra-hepatic lymphocyte mediated immune response. *PLoS One*. **10**:e0136106. doi:10.1371/journal.pone.0136106

Chua, A. C. G., R. M. Graham, D. Trinder, and J. K. Olynyk. 2007. The regulation of cellular iron metabolism. *Crit. Rev. Clin. Lab. Sci.* **44**:413–459. doi:10.1080/10408360701428257

Dalto, D. B., and J. J. Matte. 2020. Effects of different sources and levels of dietary iron and selenium on the postprandial net

portal appearance of these minerals in growing pigs. *J. Anim. Sci.* doi:10.1093/jas/skaa063

David, H., and M. D. Boldt. 1999. New perspectives on iron: an introduction. *Am. J. Med. Sci.* **318**:207–212. doi:10.1016/S0002-9629(15)40625-1

Dayani, P. N., M. C. Bishop, K. Black, and P. M. Zeltzer. 2004. Desferoxamine (DFO) – mediated iron chelation: rationale for a novel approach to therapy for brain cancer. *J. Neurooncol.* **67**:367–377. doi:10.1023/b:neon.0000024238.21349.37

Deng, Q. Q., X. Tan, H. R. Wang, Q. Y. Wang, P. F. Huang, Y. L. Li, J. Z. Li, H. S. Yang, and Y. L. Yin. 2020. Changes in cecal morphology, cell proliferation, antioxidant enzyme, volatile fatty acids, lipopolysaccharide, and cytokines in piglets during the post-weaning period. *J. Anim. Sci.* **98**:1–9. doi:10.1093/jas/skaa046

Dong, Z., D. Wan, H. Yang, G. Li, Y. Zhang, X. Zhou, and Y. L. Yin. 2020. Effects of iron deficiency on serum metabolome, hepatic histology, and function in neonatal piglets. *Animals (Basel)*. **10**:1353–1365. doi:10.3390/ani10081353

Donker, A. E., R. A. Raymakers, L. T. Vlasveld, T. van Barneveld, R. Terink, N. Dors, P. P. Brons, N. V. Knoers, and D. W. Swinkels. 2014. Practice guidelines for the diagnosis and management of microcytic anemias due to genetic disorders of iron metabolism or heme synthesis. *Blood* **123**:3873–86; quiz 4005. doi:10.1182/blood-2014-01-548776

Donovan, A., C. A. Lima, J. L. Pinkus, G. S. Pinkus, L. I. Zon, S. Robine, and N. C. Andrews. 2005. The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. *Cell Metab.* **1**:191–200. doi:10.1016/j.cmet.2005.01.003

Drakesmith, H., and A. M. Prentice. 2012. Hepcidin and the iron-infection axis. *Science* **338**:768–772. doi:10.1126/science.1224577

Feng, J., W. Q. Ma, Z. R. Xu, Y. Z. Wang, and J. X. Liu. 2007. Effects of iron glycine chelate on growth, haematological and immunological characteristics in weanling pigs. *Anim. Feed. Sci. Tech.* **134**:261–272. doi:10.1016/j.anifeedsci.2007.02.005

Flohr, J. R., J. M. Derouchey, J. C. Woodworth, M. D. Tokach, R. D. Goodband, and S. S. Dritz. 2016. A survey of current feeding regimens for vitamins and trace minerals in the US swine industry. *J. Swine Health Prod.* **24**:290–303.

