Transcriptome and metabolome analyses reveal the regulatory effects of compound probiotics on cecal metabolism in heat-stressed broilers

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ABSTRACT The effect of compound probiotics on the caecum of broilers under heat stress was assessed in this study. A total of 400 twenty-eight-day-old AA male broilers were randomly divided into 4 treatment groups, where each group had 5 replicates of 20 broilers. The 4 treatment groups were a heat stress control group (broilers receiving a normal diet) and groups HP I, HP II, and HP Ⅲ, consisting of broilers receiving 1, 5, and 10 g of compound probiotics added to each kilogram of feed, respectively. Compound probiotics (*L. casei, L. acidophilus*, and *B. lactis* at a ratio of 1:1:2) were used to formulate a compound probiotic powder, with 1×10^{10} CFU/g of effective viable bacteria. Heat stress treatment was performed at 32 ± 1°C from 9:00 to 17:00 every day from 28 d to 42 d. In d 28 to 42, compared with the HC group, the ADG of broilers in the HP II and III groups was significantly increased ($P < 0.05$); the ADFI difference between groups was not significant ($P > 0.05$); the FCR of HP II and III broilers was significantly decreased ($P < 0.05$); and the FCR of the HP I group increased, but the difference was not significant ($P > 0.05$). Transcriptome results demonstrate that 665 differential genes were screened (DEGs; upregulated: 366, downregulated: 299). The DEGs were enriched in the B cell receptor signaling pathway, the intestinal immune network for IgA synthesis, the Fc epsilon RI signaling pathway, and other signaling pathways, according to KEGG enrichment analysis. Metabolome analysis identified 92 differential metabolites (DAMs; upregulated: 48, downregulated: 44). KEGG enrichment analysis indicated significant enrichment of Pantothenate and CoA biosynthesis and beta-Alanine metabolism. The combined transcriptome and metabolome analysis revealed that the DAMs and DEGs were mostly involved in beta-alanine metabolism, arginine biosynthesis, amino sugar and nucleotide sugar, and alanine, aspartate, and glutamate metabolism. The results of this study suggest that the addition of compound probiotics has a positive effect on intestinal metabolites, improving the growth performance and contributing to the overall health of broilers under heat stress.

Key words: probiotics, transcriptome, metabolome, cecum, heat stress

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INTRODUCTION

Since the 1950s, significant progress has been made in adding antibiotics to poultry feed. Antibiotics are the most extensively utilized additions to increase traditional commercial chicken production, improving nutrient utilization, rate of growth, and poultry health (Gadde et al., 2017; Callaway et al., 2021). Yet, antibiotic residues and animal food safety issues have become a growing problem. Due to public concerns regarding the use of antibiotics in poultry feed (Hoelzer et al., 2017), as a consequence, the use of antibiotics in animal farming has been outlawed in several nations. Antibiotics have been banned in the E.U., resulting in a rise in broiler disease outbreaks in European countries. Prophylactic use of all antibiotics in food animals has been banned in China since the end of 2020 (Qiao et al., 2020). For the prevention of infectious illness in broilers, the poultry industry requires alternative substances to minimize or replace antibiotic growth boosters. Probiotics, prebiotics, and phytochemicals have gained popularity for this reason, and have been widely used to replace antibiotics (Seifi et al., 2013; Lin et al., 2019; Chen and Yu, 2020).

Probiotics are described as: "Single or mixed cultures of live microorganisms that, when applied to animals or humans, have a beneficial effect on the host by improving the properties of the native gastrointestinal microbiota" (Al-Shawi et al., 2020). Competitive exclusion and immunological modulation are 2 primary strategies

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by which probiotics maintain healthy bacteria populations. Competitive exclusion involves competition for substrates, generation of pathogen-inhibiting antimicrobial metabolites, and competition for attachment sites. As such, the possible mechanisms of action of probiotics are competition between native organisms and pathogens for adhesion receptors in the intestinal epithelium, competition for available nutrients, the establishment of environmental conditions by lowering pH, direct antibacterial effects by releasing antibacterial substances and neutralizing toxins, clustering with pathogenic bacteria, and activating the immune system (Jin et al., 1997; Simon et al., 2001; Edens, 2003).

Heat stress involves the accumulation of a number of nonspecific defensive responses generated by an animal's body in response to an increase in the temperature of its development environment (Esam et al., 2022). Research institutes have suggested that future temperature increases on Earth's surface may range from 0.3 to 4.8°C by 2100; this could affect poultry farming, through changes in forage crops and fodder, biodiversity, animal welfare, productivity, and reproductive characteristics (Stocker et al., 2014). Heat stress seems to already have become a problem for the poultry production business as a result of rising global temperatures, particularly in tropical and subtropical areas (Gregory, 2010). Due to their incapacity to shed body heat owing to their feather covering and small sweat glands, broilers are the most vulnerable to HS (Zhang et al., 2017). Broilers experiencing HS suffer from a variety of physiological challenges, including systemic immunological disorders, endocrine abnormalities, pulmonary alkalosis, and electrolyte imbalances, all of which influence their health and performance (Lara and Rostagno, 2013). Heat stress in broilers may result in yield reductions and, in the worst-case scenario, death (Rath et al., 2015). With the recent rapid development of omics, multiomics approaches have been widely used in scientific research to reveal biological processes. Metabolomics involves quantitative analysis of the overall changes of metabolites in a certain period, which can reveal whether metabolites are linked to physiological or pathological changes, as well as reflecting the metabolic state of biological systems (Wishart, 2019). The metabolome is made up of tiny molecules (typically less than 1,500 Da) with various polarities and structural variations (Schrimpe-Rutledge et al., 2016). RNA-Seq analysis can quantify gene expression levels, and has been widely used to elucidate the molecular mechanisms underlying complex traits in organisms. The rapid sequencing, high sensitivity, and cost-effectiveness of RNA-Seq help to lay the foundation for gene regulation research (Haas and Zody 2010; Mishima et al., 2014; Yang et al., 2020). Therefore, RNA-Seq provides technical support for biological research (Zhai et al., 2020).

The cecum has the highest density of bacterial flora in broilers, and plays a crucial role in nutrient absorption in poultry. The effect of adding multiple compound probiotics is typically higher than that of single probiotics, and the effect of using different probiotic combinations also differs (Luo et al., 2021). Therefore, for this study, RNA-Seq and LC-MS techniques were used to analyse the cecum of broilers fed compound probiotics and kept under heat stress conditions; furthermore, a combined analysis of these 2 techniques was also performed. Our research reveals the impact of compound probiotics on the intestinal metabolism of heat-stressed broilers, in terms of both metabolic and molecular pathways, laying the groundwork for future research on the impact of probiotics on the intestinal metabolism of heat-stressed broilers.

