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Bile acids enhance fat metabolism and skeletal muscle development in Zhijiang duck by modulating gut microbiota

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ABSTRACT

To optimize livestock production of integrated farms, dietary crude fat levels are often increased, making efficient fat utilization crucial. Bile acids are known to improve fat utilization, but their impact on growth performance and breast muscle development in Zhijiang ducks remains unclear. In this study, a total of 360 twenty-day-old Zhijiang ducks with similar body weights were divided into three groups: the control group (CN) received a basal diet; the high-fat group (FA) received the basal diet plus 1.25 % rapeseed oil; and the high-fat plus bile acids compound (BA) group (FB) received the FA diet supplemented with 250 mg/kg BA for 30 days. Results indicated that the addition of rapeseed oil and BA significantly increased ($P < 0.05$) average daily gain (ADG) and reduced ($P < 0.05$) feed conversion ratio (FCR). Slaughter data showed that BA significantly enhanced ($P < 0.05$) breast muscle weight and percentage while decreasing ($P < 0.05$) abdominal fat weight. Additionally, BA increased ($P < 0.05$) the cross-sectional area of breast muscle fibers, total bile acid content, and levels of insulin-like growth factors 1/2 (IGF1/2). Transcriptomic analysis further revealed that BA significantly upregulated ($P < 0.05$) the levels of *PPARα, CPT1α, NR1H4*, and *CETP* in breast muscle. 16S rRNA analysis showed a significant increase ($P < 0.05$) in the relative abundances of genera *Enorma*, [*Eubacterium nodatum group*], *Rikenellaceae RC9 gut group,* and *SP3-e08*. Additionally, the Spearman correlation suggested a positive correlation between the genera *Olsenella, SP3-e08, Enorma, Rikenellaceae_RC9_gut_group*, and [*Eubacterium_nodatum_group*] with *PPARα, CETP, NR1H4,* and *CPT1α*. In contrast, the genera *Christensenellaceae_R_7_group* and *Sutterella* exhibited negative correlations with *PPARα*. These findings provide new insights into the role of BA in promoting growth performance and skeletal muscle development in Zhijiang ducks fed a high-fat diet, with this effect potentially linked to changes in the gut microbiota.

Introduction

In integrated farming systems, increasing the dietary crude fat content can promote growth and improve feed efficiency (Ravindran et al., 2016). However, due to the limited ability of poultry to efficiently utilize fat, excess fat is often deposited in areas such as the abdomen and viscera (Fouad and El-Senousey, 2014). Zhijiang ducks, a prized local breed in China, continue to encounter significant challenges in commercial farming, including prolonged production cycles, inefficient feed conversion, and low meat yield (Li et al., 2023). Moreover, as the Zhijiang duck industry shifts from traditional live sales to processed products like chilled duck, convenience foods, and ready-to-eat meals, meat yield has become an increasingly vital factor, serving as a key indicator of production efficiency. Therefore, exploring effective strategies to improve feed conversion and enhance meat yield in Zhijiang ducks is essential to meet market demand and increase overall farming profitability.

The growth hormone axis plays a crucial role in regulating skeletal muscle development, mainly through insulin-like growth factors (IGFs) such as IGF1 and IGF2 (Stewart and Rotwein, 1996). Both the liver and skeletal muscle secrete IGF1/2 through endocrine, autocrine, and paracrine mechanisms, which activate multiple signaling pathways that promote cell proliferation, differentiation, and hypertrophy, thereby

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regulating muscle growth and development (Florini et al., 1991; Stewart and Pell, 2010). Study have shown that IGF1/2 promote muscle cell proliferation in rainbow trout, along with promoted DNA synthesis (Codina et al., 2008). Furthermore, higher levels of IGF1 /2 in the blood are linked to greater breast muscle mass in geese (Ma et al., 2024).

Bile acids are amphipathic molecules synthesized from cholesterol in hepatocytes through the action of various enzymes. They play a crucial role in fat emulsification and absorption, and regulate lipid metabolism, energy metabolism, and skeletal muscle development by activating specific receptors (Vítek et al., 2016). The farnesoid X receptor (FXR, encoded by NR1H4), one of the primary receptors for bile acids, is widely distributed in tissues such as the liver, ileum, and skeletal muscle (Zhou et al., 2022). Activation of FXR and its downstream genes is involved in regulating the differentiation, proliferation, and hypertrophy of skeletal muscle cells (Ma et al., 2024; Qiu et al., 2022). In a mouse model of muscle atrophy induced by antibiotics, upregulation of FXR activity promoted protein synthesis in skeletal muscle cells (Qiu et al., 2021). Dietary supplementation with bile acids has been shown to reduce abdominal fat deposition, lower blood triglyceride (TG) level, and increase breast muscle mass in broilers fed a high-fat diet, accompanied by elevated expression of PPARα and CPT1 (Ge et al., 2019). Supplementation of bile acids in the diet has been shown to promote fat metabolism and breast muscle development in broilers fed a high-fat diet through the FXR/IGF2 pathway (Chen et al., 2024). However, it is still unclear whether a similar mechanism is involved in regulating fat metabolism and skeletal muscle development in Zhijiang ducks fed a high-fat diet.

The gut microbiota plays a critical role in regulating host fat metabolism and skeletal muscle development (Han et al., 2022). It has been shown that gut microbiota modulates the effects of exercise on skeletal muscle development (Valentino et al., 2021) and fat metabolism (Yu et al., 2019). Supplementation with Clostridium butyricum has been reported to enhance muscle weight, fiber diameter, and cross-sectional area in sheep by regulating gut-derived metabolites (Dou et al., 2023). As a microbial metabolite, bile acids are involved in reshaping the gut microbiota (Gao et al., 2023; Larabi et al., 2023). Bile acids have been shown to optimize the gut microbiota in broilers fed a high-fat diet, promoting liver fat metabolism and increasing breast muscle weight (Wang et al., 2024; Hu et al., 2024). However, it remains unclear whether dietary bile acid supplementation can improve fat metabolism and skeletal muscle development in Zhijiang ducks fed a high-fat diet by modulating the gut microbiota.

This study aims to explore the effects of dietary bile acids supplementation on skeletal muscle gene expression and gut microbiota in Zhijiang ducks fed a high-fat diet, using transcriptomic and 16S rRNA sequencing techniques. Additionally, we will further explore the relationship between the gut microbiota, fat metabolism, and skeletal muscle development. This research provides new insights into the mechanisms by which bile acids influence breast muscle development in Zhijiang ducks and may offer more efficient strategies for poultry production.

Materials and methods

Ethical statement

All experimental procedures were approved by the Animal Ethics Committee of Huaihua Institute of Agricultural Sciences. The project number is 20240524101. The sampling procedures followed the "*Guidelines on Ethical Treatment of Experimental Animals*" (2006) No.398 set by the Ministry of Science and Technology, China.

