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Dietary areca nut extract supplementation modulates the growth performance and immunity of Jiaji ducks (*Cairina moschata*)

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ABSTRACT

Areca nut extract (ANE) has a variety of pharmacological effects on animals. Here, we investigated the influence of ANE on the slaughter performance and immune function of Jiaji ducks. One hundred and fifty 42-day-old healthy Jiaji ducks were randomly divided into 2 groups (5 replicates of 15 ducks each), named DCK group (control) and DNT group (treatment), respectively. Ducks in the DCK group were fed a basal diet and ducks in the DNT group were fed a basal food supplemented with 0.08 g ANE per kg of basal diet. Additionally, using proteomics, untargeted metabolomics, and metagenomics, we analyzed the impact of ANE on the protein profile of the spleen, the composition of plasma metabolites, and the structure of the cecal microbiota. The results showed that the dietary inclusion of ANE significantly increased the slaughter rate of Jiaji ducks. Proteomic analysis revealed 78 differentially expressed proteins in the spleens of ANE-treated birds, including 54 proteins up-regulated and 24 proteins down-regulated in the DNT group, mainly enriched in cell adhesion molecules and glutathione metabolic pathways. Untargeted metabolomic analysis revealed that 117 serum metabolites were differentially regulated between the ANE and DCK groups; meanwhile, KEGG pathway analysis indicated that these metabolites were mainly involved in arachidonic acid metabolism, phospholipase D signaling pathway and eicosanoids. Furthermore, a metagenomic analysis showed that the genus *Methanobrevibacter* was significantly downregulated in the ANE supplementation group. Combined, the results of the metagenomic and metabolomic analyses showed that the relative abundance of *Prevotella* was significantly lower in the ANE group than in the DCK group and that *Prevotella* was negatively correlated with the levels of the anti-inflammatory compound hydrocinnamic acid and the lipid metabolism regulator ganoderic acid A. This study provides a reference for the application of ANE as a supplement in the diet of Jiaji ducks.

Introduction

Jiaji ducks (*Cairina moschata*) originated from Jiaji Town, Qionghai County, Hainan Province, and have a history of over 200 years, making them an important genetic resource in China (Gu et al., 2020). Jiaji ducks are known for their fast growth, strong disease resistance, high survival rate, high lean meat yield, and delicious taste (Kokoszyński et al., 2020). Improving growth performance, gut health and immunity is critical to the production of Jiaji ducks. Numerous studies have shown that the traditional practice of supplementing poultry feed with antibiotics to promote weight gain has led to the development of bacterial antibiotic resistance and public health problems. This emphasizes the importance of developing new feed additives that have a favourable safety profile, low potential for inducing drug resistance and do not produce toxic residues. Natural plant extracts often possess a wide range of biological properties, including antioxidant, antiparasitic, and antibacterial effects (Connelly et al., 1996; Lee et al., 2003; Kamaraj et al., 2010). They represent important sources for the development of novel feed additives that enhance immunity in poultry.

Areca nut is a tropical crop widely grown in southeast asian countries, including China, India, and Malaysia (Murwani et al., 2022). Recent studies have shown that areca nut has a variety of

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pharmacological activities, such as antiparasitic, antioxidant, antibacterial, and anti-inflammatory effects. It is commonly used as a component of traditional Chinese medicine (Bhandare et al., 2010; Salehi et al., 2020; Mei et al., 2021). Research has also demonstrated that flavonoids present in areca nut extract (**ANE**), such as quercetin and liquiritin, are the main active components responsible for its anti-inflammatory and antioxidant biological functions (Bhandare et al., 2010). Hu et al. (2021) found that areca nut polysaccharides, which account for approximately 3.3 % of the areca nut, can inhibit the ability of lipopolysaccharide-stimulated macrophages to produce nitric oxide, indicating that these substances are also important mediators of areca nut anti-inflammatory activity. Meanwhile, Sazwi et al. (2013) demonstrated that areca nut water extract exhibits an oxygen radical clearing capacity similar to that of vitamin C. Additionally, Li et al. (2017) reported that the continuous oral administration of 3 % crude ANE for 6 months significantly mitigated ovarian resection-induced lipid peroxidation, oxidative stress damage, and osteoporosis in mice. The mechanism underlying the antioxidant effects of ANE may be related to the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway. Nrf2 is an important transcription factor that regulates cellular oxidative stress responses and contributes to the maintenance of cellular redox homeostasis. Its activation leads to the transcriptional regulation of downstream antioxidant response element-containing genes (Yi et al., 2022). These observations indicate that ANE has broad application prospects as feed additive. Accordingly, in this study, we supplemented the diets of Jiaji ducks with ANE, and subsequently evaluated the effects on slaughter performance, cecum microbial composition, spleen proteome and blood metabolome in the Jiaji ducks.

Materials and methods

Ethical approval

All animal experiments were conducted in accordance with the Guidelines for Experimental Animals established by the Ministry of Science and Technology (Beijing, China) and were approved by the Henan University of Science and Technology (Luo'yang, China). Ethical approval for the study was given by the Animal Ethics Committee of the university (No. HAUST-2022079).

