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Original Research Article

# Effect of riboflavin deficiency on intestinal morphology, jejunum mucosa proteomics, and cecal microbiota of Pekin ducks

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ABSTRACT

This study was to determine the effects of riboflavin deficiency (RD) on intestinal development, jejunum mucosa proteome, cecal short-chain fatty acids (SCFA) profiling, and cecal microbial diversity and community of starter Pekin ducks. Male white Pekin ducks (1 d old, $n = 240$) were allocated into 2 groups, with 12 replicates and 10 birds per replicate in each group. For 21 d, all ducks had ad libitum access to either an RD or a riboflavin adequate (control, CON) diet, formulated by supplementing a basal diet with 0 or 10 mg riboflavin per kg of diet, respectively. Compared to the CON group, growth retardation, high mortality, and poor riboflavin status were observed in the RD group. Furthermore, RD reduced the villus height and the ratio of villus height to crypt depth of jejunum and ileum ($P < 0.05$), indicating morphological alterations of the small intestine. In addition, dietary RD enhanced relative cecum weight and decreased cecal SCFA concentrations ($P < 0.05$), including propionate, isobutyrate, butyrate, and isovalerate. The jejunum mucosa proteomics showed that 208 proteins were upregulated and 229 proteins were downregulated in the RD group compared to those in the CON group. Among these, RD mainly suppressed intestinal absorption and energy generation processes such as glycolysis and gluconeogenesis, fatty acid beta oxidation, tricarboxylic acid cycle, and oxidative phosphorylation, leading to impaired ATP generation. In addition, RD decreased the community richness and diversity of the bacterial community in the cecum of ducks. Specifically, RD reduced the abundance of butyrate-producing bacteria in the cecum ($P < 0.05$), such as *Eubacterium coprostanoligenes*, *Prevotella* and *Faecalibacterium*. Dietary RD resulted in growth depression and intestinal hypofunction of Pekin ducks, which could be associated with impaired intestinal absorption and energy generation processes in intestinal mucosa, as well as gut microbiota dysbiosis. These findings contribute to our understanding of the mechanisms of intestinal hypofunction due to RD.



## 1. Introduction

Riboflavin is a precursor of 2 coenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The coenzymes of this vitamin participate in many biological redox reactions, such as tricarboxylic acid (TCA) cycle, fatty acid oxidation, electron transport chain, and amino acid degradation (Thakur et al., 2017; Saedisomeolia and Ashoori, 2018; Suwannasom et al., 2020; Olfat et al., 2022). The importance of riboflavin is highlighted by the adverse effects of riboflavin deficiency (RD) in mammals, including growth depression, dermatitis, anemia, gastrointestinal tract disorders, and glossitis (Saedisomeolia and Ashoori, 2018; Thakur et al., 2017; Olfat et al., 2022). Additionally, in poultry studies, RD

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results in growth retardation, dermatosis, and high mortality in chicks (Chung and Baker, 1990; Cogburn et al., 2018) and ducks (Tang et al., 2013, 2015, 2017, 2019; Zhang et al., 2021).

Riboflavin has been extensively demonstrated to be able to maintain the structure of intestinal integrity and maintain the gastrointestinal functionality of animals (Williams et al., 1995, 1996a, 1996b; Yates et al., 2001, 2003). Intestinal hypofunction was shown to be a major consequence of RD in rats. Dietary RD induced at weaning significantly changed the morphology and cytokinetics of the small intestine in rats (Williams et al., 1995, 1996a, 1996b; Yates et al., 2001, 2003), impairing the normal increase in villus number and leading to an adaptive increase in length of villi and depth of crypts (Williams et al., 1995, 1996a). Furthermore, 3 intestinal cell models of riboflavin depletion inhibited cell growth and resulted in cell cycle arrest in mitosis in vitro (Nakano et al., 2011; Lee et al., 2013).

Riboflavin could modulate multiple pathways for the maintenance of gastrointestinal function. First, riboflavin has been shown to enhance iron absorption (Powers et al., 1993), and RD can significantly increase the rate of gastrointestinal iron loss as well as decrease the mobilization of iron from stores (Adelekan and Thurnham, 1986; Powers et al., 1983, 1988, 1991). Second, riboflavin depletion leads to impaired energy generation (Lee et al., 2013) and proliferation in intestinal cells (Nakano et al., 2011). Our previous findings in ducks also support this implication that RD impaired liver energy generation processes, including fatty acid beta oxidation, TCA cycle and electron transport chain (Tang et al., 2017, 2019). Third, riboflavin is involved in the antioxidant system, and RD could cause intestinal oxidative stress both in vitro (Lee et al., 2013) and in vivo (Tang et al., 2014; Olfat et al., 2022). Furthermore, riboflavin status has been shown to modulate the gut microflora of animals, and RD could induce gut microbiota dysbiosis (Biagi et al., 2020; Pan et al., 2020, 2021; Zhu et al., 2022). Dietary RD induces genomic instability of esophageal squamous cells and esophageal epithelial atrophy by modulating the gut microbiota in rats (Pan et al., 2020, 2021). Supplementation with 100 mg/kg riboflavin in the diet significantly modulated the cecal microbiota of broilers, increasing the abundance of health-promoting bacterial groups, including *Bifidobacterium*, resulting in boosted production of butyrate in the cecal environment compared to riboflavin-adequate birds (Biagi et al., 2020).

Therefore, dietary RD could also affect the intestinal development of ducks. However, the effects of dietary RD on intestinal health in ducks, as well as the underlying molecular mechanisms, have not been well characterized. Therefore, the objectives of the present study were to investigate the effects of RD on intestinal development, jejunum mucosa protein alterations, and cecal short-chain fatty acids (SCFA) profiling, and cecal microbiota of starter Pekin ducks, using proteomic and 16S rRNA gene amplicon sequencing approaches.

## 2. Materials and methods

### 2.1. Animal ethics statement

All experimental procedures were approved by the Animal Welfare Committee of Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, and performed according to the guidelines for animal experiments established by the National Institute of Animal Health.

### 2.2. Animals, diets, and experimental design

Male white Pekin ducks (*Anas platyrhynchos;* 1 d old, $n = 240$) were obtained from the Pekin duck breeding center (Chinese Academy of Agricultural Sciences) and randomly allocated to 24 raised plastic-floor pens with 10 birds per pen. All ducks were assigned to 2 experimental groups, each containing 12 replicates with 10 birds per replicate. From hatch to 21 d of age, the ducks had ad libitum access to water and either an RD or a control diet (CON). During this interval, there was continuous light. The temperature was kept at 33 °C from 1 to 3 d of age, and then gradually reduced to approximately 25 °C at 14 d of age.

The riboflavin-deficient basal diet was formulated in line with the Ministry of Agriculture of China (2012), containing only 1.69 mg riboflavin per kg of diet (Table 1). The RD and CON diets were produced by supplementing this basal diet with 0 and 10 mg/kg diet, respectively, of crystalline riboflavin (purity, 99%; Sigma–Aldrich, St. Louis, MO, USA). The riboflavin concentration of the CON diet (11.69 mg/kg) met NRC (1994) requirements for starter ducks (4 mg/kg) and Ministry of Agriculture of China (2012) (10 mg/kg).

