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Impact of dietary protein and energy levels on fatty acid profile, gut microbiome and cecal metabolome in native growing chickens

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ABSTRACT The present study investigated the optimal concentration of dietary ME and CP for the fatty acid profile of meat, gut microbiome, and cecal metabolome in Danzhou chickens from 120 to 150 d of age. A total of seven hundred and twenty 120-d-old Danzhou female chickens, with a similar BW, were randomly allocated into 6 treatments with 6 replicates and each of 20 birds. The chickens were fed 2 levels of dietary ME (11.70 MJ/kg, 12.50 MJ/kg), and 3 levels of dietary CP (13%, 14%, and 15%). The results showed that dietary ME and CP levels didn't affect final BW, ADG, ADFI, and feed gain ratio (g: g) ($P > 0.05$). The serum concentrations of triglyceride, insulin, and glucose in the 12.50 MJ/kg group were the highest ($P < 0.05$). Dietary ME, CP levels, and their interactions affected ($P < 0.05$) the fatty acid content in the breast muscle, thigh muscle, and liver. The levels of C18:0, C20:0, C22:0, C22:1, C18:2, C18:3, C22:6, and SFA of the liver in the high ME group were higher than those in the low ME group ($P < 0.05$). The levels of C16:0, C14:1, C18:1, C22:5, SFA, MUFA and USFA in the low CP group were higher than the corresponding values in the other groups ($P < 0.05$). Dietary ME and CP levels altered the composition and relative abundance of microbiota in the cecum of chickens at various taxonomic levels to different extents. Significant effects of interactions were found between dietary ME and CP on the relative abundance of 10 species ($P < 0.05$), and among these species, 6 species belonged to the genus *Bacteroides*. Notably, the relative abundance of 2 probiotic species including *Lactobacillus crispatus* and *Lactobacillus salivarius* was significantly increased ($P < 0.05$) with increasing dietary ME level. There were 6 differential metabolites in the cecum, comprising thromboxane A2, 5,6-DHET, prostaglandin D2, 20-hydroxyeicosatetraenoic acid, 12(*S*)-HPETE and prostaglandin I2 significantly reduced ($P < 0.05$) with increasing the dietary ME level; all of them are involved in arachidonic acid metabolism. In conclusion, the present study suggested that the dietary levels of 12.50 MJ/kg ME and 14% CP enhanced meat quality in terms of fatty acid composition, and showed benefits for maintaining intestinal health via positive regulation of cecal microbiota in native growing Danzhou chickens.

Key words: metabolizable energy, crude protein, fatty acid, gut metabolism, native growing chicken

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INTRODUCTION

With the improvement of people's living standards, the demand for slow growing meat-type chickens is continually increasing due to their distinct tasting meat and excellent nutritional profile (Khumpeerawat et al., 2021; Haunshi et al., 2022). Slow-growing meat-type chickens have better meat quality and flavor than fast-growing meat-type chickens due to their long growth cycle. So, it is more popular among Chinese consumers (Gou et al., 2016; Jiang et al., 2018). Danzhou chicken is a local slow-growing yellow-feathered breed used for meat and egg production; it is often marketed at 150 to 180 d of age, with a BW range of 1.1 to 1.5 kg. This breed is mainly distributed in Hainan Province, China, and possesses features of strong disease resistance, coarse feeding resistance, and high meat quality and distinct flavor (Cai, 2018).

Generally, the level of nutrient intake, especially of dietary metabolizable energy (**ME**) and crude protein (**CP**), has critical influences on performance and meat quality of broilers (Mir et al., 2017). The optimal dietary ME and CP levels for broilers has been estimated in several previous studies (Applegate and Angel, 2014; NRC, 1994). Previous studies on the interactions of ME and CP levels showed varying results depending on different environments, production systems, breeds, etc

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(Gonzalez Ariza et al., 2022; Song et al., 2023). Little effort has been done to optimize the dietary ME and CP levels for slow-growing meat-type chickens of different breeds (Jiang et al., 2003; Gou et al., 2016), and there is no research on the nutritional requirements of Danzhou chickens. In practical production, producers usually refer to the nutritional requirements of other native chicken breeds with similar body size, such as Wenchang, and Lingnanhuang broilers. Determining the optimal dietary ME and CP levels for this breed, therefore, is of paramount importance to maximize their production parameters and meat quality.

In poultry production, the meat fatty acid composition is considered a factor that affects the meat quality and mainly influences flavor, juiciness, and tenderness (Wood et al., 2008; Li et al., 2022). Notably, recent evidence indicated that the gut microbiota plays an important role in linking the diet composition and fatty acid metabolism, the so-called "host-gut microbiota-metabolism axis" (Hyun et al., 2022; Yun et al., 2022). The current study aimed to evaluate the effects of dietary ME and CP levels on growth performance, serum parameters, and meat fatty acid composition in growing Danzhou chickens via host-gut microbiota-metabolism axis. The study results are expected to provide a useful reference for the establishment of accurate diet formulation for the Danzhou chickens during the fattening period.

MATERIAL AND METHODS

Chickens, Diet and Management

The experimental conditions for the study conformed to the standards for animal experiments and were approved by the Institutional Animal Care and Use Committee of the Chinese Academy of Tropical Agricultural Sciences (approval number CATAS-20221015-1).

A total of 720 female Danzhou chickens were weighted at 120 d of age and randomly assigned to 6 dietary treatments in a 2 × 3 factorial arrangement. A total of 6 replicates, each of 20 chickens, 2 chickens/cage, were assigned for each dietary treatment. The chickens were fed 2 levels of dietary ME (11.70 MJ/kg, 12.50 MJ/kg), and 3 levels of dietary CP (13, 14, 15%). The experimental diets were offered to the chickens between 120th and 150th d of age. The composition and nutrient levels of the diets were seen in Table 1. The dry matter, gross energy, and CP of the diets were analyzed according to the guidelines of Association of Official Analytical Chemists (2000). The other nutrient values of diet were calculated according to the data provided by Feed Database in China (2013). All chickens were housed in 3-tier wired cages (the size of each cage is 40 × 45 × 45 cm) for the 30-d feeding trial in one house and had free access to feed and water during the entire rearing period, and immunized according to normal immunization procedures. The birds were raised under artificial lighting providing 18 h light: 6 h dark. Relative humidity and average room temperature were approximately 70% and 25 °C throughout the 30-d experimental period (120 −150 d of age).

Table 1. Composition and nutrient levels of experimental diets (%, as fed basis).

Treatments	T1	T2	T3	T4	T5	T6
ME, MJ/kg	11.70	11.70	11.70	12.50	12.50	12.50
CP, %	13	14	15	13	14	15
Ingredients						
Corn	66.30	64.20	63.20	71.45	67.70	71.20
Soybean meal	10.85	13.80	17.10	12.80	15.40	19.30
Wheat	8.00	9.20	8.20	6.00	9.40	2.00
Wheat bran	9.35	7.30	6.00	2.25	/	/
Soybean oil	/	/	/	2.00	2.00	2.00
Shell powder	1.50	1.50	1.50	1.50	1.50	1.50
Premix[1]	4.00	4.00	4.00	4.00	4.00	4.00
Total	100.00	100.00	100.00	100.00	100.00	100.00
Analysis results of nutrient level[2]						
DM, %	88.62	88.35	88.46	88.38	88.55	88.65
Gross energy, MJ/kg	15.13	15.18	15.22	15.45	15.48	15.54
CP, %	13.17	13.98	15.19	13.07	13.95	15.05
Calculated results of nutrient level[3]						
ME, MJ/kg	11.72	11.75	11.72	12.58	12.60	12.54
CP, %	13.01	14.01	15.05	13.02	14.00	15.00
Ca, %	0.95	0.96	0.97	0.95	0.96	0.96
Available P, %	0.14	0.14	0.14	0.15	0.13	0.13
Total Lys, %	0.53	0.59	0.66	0.54	0.60	0.69
Total Met, %	0.21	0.25	0.24	0.23	0.24	0.25
Total Cys, %	0.24	0.26	0.27	0.25	0.26	0.27

[1]The premix provided the following per kg of diets: vitamin A, 11,000 IU; vitamin E, 20 IU; vitamin K, 3 mg; vitamin D, 3000 IU; vitamin B1, 2 mg; vitamin B2, 8 mg; vitamin B12, 0.04 mg; Fe, 65 mg; Cu, 10 mg; Mn, 77 mg; Zn, 70 mg; pantothenic acid, 19 mg; folic acid, 1.1 mg.
[2]Analyzed in triplicates.
[3]According to the data provided by Feed Database in China (2013)

Growth Performance

The amounts of provided and refused feed were measured daily on a replicate basis to calculate the average daily feed intake (**ADFI**), including adjustments for any dead birds. The initial and final body weights, average daily body weight gain (**ADG**), and feed: gain ratio (g: g) were measured on a replicate basis.