Bile acids preparation

In this study, bile acids were procured from Shandong Longchang Animal Health Care Co., Ltd. (Dezhou, China). The extraction process involved porcine bile and utilized several physicochemical techniques, such as saponification, decolorization, acidification, purification, and drying. The final product achieved a purity of 96.9 %, which includes 73.2 % hyodeoxycholic acid (HDCA), 19.8 % chenodeoxycholic acid (CDCA), and 3.9 % hyocholic acid (HCA).

Birds, diets, and experimental design

A total of 360, 20-day-old Zhijiang ducks, weighing approximately 201.37 ± 2.15 g, were reared at Hunan Zhiminfeng Animal Husbandry Technology Co., Ltd. (Huaihua, Hunan). The ducks were randomly assigned to three groups, each with five replicates, and each replicate consisted of 24 ducks (12 males and 12 females). The groups were as follows: the control group (**CN**), which received a basal diet; the fat group (**FA**), which was fed the basal diet supplemented with 1.25 % rapeseed oil; and the fat + bile acids compound (**BA**) group (**FB**), which was fed the basal diet supplemented with both 1.25 % rapeseed oil and 250 mg/kg BA. The experiment lasted 30 days, during which the ducks had unrestricted access to water and feed. The nutritional composition of the diets is provided in Table 1.

Growth performance

Feed intake was daily recorded for each replicate. At 20 days and 50 days of age, the ducks were weighed after a 9-hour fasting period. Subsequently, the total feed intake per duck, average daily feed intake (**ADFI**), average daily gain (**ADG**), and feed conversion ratio (**FCR**) were calculated.

Sample collection

At 50 days of age, one male and one female duck were selected from each replicate (with the weights of the selected ducks close to the average for their respective sex within that replicate) for slaughter, totaling 10 ducks per group. A quick decapitation method was used to collect blood. Subsequently, the breast muscle, leg muscle, abdominal fat, and liver were weighed. Tissue samples from the breast muscle for histological examination were preserved in paraformaldehyde, while molecular samples were stored at -80°C for subsequent analysis. Organ indices were calculated according to the guidelines provided in NY/T 823-2020, titled "*Nomenclature and Measurement Calculation Methods for Poultry Production Performance*".

Measuring concentrations of TBA, TG, and IGF1/2

Serum samples were analyzed to measure the concentration of total bile acids (**TBA**, #H101T), triglyceride (**TG**, #H201), **IGF1** (#YB-IGF1), and **IGF2** (#YB-IGF2), following the manufacturer's guidelines. The measurements for TBA and TG were conducted using a Hitachi 7020 automatic biochemical analyzer (Tokyo, Japan) along with the corresponding commercial assay kits from Meinkang Biotechnology Co., Ltd. (Ningbo, China). For IGF1 and IGF2, a full-wavelength microplate reader (Synergy 2, BioTek, Winooski, Vermont, USA) was utilized in conjunction with the respective ELISA kits obtained from Jiangsu Meimian Industrial Co., Ltd. (Nantong, China).

Histological evaluation of breast muscle

Three ducks from each group were randomly selected for histological examination. Fresh breast muscle samples were fixed overnight in 4 % paraformaldehyde at 4°C for 24 h and then embedded in paraffin. Sections measuring 3 micrometers were prepared and stained with hematoxylin & eosin (**H&E**). For each histological section, five images showing no signs of tissue disruption or damage were analyzed to estimate the number and cross-sectional areas of breast myofibers using an optical microscope (Olympus-BX53, Tokyo, Japan).

Table 1
Ingredients and nutrient composition of the diets for Zhijiang ducks.

Items	CON[1]	FA[2]	FB[3]	Items	CON[1]	FA[2]	FB[3]
Ingredients, %				Calculated nutrient			
Corn	46.80	46.80	46.80	Metabolizable energy (ME), MJ/kg	12.20	12.68	12.68
DDGS	24.00	24.00	24.00	Dry matter, %	84.66	85.90	85.90
Rice bran	10.00	10.00	10.00	Crude protein, %	17.60	17.60	17.60
Wheat flour	5.00	5.00	5.00	Crude ash, %	3.47	3.48	3.48
Soybean meal	5.60	5.60	5.60	Calcium, %	0.58	0.58	0.58
Wheat Gluten	3.00	3.00	3.00	Total phosphorus, %	0.69	0.69	0.69
Rapeseed meal	1.87	1.87	1.87	Phytate phosphorus, %	0.33	0.33	0.33
Limestones	1.00	1.00	1.00	Sodium chloride, %	0.25	0.25	0.25
Calcium hydrogen phosphate	0.58	0.58	0.58	Lysine, %	0.55	0.55	0.55
Premix [4]	2.15	2.15	2.15	Methionine+Cysteine, %	0.59	0.59	0.59
Rapeseed oil, %	0.00	1.25 %	1.25 %	Threonine, %	0.56	0.56	0.56
Bile acids compound, mg/kg	0.00	0.00	250	Valine, %	0.74	0.74	0.74

[1] CN, Control group;
[2] FA, Fat group;
[3] FB, Fat + bile acids compound group.
[4] The vitamin premix provided the following (per kg of diet): Vitamin A, 2 500 IU; Vitamin D_3, 400 IU; Vitamin E, 20 IU; Vitamin K_3, 10 IU; Vitamin B_1, 18 mg; Vitamin B_2, 40 mg; Vitamin B_6, 30 mg; Vitamin B_{12}, 0.71 mg; Choline chloride, 750 mg; Niacin, 55 mg; D-pantothenic acid, 11 mg; Folic acid, 0.5 mg; Iron, 80 mg; Copper, 8 mg; Manganese, 60 mg; Iodine, 0.35 mg; Zinc, 40 mg; Selenium, 0.15 mg.

16S rRNA analysis of duck cecum content

The 16S rRNA amplicon sequencing was performed by Novogene Technology Co., Ltd. (Beijing, China) to analyze the microbiomes of duck cecum content. Total genomic DNA was extracted from cecum content using the CTAB/SDS method, and its concentration, integrity and purity were assessed on 1 % agarose gels before dilution to 1 ng/μl with sterile water. The V4 region of the 16S rRNA gene was amplified using specific primers 515-F (primer sequence: GTGCCAGCMGCCGCGGTAA) and 806-R (primer sequence: GGACTACHVGGGTWTCTAAT), with barcoding. PCR reactions contained 15 μl of Phusion® High-Fidelity PCR Master Mix, 0.2 μM of each primer, and approximately 10 ng of template DNA. The amplification was followed by thermal cycling and electrophoresis on a 2 % agarose gel for detection. Sequencing libraries were prepared using the Ion Plus Fragment Library Kit (Thermo Scientific), with quality assessment performed using a Qubit® 2.0 Fluorometer and an Agilent Bioanalyzer 2100. The libraries were then sequenced on an Illumina NovaSeq 6000, generating 250-bp paired-end reads.