### 2.3. Data and sample collection

At 21 d of age, after overnight fasting, the ducks and residual diet from each pen were weighed to determine average daily weight gain (ADG) and average daily feed intake (ADFI). ADFI was corrected for mortality. One duck from each pen was randomly selected, and blood was collected from a wing vein into heparin sodium-containing tubes, centrifuged at 1,500 × *g* for 10 min, and stored at −20 °C. Thereafter, these ducks were euthanized by $CO_2$ inhalation. The breast meat, leg meat, abdominal fat, small intestine, and cecum were removed from the carcasses and weighed. The intestinal sections were divided into the duodenum, jejunum, and ileum. They were rinsed with physiological saline, cut into 1 cm length segments, and fixed in 10% neutral formalin for histological analysis. Jejunum mucosa from the remaining segment was

**Table 1**
Composition of riboflavin-deficient basal diet for Pekin ducks from hatch to 21 d of age (%, as-fed basis).

| Item | Content |
|---|---|
| Ingredients | |
| Corn | 62.95 |
| Soybean meal | 33.30 |
| Limestone | 0.80 |
| Dicalcium phosphate | 1.50 |
| Vitamin and trace mineral premix[1] | 1.00 |
| Sodium chloride | 0.30 |
| DL-Methionine | 0.15 |
| Total | 100.00 |
| Calculated nutrient levels | |
| Metabolizable energy[2], MJ/kg | 12.22 |
| Crude protein | 20.02 |
| Calcium | 0.93 |
| Nonphytate phosphorus | 0.36 |
| Lysine | 1.11 |
| Methionine | 0.45 |
| Methionine + cysteine | 0.79 |
| Threonine | 0.83 |
| Tryptophan | 0.22 |
| Arginine | 1.38 |
| Riboflavin[3], mg/kg | 1.69 |

[1] Supplied per kilogram of diet: Cu ($CuSO_4 \cdot 5H_2O$) 10 mg, Fe ($FeSO_4 \cdot 7H_2O$) 60 mg, Zn (ZnO) 60 mg, Mn ($MnSO_4 \cdot H_2O$) 80 mg, Se ($NaSeO_3$) 0.3 mg, I (KI) 0.2 mg, choline chloride 1,000 mg, vitamin A (retinyl acetate) 10,000 IU, vitamin $D_3$ (cholecalciferol) 3,000 IU, vitamin E (DL-α-tocopheryl acetate) 20 IU, vitamin $K_3$ (menadione sodium bisulfate) 2 mg, thiamin (thiamin mononitrate) 2 mg, pyridoxine hydrochloride 4 mg, cobalamin 0.02 mg, nicotinic acid 50 mg, calcium-D-pantothenate 20 mg, folic acid 1 mg, biotin 0.2 mg.

[2] The value was calculated according to the AME of ducks (Ministry of Agriculture of China, 2012).

[3] The value was based on high performance liquid chromatography.

obtained as described previously (Tan et al., 2014). Jejunum mucosa and cecal content were sampled, snap frozen in liquid nitrogen, and stored at −80 °C until further analysis.

### 2.4. Riboflavin content

The riboflavin concentrations in feed, plasma, and liver were determined by reversed-phase high performance liquid chromatography (HPLC) according to the methods described previously (Tang et al., 2013, 2014). Before HPLC analysis, feed and plasma samples were prepared according to the method described previously (Britton et al., 2003; Petteys and Frank, 2011), while liver samples were prepared according to the method for animal tissue described previously (Batey and Eckhert, 1990). The peak was identified and quantified using pure authentic standards (Sigma−Aldrich, St. Louis, MO, USA).

### 2.5. Intestinal morphology assessment

The duodenum, jejunum, and ileum sections were embedded in paraffin, transversely sectioned (4 μm thick) and stained with hematoxylin and eosin following deparaffinization and dehydration. Intestinal tissues and structures were observed using a BH2 Olympus microscope (Olympus, Tokyo, Japan) and analyzed using an image analysis system (Olympus 6.0). Villus height, crypt depth, and the ratio of villus height to crypt depth were assessed following the method described previously (Tan et al., 2014).

### 2.6. Jejunum mucosal proteomics

Six jejunum mucosal samples (3 biological replicates per group) were used to conduct the isobaric tags for relative and absolute quantification (iTRAQ) assays. Proteins were extracted and digested as previously described (Tang et al., 2019). Each digested sample was labelled with iTRAQ 8-plex reagents (AB Sciex, Foster City, CA, USA) according to the manufacturer's instructions. The RD samples were labelled with iTRAQ tags 113, 114, and 117, and the CON samples were labelled with tags 118, 119, and 121. Labelled samples were mixed and fractionated into 20 fractions by HPLC (DIONEX Ultimate 3000 BioRS, Thermo Fisher, Waltham, MA, USA) using a Durashell C18 column (5 μm, 100 Å, 4.6 × 250 mm). The LC-electrospray ionization-MS/MS analysis was conducted with a Triple TOF 5600 plus system (AB SCIEX, Framingham, MA, USA). The original MS/MS file data for identification and quantitation were analyzed against the database *UniProt_Mallard_8839* using ProteinPilot Software version 4.0 (AB SCIEX). To minimize the false discovery rate (FDR), a threshold for protein identification was applied. Only unique peptides with a confidence >95% were included in the iTRAQ labelling quantification and used for further analysis.

For analysis of proteomic results, the relative expression of identified proteins was based on the ratio of the reporter ions of the peptides between the two groups (RD versus CON). A protein was considered differentially expressed when it had both a fold change (FC) > 1.5 and *P*-value < 0.05.

### 2.7. Cecal content SCFA analysis

The concentrations of SCFA in cecal content were determined by a gas chromatography system (VARIAN CP-3800, Varian, Palo Alto, CA, USA; Capillary Column 30 m × 250 μm × 0.25 μm film thickness) according to the methods described previously (Hao et al., 2021).

### 2.8. Cecal microbiological analysis

Cecal content samples ($n = 12$) were used for total genomic DNA extraction using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Using genomic DNA with the required purity and concentration as templates, the V3−V4 region of 16S rDNA was amplified by polymerase chain reaction (PCR) using primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Purified amplicons were pooled in equal amounts and paired-end sequenced (2 × 250 bp) on an Illumina MiSeq platform at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). For sequence processing, the raw reads were demultiplexed and quality-filtered by the QIIME2 pipeline (version 1.9.1). The potential chimeric sequences were discarded using the UCHIME algorithm. The available sequences were clustered into operational taxonomic units (OTUs) according to 97% similarity against the SILVA database. Four indices (ACE, Chao1, Shannon, and Simpson) of α-diversity were computed to analyze the quantitative complexity of species diversity for each sample. α-Diversity analysis and β-diversity analysis were performed using principal coordinate analysis (PCoA) based on unweighted UniFrac distances. The significant distinction of microbial communities was estimated by ANOSIM based on unweighted UniFrac distances. The relative abundance of bacteria at the family level was analyzed by Student's *t*-test between 2 groups. The linear discriminant analysis (LDA) effect size (LEfSe) method was performed to identify the significantly abundant taxa (phylum to genera) of bacteria between the 2 groups (LDA score > 4, $P < 0.05$).

### 2.9. Statistical analyses

All data were analyzed by Student's *t*-test, $P < 0.05$ was considered significant and variability was reported as the standard deviation. The replicate pen of 10 ducks for growth performance or 1 duck for other indices served as the experimental unit. These statistical analyses were conducted using SAS software (Version 9.2; SAS Institute Inc., Cary, NC, USA).

## 3. Results

### 3.1. Mortality, growth performance, carcass traits, and relative organ weight

The mortality of RD ducks was greater than the CON birds ($P < 0.001$; Table 2). Compared to the CON group, ADG and ADFI were decreased in the RD group ($P < 0.001$; Table 2). In comparison with the CON group, the percentages of breast muscle and leg muscle were declined in the RD group, whereas the relative small intestinal weight and relative cecum weight were increased ($P < 0.001$; Table 2).

### 3.2. Tissue riboflavin content

The plasma riboflavin concentration decreased by 77% as a result of RD when compared to the CON group ($P < 0.001$; Table 3). Compared to the CON group, riboflavin concentration in plasma, liver, and ileum content were decreased in the RD group, as well as liver FMN ($P < 0.001$; Table 3). There were no significant differences in riboflavin and FMN concentrations of cecal content between the 2 groups ($P > 0.05$; Table 3). However, FAD concentration of cecal content was greater in the RD group than that in the CON group ($P < 0.001$; Table 3).

