Dietary supplementation of ferrous glycinate improves intestinal barrier function by modulating microbiota composition in Cherry Valley ducks

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ABSTRACT

Ferrous glycinate (Fe-Gly) has been increasingly used as iron fortification in the diets of weaned piglets and broilers, but the effect of Fe-Gly on intestinal barrier function in meat ducks has not been well defined. This study therefore investigated the effect of Fe-Gly on apparent nutrient utilization, hematological indices, intestinal morphological parameters, intestinal barrier function and microbial composition in meat ducks. A total of 672 one-day-old Cherry Valley ducks were randomly divided into 6 treatments (8 replicates for each treatment and 14 ducks for each replicate) and fed diets with 0 (control), 30, 60, 90 and 120 mg/kg Fe-Gly or 120 mg/kg FeSO4 for 35 d. The results showed that diets supplemented with Fe-Gly significantly increased average daily gain (ADG), average daily feed intake (ADFI), hematocrit (HCT), mean cell volume (MCV), the apparent utilization of dry matter (DM) and metabolizable energy (ME), villus height (VH) and villus height-to-crypt depth ratio (V:C) (P < 0.05). Fe-Gly also significantly up-regulated barrier-related genes including zonula occludens-1 (ZO-1), zonula occludens-2 (ZO-2), mucin 2 (MUC2) and lysozyme (LYZ) (P < 0.05), and down-regulated the mRNA expression of claudin-2 (CLDN2) and occludin (OCLN) in the jejunum (P < 0.05). The 16S rRNA sequence analysis indicated that the diet with Fe-Gly had a higher relative abundance of Intestinimonas and Romboutsia (P < 0.05), which have an ability to produce short chain fatty acids (SCFAs), especially butyric acid. It also decreased the relative abundance of pathobiont, including Megamonas, Eubacterium_coprostanoligenes_group and Plebeius (P < 0.05). Additionally, diets supplemented with 120 mg/kg Fe-Gly significantly increased the apparent utilization of DM and ME (P < 0.05) and decreased the relative abundance of Megamonas_unclassified and Bacteroides_unclassified compared with those fed 120 mg/kg FeSO4 (P < 0.05). These results revealed that diets supplemented with Fe-Gly exerted a potent beneficial effect on physical, chemical, immune and microbial barriers, thereby improving the integrity of the intestinal structure, promoting the digestion and absorption of nutrients to a certain extent, and ultimately elevating the growth performance of ducks.

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1. Introduction

China is the world’s largest producer and consumer of pork, but in 2018 the African swine fever virus broke out and spread rapidly across the country, causing a substantial loss to the pig industry, leading to a shortage of pork (Ma et al., 2021). The United Nations Food and Agriculture Organization predicts that poultry meat may be a major substitute for pork in the next few years and become the world’s largest supply of meat (Efremova, 2019). Thus, duck
Ducks were randomly allocated into 6 treatments, 8 replicates for from Sichuan Mianying Duck Industry Co., Ltd. (Mianyang, China).

2.2. Animals, experimental design, and treatments

A total of 672 one-day-old Cherry Valley ducks were purchased from Sichuan Mianying Duck Industry Co., Ltd. (Mianyang, China). Ducks were randomly allocated into 6 treatments, 8 replicates for each treatment and 14 ducks for each replicate. The dietary treatments included A (negative control, 0 mg/kg Fe-Gly), B (30 mg/kg Fe-Gly), C (60 mg/kg Fe-Gly), D (90 mg/kg Fe-Gly), E (120 mg/kg Fe-Gly) and F (positive control, 120 mg/kg FeSO₄) groups. Fe-Gly was purchased from Sichuan Jilongda Biological Technology Co., Ltd. Guanghan, China, and FeSO₄ from Chengdu Shuxing Feed Co., Ltd. Chengdu, China. The basal diet was formulated based on the NRC (1994). The ingredients and nutrient levels of basal diets (air-dry basis) are shown in Table S1. The feed was supplied in pellet form, and the diameter of the pellets was 2 mm from 1 to 14 d of age and 3 mm from 15 to 35 d of age. The iron concentrations of the 6 diets were analyzed to be 67.27, 96.72, 123.05, 151.79, 188.81 and 184.62 mg/kg for the diets from 1 to 14 d of age, and 64.95, 98.00, 128.95, 156.55, 180.69 and 180.08 mg/kg for the diets from 15 to 35 d of age. All ducks were reared in cages (2.0 m × 1.0 m) in a temperature-controlled room and maintained on a 24-h constant light schedule, and allowed free access to feed and water. The living environment of the ducks was in accordance with the animal welfare guidelines.