Transcriptomic analysis of duck breast muscle

Total RNA was extracted from duck breast muscle using TRIzol reagent (Magen, Shanghai, China). RNA quality was assessed by measuring the A260/A280 absorbance ratio using a Nanodrop ND-2000 (Thermo Scientific, Rockford, IL, USA) and by analyzing the RNA profile with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA), ensuring that only qualified samples were used for library construction. mRNA was purified from total RNA using poly-T oligo-attached magnetic beads, followed by fragmentation and first-strand cDNA synthesis with random hexamer primers, which was then followed by second-strand cDNA synthesis. Library preparation included end repair, A-tailing, adapter ligation, size selection, amplification, and purification. Library quantification was performed using Qubit and Real-time PCR, with size distribution assessed using the Bioanalyzer 2100. The prepared library was sequenced on an Illumina NovaSeq™ X Plus platform. Raw data were processed with FASTP software to obtain clean reads, followed by Q30 and GC content calculation. Clean reads were aligned to the duck reference genome (https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_015476345.1/) using HISAT2 (v2.1.5). FeatureCounts (v1.5.0-p3) was used to calculate read counts for each gene, and FPKM values were computed based on gene length. Gene expression levels were quantified using the FPKM algorithm. Differential expression analysis between groups was conducted using the DESeq2 R package (v1.20.0), adjusting *P*-values with Benjamini and Hochberg's method to control the false discovery rate. Genes were classified as differentially expressed genes (**DEGs**) if they met the criteria of $|\log2FC| \geq 1$ and padj ≤ 0.05. DEGs were further used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis with clusterProfiler (v3.8.1), applying a significance threshold of Benjamini-Hochberg padj ≤ 0.1 relative to the entire transcriptome background.

RNA isolation and real-time PCR

To verify the reliability of the DEG data, 4 DEGs were chosen for Real-time PCR analysis. Briefly, Total RNA was isolated from breast muscle samples using TRIzol Reagent obtained from Tsingke Biotech Co., Ltd. (#TSP401, Nanjing, China). The extracted RNA was then reverse-transcribed into cDNA following the protocols provided by TransGen Biotech Co., Ltd. (#AU341-02-V2, Nanjing, China). For Real-time PCR, 2 μL of diluted cDNA (1:20) was utilized, and the reactions were performed with PerfectStart Green qPCR SuperMix from TransGen Biotech Co., Ltd. (#AQ601-02, Nanjing, China) on the QuantStudio 6 Flex Real-time PCR System. All primers, detailed in Table 2, were synthesized by Tsingke Biotech Co., Ltd. (Nanjing, China), with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) designated as the housekeeping gene. The Real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method as described by Livak and Schmittgen (2001).

Statistical analysis

Statistical analyses were conducted using SPSS software (Version 20.0; SPSS, Inc). One-way ANOVA with least significant difference (LSD) was employed to assess significant differences in the data. The Pearson correlation coefficient was used to explain the correlation between these two variables. A *p*-value of less than 0.05 ($P < 0.05$) was considered indicative of a significant difference.

Results

Growth performance

The feed intake-related metrics among the three groups, including average feed intake (Fig. 1A), total feed intake (Fig. 1B), and ADFI (Fig. 1C), did not show significant differences. In comparison to the CN group, the FA group demonstrated a significant increase ($P < 0.05$) in ADG (Fig. 1D); however, the addition of BA did not result in further improvements in ADG. Furthermore, the FCR (Fig. 1E) in the FA group

Table 2
Nucleotide sequences of specific primers.

Target genes	GenBank accession	Primer sequences (5′ to 3′)	PCR product (bp)	Used for
CETP	XM_038185263.1	F: TGTCGGGATCCCAGAAGTGA R: GAGCATTTGCTCCTTCAAGAAC	218	Real-time PCR
FXR	XM_038187558.1	F: AAATGGAGGCAACTGCGAGA R: GATCTGGGGGCTGTTTCACA	173	Real-time PCR
CPT1α	XM_027457809.2	F: GGCGTGTGAGGGGAGC R: TAGGTCAGGACACGCTCAGA	237	Real-time PCR
PPARα	NM_001310383.1	F: ATGCCAAGGTCTGAGAAGGC R: CCCTGCAAGGATGACTCTGG	168	Real-time PCR
GAPDH	XM_027449740	F: AAGGCTGAGAATGGGAAAC R: TTCAGGGACTTGTCATACTTC	254	Real-time PCR

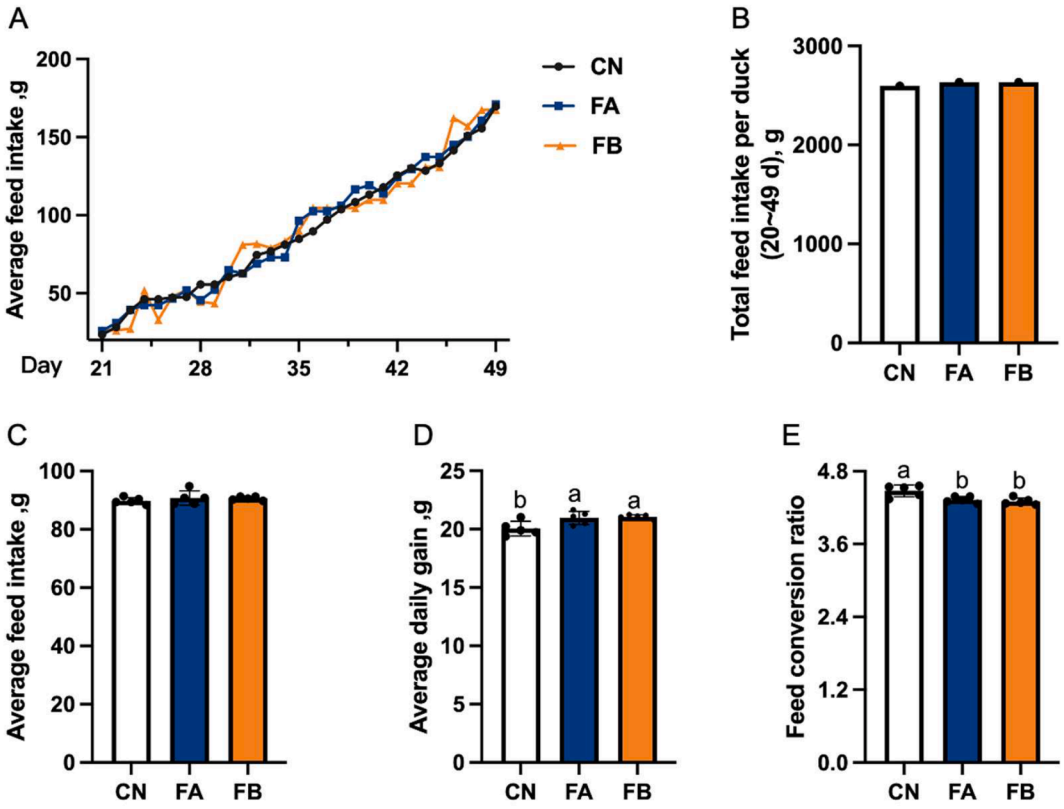

Fig. 1. Growth performance of ducks with different diets.
Ducks were treated with 1.25 % rapeseed oil with or without 250 mg/kg BA for 30 d. (A) Average feed intake; (B) Total intake per duck; (C) Average daily feed intake; (D) Average daily gain; (E) Feed conversion ratio. CN, Control group; FA, Fat group; FB, Fat + bile acids group. a, b, c: Means in different groups without common superscripts are significantly different ($P < 0.05$).

was significantly lower ($P < 0.05$) than that in the CN group, yet the inclusion of BA did not lead to a further reduction in FCR. In summary, enhancing the crude fat level in the diet significantly improved the growth performance of Zhijiang ducks during the 20 to 50-day age period.