Furugouri, K. 1972. Effect of elevated dietary levels of iron on iron store in liver, some blood constituents and phosphorus deficiency in young swine. *J. Anim. Sci.* **34**:573–577. doi:10.2527/jas1972.344573x

Garcia-Hernandez, V., M. Quiros, and A. Nusrat. 2017. Intestinal epithelial claudins: expression and regulation in homeostasis and inflammation. *Ann. N. Y. Acad. Sci.* **1397**:66–79. doi:10.1111/nyas.13360

Hansen, S. L., N. Trakooljul, H. C. Liu, A. J. Moeser, and J. W. Spears. 2009. Iron transporters are differentially regulated by dietary iron, and modifications are associated with changes in manganese metabolism in young pigs. *J. Nutr.* **139**:1474–1479. doi:10.3945/jn.109.105866

Hentze, M. W., M. U. Muckenthaler, B. Galy, and C. Camaschella. 2010. Two to tango: regulation of Mammalian iron metabolism. *Cell* **142**:24–38. doi:10.1016/j.cell.2010.06.028

Hohnholt, M., M. Geppert, and R. Dringen. 2010. Effects of iron chelators, iron salts, and iron oxide nanoparticles on the proliferation and the iron content of oligodendroglial OLN-93 cells. *Neurochem. Res.* **35**:1259–1268. doi:10.1007/s11064-010-0184-5

van Keulen, J., and B. A. Young. 1977. Evaluation of acid-insoluble ash as a natural marker in ruminant digestibility studies. *J. Anim. Sci.* **44**:282–287. doi:10.2527/jas1977.442282x

Knutson, M. D., P. B. Walter, B. N. Ames, and F. E. Viteri. 2000. Both iron deficiency and daily iron supplements increase lipid peroxidation in rats. *J. Nutr.* **130**:621–628. doi:10.1093/jn/130.3.621

Lallès, J.-P., G. Boudry, C. Favier, N. Le Floc′ h, I. Luron, and L. Montagne. 2004. Gut function and dysfunction in young pigs: physiology. *Anim. Res.* **53**:301–316. doi:10.1051/animres:2004018

Lee, J., A. Hosseindoust, M. Kim, K. Kim, Y. Choi, J. Moturi, C. Song, S. Lee, H. Cho, and B. Chae. 2019. Effects of hot melt extrusion processed nano-iron on growth performance, blood composition, and iron bioavailability in weanling pigs. *J. Anim. Sci. Technol.* **61**:216–224. doi:10.5187/jast.2019.61.4.216

Lieu, P. T., M. Heiskala, P. A. Peterson, and Y. Yang. 2001. The roles of iron in health and disease. *Mol. Aspects Med.* **22**:1–87. doi:10.1016/s0098-2997(00)00006-6

Lu, J. J., S. M. Chen, X. W. Zhang, J. Ding, and L. H. Meng. 2011. The anti-cancer activity of dihydroartemisinin is associated with induction of iron-dependent endoplasmic reticulum stress in colorectal carcinoma HCT116 cells. *Invest. New Drugs* **29**:1276–1283. doi:10.1007/s10637-010-9481-8

Luiggi, F. G., D. A. Berto, G. D. Mello, L. V. C. Girão, C. C. E. J. Villela, V. Lo Tierzo, and M. A. Trindade Neto. 2014. Relative bioavailability of iron from organic sources for weanling piglets. *Semina:Ciênc. Agrár.* **35**:2807–2816. doi:10.5433/1679-0359.2014v35n5p2807

Martina, U. M., R. Stefano, W. H. Matthias, and G. Bruno. 2017. A red carpet for iron metabolism. *Cell* **168**:344–361. doi:10.1016/j.cell.2016.12.034

McCauley, H. A., and G. Guasch. 2015. Three cheers for the goblet cell: maintaining homeostasis in mucosal epithelia. *Trends Mol. Med.* **21**:492–503. doi:10.1016/j.molmed.2015.06.003

McDowell, L. R. 1992. Iron. In: *Minerals in animal and human nutrition*. San Diego (CA): Academic Press, Inc.; p. 152–175.