2.3. Data and sample collection

The weight of the duck and the depletion of feed was determined to calculate the body weight (BW), average daily gain (ADG), average daily feed intake (ADFI) and feed-to-gain ratio (F:G). At the end of the experiment (at 14 or 35 d of age), one duck of the average pen body weight was randomly selected from each replicate (n = 8). Blood samples were collected from the jugular vein at 08:00 after 12 h of fasting. Blood was sampled into 2-mL Vacutest tubes containing K3EDTA anticoagulant. Then, 48 ducks were anesthetized by intravenous injection with sodium pentobarbital (30 mg/kg BW) and slaughtered. The length and weight of the full jejunum were measured. Jejunum segments 1.5 cm in length (midway between the point of entry of the bile ducts and Meckel’s diverticulum) were flushed with saline (0.9% NaCl) and fixed in 100 g/L buffered formalin (pH = 7.0) for histomorphological analysis. Then, jejunal tissue was collected and immediately frozen with liquid nitrogen and stored at −80 °C for RNA isolation. Cecal contents were collected in sterile 1.5-mL tubes and stored at −80 °C DNA isolation for microbial community analysis. The feed offered to the ducks and excreta was sampled and frozen at −20 °C for analysis of apparent nutrient utilization.

2.4. Apparent nutrient utilization

Apparent utilization of nutrients was measured using acid-insoluble ash (AIA) as a marker (Young, 1977). Feed and excreta samples were dried at 65 °C for 48 h in a forced-air oven, then ground and passed through a 1-mm sieve and subjected to chemical analyses. Samples were analyzed for dry matter (DM), crude protein (CP) and ether extract (EE) according to the AOAC International guidelines (Horwitz, 2010). The energy was determined using an adiabatic oxygen bomb calorimeter (Parr 6400 calorimeter, Moline, IL, USA).

2.5. Hematological analyses

On the day of the experiment, the ducks were fasted for 12 h from midnight and blood sampled into 2-mL Vacutest tubes containing K3EDTA anticoagulant. Hematological indices including total red blood cell count (RBC), hematocrit (HCT), hemoglobin (Hb) and mean corpuscular volume (MCV) were measured using an automated blood cell counter (FORCYTE; Oxford Science, Las Vegas, NV, US). Mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were calculated by the formulas:
MCH (pg) = Hb/HCT; MCHC (g/L) = MCH × MCV (Park and Park, 2017).

2.6. Histomorphological analysis

Jejunal segments 1.5 cm in length were removed, flushed gently with saline (0.9% NaCl) and then fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin, and 20 villi per section were examined by a light microscope (Olympus CX31, Tokyo, Japan) (El-Shall et al., 2020). The villus height (VH), crypt depth (CD) and villus height-to-crypt depth ratio (V/C) were determined according to the method of Qin et al. (2020).

2.7. Quantitative real-time PCR

Total RNA was isolated from frozen jejunal tissue using trizol reagent (Takara), according to the manufacturer’s instructions. Real-time quantitative PCR was conducted using the primers listed in Table S2. The PCR system consisted of 5.0 μL of SYBR Green qPCR Mix, 0.2 μL of cDNA, 0.3 μL of each primer, and 4.2 μL of double distilled water in a final volume of 20 μL. The housekeeping gene β-actin served as a control to normalize the mRNA expression level. Relative quantities of mRNA were calculated using the 2^(-DCt) method according to Zhang et al. (2014).