Preparation of ANE

The areca nut samples used in this study were provided by the Institute of Agricultural Product Processing Design and Research of the Hainan Academy of Agricultural Sciences. Dried areca nuts were ground to a powder using a high-speed pulverizer. For extraction, the nut powder was soaked in 75 % ethanol (1:20 *wt/vol* [g/mL]) at 40 °C for 24 h. After extraction, the mixture was filtered through an 80-mesh sieve, followed by rotary evaporation at 60 °C. Finally, the residue was dried in an oven and pulverised.

Quantification of arecoline and polyphenols in ANE

The content of arecoline in areca nut was quantified using high-performance liquid chromatography (**HPLC**) as previously described (Cox et al., 2004), but with some modifications. A Waters UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm) was used for chromatographic separation (wavelength: 215 nm, column temperature: 30 °C, flow rate: 0.15 mL/min). The sample volume was 10 μL and the mobile phase consisted of 0.1 % phosphoric acid and acetonitrile (65:35). In addition, areca nut polyphenols were detected using a spectrometry kit (Suzhou Michy Biology Technology Ltd, china).

Experimental Design and Samples Collection

A total of 150 healthy Jiaji ducks (42 days old) of similar genetic backgrounds and body weight were selected as experimental subjects. The ducks, obtained from Hainan Chuanwei Muscovy Duck Breeding Co., Ltd., were randomly divided into 2 groups (5 replicates per group, 15 ducks per replicate), namely, a DCK group (control), in which the ducks were fed a basal diet, and DNT group, in which the animals were provided with the basal diet supplemented with 0.08 g of ANE per kg of basal diet (Wang et al., 2023). The composition and nutritional level of the basal diet are shown in Table 1. The feeding trial lasted for 28 days. All the Jiaji ducks used in the experiment had free access to feed and water and received routine immunization. At the end of the experiment, 5 mL of fasting blood was taken from the wing veins of 2 Jiaji ducks from each replicate with body weights close to the average body weight. The collected blood was placed in a centrifuge tube, left to stand for 30 min, and centrifuged at 3,000 rpm for 5 min, and the plasma was prepared and stored at −20 °C for metabolomic analysis. In addition, 2 duck from each replicate in treatment group (with a body weight close to the average body weight of the treatment group) was used to determine the slaughter rate, semi-evisceration rate, full-evisceration rate, leg muscle yield, breast muscle yield, abdominal fat yield, and skin fat yield. Then, the cecum of each selected duck was immediately ligated with a sterile suture to ensure sufficient cecal contents, and the ligated cecal segments were removed. Simultaneously, spleen tissue was collected from each duck. The collected samples were labeled and immediately frozen in liquid nitrogen and then stored at −80 °C. Eight samples of cecum contents were used for macrogenomic analysis and spleen tissue for proteomic analysis.

Untargeted metabolomic analysis

Eight serum samples from each of the DCK and DNT groups were used for untargeted metabolomics analysis. Liquid chromatography-mass spectrometry was conducted using the Thermo Fisher Scientific Vanquish Flex UHPLC system (Thermo Fisher, USA) coupled with a Q-Exactive high-resolution tandem mass spectrometer (Thermo Fisher) (Cao et al., 2020). Chromatographic separation was performed on an

Table 1
Composition and nutrient levels of basal diet (%, air-dry basis).

Items	Content
Ingredients	
Corn	55.40
Soybean meal	23.00
Rice bran	5.00
Corn gluten meal	1.60
Sesame meal	3.00
wheat shorts	2.00
Limestone	5.80
Fine stone	2.00
Vitamin premix[1]	1.40
Calcium hydrogen phosphate	0.80
Nutrient levels	
Metabolic energy (Mcal/kg)	2.60
Crude protein	17.06
Crude fat	2.61
Ca	4.35
Lysine	0.89
Methionine	0.26
Methionine + Cystine	0.53
Available phosphorus	0.45

[1] Provided per kg of feed: Vitamin A, 10,000 IU; Vitamin D, 32,500 IU; Vitamin E, 30 mg; Vitamin B_1, 1 mg; Vitamin B_2, 4 mg; Vitamin B_6, 3 mg; Vitamin B_{12}, 15 mg; Pantothenic acid, 8 mg; Niacin 30 mg; Folic acid, 0.5 mg; Biotin 25 μg; Fe, 25 mg; Cu, 5 mg; Mn, 100 mg; Zn, 60 mg; Se, 0.2 mg; I, 0.5 mg; Co, 0.1 mg.

ACQUITY UPLC T3 column with a column temperature of 35 °C. The mass spectrometer was operated in positive and negative ion modes. Precursor ion spectra were collected from *m/z* 70 to −1,050 at a resolution of 70,000, with the automatic gain control (**AGC**) target set at 3e6. Fragment ion spectra were collected at a resolution of 17,500 and an AGC target of 1e5. To evaluate system stability, 1 QC sample was applied for every 10 analytical samples analyzed. The preprocessing of LC-MS data was performed as previously described (Pu et al., 2021). Data processing was performed with XCMS software and included peak identification, grouping, retention time correction, and annotation of isotopes and adducts. The MS data were transformed to mzXML format and processed using the XCMS, CAMERA, and metaX toolboxes in R software (Version 4.2.2). Metabolite identification was based on retention time, *m/z* data, and peak intensity. Kyoto Encyclopedia of Genes and Genomes (**KEGG**) (https://www.kegg.jp/) and the Human Metabolome Database (**HMDB**) (http://www.hmdb.ca/) were used for metabolite annotation with a mass difference threshold of 10 ppm. *T*-tests with Benjamini-Hochberg false discovery rate (**FDR**) correction were used to detect significant differences in measured concentrations. Partial least squares-discriminant analysis (**PLS-DA**) with a variable importance in projection (**VIP**) cutoff of 1 was employed to differentiate features between groups.

