Effects of fermented *Andrographis paniculata* on growth performance, carcass traits, immune function, and intestinal health in Muscovy ducks

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ABSTRACT The study aimed to examine the effects of unfermented and fermented *Andrographis paniculata* on growth performance, carcass traits, immune function, and intestinal health in Muscovy ducks. A total of 450 (16-day-old) Muscovy ducks weighing 271.44 ± 8.25 g were randomly assigned to 5 dietary treatments (6 replicate pens of 15 ducks per treatment), consisting of one control treatment (basal diet without *A. paniculata*), one unfermented *A. paniculata* treatment (basal diet plus 30 g/kg unfermented *A. paniculata*) and 3 fermented *A. paniculata* treatments (basal diet plus 10, 30, and 50 g/kg). 30 g/kg unfermented *A. paniculata* increased the ADG, thymus index, peripheral blood lymphocyte conversion rate, villi height, intestinal thickness, villi surface area, intraepithelial lymphocytes rate, while decreased the FCR. 10 g/kg fermented *A. paniculata* markedly boosted ADG, bursa of fabricius index, thymus index, serum lysozyme, lymphocyte conversion rate, villi height, vilii width, intestinal thickness, villi surface area, while decreased the FCR. 30 g/kg fermented *A. paniculata* clearly improved ADG, bursa of fabricius index, thymus index, serum lysozyme, lymphocyte conversion rate, villi height, vilii width, intestinal thickness, villi surface area, intraepithelial lymphocytes, while decreased FCR. 50 g/kg fermented *A. paniculata* significantly increased villi height, vilii width, and villi surface area, while clearly reduced BW. Additionally, compared to 30 g/kg unfermented *A. paniculata,* 30 g/kg fermented *A. paniculata* obviously increased bursa of fabricius indices, lymphocyte conversion rate, vilii width, villi surface area. On top of that, supplementation with unfermented and fermented *A. paniculata* (30 g/kg each) decreased the relative abundance of harmful bacteria (*Succinivibrio, Succinatimonas, Sphaerochaeta*, and *Mucispirillum*) and increase the abundance of beneficial bacteria (*Rikenellaceae, Methanocorpusculum, Fournierella, Ruminococcaceae*) in the ceca of the ducks. However, fermented *A. paniculata* had considerable better effects than unfermented *A. paniculate* on all above measured indices. Overall, these results revealed that supplementation with unfermented and fermented *A. paniculata* across different treatments improved growth, immune status, intestinal morphology, and intestinal microbiota composition and structure in Muscovy ducks, making it a potential alternative to antibiotics in poultry production.

Key words: fermented *A. Paniculata*, growth performance, carcass traits, immune function, intestinal health

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INTRODUCTION

Problems with drug resistance and residues in livestock products have resulted in the recent ban of antibiotic use in livestock and poultry breeding in several countries (Liu et al., 2020). Therefore, it is important to identify and evaluate alternatives to antibiotics, including "green additives". Chinese herbal feed additives have been shown to improve feed quality and solve the problem of drug residues in livestock products (Lin et al., 2020). *A. paniculata* is a widely cultivated herb in East and Southeast Asia, belonging to *Acanthaceae* family (Jiang et al., 2021). It is commonly known as the "king of bitter" due to and has the ability to grow in most soil types and under shaded conditions. *A. paniculata* possess anti-inflammatory, antibacterial, antipyretic, and immunosuppressive properties (Julaton et al., 2022). Traditionally, it is used to treat several infectious diseases, including colds, fevers, laryngitis, malaria, dysentery, and diarrhea, in China, India, and other Southeast Asian countries. Additionally, it has been shown to significantly improve tibial length and body weight, prevent the occurrence of gastrointestinal diseases, improve antioxidant and immune functions, and promote growth in poultry (Arify et al., 2019; Aneesh et al., 2021). *A.*

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paniculata also possesses several bioactive compounds with complex structures often consisting of multiple asymmetric carbon atoms. Structural modifications using chemical reactions have demonstrated some associated disadvantages, such as low yield of bioactive compounds, poor reaction specificity, and the production of several by-products. However, fermentation of *A. paniculata* with probiotics has the potential to increase the production of active substances, promote the synthesis of specific active compounds, and facilitate the degradation of antinutritional factors and toxins in *A. paniculata*. Additionally, microorganisms used for fermentation can produce organic acids in the intestinal tract of animals, thereby inhibiting the growth of harmful bacteria and enhancing intestinal immunity. However, studies on the medicinal and growth promoting effects of fermented *A. paniculata* are limited.

Therefore, this study aimed to examine the effect of fermented *A. paniculata* on growth performance, immune function, intestinal morphology, and intestinal microbial composition in poultry. Microorganisms were firstly used to ferment *Andrographis paniculata*, and the Muscovy duck was used as the research poultry species. It is anticipated that our findings will provide scientific basis for the use of Chinese herbal medicinal technology in livestock and will contribute to the development of an effective alternative to antibiotics in poultry production.

MATERIALS AND METHODS

Preparation of Fermented A. Paniculata

To prepare the fermentation medium, 4% compound bacterial agent (mass ratio of Saccharomycetes to Lactobacillus was 1: 1), sugar, and other raw materials were mixed in water. The fermentation material and water were added into a vat containing *A. paniculata* powder, and evenly mixed. The moisture was controlled at 35 to 45%. The mixture was loaded into a breathing bag, sealed, and fermented at 30°C for 7 d. The number of viable bacteria after fermentation was $\geq 2.0 \times 10^8$ CFU/g.

Experimental Design

A total of 450 (16-day-old) Muscovy ducks weighing 271.44 ± 8.25 g (Ganzhou, Jiangxi, China) were randomly assigned to 5 dietary treatments (6 replicate per groups, 15 ducks per replicate), consisting of one control treatment (basal diets without *A. paniculata*/CG), one unfermented *A. paniculata* treatment(basal diet plus 30 g/kg unfermented *A. paniculata*/30 g/kg APG) and three fermented *A. paniculata* treatments.(basal diets plus 10, 30, and 50 g/kg of fermented *A. paniculate*/ 10 g/kg FAPG, 30 g/kg FAPG and 50 g/kg FAPG). Composition and nutrient levels of basal diets was shown in Table 1. Muscovy duck trial protocols used in this study and all experimental stages were conducted in accordance with the requirements of National Research Council's Guide for the Care and Use of Laboratory Animals.

Table 1. Composition and nutrient levels of basal diets (air-dry basis).

	Diet content	
Items	Ducklings (18−21 days)	Fattening stage ducks (22−70 days)
Ingredients (%)		
Corn	61.80	62.00
Soybean meal	26.00	20.00
Fish meal	4.20	0
Wheat bran	4.00	14.00
Met	0.13	0.15
Limestone	1.22	1.50
$CaHPO_4$	0.95	1.07
NaCl	0.20	0.28
Premix[1]	1.50	1.50
Total	100	100
Nutrient levels[2]		
ME (MJ/Kg)	11.69	11.29
CP (%)	20.00	15.50
CF (%)	2.80	3.12
Ca (%)	1.25	1.74
P (%)	0.70	0.65
Met (%)	0.45	0.40
Met+Cys (%)	0.80	0.68
Lys (%)	1.05	0.75

[1]The premix provided the following per kg of diets: VA 8,000 IU, VD3 3,000 IU, VE 20 IU, VK3 2 mg, VB1 4 mg, VB2 3.6 mg, VB5 40 mg, VB6 4 mg, VB12 0.02 mg, biotin 0.15 mg, folic acid 1.0 mg, D-pantothenic acid 11 mg, nicotinic acid 10 mg, antioxidant 100 mg, Cu (as copper sulfate) 10 mg, Fe (as ferrous sulfate) 80 mg, Mn (as manganese sulfate) 80 mg, Zn (as zinc sulfate) 75 mg, I (as potassium iodide) 0.40 mg, Se (as sodium selenite) 0.30 mg.
[2]ME was a calculated value, while the others were measured values.