2.8. 16S rRNA gene sequencing and annotation analysis

Total genomic DNA from the cecal contents was extracted using the E.Z.N.A. Stool DNA Kit (D4015, Omega, Inc., USA) according to the manufacturer’s instructions. DNA integrity was checked using a NanoDrop Spectrophotometer. DNA was diluted to 10 ng/μL using sterile ultrapure water and stored at -80°C until measured by PCR (LC-Bio Technology Co., Ltd, Hang Zhou, China). The V3–V4 regions (Escherichia coli position 341 to 806) of the 16S ribosomal DNA genes (Forward primer, 341F: CCTACGGGNGGCWGCAG; Reverse primer, 805R: GACTACHVGGGTATCTAATCC) were amplified by PCR according to the method of Sun et al. (2017). Then, the PCR products were purified by AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by Qubit (Invitrogen, USA). The amplicon pools were prepared for sequencing and the size and quantity of the amplicon library were assessed with an Agilent 2100 Bioanalyzer (Agilent, USA) and with the Library QuantiGraphs were drawn with R package (v3.5.2). Blast was used for sequence alignment and the feature sequences were annotated with the SILVA database for each representative sequence. Other diagrams were implemented using R package (v3.5.2).

2.8. Statistical analysis

All data were analyzed using one-way analysis of variance (ANOVA) and protected Least Significant Difference Test (SPSS 21.0, IBM Corp., Armonk, NY, USA). Data were expressed as the mean, with P < 0.05 considered statistically significant. Estimation of maximum responses to additive dietary iron was done using a quadratic polynomial (QP) regression model for ducks of different ages according to Taschetto et al. (2017). The QP model (Y = β1 + β2 × Fe + β3 × (Fe)^2) had Y as the dependent variable as a function of dietary level of iron, β1 as the intercept, β2 as the linear coefficient and β3 as the quadratic coefficient. The maximum response to iron was defined as iron = -β2/2β3, 120 mg/kg as Fe-Gly group and 120 mg/kg as FeSO4 group were subjected to analysis by T-test. R software was used to perform Metastat analysis to determine the differences in the relative abundance of fecal microbiomes. Pearson correlation analysis (SPSS 21.0, IBM Corp., Armonk, NY, USA) was used to analyze the strength and significance of relationships between cecal microbiota and the tested trait (White et al., 2009).
Results of apparent nutrient utilization are shown in Table 3. The apparent utilization of CP and EE were no different among the Fe-Gly supplementation groups (P > 0.05). The apparent utilization of ME and DM revealed linear and quadratic increases in response to Fe-Gly supplementation in the diet (P < 0.05), with optimal levels of Fe-Gly calculated at 74.01 and 56.17 mg/kg based on the quadratic analysis of the apparent utilization of ME and DM, respectively. In addition, the apparent utilization of ME and DM were significantly elevated in the 120 mg/kg Fe-Gly group compared with the 120 mg/kg FeSO₄ group (P < 0.05).

The effects of Fe-Gly on the length, weight, VH, CD and V:C of the jejunum of Cherry Valley ducks were evaluated, as shown in Table 4 and Fig. 1. Supplementation with 90 mg/kg Fe-Gly significantly increased the weight, VH and V:C compared with those fed the 0 mg/kg Fe-Gly diet (P < 0.05). The length of jejunum was significantly increased by the inclusion of 120 mg/kg Fe-Gly diet in the present study (P < 0.05). Additionally, the length, weight, VH and V:C of the jejunum in the 120 mg/kg Fe-Gly group were higher than that of the 120 mg/kg FeSO₄ group, but there was no significant difference (P > 0.05).

3.3. Effect of Fe-Gly supplementation on apparent nutrient utilization of Cherry Valley ducks

3.4. Effect of Fe-Gly supplementation on the jejunal parameters and morphology of Cherry Valley ducks
3.5. Effect of Fe-Gly supplementation on expression of intestinal barrier-related genes in Cherry Valley ducks

The intestinal barrier-related gene mRNA expression levels of Cherry Valley ducks are presented in Fig. 2. Supplementation with Fe-Gly diets significantly up-regulated the mRNA expression of \textit{ZO-1} and \textit{ZO-2} and down-regulated the mRNA expression of \textit{CLDN 2} \((P < 0.05)\). When compared with the 0 mg/kg Fe-Gly diet, the 120 mg/kg Fe-Gly diets induced a significantly higher \textit{CLDN 1} mRNA level \((P < 0.05)\) (Fig. 2A). In addition, the mRNA levels of \textit{OCLN} in Cherry Valley ducks was significantly downregulated by the Fe-Gly supplementation diet in the present study \((P < 0.05)\). There were also clear changes \((P < 0.05)\) in the mRNA levels of \textit{MUC2} and \textit{LYZ} in response to 60, 90 and 120 mg/kg Fe-Gly supplementation in the diet (Fig. 2B). However, no differences in the intestinal barrier-related gene mRNA levels of Cherry Valley ducks were observed between the 120 mg/kg Fe-Gly group and 120 mg/kg FeSO4 group \((P > 0.05)\).