MATERIALS AND METHODS

Test Materials

Shanxi Agricultural University's Animal Care and Use Committee approved the study protocol (registered number, SXAU-EAW-2021C0630). Broilers were euthanized with intraperitoneal injection of sodium pentobarbital (100−200 mg/kg). The test strains (*Lactobacillus casei, L. acidophilus*, and *Bifidobacterium lactis*) were purchased from Shanghai Danisco Additives Co., Ltd. The strain numbers were LC-12, JYLA-16, and JYBR-190, respectively, and the density of viable probiotics was 1×10^{10} CFU/g. Compound probiotics (*L. casei*, *L. acidophilus*, and *B. lactis,* in the ratio of 1:1:2) were used to formulate a compound probiotic powder.

Experimental Design and Animal Feeding

A total of 500 one-day-old AA male broilers were selected and raised to 28 d of age, and 400 healthy broilers of similar weight were randomly divided into 4 treatment groups, with 5 replicates in each group, with 20 broilers in each replicate. The 4 treatment groups were a heat stress control group (broilers receiving a normal diet) and groups HP I, HP II, and HP Ⅲ, consisting of broilers receiving 1, 5, and 10 g compound probiotics added to each kilogram of feed, respectively. All groups were fed a basic diet, with nutritional components as shown in Table 1. The feeding period was divided into 2 stages: 1−21 and 22−42 days.

The broilers were housed in 3-layer vertical cages, which were disinfected regularly. Feed was provided at 8:00 am and 8:00 pm every day, and drinking water was changed every 8 h. Continuous light was provided for 23 h, with a dark interval of 1 h, in order to ensure that the broilers fed and drank freely. During the first week of brooding, the temperature was maintained at 35℃, and then gradually decreased to 23℃ by the end of the experiment. The food intake and health status of the broilers were recorded daily. When the broilers grew to 28 d of age, all groups were treated with heat stress; that is, from 28 d to 42 d, the temperature of the chicken house was increased to 32 ± 1°C from 9:00 to 17:00 every day (i.e., the heat stress treatment was performed for 8 h). The temperature was 23 ± 1°C the rest of the time, and the test period was 14 d.

Table 1. Composition and nutrient levels of the basic diet.

Item	1−21 d	22−42 d
Diet composition (%)		
Corn	56.49	61.42
Soybean oil	2.22	3.00
Soybean meal	30.24	25.30
Cotton seed meal	5.00	5.00
Fishmeal	2.43	1.98
$CaHCO_3$	1.60	1.39
Limestone	1.16	1.10
Methionine	0.15	0.05
NaCl	0.30	0.35
Choline	0.19	0.19
Premix[1]	0.22	0.22
Nutrient[2] (%)		
ME ($MJ \cdot kg^{-1}$)	12.12	12.54
Crude protein	21.00	19.00
Lysine	1.12	0.98
Methionine + Cystine	0.84	0.68
Calcium	1.00	0.90
Available phosphorus	0.30	0.30

[1]The premix contained 0.2% trace elements and provided the following nutrients per kilogram feed: Fe 80 mg, Mn 80 mg, Zn 80 mg, I 0.35 mg, and Se 0.15 mg. The premix contained 0.02% vitamins per kilogram feed: vitamin D_3 3,000.00 IU, vitamin E 30.00 IU, vitamin K_3 1.00 mg, vitamin B_1 2.00 mg, vitamin B_2 6.00 mg, pantothenic acid 9.00 mg, pyridoxine 5.00 mg, niacin 30.00 mg, vitamin B_{12} 0.01 mg, biotin 0.10 mg, and folic acid 0.30 mg.

[2]Nutrition level is a calculated value.

Determination of Growth Performance

The broilers were weighed at the end of the feeding period, and were prevented from eating for 10 h but could drink water freely before weighing. The growth data were recorded, and the average daily gain (**ADG**), average daily feed intake (**ADFI**), and feed conversion rate (FCR = ADFI/ADG) of the broilers were calculated.

Sample Collection

At the age of 42 d, the broilers were slaughtered and dissected, following which the cecum was cut and rinsed with pre-cooled normal saline. A 2 cm^2 sample of cecum tissue was cut and stored in liquid nitrogen for subsequent analysis. The cecal samples of the HC and HPⅢ groups were selected and sent to Shanghai Majorbio Bio-pharm Technology Co., Ltd. for transcriptome sequencing and LC-MS non-targeted metabolome analysis.

Transcriptome Data Analysis and Validation

Extraction of Total RNA From Transcriptome Samples Total RNA was extracted from cecal tissue using Trizol reagent (Solarbio, Beijing, China), and the complete extraction process was performed on an ultra-clean workbench. The concentration, purity, and RNA integrity number value of total RNA were then detected using NanoDrop 2000 and Agilent 2100 bioanalyzers.

Construction of cDNA Library of Transcriptome Samples Using 1 μg of the qualified total RNA sample, mRNA was enriched with magnetic beads and broken into 300 bp fragments. Then, the mRNA fragments were reverse-transcribed into single-strand and double-strand cDNA and their ends were repaired. A tail was added at the 3′ end, followed by adapter ligation. The final cDNA library was obtained after PCR amplification and purification. The generated library was paired-end sequenced using an Illumina NovaSeq 6000 platform.

Transcriptome Sequencing Data Analysis and Quality Control By sequencing the cDNA library on the Illumina platform, the image data obtained through sequencing were stored in Fastq format as raw data. The raw reads of each sample were evaluated for sequencing-related quality and filtered to obtain high-quality clean data for subsequent analysis.

Alignment With the Reference Genome Using Ensembl as the reference genome, the obtained clean data were compared with the reference genome. These mapped data were then further analyzed.

Differentially Expressed Gene Screening and Functional Enrichment Differential gene expression analysis between sample groups was carried out using DESeq2. The differential expression fold (fold change, **FC**) of each gene was calculated, and the differentially expressed genes (**DEGs**) were screened by using $|\log_2 FC| \geq 1$ and $P < 0.05$ as the standard. GO and KEGG pathway enrichment analyses were conducted using the topGO R package and Orthology-Based Annotation System (**KOBAS**) software, respectively.

Real-Time PCR Validation To verify the reproducibility and accuracy of gene expression data in RNA-Seq of broiler control and probiotic groups, several genes (*LIPG, FABP1, CDCA9, IL-1β, ADIPOQ*) were selected for real-time quantitative PCR. The broiler *β-actin* gene was used as the internal reference gene, which was amplified along with the target gene. The primer information is provided in Table 2. The $2^{-\triangle\triangle CT}$ approach was used to calculate the relative expression levels of differential genes among the groups.

Metabolome Data Processing and Analysis

Metabolome Sample Processing Accurately weighed (50 mg) cecal material, 6 mm grinding beads, 400 mL of extraction solution (methanol:water = 4:1 v:v), and 0.02 mg/mL standard (L-2-chlorophenylalanine) were

Table 2. Primers for real-time quantitative PCR.