Slaughter performance

Compared to the CN group, the FA group showed a significant increase ($P < 0.05$) in body weight; however, the addition of BA did not lead to any further increase in body weight. The dietary supplementation of FA showed no significant effect on breast muscle weight and rate, while BA notably improved ($P < 0.05$) both of these parameters compared to the FA groups. Additionally, A diet with high levels of crude fat significantly increased ($P < 0.05$) abdominal fat weight without affecting the abdominal fat rate, whereas the addition of BA significantly reduced ($P < 0.05$) both abdominal fat weight and rate. Furthermore, neither the addition of crude fat nor BA had a significant effect on any other parameters in this table. The detailed results are presented in Table 3.

Metrics of skeletal muscle development

The histological analysis of breast muscle tissue revealed no significant differences in fiber cross-sectional area (Fig. 2A, B) and number (Fig. 2A, C) between the CN and FA groups. However, the fiber cross-sectional area in the FB group was significantly greater ($P < 0.05$) than that in the FA group, while fiber number remained unchanged. Additionally, there were no significant changes in serum levels of TBA (Fig. 2D), IGF1 (Fig. 2E), and IGF2 (Fig. 2F) between the CN and FA groups, but these levels were significantly higher ($P < 0.05$) in the FB group compared to the FA group. The TG (Fig. 2G) level in the FA group was significantly higher ($P < 0.05$) than that in the CN group, while no significant difference was observed between the FA and FB groups.

Table 3
Slaughter characteristics of Zhijiang duck with different diets.

Items	CN[1]	FA[2]	FB[3]	*P* value
Body weight (g)	823.70 ± 14.61[b]	872.50 ± 13.02[a]	877.40 ± 16.63[a]	0.03
Breast muscle weight (g)	16.57 ± 1.54[b]	21.47 ± 1.68[b]	28.26 ± 1.85[a]	0.001
Thigh muscle (g)	76.13 ± 4.64	87.56 ± 3.27	83.74 ± 4.10	0.15
Abdominal fat (g)	2.15 ± 0.25[b]	3.23 ± 0.41[a]	1.57 ± 0.41[b]	0.02
Liver weight (g)	32.01 ± 1.91	36.44 ± 1.52	36.88 ±1.87	0.12
Breast muscle weight/ body weight (%)	2.01 ± 0.17[b]	2.51 ± 0.20[b]	3.21 ± 0.19[a]	0.001
Thigh muscle/body weight (%)	9.30 ± 0.63	10.05 ± 0.40	9.50 ± 0.50	0.59
Abdominal fat weight/ body weight (%)	0.26 ± 0.03[a]	0.37 ± 0.06[a]	0.18 ± 0.05[b]	0.03
Liver weight/body weight (%)	3.90 ± 0.24	4.20 ± 0.22	4.20 ± 0.21	0.55

[1] CN, Control group;
[2] FA,
[3] Fat group; FB, Fat + bile acids compound group. Ducks were treated with 1.25 % rapeseed oil with or without 250 mg/kg feed BA for 30 d. a, b, c: Means in different groups without common superscripts are significantly different ($P < 0.05$).

Transcriptome analysis of duck breast muscle

RNA sequencing (RNA-seq) technology was employed to assess the gene expression levels in the breast muscle of duck from CN, FA, and FB groups. The sequencing data underwent quality control and annotation, achieving an average mapping rate of 87.51 % for all clean reads against the reference genome (Table S1). An average of 6.39 Gb of clean reads was obtained from 58.53 Gb of raw reads across all samples, with a GC content of 49.15 % and a Q30 value of 93.35 % (Table S2). A 3D plot of principal component analysis (Fig. 3A) clearly separated the gene expression patterns among the CN, FA, and FB. A total of 395 DEGs were identified through pairwise comparisons, as depicted in the volcano plots (Fig. 2B, C). Detailed results for the CON *vs.* FA comparison are provided in Table S3, and for the FA *vs.* FB comparison in Table S4. Specifically, A total of 217 genes (125 upregulated and 92 downregulated) were identified in the comparison between CN and FA group, while 179 genes (91 upregulated and 88 downregulated) were revealed in the comparison between FA and FB group (Fig. 3D). Hierarchical clustering analysis demonstrated that the gene expression patterns between ducks treated with crude fat and BA were markedly different (Fig. 3E), further confirming the effects of these treatments. Subsequently, the enrichment of DEGs following the two treatments was annotated using the KEGG pathway database (Fig. 3E, F). The analysis revealed that pathways associated with fatty acid metabolism, the cell cycle, steroid hormone biosynthesis, motor proteins, and the PPAR signaling pathway were shared between the crude fat and BA treatments. Notably, the addition of a crude fat did not significantly impact the enriched fatty acid metabolism or the PPAR signaling pathway. In contrast, BA supplementation significantly increased the expression levels of genes associated with these metabolic pathways, providing further evidence of the close relationship between fat metabolism and the physiological effects of bile acids.

Analysis of microbiota in cecum content

The cecum content in the three groups was analyzed using 16S rRNA sequencing and identify the intestinal microbiota diversity of Zhijiang duck. A total of 1,501,796 paired-end reads (PE) and 1,493,193 effective tags were obtained from 15 samples, with a Q30 percentage of 97.98 % and a GC content of 51.73 % (Table S5). To further investigate the similarity (or dissimilarity) in microbiota communities among the three groups, the number of observed amplicon sequence variants (ASVs) were subjected to principal coordinate analysis (PCoA) based on unweighted_unifrac. The scatter plot (Fig. 4A) shows that axis 1 represents 25.84 % and axis 2 represents 24.79 % of the diversity, indicating

