Dietary 5-aminolevulinic acid supplementation improves growth performance, nutrient utilisation, iron status and antioxidant capacity of broilers

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ABSTRACT
This study aimed to investigate the effects of dietary supplementation with 5-aminolevulinic acid (5-ALA) on growth performance, blood parameters, iron status, nutrient digestibility, and antioxidant capacity in broilers. A total of 600 one-day-old Arbour Acres broilers were distributed into 5 groups with 6 replicates of 20 birds by a completely randomised design. The birds in the 5 groups were fed the basal diets with 0, 15, 30, 45, and 60 mg/kg 5-ALA, respectively. Dietary supplementation with 60 mg/kg 5-ALA significantly reduced the feed to gain ratio during days 1–21 and days 22–42. Moreover, compared with the control group, dietary supplementation with 30 mg/kg 5-ALA elevated haemoglobin concentrations during days 1–21. Furthermore, dietary supplementation with 45 and 60 mg/kg 5-ALA increased the digestibility of crude protein and decreased serum uric acid levels. At 21 days of age, dietary supplementation with 45 and 60 mg/kg 5-ALA increased liver catalase activity and decreased liver malondialdehyde concentrations, and upregulated the liver mRNA expression of ferritin light chain and nuclear factor erythroid 2-related 2. In addition, the liver mRNA expression of haem oxygenase-1 and divalent metal transporter were upregulated in the 60 mg/kg 5-ALA supplementation group, while the liver mRNA expression of Kelch-like ECH-associated protein 1 was decreased in the 5-ALA supplementation groups. In conclusion, dietary supplementation with 60 mg/kg 5-ALA improved the growth performance, nutrient digestibility, iron status and antioxidant capacity of 21-d-old broilers.

HIGHLIGHTS
- Addition of 60 mg/kg 5-ALA to broiler diets reduced the feed to gain ratio.
- Addition of 45 and 60 mg/kg 5-ALA to broiler diets improved crude protein digestibility and decreased serum uric acid levels.
- 5-ALA exhibited antioxidant properties by enhancing the Nrf2 and HO-1 expression

Introduction
In poultry production, broilers are always subjected to different degrees of oxidative stress including nutrition, disease, high stocking density, hot and humid environments and other factors. Oxidative stress could impair the health and growth performance of broilers and lead to serious economic losses. Previous studies showed that oxidative stress could be prevented by adding feed additives in broiler diets, such as vitamins, sulfur-containing amino acids, and plant extracts (Jang et al. 2014; Jiang et al. 2016; Miao et al. 2021). Thus, it is of great interest to identify a novel and effective feed additive that could modulate the antioxidant system to protect broilers from oxidative stress. 5-Aminolevulinic acid (5-ALA) is an endogenous non-protein amino acid that is involved in haem synthesis (Nishio et al. 2014). Haem has important physiological functions as a prosthetic group of haemoglobin (HGB), myoglobin, catalases (CAT), peroxidase, cytochrome c, cytochrome p450, etc. (Gozzelino et al. 2010; Ogura et al. 2011). 5-ALA has received widespread attention as a novel additive...
for livestock and poultry. Studies have reported that dietary supplementation with 10 or 15 mg/kg 5-ALA increases HGB levels, red blood cell (RBC) counts, and iron (Fe) status in broilers (Chen et al. 2008b; Wang et al. 2011a). In addition, dietary supplementation with 5-ALA enhanced immune responses and anti-inflammatory effects in broilers and Pacific white shrimp (Sato et al. 2012; Pedrosa-Geramnio et al. 2019). However, very few studies exploring the antioxidant effects of 5-ALA in poultry, especially the antioxidant mechanism, have been conducted.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important transcription factor and is demonstrated to be responsible for regulating antioxidant and cellular oxidative stress responses (Iranshahy et al. 2018). Normally, the inactive Nrf2-Kelch-like ECH-associated protein 1 (Keap-1) complex exists in the cytoplasm. Under oxidative stress or specific chemical reagent induction, Nrf2 dissociates from this complex to translocate into the nucleus, where it induces the expression of antioxidant response elements, such as haem oxygenase-1 (HO-1, Huang et al. 2021; Campbell et al. 2021). HO-1 has been shown to induce cytoprotective effects against oxidative injury and inflammatory insults in RAW 264.7 macrophages and zebrafish (Ji et al. 2021). HO-1 is involved in the degradation of haem and generates carbon monoxide, ferrous Fe, and bilirubin, which are also prominent endogenous antioxidant cytoprotectants (Sugiyama et al. 2018). Exogenous 5-ALA administration leads to the upregulation of HO-1 expression in the murine tubular epithelial cells (Liu et al. 2021; Ji et al. 2021). In addition, Zhao et al. (2015) reported that 5-ALA ameliorated tissue injury and cardiomyocyte damage by activating the Nrf2/HO-1 signalling pathway. Based on this information, we hypothesised that dietary supplementation with 5-ALA would improve the antioxidant capacity of broilers by regulating the Nrf2/HO-1 signalling pathway. In this experiment, we investigated the effect of dietary supplementation with 5-ALA on the growth performance, nutrient digestibility, blood characteristics, iron status, and antioxidant capacity of broilers.