Cecal metagenomic analysis

Microbiome DNA was extracted from eight cecum samples from each of the DCK and DNT groups using the E.Z.N.A(Omega Bio-tek). Stool DNA Kit (D4015-02, Omega, Inc., Norcross, GA). DNA quality and quantity were assessed using the Qubit v.2.0 Fluorometer. Following random fragmentation of the genomic DNA, libraries were constructed using the TrueLib DNA Library Rapid Prep Kit for Illumina (ExCell Bio, Shanghai, China). After passing QC, the libraries were paired-end sequenced on the NovaSeq 6000 platform in PE15 mode. Trimmomatic (v0.36) was used to trim low-quality bases (quality score < 20) and remove short reads (< 50 bp). MEGAHIT (version 1.1.2) and QUAST (version 5.0.2) were used to assemble and evaluate filtered reads for each sample. MetaGeneMark (version 2) was used to predict coding regions from contigs longer than 500 bp. MMseqs (version 2) was used to cluster assembled contigs and build a non-redundant database (95 % identity) based on the same contigs. The predicted genes were aligned against the original sequences and abundance was estimated using DIAMOND (version 3). Subsequently, the taxonomic and functional annotation of gut metagenomes was performed using DIAMOND to classify the intestinal microbiota against the non-redundant protein sequence database. Classification analysis was conducted at the phylum, class, order, family, genus, and species levels and relative abundances were calculated at each level. KEGG (2017-03) annotation was performed using DIAMOND.

Proteomic analysis and quantification of TMT-labeled proteins in Jiaji duck spleen tissue

The proteomic profiles of three spleen samples from each of the DCK and DNT groups were analysed using the TMT labeling method. First, the spleen samples were weighed and transferred to centrifuge tubes, followed by the addition of urea lysis buffer containing Tris-HCl and the Roche mixture. The tubes were then placed on ice for 10 min and homogenized using a tissue homogenizer. After centrifugation, dithiothreitol was added to the collected supernatants, and the solution was incubated at 37 °C for 1 h. Finally, iodoacetamide was added to the samples, followed by incubation in the dark for 30 min. After the determination of protein concentrations using the Bradford method, proteins were separated using SDS-PAGE. For TMT labeling, TMTpro-16plex labels (Cat. No. A44521, Thermo Fisher) were separately dissolved and added to each sample at specific ratios. The labeled peptides of each sample were mixed and diluted. After fractionation and freeze-drying, the peptide samples were reconstituted in 0.1 % formic acid and centrifuged, and the supernatants were injected into an Agilent ZORBAX 300Extend-C18 column (Agilent Technologies, Santa Clara, CA, USA) for separation. The separated peptides were ionized and transferred to the mass spectrometer for detection. MaxQuant (v2.0.1.0) software was used for data analysis. Protein sequences were searched against the UniProt database and contaminants or reverse sequences were removed. The Student's *t*-test was used to assess the significance of differences between groups. Proteins with fold changes in abundance > 1.2 or < 0.8 and *P*-values < 0.05 were considered to be differentially expressed. Gene Ontology (**GO**) mapping and annotation were performed using the NCBI database (www.ncbi.nlm.nih.gov) and Blast2GO software (v6.0). KEGG pathway enrichment analysis was conducted for the identification of relevant biological pathways.

Data processing

All data were analyzed using SPSS 25.0 (IBM, NY). The effects of dietary ANE addition on the slaughter performance of Jiaji ducks were evaluated using two-tailed unpaired Student's *t*-test. The data are presented as means ± SE, with $P < 0.05$ indicating statistical significance.

Results

The content of arecoline and polyphenols in ANE

HPLC and ELISA analysis showed that the ANE contained arecoline, at a determined concentration of 8.75 mg/g, and polyphenols, at a concentration of 8.86 mg/g.

The effects of adding ANE to feed on the slaughter performance of Jiaji ducks

The effects of ANE supplementation on the slaughter performance of Jiaji ducks are shown in Table 2. The results showed that the addition of ANE to the diet significantly increased the slaughter rate of Jiaji ducks ($P < 0.05$).

Table 2
The effect of ANE on the slaughter performances of Jiaji duck.