Gene	Primer sequence(5′-3′)	DNA fragment (bp)
LIPG	F: TGCTTGAGCAAATTGGCCTT R: GCTCCTTAGCTCCTTCCACA	140
FABP1	F: GAAGAGTGTGAGATGGAGCTGCTG R: GGTGATGGTGTCTCCGTTGAGTTC	132
CDCA9	F: AGTCGCTGGTTTCATTGAGC R: ATTTGTGGCACTGCAGTCAG	125
β-actin	F: GCCCTGGCACCTAGCACAATG R: CTCCTGCTTGCTGATCCACATCTG	129
IL-1β	F: TTCATCTTCTACCGCCTGGA R: AGCTTGTAGGTGGCGATGTT	155
ADIPOQ	F: GCTCAGTAGGCTTCCTCCTT R: CCATCCAGTTGGCACATGAC	108

added into a 2 mL centrifuge tube. After 6 min of grinding in a frozen tissue grinder (−10°C, 50 Hz), ultrasonic extraction was performed for 30 min at low temperature (5°C, 40 kHz). Then, the sample was held at −20°C for 30 min and centrifuged for 15 min (13,000 rpm, 4°C). The supernatant (20 μL) from each sample was pipetted into a sample vial with an inner cannula for computer analysis. Every 6 samples were interspersed with one QC sample injection. All samples were analyzed through LC-MS, and each group of samples included 6 biological replicates.

LC-MS Detection of Metabolites The UHPLC-Q Exactive system from Thermo Fisher Scientific was used for the LC-MS analysis.

The chromatographic conditions were as follows: An HSS T3 column (100 mm × 2.1 mm id, 1.8 μm) was used for chromatography. Mobile phase A was 95% water + 5% acetonitrile (containing 0.1% formic acid), and mobile phase B was 47.5% acetonitrile + 47.5% isopropyl alcohol + 5% water (with 0.1% formic acid). The flow rate was 0.40 mL/min, the injection volume was 2 μL, and the column temperature was 40°C.

Electrospraying was used to ionize the materials for mass spectrometry (**MS**), and positive and negative ion scanning modes were used to acquire the mass spectrum signals of the samples, with a mass scanning range of 70 to 1,050 m/z. The operating parameters used were as follows: positive ion voltage, 3500 V; negative ion voltage, 2,800 V; sheath gas, 40 psi; auxiliary heating gas, 10 psi; ion source heating temperature, 400°C; 20-40-60 V cyclic collision energy; primary MS (MS1) resolution, 70,000; and secondary MS (MS2) resolution, 17,500.

Data Pre-processing and Database Searching To reduce errors caused by sample preparation and instrument instability, the Progenesis QI software was used to process the raw data. Then, the response intensity of the sample mass spectrum peaks was normalized. QC samples with a relative standard deviation (**RSD**) greater than 30% were deleted. Logarithmic transformation of the variables were performed to obtain the final data matrix for subsequent analysis. Finally, the information from MS1 and MS2 were simultaneously combined with information from the metabolic public databases HMDB (http://www.hmdb.ca/) and Metlin (https://metlin.scripps.edu/), in order to obtain metabolite information.

Principal Component Analysis and Orthogonal Projections to Latent Structures Discriminant Analysis To understand the overall metabolic differences between samples in each group and those between samples within a group, principal component analysis (**PCA**) and orthogonal projections to latent structures discriminant analysis (**OPLS-DA**) were performed. Significantly different metabolites were initially selected on the basis of the variable importance in projection (**VIP**) and Student's *t* test *P*-values. Metabolites with VIP ≥ 1 and $P < 0.05$ were generally considered to significantly differ. Finally, through metabolic pathway annotation in the KEGG database (https://www.kegg.jp/kegg/pathway.html), the pathways in which differential metabolites were involved were obtained. KEGG pathways were considered significantly enriched for $P < 0.05$.

Statistical Analysis

After the preliminary arrangement of data in Microsoft Excel 2018, ANOVA was performed. Duncan's test was used to determine differences among treatments, using the SPSS 24.0 statistical software. The histogram was built using GraphPad Prism 8.

RESULTS

Growth Performance

The effect of heat stress on the growth performance of broiler chicks is shown in Table 3. At 28 to 42 d, compared with the control group, the ADG of broilers in HP II and III group was increased significantly ($P<0.05$), while the ADG of HP I group was increased, but the difference was not significant ($P>0.05$). There was no significant difference in ADFI among the groups ($P>0.05$). Compared with the control group, the FCR of broilers in HP II and III groups was decreased significantly ($P<0.05$), while the FCR of the HP I group increased, but the difference was not significant ($P>0.05$).

Transcriptome Analysis

Summary of the Raw Sequence Reads Table 4 summarizes the RNA-Seq results of the 6 cecal samples, including Raw and Clean reads. Q20 was greater than 97%, Q30 was greater than 93%, and the percentage of GC content was between 50.45 and 51.7%. No separation phenomena were observed, indicating that the quality of the sequencing data was high and met the sequencing requirements, such that the next step could be carried out.

Gene Differential Expression Analysis A total of 665 genes were differentially expressed with $|\log_2 FC| \geq 1$ at $P < 0.05$. Of these, 299 and 366 genes were downregulated and upregulated, respectively (Figure 1A).

Table 3. Growth performance of broilers under heat stress.

Index	Heat stress Control	Heat stress Probiotics Ⅰ	Heat stress Probiotics Ⅱ	Heat stress Probiotics Ⅲ	*P*-value
28~42 days					
ADG (g/bird)	73.46 ± 5.49[c]	78.68 ± 2.10[c]	84.98 ± 1.26[b]	92.45 ± 1.34[a]	0.001
ADFI (g/bird)	151.99 ± 18.2	153.74 ± 18.7	154.93 ± 11.7	167.92 ± 23.7	0.717
FCR(g/g)	2.08 ± 0.16[b]	1.95 ± 0.05[ab]	1.82 ± 0.03[a]	1.81 ± 0.03[a]	0.016

[abc]Different superscript letters in the same row indicate statistically significant differences ($P < 0.05$).

Table 4. Summary statistics for sequence quality and alignment information of 6 cecal samples in two groups.

Sample	HC1	HC2	HC3	HP1	HP2	HP3
Raw reads	46432436	45547564	45510350	46416688	46356152	43522658
Raw bases	7011297836	6877682164	6872062850	7008919888	6999778952	6571921358
Clean reads	45626058	44874338	44849600	45684628	45630120	42852980
Clean bases	6746702315	6630217774	6643740686	6737791062	6752081444	6331100604
Error rate	0.026	0.0257	0.0259	0.0258	0.0259	0.0256
Q20	97.45	97.6	97.53	97.53	97.52	97.61
Q30	93.36	93.66	93.46	93.52	93.5	93.72
GC content	51.7	51.07	50.53	50.45	50.9	51.24

Gene Ontology Annotation Analysis of DEGs The functionalities of DEGs were assessed using Gene Ontology (**GO**) analysis. The 3 components of GO are biological process (**BP**), cellular component (**CC**), and molecular function (**MF**). GO-BP including activation of immune response and B cell receptor signaling pathway were significantly enriched. The main cellular components enriched in GO-CC were the immunoglobulin complex and circulation. The molecular functions enriched in GO-MF included immunoglobulin receptor binding and antigen binding (Figure 1B).