Materials and methods

Animals used in this experiment were approved by the Animal Ethics Committee of the Chinese Academy of Agricultural Sciences. All experimental operations complied with the guidelines for animal experiments set by the National Institute of Animal Health (Statement no. AEC-CAAS-20190120).

Animal management and experimental design

A total of 600 one-day-old Arbour Acres broilers were randomly assigned to five dietary treatments, with six replicates per group and 20 birds per replicate (half male and half female). The temperature was set at 32°C for the first 3 days and then decreased by 2°C each successive week until it reached 24°C. The chicken house was kept under 24 h continuous light for the first 3 days, followed by 18 h continuous light and 6 h darkness from days 4 to 42. The birds were provided feed and water ad libitum and vaccinated according to the local vaccination schedule during the feeding trial.

Four different concentrations of 5-ALA (15, 30, 45, and 60 mg/kg; 5-ALA) were produced by adding 5-ALA to the control diet. The 5-ALA used in this study was provided by Challenge Corporation (Beijing, China). The control group was fed a basal diet (0 mg/kg). Diets were free from antibiotics and coccidiostat but were pelleted before feeding. All nutrients contained in the basal diet met or exceeded the feeding standard of China (NY/T 2004) for broilers. The ingredient

Table 1. Ingredient composition and nutrient content of basal diets (as-fed basis).

| Items                  | Start phase | Finish phase |
|------------------------|-------------|--------------|
| Ingredient (%)          |             |              |
| Corn                   | 53.25       | 52.44        |
| Soybean meal           | 28.74       | 18.28        |
| Cottonseed meal        | 3.00        | 6.00         |
| Rapeseed meal          | 2.00        | 4.00         |
| DDGS                   | 5.00        | 10.00        |
| Vegetable oil          | 3.42        | 5.31         |
| Salt                   | 0.32        | 0.32         |
| Calcium hydrogen phosphate | 1.60    | 0.91         |
| Limestone              | 1.48        | 1.62         |
| L-lysine-HCl 79%       | 0.40        | 0.42         |
| DL-methionine, 99%     | 0.33        | 0.27         |
| L-Threonine, 98.5%     | 0.01        | 0.03         |
| Choline chloride, 50%  | 0.20        | 0.15         |
| Premix*                | 0.25        | 0.25         |

Analysed nutrient composition

| Crude protein (%)      | 20.76       | 19.13        |
| Total phosphorus (%)   | 0.67        | 0.55         |
| Total fat (%)          | 6.32        | 9.04         |
| Calcium                | 0.99        | 0.88         |

Calculated nutrient composition

| Metabolizable energy (kcal/kg) | 3000 | 3100 |
| Lysine (%)                  | 1.25  | 1.10 |
| Methionine (%)              | 0.65  | 0.57 |
| Methionine + Cysteine (%)   | 1.00  | 0.90 |
| Threonine (%)               | 0.80  | 0.72 |

* The premix provided the following per kg of diets: vitamin A 8000 U, vitamin D3 1000 U, vitamin E 20 mg, vitamin K3 0.5 mg, vitamin B1 2.0 mg, vitamin B2 8 mg, vitamin B6 3.5 mg, vitamin B12 0.01 mg, pantothenic acid 10 mg, nicotinic acid 35 mg, folic acid 0.55 mg, biotin 0.18 mg, Cu 8 mg, Fe 100 mg, Zn 80 mg, Mn 80 mg, I 0.7 mg.
The basal diets and nutrient compositions are shown in Table 1.