**Table 2**
Growth performance, carcass trait, and relative organ weight of 21-d-old ducks in the riboflavin-deficient (RD) and control (CON) groups.

| Item | RD | CON | *P*-value |
|---|---|---|---|
| Mortality, % | 34.17 ± 25.39[a] | 0.83 ± 2.89[b] | <0.001 |
| ADG, g/d | 18.07 ± 6.23[b] | 40.45 ± 8.11[a] | <0.001 |
| ADFI, g/d | 40.85 ± 6.47[b] | 84.02 ± 7.26[a] | <0.001 |
| Breast muscle yield, % | 0.74 ± 0.25[b] | 1.67 ± 0.36[a] | <0.001 |
| Leg muscle yield, % | 4.19 ± 0.59[b] | 4.89 ± 0.43[a] | <0.001 |
| Abdominal fat yield, % | 0.56 ± 0.61 | 0.86 ± 0.23 | 0.102 |
| Relative small intestinal weight, % | 6.74 ± 0.96[a] | 4.56 ± 0.30[b] | <0.01 |
| Relative cecum weight, % | 0.45 ± 0.12[a] | 0.35 ± 0.07[b] | 0.013 |

ADG = average daily weight gain; ADFI = average daily feed intake.
Data are means of 12 replicate pens with 10 birds per pen.
The carcass trait or relative organ weight is calculated using the following equations. Breast meat, leg meat, or abdominal fat yield = (breast meat, leg meat, or abdominal fat weight) × 100%/live body weight.
Relative organ weight = (small intestinal or cecum weight) × 100%/live body weight.
[a, b] Within a row, mean without a common superscript differed ($P < 0.05$).

### 3.3. Intestinal mucosal histomorphology

Dietary RD resulted in morphological alterations of the small intestine of Pekin ducks. Compared to the CON group, RD reduced the villus height of jejunum and ileum ($P < 0.05$; Table 4), but enhanced the crypt depth of duodenum and ileum ($P < 0.05$; Table 4). Compared to the CON group, RD decreased the ratio of villus height to crypt depth of duodenum, jejunum, and ileum ($P < 0.05$; Table 4).

### 3.4. Cecal SCFA profiling

Compared to the CON group, RD decreased cecal propionate, isobutyrate, butyrate, and isovalerate concentrations ($P < 0.05$; Table 5). There were no significant differences in cecal acetate and valerate between the RD and CON groups ($P > 0.05$; Table 5).

### 3.5. Changes in the mucosal proteomics of ducks in response to RD

Using iTRAQ analysis, a total of 31,678 peptide spectral matches were identified, from which 3,086 proteins were identified in the mucosa of the 2 groups. In comparisons of the relative abundance of proteins from the mucosa of RD versus CON ducks, a total of 437 proteins had an FC > 1.5, of which 208 proteins were upregulated and 229 proteins were downregulated. Among these proteins, energy metabolism (glycolysis and gluconeogenesis, fatty acid beta oxidation, TCA cycle, oxidative phosphorylation) and intestinal absorption were selected in the present study and the proteins involved in these processes are listed in Table 6.

**Table 3**
Tissue riboflavin content of 21-d-old ducks in the riboflavin-deficient (RD) and control (CON) groups.

| Item | RD | CON | *P*-value |
|---|---|---|---|
| Plasma riboflavin, nmol/L | 46.9 ± 15.4[b] | 208.3 ± 73.0[a] | <0.001 |
| Liver riboflavin, μg/g | 2.78 ± 0.54[b] | 4.11 ± 0.50[a] | <0.001 |
| Liver FMN, μg/g | 0.99 ± 0.22[b] | 1.19 ± 0.16[a] | <0.001 |
| Ileum riboflavin, μg/g | 0.76 ± 0.45[b] | 2.93 ± 0.99[a] | <0.001 |
| Ileum FMN, μg/g | 0.086 ± 0.066 | 0.114 ± 0.077 | 0.275 |
| Ileum FAD, μg/g | 0.146 ± 0.066 | 0.122 ± 0.024 | 0.221 |
| Cecal riboflavin, μg/g | 4.11 ± 2.63 | 4.12 ± 1.01 | 0.984 |
| Cecal FMN, μg/g | 0.324 ± 0.098 | 0.328 ± 0.110 | 0.914 |
| Cecal FAD, μg/g | 0.192 ± 0.062[a] | 0.113 ± 0.036[b] | <0.001 |

FMN = flavin mononucleotide; FAD = flavin adenine dinucleotide.
Data are means of 12 replicate pens with 1 bird per pen.
[a, b] Within a row, mean without a common superscript differed ($P < 0.05$).

**Table 4**
Intestinal mucosal histomorphology of 21-d-old ducks in the riboflavin-deficient (RD) and control (CON) groups.

| Item | RD | CON | *P*-value |
|---|---|---|---|
| Duodenum | | | |
| Villus height, μm | 516 ± 116 | 700 ± 182 | 0.110 |
| Crypt depth, μm | 208.0 ± 37.5[a] | 156.6 ± 17.2[b] | 0.021 |
| Villus height to crypt depth ratio | 2.57 ± 0.83[b] | 4.57 ± 1.45[a] | 0.042 |
| Jejunum | | | |
| Villus height, μm | 473.5 ± 49.3[b] | 668.7 ± 107.3[a] | 0.020 |
| Crypt depth, μm | 197.0 ± 31.0 | 178.0 ± 21.5 | 0.350 |
| Villus height to crypt depth ratio | 2.45 ± 0.41[b] | 4.02 ± 0.87[a] | 0.021 |
| Ileum | | | |
| Villus height, μm | 427.8 ± 83.6[b] | 592.2 ± 72.1[a] | 0.005 |
| Crypt depth, μm | 201.0 ± 28.9[a] | 159.1 ± 17.4[b] | 0.013 |
| Villus height to crypt depth ratio | 2.15 ± 0.46[b] | 3.75 ± 0.49[a] | <0.001 |

Data are means of 12 replicate pens with 1 bird per pen.
[a, b] Within a row, mean without a common superscript differed ($P < 0.05$).

Of the proteins enriched in glycolysis and gluconeogenesis, 8 proteins were downregulated in the RD group (acetyl-coenzyme A synthetase [ACSS1], Aldedh domain-containing protein [ALDH1A2], aldehyde dehydrogenase 9 family member A1 [ALDH9A1], fructose-bisphosphate aldolase [ALDOB], dihydrolipoyl dehydrogenase [DLD], fructose-bisphosphatase 1 [FBP1], hexokinase domain containing 1 [HKDC1], and triosephosphate isomerase [TPI1]), while 2 proteins were upregulated (aldehyde dehydrogenase [ALDH1A3] and L-lactate dehydrogenase B chain [LDHB]).

Dietary RD downregulated 10 proteins involved in fatty acid beta oxidation, including acetyl-CoA acyltransferase 1 (ACAA1), acyl-CoA dehydrogenase long chain (ACADL), medium-chain specific acyl-CoA dehydrogenase (ACADM), acyl-CoA dehydrogenase short chain (ACADS), acyl-CoA dehydrogenase family member 9 (ACAD9), electron transfer flavoprotein-ubiquinone oxidoreductase (ETFDH), aldedh domain-containing protein (ALDH2), aldehyde dehydrogenase 9 family member A1 (ALDH9A1), hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta (HADHB), and hydroxyacyl-coenzyme A dehydrogenase (HADH).