Sampling

At completion of treatments at 150 d of age, one bird was selected randomly from each replicate after 12 h of feed-withdrawal. Blood samples were collected in 10 mL tubes from the jugular vein, and centrifuged at 4,000 × *g* for 10 min for separation of serum, and stored at -20 °C until analysis. The birds were immediately slaughtered via cervical dislocation. Tissue samples were obtained from the liver, the right breast muscle, and the right thigh muscle and then frozen at -20 °C for fatty acid profile determination. The cecal contents were collected and stored at -80°C for microbiome and metabolomics analysis.

Blood Sample Analysis

The serum levels of insulin (**INS**), triglycerides (**TG**) and glucose (**GLU**) were determined using a microplate

reader (SP-Max 2300A, Shanghai Flash Spectrum Biological Technology CO., Ltd, Shanghai, China) and commercial kits (Shanghai Enzyme-linked Biotechnology Inc., Shanghai, China) according to the manufacturer's instructions.

Fatty Acid Composition Determination

Tissue samples were collected (approximately 50 mg) into 2 mL EP tubes, extracted with 500 μL extracting solution [$V_{Isopropanol}$: $V_{n\text{-}Hexane}$= 2: 3, containing 0.2mg/L internal standard (Stearic-d35 acid)], mixed with vortex for 30s, homogenized in a ball mill for 4 min at 40 Hz, then sonicated for 5 min (incubated in ice water), and centrifuged for 15 min at 13,800 × *g*, 4°C. The supernatant was transferred into a fresh 1.5 mL EP tube. The extraction step was repeated another 2 times, and all supernatants were then combined. 800 μL of the combined supernatant and nitrogen blow-dry were taken, 500 μL of methanol: trimethylsilyl diazomethane solution (1:2, v/v) were added, and equilibrated at room temperature for 30 min, and then were dried by blowing nitrogen. The resulting solution was redissolved by adding 160 μL of n-hexane, then centrifuged for 1 min at 13,800 × *g*, and the final supernatant was transferred into a fresh vial for GC-MS analysis.

GC-MS analysis was performed using an Agilent 7890B gas chromatograph system coupled with an Agilent 5977B mass spectrometer. The system utilized a DB-FastFAME capillary column (90 m × 250 μm × 0.25 μm film thickness; Agilent, Beijing, China). A 1 μL aliquot of the analyte was injected in split mode (5:1). Helium was used as the carrier gas, the front inlet purge flow was 3 mL/min, and the gas flow rate through the column was 46 psi with constant pressure. The initial temperature was kept at 50°C, held on for 1 min; raised to 200°C at a rate of 50°C/min, held on for 15 min; raised to 210°C at a rate of 2°C/min, held on for 1 min; raised to 230°C at a rate of 10°C/min, held on for 15 min. The injection, transfer line, quad and ion source temperatures were 240°C, 240°C, 230°C and 150°C, respectively. The energy was -70 eV in electron impact mode. The mass spectrometry data were acquired in Scan/SIM mode with the *m/z* range of 33-400 after a solvent delay of 7 min. The mass-to-charge ratios (*m/z*) of fatty acids detected are included in Supplementary Table S1.

Cecal Microbiome

Microbiome characterization was performed by Biotree Inc. (Shanghai, China). The total DNA was extracted from cecal content samples using the Fecal Genome DNA Extraction Kit (AU46111-96, BioTeke, China). DNA libraries were constructed using the TruSeq Nano DNA Library Preparation Kit-Set (#FC-121−4001, Illumina, San Diego, CA) following the manufacturer's instructions. Metagenome libraries were then sequenced on an Illumina NovaSeq 6000 platform with PE150. Sequencing adapters were removed from demultiplexed raw sequences using cutadapt (v1.9). Then, the low-quality reads (quality scores < 20), short reads (<100 bp), and reads containing more than 5% "N" records were trimmed by using the sliding-window algorithm method in fqtrim (v0.94). Quality filtered reads were first aligned to host genome by using bowite (v2.2) to filter out host contaminations. Then, the remaining reads were subjected to denovo assembly for each sample using MEGAHIT (v1.2.9) and used to assign microbial function and taxonomy. MetaGeneMark (v3.26) was used to predict the coding regions (CDS) of the assembled contigs, and CDS sequences of all samples were clustered using CD-HIT (v4.6.1) to obtain unigenes. DIAMOND (v0.9.14) was used to perform a taxonomic assessment of microbiota based on the NR database. The alpha diversity (Observed species, Gini-Simpson, Chao1, and Shannon indexes) and beta diversity [principal coordinate analysis (**PCoA**) based on Bray−Curtis distance] metrics were calculated using QIIME (v1.9.1). For metagene sequencing, the top 20 microbiomes at the taxonomic levels of phylum, class, order, family, and genus, as well as the top 300 microbiomes at the species level were chosen to compare their differences in relative abundance, respectively.

Metabolomics of Cecal Contents

A total of 50 mg of cecal content sample was weighted to an EP tube, and 1,000 μL extract solution (methanol: acetonitrile: water = 2: 2: 1, with isotopically-labeled internal standard mixture) was added. Then the samples were homogenized at 35 Hz for 4 min and sonicated for 5 min in an ice-water bath. The homogenization and sonication cycle were repeated 3 times. Then the samples were incubated for 1 h at -40 °C and centrifuged at 13,800 × *g* for 15 min at 4 °C. The resulting supernatant was transferred to a fresh glass vial for analysis. The quality control (**QC**) sample was prepared by mixing an equal aliquot of the supernatants from all of the samples.

LC-MS/MS analyses were performed using an UPLC system (Vanquish, Thermo Fisher Scientific, Shanghai, China) with an ACQUITY UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 μm; Waters, Shanghai, China) coupled to Orbitrap Exploris 120 mass spectrometer (Orbitrap MS, Thermo, Shanghai, China). The mobile phase consisted of 25 mmol/L ammonium acetate and 25 mmol/L ammonia hydroxide in water (pH = 9.75) (A) and acetonitrile (B). The auto-sampler temperature was 4 °C, and the injection volume was 2 μL. The Orbitrap Exploris 120 mass spectrometer was used for its ability to acquire MS/MS spectra on information-dependent acquisition (**IDA**) mode in the control of the acquisition software (Xcalibur, Thermo). In this mode, the acquisition software continuously evaluates the full scan MS spectrum. The electrospray ion source (**ESI**) conditions were set as follows: sheath gas flow rate as 50 arb, aux gas flow rate as 15 arb, capillary temperature 320 °C, full MS resolution as 60,000, MS/MS resolution as

15,000 collision energy as 10/30/60 in NCE mode, spray voltage as 3.8 kV (positive) or -3.4 kV (negative), respectively.

The raw data were converted to the mzXML format using ProteoWizard and processed with an in-house program, which was developed using R and based on XCMS, for peak detection, extraction, alignment, and integration. Then an in-house MS^2 database (Biotree DB, Shanghai, China) was applied to metabolite annotation. The cutoff for annotation was set at 0.3. Statistically significant pathways (i.e., top enriched pathways) were selected using a cutoff of false discovery rate (**FDR**) ≤ 0.25 and $P < 0.05$. All metabolite-related statistical analyses were performed using the MetaboAnalyst 5.0 (http://www.metaboanalyst.ca/) platform. Multivariate statistics including unsupervised principal component analysis (**PCA**) and supervised partial least squares-discriminant analysis (**PLS-DA**) were conducted on the metabolic data to obtain an overview of the variation among groups. Subsequently, statistical differences were calculated using a Student's *t*-test for comparison within 2 groups or one-way ANOVA with Fisher's LSD post hoc test for comparison within more than 2 groups. A difference where $P < 0.05$ was considered statistically significant.

Statistical Analysis

Six replicates were used as the experimental units. The main effects of dietary ME and CP levels as well as their interactions were analyzed by 2-way ANOVA using SPSS 23.0 (IBM-SPSS Inc., Chicago, IL). The data are expressed as means with SEM in tables and SD in figures, respectively. Where the ANOVA indicated significant main effects, the corresponding means were compared using Tukey's multiple comparisons test. $P < 0.05$ indicated a significant difference, and $0.05 < P < 0.10$ indicated a tendency.

RESULTS

Growth Performance

Table 2 showed that dietary ME and CP levels had no significant effects on final weight, ADG, ADFI, and feed gain ratio (g: g) of chickens ($P > 0.05$).

Serum Characteristics

As shown in Table 3, the chickens in T4 group (ME level of 12.50 MJ/kg and CP level of 13%) had the highest serum concentration of TG. The serum level of INS in the medium CP group was higher than that in the low CP level group ($P < 0.05$). The high ME level significantly increased the serum levels of INS, TG, and GLU compared with the low ME level ($P < 0.05$). There were significant interactions between ME and CP on serum levels of TG and INS ($P < 0.05$).