**Growth performance**

After 8 hours of fasting, the body weight (BW) of broilers was measured for each replicate on the morning of d 21 and d 42. Feed intake was recorded on a cage basis. Subsequently, the average daily gain (ADG), average daily feed intake (ADFI), and feed to gain ratio (F/G) were calculated.

**Apparent total tract digestibility**

Broilers aged 28 to 35 d were fed diets mixed with titanium dioxide (0.4%) as an inert marker to determine the total tract nutrient digestibility coefficients of dry matter (DM), gross energy (GE) and crude protein (CP; Short et al. 1996). Fresh excreta samples from each replicate were collected daily for 3 days after a 5-day (d 28–32) adaptation period and then stored at −20°C. Feathers, scales, and feed were removed from the excreta samples. Before chemical analysis, excreta samples were dried at 65°C and then ground to a particle size of 0.45 mm. All feed and excreta samples were analysed for DM. The CP content was determined by the Kjeldahl method (KDY-9830, Ketuo, China). The GE content of the samples was determined in a bomb calorimeter (C2000, IKA, Guangzhou, China) using benzoic acid as the standard. The apparent nutrient digestibility coefficients were calculated using the following formula:

\[ \frac{1-\left(\frac{NE \times Td}{Nd \times TE}\right)}{Nd \times TE} \times 100 \]  

where NE = nutrient concentration in excreta (%), Nd = nutrient concentration in diet (%), Td = titanium dioxide concentration in diet (%), and TE = titanium dioxide concentration in excreta (%).

**Determination of biochemical parameters**

At 21 d and 42 d, two birds (one male and one female) with approximately average weight from each replicate group were euthanized by head-only electrical stunning, and then ten-millilitre blood samples were immediately obtained from the left jugular vein. Two-millilitre blood samples were stored in anticoagulant tubes for the analysis of RBCs and haemoglobin concentration. The remaining blood samples (8 mL) were centrifuged at 1500 xg for 10 min at 4°C. The serum samples were kept at −20°C until analysis.

RBC counts and HGB levels were analysed by a TEK-II mini automatic blood cell analyser (TEK5000P, Tecom Technologies Co., Jiangxi, China). The concentrations of serum total protein (TP), albumin (ALB), globulin (GLB), uric acid (UA), creatinine (CRE), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and Fe; total Fe-binding capacity (TIBC); and total bilirubin (TBIL) were analysed by an automated system (7600 analyser, Hitachi High Technologies Co., Tokyo, Japan) or ELISA plate reader (Multiskan SkyHigh, Thermo Fisher Scientific, Massachusetts, USA) with standard commercial kits following the protocols recommended by the manufacturer (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

**Liver tissue collection and antioxidants analysis**

Liver samples from 12 birds per group (2 subsamples/replicate cage) were taken from the same lobe or side, washed with sterile saline solution to remove blood residue, immediately preserved in liquid nitrogen and then stored at −80°C until analysis.

### Table 2. Specific sequences primers were used for RT-PCR in this study.

| Gene target | Primer sequence (5’ to 3’) | Accession no. | Amplicon size (bp) |
|-------------|---------------------------|---------------|-------------------|
| β-actin     | F: GAGAAATTTGCGTGACATCA   | NM_205518.1   | 152               |
|             | R: CCTGAACCTCTACCTTGCCA   |               |                   |
| HO-1        | F: AGAGAGACAGGCAGATGCAG   | HM237181.1    | 151               |
|             | R: GAGGGAAGGCAGAAGAGAAA   |               |                   |
| FECH        | F: CTGCTCATCACCTCTGTGCC   | NM_204196.1   | 134               |
|             | R: TACGGATCACCAAGGCTTACA  |               |                   |
| Nrf2        | F: ATACAGGCCCTGGAAACCAA   | NM_205117.1   | 143               |
|             | R: GGCTGCAAATGCTGGAAAAA   |               |                   |
| Keap1       | F: TCAACTGGGGTGAGTACGAC   | KU321503.1    | 162               |
|             | R: TCCTGCCCAGTAATCTCTTG   |               |                   |
| FTL         | F: GCCAGCATACTCAAGAACA    | NM_204383     | 137               |
|             | R: GGTCCTATAAGAATACCC     |               |                   |
| DMT         | F: AGCGGTCAACACTATTTCTGC  | NM_001128102.1| 129               |
|             | R: GGTCCTATAAGGCGATGCT    |               |                   |
| CYP1A1      | F: TAAGGGACACATTCGGGAGC   | NM_205147.1   | 184               |
|             | R: CAAGGCCAGCAGTACATACGC  |               |                   |