In RD ducks, there was downregulation of 7 proteins involved in the TCA cycle, including aconitate hydratase (ACO2), DLD, succinate dehydrogenase [ubiquinone] flavoprotein subunit (SDHA), succinate dehydrogenase [ubiquinone] iron-sulfur subunit (SDHB), succinate dehydrogenase complex subunit C (SDHC), succinate-CoA ligase subunit beta (SUCLA2), and succinate–CoA ligase [ADP/GDP-forming] subunit alpha (SUCLG1).

A total of 23 proteins involved in oxidative phosphorylation were all downregulated in the RD group, including cytochrome *c* oxidase subunit 4I1 (COX4I1), cytochrome *c* oxidase subunit 5A (COX5A), cytochrome *c* oxidase subunit 6A (COX6A1), cytochrome *c* oxidase polypeptide 7A2 (COX7A2), NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10 (NDUFA10), NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12

**Table 5**
Cecal short-chain fatty acids (SCFA) of 21-d-old ducks in the riboflavin-deficient (RD) and control (CON) groups.

| Item | RD | CON | *P*-value |
|---|---|---|---|
| Acetate, μmol/g | 53.41 ± 16.02 | 50.16 ± 8.56 | 0.601 |
| Propionate, μmol/g | 12.06 ± 2.02[b] | 19.81 ± 3.03[a] | <0.001 |
| Isobutyrate, μmol/g | 0.43 ± 0.15[b] | 0.91 ± 0.50[a] | 0.039 |
| Butyrate, μmol/g | 3.45 ± 0.88[b] | 6.78 ± 3.27[a] | 0.030 |
| Isovalerate, μmol/g | 0.50 ± 0.34[b] | 1.47 ± 0.95[a] | 0.032 |
| Valerate, μmol/g | 0.97 ± 0.36 | 1.59 ± 0.71 | 0.067 |

Data are means of 12 replicate pens with 1 bird per pen.
[a, b] Within a row, mean without a common superscript differed ($P < 0.05$).

**Table 6**
Selected differentially expressed proteins in duck mucosa caused by riboflavin deficiency (RD).

| UniProtKB ID | Protein name | Short name | Fold change | *P*-value |
|---|---|---|---|---|
| **Glycolysis and gluconeogenesis** | | | | |
| U3I693 | Acetyl-coenzyme A synthetase | ACSS1 | −1.64 | 6.40E-04 |
| U3IM27 | Aldedh domain-containing protein | ALDH1A2 | −2.17 | 2.55E-03 |
| U3IKG5 | Aldehyde dehydrogenase 9 family member A1 | ALDH9A1 | −2.64 | 5.85E-05 |
| B4Z854 | Fructose-bisphosphate aldolase | ALDOB | −2.05 | 6.21E-04 |
| U3IR48 | Dihydrolipoyl dehydrogenase | DLD | −3.48 | 1.40E-05 |
| U3J2H8 | Fructose-bisphosphatase 1 | FBP1 | −2.58 | 2.24E-04 |
| U3IVG9 | Hexokinase domain containing 1 | HKDC1 | −2.75 | 1.22E-03 |
| U3I8D8 | Triosephosphate isomerase | TPI1 | −2.20 | 4.13E-05 |
| U3IF86 | Aldehyde dehydrogenase | ALDH1A3 | 1.81 | 4.00E-03 |
| P13743 | L-lactate dehydrogenase B chain | LDHB | 1.61 | 4.93E-03 |
| **Fatty acid beta oxidation** | | | | |
| U3IF09 | Acetyl-CoA acyltransferase 1 | ACAA1 | −1.71 | 2.91E-04 |
| U3IAY7 | Acyl-CoA dehydrogenase long chain | ACADL | −2.80 | 2.14E-07 |
| U3ITA9 | Medium-chain specific acyl-CoA dehydrogenase, mitochondrial | ACADM | −1.85 | 5.84E-04 |
| U3J8W0 | Acyl-CoA dehydrogenase short chain | ACADS | −2.08 | 2.18E-05 |
| U3J1J0 | Acyl-CoA dehydrogenase family member 9 | ACAD9 | −2.42 | 1.00E-04 |
| R0LSV8 | Electron transfer flavoprotein-ubiquinone oxidoreductase | ETFDH | −2.31 | 3.88E-05 |
| U3IM27 | Aldedh domain-containing protein | ALDH2 | −2.17 | 2.55E-03 |
| U3IKG5 | Aldehyde dehydrogenase 9 family member A1 | ALDH9A1 | −2.64 | 5.85E-05 |
| U3I6S1 | Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit beta | HADHB | −1.86 | 1.08E-06 |
| R0LHP1 | Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial | HADH | −1.64 | 9.17E-04 |
| **TCA cycle** | | | | |
| U3IC15 | Aconitate hydratase, mitochondrial | ACO2 | −2.05 | 8.30E-05 |
| U3IR48 | Dihydrolipoyl dehydrogenase | DLD | −3.48 | 1.40E-05 |
| U3IHF6 | Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial | SDHA | −4.56 | 4.97E-08 |
| U3J5X3 | Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial | SDHB | −3.02 | 3.76E-06 |
| U3J0A4 | Succinate dehydrogenase complex subunit C | SDHC | −1.74 | 3.24E-04 |
| R0KC12 | Succinate-CoA ligase subunit beta | SUCLA2 | −1.57 | 3.11E-04 |
| U3J6J0 | Succinate–CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial | SUCLG1 | −2.20 | 6.66E-04 |
| **Oxidative phosphorylation** | | | | |
| U3IRR0 | Cytochrome *c* oxidase subunit 4I1 | COX4I1 | −1.72 | 1.13E-02 |
| U3J5F9 | Cytochrome *c* oxidase subunit 5A | COX5A | −1.93 | 2.17E-06 |
| R0JG80 | Cytochrome *c* oxidase subunit 6A, mitochondrial | COX6A1 | −1.55 | 8.12E-03 |
| R0JCU5 | Cytochrome *c* oxidase polypeptide 7A2, mitochondrial | COX7A2 | −1.69 | 1.09E-04 |
| U3I8R9 | NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial | NDUFA10 | −2.68 | 3.08E-07 |
| U3J741 | NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12 | NDUFA12 | −1.62 | 9.56E-05 |
| R0LRI8 | NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2 | NDUFA2 | −2.36 | 1.28E-06 |
| U3J532 | NADH:ubiquinone oxidoreductase subunit A5 | NDUFA5 | −2.50 | 5.73E-06 |
| R0LLX6 | NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6 | NDUFA6 | −1.58 | 1.14E-02 |
| U3J5K2 | NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8 | NDUFA8 | −1.74 | 9.28E-05 |
| R0JTT3 | NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9 | NDUFB9 | −2.56 | 3.99E-07 |
| U3I998 | NADH:ubiquinone oxidoreductase core subunit S1 | NDUFS1 | −2.58 | 2.19E-05 |
| R0KZ49 | NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial | NDUFS2 | −1.99 | 4.03E-04 |
| U3IMS0 | NADH:ubiquinone oxidoreductase core subunit S3 | NDUFS3 | −1.80 | 1.33E-05 |
| U3J3R1 | NADH:ubiquinone oxidoreductase subunit S4 | NDUFS4 | −1.59 | 4.93E-04 |
| U3IE92 | NADH:ubiquinone oxidoreductase core subunit S7 | NDUFS7 | −1.76 | 9.68E-04 |
| U3J3L1 | NADH:ubiquinone oxidoreductase core subunit V1 | NDUFV1 | −1.95 | 3.43E-05 |
| U3IKH0 | NADH:ubiquinone oxidoreductase core subunit V2 | NDUFV2 | −1.55 | 1.93E-03 |
| U3IHF6 | Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial | SDHA | −4.56 | 4.97E-08 |
| U3J5X3 | Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial | SDHB | −3.02 | 3.76E-06 |
| U3J0A4 | Succinate dehydrogenase complex subunit C | SDHC | −1.74 | 3.24E-04 |
| U3I342 | Ubiquinol-cytochrome c reductase core protein 2 | UQCRC2 | −1.69 | 1.56E-05 |
| U3IHB0 | ATP synthase membrane subunit f | ATP5MF | −1.98 | 1.79E-04 |
| **Intestinal absorption** | | | | |
| U3IMH5 | Triacylglycerol lipase | PNLIP | 4.39 | 8.68E-06 |
| U3I073 | Fatty acid binding protein 1 | FABP1 | −2.03 | 2.97E-03 |
| R0K6H7 | Fatty acid binding protein 2 | FABP2 | −5.83 | 9.07E-06 |
| U3IGY5 | Aspartate aminotransferase | GOT2 | −1.54 | 7.41E-03 |
| U3I559 | Microsomal triglyceride transfer protein | MTTP | −2.12 | 2.08E-03 |
| U3J0H0 | Scavenger receptor class B member 1 | SCARB1 | −2.26 | 2.51E-03 |
| U3IM39 | Solute carrier family 27 member 4 | SLC27A4 | −1.62 | 4.56E-06 |
| U3IFN5 | Villin 1 | VIL1 | −3.12 | 3.80E-05 |
| U3IY96 | Ezrin | EZR | −1.65 | 2.36E-04 |