Items	Addition level of betel nut extract (g/kg)		
	DCK	DNT	*P*-value
Carcass Weight(g)	1942.50 ±53.44	2076.00 ±30.43	0.398
Live Weight Before Slaughter (g)	2147.50 ±70.87	2312.00 ±46.30	0.356
Semi-Eviscerated Carcass Weight (g)	1815.00 ±32.79	1928.00 ±21.54	0.547
Fully-Eviscerated Carcass Weight (g)	1642.50 ±41.71	1716.00 ±16.00	0.219
Leg Muscle Weight (g)	91.47±9.34	88.64±7.46	0.686
Breast Muscle Weight (g)	129.65±4.48	133.94±7.11	0.492
Abdominal Fat Weight (g)	53.73±18.05	84.56±9.35	0.371
Skin Fat Weight (g)	354.38±47.02	413.40±30.27	0.581
Slaughter rate(%)	90.51±1.61	93.58±1.99	0.042
Half-dressed carcass rate(%)	84.65±3.05	83.46±2.09	0.506
Fully-dressed carcass rate(%)	76.56±2.37	74.30±2.46	0.207
Leg muscle rate(%)	5.56±1.03	5.16±0.91	0.558
Breast muscle rate(%)	7.91±0.65	7.80±0.87	0.847
Abdominal fat rate(%)	3.16±2.14	4.68±1.08	0.204
Skin fat rate (%)	21.44±4.65	24.11±4.05	0.388

Values are presented as the mean ± SE; n = 10.
DCK, DCK group; DNT, basal diet + 0.08 g/kg ANE. Lowercase letters in superscript indicate significant differences ($P < 0.05$)

Metabolic differences between the DCK and the DNT groups

The metabolites detected in this experiment were mainly classified as alkaloids and their derivatives, benzene ring compounds, hydrocarbons, lipids and lipid-like molecules, nucleotides and analogs, and organic acids and their derivatives. PLS-DA model score plot showed a clear separation between the DCK and the DNT groups, indicating that the model could effectively explain and predict the differences between the samples of the 2 groups (Fig. 1). To determine the expression patterns of the differential metabolites in the different samples, hierarchical clustering was performed based on the expression levels of the differential metabolites. The criteria for selecting significantly different metabolites were VIP > 1 and $P < 0.05$. A total of 117 differential metabolites were detected, including 63 up-regulated and 54 down-regulated metabolites (Table S1). Deviation plot analysis showed that, compared with the DCK group, the DNT group showed significant downregulation of differential metabolites such as lyso PG 16:1, lyso PE 21:1, propachlor ESA, and 8-isoprostane E1, and significant upregulation of metabolites such as ganoderic acid A and hydrocinnamic acid (Fig. 2). The results of the metabolic pathway showed that these differential metabolites were mainly involved in arachidonic acid metabolism, phospholipase D signaling pathway and eicosanoids (Fig. 3).

Differences in the cecal microbiome between the DCK and DNT groups

After quality control and data filtering, a total of 91.84 Gb of clean metagenomic data was obtained, representing 1,063,285 genes. The raw data of the macrogenome was submitted to the SRA database and obtained the accession number PRJNA1221121. The result showed that 53,391 genes were unique to the DCK group, whereas 23,979 genes were unique to the DNT group. Analysis of α-diversity using indices such as Chao1 and Simpson showed that there were no significant differences in microbial diversity between the DCK and the DNT groups (Table 3). Further analysis of the community structure at the phylum level revealed that Bacteroidetes, Firmicutes, and Proteobacteria were the dominant phyla in both groups (Table S2). Compared with the DCK group, the relative abundance of Bacteroidetes and Firmicutes showed a decreasing trend in the DNT group, resulting in a lower Bacteroidetes to Firmicutes ratio. The proportion of Proteobacteria also decreased in the DNT group. Additionally, the relative abundance of Candidatus Jorgensenbacteria and Candidatus Diapherotrites was significantly lower in the DNT samples than in the DCK samples ($P < 0.05$), but that of Candidatus Kaiserbacteria was significantly higher ($P < 0.05$) (Fig. 4 A). At the genus level, *Bacteroides* and *Oscillibacter*, both belonging to the phylum Bacteroidetes, were the dominant genera in the DCK and DNT groups (Table S3). Relative to the DCK group, the proportion of *Bacteroides* was decreased in the DNT group, whereas that of *Oscillibacter* was increased. The relative abundance of *Methanocorpusculum* was significantly decreased in DNT group (Fig. 4 B). At the species level, we selected the 20 species with the largest differences in relative abundance and visualized their abundance across the different groups using box plots. Among these 20 species, the abundance of uncultured *Bacteroides* sp and *Methanocorpusculum labreanum* was significantly downregulated in the cecal microbiota of Jiaji ducks fed with ANE compared with that in DCK Jiaji ducks (Supplementary Figure).

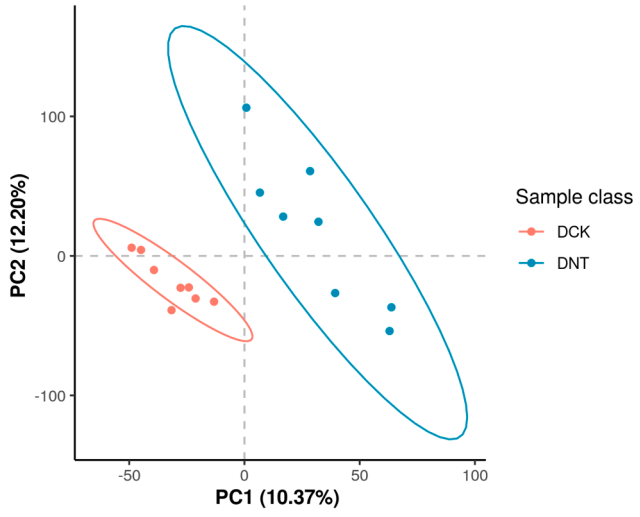

Fig. 1. Partial least-squares-discriminant analysis (PLS-DA) for DCK and DNT ducks.