Fatty Acid Composition in Peripheral Tissues

Table 4A showed that dietary ME level and CP level affected the levels of C22:1, C22:4, and C22:6 in the breast muscle significantly ($P < 0.05$), and T1 group (ME level of 11.70 MJ/kg and CP level of 13%) showed the lowest ($P < 0.05$) levels of C22:1 and C22:6 in the breast muscles. Table 4B showed that the levels of C22:1, C20:2, and C22:6 in the high ME group were higher than those in the low ME group ($P < 0.05$). The low CP group had the highest level of C22:1 ($P < 0.05$). The content of C22:1 in the breast muscle was affected by interaction effects of dietary ME and CP ($P < 0.05$).

Table 2. Effect of dietary ME and CP levels on performance of Danzhou chickens aged 120 to 150 d.

Treatments	ME/ (MJ/kg)	CP/%	Initial weight/g	Final weight/g	Average daily gain (ADG)/g	Average daily feed intake (ADFI)/g	Feed gain ratio (g: g)
T1	11.70	13	951.90	1,088.80	4.40	57.25	13.15
T2	11.70	14	963.30	1,080.40	3.80	55.81	14.91
T3	11.70	15	948.40	1,101.70	4.90	57.44	11.78
T4	12.50	13	947.00	1,075.80	4.20	56.48	14.08
T5	12.50	14	957.00	1,079.90	4.00	56.49	16.95
T6	12.50	15	956.40	1,111.50	5.00	55.44	11.82
SEM			12.30	22.00	0.60	1.53	1.98
Main effect							
Low ME	11.70	/	954.50	1,090.30	4.40	56.83	13.28
High ME	12.50	/	953.50	1,089.10	4.40	56.14	14.28
SEM			6.60	12.10	0.30	0.82	1.16
P-value			0.919	0.948	0.988	0.584	0.540
Low CP		13	949.40	1,082.30	4.30	56.90	13.61
Medium CP		14	960.10	1,080.10	3.90	56.10	15.93
High CP		15	952.40	1,106.60	5.00	56.40	11.80
SEM		/	8.20	14.53	0.40	1.00	1.32
P-value		/	0.675	0.428	0.188	0.895	0.141
ME × CP *P*-value			0.818	0.875	0.926	0.686	0.880

Low ME level = 11.70 MJ/kg ME and CP of 13%, 14% and 15% (n= 18); High ME level = 12.50 MJ/kg ME and CP of 13%, 14% and 15% (n= 18); Low CP level = 13% CP and ME of 11.70 MJ/kg and 12.50 MJ/kg (n= 12); Medium CP level = 14% CP and ME of 11.70 MJ/kg and 12.50 MJ/kg (n = 12); High CP level = 15% CP and ME of 11.70 MJ/kg and 12.50 MJ/kg (n = 12). The below is the same.

Table 3. Effect of dietary ME and CP levels on the serum concentration of TG, INS and GLU.

Treatments	ME/(MJ/kg)	CP/%	TG/(mmol/L)	INS/(mU/L)	GLU/(mmol/L)
T1	11.70	13	2.46^{ab}	38.96^{ab}	12.75
T2	11.70	14	2.10^{b}	39.05^{ab}	11.88
T3	11.70	15	2.99^{ab}	36.10^{b}	13.12
T4	12.50	13	3.34^{a}	36.31^{b}	14.73
T5	12.50	14	2.80^{ab}	44.37^{a}	16.10
T6	12.50	15	2.83^{ab}	44.69^{a}	16.48
SEM	/	/	0.21	1.53	1.20
Main effect					
Low ME	11.70	/	2.52^{b}	38.03^{b}	12.59^{b}
High ME	12.50	/	2.99^{a}	41.79^{a}	15.77^{a}
SEM			0.14	0.94	0.67
P-value			0.011	0.005	0.003
Low CP	/	13	2.90	37.63^{b}	13.74
Medium CP	/	14	2.45	41.71^{a}	13.99
High CP	/	15	2.91	40.39^{ab}	14.80
SEM		/	0.17	1.37	0.95
P-value		/	0.066	0.036	0.656
ME × CP *P*-value		/	0.046	0.003	0.647

Within a column, means not sharing a common superscript letter are significantly different at $P < 0.05$. TG, triglyceride; INS, insulin; GLU, glucose.

Table 4A. Effect of dietary ME and CP levels on fatty acid composition in the breast muscle of Danzhou chickens (μg/g).

	Treatments							
Items	T1	T2	T3	T4	T5	T6	SEM	*P*-value
C8:0	0.04	0.04	0.04	0.04	0.05	0.05	0.00	0.058
C12:0	0.02	0.03	0.01	0.09	0.06	0.27	0.10	0.406
C13:0	0.09	0.12	0.09	0.17	0.11	0.08	0.03	0.207
C14:0	1.59	1.41	1.73	1.82	1.54	4.42	1.29	0.560
C15:0	0.80	0.88	0.66	0.64	0.76	1.11	0.17	0.407
C16:0	181.33	158.72	194.29	170.85	169.29	280.31	49.70	0.553
C17:0	1.10	1.09	1.13	1.11	1.12	1.61	0.29	0.771
C18:0	108.33	99.51	104.27	96.53	107.55	123.10	12.78	0.752
C20:0	0.97	0.98	0.92	0.89	0.86	1.20	0.16	0.735
C21:0	0.45	0.42	0.42	0.41	0.40	0.41	0.01	0.113
C22:0	0.55	0.47	0.47	0.48	0.46	0.50	0.05	0.733
C14:1	0.40	0.40	0.56	0.70	0.47	1.38	0.30	0.188
C16:1	11.86	7.30	14.81	11.45	7.52	36.96	12.42	0.556
C18:1	170.34	155.88	188.24	188.30	148.45	377.55	92.07	0.509
C20:1	0.99	0.82	1.01	1.06	0.83	2.56	0.74	0.555
C22:1	1.21^{b}	1.45^{b}	1.84^{b}	2.95^{a}	1.56^{b}	1.36^{b}	0.19	< 0.001
C18:2	97.88	87.23	119.06	116.53	111.73	198.77	46.28	0.606
C18:3	10.89	8.70	11.00	10.37	9.45	16.33	2.98	0.546
C20:2	2.86	2.77	3.36	3.59	3.97	3.74	0.49	0.119
C20:4	126.46	122.39	143.36	120.85	133.41	111.57	9.62	0.288
C20:5	1.02	0.71	0.86	0.88	0.92	0.79	0.08	0.155
C22:4	14.66^{a}	12.09^{ab}	15.51^{a}	10.58^{b}	12.90^{ab}	10.68^{b}	1.22	0.034
C22:5	25.41	23.45	25.42	22.38	22.58	19.54	2.15	0.409
C22:6	13.79^{b}	15.53^{ab}	16.56^{ab}	18.45^{ab}	23.52^{a}	18.64^{ab}	2.09	0.046
SFA	295.27	263.68	304.03	273.02	282.20	413.07	63.95	0.603
MUFA	184.80	165.86	206.45	204.47	158.83	419.82	105.47	0.515
PUFA	292.98	272.87	335.12	303.64	318.47	380.06	51.47	0.752
USFA	477.78	438.73	541.57	508.11	477.31	799.88	155.38	0.615
USFA/SFA	1.61	1.65	1.79	1.83	1.66	1.75	0.07	0.241

Within a row, means not sharing a common superscript letter are significantly different at $P < 0.05$. SFA, saturated fatty acid; USFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; USFA/SFA, the ratio of unsaturated fatty acids to saturated fatty acid.

There was a tendency for C20:4, C20:5 and C22:4 affected by interactions of dietary ME and CP ($0.05 < P < 0.10$).

The fatty acid content in the thigh muscle was summarized in Table 5A and Table 5B. Dietary ME level and CP level affected the contents of C13:0, C22:1, and C22:6 significantly ($P < 0.05$) (Table 5A), and the highest ($P < 0.05$) levels of C13:0 and C22:1 were observed in T4 group (ME level of 12.50 MJ/kg and CP level of 13%). Additionally, T1 group (ME level of 11.70 MJ/kg and CP level of 13%) had the lowest C22:6 concentrations ($P < 0.05$). Table 5B showed that the levels of C22:1, C22:5, C22:6, and USFA/SFA in the high ME group were higher than those in the low ME group ($P < 0.05$). The contents of C13:0 and C22:1 in the thigh muscle were influenced by interaction effects of dietary ME and CP ($P < 0.05$).