Growth Performance and Carcass Traits Measurements

Body weight (**BW**) at 16-, 21-, 42-, and 70-day-old and daily feed consumption of the ducks were recorded to calculate average daily gain (**ADG**), average daily feed intake (**ADFI**), and feed conversion ratio (**FCR, ADFI/ADG**). Carcass traits (dressed percentage, percentage of half-eviscerated yield with giblet, percentage of eviscerated yield, percentage of breast muscle yield, and percentage of leg muscle yield) were determined according to NY/T 823-2020. Additionally, visceral organ parameters (heart, liver, lung, kidney, gizzard, proventriculus, and pancreas index) and immune organ indices (spleen, bursa of Fabricius, and thymus index) were calculated using the following formula: Organs parameters (visceral organ parameters and immune organ indices) = the weight of organ (mg)/BW (g).

Immune Index

Serum samples (30) were used for the determination of 2 immune parameters, including serum lysozyme and lymphocyte conversion rate. Serum lysozyme and lymphocyte conversion rate were analyzed using standard kits (Ganzhou Beisite Biological Co. Ltd, Ganzhou, China) according to the manufacturer's instructions.

Histological Assay

Frozen sections were thawed, dried, and fixed with 4% paraformaldehyde for 15 min. They were differentiated using 75% alcohol, dyed for 3 to 5 min using

hematoxylin, differentiated using hydrochloric acid alcohol, and dyed using ammonia solution. Subsequently, the stained sections were scanned using a panoramic scanner (3DHISTECH, Budapest, Hungary). Villi length, crypt depth, villi width, villi length/crypt depth ratio, and intestinal thickness were determined using Caseviewer Systems. Villi surface area was calculated as follows: Villi surface area = $2\cdot\pi\cdot r\cdot h$ (where r is half of villi width; h is villi height).

Immunohistochemistry

All formalin-fixed, paraffin-embedded duodenal sections (5−6 μm) were placed on coated object-slides. Thereafter, the sections were deparaffinized in light and incubated with rabbit anti-human CD3 monoclonal antibody (1: 200; Proteintech, Chicago, Illinois), followed by incubation with secondary antibody (HRP-Goat anti-rabbit IgG [1:200; Solaribio, China]). Immunohistochemical staining was in accordance with the step of Wu et al. (2019). Positive cells ratio (Average Optical Density/AOD) were calculated as follows. AOD = Integrated option density (**IOD**)/the area of the target protein distribution region.

Microbial DNA Extraction, PCR, and 16s rRNA Sequencing

Total genome DNA from cecal samples was extracted using the CTAB/SDS method. DNA concentration and purity were monitored on 1% agarose gels. PCR amplification of the bacterial 16S rRNA gene V4 region was performed using the forward and reverse primers (515F/GTGCCAGCMGCCGCGGTAA and 806R/GGACTACHVGGGTWTCTAAT). Sequencing adapters were appended to the end of primers. PCR was performed to amplify the target genes, and the PCR products were purified using a Qiagen Gel Extraction Kit (Qiagen, Germany). Sequencing libraries were generated using TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, California) following the manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific, Waltham, Massachusetts) and Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated.

Processing of Sequencing Data

High-quality reads were assembled using FLASH v1.2.7 software. Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags according to the QIIME V1.9.1 software quality-controlled process. UCHIME v4.2 software was used to identify and remove the chimeric sequences, and effective reads were generated. Further bioinformatic analyses were performed by Consure Biotechnology Co.Ltd (Beijing, China).

Statistical Analysis

All data were analyzed using SPSS software 17.0 software and were expressed as the mean ± standard deviation (**SD**). A multiple t test was performed to determine significant differences among the 5 groups. Means were considered statistically significant and highly significant at $P < 0.05$ and < 0.01, respectively.

RESULTS

Growth Performance

As shown in Table 2, the ADG of 22 to 42 d in 30 g/kg APG was increased ($P < 0.05$), but the FCR of 22 to 42

Table 2. Effects of fermented *A. paniculata* on growth performance in Muscovy ducks.

Items	CG	30 g/kg APG	10 g/kg FAPG	30 g/kg FAPG	50 g/kg FAPG	*P*-value
Body weight (g)						
16 d	267.33 ± 9.50	274.33 ± 8.50	270.00 ± 9.85	258.67 ± 7.51	277.33 ± 16.01	0.32
21 d	386.69 ± 19.54	386.64 ± 18.39	388.57 ± 16.85	360.86 ± 15.36	375.19 ± 16.06	0.09
42 d	1,089.25 ± 49.16^{AB,a}	1,106.63 ± 45.89^{A,a}	1,091.25 ± 75.36^{AB,a}	1,077.89 ± 41.98^{AB,ab}	1,035.54 ± 47.41^{B,b}	0.05
70 d	2,220.00 ± 82.46^{A,a}	2,211.25 ± 68.07^{AB,a}	2,230.63 ± 90.85^{A,a}	2,205.00 ± 61.18^{AB,a}	2,106.34 ± 94.04^{B,b}	0.03
Average daily gain (g)						
16-21 d	21.68 ± 4.36	24.60 ± 3.11	24.36 ± 3.72	22.34 ± 2.24	20.68 ± 4.25	0.63
22-42 d	31.95 ± 0.54^{AB,b}	36.32 ± 0.72^{A,a}	33.96 ± 3.03^{AB,ab}	35.78 ± 1.31^{AB,a}	31.73 ± 1.58^{B,b}	0.02
42-70 d	40.06 ± 3.25	39.22 ± 2.88	41.22 ± 3.99	40.24 ± 2.58	38.54 ± 4.17	0.59
16-70 d	34.50 ± 1.21^{B,c}	35.34 ± 0.11^{AB,bc}	37.24 ± 1.11^{A,a}	36.27 ± 0.70^{AB,ab}	34.80 ± 0.88^{B,c}	0.02
Average daily feed intake (g)						
16-21 d	43.22 ± 0.81	44.11 ± 1.27	43.04 ± 1.63	42.51 ± 1.01	43.47 ± 1.25	0.62
22-42 d	89.11 ± 3.93	85.71 ± 2.16	88.87 ± 2.92	89.14 ± 1.50	87.99 ± 2.39	0.52
42-70 d	160.95 ± 2.23	156.38 ± 2.07	161.15 ± 3.82	157.88 ± 2.44	163.35 ± 2.55	0.06
16-70 d	120.68 ± 1.42	117.15 ± 1.93	120.65 ± 3.16	119.05 ± 1.80	121.50 ± 2.33	0.20
Feed conversion ratio						
16-21 d	2.05 ± 0.40	1.81 ± 0.22	1.80 ± 0.30	1.91 ± 0.20	2.17 ± 0.45	0.60
22-42 d	2.79 ± 0.04^{A,a}	2.36 ± 0.05^{B,c}	2.63 ± 0.05^{AB,ab}	2.50 ± 0.10^{AB,bc}	2.78 ± 0.14^{A,a}	*P < 0.01*
42-70 d	3.94 ± 0.22	3.75 ± 0.19	3.56 ± 0.16	3.74 ± 0.07	3.83 ± 0.21	0.31
16-70 d	3.50 ± 0.12^{A,a}	3.31 ± 0.01^{AB,b}	3.24 ± 0.10^{B,b}	3.28 ± 0.07^{AB,b}	3.49 ± 0.09^{A,a}	0.01

In the same row, no letter or the same letter superscripts: no significant difference ($P > 0.05$).
^{abc}Different small letter superscripts: significant difference ($P < 0.05$).
^{AB}Different capital letter superscripts: significant difference ($P < 0.01$). The same as below.

Table 3. Effects of fermented *A. paniculata* on carcass traits in Muscovy ducks.