KEGG Pathway Analysis of DEGs Figure 1C shows the significantly enriched pathways from the KEGG enrichment analysis. In total, 665 DEGs were subjected to KEGG enrichment analysis, and the top 20 significantly enriched KEGG pathways ($P < 0.05$) were selected for display. Among them, the B cell receptor signaling pathway, Intestinal immune network for IgA production, and Fc epsilon RI signaling pathway are related to immunity.

Confirmation of RNA-Seq Results Five DEGs (*LIPG, FABP1, CDCA9, IL-1β, ADIPOQ*) were randomly selected for qRT-PCR verification, in order to determine whether the transcriptome sequencing data were accurate. A high level of consistency was noted between the RNA-Seq and qPCR methodologies (Figure 2), confirming that the relative gene expression obtained by RNA-Seq was reliable.

Metabolome Analysis

PCA Analysis PCA analysis was carried out for 12 cecum samples from the heat stress control group and the heat stress probiotic group, using the non-targeted LC-MS metabolomic analysis method (Figure 3). The QC samples were concentratedly distributed in both positive ion mode (a) and negative ion mode (b), demonstrating that the analytical system was stable and the data were dependable enough to be used for further analysis.

OPLS-DA Analysis The metabolites were subjected to OPLS-DA analysis, and the HC group and the HP group were effectively divided into 2 categories in both positive ion mode and negative ion mode (Figure 4). It can be seen that the duplicate points of the 2 groups of samples were close to each other and that they were clustered into one category, indicating that the data repeatability is good. In order to avoid over-fitting, we also performed OPLS-DA model validation (replacement test, n = 200), and the R2 and Q2 intercepts in the positive and negative ion modes reached 0.9926 and −0.0439, and 0.9449 and 0.0304, respectively. In theory, the closer the R2 values are to 1, the more reliable the model is. Therefore, the results indicated that the 2 models had good stability and did not suffer from over-fitting, demonstrating that the material is sufficiently reproducible to be suitable for the following qualitative and quantitative assay validation.

Differential Metabolite Analysis A total of 1,934 metabolites with known structures were identified in this study in positive and negative ion mode. Differential metabolites were screened from all metabolites according to the VIP value (VIP > 1) of the OPLS-DA model and the P value ($P < 0.05$) of the independent samples t test. Compared with the control group (HC), in the HP group, we preliminarily identified 92 differential metabolites (57 in positive ion mode and 35 in negative ion mode; Figure 5A). After annotating the differential metabolites into the HMDB database for classification, Figure 5 shows that lipids and lipid-like molecules accounted for 46.58% of the differential metabolites in the HC and HPⅢ groups, organic acids and derivatives accounted for 24.66%, organic oxygen compounds accounted for 12.33%, organic heterocyclic compounds accounted for 9.59%, and the rest accounted for 6.84% (Figure 5C).

KEGG Enrichment Analysis of Differential Metabolites Differential metabolites were enriched according to KEGG pathway analysis, with P-value < 0.05 as the screening criterion. They were mainly enriched in beta-Alanine metabolism (beta-Alanine metabolism), Phosphatidylinositol signaling (Phosphatidylinositol signaling system), sub-Oleic acid metabolism (Linoleic acid metabolism), Inositol phosphate metabolism (Inositol phosphate), Galactose metabolism (Galactose), and so on (see Figure 5B).

Integrated Transcriptome and Metabolome Analysis

In order to further analyze the effect of the 3 considered beneficial bacteria on the broiler caecum under heat stress, Pearson correlation analysis was performed on differential genes and differential metabolites. Figure 6 shows the correlation between the top 50 differential genes and differential metabolites, which further