Table 6A showed that in the liver, the dietary levels of ME and CP affected most of fatty acids content, including C14:0, C16:0, C18:0, C20:0, C22:0, C16:1, C18:1, C20:1, C22:1, C18:2, C22:5, C22:6, SFA, MUFA, and USFA ($P < 0.05$). Table 6B showed that levels of C18:0,

Table 4B. Effect of dietary ME and CP levels on fatty acid content in the breast muscle of Danzhou chickens (μg/g).

	ME (MJ/kg)			CP (%)				
Items	11.70	12.50	*P*-value	13	14	15	*P*-value	ME × CP *P*-value
C8:0	0.04^{b}	0.05^{a}	0.036	0.04	0.04	0.05	0.262	0.122
C12:0	0.02	0.14	0.139	0.05	0.05	0.14	0.546	0.433
C13:0	0.10	0.12	0.331	0.13	0.12	0.08	0.225	0.183
C14:0	1.58	2.59	0.342	1.71	1.47	3.07	0.419	0.538
C15:0	0.78	0.83	0.710	0.72	0.82	0.88	0.619	0.144
C16:0	178.12	206.82	0.485	176.09	164	237.3	0.301	0.599
C17:0	1.10	1.28	0.465	1.10	1.11	1.37	0.576	0.660
C18:0	104.04	109.06	0.634	102.43	103.53	113.69	0.629	0.486
C20:0	0.96	0.98	0.834	0.93	0.92	1.06	0.655	0.406
C21:0	0.43^{a}	0.40^{b}	0.024	0.43	0.41	0.42	0.379	0.357
C22:0	0.49	0.48	0.719	0.51	0.46	0.49	0.566	0.485
C14:1	0.45	0.85	0.110	0.55	0.44	0.97	0.182	0.435
C16:1	11.32	18.64	0.476	11.65	7.41	25.88	0.311	0.591
C18:1	171.49	238.10	0.383	179.32	152.17	282.9	0.339	0.516
C20:1	0.94	1.48	0.378	1.03	0.82	1.79	0.407	0.511
C22:1	1.50^{b}	1.96^{a}	0.006	2.08^{a}	1.51^{b}	1.60^{b}	0.011	< 0.001
C18:2	101.39	142.34	0.287	107.2	99.48	158.91	0.389	0.769
C18:3	10.20	12.05	0.453	10.63	9.07	13.67	0.308	0.592
C20:2	3.00^{b}	3.77^{a}	0.038	3.23	3.37	3.55	0.342	0.274
C20:4	130.74	121.94	0.272	123.65	127.9	127.47	0.889	0.098
C20:5	0.86	0.86	0.967	0.95	0.81	0.82	0.211	0.082
C22:4	14.09^{a}	11.39^{b}	0.011	12.62	12.50	13.09	0.876	0.056
C22:5	24.76	21.50	0.074	23.90	23.01	22.48	0.803	0.514
C22:6	15.29^{b}	20.20^{a}	0.007	16.12	19.52	17.60	0.279	0.379
SFA	287.66	322.76	0.507	284.14	272.94	358.55	0.360	0.581
MUFA	185.71	261.04	0.389	194.63	162.35	313.14	0.335	0.529
PUFA	300.32	334.06	0.428	298.31	295.67	357.59	0.407	0.928
USFA	486.03	595.10	0.397	492.94	458.02	670.73	0.353	0.710
USFA/SFA	1.68	1.75	0.293	1.82	1.95	2.20	0.329	0.174

Within a row, means not sharing a common superscript letter are significantly different at $P < 0.05$. SFA, saturated fatty acid; USFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; USFA/SFA, the ratio of unsaturated fatty acids to saturated fatty acid.

Table 5A. Effect of dietary ME and CP levels on fatty acid content in the thigh muscle of Danzhou chickens (μg/g).

	Treatments							
Items	T1	T2	T3	T4	T5	T6	SEM	*P*-value
C8:0	0.07	0.06	0.07	0.07	0.06	0.06	0.01	0.396
C12:0	0.09	0.24	0.26	0.11	0.12	0.18	0.11	0.827
C13:0	0.05^{b}	0.07^{ab}	0.12^{ab}	0.13^{a}	0.08^{ab}	0.06^{ab}	0.02	0.008
C14:0	2.36	3.34	3.38	1.68	1.96	3.17	1.16	0.837
C15:0	1.02	1.19	1.22	1.00	0.68	0.87	0.15	0.126
C16:0	144.27	171.15	176.58	128.06	137.86	189.56	35.64	0.793
C17:0	1.24	1.46	1.66	1.45	1.20	1.35	0.27	0.852
C18:0	92.40	96.26	105.00	93.25	89.55	97.26	11.66	0.955
C20:0	0.88	0.95	1.09	0.90	0.85	1.02	0.17	0.915
C21:0	0.40	0.40	0.42	0.40	0.39	0.42	0.02	0.877
C22:0	0.43	0.42	0.49	0.50	0.46	0.53	0.06	0.782
C14:1	0.31	0.36	0.48	0.16	0.24	0.58	0.24	0.824
C16:1	12.91	15.75	19.50	6.62	10.19	23.96	8.30	0.718
C18:1	129.40	174.59	186.13	112.10	126.98	232.19	64.42	0.772
C20:1	1.62	1.95	2.56	1.59	1.47	2.31	0.72	0.863
C22:1	0.93^{b}	1.21^{ab}	1.33^{ab}	2.27^{a}	1.84^{ab}	1.33^{ab}	0.25	0.011
C18:2	95.66	121.90	146.05	125.59	112.96	167.44	36.42	0.787
C18:3	8.01	8.33	9.36	8.04	8.06	12.11	2.06	0.692
C20:2	1.88	2.14	2.09	1.98	2.18	2.58	0.42	0.895
C20:4	75.85	64.81	75.54	79.76	75.37	85.47	6.81	0.441
C20:5	0.63	0.52	0.61	0.68	0.68	0.73	0.07	0.487
C22:4	8.73	7.04	8.58	7.71	7.17	8.30	0.76	0.484
C22:5	12.39	11.08	11.72	12.75	12.39	14.55	0.88	0.148
C22:6	6.53^{b}	6.95^{ab}	7.30^{ab}	9.87^{ab}	12.13^{ab}	13.12^{a}	1.44	0.008
SFA	243.22	275.55	290.29	227.56	233.21	294.48	48.04	0.858
MUFA	145.18	193.85	210.01	122.73	140.72	260.37	73.56	0.772
PUFA	209.67	222.77	261.25	246.37	230.93	304.29	42.08	0.667
USFA	354.85	416.62	471.25	369.10	371.64	564.67	113.85	0.771
USFA/SFA	1.43	1.44	1.52	1.58	1.57	1.76	0.10	0.212

Within a row, means not sharing a common superscript letter are significantly different at $P < 0.05$. SFA, saturated fatty acid; USFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; USFA/SFA, the ratio of unsaturated fatty acids to saturated fatty acid.

Table 5B. Effect of dietary ME and CP levels on fatty acid content in the thigh muscle of Danzhou chickens (μg/g).

	ME (MJ/kg)			CP (%)				
Items	11.70	12.50	*P*-value	13	14	15	*P*-value	ME × CP *P*-value
C8:0	0.07	0.06	0.255	0.07	0.06	0.07	0.672	0.218
C12:0	0.20	0.14	0.490	0.10	0.18	0.22	0.555	0.804
C13:0	0.08	0.09	0.329	0.09	0.08	0.09	0.497	0.001
C14:0	3.03	2.27	0.431	2.02	2.65	3.27	0.564	0.881
C15:0	1.14^{a}	0.85^{b}	0.020	1.01	0.94	1.04	0.755	0.255
C16:0	164.00	151.83	0.679	136.16	154.51	183.07	0.425	0.807
C17:0	1.46	1.33	0.591	1.35	1.33	1.51	0.769	0.577
C18:0	97.88	93.36	0.638	92.83	92.90	101.13	0.718	0.923
C20:0	0.97	0.92	0.725	0.89	0.90	1.05	0.564	0.925
C21:0	0.41	0.40	0.737	0.40	0.40	0.42	0.461	0.972
C22:0	0.45	0.49	0.352	0.46	0.44	0.51	0.493	0.950
C14:1	0.38	0.33	0.784	0.23	0.30	0.53	0.431	0.844
C16:1	16.05	13.59	0.719	9.77	12.97	21.73	0.342	0.772
C18:1	163.38	157.09	0.906	120.75	150.78	209.16	0.389	0.761
C20:1	2.04	1.79	0.666	1.61	1.71	2.44	0.463	0.954
C22:1	1.15^{b}	1.81^{a}	0.004	1.60	1.52	1.33	0.561	0.045
C18:2	121.20	135.33	0.638	110.62	117.43	156.75	0.404	0.855
C18:3	8.57	9.40	0.625	8.03	8.20	10.74	0.352	0.726
C20:2	2.04	2.25	0.551	1.93	2.16	2.33	0.639	0.844
C20:4	72.06	80.20	0.154	77.80	70.09	80.50	0.299	0.866
C20:5	0.59	0.69	0.094	0.65	0.60	0.67	0.601	0.765
C22:4	8.12	7.72	0.530	8.22	7.10	8.44	0.185	0.742
C22:5	11.73^{b}	13.23^{a}	0.046	12,57	11.73	13.13	0.295	0.384
C22:6	6.92^{b}	11.71^{a}	<0.001	8.20	9.54	10.21	0.374	0.671
SFA	269.69	251.75	0.651	235.39	254.38	292.38	0.490	0.889
MUFA	183.01	174.61	0.890	133.95	167.28	235.19	0.386	0.772
PUFA	231.23	260.53	0.400	228.02	226.85	282.77	0.329	0.907
USFA	414.24	435.14	0.824	361.98	394.13	517.96	0.364	0.831
USFA/SFA	1.47^{b}	1.64^{a}	0.040	1.51	1.51	1.64	0.287	0.823

Within a row, means not sharing a common superscript letter are significantly different at $P < 0.05$. SFA, saturated fatty acid; USFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; USFA/SFA, the ratio of unsaturated fatty acids to saturated fatty acid.