Items	CG	30 g/kg APG	10 g/kg FAPG	30 g/kg FAPG	50 g/kg FAPG	*P*-value
Dressed percentage (%)	83.89 ± 2.51^{bc}	83.69 ± 0.77^{c}	86.00 ± 0.85^{a}	85.67 ± 0.71^{ab}	84.48 ± 1.71^{abc}	0.04
Percentage of half-eviscerated yield with giblet (%)	75.96 ± 1.72	76.82 ± 1.19	78.03 ± 1.47	76.86 ± 3.15	77.45 ± 0.74	0.40
Percentage of eviscerated yield (%)	66.39 ± 7.11	68.51 ± 3.49	71.22 ± 1.79	69.92 ± 3.15	70.14 ± 0.78	0.26
Percentage of breast muscle yield (%)	6.91 ± 1.13^{b}	8.69 ± 1.41^{a}	7.32 ± 0.99^{ab}	7.43 ± 0.72^{ab}	7.07 ± 1.45^{b}	0.10
Percentage of leg muscle yield (%)	17.80 ± 4.90	17.00 ± 5.09	16.01 ± 2.76	17.17 ± 1.26	15.90 ± 1.86	0.75
Heart index (mg/g)	4.79 ± 0.29	5.09 ± 0.38	4.55 ± 2.24	4.24 ± 2.12	5.03 ± 0.46	0.83
Liver index (mg/g)	21.09 ± 0.98	20.59 ± 0.74	22.40 ± 1.75	20.95 ± 2.30	21.91 ± 1.50	0.33
Lung indexs (mg/g)	7.18 ± 0.32^{b}	9.07 ± 1.66^{a}	8.87 ± 1.89^{ab}	9.79 ± 1.38^{a}	9.65 ± 1.45^{a}	0.04
Kidney index (mg/g)	6.20 ± 0.57	6.43 ± 1.11	6.87 ± 1.02	6.97 ± 0.95	7.57 ± 1.17	0.22
Gizzard index (mg/g)	24.61 ± 4.05	26.18 ± 4.42	22.87 ± 2.10	22.79 ± 3.44	25.06 ± 2.41	0.38
Proventriculus index (mg/g)	3.54 ± 0.56	3.65 ± 0.30	3.46 ± 0.42	3.52 ± 0.36	3.74 ± 0.58	0.85
Pancreas index (mg/g)	2.13 ± 0.36	2.26 ± 0.14	2.14 ± 0.89	1.76 ± 1.54	1.91 ± 0.98	0.88

In the same row, values with no letter or the same small letter superscripts mean no significant difference ($P > 0.05$), while with different small letter superscripts mean significant difference ($P < 0.05$), and with different capital letter superscripts mean significant difference ($P < 0.01$).

d ($P < 0.01$) and 16 to 70 d ($P < 0.05$) in 30 g/kg APG was decreased compared with CG. Additionally, the ADG of 16 to 70 d in 10 g/kg FAPG was increased ($P < 0.01$), but the FCR of 16 to 70 d in 10 g/kg FAPG was decreased ($P < 0.01$) compared with CG. Moreover, the ADG of 22 to 42 d ($P < 0.05$) and 16 to 70 d ($P < 0.05$) in 30 g/kg FAPG were increased, but the FCR of 22 to 42 d ($P < 0.05$) and 16 to 70 d ($P < 0.05$) in 30 g/kg FAPG were decreased compared with CG. Furthermore, 50 g/kg of fermented *A. paniculata* reduced the BW of 42 d ($P < 0.05$) and 70 d ($P < 0.01$) in 50 g/kg FAPG compared with CG.

Carcass Traits

Compared with CG, dressed percentage in 10 g/kg FAPG was increased ($P < 0.05$); percentage of breast muscle yield in 30 g/kg APG was increased ($P < 0.05$); lung index in 30 g/kg APG, 30 g/kg FAPG and 50 g/kg FAPG were increased ($P < 0.05$), as shown in Table 3.

Immune Function

In 30 g/kg APG, thymus index ($P < 0.05$) and lymphocyte conversion rate ($P < 0.01$) was increased compared with CG (Tables 4 and 5). However, in 10 g/kg FAPG and 30 g/kg FAPG, the bursa of Fabricius and thymus index, serum lysozyme level, and lymphocyte conversion rate were increased ($P < 0.05$) compared with CG. Additionally, in 30 g/kg FAPG, bursa of Fabricius index ($P < 0.05$) and lymphocyte conversion rate ($P < 0.01$) were increased compared with 30 g/kg APG.

Intestinal Parameters

In 30 g/kg APG, villi height, intestine thickness, intraepithelial lymphocytes (**iELs**) ($P < 0.01$), and villi surface area ($P < 0.05$) in the ducks were increased compared with CG (Table 6). However, in 10 g/kg FAPG, villi height, intestine thickness, villi surface area ($P < 0.01$), and villi width ($P < 0.05$) in the ducks were elevated compared with CG. Similarly, in 30 g/kg FAPG, villi height, villi width, intestine thickness, villi surface area, and iELs were increased ($P < 0.01$) in the ducks compared with CG. Additionally, in 50 g/kg FAPG, villi height, villi width, and villi surface area were increased ($P < 0.01$) compared with CG.

16S rRNA Sequencing Data Statistics

To further verify the effect of fermented *A. paniculata* on the intestinal health of Muscovy ducks, we collected the cecal contents from the control treatments (basal diets without *A. paniculata*/*CG*), one unfermented *A. paniculata* treatment (30 g/kg APG) and one fermented *A. paniculata* treatment (30 g/kg FAPG) for 16S rRNA

Table 4. Effects of fermented *A. paniculata* on immune organ indices in Muscovy ducks.

Items	CG	30 g/kg APG	10 g/kg FAPG	30 g/kg FAPG	50 g/kg FAPG	*P*-value
Spleen index (mg/g)	0.71 ± 0.05	0.80 ± 0.23	1.07 ± 0.47	0.87 ± 0.11	0.85 ± 0.16	0.50
Bursa of Fabricius index (mg/g)	0.76 ± 0.20^{Bc}	1.00 ± 0.20^{ABbc}	1.12 ± 0.29^{ABab}	1.45 ± 0.12^{Aa}	0.93 ± 0.10^{Bbc}	0.02
Thymus index (mg/g)	1.76 ± 0.14^{Bc}	2.82 ± 0.56^{ABab}	2.76 ± 0.70^{ABab}	3.63 ± 0.57^{Aa}	2.31 ± 0.48^{ABbc}	0.02

In the same row, values with no letter or the same small letter superscripts mean no significant difference ($P > 0.05$), while with different small letter superscripts mean significant difference ($P < 0.05$), and with different capital letter superscripts mean significant difference ($P < 0.01$).

Table 5. Effects of fermented *A. paniculata* on immune-related indices in Muscovy ducks.

Items	CG	30 g/kg APG	10 g/kg FAPG	30 g/kg FAPG	50 g/kg FAPG	*P*-value
Lysozyme (μg/L)	51.15 ± 2.55^{b}	54.42 ± 1.33^{ab}	56.69 ± 1.79^{a}	55.11 ± 2.98^{a}	53.47 ± 0.31^{ab}	0.07
Lymphocyte conversion rate (%)	59.95 ± 1.38^{Cd}	67.45 ± 3.10^{Bb}	63.12 ± 2.33^{Cc}	74.59 ± 2.58^{Aa}	61.13 ± 1.38^{Ccd}	$P < 0.01$

In the same row, values with no letter or the same small letter superscripts mean no significant difference ($P > 0.05$), while with different small letter superscripts mean significant difference ($P < 0.05$), and with different capital letter superscripts mean significant difference ($P < 0.01$).

Table 6. Effects of fermented *A. paniculata* on intestinal morphology in Muscovy ducks.