Correlation between microbial communities and serum metabolites

Next, to determine the relationship between the differential serum metabolites and gut microbial species, we performed a Spearman's correlation analysis between the significantly differential metabolites identified based on the metabolomics data and the species displaying differential abundance identified in the metagenomic sequencing analysis. The results showed that *Odoribacter splanchnicus* CAG:14 was positively correlated with hydrocinnamic acid and *Xenorhabdus nematophila* and *Sphingomonas* sp. 2SG showed a negative correlation with hydrocinnamic acid. *Ralstonia* virus RSL2s and *Stenotrophomonas* phage YB07 showed a positive correlation with lyso PE 21:1, whereas *Methanosphaera stadtmanae* demonstrated negative correlations with lyso PG 16:1 and lyso PE 21:1. *Pseudomonas otitidis* and *Prevotella* sp. P5-60s were negatively correlated with both hydrocinnamic acid and Ganoderic acid A. *Sphingomonas* sp. 2SG showed a negative correlation with hydrocinnamic acid and a positive correlation with lyso PG 16:1 (Fig. 5).

Quantitative proteomic analysis of TMT-labeled spleen proteins

To elucidate the mechanism by which the dietary addition of ANE affects the immune performance of Jiaji ducks at the protein level, we conducted a quantitative proteomic analysis of TMT-labeled proteins from the spleen tissue of ducks from both the DCK and DNT groups and identified a total of 4,817 proteins with high confidence. Using fold change > 1.2 and a P-value < 0.05 as screening criteria, we identified a total of 78 differentially expressed proteins, including 54 proteins that were upregulated and 24 proteins that were downregulated in the DNT group (Table S4). GO functional enrichment analysis showed that the differential proteins in the spleen were mostly associated with cellular components such as focal adhesion and biological processes such as cell matrix adhesion (Fig. 6 A). KEGG enrichment analysis showed that the differentially expressed proteins were mainly enriched in the immune networks of cell adhesion molecules and Glutathione metabolism (Fig. 6 B).

Discussion

Metabolic profiling data showed that the hydrocinnamic acid and ganoderic acid A were significantly upregulated with dietary ANE supplementation, whereas lyso PG 16:1, Lyso PE 21:1, 8-iso-prostaglandin E1, and propachlor ESA were significantly downregulated. Ma et al. (2021) found that ganoderic acid A can alleviate carbon tetrachloride-induced renal inflammation and oxidative stress in mice. Meanwhile, Lu et al. (2021) investigated the effects of ganoderic acid A on ovalbumin-induced asthma in mice and found that the inflammatory cell count and the expression of IL-4, IL-5, and IL-13 were lower in the ganoderic acid A treatment group than in the model group. The authors further reported that ganoderic acid A ameliorated asthma-induced pulmonary inflammation by inhibiting the TLR/NF-kB signaling pathway. Hydroxycinnamic acid, a phenolic acid, is the main product of phenylalanine metabolism (Xu et al., 2022). Studies have shown that hydroxycinnamic acid has inhibitory effects on *E. coli, Salmonella, Pseudomonas aeruginosa*, and *Clostridium difficile* (Cueva et al., 2010). It

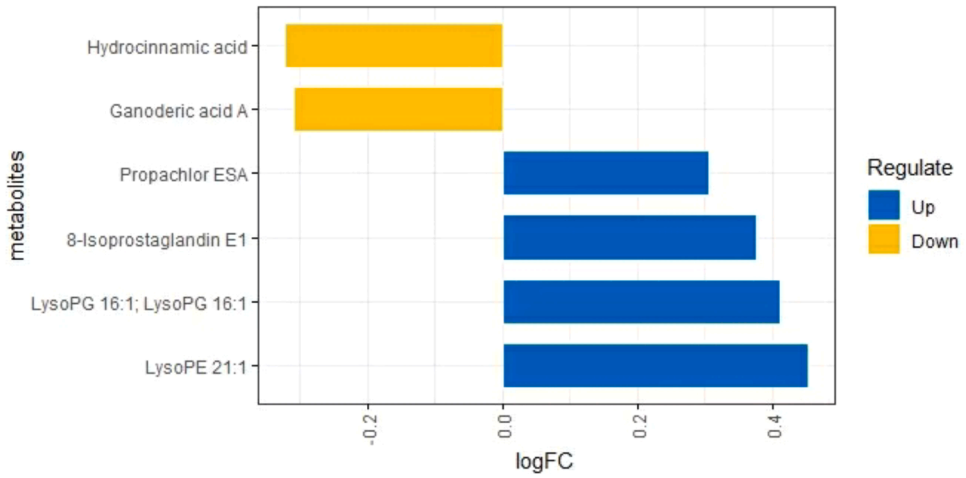

Fig. 2. Differential metabolite deviation plot between DNT and DCK ducks.

Fig. 3. KEGG enrichment analysis of differential metabolites.

Table 3
Effects of ANT on alpha diversity index of gut microbiota in Jiaji ducks.