**Abbreviations:** F, forward primer; R, reverse primer; HO-1, Haem oxygenase-1; FECH, Ferrochelatase; 5-ALA, 5-aminolevulinate dehydratase; Nrf2, nuclear factor erythroid 2-related 2; Keap1, kelch-like ECH-associated protein 1; FTL, ferritin light polypeptide; DMT, divalent metal transporter; CYP1A1, cytochrome P450, family 1, subfamily A.
then kept at \(-70\,^\circ C\) for mRNA and antioxidant capacity analysis. A 10% liver homogenate was prepared using phosphate buffer solution at a weight (g)-to-volume (mL) ratio of 1:9. The homogenate supernatant was obtained by centrifugation (3500 xg) for 10 min at 4\(^{\circ}C\). The protein concentration of the homogenate was determined with the Bradford method.

The levels of total antioxidant capacity (T-AOC), malondialdehyde (MDA), superoxide dismutase (SOD), and CAT were measured in the homogenate supernatant of the live tissue using their respective commercial assay kits (T-AOC, A015-2-1; MDA, A003-1-2; SOD, A001-1-1; CAT, A007-1-1) purchased from Jiancheng Bioengineering Research Institute (Nanjing, China).

**Real-time quantitative PCR analysis**

Total RNA was extracted from liver tissue of broilers at 21 days of age, following the TRIzol method protocol. The quality and concentration of RNA samples were measured in a NanoDrop-2000 spectrophotometer (Thermo Fisher Scientific Co., Waltham, MA). The optical density at 260/280 nm was analysed, and RNA gel imaging detection was performed.

The RNA was reverse transcribed into cDNA with a transcription first strand cDNA Synthesis Kit (Tiangen, China) following the procedure recommended by the manufacturer. Primers were designed and checked for specificity using GenBank from the National Centre for Biotechnology Information. The primers were synthesised by Sangon Biotech Co. Ltd. (Shanghai, China). The primer sequences and accession numbers are listed in Table 2.

The cDNA samples were amplified by real-time quantitative polymerase chain reaction with SuperReal PreMix (Probe) reagents (Tiangen, China). The RT–PCR conditions were as follows: 95\(^{\circ}C\) for 5 min, followed by 40 cycles of 95\(^{\circ}C\) for 10 s and 60\(^{\circ}C\) for 30 s, and a final extension at 72\(^{\circ}C\) for 5 min.

The specificity of qPCR products was evaluated by melting curve analysis. \(\beta\)-actin was utilised as an internal reference gene, broilers fed the basal diet were used as a calibrator in this study, and the relative expression of the target gene was calculated by the \(2^{-\Delta\Delta CT}\) method (Livak and Schmittgen 2001).

**Statistics analysis**

All data were analysed by one-way analysis of variance (ANOVA) with the SPSS 19.0 software package for Windows (SPSS, Chicago, IL). The replicates were considered the experimental unit. Differences in means among treatments were separated by Tukey’s multiple comparisons. \(P < .05\) was used to judge statistical significance. The statistical model is as follows:

\[
Y_{ij} = \mu + \alpha_i + e_{ij}
\]

where \(Y\) = the dependent variables, \(\mu\) = general mean, \(\alpha_i\) = fixed effect of treatment \(i\), and \(e_{ij}\) = random error.