Items	CG	30 g/kg APG	10 g/kg FAPG	30 g/kg FAPG	50 g/kg FAPG	*P*-value
Villi height (μm)	995.82 ± 160.68^{B}	$1{,}430.83 \pm 139.16^{A}$	$1{,}263.66 \pm 151.11^{A}$	$1{,}373.20 \pm 198.41^{A}$	$1{,}157.08 \pm 125.78^{A}$	$P < 0.01$
Crypt depth (μm)	213.80 ± 51.94	246.49 ± 38.92	239.09 ± 40.73	251.55 ± 60.52	198.75 ± 49.57	0.31
Vilii width (μm)	200.90 ± 63.91^{Bb}	227.28 ± 40.84^{Bab}	265.46 ± 32.56^{Ba}	406.27 ± 59.07^{Aa}	382.59 ± 47.04^{Aa}	$P < 0.01$
V/C ratio (μm)	4.56 ± 1.13	5.79 ± 0.99	5.37 ± 0.85	5.68 ± 1.42	6.08 ± 1.38	0.30
Intestine thickness (μm)	254.74 ± 16.40^{C}	351.22 ± 65.10^{A}	329.85 ± 34.73^{A}	333.27 ± 19.25^{A}	298.32 ± 29.73^{AB}	0.35
Villi surface area (mm^2)	0.65 ± 0.29^{Cc}	1.05 ± 0.27^{BCb}	1.13 ± 0.11^{Bb}	1.61 ± 0.33^{Aa}	1.40 ± 0.27^{AaBb}	$P < 0.01$
Intraepithelial lymphocytes (%)	3.34 ± 0.19^{B}	6.76 ± 1.80^{A}	4.36 ± 0.52^{B}	6.49 ± 1.06^{A}	5.23 ± 1.36^{AB}	$P < 0.01$

In the same row, values with no letter or the same small letter superscripts mean no significant difference ($P > 0.05$), while with different small letter superscripts mean significant difference ($P < 0.05$), and with different capital letter superscripts mean significant difference ($P < 0.01$).

Table 7. Summary on raw data processing.

Items	CG	30 g/kg APG	30 g/kg FAPG	*P*-value
Raw Reads	$71{,}796.33 \pm 6{,}946.93$	$64{,}127.33 \pm 8916.69$	$66{,}905.00 \pm 4{,}784.33$	0.46
Clean Reads	$66{,}592.33 \pm 6{,}415.54$	$58{,}883.33 \pm 7077.94$	$62{,}399.00 \pm 4{,}470.57$	0.36
Effective Reads	$62{,}356.33 \pm 6{,}123.90$	$54{,}716.67 \pm 6210.68$	$58{,}169.33 \pm 4{,}152.56$	0.31
AvgLen (bp)	252	252	252	
GC (%)	53.22 ± 0.15	53.15 ± 0.89	52.73 ± 0.20	0.52
Q20 (%)	99.83 ± 0.01	99.83 ± 0.01	99.84 ± 0.01	0.37
Q30 (%)	99.09 ± 0.01	99.11 ± 0.03	99.13 ± 0.03	0.13
Effective (%)	86.84 ± 0.20	85.52 ± 2.15	86.95 ± 0.17	0.36

Note: Raw Reads: Counts of raw reads; Clean Reads: Counts of clean reads(post quality control and assembly); Effective Reads: Counts of effective reads after chimeric reads removal; AvgLen (bp): Average read length of each sample; GC(%): GC content, i.e. proportion of G and C in all bases; Q20 (%): Percentage of bases with Q-score larger or equal to Q20; Q30(%): Percentage of bases with Q-score larger or equal to Q30; Effective(%): Percentage of effective reads in raw reads.

Sequencing. A total of 608,486 raw reads were generated from 9 samples, of which 563,624 clean reads were obtained after quality control. A minimum of 52,637 and an average of 62,625 clean reads were generated for each sample. Table 6 shows basic information for each sample. The results of raw data processing, including raw reads, clean reads, effective reads, avgLen, GC, Q20, and Q30 are shown in Table 7. The effective rates of each group were >80%, indicating that the data were reliable.

OTU/ASV Analysis

Operational taxonomic unit (**OTU**) refers to a cluster of sequences used to define a group (e.g., species, genus, strain, etc.) in phylogenetic or population genetic studies. These DNA sequences are clustered according to sequence similarity. Each OTU corresponds to a representative sequence.

The results showed that adding unfermented and fermented *A. paniculata* to the diet significantly affected the number of unique OTUs in the intestines of Muscovy ducks. There were 797 common OTUs among CG, 30 g/kg APG and 30 g/kg FAPG, and 18 unique OTUs in CG, 21 unique OTUs in 30 g/kg APG and 4 unique OTUs in the 30 g/kg FAPG (Figure 1).

α-Diversity Index Statistics

The results of α-diversity analysis showed that there was a significant increase ($P = 0.05$) in the Shannon index in the 30 g/kg APG and 30 g/kg FAPG compared with CG (Table 8).

β-Diversity Analysis

Sample clustering trees showing species between different samples and species compositions at the genus level are shown in a histogram (Figure 2A). Notably, between group differences were higher than within group differences (Figure 2A). Species compositions in genus level are shown using a histogram (Figure 2A). *Bacteroides* and *Desulfovibrio* were the dominant bacterial genera in each group.

Analysis of similarity (**ANOSIM**) indicated that between group differences were higher than within group differences ($R = 0.901$, $P = 0.007$), and confirmed the high reliability of the test (Figure 2B).

Differential Analysis Between Groups

Differences in microbial composition between the experimental groups are shown in Figure 3. The addition of 30 g/kg of unfermented and fermented *A. paniculata* to the diet (30 g/kg APG) significant decreased the relative

Table 8. Summary of alpha diversity metrics.

Items	CG	30 g/kg APG	30 g/kg FAPG	*P*-value
Feature	897.00 ± 17.09	904.33 ± 28.75	885.67 ± 17.50	0.40
ACE	917.10 ± 12.28	931.16 ± 21.64	912.97 ± 17.12	0.46
Chao1	934.71 ± 7.01	948.51 ± 25.33	922.42 ± 19.55	0.31
Simpson	0.96 ± 0.01	0.97 ± 0.01	0.96 ± 0.01	0.24
Shannon	6.45 ± 0.17^{b}	7.14 ± 0.42^{a}	7.01 ± 0.20^{a}	0.05
PD-wholetree	52.13 ± 0.64	53.52 ± 0.56	53.37 ± 0.83	0.09
Coverage	0.999 ± 0.003	0.999 ± 0.000	0.999 ± 0.001	0.60

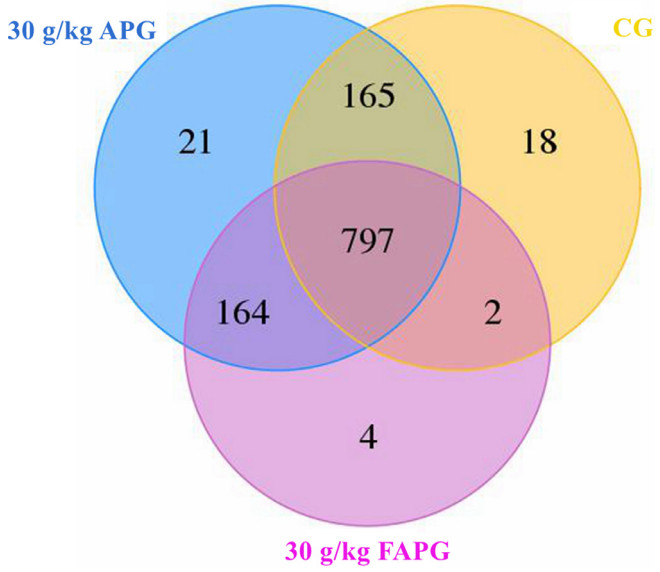

Figure 1. Venn Diagram on operational taxonomic units (OTUs). Note: The number in each independent or overlapped area stands for number of unique or common features in each corresponding collection.

abundance of *uncultured-bacterium-o-WCHB-41, uncultured-bacterium-o-Rhodospirillales, uncultured-bacterium-o-Bacteroidales, uncultured-bacterium-f-Clostridiales-vadinBB60-group, Succinivibrio, Succinatimonas, Sphaerochaeta, Ruminococcaceae-UCG-007, Mucispirillum, Methanobrevibacter, Elusimicrobium, Coprobacter,* and *Butyricicoccus*, but increased the abundance of *Skermanella, Ruminococcaceae-UCG-004, Rikenellaceae-RC9-gut-group, Methanocorpusculum, Fournierella, Erysipelotrichaceae-UCG-004,* and *Adhaeribacter* ($P < 0.05$) compared with CG (Figure 3A).