TCA = tricarboxylic acid; NADH = reduced nicotinamide adenine dinucleotide.
Fold change is expressed as the ratio of the riboflavin-deficient (RD) to the control (CON) group. For downregulated proteins, the fold change was transformed to the corresponding negative value.

(NDUFA12), NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2 (NDUFA2), NADH:ubiquinone oxidoreductase subunit A5 (NDUFA5), NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6 (NDUFA6), NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8 (NDUFA8), NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9 (NDUFB9),

**Table 7**
Cecal microbial diversity of 21-d-old ducks in the riboflavin-deficient (RD) and control (CON) groups.

| Item | RD | CON | *P*-value |
|---|---|---|---|
| ACE | 277 ± 70[b] | 353 ± 31[a] | 0.003 |
| Chao1 | 279 ± 74[b] | 359 ± 36[a] | 0.003 |
| Shannon | 3.34 ± 0.34[b] | 3.94 ± 0.30[a] | <0.001 |
| Simpson | 0.092 ± 0.034[a] | 0.049 ± 0.023[b] | 0.002 |

Data are means of 12 replicate pens with 1 bird per pen.
[a, b] Within a row, mean without a common superscript differed ($P < 0.05$).

NADH:ubiquinone oxidoreductase core subunit S1 (NDUFS1), NADH dehydrogenase [ubiquinone] iron-sulfur protein 2 (NDUFS2), NADH:ubiquinone oxidoreductase core subunit S3 (NDUFS3), NADH:ubiquinone oxidoreductase subunit S4 (NDUFS4), NADH:ubiquinone oxidoreductase core subunit S7 (NDUFS7), NADH:ubiquinone oxidoreductase core subunit V1 (NDUFV1), NADH:ubiquinone oxidoreductase core subunit V2 (NDUFV2), SDHA, SDHB, SDHC, ubiquinol-cytochrome c reductase core protein 2 (UQCRC2), ATP synthase membrane subunit f (ATP5MF).

Of the proteins involved in intestinal absorption, 1 protein was upregulated (triacylglycerol lipase [PNLIP]) in response to dietary RD, while 8 proteins were downregulated (fatty acid binding protein 1 [FABP1], fatty acid binding protein 2 [FABP2], aspartate aminotransferase [GOT2], microsomal triglyceride transfer protein [MTTP], scavenger receptor class B member 1 [SCARB1], solute carrier family 27 member 4 [SLC27A4], villin 1 [VIL1], and ezrin [EZR]).

### 3.6. Changes in the cecal microbial diversity and community of ducks in response to RD

Compared to the CON group, the α-diversity estimators of richness, including ACE, Chao1, and Shannon, were all significantly decreased in the RD group ($P < 0.05$; Table 7), while Simpson was markedly increased ($P < 0.05$; Table 7).

The microbial profile was clustered using PCoA (Fig. 1) based on unweighted UniFrac distance, by which cecal microbial communities of ducks from the 2 groups were distributed into 2 detached clusters. The ANOSIM method based on unweighted UniFrac distance ulteriorly illustrated that microbial communities were significantly distinguished in different contents of the 2 groups ($R = 0.557$, $P = 0.001$), indicating that RD exerted an effect on microbial composition.

To analyze the microbial composition, phyla, family, and genus were selected as taxonomic levels. Bacteroidota, Firmicutes, Actinobacteriota, Proteobacteria, Spirochaetota, and Deferribacterota were the main phyla in the cecum of ducks, and Bacteroidota and Firmicutes were the dominant phyla in both groups (Fig. 2A). At family level, the duck cecal microbial communities of the 2 groups were predominated by sequences representative of Bacteroidaceae, Rikenellaceae, Ruminococcaceae, Lachnospiraceae (Fig. 2B). *Bacteroides, Alistipes*, and *Subdoligranulum* were prominent genus in the cecum of ducks (Fig. 2C).

Compared to the CON group, the relative abundance of *Alistipes, Corynebacterium, Enteroboccus, Butyricicoccus,* and *Streptococcus* at genus level was significantly increased in the RD group, while *Eubacterium coprostanoligenes, Olsenella, Corynebacterium, Prevotella,*

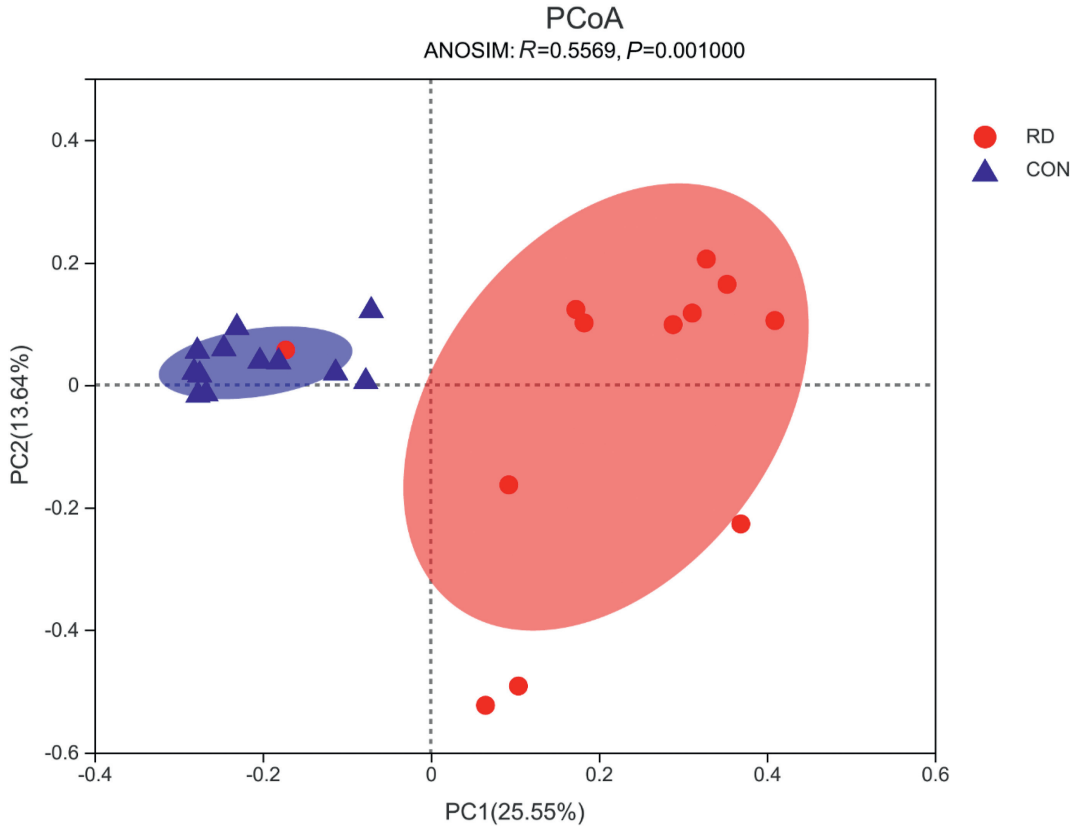


**Fig. 1.** Effects of riboflavin deficiency on β-diversity based on unweighted UniFrac distance calculated from OTU abundance matrix of Pekin ducks on d 21. RD = riboflavin deficiency group; CON = control group.