3.6. 16S rRNA gene sequencing and annotation analysis

After DNA extraction, the hypervariable V3–V4 regions of the 16S ribosomal DNA were enriched in each sample, and subsequent high-throughput analysis generated a total of 2,814,672 raw reads. Each sample produced approximately 82,784 joined tags, which were assembled using PandaSeq v2.8 (min = 55,853, max = 87,828).
Over 78.00% ± 4.90% of the total joined tags from each sample passed quality control and were processed for further analysis (Table S3). Venn diagrams analysis of the high-quality tags yielded 5,151 unique OTU candidates at 97% sequence similarity, and 521 candidates that were shared across all samples (A, B, C, D and E groups) were defined as core OTU. The core OTU comprised approximately 10.12% of the total candidates, while only 528, 885, 654, 679 and 695 OTU were identified uniquely in the A (Control, 0 mg/kg Fe-Gly), B (30 mg/kg Fe-Gly), C (60 mg/kg Fe-Gly), D (90 mg/kg Fe-Gly) and E (120 mg/kg Fe-Gly) groups, respectively (Fig. 3A). Between E (120 mg/kg Fe-Gly) and F (120 mg/kg FeSO₄) groups, Venn diagram analysis of the high-quality tags yielded 2,835 unique OTU candidates at 97% sequence similarity, and 863 candidates that were shared across all samples were defined as core OTU. The core OTU comprised approximately 30.44% of the total candidates (Fig. 3B). Additionally, the microbial diversity in the cecal digesta of Cherry Valley ducks was assessed using the QIIME pipeline, based on the OTU annotation, which identified the top 20 phyla (Fig. 4A and B). The most abundant phylum in the cecal contents of Cherry Valley ducks was Firmicutes, which accounted for approximately 52.32% of all sequences, followed by Bacteroidetes (31.54%) (Table S4A). Among the 277 genera detected, eleven genera had a relative abundance greater than 2.0%, such as Bacteroides, Megamonas, unclassified, Faecalibacterium, Intestimonas, Alistipes, Romboutsia, Fournierella, Ruminococcaceae_UCG-014, Ruminococcaceae_UCG-005 and Eubacterium_coprostanoligenes_group (Table S4B).

3.7. Microbial diversity in the cecal digesta of Cherry Valley ducks

To compare the alpha-diversity (within-sample diversity or an estimate of species richness and evenness) of each sample with differing sequence counts/sampling efforts directly, we rarified the data using QIIME. Rarefaction curve analysis indicated that the sequencing depth and samples were sufficient to wholly capture the diversity present, as displayed by the microbial diversity index Chao1 (Fig. 5). In addition, we further used Chao1, Shannon, Simpson and Observed_ottus estimator between different groups to evaluate the cecal digesta diversity of Cherry Valley ducks affected by Fe-Gly or FeSO₄ supplementation. As shown in Fig. 6, there was a trend toward increased alpha diversity in the Fe-Gly supplementation groups compared with those of the control, but these differences did not significantly affect the species-level microbial diversity as assessed using Chao1 (P = 0.13) and Observed_ottus (P = 0.13). However, the Shannon (P = 0.042) and Simpson (P = 0.029) diversity index showed a significant increase in the diversity of the B and E groups compared with that of the NC group. To compare the cecal microbiome profiles of the different groups, we performed a beta diversity analysis by hierarchical clustering, principal coordinates analysis (PCoA), and non-metric multidimensional scaling analysis (NMDS). The result revealed that there was a separation (PCoA-PC1:11.73% vs PC2:7.9%) in the microbial composition of the A group and the B, C, D, E, F groups (Fig. 7A). NMDS also displayed distinct diversity differences between the groups (Stress = 0.16) (Fig. 7B).