LEfSe analysis showed that *s-uncultured-bacterium-o-Bacteroidales, g-uncultured-bacterium-o-Bacteroidales, f-uncultured-bacterium-o-Bacteroidales, s-uncultured-bacterium-f-Clostridiales-vadinBB60-group, g-uncultured-bacterium-f-Clostridiales-vadinBB60-group, f-Clostridiales-vadinBB60-group,* and *o-Clostridiales* were enriched in CG, whereas *s-uncultured-bacterium-g-Erysipelotrichaceae-UCG-004* and *g-Erysipelotrichaceae-UCG-004* were enriched in 30 g/kg APG (Figure 3B).

The length of the histogram in Figure 3C represents the impact of different species. The results showed that the most influential microorganisms in CG are *o-Clostridiales, c-Clostridia, f-uncultured-bacterium-o-Bacteroidales, g-uncultured-bacterium-o-Bacteroidales, s-uncultured-bacterium-o-Bacteroidales, g-uncultured-bacterium-f-Clostridiales-vadinBB60-group* and *s-uncultured-bacterium-f-Clostridiales-vadinBB60-group*. In 30 g/kg APG, the most influential microorganisms were *s-uncultured-bacterium-g-Erysipelotrichaceae-UCG-004, g-Erysipelotrichaceae-UCG-004* and *c-Gammaproteobacteroa.*

Kyoto Encyclopedia of Genes and Genomes Pathway Enrichment of Intestinal Microbes

As shown in Figure 4, in 30 g/kg APG, pathways of *Metabolism of other amino acids, Xenobiotics biodegradation and metabolism, Aging, Drug resistance: Antineoplastic, Neurodegenerative diseases,* and *Circulatory system* were upregulated ($P < 0.05$), but pathways of *Metabolism of cofactors and vitamins, Translation* and *Signaling molecules and interaction* were downregulated ($P < 0.05$) compared with CG. In 30 g/kg FAPG, pathways of *Cancers: Specific types, Drug resistance: Antineoplastic, Infectious diseases: Parasitic, Xenobiotics biodegradation and metabolism, Neurodegenerative diseases, Substance dependence, Amino acid metabolism, Aging, Metabolism of other amino acids, Circulatory system and Lipid metabolism* were upregulated ($P < 0.05$), but pathways of *Global and overview maps, Folding, sorting and degradation* and *Translation* were downregulated ($P < 0.05$) compared with CG.

COG Analysis

COG (Clusters of Orthologous Groups of proteins) is a commonly used function classification database of proteins in prokaryotes. In 30 g/kg FAPG, *Gerneral function prediction only, Secondary metabolites biosynthesis, transport and catabolism, Extracellular structures* were upregulated ($P < 0.05$; Figure 5), but *Cell cycle control, cell division, chromosome partitioning* and *Nucleotide transport and metabolism* were downregulated ($P < 0.05$) compared with the CG.

DISCUSSION

In traditional herbal medicine, *A. paniculata* has been reported to possess antihepatotoxic, antibiotic, antimalarial, antihepatic, antithrombogenic, anti-inflammatory, antivenom, and antipyretic properties, in addition to its use as an immunostimulant (Kumar et al., 2021). When used as a feed additive, *A. paniculata* has the potential to improve the nutritional composition of feed, increase the body weight of poultry, and reduce mortality of domestically raised poultry (Jahja et al., 2022).

The enhancement of ADG and reduction of FCR (**ADFI/ADG**) has been shown to improve in growth performance. Consistent with these findings, our results showed that both unfermented and fermented *A. paniculata* promoted growth, with a more distinct effect observed using fermented *A. paniculata*. However, we found that Muscovy duck body weight significantly reduced following consumption of feed containing 50 g/kg of fermented *A. paniculata*. This may be attributed to the presence of toxic factors and bitter taste of *A. paniculata* at a large dose. Moreover, excessive introduction of live bacteria into the animal intestines through the intake of fermented products may affect nutrient absorption and utilization and growth in the host animals.

Carcass traits are indicators of meat quality (Gungor et al., 2020). It is generally acknowledged that meat quality is optimal when the dressed percentage and percentage of eviscerated yield are over 80% and

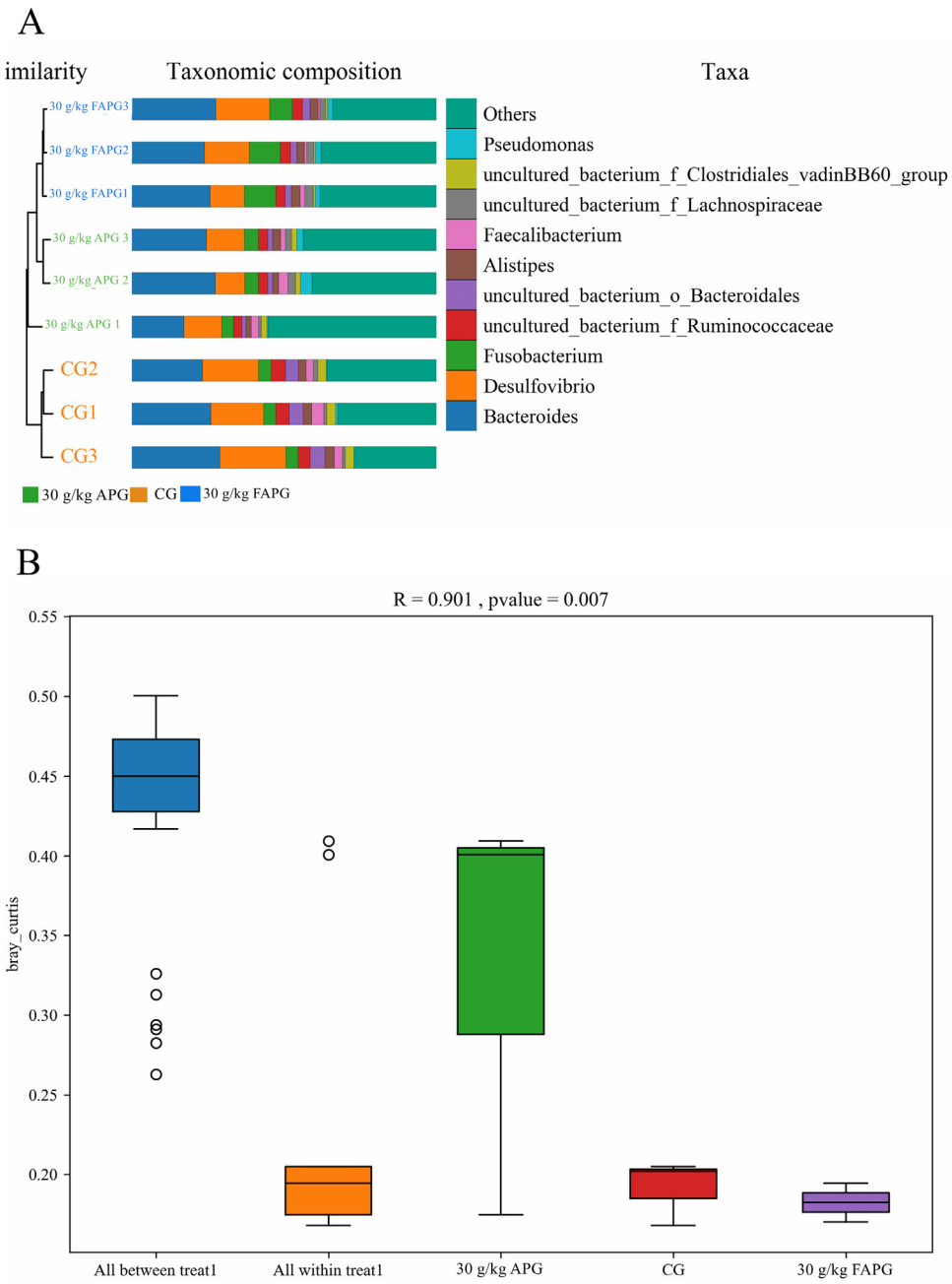

Figure 2. Intestinal microbiota β-diversity (A) Clustering tree and histogram. (B) PERMANOVA/Anosim analysis box plot.