*Faecalibacterium, Brachyspira, Mucispirillum, and Elusimicrobium* were markedly decreased (Fig. 3). Furthermore, LEfSe analysis (Fig. 4) was explored to identify significant taxa in phylotypes. The genus *Alistipes, Enterococcus,* and *Corynebacterrium* were biomarkers in the RD group (Fig. 4).

## 4. Discussion

Growth depression and high mortality occurred in RD ducks in the present study, consistent with previous studies (Tang et al., 2013, 2014, 2015, 2017). Although we have not established a clear cause–effect relationship with regard to the death of the birds due to RD, it is most likely that their death stems from the impaired primary metabolic processes, such as fatty acid beta oxidation, TCA cycle, and oxidative phosphorylation (Tang et al., 2017). Furthermore, poor riboflavin status in RD ducks was confirmed by a marked reduction of riboflavin contents in plasma, liver, and ileum, as tissue riboflavin is a useful biomarker for riboflavin status. A severe RD animal model was successfully established. In the present study, we investigated the effects of RD on intestinal development, jejunum mucosa protein alterations, cecal content SCFA profiling, and cecal microbiota of starter Pekin ducks, using proteomic and 16S rRNA gene amplicon sequencing approaches.

Dietary RD induced significant changes in the morphology and cytokinetics of the small intestine in weaning rats (Williams et al., 1995, 1996a, 1996b; Yates et al., 2001, 2003), impairing the normal increase in villus number and leading to an adaptive increase in length of villi and depth of crypts (Williams et al., 1995, 1996a). In agreement with previous studies, dietary RD impaired the small intestinal morphology, decreased the villus height of jejunum and ileum, and increased the crypt depth of duodenum and ileum in ducks in the present study. The discrepancy in the results on the villus height of small intestine in response to RD between rats and ducks may be due to the different species and growth periods.

The present study was apparently the first to use a proteomic and 16S rRNA gene sequencing approach to investigate intestinal damage in response to RD. Based on jejunum mucosa proteomics, RD mainly suppressed glycolysis and gluconeogenesis, fatty acid beta oxidation, TCA cycle, oxidative phosphorylation, and intestinal absorption processes. Notably, almost all flavoproteins or subunits of flavin-dependent enzymes among the RD altered proteins were reduced in the mucosa from RD ducks, such as ACADS, ACADM, ACADL, ACAD9, SDHA, ETFDH, and DLD, confirming our previous findings in duck liver (Tang et al., 2017, 2019; Zhang et al., 2021). This finding is consistent with the hypothesis that flavoprotein expression may be downregulated owing to reduced supply of riboflavin in the diet and is in line with previous studies in humans (Gianazza et al., 2016) and ducks (Tang et al., 2017, 2019; Zhang et al., 2021).

Dietary RD downregulated 8 proteins in glycolysis and gluconeogenesis, and upregulated 2 proteins in this pathway. That 8 of 10 proteins were downregulated in the RD group implied that mucosa glycolysis and gluconeogenesis were impaired. In addition, dietary RD downregulated ten proteins involved in fatty acid beta oxidation, 7 proteins involved in the TCA cycle, and 23 proteins involved in oxidative phosphorylation, indicating that all these processes were impaired by RD. These results are consistent with our previous findings in the liver of ducks that RD impaired energy generation processes, including fatty acid beta-oxidation, TCA cycle, and oxidative phosphorylation (Tang et al., 2017, 2019). Together, the energy generation processes in the jejunum mucosa, including glycolysis, fatty acid beta oxidation, TCA cycle, and oxidative phosphorylation, were all inhibited by RD, probably resulting in insufficient ATP production and subsequent intestinal cell arrest and virtually leading to abnormal intestinal morphology. This implication was supported by previous findings that riboflavin depletion leads to impaired energy generation (Lee et al., 2013) and proliferation in intestinal cells (Nakano et al., 2011).

A novel and important finding of this study is that dietary RD downregulated 8 proteins involved in the intestinal absorption, such as FABP1, FABP2, GOT2, MTTP, SCARB1, SLC27A4, VIL1, and EZR, implying suppression of this process. VIL1 and EZR (also known as villin 2; VIL2) are microvillar proteins in intestinal epithelial cells that are involved in the absorptive and secretory function of epithelial cells by modulating F-actin polymerization/depolymerization (Saleh et al., 2009). Specifically, villin-depleted mice showed a reduction in intestinal glucose absorption (Mziaut et al., 2016), whereas EZR absence resulted in abnormal villus morphogenesis (Saotome et al., 2004). FABP1 and FABP2 belong to fatty acid binding proteins (FABPs) family, which participate in the uptake, intracellular metabolism and/or transport of long-chain fatty acids (Storch et al., 2000). SLC27A4 is the principal fatty acid transporter in enterocytes, and reduction of SLC27A4 expression in primary enterocytes inhibits fatty acid uptake by 50% (Stahl et al., 1999). MTTP is involved in lipid transfer during the assembly of lipoproteins in the liver and intestine (Hussain et al., 2003). Therefore, decreased expression of the aforementioned proteins due to RD probably impair glucose and fat absorption in the small intestine, which may lead to growth depression.

Riboflavin status has been shown to modulate the gut microflora of animals, and RD induces gut microbiota dysbiosis (Biagi et al., 2020; Pan et al., 2020, 2021; Zhu et al., 2022). In the present study, based on α-diversity measurements, dietary RD decreased the community richness and diversity of the bacterial community in the cecum, as indicated by the reduced ACE, Chao1, and Shannon index values and increased Simpson index. In line with a previous study, Zhu et al. (2022) reported that dietary RD reduced the diversity of microbial species in the colonic content of mice. In addition, the beta-diversity analysis showed significant clustering between the 2 groups, indicating that the cecal microbiota community structure was markedly altered by RD in the current study. Emerging evidence suggests that gut microbiota dysbiosis has many consequences, which can be divided into disruption of the gut barrier and imbalance of the host immune and metabolic systems (Hrncir, 2022). Dietary RD induces genomic instability of esophageal squamous cells and esophageal epithelial atrophy by modulating the gut microbiota in rats (Pan et al., 2020, 2021). Riboflavin bioenriched fermented soymilk can effectively alleviate deleterious ariboflavinosis associated with oxidative stress mediated chronic intestinal inflammation and gut dysbiosis in the riboflavin depletion-repletion mouse model (Zhu et al., 2022).