3.8. Fe-Gly supplementation changed the composition of the cecal microbiota

As shown in Table S5A and Fig. 8A, a total of 12 genera displayed a significant difference among the Fe-Gly supplementation groups. In detail, compared with the NC group, the genus-taxa level of Olsenella and Desulfovibrionaceae_unclassified were increased, and Prevotellaceae_NK3B31_group, Megamonas, Bacteroides and Eubacterium_coprostanoligenes_group were decreased (P < 0.05) in the Fe-Gly supplementation groups. Additionally, the 30 mg/kg Fe-Gly group significantly increased the genus-taxa level of Fournierella and Intestimonas (P < 0.05). The 60 mg/kg Fe-Gly group significantly increased the genus-taxa level of Romboutsia and Intestimonas (P < 0.05). Dietary 90 mg/kg Fe-Gly supplementation significantly increased the genus-taxa level of Faecalibacterium (P < 0.05). And dietary 120 mg/kg Fe-Gly supplementation significantly increased the genus-taxa level of Fournierella, Intestimonas and Subdoligranum, and decreased the genus-taxa level of Alistipes (P < 0.05). Furthermore, the 120 mg/kg Fe-Gly group significantly increased the genus-taxa level of Prevotellaceae, Intestimonas and Subdoligranum, and decreased the genus-taxa level of Megamonas, Subdoligranum and Alistipes compared with 120 mg/kg FeSO₄ group (P < 0.05). At the species level, a total of 17 species displayed a significant difference among the Fe-Gly supplementation groups (P < 0.05) (Table S5B and Fig. 8B). Among them, the species-taxa level of Olsenella_unclassified and Desulfovibrionaceae_unclassified were increased, and the species-taxa level of Plebeius, Megamonas_unclassified, Prevotellaceae_NK3B31_group_unclassified and Eubacterium_coprostanoligenes_group_unclassified were decreased.
in the Fe-Gly supplementation groups compared with the NC group ($P < 0.05$). In addition, there were also obvious changes ($P < 0.05$) in the species-taxa level of Alistipes_unclassified, Fusobacterium_unclassified, Prevotellaceae_NK3B31_group_unclassified, Barnesiaceae, Bacteroides_unclassified and Megamonas_unclassified between the 120 mg/kg Fe-Gly group and 120 mg/kg FeSO₄ group.

3.9. Correlations of cecal microbiota with the nutrient apparent utilization

As shown in Fig. S1, to further identify genera that significantly correlated with the nutrient apparent utilization of Cherry Valley ducks, we used the Pearson correlation test and found that the relative abundance of Romboutsia correlated positively ($P < 0.05$) with the apparent utilization of ME and DM. The apparent utilization of ME displayed a strong negative correlation ($P < 0.01$) with relative abundance of Megamonas and Eubacterium_coprostanoligenes_group. At the species level (Fig. S2), the apparent utilization of ME and DM displayed a strong positive correlation with the relative abundance of Romboutsia_unclassified ($P < 0.01$) and a negative correlation with the relative abundance of Plebeius ($P < 0.05$). The apparent utilization of ME also correlated negatively with the relative abundance of Megamonas_unclassified and Eubacterium_coprostanoligenes_group_unclassified ($P < 0.01$).
3.10. Correlations of cecal microbiota with hematological indices

At the genus level (Fig. S1), the relative abundance of *Megamonas* correlated negatively with HCT and MCV ($P < 0.01$). Additionally, HCT displayed a strong positive correlation with the relative abundance of *Subdoligranulum* ($P = 0.019$) and a negative correlation with the relative abundance of *Alistipes* ($P = 0.010$). MCV correlated negatively with the relative abundance of *Bacteroides* ($P = 0.043$). At the species level (Fig. S2), HCT and MCV showed significantly negative correlations with the relative abundance of *Megas微微monas_unclassifed* ($P < 0.01$) and *Plebeius* ($P < 0.05$). HCT also correlated positively with the relative abundance of *Bacteroides_unclassified* ($P < 0.01$) and *Intestinimonas_unclassified* ($P < 0.05$).