Items	DCK	DNT	*P*-value
Observed_species	7958.75±714.96	7767.13±856.66	0.96
Shannon	7.58±0.29	7.57±0.40	0.96
Simpson	0.97±0.01	0.97±0.01	0.88
Chao1	9469.17±915.24	8965.77±1241.50	0.44
Goods_coverage	0.9987±0.00	0.9989±0.00	0.13

Values are presented as the mean ± SE; n = 8.

has also been reported that hydroxycinnamic acid can inhibit the NLR family pyrin domain containing 3 (NLRP3) inflammasome signaling pathway, downregulate the MAPK and NF-κB signaling pathways in rats (Abozaid et al., 2020). Here, we found that these 2 differential metabolites were significantly upregulated in the ANE group. This indicated that the extract can enhance the levels of anti-inflammatory substances in Jiaji ducks, thereby enhancing their anti-inflammatory and antimicrobial abilities through the phenylalanine metabolic pathway. Meanwhile, lyso PG 16:1 and lyso PE 21:1 were found to be significantly downregulated in the DNT group. Both metabolites are glycerophospholipids and disturbances in glycerophospholipid metabolism are typically associated with inflammation, oxidative stress, and the induction of cell apoptosis (Knuplez and Marsche, 2020). Studies have shown that lyso PG is a pro-inflammatory factor that induces inflammation, activate macrophages and T lymphocytes, and alter the permeability of vascular smooth muscle. Lyso PG can also activate intracellular protein kinase C, leading to an increase in the levels of

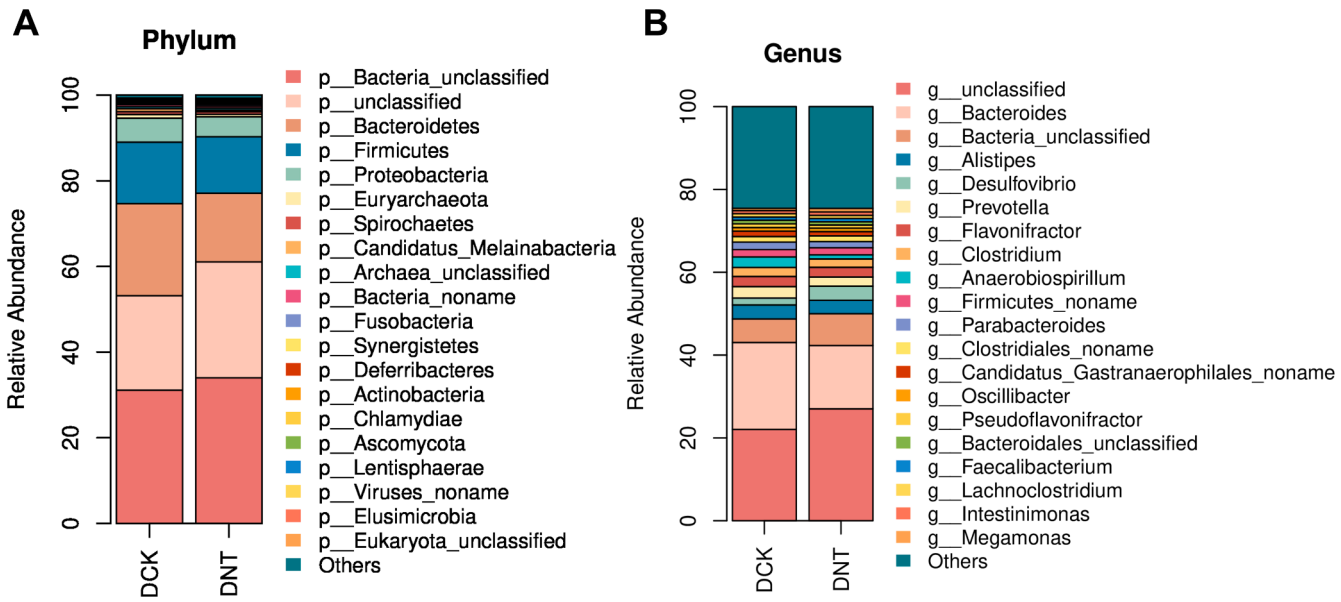

Fig. 4. Relative abundance of the top phyla (A) and genus (B) in the samples.