**Results**

**Growth performance**

As shown in Table 3, compared with the control group (0 mg/kg 5-ALA), dietary supplementation with 60 mg/kg 5-ALA significantly \((P < .05)\) reduced the F/G at 1–21 and 22–42 d of age. In addition, dietary supplementation with 60 mg/kg 5-ALA increased \((P < .05)\) the BW of 42-d-old broilers. There were no significant effects on ADG, ADFI or mortality with increasing levels of 5-ALA.

**Table 3. Effects of dietary supplementation with 5-aminolevulinic acid on growth performance of broiler chickens.**

| Items          | 5-ALA level (mg/kg) |
|----------------|---------------------|
|                | 0       | 15      | 30      | 45      | 60      | SEM | P-value |
| 21d BW (g)     | 864     | 873     | 884     | 872     | 893     | 3.633 | .161    |
| ADG (g/d)      | 39.22   | 39.63   | 40.15   | 39.59   | 40.61   | 0.172 | .166    |
| ADFI (g/d)     | 49.84   | 50.32   | 50.73   | 50.34   | 50.06   | 0.237 | .835    |
| F/G (g/g)      | 1.28\(a\) | 1.27\(a\) | 1.26\(a\) | 1.27\(a\) | 1.23\(b\) | 0.004 | <.001   |
| Mortality (%)  | 1.67    | 1.67    | 0.83    | 0.00    | 0.83    | 0.009 | .615    |
| 22–42d         |         |         |         |         |         |      |
| 42d BW, g      | 2528\(a\) | 2557\(a\) | 2586\(ab\) | 2566\(ab\) | 2629\(b\) | 10.98 | .039    |
| ADG (g/d)      | 79.75   | 80.59   | 81.35   | 81.52   | 82.70   | 0.458 | .380    |
| ADFI (g/d)     | 139.24  | 139.64  | 140.41  | 140.06  | 140.79  | 0.653 | .962    |
| F/G (g/g)      | 1.75\(a\) | 1.73\(ab\) | 1.73\(ab\) | 1.72\(ab\) | 1.70\(b\) | 0.005 | .038    |
| Mortality (%)  | 0.93    | 1.85    | 1.85    | 0.93    | 0.93    | 0.012 | .651    |

Note: \(a,b\)Mean values with different superscripts within a row differ significantly

Abbreviations: 5-ALA, 5-aminolevulinic acid; SEM, standard error of the mean; BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F/G, feed to gain.
Table 4. Effects of dietary supplementation with 5-aminolevulinic acid on nutrient utilisation in broiler chickens.

| S-ALA level (mg/kg) | Items | 0  | 15 | 30 | 45 | 60 | SEM | P-value |
|---------------------|-------|----|----|----|----|----|-----|---------|
| Dry matter (%)      |       | 68.61 | 68.91 | 69.07 | 69.40 | 69.11 | 0.121 | .349 |
| Gross energy (%)    |       | 71.12 | 71.70 | 71.79 | 71.58 | 72.14 | 0.139 | .218 |
| Crude protein (%)   |       | 61.88a | 62.01a | 63.30ab | 63.62b | 64.22b | 0.262 | .011 |

Abbreviations: S-ALA, 5-aminolevulinic acid; SEM, standard error of the mean.

Table 5. Effects of dietary supplementation with 5-aminolevulinic acid on the haematological iron status of the blood of broiler chickens.

| S-ALA level (mg/kg) | Items | 0  | 15 | 30 | 45 | 60 | SEM | P-value |
|---------------------|-------|----|----|----|----|----|-----|---------|
| RBC (10^{12}/L)     |       | 2.40 | 2.53 | 2.62 | 2.49 | 2.59 | 0.027 | .089 |
| HGB (g/L)           |       | 94.75a | 99.64ab | 105.92b | 98.25b | 102.10b | 1.018 | .024 |
| Iron (umol/L)       |       | 26.68 | 29.01 | 31.13 | 29.25 | 30.47 | 0.889 | .592 |
| TIBC (umol/L)       |       | 33.77 | 42.70 | 46.52 | 42.26 | 46.84 | 1.638 | .097 |

Abbreviations: S-ALA, 5-aminolevulinic acid; SEM, standard error of the mean; RBC, red blood cells; HGB, haemoglobin; TIBC, total iron binding capacity.