3.11. Correlations of cecal microbiota with jejunal histomorphology

As shown in Fig. S2, VH and V:C showed significant positive correlations with the relative abundance of *Intestinimonas* ($P < 0.05$), which correlated negatively with the value of CD at the genus level ($P < 0.01$). At the species level (Fig. S2), VH and V:C showed significantly positive correlations with the relative abundance of *Faecalibacterium_unclassified* ($P < 0.05$) and negative correlations with the relative abundance of *Prevotellaceae_NK3B31_group*.
Fig. 7. Microbial diversity indices in the cecal microbiome. (A) The principal coordinates analysis (PCoA); (B) the (non-metric) multi-dimensional scaling (NMDS). Each point in the figure represents a sample. Capital letters from A to E refer to 0, 30, 60, 90 and 120 mg/kg ferrous glycinate (Fe-Gly) groups, respectively, and F refers to 120 mg/kg FeSO₄ group.

Fig. 8. Community bar-plots analysis shows relative abundance of cecal microbiota (A) at the genus and (B) species levels in each group. Capital letters from A to E refer to 0, 30, 60, 90 and 120 mg/kg ferrous glycinate (Fe-Gly) groups, respectively, and F refers to 120 mg/kg FeSO₄ group.
unclassified ($P < 0.05$). Lastly, CD displayed a significantly negative correlation with the relative abundance of *Intestinimonas_unclassified* ($P < 0.01$).

### 3.12. Correlations of cecal microbiota with expression of intestinal barrier-related genes

The correlations of cecal microbiota with expression of intestinal barrier-related genes in Cherry Valley ducks are presented in Fig. S1 and Fig. S2. At the genus level, the mRNA expression of *ZO-1*, *ZO-2* and *CLDN1* showed significantly negative correlations ($P < 0.05$) with the relative abundance of *Bacteroides* and *Eubacterium_coprostanoligenes_group*. The bacterial abundance of *Intestinimonas* correlated positively ($P < 0.05$) with the mRNA expression of *ZO-2*, *CLDN1* and *LYZ*, and correlated negatively ($P < 0.05$) with the mRNA expression of *CLDN2*. *LYZ* mRNA level showed significantly positive correlations with the relative abundance of *Intestinimonas* ($P < 0.05$) and negative correlations ($P < 0.05$) with the relative abundance of *Meganonas* and *Eubacterium_coprostanoligenes_group* (Fig. S1). At the species level, the mRNA expression of *ZO-1*, *ZO-2* and *CLDN1* showed significantly negative correlations ($P < 0.01$) with the relative abundance of *Plebeius*. The relative abundance of *Intestinimonas_unclassified* correlated positively ($P < 0.05$) with the mRNA expression of *ZO-2*, *CLDN1*, *MUC2* and *LYZ*, and correlated negatively ($P < 0.05$) with the mRNA expression of *CLDN2*. Additionally, expression of *OCLN* mRNA displayed significantly positive correlations ($P < 0.01$) with the relative abundance of *Plebeius*. *LYZ* mRNA level also displayed significantly negative correlations ($P < 0.01$) with the relative abundance of *Meganonas_unclassified* and *Eubacterium_coprostanoligenes_group_unclassified* (Fig. S2).

### 4. Discussion

In recent years, the poultry industry has developed rapidly, and the growth rate of poultry has also increased greatly since the introduction of African swine fever (Woonwong et al., 2020). However, the standard requirements of trace minerals in poultry have not been adjusted according to present conditions, especially in meat ducks with the current nutritional feed parameters for duck being referenced from that of chicken. Ma et al. (2014) showed that the amount of iron recommended by the NRC (1994) cannot meet the rapid growth demands of poultry. Therefore, iron supplementation is necessary to meet these requirements. Our study showed that 90 mg/kg of dietary Fe-Gly supplementation significantly increased the BW, ADG and ADFI of ducks, possibly because Fe-Gly has a lower molecular weight and therefore better absorption, which has a positive effect on the growth performance of ducks (Kulkarni et al., 2011). Similar results were reported that the growth performance of broilers was improved by Fe-Gly supplementation (Ma et al., 2012). Additionally, we discovered that ducks fed Fe-Gly displayed no significant difference in growth performance compared to those fed FeSO₄, which may be due to duck growth being unaffected by iron source under conditions of sufficient of iron intake (Wang et al., 2008).