Table 6A. Effect of dietary ME and CP levels on fatty acid content in the liver of Danzhou chickens (μg/g).

	Treatments							
Items	T1	T2	T3	T4	T5	T6	SEM	*P*-value
C8:0	0.03	0.05	0.06	0.04	0.05	0.05	0.01	0.866
C12:0	0.21	0.16	0.21	0.25	0.24	0.20	0.04	0.769
C13:0	0.04	0.05	0.07	0.06	0.05	0.06	0.02	0.694
C14:0	13.59^{ab}	6.78^{b}	8.10^{ab}	11.40^{ab}	13.93^{a}	9.14^{ab}	1.63	0.018
C15:0	2.05	1.54	1.64	1.87	1.96	1.80	0.22	0.597
C16:0	919.44^{ab}	615.62^{c}	675.78^{bc}	824.31^{abc}	976.51^{a}	711.11^{abc}	68.71	0.004
C17:0	5.58	5.04	4.86	6.24	6.91	5.11	0.67	0.240
C18:0	504.50^{ab}	424.31^{b}	409.02^{b}	483.42^{ab}	578.32^{a}	505.30^{ab}	30.97	0.007
C20:0	2.32^{ab}	2.05^{b}	1.85^{b}	2.55^{ab}	3.06^{a}	2.55^{ab}	0.22	0.008
C21:0	0.76	0.69	0.59	0.67	0.76	0.66	0.05	0.101
C22:0	0.94^{b}	0.91^{b}	0.82^{b}	1.16^{ab}	1.46^{a}	1.17^{ab}	0.10	0.001
C14:1	3.25	1.23	1.55	2.06	2.19	1.19	0.51	0.065
C16:1	173.43^{a}	65.04^{b}	93.73^{ab}	92.05^{ab}	116.52^{ab}	73.99^{b}	22.31	0.024
C18:1	1331.70^{ab}	778.09^{c}	857.89^{bc}	1064.99^{abc}	1385.74^{a}	904.05^{abc}	121.59	0.004
C20:1	9.47^{ab}	6.05^{ab}	5.83^{b}	6.47^{ab}	10.15^{a}	7.21^{ab}	0.97	0.011
C22:1	2.02^{ab}	1.72^{b}	2.23^{ab}	3.13^{ab}	3.58^{ab}	3.97^{a}	0.48	0.011
C18:2	461.68^{ab}	347.36^{b}	323.27^{b}	479.87^{ab}	540.21^{a}	385.94^{ab}	50.52	0.035
C18:3	44.46	31.95	29.90	41.23	45.38	42.01	4.20	0.055
C20:2	15.85	14.10	11.91	13.15	14.57	12.66	1.29	0.326
C20:4	338.71	277.46	251.70	291.58	314.32	296.52	38.79	0.704
C20:5	2.55	1.38	1.27	1.84	2.68	2.92	0.63	0.305
C22:4	24.41	18.85	16.39	18.11	21.85	21.81	3.25	0.539
C22:5	105.74^{a}	90.87^{ab}	73.61^{b}	79.15^{ab}	73.75^{b}	67.59^{b}	7.40	0.011
C22:6	91.64^{ab}	81.08^{b}	70.76^{b}	110.36^{ab}	125.37^{a}	126.72^{a}	13.61	0.027
SFA	1449.45^{ab}	1057.21^{c}	1103.01^{bc}	1331.97^{abc}	1583.24^{a}	1237.15^{abc}	83.85	0.001
MUFA	1519.87^{a}	852.14^{b}	961.23^{ab}	1168.70^{ab}	1518.18^{a}	990.40^{ab}	137.23	0.004
PUFA	1085.05	863.05	778.81	1035.30	1138.13	956.17	91.37	0.075
USFA	2604.92^{a}	1715.18^{b}	1740.04^{b}	2204.00^{ab}	2656.31^{a}	1946.58^{ab}	176.13	0.001
USFA/SFA	1.80	1.62	1.58	1.64	1.67	1.57	0.07	0.180

Within a row, means not sharing a common superscript letter are significantly different at $P < 0.05$. SFA, saturated fatty acid; USFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; USFA/SFA, the ratio of unsaturated fatty acids to saturated fatty acid.

Table 6B. Effect of dietary ME and CP levels on fatty acid content in the liver of Danzhou chickens (μg/g).

	ME (MJ/kg)			CP (%)				
Items	11.70	12.50	*P*-value	13	14	15	*P*-value	ME × CP *P*-value
C8:0	0.05	0.04	0.671	0.04	0.05	0.05	0.512	0.867
C12:0	0.19	0.23	0.312	0.23	0.20	0.21	0.764	0.632
C13:0	0.05	0.06	0.748	0.05	0.05	0.07	0.379	0.633
C14:0	9.49	11.49	0.143	12.49	10.36	8.62	0.075	0.024
C15:0	1.74	1.88	0.454	1.96	1.75	1.72	0.516	0.419
C16:0	736.95	837.31	0.084	871.87^{a}	796.06^{ab}	693.45^{b}	0.047	0.007
C17:0	5.16	6.09	0.101	5.91	5.98	4.99	0.269	0.464
C18:0	445.94^{b}	522.34^{a}	0.005	493.96	501.31	457.16	0.325	0.026
C20:0	2.08^{b}	2.72^{a}	0.001	2.43	2.56	2.20	0.269	0.213
C21:0	0.68	0.70	0.666	0.72	0.73	0.63	0.065	0.153
C22:0	0.89^{b}	1.26^{a}	<0.001	1.05	1.19	0.99	0.150	0.276
C14:1	2.01	1.81	0.632	2.65^{a}	1.71^{ab}	1.37^{b}	0.044	0.118
C16:1	110.73	94.19	0.371	132.74	90.78	83.86	0.076	0.020
C18:1	989.23	1118.26	0.204	1198.34^{a}	1081.92^{ab}	880.97^{b}	0.043	0.004
C20:1	7.12	7.94	0.305	7.97	8.10	6.52	0.210	0.004
C22:1	1.99^{b}	3.56^{a}	<0.001	2.57	2.65	3.10	0.503	0.705
C18:2	377.44^{b}	468.67^{a}	0.035	470.78	443.78	354.61	0.071	0.216
C18:3	35.44^{b}	42.87^{a}	0.038	42.84	38.67	35.96	0.270	0.104
C20:2	13.95	13.46	0.643	14.50	14.33	12.29	0.178	0.345
C20:4	289.29	300.81	0.719	315.14	295.89	274.11	0.577	0.432
C20:5	1.73	2.48	0.154	2.20	2.03	2.10	0.964	0.146
C22:4	19.89	20.59	0.792	21.26	20.35	19.10	0.801	0.180
C22:5	90.07^{a}	73.50^{b}	0.010	92.44^{a}	82.31^{ab}	70.60^{b}	0.022	0.391
C22:6	81.16^{b}	120.82^{a}	0.001	101.00	103.23	98.74	0.947	0.388
SFA	1203.23^{b}	1384.12^{a}	0.013	1390.71^{a}	1320.23^{ab}	1170.08^{b}	0.039	0.002
MUFA	1111.08	1225.76	0.314	1344.28^{a}	1185.16^{ab}	975.82^{b}	0.039	0.003
PUFA	908.97	1043.20	0.082	1060.17	1000.59	867.49	0.115	0.207
USFA	2020.05	2268.96	0.094	2404.46^{a}	2185.75^{ab}	1843.31^{b}	0.012	0.003
USFA/SFA	1.67	1.63	0.444	1.72	1.64	1.58	0.100	0.287

Within a row, means not sharing a common superscript letter are significantly different at $P < 0.05$. SFA, saturated fatty acid; USFA, unsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; USFA/SFA, the ratio of unsaturated fatty acids to saturated fatty acid.