60%, respectively. The meat quality within our study was considered "excellent", as the dressed percentage and percentage of eviscerated yield exceeded the aforementioned standards. Additionally, the marked increase in other carcass traits, including dressed percentage of 10 g/kg FAPG, percentage of breast muscle yield of 30 g/kg APG, lung index of 30 g/kg APG, 30 g/kg, and 50 g/kg FAPG, indicated an improvement in the productive performance of the experimental Muscovy duck groups compared with CG.

The immune organ indices are measures of the immune function of the body in poultry (Zhang et al., 2021). The spleen is the largest peripheral immune organ in poultry and is involved in humoral and cellular immunity (Madej et al., 2020). Bursa of Fabricius, containing various bursal-derived peptides, is a unique humoral immune central organ in poultry (Feng et al., 2012). The thymus is the main site of T cell production, and its immune function plays an important role in anti-infection, antitumor, and autoimmune effects (Amirghofran et al., 2012). Our results showed that supplementation with 30 g/kg fermented *A. paniculata* significantly increased the bursa index by 90.79% compared with CG. Additionally, supplementation with 30 g/kg unfermented *A. paniculata* and fermented *A. paniculata* clearly increased the thymus index of the ducks by 60.23% and 106.25%, respectively, compared with CG. These results indicated that both unfermented and fermented *A. paniculata* improved the immune organ indices of Muscovy ducks; however, fermented *A.*

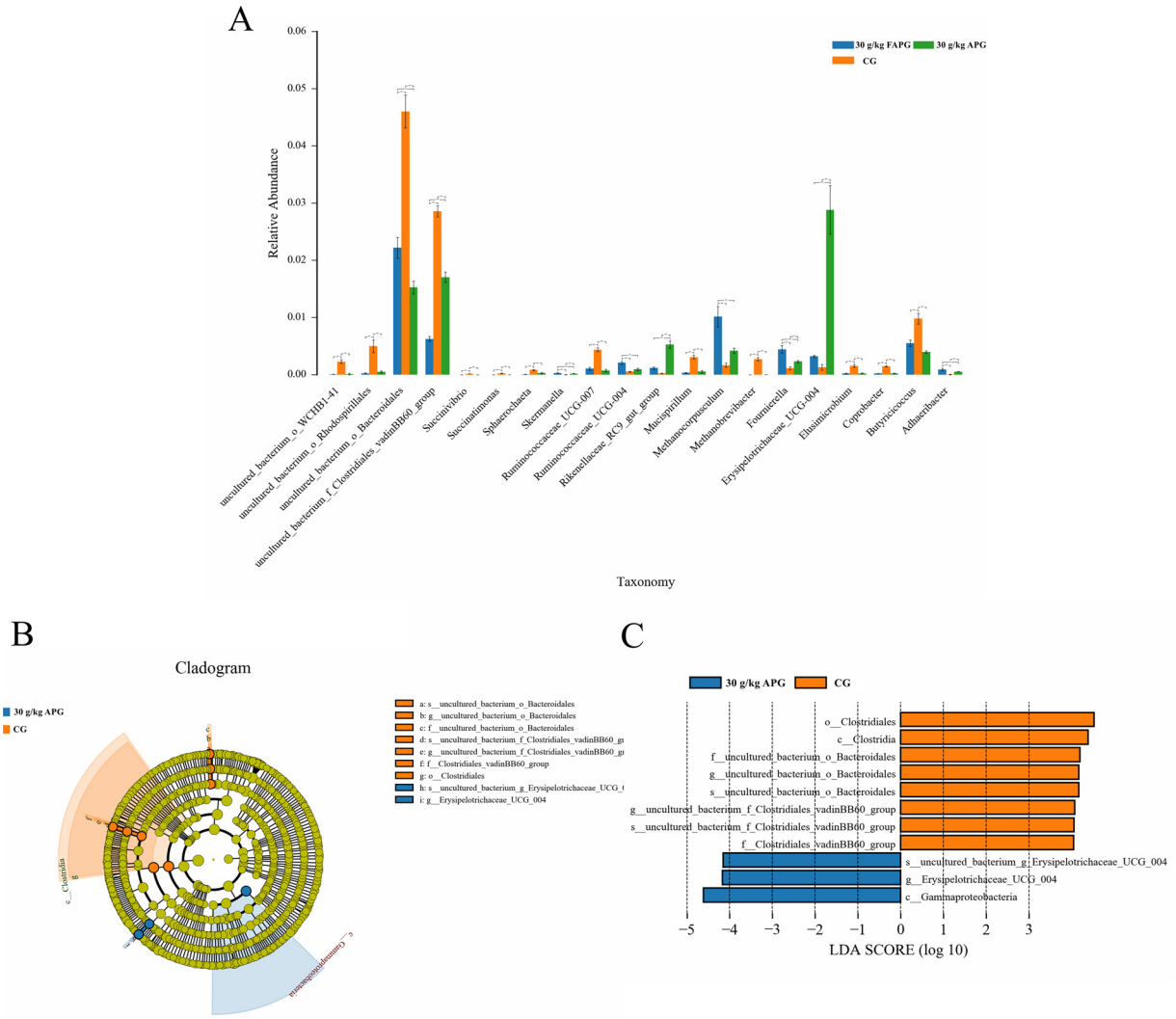

Figure 3. Analysis of differences between groups. (A) Histogram of ANOVA between groups. (B) Cladogram based on LEfSe analysis (C) LDA value distribution histogram.

paniculata had a more distinct effect on the 2 organ immune indices.

In the present study, we found that supplementation with fermented *A. paniculata* significantly increased serum lysozyme activity in Muscovy ducks, consistent with Deng et al. (2016) who found that using the ultrafine powder of *A. paniculata* as a diet supplement increased serum lysozyme activity in Sanhuang chickens. Lysozyme is an alkaline enzyme that can hydrolyze polysaccharides on the surface of pathogenic bacteria, and possesses anti-infective, antiviral, and immune-enhancing effects (Khlyustova et al., 2022). Additionally, serum lysozyme has shown to be an important indicator of nonspecific immunity in the body. Therefore, our results suggest that fermented *A. paniculata* supplementation may have improved innate immunity in the Muscovy ducks by increasing serum lysozyme activity.

Most of the peripheral blood lymphocytes of Muscovy ducks are T cells, which are important in mediating cellular immunity (Wu et al., 2019). After stimulation with nonspecific antigen ConA in vitro, they can transform into lymphoblasts for lymphocyte proliferation (Alekseeva et al., 2021). Therefore, the degree of T lymphocyte transformation and proliferation reflects the level of cellular immunity in the body (Hafez et al., 2020). In the present study, supplementation with unfermented and fermented *A. paniculata* observably increased the conversion rate of peripheral blood lymphocytes in Muscovy ducks. This indicated that *A. paniculata* can improve the cellular immunity of the body, which was consistent with the findings of Hu et al. (2006). Notably, fermented *A. paniculata* had a considerably better effect than unfermented *A. paniculata*, which may be due to the further release of active substances that occurs following fermentation.