Nutrient utilisation

Table 4 shows the nutrient utilisation of DM, GE and CP measured during d 28 to 35. Compared with the control group, dietary supplementation with 45 and 60 mg/kg S-ALA significantly (P < .01) increased the digestibility coefficient of CP. No significant differences were observed in DM and GE in response to S-ALA supplementation in the diets.

Haematological iron status

As shown in Table 5, at the age of 21 d, the HGB concentrations in the 30 mg/kg S-ALA supplementation group was significantly higher than that of those in the control group; dietary supplementation with S-ALA showed a tendency to improve serum TIBC concentrations (P = .097) and RBC counts (P = .089). However, S-ALA supplementation had no effect on RBC counts, HGB, serum Fe, and serum TIBC concentrations at the age of 42 d.

Serum biochemical parameters

The serum biochemical characteristics are presented in Table 6. At the age of 21 d, a significantly (P < .05) lower UA level was observed in the groups fed 45 and 60 mg/kg S-ALA than in the control; dietary S-ALA supplementation had no significant effects on AST, ALT, CRE, TP, ALB, and TBIL.

Antioxidant indexes of the liver

As indicated in Table 7, compared with the control group, dietary supplementation with 60 mg/kg S-ALA significantly increased (P < .05) T-AOC activities. CAT activity was improved (P < .05) in the 5-ALA-supplementation groups, while MDA concentrations and MDA/T-AOC ratios were reduced (P < .05) in the 45 and 60 mg/kg S-ALA groups in 21-d-old broilers. There were no significant effects on T-AOC, CAT, SOD activity, and MDA concentrations in 42-d-old broilers, while dietary supplementation with 45 mg/kg S-ALA significantly reduced (P < .05) the MDA/T-AOC ratio.

Relative mRNA expression in liver

As shown in Figure 1, compared with the control diet, dietary supplementation with 5-ALA improved (P < .05) the cytochrome P450 family 1 (CYP1A1) mRNA expression levels; dietary supplementation with 30 and 45 mg/kg S-ALA significantly increased (P < .05) the expression level of ferrochelatase (FECH) gene. The expression levels of the ferritin light chain (FTL) and divalent metal transporter (DMT) genes were upregulated (P < .05) when the diet was supplemented with 45 and 60 mg/kg S-ALA. The gene expression of Nrf2 and HO-1 was analysed in the liver (Figure 2). Dietary supplementation with 45 and 60 mg/kg S-ALA significantly decreased (P < .05) the expression level of the Keap1 gene but upregulated the expression of the Nrf2 gene. In addition, dietary supplementation with
60 mg/kg 5-ALA significantly increased the expression level of the HO-1 gene.

Discussion

Previous studies reported that dietary supplementation with 10 or 15 mg/kg had no significant effect on growth performance of broilers (Chen et al. 2008b; Wang et al. 2011a). In our study, dietary supplementation with 15 mg/kg 5-ALA also had no significant effect on growth performance, but dietary supplementation with 60 mg/kg 5-ALA reduced the F/G at 1–21 (3.91%) and 1–42 d (2.86%). Similarly, there was a beneficial effect on BW, ADG, and F/G when weanling

Table 7. Effects of dietary supplementation with 5-aminolevulinic acid on antioxidant capacity in the liver of broiler chickens.

| Items                  | 5-ALA level (mg/kg) | 0   | 15  | 30  | 45  | 60  | SEM | P-value |
|------------------------|---------------------|-----|-----|-----|-----|-----|-----|---------|
| T-AOC (U/mgprot)       | 21d                 | 0.33a| 0.38ab| 0.39ab| 0.45b| 0.57b| 0.029| .026    |
| CAT (U/mgprot)         | 12.68a              | 18.22b| 18.66b| 19.49b| 18.41b| 0.795| .051    |
| SOD (U/mgprot)         | 348.36              | 373.45| 359.07| 376.15| 388.58| 6.246| .275    |
| MDA (nmol/mgprot)      | 7.97b               | 6.95a6b| 6.856b| 5.78a| 5.80a| 0.245| .015    |
| MDA/T-AOC ratio        | 24.12b              | 18.29ab| 17.51ab| 12.84ab| 10.36a| 1.683| .012    |
| T-AOC (U/mgprot)       | 42d                 | 0.34a| 0.37  | 0.38 | 0.50 | 0.41 | 0.029| .364    |
| CAT (U/mgprot)         | 12.92a              | 13.92 | 13.40 | 13.65 | 16.80 | 0.562| .183    |
| SOD (U/mgprot)         | 169.07              | 187.24| 172.26| 176.82| 186.97| 2.823| .116    |
| MDA (nmol/mgprot)      | 12.35               | 8.46  | 8.46  | 8.95  | 9.79  | 0.528| .097    |
| MDA/T-AOC ratio        | 36.29b              | 25.54ab| 20.57ab| 19.62a| 26.00ab| 1.881| .042    |