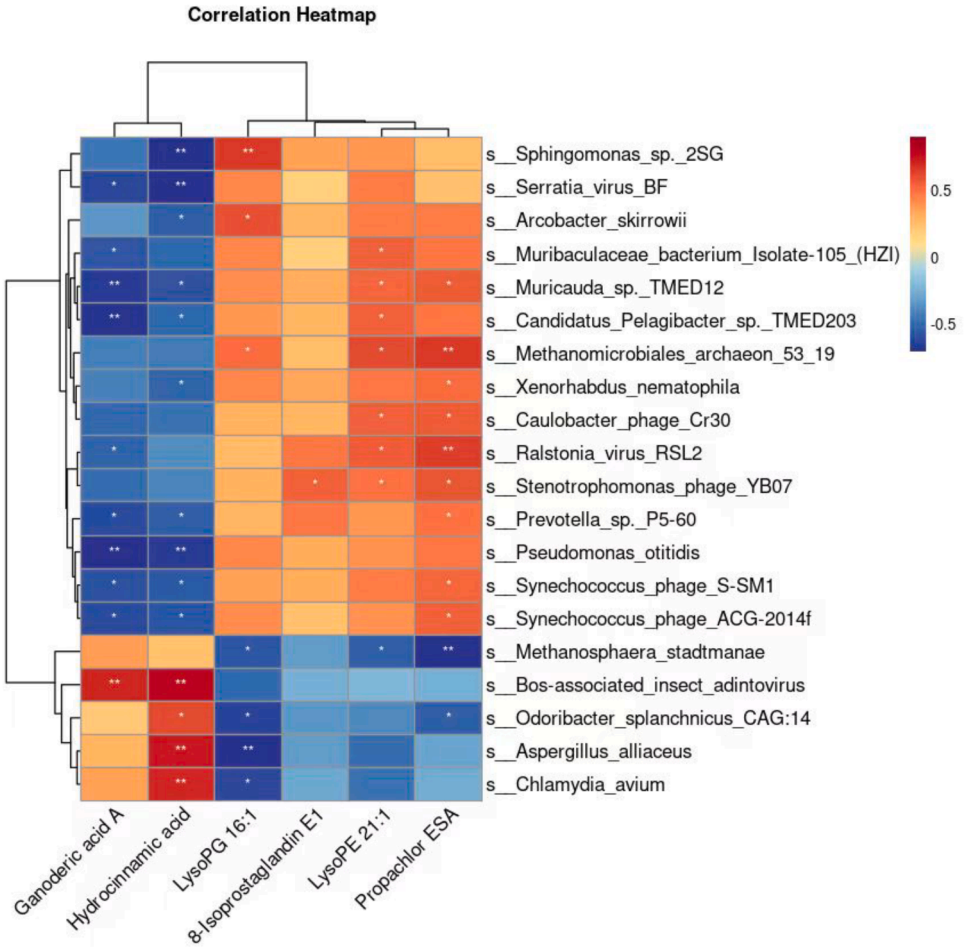

Fig. 5. Pearson's correlation analysis between cecal species and metabolites.
Note:One asterisk indicates a significant Pearson's correlation and 2 asterisks indicate a highly significant Pearson's correlation.

oxygen free radicals and the induction of inflammatory reactions in myocardial vascular cells. In addition, evidence suggests that, in mice, lyso PE is positively correlated with cytokines and oxidative stress markers, and negatively correlated with antioxidant enzymes and anti-inflammatory cytokines. 8-Iso-prostaglandin E1 is a metabolite of arachidonic acid that is mainly generated through oxygen free

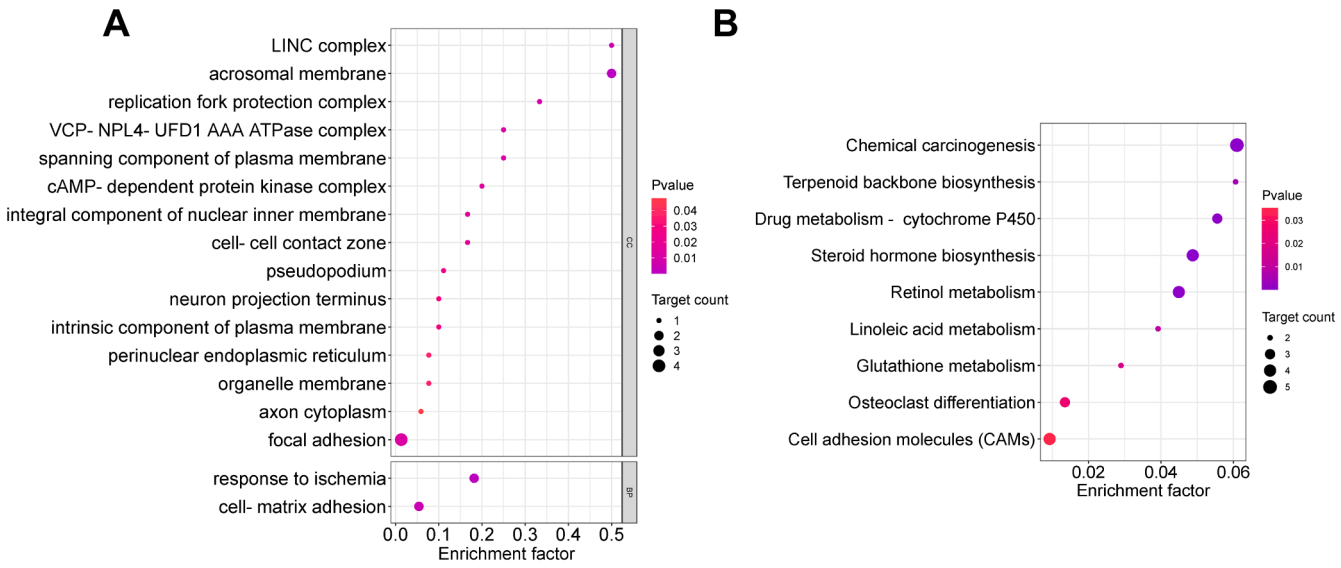

Fig. 6. Differentially expressed proteins enrichment analysis. (A) GO annotation enrichment analysis. (B) KEGG pathway enrichment analysis.

radical-mediated lipid peroxidation and is often used as a marker of lipid peroxidation-induced oxidative stress levels. In summary, the addition of ANE to the diet of Jiaji ducks can alleviate oxidative stress and inflammatory responses, enhance the anti-inflammatory and antimicrobial abilities of the birds, and improve their immune performance by regulating arachidonic acid metabolic pathway.