The growth performance of poultry is closely related to the morphological structure of the intestinal mucosa, and the integrity of its structure and function is important for poultry health (Wang et al., 2021). In the present study, supplementation with both fermented and unfermented *A. paniculata* significantly improved the villi height and villi surface area of the ducks, indicating that both unfermented and fermented *A. paniculata* can improve intestinal function of digestive absorption. However, supplementation with 30 g/kg fermented *A. paniculata* had the best effects on villi surface area and

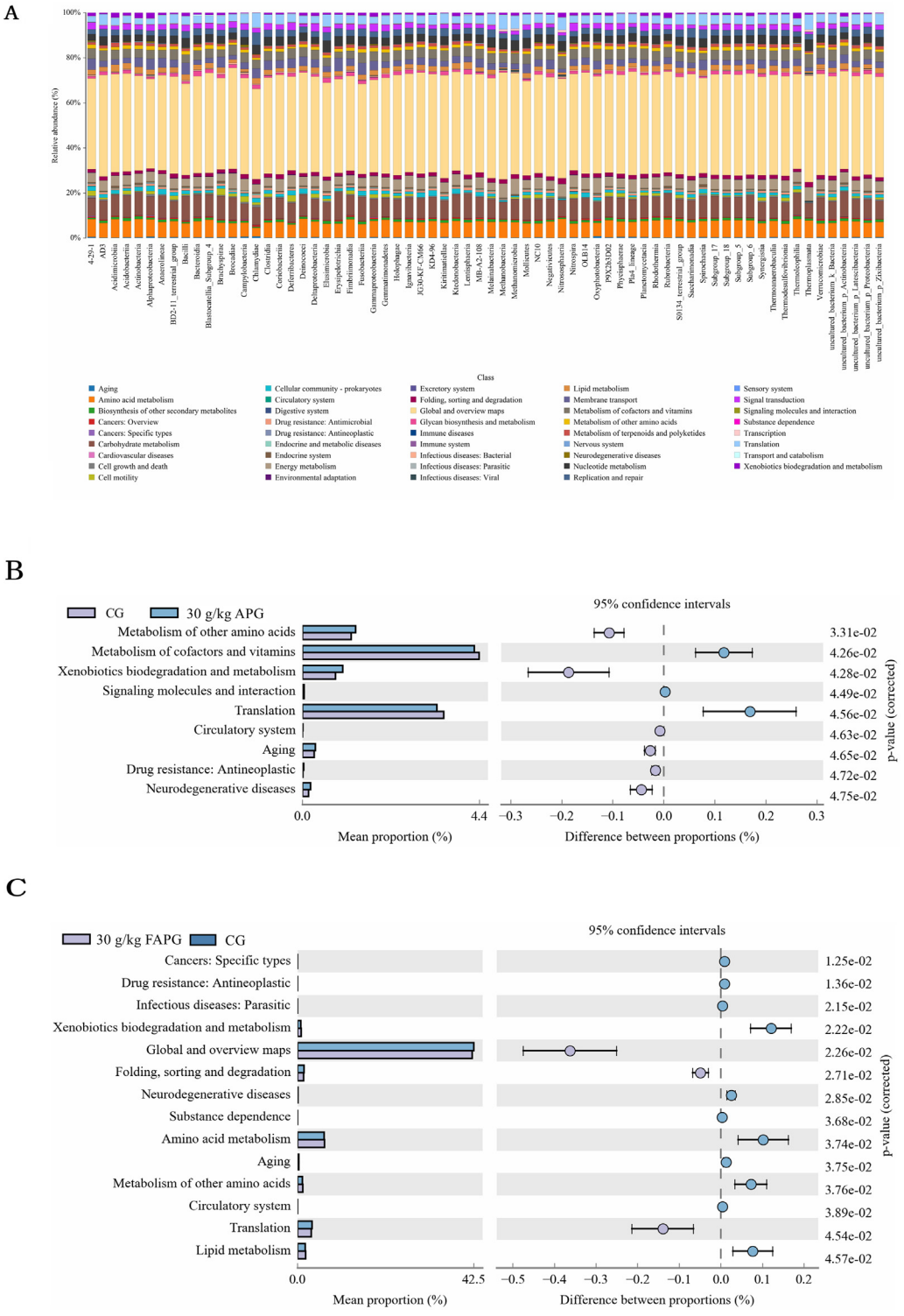

Figure 4. KEGG pathway enrichment analysis. (A) Histogram of KEGG pathways. (B) KEGG metabolic pathways difference between the control and 30 g/kg unfermented *A. paniculata* group (C) KEGG metabolic pathways difference between control and 30 g/kg fermented *A. paniculate* group.

villi width, indicating that fermented *A. paniculata* is preferable for improving intestinal health in Muscovy ducks. These results could explain the observed improvement in growth performance of Muscovy ducks fed fermented *A. paniculata* compared with those fed unfermented *A. paniculata*. Intestine thickness is closely related to small intestinal motility, which directly affects the tonic contraction, rhythmic segmental motility, and peristalsis of the small intestine (Maged et al., 2022). The thicker the intestinal walls, the stronger the intestinal peristaltic function, and the faster the excretion of pathogenic microorganisms, such as bacteria and viruses. In the present study, supplementation with both 30 g/kg of unfermented and fermented *A. paniculata* significantly increased the intestine thickness of Muscovy ducks, which further improved intestinal

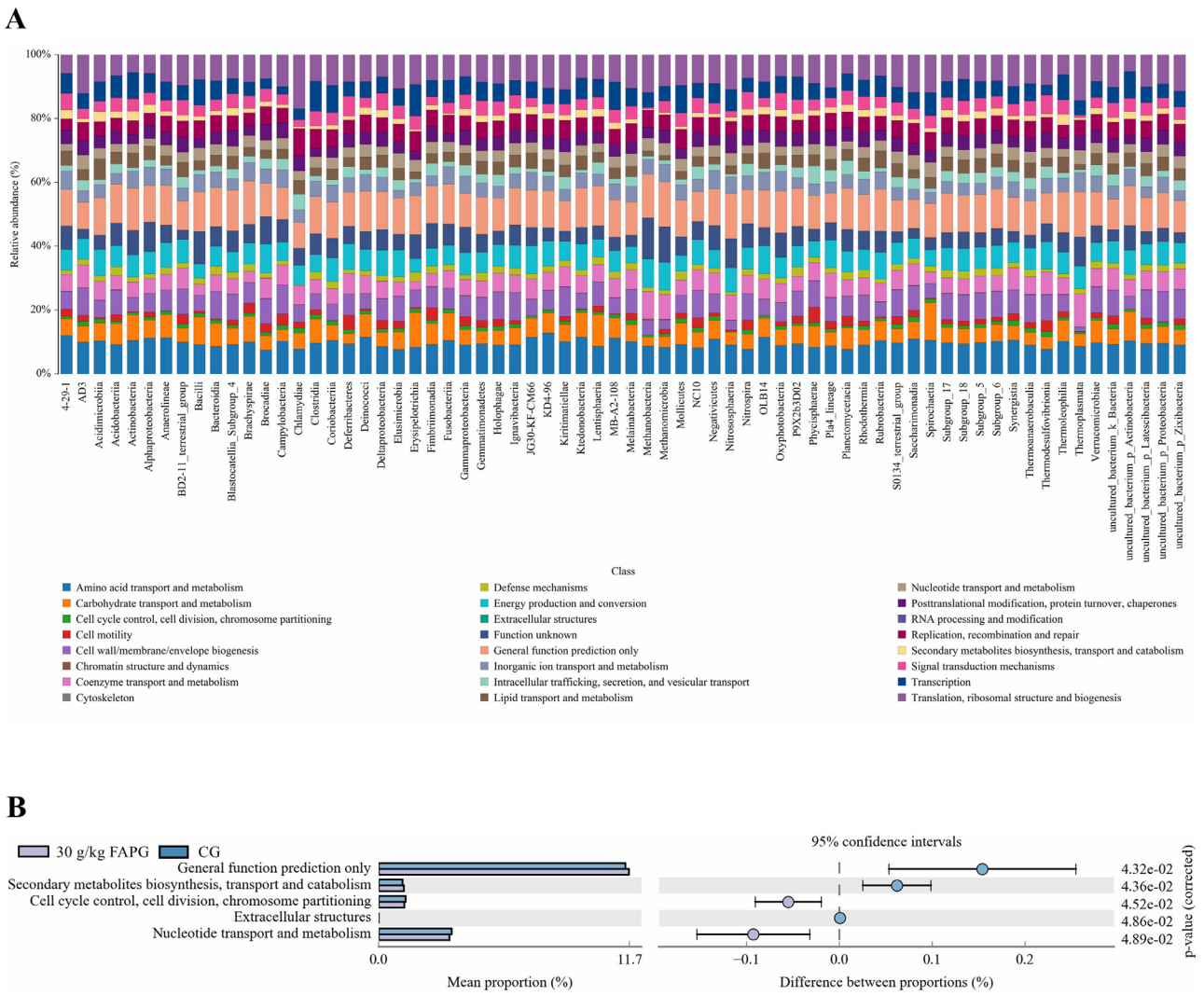

Figure 5. COG pathways. (A) Histogram of COG pathways. (B) Statistical graph of COG function classification.

function and health in Muscovy ducks. Additionally, intestinal mucosal immune-related cells, such as intraepithelial lymphocytes, play an important role in maintaining the integrity of intestinal epithelial cells and immune responses, and acts as the body's first defense barrier against pathogens (Tian et al., 2021). The local immune function of the intestine can be reflected by the changes in the number of these immune-related cells (Wang et al., 2019). In the present study, supplementation with either 30 g/kg of unfermented or fermented *A. paniculata* improved intestinal immune function, in addition to intestine thickness, which improved intestinal function and health in the Muscovy ducks. These results indicate that *A. paniculata* could be used as a potential alternative to antibiotic growth promoters.