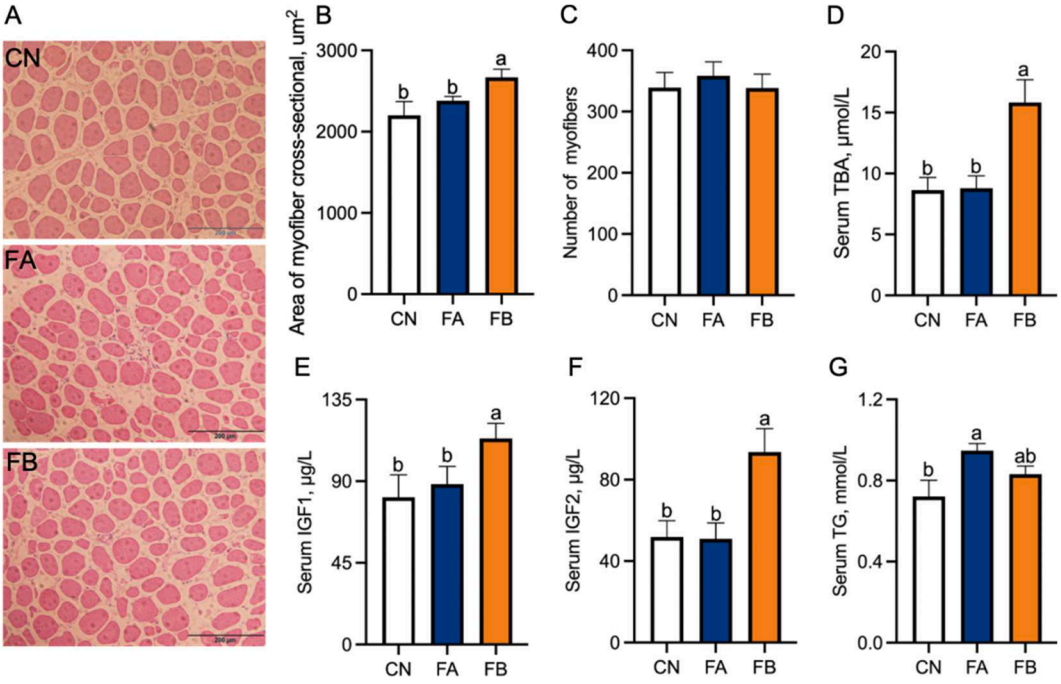

Fig. 2. Skeletal muscle development indicators of ducks with different diets.
Ducks were treated with 1.25 % rapeseed oil with or without 250 mg/kg feed BA for 30 d. (A) H&E staining of breast muscle sections, Scale bar = 200 μm, n = 3; (B) Areal of myofiber cross-sectional; (C) Number of myofibers; (D) Serum TBA; (E) Serum IGF1; (F) Serum IGF2; (G) Serum TG. CN, Control group; FA, Fat group; FB, Fat + bile acids compound group. a, b, c: Means in different groups without common superscripts are significantly different ($P < 0.05$).

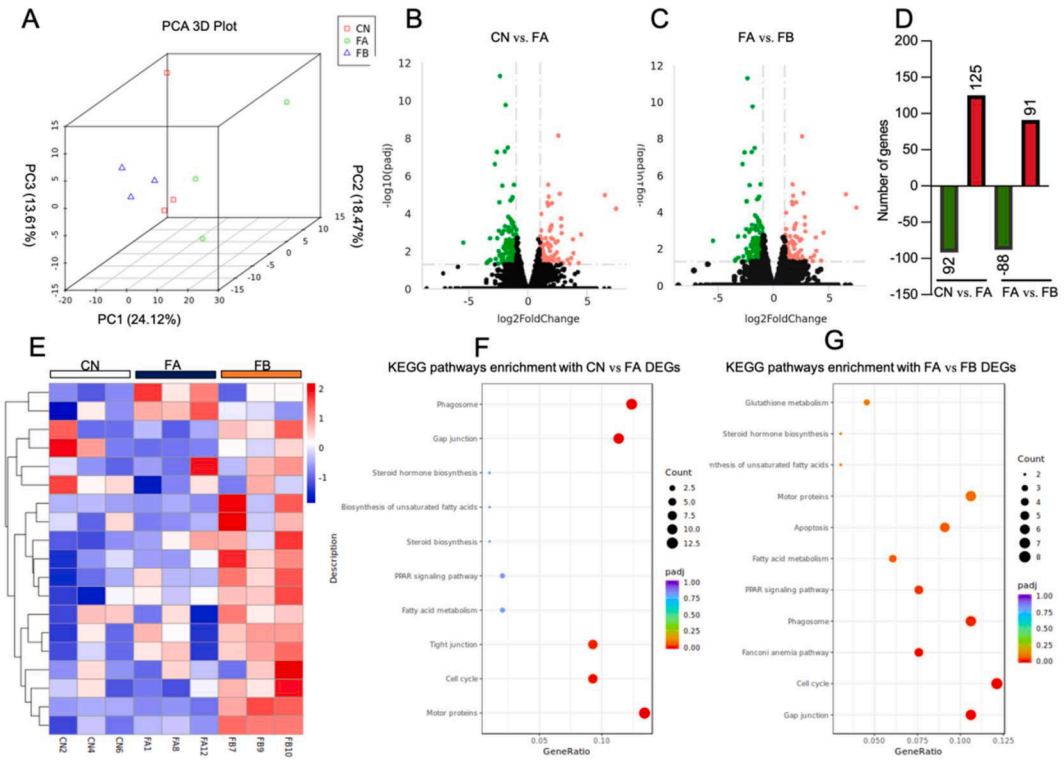

Fig. 3. Changes in the breast muscle transcriptional profile in duck with different diets.
Ducks were treated with 1.25 % rapeseed oil with or without 250 mg/kg feed BA for 30 d. (A) 3D PCA score plots of breast muscle tissues of the CN, FA, and FB; (B) Volcano plot indicating the genes in breast muscle with significantly increased (red dots) or decreased (green dots) expression in CN vs FA group; (C) Volcano plot indicating the genes in breast muscle with significantly increased (red dots) or decreased (green dots) expression in FA vs FB group; (D) Number of DEGs up (red) and downregulated (green) in liver tissues revealed via pairwise comparisons; (E) Heatmap of hierarchical clustering indicates differentially expressed genes in breast muscle of ducks from CN, FA, and FB; (F) Significantly enriched KEGG pathways in the CN vs FA;(G) Significantly enriched KEGG pathways in the FA vs FB. CN, Control group; FA, Fat group; FB, Fat + bile acids compound group.