Short-chain fatty acids (SCFA) are the principal end products of carbohydrate fermentation by gut microbes and serve as indicators of microbial activity. In the present study, RD decreased cecal SCFA concentrations in ducks, such as propionate, isobutyrate, butyrate, and isovalerate. The results are consistent with previous findings in mice that RD resulted in a significant reduction in SCFA release except for acetic acid and valeric acid (Zhu et al., 2022). Meanwhile, a previous study in broilers found that supplementation with 100 mg/kg riboflavin in the diet significantly boosted butyrate production in the cecal environment compared to riboflavin-adequate birds (Biagi et al., 2020). The decreased SCFA concentrations suggest that RD could reduce carbohydrate fermentation by bacteria. Among the various SCFA, butyrate is the major energy source of intestinal epithelial cells, and it plays an important role in maintaining mucosal integrity and genomic stability and improving intestinal inflammation. Thus, the decreased cecal SCFA concentrations in RD ducks may be responsible for intestinal morphology alterations in the present study. Furthermore, RD decreased the

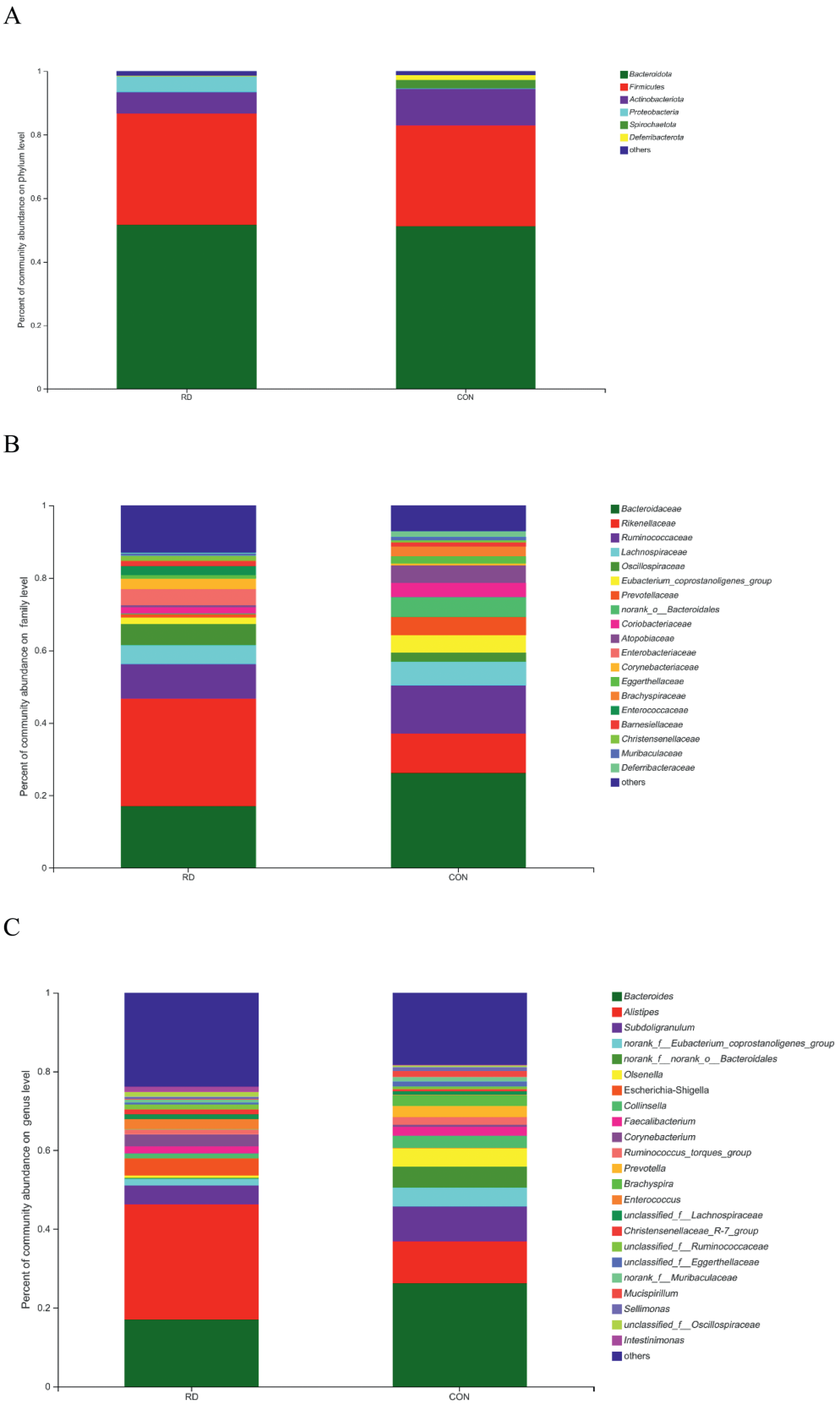


**Fig. 2.** Effects of riboflavin deficiency on composition of cecal microbiota identified at phylum (A), family (B), and genus (C) level of Pekin ducks on d 21. RD = riboflavin deficiency group; CON = control group.

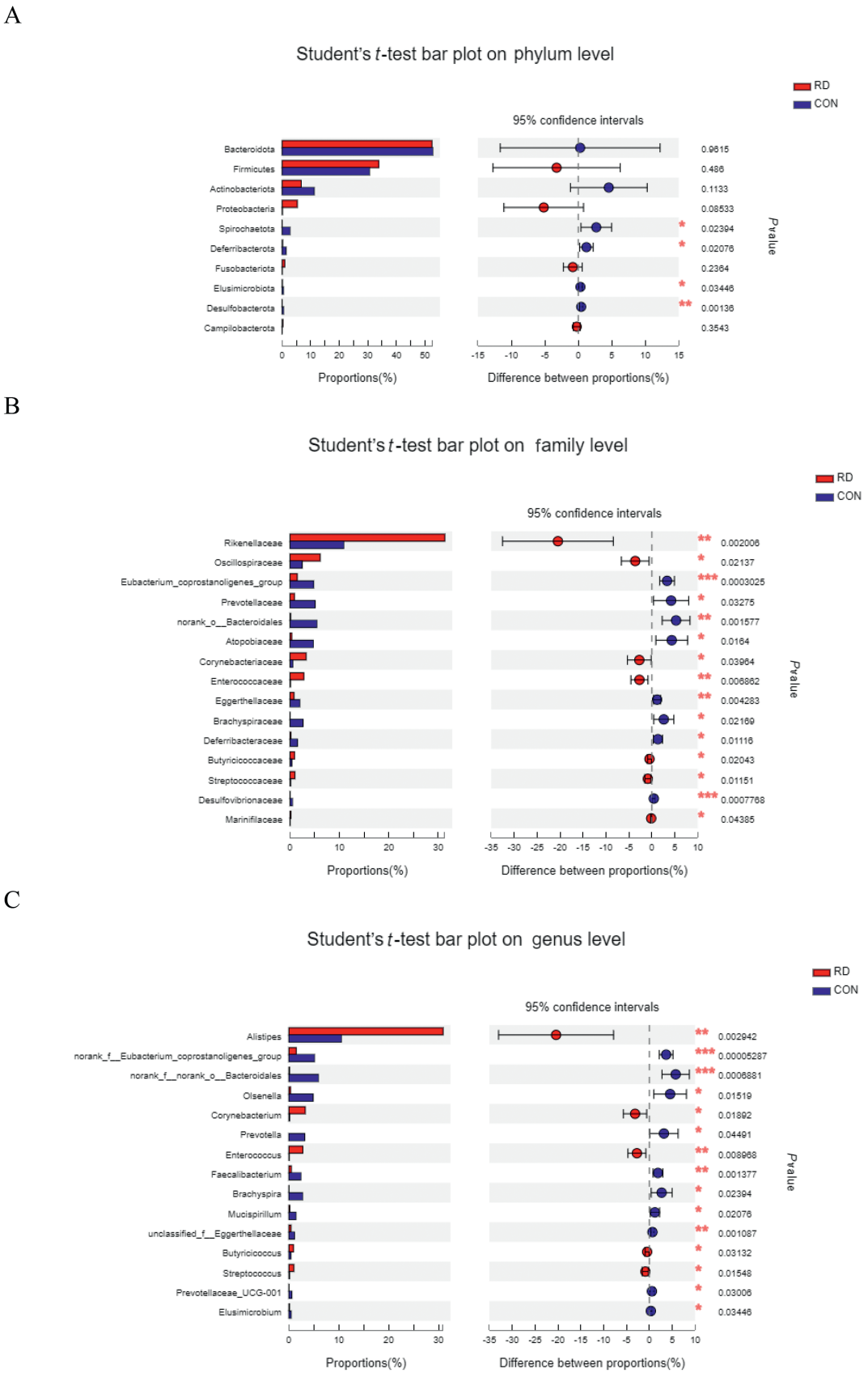


**Fig. 3.** Effects of riboflavin deficiency on differential species identified of cecal microbiota at phylum (A), family (B), and genus (C) level of Pekin ducks on d 21. RD = riboflavin deficiency group; CON = control group.