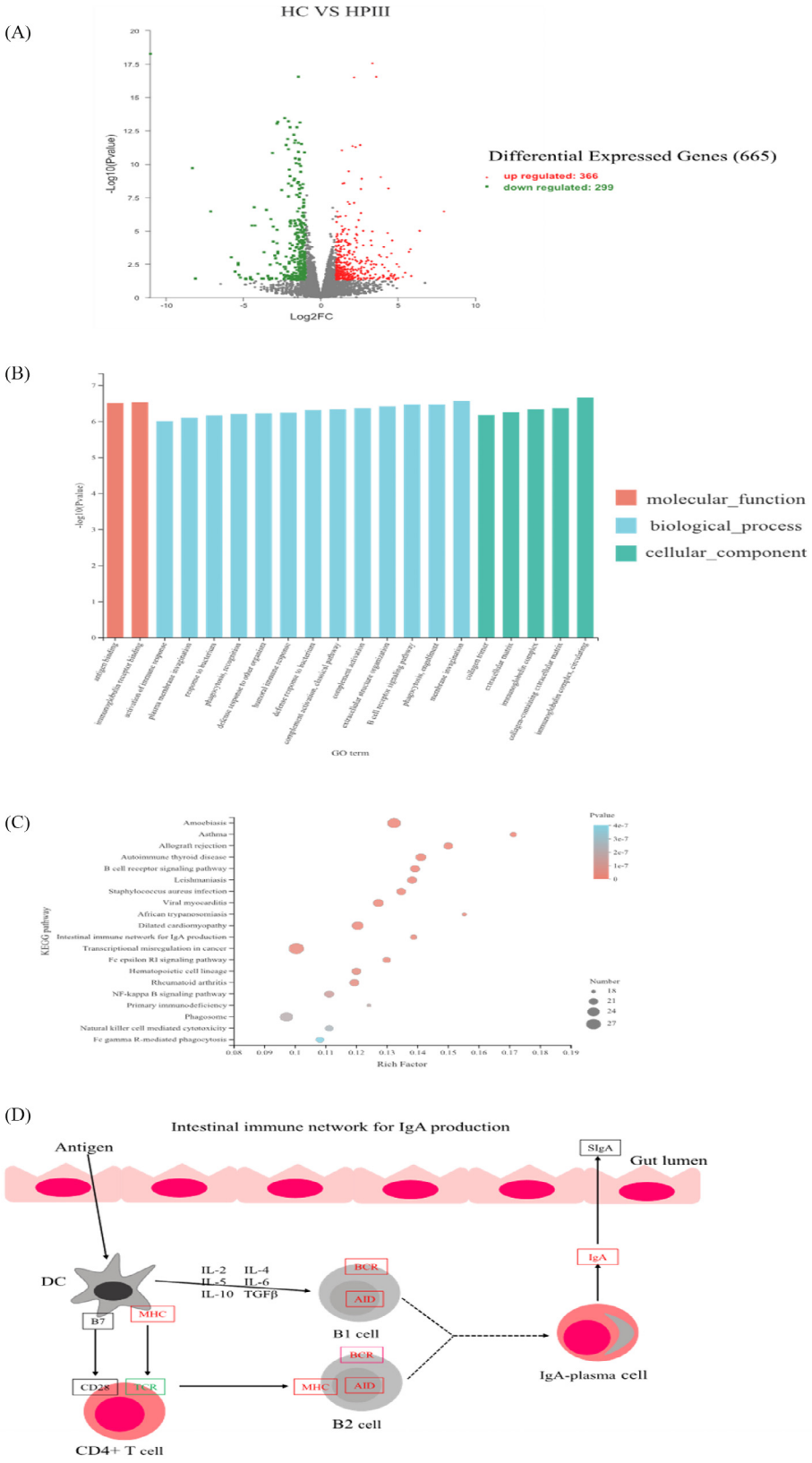

Figure 1. Transcriptome analysis of cecum in HC and HPⅢ groups. (A) Differential gene expression analysis between the HC and HPⅢ groups. Red dots (Up) represent significantly upregulated genes ($P < 0.05$, FC ≥ 2); green dots (Down) represent significantly downregulated genes ($P < 0.05$, FC ≤ 0.5); and gray dots (No) represent insignificant DEGs. (B) GO classification map of DEGs between the HC and HPⅢ groups. (C) Bubble map of DEGs categorized through KEGG pathway enrichment analysis (top 20). (D) Influence of probiotics on genes downstream the Intestinal immune network for IgA synthesis in the cecum of heat-stressed broilers. Inside the text box is the gene; red represents significantly upregulated genes; green represents significantly downregulated genes; and black represents insignificant DEGs. Abbreviations: DEGs, differentially expressed genes; GO, Gene Ontology.

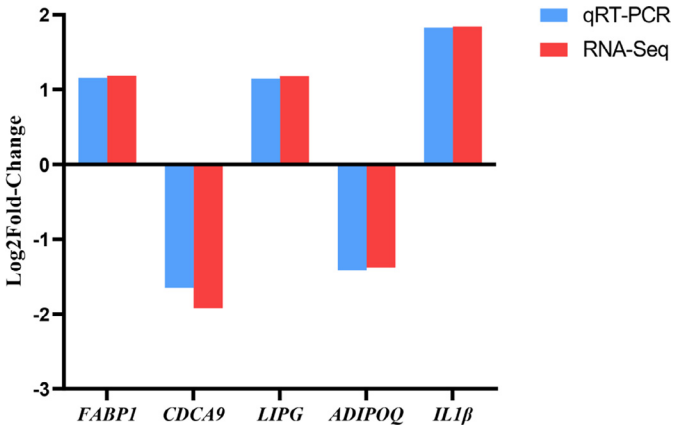

Figure 2. Validation of DEGs by qRT-PCR. Abbreviation: DEGs, differentially expressed genes.

highlights the obtained results; in particular, the association of each gene with each metabolite was determined (Figure 6A).

To further analyse the effects of the probiotics on the heat-stressed broiler caecum, a comprehensive analysis of metabolomic and transcriptomic data was performed. DAMs and DEGs were annotated into 10 pathways. Of them, the lipid metabolism and amino acid biosynthesis pathways were the most annotated, suggesting that these pathways play pivotal roles in the regulation of cecal metabolism in heat-stressed broilers (Figure 6B).

DISCUSSION

Heat stress is a major issue in the poultry industry, slowing chicken growth and potentially causing huge financial losses. In this study, we investigated the effect of probiotics on broilers under heat stress and showed that probiotics increased ADG and decreased FCR in broilers, while having no effect on ADFI. This finding is similar to that presented in a previous study (Wang et al., 2018), where feeding *Bacillus subtilis* at 1×10^6 CFU/g led to a similar positive effect on the growth performance of broilers under heat stress. Abdelqader et al. (2020) showed that supplementation with 3×10^7 CFU/kg of *Bacillus subtilis* probiotics in broiler diets reduced FCR and increased broiler body weight. Quinteiro-Filho et al. (2012) and Alhenaky et al. (2017) suggested that the effects of heat stress on broiler performance may be the result of damage to the gut epithelium, resulting in compromised gut health, loss of mucosal structure and integrity, and nutrient malabsorption. Heat stress may reduce digestive enzyme activity in chickens (Lin et al., 2006), while probiotics accelerate feed metabolism by increasing the production of digestive enzymes in the small intestine (Chen et al., 2009). Martin et al. (2008) and Song et al. (2014) suggested that a possible mechanism by which probiotics mitigate the harmful effects of heat stress is their role in repairing damaged gut mucosal structures and improving the gut microbiota balance. In this study, the probiotic viable density used was 10^{10} cfu/g, which is several times higher than the commonly recommended density of 10^8 cfu/g product for commercial probiotic feed additives. Compared with commercial probiotics, the probiotics used in this study had better effects, possibly as a larger number of viable probiotics is more conducive to colonizing the intestine and, in turn, a larger number of probiotics colonizing the intestine has a more obvious probiotic effect. The effects of probiotics as feed additives may vary for different reasons, including the viability of the probiotic species, differences in probiotic densities and probiotic delivery methods or dosages, broiler selection, and varying growth environments. Probiotics can reduce the intestinal damage caused by heat stress in broilers, allowing the intestines to develop greater villus height and surface area which, in turn, promotes the growth and development of broilers.

Transcriptomics can reveal complex biological pathways by comprehensively studying gene expression and regulation (Haas and Zody, 2010). From the transcriptome results, 665 differential genes were screened, of

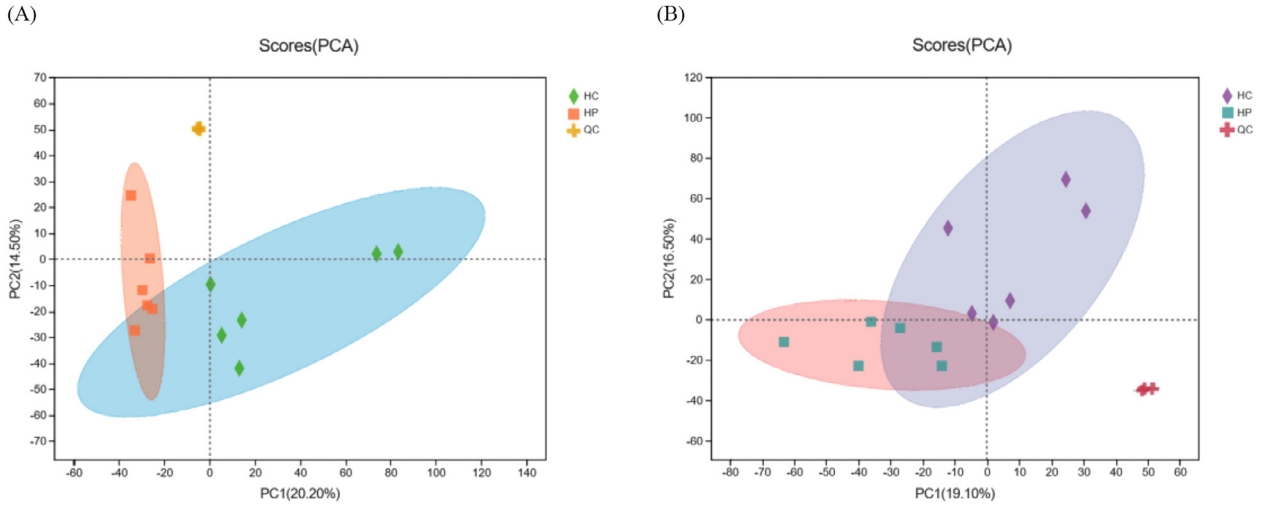

Figure 3. PCA results of two groups of samples and QC samples in the positive ion mode (A) and negative ion mode (B). Abbreviation: PCA, principal component analysis.