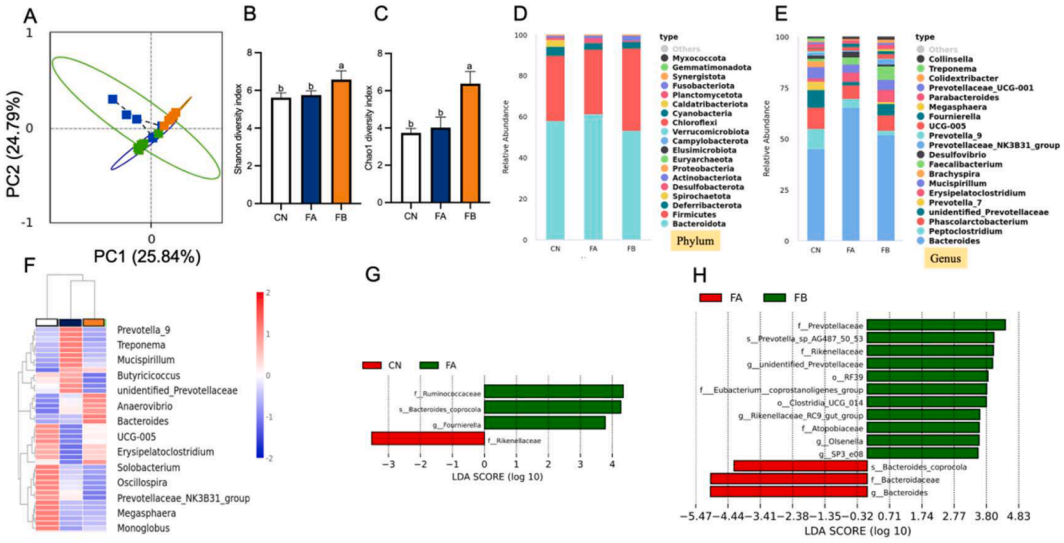

Fig. 4. Altered microbiota of cecum content from duck with different diets.
Ducks were treated with 1.25 % rapeseed oil with or without 250 mg/kg feed BA for 30 d. (A) Principal component analysis (PCA) of difference in microbiota of duck cecum content, with plots based on the Bray–Curtis distance. Each point represents a sample and the colors represent different groups; (B) α diversity was determined using the Shannon diversity index on raw ASV abundance after filtering out; (C) β diversity was determined using the Chao1 diversity index on raw ASV abundance after filtering out; (D) Community bar plot analysis of the microbiota of duck at the phylum level; (E) Community bar plot analysis of the microbiota of duck at the genus level; (F) Heatmap of hierarchical clustering indicates differentially microbiota at genus levels from CN, FA, and FB; (G) The LDA analysis of difference in the cecum content microbes between CN and FA, LDA score ≥ 3.5. Red means the CN group; green means the FA group; (H) The LDA analysis of difference in the cecum content microbes between FA and FB, LDA score ≥ 3.5. Red means the FA group; green means the FB group. Control group; FA, Fat group; FB, Fat + bile acids compound group.

similar contributions to the gut microbiota community structure driven by crude fat and BA supplementation. Also, the Shannon index (α-diversity) (Fig. 4B) and Chao 1 index (β-diversity) (Fig. 4C) were compared across the three groups. The results indicated no significant differences in the Shannon and Chao 1 indices between the CN and FA groups. However, BA supplementation significantly increased both indices ($P < 0.05$) compared to the FA group.

The bar plots of the top 20 microbial communities at the phylum level (Fig. 4D) revealed that *Bacteroidota* was the dominant phylum in both the CN (58.03 %) and FA (61.33 %) groups, with *Firmicutes* following closely at 31.88 % in the CN group and 31.48 % in the FA group. Meanwhile, the FB group displayed proportions of *Bacteroidota* (53.19 %) and *Firmicutes* (40.26 %). At the genus level (Fig. 4E), *Bacteroides* was the dominant taxon in all groups, with the highest relative abundance in the FA group (51.85 %), followed by the CN group (35.96 %) and the FB group (33.27 %). In the CN group, the next most abundant genera were *Phascolarctobacterium* (8.10 %) and *Peptoclostridium* (8.07 %). In the FA group, *Phascolarctobacterium* accounted for 5.02 %, and *Peptoclostridium* made up 3.47 %. In the FB group, *Phascolarctobacterium* was present at 4.83 %, and *Peptoclostridium* at 1.36 %. Hierarchical clustering analysis of the relative abundance of the top 20 genera (Fig. 4F) revealed distinct microbiota composition patterns across the different diets, further supporting the aforementioned results.

Additionally, linear discriminant analysis effect size (LEfSe) (LDA $\geq$ 3.5) was performed to identify bacterial taxa that exhibited dominant genera in each group, based on comparisons between CN *vs.* FA and FA *vs.* FB. In CN *vs.* FA comparison (Fig. 4G), 3 taxa were enriched in the FA group: the family *Ruminococcaceae*, the species *Bacteroides_coprocola*, and the genus *Fournierella*. Meanwhile, the family *Rikenellaceae* were enriched in the CN group. In FA *vs.* FB comparison (Fig. 4H), 11 bacterial taxa were enriched in the FB group, including the order of *Clostridia_UCG_014* and *RF39*, the families *Prevotellaceae, Rikenellaceae, Atopobiaceae,* and *Eubacterium_coprostanoligenes_group*, the genera of *unidentified Prevotellaceae, Rikenellaceae RC9 gut group, Olsenella*, and *SP3-e08*, and species *Prevotella sp AG487_50_53*. In contrast, the family *Bacteroidaceae*, the genera *Bacteroides*, and the species *Bacteroides_coprocola* were enriched in the FA group.

Conjoint network of microbiota in cecum content and gene expression in breast muscle

After KEGG analysis, the FPKM values of *PPARα* remained unchanged among the three groups. Similarly, the FPKM values of *CPT1α, NR1H4*, and *CETP* did not differ between the CN and FA groups; however, the FPKM values of these genes in the FB group were significantly higher ($P < 0.05$) than those in the FA group (Fig. 5A). To validate the DEGs library, 4 DEGs were selected for Real-time PCR analysis. The results (Fig. 5B) indicated that the Real-time PCR outcomes were consistent with the expression trends observed in the high-throughput sequencing, despite some quantitative differences in expression levels.