C20:0, C22:0, C22:1, C18:2, C18:3, C22:6, and SFA in the liver of the high ME treatment were higher than those of the low ME treatment ($P < 0.05$). The levels of C16:0, C14:1, C18:1, C22:5, SFA, MUFA and USFA in the low CP treatment were higher than the corresponding values in the high CP treatments ($P < 0.05$), but their difference between the low and medium CP treatments was not significant ($P > 0.05$). The interaction effects of dietary ME and CP on C14:0, C16:0, C18:0, C16:1, C18:1, C20:1, SFA, MUFA, and USFA yields in the liver were significant ($P < 0.05$).

Cecal Microbiota Analysis

The observed species and Chao1 indices reflect community richness, while Shannon and Gini-Simpson indices represent community diversity. The alpha diversity measurements showed that the observed species, Chao1 and Gini-Simpson indices of 11.70 MJ/kg ME treatment were higher than those of 12.50 MJ/kg treatment ($P < 0.05$) (Supplementary Table S2). The beta diversity metrics evaluated the overall differences among the treatments, and the results were observed with the PCoA analysis based on the Bray-Curtis dissimilarity matrix. The results revealed that first 2 axes (PCo1 and PCo2) explained 49.03% of the composition variation among the 6 treatments (Supplementary Figure S1 A), and explained 47.35% between low and high ME treatments (Supplementary Figure S1 B), and explained 48.98% among low, medium, and high CP treatments (Supplementary Figure S1 C), respectively. In addition, the axis 2 (PCo2) of PCoA analysis separated the T1 group from the T2 and T4 groups (Supplementary Figure S1 A).

As displayed in Supplementary Table S3 A and B, dietary ME and CP levels altered the top 20 relative abundance of microbiota composition in the cecum of chickens at various taxonomic levels (phylum, class, order, family, and genus) to different extents.

Taxonomically, species are fundamental units of biodiversity. At the species level, the relative abundance of the top 300 species in this study represented about 85% of the total relative abundance of microorganisms present in the cecum of chickens. Supplementary Table S4A and B showed changes in the relative abundance of the top 300 gut microbiota at the species level among the 6 treatments, or between low and high ME treatments, or among low, medium, and high CP treatments, respectively. Statistical analysis illustrated that 12 species exhibited significant shifts in abundance among the 6 treatments (Figures 1A and 1B, Supplementary Table S4 A), 33 between low and high ME treatments (Figures 1C and D, Supplementary Table S4 B), and 10 among low, medium, and high CP treatments (Figure 1E, Supplementary Table S4 B). Specifically, the 12 of 33 species with differential abundance ($P < 0.05$) between low and high ME levels belonged to genera *Bacteroides* and *Oscillibacter*, respectively, and a trend for lower abundance of these 12 species was observed in the high ME

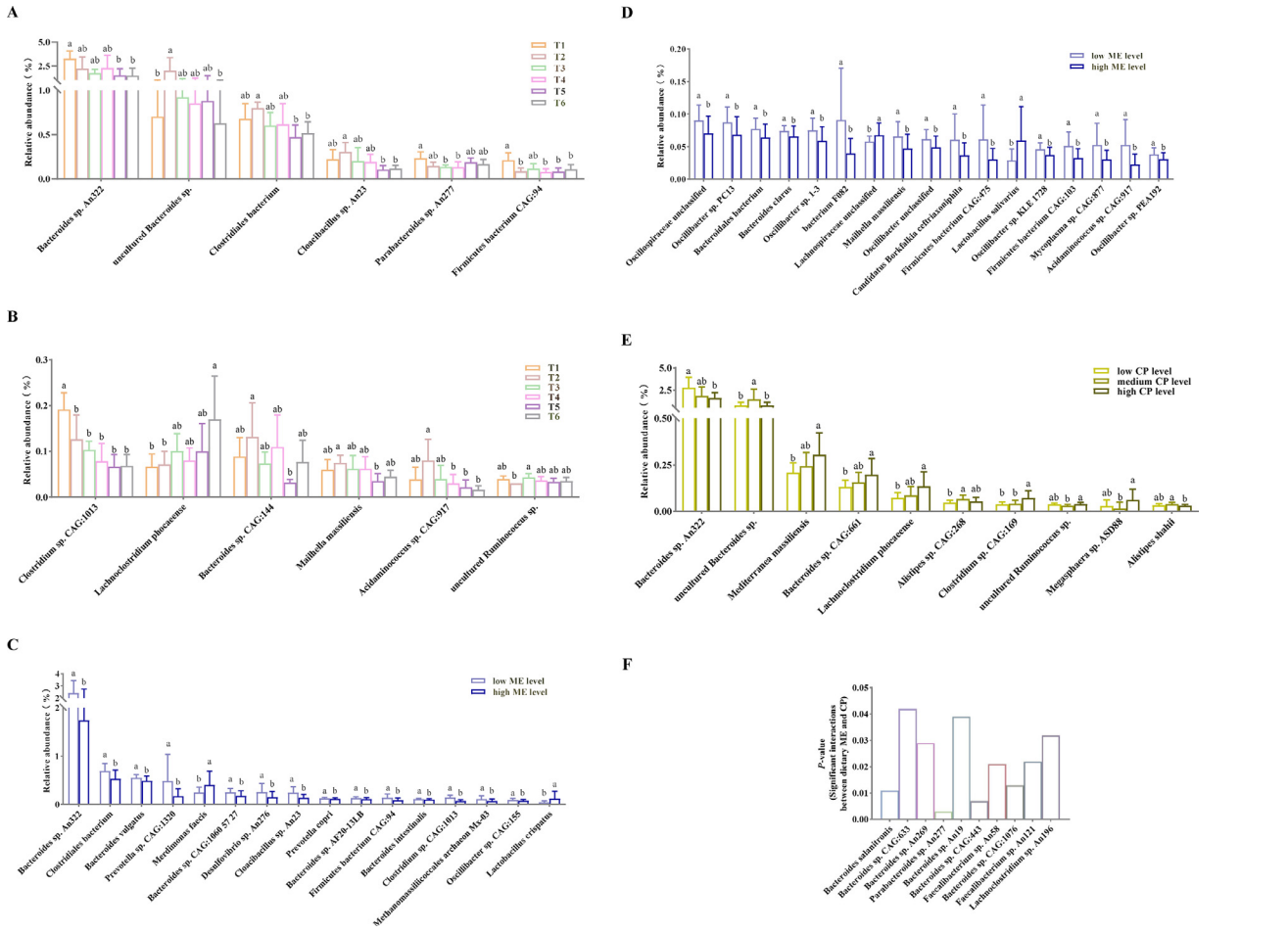

Figure 1. Changes in gut microbiota composition at the species level. (A-E) Species with relative abundance that changed significantly in the top 300 gut microbiota among 6 groups (A-B), or between low and high ME levels (C-D), or among low, medium, and high CP levels (E). (F) Significantly interactions ($P < 0.05$) between dietary ME and CP on gut microbiota (top 300) of birds. Columns not sharing a common superscript letter are significantly different at $P < 0.05$.

treatment compared to those in the low ME treatment ($P < 0.05$) (Figures 1C and 1D). Additionally, significant effects of interactions were found between dietary ME and CP on the relative abundance of 10 species ($P < 0.05$), and among these species, 6 species belonged to the genus *Bacteroides* (Figure 1F). Notably, the relative abundance of 2 probiotic species including *Lactobacillus crispatus* and *Lactobacillus salivarius* was significantly increased ($P < 0.05$) with increasing dietary ME level from 11.70 MJ/kg to 12.50 MJ/kg (Figures 1C and 1D).