Abbreviations: 5-ALA, 5-aminolevulinic acid; SEM, standard error of the mean; T-AOC, total antioxidant capacity; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase.

Different superscripts letters within a row indicate significant differences at $P < 0.05$. 

Figure 1. The effects of 5-aminolevulinic acid (5-ALA) supplementation on the mRNA expression of substances related to Fe status, haem synthesis and metabolism in the liver of broilers. DMT, divalent metal transporter; FTL, ferritin light polypeptide; FECH, ferrochelatase; CYP1A1, cytochrome P450, family 1, subfamily A.
pigs were fed a high (1000/kg) level of 5-ALA (Hossain et al. 2016), while dietary supplementation with 5, 10, 15 or 50 mg/kg 5-ALA had no significant effect on the growth performance of weanling pigs (Mateo et al. 2005; Chen et al. 2008a). The above results indicated that dietary supplementation with 5-ALA had a dose-response effect on growth performance, and proper dosage is beneficial to improve growth performance. In addition, dietary supplementation with 60 mg/kg 5-ALA increased the digestibility of CP (2.34%) at 28–35 d, which can be used to explain the positive effect on the growth performance of broilers. Hossain et al. (2016) also demonstrated that weanling pigs fed diets supplemented with 5-ALA (500 or 1000 mg/kg) tended to have increased DM and CP digestibility. However, why CP digestibility was improved by supplementation with 5-ALA could not be clearly elucidated. 5-ALA participates in oxygen metabolism and energy generation via the intermediate product haem, which might explain why CP digestibility was improved by supplementation with 5-ALA.

RBC count and HGB concentration are commonly used to assess iron status because iron concentration is associated with the HGB concentration (Southern and Baker 1982). Previous studies have indicated that dietary supplementation with exogenous 5-ALA increased the RBC count, HGB concentration, and TIBC of laying hens and weaned pigs (Yan and Kim 2011; Wang et al. 2011b). In the current study, dietary supplementation with 30 mg/kg 5-ALA improved HGB concentration at 21 days of age. Similarly, Chen et al. (2008b) reported that the HGB concentration tended to increase with increasing 5-ALA (5, 10, and 15 mg/kg) supplementation in broiler diets. However, a positive correlation did not exist between the HGB concentration and the dosage of 5-ALA in the present study. This may be ascribed to the feedback mechanism of haem synthesis. Haem is essential for the formation of haemoglobin. In turn, haem synthesis is regulated by haem oxidase, ferrochelatase, and 5-ALA synthase, which are reaction limiting enzymes during the formation of HGB (Fujino et al. 2016). As expected, dietary supplementation with 45 and 60 mg/kg 5-ALA upregulated the liver mRNA expression of light chain ferritin (FTL), and supplementation with 60 mg/kg 5-ALA upregulated the liver mRNA expression of divalent metal transporters (DMT). Similarly, the serum TIBC tended to increase with 5-ALA supplementation on d 21. Divalent metal transporters (DMT) play a very important role in maintaining Fe homeostasis by mediating Fe absorption and transport in the small intestine (Hentze et al. 2010). Ferritin and TIBC are important indicators for Fe storage (Harrison and Arosio 1996). These results suggested that 5-ALA, especially at 45 and 60 mg/kg, is beneficial to Fe utilisation of 21-d-old broilers. However, the current results showed that dietary supplementation with 5-ALA had no significant effect on Fe status or haematological profiles of 42-day-old broilers. This discrepancy may be attributed to Fe overload in the grower phase. In the current study, the premix provided 100 mg Fe per kg of basal diets during both feeding phases, while tissue Fe deposition decreases as broilers age. Cao et al. (1996) showed that the Fe deposition in the liver, kidney, spleen and bones of broilers at the third week was less than that in the second week. Chen et al. (2008b) also suggested that 5-ALA supplementation had no significant effect on Fe status when chickens were subjected to Fe overload situations, which is in agreement with the results of the current study.