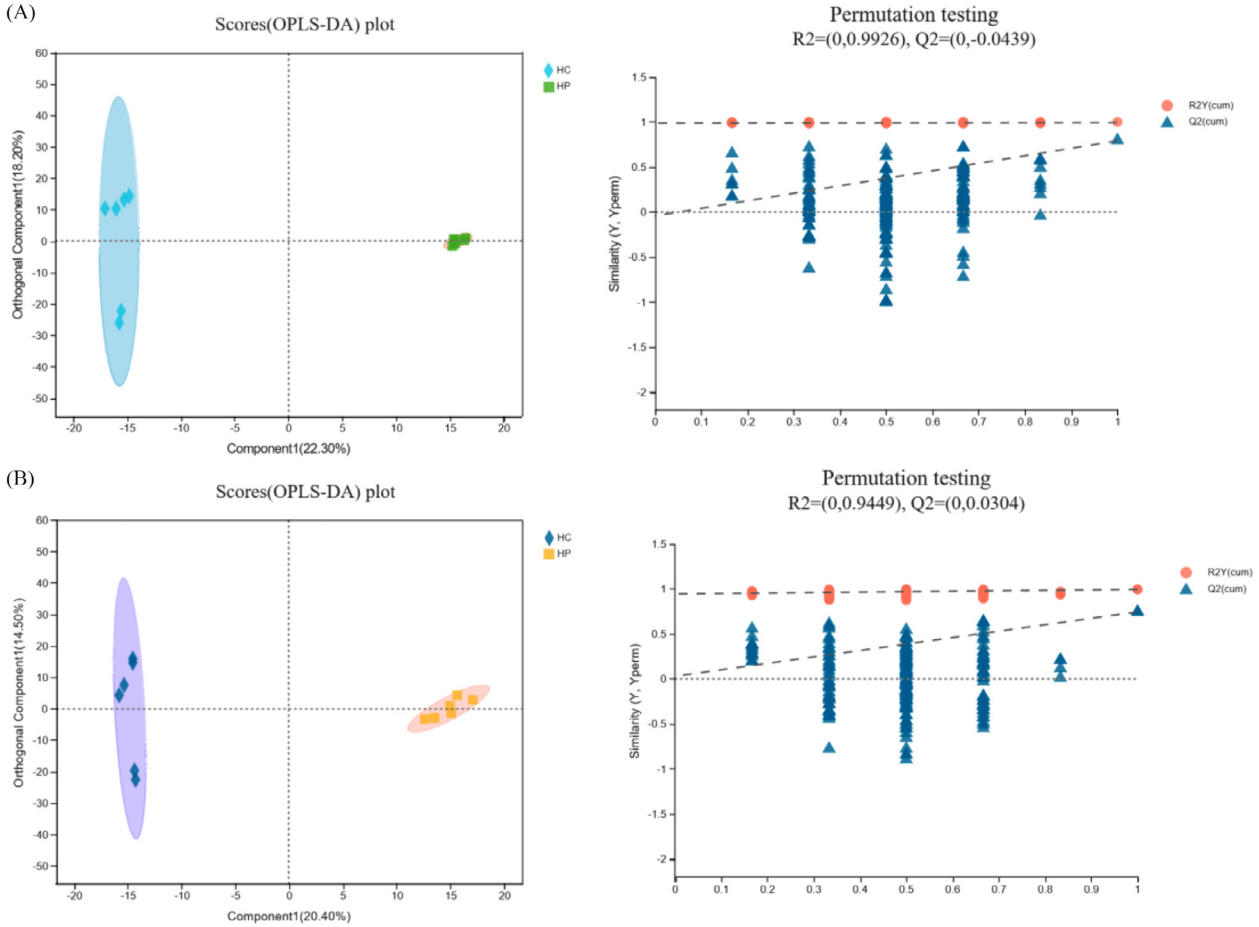

Figure 4. OPLS-DA model score diagram (left) and model validation diagram (right) for the two groups of samples in the positive ion mode (A) and negative ion mode (B). Abbreviation: OPLS-DA, orthogonal projections to latent structures discriminant analysis.

which 299 were downregulated and 366 were upregulated. They were mostly enriched in immune-related GO terms, according to the GO enrichment analysis. DEGs were primarily abundant in the B cell receptor signaling route, the Intestinal immune network for IgA synthesis, the Fc epsilon RI signaling pathway, and other signaling pathways, according to KEGG enrichment analysis. MHC is a crucial cell surface protein of the adaptive immune system. TCR is a protein complex on the surface of T cells and T lymphocytes that recognizes antigen pieces as peptides coupled to MHC molecules (Davis and Bjorkman, 1988). The BCR (B-cell receptor) is a molecule on the surface of B cells that identifies and binds certain antigens. The *AID* (activated cytidine deaminase) gene can control the body's immune system to produce antibodies. With the largest lymphoid tissue, neuroendocrine system, and trillions of commensal bacteria in the body, the intestine is also an important organ regarding the host mucosal immune system. Dendritic cells (**DCs**) are specialized antigen-presenting cells (**APCs**) that play a key role in activating the immune response (Rajput et al., 2014). CD4+ T cells are associated with major histocompatibility complex (**MHC**) class II molecules, and act as helper T cells or inflammatory T cells in response to exogenous antigens (Brisbin et al., 2008). Avian immunoglobulin A is present in most intestinal cells, similar to mammalian immunoglobulin A, and is released into the intestinal lumen through transepithelial transport of SIgA (Lindner et al., 2012). Immunoglobulins are widely considered to be essential in protecting the mucosal surface from toxins, viruses, and bacteria, by neutralising or preventing these pathogens from binding to the mucosal surface; SIgA also plays a role in maintaining intramucosal homeostasis (Lammers et al., 2010). Gyawali et al. (2022) showed that the addition of *Lactobacillus paracaesi* microcapsules to broiler diets increased SIgA levels in the cecum and reduced inflammation, affecting the immune system through increasing the secretion of SIgA. As can be seen from the diagram in Figure 1D, antigens enter the gut through epithelial cells and are recognized by dendritic cells. They are presented to B1 cells by IL2 (or similar) and then to IgA plasma cells; on the other hand, they are presented to CD4+ T cells by *MHC* and are recognized by *TCR* on CD4+ T cells, the CD4+ T cells are presented to B2 cells by *TCR*, the *MHC* of the B2 cells recognizes and delivers to IgA plasma cells, IgA plasma cells produce IgA, and SIgA is released into the gut by IgA plasma cells. We observed that heat stress could activate intestinal immune