The effect of *A. paniculata* on the intestinal microbial composition and structure was examined and the richness and diversity of intestinal bacteria in the cecum of the Muscovy ducks were assessed using α-diversity indices, including Shannon index (Zengin et al., 2022). Supplementation with either 30 g/kg unfermented or 30 g/kg fermented *A. paniculata* significantly increased the Shannon index, indicating that both treatments can improve species richness and diversity in the cecum of Muscovy ducks. Additionally, β-analysis showed that the experimental and control groups form 2 distinct clusters, suggesting that both fermented and unfermented *A. paniculata* had considerable effects on intestinal microbiota composition and structure.

Trillions of bacteria inhabit the gastrointestinal tract of animals. In most hosts, these symbionts play a large role in promoting microbe-host internal environmental balance (Wang et al., 2022). Among them, the main metabolic end products of *Succinivibrio and Succinatimonas* are acetic acid and succinic acid (Li et al., 2020). Succinic acid is harmful to the environment to some extent and can cause pollution to water bodies and the atmosphere. *Sphaerochaeta* has a helical morphology and motility conferred by flagella around the axial cytosol, and has some pathogenicity (Bidzhieva et al., 2020). *Mucispirillum* is a spiral, flagellated, gram-negative bacteria, and obligate anaerobes, which live in the intestinal mucus layer, and is associated with intestinal inflammation development (Loy et al., 2017). In the present study, supplementation with unfermented and fermented *A.*

paniculata decreased the abundance of *Succinivibrio, Succinatimonas, Sphaerochaeta*, and *Mucispirillum*, indicating the animal and environmental health promoting effects of *A. paniculata*.

In the present study, supplementation with unfermented and fermented *A. paniculata* increased the abundance of *Skermanella. Skermanella* possess antimony resistance properties (Luo et al., 2012); therefore, contributing to improved heavy metal tolerance, health status, and meat quality in Muscovy ducks. We observed an increase *Ruminococcaceae* abundance, which are the main microorganisms that convert primary bile acids into secondary bile acids, and thus play an important role in lipid digestion and absorption (Gu et al., 2022). Therefore, these results indicate that *A. paniculata* can promote lipid digestion and absorption in Muscovy ducks. We observed an increase in the abundance of the *Rikenellaceae, Methanocorpusculum*, and *Fournierella genera. Rikenellaceae* reduces the negative associated effects of IBD enteritis (Huang et al., 2019), *Methanocorpusculum* is involved in the mediation of abdominal fat deposition (Dong et al., 2019), and *Fournierella* is positively associated with muscle and bone health (Farkas et al., 2022). Therefore, the increased abundance of these 3 genera indicated that both unfermented and fermented *A. paniculata* can improve intestinal health, reduce body fat levels, and improve carcass quality and the health status of Muscovy ducks.

Overall, supplementation with unfermented and fermented *A. paniculata* inhibited the abundance of harmful bacteria (*Succinivibrio, Succinatimonas, Sphaerochaeta*, and *Mucispirillum*) and increased the abundance of beneficial bacteria (*Rikenellaceae, Methanocorpusculum, Fournierella*). We found that supplementation with unfermented and fermented *A. paniculata* increased *Adhaeribacter* abundance, which could reduce antibiotic residues and promote chlortetracycline degradation (**REF**). The transfer of antibiotics through the food chain pose considerable threats to human health; thus, the use of *A. paniculata* as a natural alternative to antibiotics provides important implications for reducing the risks to human health from poultry consumption.

Based on KEGG pathway enrichment analysis database, supplementation with unfermented and fermented A. paniculata upregulated the pathways of *Metabolism of other amino acids, Xenobiotics biodegradation and metabolism, Aging, Drug resistance: Antineoplastic, Neurodegenerative diseases*, and *Circulatory system. Metabolism of other amino acids* is associated with the metabolism of amino acids, such as taurine, hypotaurine, phosphonate, hypophosphite, selenoamino acids, and cyanoamino acids (Hu et al., 2003). *Xenobiotics biodegradation and metabolism* are associated with benzoic acid aminobenzoic acid, orthofluorobenzoic acid, chloride paraffin, chloroalkene, methylbenzene (Nakov et al., 2020). Additionally, supplementation with 30 g/kg fermented *A. paniculata* promoted pathways related to amino acid and lipid metabolism, of which *Amino acid metabolism* pathways are related to the metabolism of alanine, aspartic acid, glutamic acid, glycine, serine, threonine, valine, leucine, lysine, and isoleucine, as well as the biosynthesis of valine, leucine, isoleucine, lysine, and arginine (Cui et al., 2020). *Lipid metabolism* pathways are related to biosynthesis of fatty acids, elongation of fatty acid, synthesis and degradation of ketones, biosynthesis of cork and wax, steroids, primary bile acid, secondary bile acid, and steroid hormone, and metabolism of glycerides, glycerophospholipids, ether lipid, sphingoglycolipid, arachidonic acid, linoleic acid, and linolenic acid (Russell, 2018). Overall, compared with 30 g/kg unfermented *A. paniculata*, 30 g/kg fermented *A. paniculata* had a stronger effect on the metabolism and degradation of amino acids and lipids in Muscovy ducks.

COG analysis showed that supplementation with 30 g/kg fermented *A. paniculata* improved secondary metabolites biosynthesis, transport, and catabolism. Secondary metabolites are a class of nonessential small organic compounds necessary for the normal operation of cell activities, growth, and development of body (Adebayo et al., 2019). Therefore, our results suggest that fermented *A. paniculata* supplementation may play an important role in promoting the growth and development of Muscovy ducks through improving secondary metabolite biosynthesis.

Overall, these results suggest that fermented *A. paniculata* can maintain normal body activities, promote protein synthesis, and maintain the extracellular structure of eukaryotic cells. Moreover, COG and KEGG enrichment analysis of differentially expressed genes among the 3 groups confirmed that unfermented and fermented *A. paniculata* can regulate microbial diversity, abundance, and structure. However, fermented *A. paniculata* had considerably stronger effects than unfermented *A. paniculata* on pathways associated with amino acid and lipid metabolism, and secondary metabolites biosynthesis, transport, and catabolism. Therefore, it was speculated fermented *A. paniculata* may improve nutrient metabolism by bacteria, improve their adaptability to the environment, and regulate the immune and digestive system by regulating the genes in these pathways.

These results indicate that the observed improvement in duck growth performance, immune status, and intestinal morphology can be attributed to the consumption of diets supplemented with fermented *A. paniculata*; however, further studies are necessary to elucidate the specific molecular mechanism of fermented *A. paniculata*.

CONCLUSIONS

In summary, supplementation with fermented *A. paniculata* improved growth performance, immune status, intestinal morphology, and gut microbiota composition and structure in Muscovy ducks. Therefore, fermented *A. paniculata* have potential application in the production of Muscovy ducks, and could be used as potential alternative for antibiotics.

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DISCLOSURES

All authors declare they have no conflicts of interest.

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