Erythrocyte precursors in bone marrow need to use large amounts of iron from blood to synthesize HB to meet the requirements of rapid animal growth (Lipinski et al., 2010). Xie et al. (2019) reported that about 60% to 80% of iron in the animal is used to synthesize HB and RBC. Therefore, researchers usually use HB level as a reliable indicator to reflect the iron status of animals (Abbasi et al., 2015; Shi et al., 2015). In addition, HCT and RBC are closely related to HB and are also used as markers to evaluate biological responses to iron (Tako et al., 2010). The NRC (2012) standard stipulates that when the HB level is 90, 80 and 70 g/L, this indicates sufficient iron levels, borderline anemia and that the body is in an anemic state, respectively. In our experiment, the values of HB, RBC, HCT, MCV, MCH and MCHC were within physiological limits. Additionally, HCT and MCV were affected by supplementing diets with Fe-Gly, consistent with Ma et al. (2012) who observed Fe-Gly improved hematological indicators in the feeding of broilers. However, no differences in all hematological parameters were observed between Fe-Gly and FeSO₄, which also may be attributed to the duck having sufficient iron intake (Wang et al., 2008).

There is little information on the effects of the Fe-Gly supplementation on apparent utilization in poultry. In this study, the Fe-Gly had a positive effect on the utilization of ME and DM, suggesting that more nutrients were absorbed in the gut and may explain the significant increase in ADG and ADFI of ducks. In addition, we also observed the apparent utilization of ME and DM was significantly increased in the 120 mg/kg Fe-Gly group compared with the 120 mg/kg FeSO₄ group. The reason may be that glycine is easily soluble in water and has the lowest molecular weight of all the amino acids (Kulkarni et al., 2011). In theory, Fe-Gly, iron chelated by glycine, is more easily passed through the small intestinal epithelial cells and absorbed. The normal development of microarchitecture in the small intestine is the foundation for promoting the digestion and absorption of nutrients, which plays an important role in an individual's growth and development. The value of VH and ADG effects is more significant as a measure to reflect gross intestinal morphology; a higher VH value and lower CD enable the intestines to have a stronger ability to digest and absorb nutrients (Jia et al., 2010). In our study, an increase in length, height, VH and V/C and a decrease in CD of ducks were observed following Fe-Gly supplementation and indicated an enhanced absorptive capacity and intestinal health. This may explain why Fe-Gly had a positive and significant effect on the apparent utilization of ME and DM in ducks. Moreover, the jejunal parameters and morphology of the Fe-Gly supplementation group were better compared with the FeSO₄ group. This is in agreement with recent observations in piglets fed complexed glycines (Pei et al., 2020). However, the specific mechanism is currently unclear.

As we know, the animal intestine is the organ with the largest surface area in contact with the external environment. In addition to digestion and absorption of nutrients, it is also an important barrier to prevent pathogenic substances from entering the animal body (Wang et al., 2013). The intestinal barrier includes physical, chemical and immune barriers (Anderson et al., 2012). Among them, the integrity and function of the intestinal physical barrier is primarily maintained by the tight junction structure, which is a multi-protein complex including the occludin family, the claudin family and the zonula occludens family (Camara-Lemarry et al., 2018). The chemical barrier is composed of various substances in the intestine, such as mucus, bile and digestive enzymes. Among them, the main component of mucus is mucoprotein, which can effectively prevent the invasion of bacteria or toxins (Birchenough et al., 2015). *LYZ* is an important part of the intestinal immune barrier (Wang et al., 2016). Studies have shown that disruption of the intestinal barrier function led to an increase in cell bypass permeability, allowing pathogenic substances and intestinal bacteria from the intestinal lumen to enter the animal (Guo et al., 2015; Chen et al., 2015). In the present study with meat ducks, Fe-Gly supplementation significantly increased the mRNA expression of *ZO-1*, *ZO-2*, *MUC2* and *LYZ*, and down-regulated the mRNA expression of *CLDN2* and *OCLN*. Therefore, we hypothesized that the Fe-Gly might improve intestinal physical, chemical and immune barrier functions of ducks via an as-yet-unidentified mechanism. Additionally, no differences in the expression of any intestinal barrier-related genes in Cherry Valley ducks were observed between Fe-Gly group and FeSO₄ group.