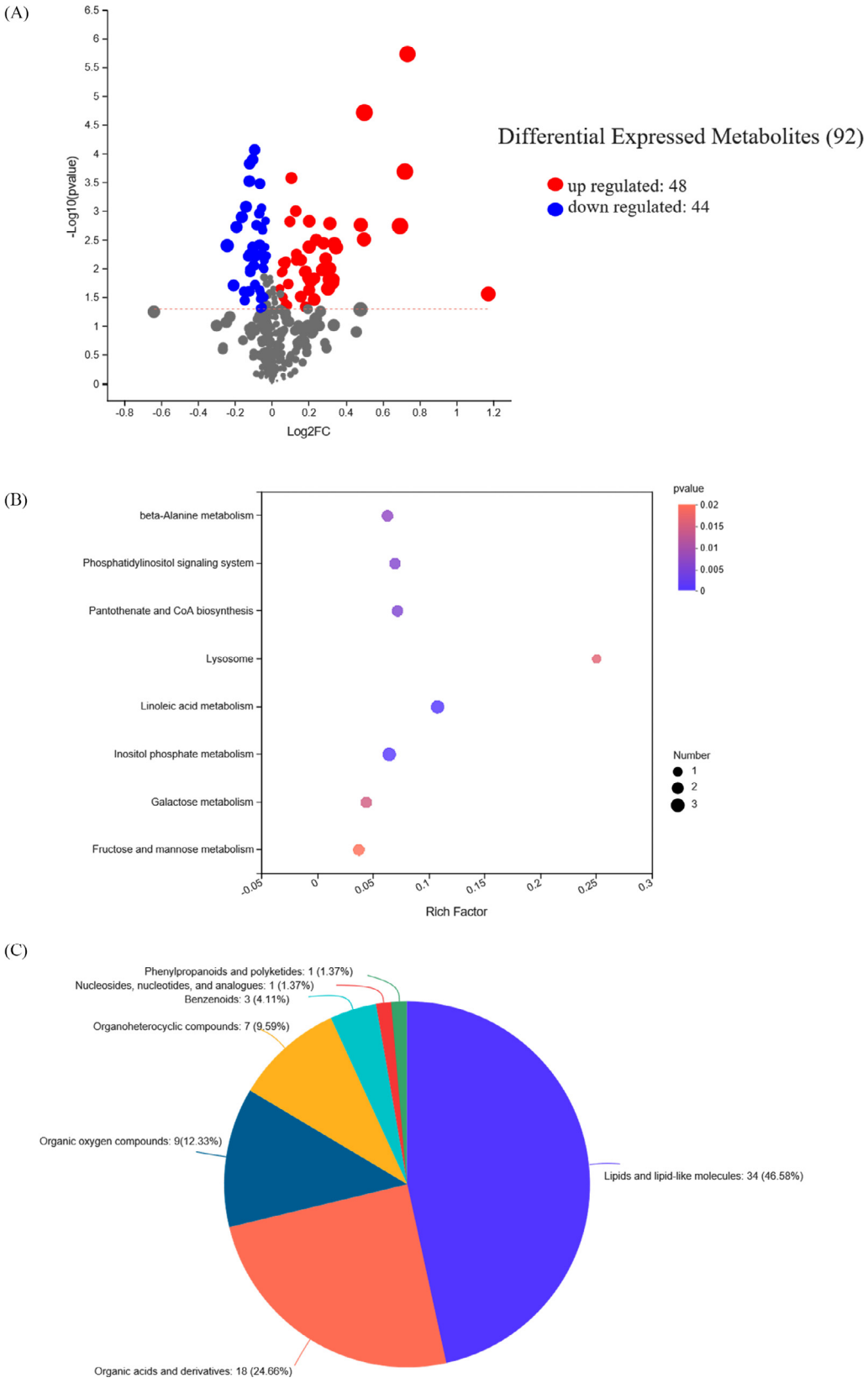

Figure 5. Metabolome analysis of cecum in the HC and HPⅢ groups. (A) Analysis of differential metabolite expression between the HC and HPⅢ groups. Red indicates the increase in metabolite content; blue indicates the decrease in metabolite content; and gray indicates no significant difference. (B) Pathway enrichment of differentially expressed metabolites was analyzed using the pathway enrichment statistical scatterplot. (C) Classification of differential metabolites in the HMDB database.

response. Probiotics may increase intestinal IgA content by upregulating IgA gene expression, thus reducing intestinal inflammation.

Metabolomics can aid in the analysis and identification of metabolites in cells and tissues and, as such, is crucial for biological research (Johnson et al., 2016).