McKie, A. T. 2008. The role of Dcytb in iron metabolism: an update. *Biochem. Soc. Trans.* **36**(Pt 6):1239–1241. doi:10.1042/BST0361239

Minamiyama, Y., S. Takemura, S. Kodai, H. Shinkawa, T. Tsukioka, H. Ichikawa, Y. Naito, T. Yoshikawa, and S. Okada. 2010. Iron restriction improves type 2 diabetes mellitus in Otsuka Long-Evans Tokushima fatty rats. *Am. J. Physiol. Endocrinol. Metab.* **298**:E1140–E1149. doi:10.1152/ajpendo.00620.2009

Mitchell, S. E., Z. Tang, C. Kerbois, C. Delville, D. Derous, C. L. Green, Y. Wang, J. J. Han, L. Chen, A. Douglas, *et al*. 2017. The effects of graded levels of calorie restriction: VIII. Impact of short term calorie and protein restriction on basal metabolic rate in the C57BL/6 mouse. *Oncotarget* **8**:17453–17474. doi:10.18632/oncotarget.15294

Neto, L. G. R., J. E. dos Santos Neto, N. B. Bueno, S. L. D. Oliveira, and T. D. R. Ataide. 2019. Effects of iron supplementation versus dietary iron on the nutritional iron status: systematic review with meta-analysis of randomized controlled trials. *Crit. Rev. Food Sci.* **59**:2553–2561. doi:10.1080/10408398.2018.1459469

Nilaweera, K. N., and J. R. Speakman. 2018. Regulation of intestinal growth in response to variations in energy supply and demand. *Obes. Rev.* **19**(Suppl 1):61–72. doi:10.1111/obr.12780

NRC. 2012. *Nutrient requirements of swine*. 11th rev. ed. Washington (DC): National Academy Press.

Pluske, J. R., D. J. Hampson, and I. H. Williams. 1997. Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livest. Prod. Sci.* **51**: 215–236. doi:10.1016/s0301-6226(97)00057-2

Pluske, J. R., M. J. Thompson, C. S. Atwood, P. H. Bird, I. H. Williams, and P. E. Hartmann. 1996b. Maintenance of villus height and crypt depth, and enhancement of disaccharide digestion and monosaccharide absorption, in piglets fed on cows' whole milk after weaning. *Br. J. Nutr.* **76**:409–422. doi:10.1079/bjn19960046.

Pluske, J. R., I. H. Williams, and F. X. Aherne. 1996a. Maintenance of villous height and crypt depth in piglets by providing continuous nutrition after weaning. *Anim. Sci.* **62**:131–144. doi:10.1017/S1357729800014417

Qi, X., Y. Zhang, H. Guo, Y. Hai, Y. Luo, and T. Yue. 2019. Mechanism and intervention measures of iron side effects on the intestine. *Crit. Rev. Food Sci. Nutr.* **2019**:1–13. doi:10.1080/10408398.2019.1630599

Ranasinghe, A. W., N. W. Johnson, and M. A. Scragg. 1987. Iron deficiency depresses cell proliferation in hamster

cheek pouch epithelium. *Cell Tissue Kinet*. **20**:403–412. doi:10.1111/j.1365-2184.1987.tb01325.x

Rao, R., and M. K. Georgieff. 2007. Iron in fetal and neonatal nutrition. *Semin. Fetal Neonatal Med*. **12**:54–63. doi:10.1016/j.siny.2006.10.007

Rhoads, J. M., W. Chen, J. Gookin, G. Y. Wu, Q. Fu, A. T. Blikslager, R. A. Rippe, R. A. Argenzio, W. G. Cance, E. M. Weaver, *et al*. 2004. Arginine stimulates intestinal cell migration through a focal adhesion kinase dependent mechanism. *Gut* **53**:514–522. doi:10.1136/gut.2003.027540

Rincker, M. J., G. M. Hill, J. E. Link, and J. E. Rowntree. 2004. Effects of dietary iron supplementation on growth performance, hematological status, and whole-body mineral concentrations of nursery pigs[1]. *J. Anim. Sci*. **82**:3189–3197. doi:10.2527/2004.82113189x