The microbial barrier formed by the gut microbiota is also a critical constituent of the intestinal barrier function besides the

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A total of 2,191,190 high quality valid tags were obtained across all samples, and the sequence size of each sample was 64,447, which was greater than those reported in previous studies of Cherry Valley ducks (Shi et al., 2020). Clustering analysis revealed that the Firmicutes and Bacteroidetes were the most dominant phyla among the total sequences. This finding was consistent with studies conducted by He et al. (2019). Additionally, in this study, the composition and structure of the cecal microbial community was affected by Fe-Gly supplementation. At the genus level, the abundance of Intestinimonas and Romboutsia increased, and the abundance of Megamonas and Eubacterium coprostanoligenes_group decreased in the cecal digesta of ducks fed diets with Fe-Gly supplementation. Additionally, the 120 mg/kg Fe-Gly group significantly decreased the genus-taxa level of Megamonas compared with the 120 mg/kg FeSO4 group. Our research found that, at the species level, the abundance of Intestinimonas_unclassified and Romboutsia_unclassified increased, whilst the abundance of Plebeius and Megamonas_unclassified decreased in the cecal digesta of ducks fed diets with Fe-Gly supplementation. Intestinimonas produced butyric acid to maintain the integrity of the intestinal tract and enhance the barrier function of the damaged intestinal tract (Bui et al., 2015). Recent studies have revealed that Romboutsia has an ability to produce SCFAs, especially butyric acid (Qin et al., 2021), which is considered to have beneficial effects for gut health through immunoregulatory effects and regulation of intestinal homeostasis (Wang et al., 2020). Yachida et al. (2019) reported that the relative abundance of Megamonas correlated with colorectal cancer. However, very few studies have focused on the relationship of Eubacterium coprostanoligenes_group. Plebeius belongs to the genus Bacteroides and is also a colorectal cancer-associated pathobiont (Liu et al., 2021). Based on the above research results, it is suggested that ferrous glycinate can change the composition of the intestinal microflora and enhance the microbial barrier function of meat ducks. Interestingly, we found that these cecal microbiota have a certain degree of positive or negative correlations with duck characteristics, including apparent nutrient utilization, hematological indices, intestinal morphology and expression of intestinal barrier-related genes, indicating that the microbial, immune, chemical and physical barriers have a complex and beneficial interaction with each other in order to maintain intestinal homeostasis (Maloy and Powrie, 2011). Disruption or alteration of one of them can lead to changes in the others (Peterson and Artis, 2014).

Given that Fe-Gly exerts a potent beneficial effect on intestinal microbial barrier, we believe it also has great potential to modulate the physical, chemical and immune barriers. In this way, it can improve the integrity of the intestinal structure, promote the digestion and absorption of nutrients, and ultimately improve the growth performance of meat ducks.

5. Conclusions

We demonstrated that Fe-Gly supplementation in the diet could enhance barrier function of Cherry Valley ducks. Dietary supplementation of 96.85 to 106.30 mg/kg Fe-Gly and 56.17 to 74.01 mg/kg Fe-Gly were suggested to improve growth performance and apparent nutrient utilization of Cherry Valley ducks, respectively. Significant characteristics including improved apparent nutrient utilization, increased hematological indices, improved intestinal morphological parameters, changed expression of intestinal barrier-related genes and altered microbial composition were identified and influenced by supplementing dietary Fe-Gly. Additionally, we found that these significantly changed cecal microbiota displayed positive or negative correlations with certain duck characteristics. These results also strongly indicated that Fe-Gly improved the integrity of the intestinal structure, promoted the digestion and absorption of nutrients, and ultimately increased the growth performance of ducks by exerting a potent beneficial effect on the physical, chemical, immune and microbial barriers. However, the exact mechanism needs further investigation.

Declarations of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix. supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.j.aninu.2022.07.007.

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