Figure 2. The effects of 5-aminolevulinic acid (5-ALA) supplementation on the mRNA expression of substance-related antioxidant signalling pathways in the liver of broilers. Nrf2, nuclear factor erythroid 2-related 2; HO-1, haem oxygenase-1; Keap1, Kelch-like ECH-associated protein 1.
UA is a metabolite of proteins and nucleic acids. Therefore, serum UA or excreta UA should be viable response variables to determine the efficiency of nitrogen utilisation in broilers (Donsbough et al. 2010). In our study, the concentration of UA was significantly lower in the 45 and 60 mg/kg 5-ALA groups than in the control group, which is consistent with the results that dietary supplementation with 60 mg/kg 5-ALA improved protein utilisation in broilers. Under normal physiological conditions in animals, serum ALT and AST are maintained at low levels, but dramatic increases in enzyme activity or levels occur in response to tissue injury and inflammation. In our study, dietary 5-ALA supplementation tended to reduce AST and ALT levels. These results indicate that supplementation with 5-ALA is beneficial for improving liver function, which may be related to the anti-inflammatory effect of 5-ALA (Sugiyama et al. 2018). In addition, dietary supplementation with 5-ALA contributed to the upregulation of the expression of CYP1A1, which is one of the cytochrome P450 superfamilies. Cytochrome P450 is generally recognised to have the functions of detoxification, cellular metabolism and homeostasis (Manikandan and Nagini 2018). Accordingly, this may be another reason that 5-ALA is beneficial for improving liver function.

Cellular redox homeostasis is mainly maintained by an elaborate endogenous antioxidant defence system, which includes endogenous antioxidant enzymes such as SOD, CAT, FTL, and transferrin (Poljsak et al. 2013). In our study, dietary supplementation with 45 and 60 mg/kg 5-ALA improved T-AOC and CAT activities of broilers at 21 d. Meanwhile, dietary supplementation with 45 and 60 mg/kg 5-ALA reduced the MDA content and MDA/T-AOC ratio (an indication of antioxidant balance). This decrease reflected the significant decrease in the antioxidant (T-AOC) for use in the protection of cells from free radicals (Attia et al. 2020). In addition, our results showed that dietary supplementation with 45 and 60 mg/kg 5-ALA upregulated the mRNA expression levels of Nrf2 and downregulated the expression of the Keap1 gene. Nrf2 and Keap1 mediate the downstream antioxidant enzyme genes and are considered the most important cellular antioxidant pathways (Nguyen et al. 2009; Balogh et al. 2021). Among the target genes, HO-1 has a cytoprotective effect against oxidative injury and other cellular stresses (Waza et al. 2018; Ishfaq et al. 2019). Studies have reported that 5-ALA can regulate redox homeostasis by inducing the upregulation of HO-1 expression. Sugiyama et al. (2018) reported that exogenous addition of 5-ALA induces HO-1 expression and leads to the downregulation of the expression of NO and some proinflammatory cytokines in raw macrophages. Pedrosa-Geramio et al. reported that 5-ALA upregulated HO-1 gene expression and protected shrimp against pathogens and environmental stress (Pedrosa-Geramio et al. 2019). In this study, dietary supplementation with 60 mg/kg 5-ALA upregulated the mRNA expression of HO-1. Our results are consistent with the results of these studies. The results indicate that dietary supplementation with 60 mg/kg 5-ALA enhances antioxidative status by upregulating the Nrf2/HO-1 signalling pathway.

**Conclusion**

In conclusion, 5-ALA supplementation, especially at the level of 60 mg/kg, improved the growth performance, CP digestibility, Fe status and liver function of broiler chickens. Moreover, 5-ALA exerted its antioxidant effect by enhancing the Nrf2/HO-1 signalling pathway at the transcriptional level.

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**Disclosure statement**

We declare that no competing interests exist.

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