Su, W., H. Zhang, Z. Ying, Y. Li, L. Zhou, F. Wang, L. Zhang, and T. Wang. 2018. Effects of dietary L-methionine supplementation on intestinal integrity and oxidative status in intrauterine growth-retarded weanling piglets. *Eur. J. Nutr*. **57**:2735–2745. doi:10.1007/s00394-017-1539-3

Tan, T. C. H., D. H. G. Crawford, L. A. Jaskowski, V. N. Subramaniam, A. D. Clouston, D. I. Crane, and L. M. Fletcher. 2013. Excess iron modulates endoplasmic reticulum stress-associated pathways in a mouse model of alcohol and high-fat diet-induced liver injury. *Lab. Invest*. **93**:1295–1312. doi:10.1038/labinvest.2013.121

Thompson, R., C. Boessen, B. Knopf, L. Greiner, and K. D. Haydon. 2019. 181 Evaluation of feeding arginine in gestation and lactation on sow and litter performance. *J. Anim. Sci*. **97**: 105–105. doi:10.1093/jas/skz122.186

Wijtten, P. J. A., J. van der Meulen, M. W. A. and Verstegen. 2011. Intestinal barrier function and absorption in pigs after weaning: a review. *Brit. J. Nutr*. **105**:967–981. doi:10.1017/s0007114510005660

Xie, Y. H., C. Y. Zhang, L. X. Wang, Q. H. Shang, G. G. Zhang, and W. R. Yang. 2018. Effects of dietary supplementation of *Enterococcus faecium* on growth performance, intestinal morphology, and selected microbial populations of piglets. *Livest. Sci*. **210**: 111–117. doi:10.1016/j.livsci.2018.02.010

Yilmaz, Ö. H., P. Katajisto, D. W. Lamming, Y. Gültekin, K. E. Bauer-Rowe, S. Sengupta, K. Birsoy, A. Dursun, V. O. Yilmaz, M. Selig, *et al*. 2012. mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake. *Nature* **486**:490–495. doi:10.1038/nature11163

Yin, J., W. Ren, G. Yang, J. Duan, X. Huang, R. Fang, C. Li, T. Li, Y. Yin, Y. Hou, *et al*. 2016. L-Cysteine metabolism and its nutritional implications. *Mol. Nutr. Food Res*. **60**:134–146. doi:10.1002/mnfr.201500031

Yu, B., W. Huang, and P. W.-S. Chiou. 2000. Bioavailability of iron from amino acid complex in weanling pigs. *Anim. Feed Sci. Tech*. **86**:39–52. doi:10.1016/S0377-8401(00)00154-1

Zhang, Y., X. Sun, C. Xie, X. Shu, and Y. Yin. 2015. Effects of ferrous carbamoyl glycine on iron state and absorption in an iron-deficient rat model. *Genes Nutr*. **10**:54–61. doi:10.1007/s12263-015-0504-0

Zhang, Y., D. Wan, X. Zhou, C. Long, X. Wu, L. Li, L. He, P. Huang, S. Chen, B. Tan, *et al*. 2017. Diurnal variations in iron concentrations and expression of genes involved in iron absorption and metabolism in pigs. *Biochem. Biophys. Res. Commun*. **490**:1210–1214. doi:10.1016/j.bbrc.2017.06.187

Zhu, H., Y. Liu, X. Xie, J. Huang, and Y. Hou. 2012. Effect of L-arginine on intestinal mucosal immune barrier function in weaned pigs after *Escherichia coli* LPS challenge. *Innate. Immun*, **19**:242–252. doi:10.1177/1753425912456223

Zhuo, Z., X. Yu, S. Li, S. Fang, and J. Feng. 2018. Heme and non-heme iron on growth performances, blood parameters, tissue mineral concentration, and intestinal morphology of weanling pigs. *Biol. Trace Elem. Res*. **2018**:1–7. doi:10.1007/s12011-018-1385-z