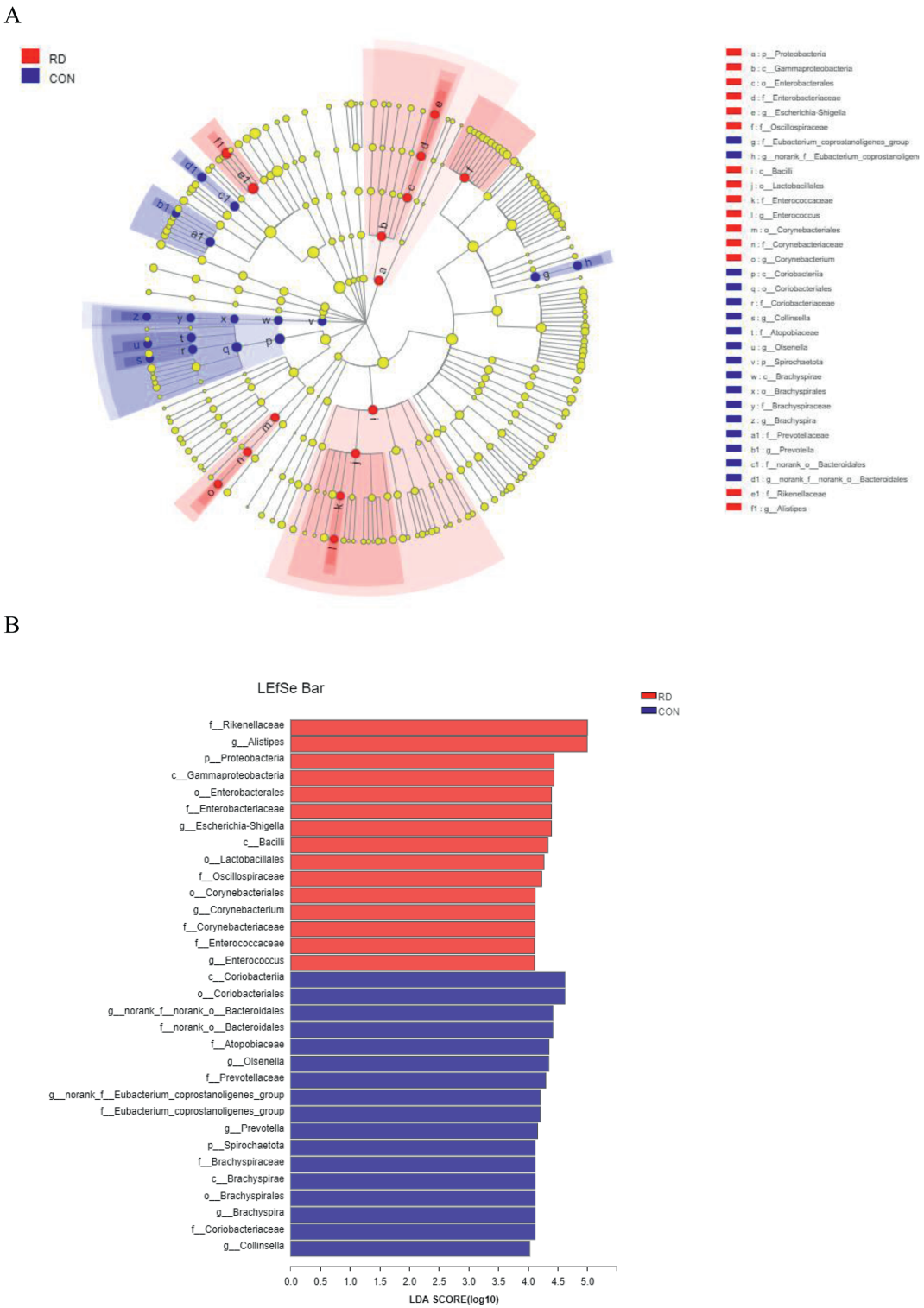


**Fig. 4.** Effects of riboflavin deficiency on linear discriminant analysis effect size (LEfSe) to detect the most significantly abundant cecal microbiota of Pekin ducks on d 21. (A) Cladoram measured from LEfSe analysis; (B) LDA score generated from differentially abundant microbiota (LDA > 4, $P < 0.05$). RD = riboflavin deficiency group; CON = control group.

abundance of *E. coprostanoligenes, Prevotella,* and *Faecalibacterium* in the cecal content in the present study, and these bacteria are considered butyrate producers (Rychlik, 2020). The reduction of abundance of these bacteria due to RD may resulted in a decreased cecal butyrate concentration. Consistent with our study, riboflavin supplementation increased the abundance level of *Prevotella* in mice (Zhu et al., 2022). In contrast with our study, riboflavin supplementation caused a significant decrease in *Faecalibacterium* abundance in the colon of humans (Pham et al., 2021), which may be due to different species and different gut segments.

Notably, RD increased the abundance of 2 riboflavin producers in the cecum, *Enterococcus* and *Sreptococcus,* which may provide an

explanation for the elevated cecal FAD concentration in the present study. The increased abundance of riboflavin-producing bacteria in the cecum was presumably a compensatory mechanism in response to RD. However, the underlying interaction mechanisms between microbes and hosts are still unknown and needed to be further investigated. This implication is supported by previous findings in the human intestine that riboflavin can be produced by certain microorganisms (LeBlanc et al., 2013). However, the concentrations of produced riboflavin by gut bacteria were insufficient to prevent RD in ducks in the present study, which was also observed in humans (LeBlanc et al., 2013).

## 5. Conclusions

Dietary RD caused growth retardation, high mortality, poor riboflavin status, and morphological alterations of the small intestine in ducks. In addition, RD increased the relative cecum weight and decreased SCFA concentrations, such as propionate, isobutyrate, butyrate, and isovalerate. Jejunum mucosa proteomics showed that RD mainly suppressed energy generation processes, such as glycolysis and gluconeogenesis, fatty acid beta oxidation, TCA cycle, and oxidative phosphorylation, leading to insufficient ATP production. Furthermore, RD probably also impaired intestinal absorption in the small intestine, as indicated by the eight diminished proteins involved. In addition, RD decreased the community richness and diversity of the bacterial community in the cecum of ducks. Specifically, RD reduced the abundance of butyrate-producing bacteria in the cecum, such as *E. coprostanoligenes, Prevotella,* and *Faecalibacterium,* which may lead to decreased cecal butyrate concentrations. These findings add to our understanding of the mechanisms of intestinal hypofunction as a result of RD.

## Author contributions

**Yaxi Xu:** Investigation, Conceptualization, Methodology, Data curation; **Bo Zhang:** Investigation, Data curation; **Rui Zhao:** Methodology; **Kexin Gao:** Methodology; **Suyun Liang:** Methodology; **Yongbao Wu:** Methodology; **Yongsheng Hao:** Formal analysis; **Dapeng Liu:** Methodology; **Zhanbao Guo:** Investigation; **Jian Hu:** Investigation; **Zhengkui Zhou:** Project administration; **Ming Xie:** Supervision; **Jing Tang:** Supervision, Conceptualization, Data curation, Writing – original draft & Writing – review & editing.

## Declaration of conflict of 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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