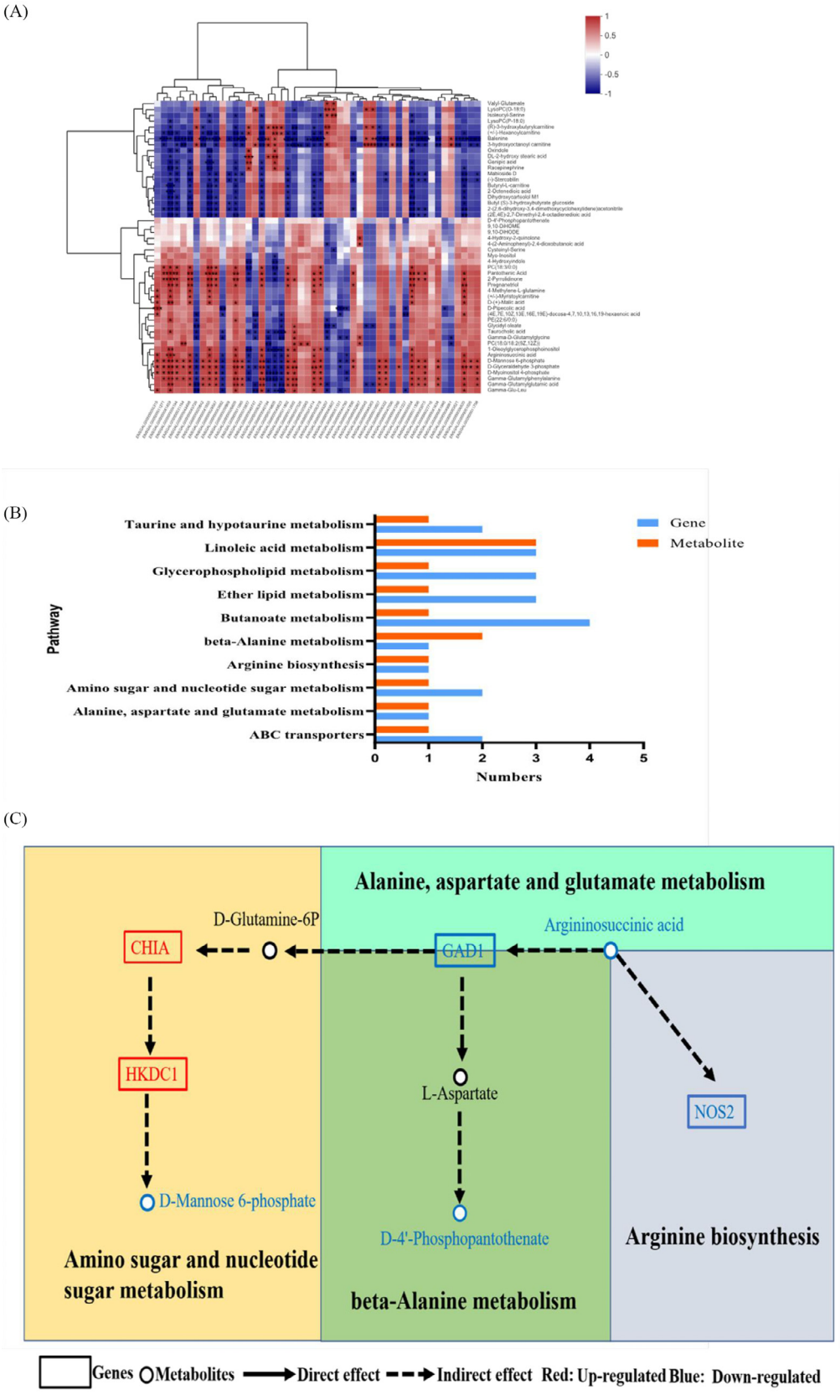

Figure 6. Combined transcriptome and metabolome analysis. (A) Heatmap of correlations between differential genes and differential metabolites. The red and blue colors represent positive and negative correlations between transcriptomics and metabolomics, respectively. "*," "**," and "***" represents $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively, and indicate the significant difference between genes and metabolites. (B) Transcriptome and metabolome coenrich the KEGG pathway. KEGG analysis of DAMs and DEGs between the control and probiotics groups. (C) Probiotics regulate cecal metabolic pathways in heat-stressed broilers. Boxes indicate genes, circles indicate metabolites, red indicates upregulation, blue indicates downregulation, and black indicates no difference. Abbreviation: DEGs, differentially expressed genes

Metabolites mainly measured by metabolomics include small molecular substances, such as glycolipids, short peptides, and nucleotides, having a molecular mass of <1.5 kDa (Johnson et al., 2012). In this paper, 92 differential metabolites were identified, 48 of which were upregulated, while 44 were down-regulated. In the KEGG enrichment analysis, beta-Alanine metabolism (Pantothenic Acid, D-4′-Phosphopantothenate) was found to be significantly enriched. Pantothenic acid is a type of vitamin B that is the prosthetic group of coenzyme A and a part of acyl carrier proteins (ACPs) (Lanska, 2012). Pantothenic acid is involved in the metabolism of carbohydrates, fats, and proteins in the body, particularly in fat synthesis and metabolism, while coenzyme A transfers acyl during metabolism(Ragaller et al., 2011). In geese, dietary supplementation with pantothenate acid significantly affected lipids (Wang et al., 2016). Fat and energy stores were significantly reduced when chicks were fed diets deficient in pantothenic acid (Beagle and Begin, 1976). Zhang et al. (2021) found that alterations to pantothenic acid metabolites in caecal microorganisms were significantly correlated with lipogenesis. D-4′-Phosphopantothenate is an intermediate in the coenzyme A (**CoA**) biosynthetic pathway, which is required in numerous enzymatic reactions in intermediate metabolic centres, including the oxidation of fatty acids, carbohydrates, and amino acids. The last product of beta-alanine metabolism is acetic acid. Probiotics can control the acetic acid content by regulating the beta-Alanine metabolism pathway in the cecum of broilers, thus playing a regulatory role. Hou et al. (2016) screened out the biosynthetic metabolic pathways of pantothenic acid and coenzyme A in the post-genomics of lean and fatty chickens, which may be related to fat deposition. Compound probiotics may alter lipid metabolism to mitigate the adverse effects of heat stress by regulating the levels of pantothenic acid and D-4′-Phosphopantothenate metabolites.

A comprehensive analysis of transcriptomic and metabolomic data was performed to further analyse the effect of probiotics on the caecum of heat-stressed broilers, and differential metabolites and genes were correlated using Pearson correlation analysis. Differential metabolites and genes were analysed for co-enrichment, including enriched taurine and hypotaurine metabolism; linoleic acid; glycerophospholipid metabolism; ether lipid metabolism; butanoate metabolism; beta-alanine metabolism; arginine biosynthesis; amino sugar and nucleotide sugar metabolism; alanine, aspartate, and glutamate metabolism; and ABC transporters. Through a comprehensive analysis of the transcriptome and metabolome, gene and metabolite regulatory networks were mapped. Combined transcriptome and metabolome analysis allowed us to further elucidate how probiotics alleviate heat stress in broilers. We discovered that the differential metabolites and genes were mostly connected to beta-alanine metabolism, arginine biosynthesis, and amino sugar and nucleotide sugar metabolism, as well as alanine, aspartate, and glutamate metabolism. *NOS2* is a nitric oxide-producing protein-coding gene (**NO**), which is involved in inflammation, as it increases the production of pro-inflammatory mediators such IL6 and IL8 (Drutman et al., 2020). It is currently believed that the high expression of *NOS2* is mainly limited to the adaptive stage of the immune response (Bogdan et al., 2000). Argininosuccinic acid is a non-proteinogenic amino acid that is an essential intermediate in the urea cycle. It is a basic amino acid (Barzał et al., 2014). GAD1 (Glutamate decarboxylase 1) is a brain-specific enzyme that is encoded by the *GAD1* gene, which is a protein that transforms glutamate to gamma aminobutyric acid (**GABA**). The GABA molecule is the body's major inhibitory neurotransmitter, which inhibits the neurological system (Trifonov et al., 2014). The hexokinase protein family is encoded by the *HKDC1* gene, which play roles in glucose metabolism. (Irwin and Tan, 2008; Hayes et al., 2013). In the immune system, mannose-6-phosphate (**M6P**) is a lectin-bound molecule (Sigdel et al., 2015). In this study, we found that probiotics may reduce the production of NO and reduce intestinal inflammation by downregulating the *NOS2* gene; downregulation of *GAD1* gene reduces GABA production and prevents inhibition of the nervous system; and upregulation of *HKDC1* gene and downregulation of D-Mannose 6-phosphate serve to regulate glucose metabolism, in order to alleviate the adverse effects of heat stress on broilers.

CONCLUSIONS

We conducted transcriptome and metabolome analyses to assess the regulatory effect of compound probiotics on caecal metabolism in broilers. Co-enrichment analysis was performed on differential genes and differential metabolites, and 10 pathways were found to be enriched. Our analysis indicated that, compared with the HC group, probiotics regulated immune-related genes, glucose metabolism, and lipid metabolism-related genes and metabolites in the HPIII group, which may indicate the regulatory mode by which probiotics act to alleviate heat stress in broilers. This study provides valuable information for studying the regulation and gene −metabolism network of probiotics acting in the ileocecal metabolism of heat-stressed broilers.

DISCLOSURES

All authors declare no conflict of interest.

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