Metabolomics Analysis of Cecal Contents

The PCA score plots indicated various degrees of overlap in different groups (Supplementary Figure S2 A1, B1 and C1). PLS-DA was further performed to enhance inter-group separation (Supplementary Figure S2 A2, B2 and C2). Results disclosed a good separation between the low and high ME levels (11.70 MJ/kg, 12.50 MJ/kg) (Supplementary Figure S2 B2). For the cecal content set, 11 differential metabolites were affected by the interaction of dietary ME and CP levels (Supplementary Table S5 A), as well as 205 metabolites were affected by dietary ME level (Supplementary Table S5 B). Subsequently, we carried out enrichment analysis for the aforementioned all differential metabolites by MetaboAnalyst 5.0 associated with the Kyoto Encyclopedia of Genes and Genomes (**KEGG**) database. All top enriched pathways (FDR ≤ 0.25 and $P < 0.05$) and their related differential metabolites are shown in Supplementary Table S6 A, B, respectively. Interestingly, arachidonic acid metabolism (top enriched pathway; FDR ≤ 0.25 and $P < 0.05$) was found to be the only significantly affected pathway between low and high ME treatments and among the 6 groups (Supplementary Table S6 A, B and Figure 2). The results indicated that the group with 11.70 MJ/kg ME and 13% CP (T1) had the highest levels of thromboxane A2 and 5,6-DHET while the group with 12.50 MJ/kg ME and 15% CP (T6) had the lowest levels of both (Figure 3A and B). Notably, increasing the dietary ME level from 11.70 MJ/kg to 12.50 MJ/kg significantly reduced ($P < 0.05$) the levels of 6 differential metabolites including thromboxane A2, 5,6-DHET, prostaglandin D2, 20-hydroxyeicosatetraenoic acid, 12(*S*)-HPETE, and prostaglandin I2 (Figures 3C−3H); all of them are involved in arachidonic acid metabolism (Figure 2).

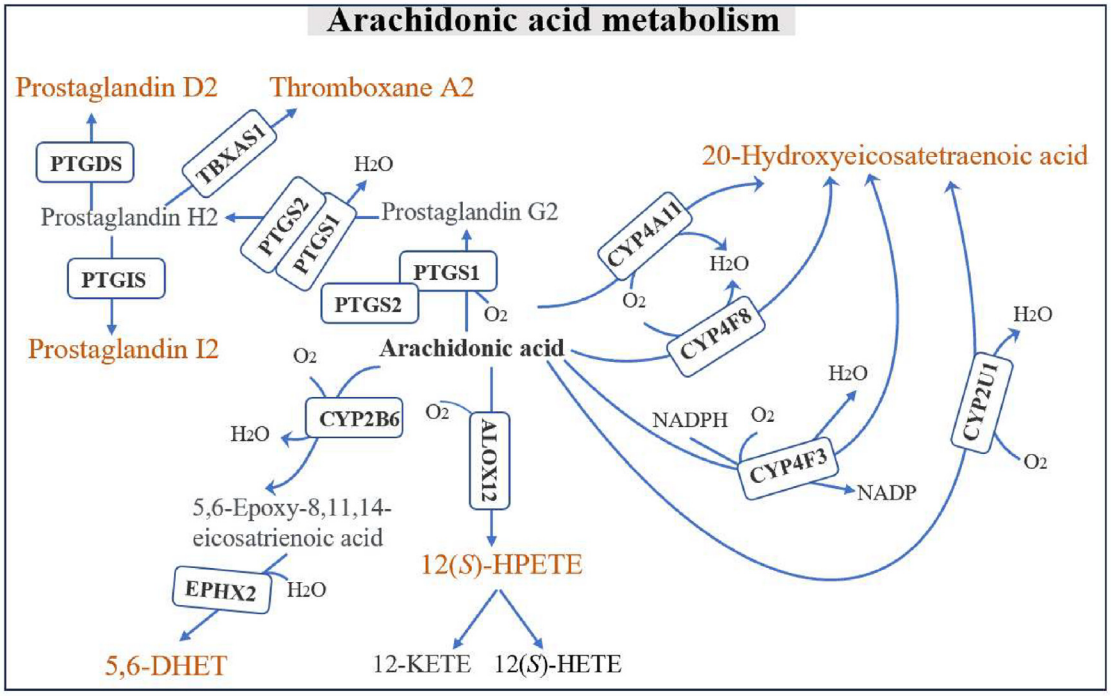

Figure 2. Common top pathway analysis plot obtained by MetaboAnalyst 5.0, based on differential metabolites from both among the 6 treatments (T1, T2, T3, T4, T5 and T6) and between low and high ME treatments. Abbreviations: ALOX12: arachidonate 12-lipoxygenase, 12*S*-type; CYP2B6: cytochrome P450 2B6; CYP2U1: cytochrome P450 2U1; CYP4A11: cytochrome P450 4A11; CYP4F3: cytochrome P450 4F3; CYP4F8: cytochrome P450 4F8; EPHX2: bifunctional epoxide hydrolase 2; PTGDS: prostaglandin-H2 *D*-isomerase; PTGIS: prostacyclin synthase; PTGS1: prostaglandin G/H synthase 1; PTGS2: prostaglandin G/H synthase 2; TBXAS1: thromboxane-A synthase.

DISCUSSION

Suitable levels of ME and CP are known to be key factors promoting the growth and development of poultry (Musigwa et.al., 2021). In this study, dietary ME and CP levels did not affect the performance of growing Danzhou chickens aged 120 to 150 d. In Beijing You Chicken, Chang et al. (2023) also reported that the performance of birds was not affected by the dietary ME (11.31 MJ/kg, 11.51 MJ/kg, 11.71 MJ/kg) and CP (14%, 15%, 16%) levels at 10 to 16 wk of age. In a like manner, Chrystal et al. (2020) found that reducing dietary CP levels from 20 to 17.2% did not affect the growth performance of Ross 308 male broilers from 14 to 35 d post hatch. Our results suggested that the current dietary ME and CP levels may be adequate for the growth of Danzhou Chicken at 120 to 150 d of age.

Fatty acids are important chemical substances that make up fats and tissue cells which determine the physicochemical properties of fats and influence the flavor of meat (Zhan et al., 2022). The diet nutritional level can regulate the fatty acid composition of chicken meat directly (Wood et al., 2008; Kouba et al., 2011). Liver is mainly responsible for chicken lipid synthesis, metabolism and transportation, and constitutes approximately 90% of fatty acids de novo synthesis in chicken (Yang et al., 2019). Our results indicated that both SFA and USFA contents of the liver in the T5 (12.50 MJ/kg ME and 14% CP) increased significantly compared to those in T2 (11.70 MJ/kg ME and 14% CP) and T3 (11.70 MJ/kg ME and 15% CP), while no significant differences were found between T5 and the other 3 treatments (T1, T4 and T6 groups). The trend of the above results is nearly consistent with that of the comparison results between the low and high ME treatments and among the low, medium, and high CP treatments. Of note, interactive effects existed ($P < 0.05$) between ME and CP on the contents of SFA, MUFA, and USFA in the liver, and suggested increasing the dietary ME level is favorable to the synthesis of those when diets were supplemented with 14% CP level. These findings may allude to dietary levels of 12.50 MJ/kg ME and 14% CP is mostly preferred to promote the synthesis of liver fatty acids in Danzhou chickens.

Furthermore, we found that chickens on the high ME diet had a higher USFA/SFA ratio in the thigh muscle than those fed the low ME diet, suggesting a greater effect of dietary ME levels on fat deposition and flavor of thigh meat, compared to its effect on those of breast meat (Yang et al., 2018; Wang et al., 2022a). Meanwhile, the high ME level significantly increase serum levels of TG, INS, and GLU in chickens compared to the low ME level, and it was a hint that high ME level in diets can provide sufficient nutrition and energy to enhance the lipid metabolism and synthesis of fatty acids in the liver and muscle tissues of chickens (Wagner et al., 2017). Additionally, it was also reported that INS facilitates the synthesis of fatty acids in the liver and inhibits the decomposition of fat in adipose tissue (Saltiel et al., 2001; Titchenell et al.,2017).

The presence of C=C double bonds in fatty acids chains of the chemical structure of USFA also contributes to the antioxidant effect, and thus enhances the health-promoting quality of chicken meat (Mas et al.,