The health and productivity of ducks are closely related to their gastrointestinal microbiota (Dai et al., 2018). Indeed, the gastrointestinal tract of animals is colonized by a large number of bacterial species that have been shown to play crucial roles in digestion, immune regulation, pathogen exclusion, and endocrine activities (Li et al., 2017). Disruption of the gut microbiota in poultry can increase their susceptibility to pathogens and infectious diseases (Corrigan et al., 2015). In this study, the dietary inclusion of ANE reduced the number of genes detected in the intestinal microbial community of the ducks, possibly due to the observed decrease in the relative abundance of the phylum Bacteroidetes. Additionally, our findings revealed that Bacteroidetes, Firmicutes, and Proteobacteria were the dominant phyla in the intestinal microbiota of Jiaji ducks from the DCK and DNT groups, which is consistent with the results of the study by Chang et al. (2024). Several studies have indicated that members of the phylum Bacteroidetes play a role in the normal development of the gastrointestinal tract (Guan et al., 2024) and influence the health of their hosts by enhancing intestinal barrier function (Yang et al., 2024).

An increasing body of evidence indicates that alterations in microbial communities and metabolic products are closely associated with organismal health and growth. We identified the 20 genera and species of microorganisms exhibiting the greatest differences in relative abundance between the DNT and DCK groups and analyzed the correlation between the differential metabolites and these microbes. The correlation analysis revealed that *Prevotella* was negatively correlated with hydrocinnamic acid and ganoderic acid A. It has been found that *Prevotella* can enhance inflammatory responses by promoting the release of inflammatory mediators by immune cells and a variety of stromal cells, making it an important pathogenic factor in chronic inflammation (Larsen, 2017). Guo et al. (2020) found that intervention with ganoderic acid A helps to positively regulate the composition of the gut microbial ecosystem primarily by contributing to the adjustment of the relative abundance of certain beneficial microbial groups, thus alleviating gut dysbiosis and disordered lipid metabolism. *Xenorhabdus nematophila* and *Sphingomonas* exhibited a negative correlation with hydrocinnamic acid. *Xenorhabdus nematophila* is a member of the Enterobacteriaceae family. It can kill a variety of insects by strongly inhibiting their immune system and colonizing their entire bodies (Vigneux et al., 2007). *Ralstonia* spp are non-fermenting, Gram-negative rods that fall within the category of human opportunistic pathogens (Shi et al., 2019). Here, we observed that ANE reduced *Ralstonia* abundance, suggesting that this extract can downregulate the levels of pathogenic bacteria in Jiaji ducks, thus enhancing their disease resistance. *Pseudomonas* and *Shewanella* showed a negative correlation with hydrocinnamic acid. Guo et al. (2021) found that the enrichment of certain gut microbial genera, including *Pseudomonas* and *Shewanella*, showed positive associations with oncogenic processes, such as KRAS and MAPK signaling and epithelial-mesenchymal transition. *Stenotrophomonas* displayed a positive correlation with lyso PE 21:1. It has been reported that *Stenotrophomonas* can induce T-cell exhaustion by activating the PD-1/PDL1 signaling pathway, thereby suppressing the host T cell immune response (Wang et al., 2021). Meanwhile, *Odoribacter* showed a positive correlation with hydrocinnamic acid. Members of this genus are beneficial bacteria and can produce butyric acid, regulate the immune system, and increase the production of mucin and tight junction proteins, thereby improving intestinal barrier function. These findings indicated that ANE supplementation can improve the intestinal microbial community structure of Jiaji ducks by increasing the abundance of beneficial microbes in their intestines and exerting inhibitory effects on the growth and proliferation of harmful microbial communities. In summary, we undertook a comprehensive analysis of the metabolites and intestinal microbial communities in Jiaji ducks and found that ANE can modulate intestinal microbial communities such as *Prevotella, Pseudomonas*, and *Sphingomonas* and alter the abundance of relevant host metabolites, thereby exerting anti-inflammatory and antibacterial effects and improving lipid metabolism disorders.

The dietary inclusion of ANE resulted in the upregulation of several proteins, including GTP-binding nuclear protein Ran, glutaminase 2, and colony-stimulating factor-1 receptor, which are involved in immune responses. GTP-binding nuclear protein Ran, encoded by the RAN gene, plays a role in the immune response to viral infection in shrimp (Han and Zhang, 2007). Glutaminase 2 is a ubiquitously expressed multifunctional enzyme involved in many physiological processes, including apoptosis, signal transduction, and cell adhesion (Deguchi-Horiuchi et al., 2023). The main function of colony-stimulating factor-1 is to stimulate the differentiation of mononuclear phagocyte lineage cells into adherent, motile, and mature macrophages. In brief, these findings suggest that ANE can impact the immune function of Jiaji ducks by increasing the expression of immune-related proteins in the spleen, thereby enhancing immune resistance in the birds.

Disclosure statement

No conflict of interest exits in this manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.104971.

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