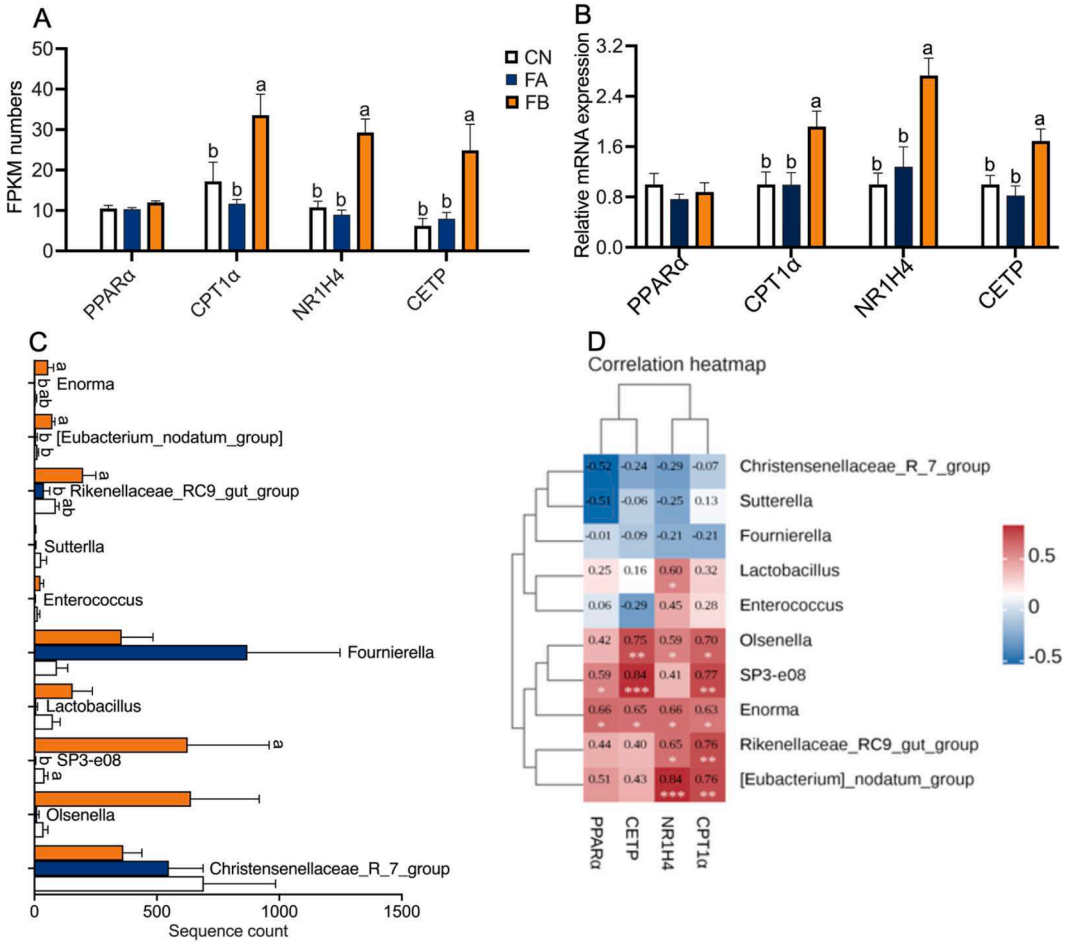

Fig. 5. Correlations between cecal microbiota and gene expression in breast muscle.
Ducks were treated with 1.25 % rapeseed oil with or without 250 mg/kg feed BA for 30 d. (A) FPKM values for *PPARα, CPT1α, NR1H4*, and *CETP*. (B) mRNA expression levels of *PPARα, CPT1α, NR1H4*, and *CETP*. (C) ASV count of microbiota at the genus level. (D) Correlation heatmap illustrating the relationship between gene expression and gut microbiota. Control group; FA, Fat group; FB, Fat + bile acids compound group. a, b, c: Means in different groups without common superscripts are significantly different ($P < 0.05$).

Additionally, based on the results of the LEfSe analysis (Fig. 5C), the ASV counts of genera *Enorma, [Eubacterium_nodatum_group]*, and *Rikenellaceae_RC9_gut_group* did not show significant changes between the CN and FA groups, while the relative abundance in the FB group was significantly higher ($P < 0.05$) than that in the FA group. Furthermore, the relative abundance of genus *SP3-e08* in the FA group was significantly greater ($P < 0.05$) than that in the CN group, and the relative abundance in the FB group was significantly higher ($P < 0.05$) than that in the FA group. No significant differences were found among the three groups for the remaining microbial genera, including *Sutterella, Enterococcus, Fournierella, Olsenella, Christensenellaceae_R_7group*, and *Lactobacillus*.

Taking the changes in gut microbiota and lipid metabolism related to skeletal muscle development into account, we further explored their potential interrelationships. Spearman correlation analysis (Fig. 5D) revealed moderate to strong positive correlations between *PPARα, NR1H4, CPT1α*, and *CETP* and several microbial genera, including *Olsenella, SP3-e08, Enorma, Rikenellaceae_RC9_gut_group*, and *Eubacterium nodatum group*, with R^2 values ranging from 0.40 to 0.84. Notably, *NR1H4* showed moderate positive correlations with *Enterococcus* ($R^2 = 0.45$), and *Lactobacillus* ($R^2 = 0.60$), while *CPT1α* exhibited a moderate positive correlation with *Lactobacillus* ($R^2 = 0.32$). Additionally, *PPARα* was found to have moderate negative correlations with *Christensenellaceae_R_7_group* and *Sutterella and Sutterlla* (R^2 = 0.52 and 0.51, respectively).

Discussion

The economic viability of poultry production largely depends on the efficiency of fat metabolism. This study aims to investigate the effects of BA supplementation on fat metabolism and breast muscle development of Zhijiang ducks fed a high-fat diet, while also exploring the roles of gut microbiota in this process. The findings will contribute to promoting the application of BA in feed formulations for Zhijiang ducks, ultimately enhancing their growth performance and increasing meat yield.

Enhancing fat utilization efficiency improves both poultry production performance and slaughter quality (Ge et al., 2019). A study indicated that incorporating bile acids with crude fat into the diet can significantly enhance average daily gain (ADG) and reduce feed conversion ratio (FCR) in broilers aged 1 to 21 days (Chen et al., 2024). Our findings align with previous reports that supplementation with crude fat significantly increased the ADG and decreased the FCR of Zhijiang ducks during the 20 to 50-day period. It is important to note that while excessive fat intake in poultry can reduce FCR, it may also lead to increased abdominal fat weight due to inadequate utilization, ultimately diminishing overall production efficiency (Fouad and El-Senousey, 2014). However, adding 80 mg/kg of bile acids to the diet can significantly enhance the efficiency of AA broilers in utilizing lard, thereby increasing breast muscle weight and reducing abdominal fat percentage (Geng et al., 2022). Additionally, incorporating 300 mg/kg of bile acids and 300 mg/kg of lipase into a basal diet significantly increased breast muscle ratio while reducing both abdominal fat weight and abdominal fat percentage in Cherry Valley ducks (Li et al., 2021). Consistent with these findings, our research demonstrates that exogenous BA supplementation decreases abdominal fat mass, and breast muscle mass and percentage in ducks fed a high-energy diet, underscoring the role of BA in regulating fat metabolism in ducks.

Skeletal muscle development is influenced by various hormones, including thyroid hormones, growth hormone, IGF1, and IGF2. In vitro studies have shown that IGF1 and IGF2 promote DNA synthesis and hypertrophy in rainbow trout skeletal muscle cells by activating the PI3K/AKT pathway (Codina et al., 2008). In vivo studies have also demonstrated that upregulating IGF2 expression in porcine embryonic fibroblasts using gene editing techniques enhances skeletal muscle development in Liang Guang Small Spotted pigs (Liu et al., 2019). Notably, the expression levels of IGFs are closely related to bile acids. Increasing evidence suggests that bile acids upregulate IGF1 levels, promoting protein synthesis in skeletal muscle and consequently promoting muscle hypertrophy in mice (Tamai et al., 2022). For instance, infusion of 2 mg/kg body weight of cholic acid via the jugular vein in ewes significantly increased serum IGF2 levels (Chen et al., 2002). Additionally, the inclusion of 250 mg/kg of bile acids in the diet significantly elevated IGF2 levels in the blood, liver, and breast muscle of Ross 308 broilers, along with an increase in skeletal muscle weight (Chen et al., 2024). In this study, the addition of 250 mg/kg of BA to the diet resulted in a significant increase in serum total bile acids, which corresponded to elevated concentrations of serum IGF1 and IGF2. Furthermore, the effect of BA significantly increased the cross-sectional area of muscle fibers, further highlighting their role in promoting skeletal muscle development.