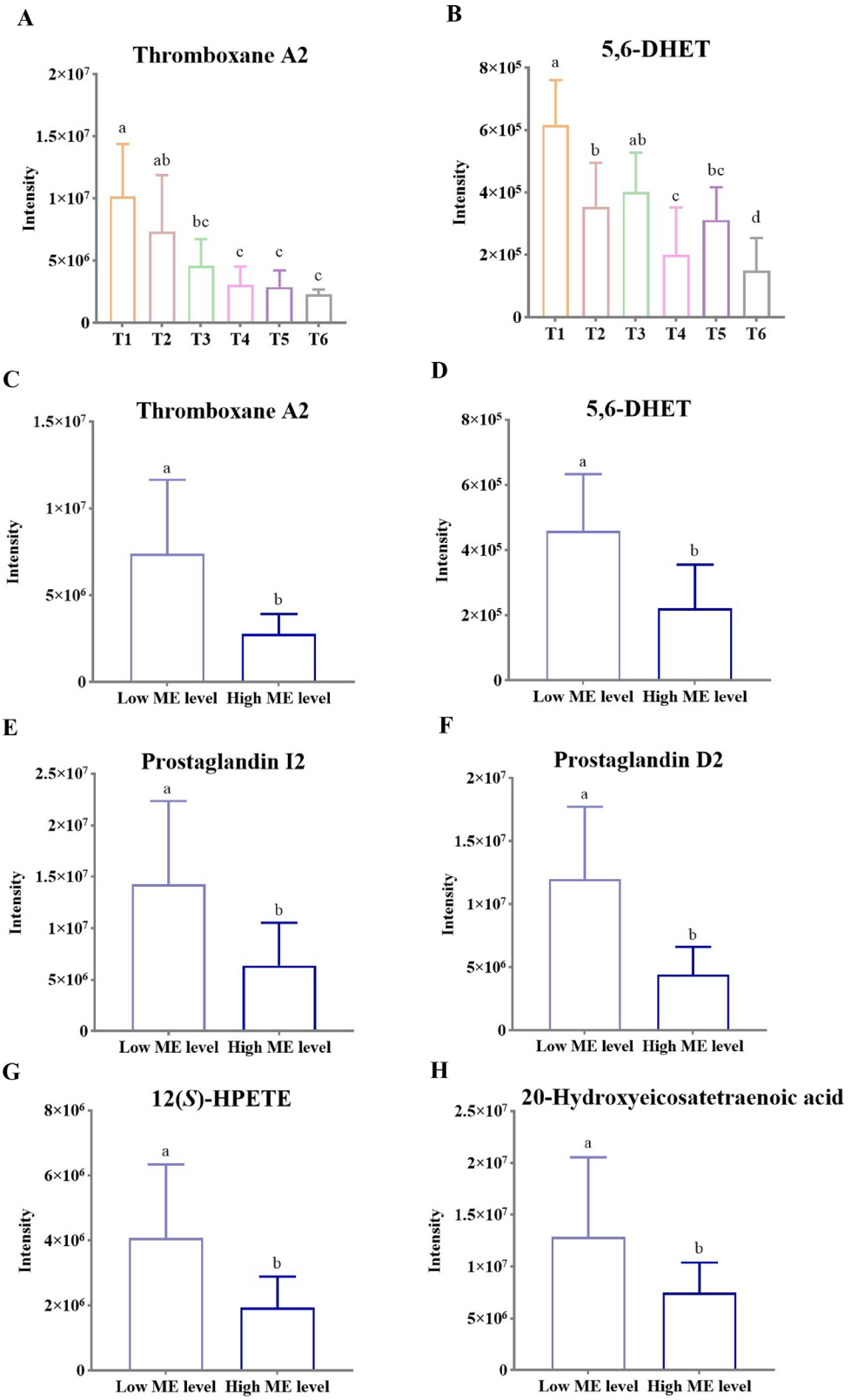

Figure 3. Differential metabolites detected in the cecal contents of chickens which associated with arachidonic acid metabolism among the 6 treatments (A, B), or between low and high ME treatments (C-H). Columns not sharing a common superscript letter are significantly different at $P < 0.05$.

2011; Hughbanks-Wheaton et al., 2014; Kim et al., 2023). The high ME level in the diet significantly increased the content of both C22:1 (mono-unsaturated erucic acid) and C22:6 (poly-unsaturated docosahexaenoic acid) in all 3 peripheral tissues of chickens (i.e., liver, thigh muscle and breast muscle) compared to the low ME level. This can further imply that the dietary level of 12.50 MJ/kg ME can effectively promote the biosynthesis of USFA in the liver. Furthermore, C22:1 content in the thigh and breast muscles was found to be affected by interactions between ME and CP ($P < 0.05$), indicating that under the low and medium CP levels

(13% and 14%), the increase of ME level was beneficial for the improvement of C22:1 content. Interestingly, there was a trend ($0.05 < P < 0.10$) for 2-way interaction between ME and CP on the contents of C20:4 (poly-unsaturated arachidonic acid), C20:5 (poly-unsaturated eicosapentaenoic acid) and C22:4 (poly-unsaturated adrenic acid) in breast muscle. At medium CP level (14%), increased level of ME improved the contents of C20:4, C20:5 and C22:4 in breast muscle; conversely, at low and high CP levels (13% and 15%), increased level of ME led to a decreased content of those. Here, it was speculated that T5 (12.50 MJ/kg ME and 14% CP) is better for the deposition of USFA in muscle tissue (especially in the breast muscle) than the other 5 treatments.

In this study, the results suggested that the dietary ME levels altered the gut microbial community abundance from phylum to species taxonomic levels and cecal metabolic phenotype, more than those of the dietary CP levels. It was well known that *Lactobacillus crispatus* and *Lactobacillus salivarius* were 2 probiotic lactic acid bacteria, which exerted an important role in regulation of intestinal immunity, inflammation as well as inhibition of oxidative stress (Rizzo et al., 2015; Sun et al., 2020). The results of metagenomics analysis revealed that birds fed diet containing high ME level had significantly higher abundance of *Lactobacillus crispatus* and *Lactobacillus salivarius* than those of birds fed diet containing low ME level, which help to improve intestinal health and maintain gut homeostasis of birds. Meanwhile, we also found that the low ME treatment significantly increased the relative abundance of 6 species from genus *Oscillibacter* when compared to the high ME treatment in this study. Currently, some studies have shown positive correlations between increased abundance of *Oscillibacter* and degrees of intestinal inflammation (Lam et al., 2012; Wu et al., 2019). On the other hand, metabolomic analysis revealed that the cecal levels of 6 differential metabolites, which comprised thromboxane A2, 5,6-DHET, prostaglandin D2, 20-hydroxyeicosatetraenoic acid, 12(*S*)-HPETE and prostaglandin I2, were reduced significantly in birds fed with the high ME diet compared to those in birds fed the low ME diet, suggesting that the high ME level in chicken diets can effectively reduce intestinal oxidative stress and inflammatory response potentially by downregulating arachidonic acid metabolism (Zhang et al., 2023). Therefore, integrated metagenomics and metabolomics analysis showed that the high ME diet (12.50 MJ/kg) may be more beneficial for maintaining the intestinal health of the chickens than the low ME diet (11.70 MJ/kg).

Additionally, some microorganisms from *Bacteroides, Lactobacillus* and *Firmicutes* at the species level, are major classes of microbiota affected by dietary ME and CP levels, as well as interactions between them. It was also identified that dietary ME and CP levels had a marked effect on liver and muscle fatty acid profiling, but not on growth parameters. These observations might imply that, for slow-growing Danzhou chickens, these microbiotas affected by dietary ME and CP levels are one of the main factors affecting the synthesis and deposition of fatty acids in liver and muscle (Bassett, et al., 2019; Wu et al., 2022; Gatya et al., 2022).

It was also reported that members of the genus *Bacteroides* are potential colonizers of the colon, and some of them in the intestine contribute to a decomposition of foods and produce energy and nutrients required for the host (Zafar et al., 2021). Notably, the results indicated that some species from genus *Bacteroides* are one of the most classes of microbiota affected by ME and CP levels in the diet, as well as interactions between them. Although the relative abundance of *Bacteroides sp. An322, Bacteroides vulgatus, Bacteroides sp. CAG:1060 57 27, Bacteroides sp. AF20-13LB, Bacteroides intestinalis* and *Bacteroides clarus* was significantly increased in the low ME treatment compared to those in high ME treatment, the differences in growth parameters were not significant between different ME treatments, and it was noted that the higher abundance of *Bacteroides vulgatus* and *Bacteroides clarus* in the low ME treatment, may also increase the risk of infection to some extent for the body (Wang et al., 2022b; Lei et al., 2023). Meanwhile, the abundance of *uncultured Bacteroides sp.* was significantly enriched in the medium CP treatment compared to the other CP treatments. Compared with the high CP treatment, the low CP treatment obviously increased the abundance of *Bacteroides sp. An322* and decreased the abundance of *Bacteroides sp. CAG:661*, however, the abundance of the 2 species was not distinguished in the medium CP treatment compared with the low- and high CP treatments, implying the medium CP treatment (14% CP level in the diet) might be more beneficial for the growth of these 3 species belonged to the *Bacteroides* genus. Interestingly, it was also found that the interactions between ME and CP have a significant effect mainly on the abundance of *Bacteroides* species. Except for *Bacteroides sp. CAG:633*, the abundance of the other 5 species (*Bacteroides salanitronis, Bacteroides sp. An269, Bacteroides sp. An19, Bacteroides sp. CAG:443* and *Bacteroides sp. CAG:1076*) increased with rising ME level when diets were supplemented with 14% CP level, which showed the dietary levels of 12.50 MJ/kg ME and 14% CP might be the appropriate concentrations for increasing the abundance of these 5 species within the genus *Bacteroides*.

CONCLUSIONS

The present study suggested that the dietary levels of 12.50 MJ/kg ME and 14% CP displayed beneficial roles in improving meat quality in terms of fatty acid composition and in maintaining intestinal health in terms of cecal microbiota in native growing Danzhou chickens.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.103917.

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