To further elucidate the regulatory mechanisms of BA on fat metabolism, KEGG pathway enrichment analysis revealed that diets enriched with crude fat and BA significantly altered several metabolic pathways in ducks. Notably, the pathways related to PPARα, steroid hormone biosynthesis, and fatty acid metabolism were prominently enriched. PPARs, important nuclear transcription factors involved in fat and cholesterol metabolism, have been shown in vitro to enhance long-chain fatty acid oxidation by 6-fold in 293T human embryonic kidney cells overexpressing CPT1α (Jambor de Sousa et al., 2005). Furthermore, feeding broilers a diet containing bile acids (4 mg to 32 mg per day) for 35 days significantly increased hepatic PPARα gene expression while simultaneously lowering serum TG levels (Hu et al., 2024). The steroid hormone synthesis pathway is also crucial for fatty acid oxidation and bile acid metabolism. Hepatic FXR not only enhances CPT1α expression through PPARα but may also help maintain the homeostasis of fats and cholesterol via CETP regulation (Kinoshita et al., 2004; Zhou et al., 2022). Additionally, feeding laying hens a diet containing 100 mg/kg of bile acids significantly reduced hepatic TG levels, while simultaneously upregulating the expression of FXR, CYP7A1, and CYP8B1 (Sun et al., 2023). In broilers fed a diet with 250 mg/kg of bile acids, there was a significant increase in FXR expression in breast muscle and a notable decrease in hepatic TG content (Chen et al., 2024). These findings collectively indicate that bile acids regulate fat metabolism in animals by influencing receptor activity and cholesterol metabolic pathways. The result of present study also showed that BA supplementation significantly upregulated the mRNA levels of *FX*R, *CPT1α, CETP*, and *PPARα* repeat identified by Real-time PCR. The gut microbiota is closely linked to the signaling pathways mediated by bile acids that influence fat metabolism and skeletal muscle development. A study demonstrated that feeding high-fat diets to mice increases the relative abundances of *Mucispirillum, Desulfovibrio, Anaerotruncus*, and *Desulfovibrionaceae*, while the abundances of *Bifidobacterium* and *Bacteroides* decrease, leading to hepatic fat metabolism disorders (Zhang et al., 2021). Additionally, supplementation with *Lactobacillus johnsonii* strain BS15 has been shown to reduce fat deposition and optimize gut microbiota structure in Cobb broilers (Wang et al., 2017). Furthermore, incorporating 60 mg/kg of bile acids into the diet decreases the abundance of *Bacteroides* in the cecum while increasing the abundances of *Bifidobacterium, Escherichia*, and *Lactobacillus*, which aids in reducing hepatic fat deposition associated with high-fat diets (Wang et al., 2024).

In this study, the addition of BA to a high-fat diet significantly increased both the Shannon (5.81 ± 0.11 *vs.* 6.56 ± 0.65, $P = 0.011$) and Chao1 (4.03 ± 0.57 *vs.* 6.38 ± 0.65, $P = 0.025$) indices of the duck gut microbiota, indicating that BA supplementation may enhance gut microbial diversity and optimize community structure. LEfSe analysis further revealed that BA significantly enriched the abundances of [*Eubacterium*] *nodatum group* (LDA = 3.02, $P = 0.02$), *Rikenellaceae RC9 gut group* (LDA = 3.59, $P = 0.02$), *SP3-e08* (LDA = 3.53, $P = 0.02$), and *Enorma* (LDA = 3.03, $P = 0.02$) in the duck gut. Although these differentially abundant genera differ from those reported in previous studies (Hu et al., 2024; Yin et al., 2021; Yang et al., 2022), such variations may be attributed to differences in growth stages, rearing environments, or duck breeds. In a human disease model, *Enorma* abundance was

significantly increased and showed a negative correlation with metabolites such as 3-hydroxy-3-methyl-2-oxo-butanoic acid, glycocholic acid (GCA), and chenodeoxycholic acid glycine conjugate (GCDCA) (Feng et al., 2023). As conjugated bile acids, GCA and GCDCA inhibit the bile acid receptor FXR (Dai et al., 2011), which is crucial for fat metabolism and skeletal muscle development. Additionally, the [*Eubacterium*] *nodatum group* is linked to the production of various secondary metabolites in the gut. Studies have shown that during the recovery phase in patients with type 2 diabetes, the abundance of [*Eubacterium*] *nodatum group* increases and positively correlating with metabolites such as deoxycholic acid (DCA) and cholanic acid (CA) (Xiao et al., 2020). CA, a primary bile acid synthesized via the classical pathway, is converted into DCA by specific gut microbes. Both CA (Lew et al., 2004) and DCA (Xu et al., 2022) have been reported to upregulate FXR activity. Interestingly, in a disease model, the abundance of *Rikenellaceae_RC9_gut_group* increased and showed positive correlations with the expression of genes involved in gastric fat digestion and absorption, as well as with elevated levels of deoxycholic acid (DCA) and tauroursodeoxycholic acid (TCDCA); notably, FXR expression levels were also significantly upregulated (Xu et al., 2023). Multiple studies have identified TCDCA and DCA as FXR agonists. Although limited research is available on *SP3-e08*, some evidence suggests its involvement in feed efficiency and short-chain fatty acid production in ruminants (Huang et al., 2024).

In this study, Bile acid supplementation in a high-fat diet significantly enriched cecal bacterial genera compared to the FA group, with *Enorma* increasing by 56.3-fold, *SP3-e08* by 178.8-fold, *Rikenellaceae_RC9_gut_group* by 10.5-fold, and *[Eubacterium]_nodatum_group* by 5.1-fold. These substantial microbial shifts were closely associated with alterations in the expression of genes involved in lipid metabolism and skeletal muscle development. For example, the expression of *CPT1α* was positively correlated with the abundances of *Olsenella, SP3-e08, Enorma, Rikenellaceae_RC9_gut_group*, and *[Eubacterium]_nodatum_group*. Similarly, *NR1H4* expression was associated with higher levels of *Lactobacillus, Olsenella, Rikenellaceae_RC9_gut_group*, [*Eubacterium*]*_nodatum_group*, and *Enorma*. In addition, *CETP* expression was positively linked to *Olsenella, SP3-e08*, and *Enorma*, while *PPARα* expression showed positive correlations with *SP3-e08* and *Enorma*. Collectively, these findings suggest that BA supplementation not only reshapes the cecal microbial community in a quantitative manner but also influence host lipid metabolism and skeletal muscle development by modulating specific microbial populations and their associated metabolic activities.

Conclusion

In conclusion, the inclusion of porcine bile acids to high-fat diets significantly improved the slaughter performance in Zhijiang ducks, in terms of breast muscle weight. This underlying mechanism may be attributed to the optimization of gut microbiota structure, which enhances FXR-mediated fatty acid oxidation and promotes skeletal muscle development. These findings indicate that bile acids hold considerable potential to improve the economic efficiency of large-scale farming of Zhijiang ducks, especially under high-fat dietary conditions.

Declaration of competing interest

This manuscript is an original, unpublished work and has not been submitted to any other journals for publication, in whole or in part. All authors listed have read and approved this manuscript, and there is no conflict of interest in the submission of this manuscript.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.105319.

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