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Comprehensive analysis of differentially expressed mRNA profiles in chicken jejunum and cecum following *Eimeria maxima* infection

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ABSTRACT Coccidiosis, a protozoan disease that substantially impacts poultry production, is characterized by an intracellular parasite. The study utilized 48 one-day-old Horro chickens, randomly divided into the infected (**I**) and control (**C**) groups. The challenge group of chickens were administered *Eimeria maxima* oocysts via oral gavage at 21-days-old, and each chicken received 2 mL containing 7×10^4 sporulated oocysts. The total RNAs of chicken jejunum and cecum tissues were isolated from three samples, each from I and C groups. Our study aimed to understand the host immune-parasite interactions and compare immune response mRNA profiles in chicken jejunum and cecum tissues at 4 and 7 days postinfection with *Eimeria maxima*. The results showed that 823 up- and 737 down-regulated differentially expressed mRNAs (**DEmRNAs**) in jejunum at 4 d infection and control (J4I vs. J4C), and 710 up- and 368 down-regulated DEmRNAs in jejunum at 7 days infection and control (J7I vs. J7C) were identified. In addition, DEmRNAs in cecum tissue, 1424 up- and 1930 down-regulated genes in cecum at 4 days infection and control (C4I vs. C4C), and 77 up- and 191 down-regulated genes in cecum at 7 days infection and control (C7I vs. C7C) were detected. The crucial DEmRNAs, including *SLC7A5, IL1R2, GLDC, ITGB6, ADAMTS4, IL1RAP, TNFRSF11B, IMPG2, WNT9A*, and *FOXF1*, played pivotal roles in the immune response during *Eimeria maxima* infection of chicken jejunum. In addition, the potential detection of *FSTL3, RBP7, CCL20, DPP4, PRKG2, TFPI2*, and *CDKN1A* in the cecum during the host immune response against *Eimeria maxima* infection is particularly noteworthy. Furthermore, our functional enrichment analysis revealed the primary involvement of DEmRNAs in small molecule metabolic process, immune response function, inflammatory response, and toll-like receptor 10 signaling pathway in the jejunum at 4 and 7 days postinfection. Similarly, in the cecum, DEmRNAs at 4 and 7 days postinfection were enriched in processes related to oxidative stress response and immune responses. Our findings provide new insights and contribute significantly to the field of poultry production and parasitology.

Key words: mRNA, chicken, differential expression, Eimeria maxima, jejunum and cecum

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INTRODUCTION

Eimeria coccidian is one of the most significant infectious diseases in modern poultry rearing, with a considerable impact on animal welfare, animal health, and the industrial economy. *Eimeira* species are distributed worldwide, significantly destroying chicken production and resulting in extensive economic losses (Blake and Tomley, 2014a; Blake et al., 2020). Seven recognized specific genus *Eimeria* species cause coccidiosis disease. However, Blake et al revealed that three new *Eimeria* parasite species, namely OTUs x, y, and z have been explored to affect domestic chickens (Blake et al., 2021). The *Eimeria* species invade different host intestinal regions, leading to considerable challenges in chicken production (Fatoba and Adeleke, 2020), and their strong resistance to environmental conditions makes the

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infection difficult to eliminate (Chapman and Jeffers, 2014).

The host immune-related gene response plays a vital role in combating coccidia infection, and mRNA expression related to the immune response is a crucial aspect of this process. The host immune protective response can be performed through genetic selection (Kachuri et al., 2020). Hostparasite interaction in the gut is crucial for the design of new approaches against coccidiosis control (Fredensborg et al., 2020). The immune evasion mechanisms and proteins recognized by the host immune system are still inadequate (Sandholt et al., 2021). Cytokines produce varied host defense mechanisms, especially from epithelial cells, during infection (Ouyang et al., 2011). Defensive immunity powerfully relies on a Th1-type T-cell response with (***IFN***) - γ as a key component (Kim et al., 2019). The host's humoral and cellular immunity mainly respond to coccidian disease (Min et al., 2013). Cell-mediated immunity has been promoted during *Eimeria* infection of the host, such as macrophages and T lymphocytes have significant roles in immunity (Yun et al., 2000; Lee et al., 2022).

Interleukin (***IL-12***) and *IL-10* are early immune regulatory cytokines that provide functional link between the host's innate and adaptive immune responses (Ma et al., 2015). Henceforth, *IL-10* is an anti-inflammatory cytokine and performances as a stand-down signal to reduce inflammation and host pathology during disease (Couper et al., 2008; Eddie et al., 2017). *Eimeria* infection of the host has a resilient cell-mediated immune response induction and is central to developing protective immunity against coccidiosis (Rafiqi et al., 2018). Genes associated with cytokine-cytokine receptor interactions, such as *CXCR5, GDF15, IL7R, IL12RB2, IL22*, and *IFNGR1* play key roles in initiating the immune response by facilitating the migration of T and B lymphocytes in the inflammatory pathway (Kim et al., 2022). Previous study has reported mRNA expression responses on cecum tissue, most of the effects associated with *Eimeria tenella* infection (Kim et al., 2022). Contrarily, *Eimeria maxima* infection also affects differential gene expression in the cecum tissue of chicken (Li et al., 2019).

Next-generation mRNA sequence analysis is extensively used for pinpointing host immune response gene expression during coccidian infection of chicken (Wang et al., 2019). However, there are few studies on host immune response gene information concerning the different time points of coccidian infection of chicken. The molecular functions of host immune mRNAs related to Horro chicken have not yet been described during *Eimeria maxima* challenge. Thus, this study used mRNA sequences to understand the host immune-parasite interactions in the jejunum and cecum tissue at 4 and 7 d postinfection with *Eimeria maxima*. Our findings revealed new information on the expression changes of mRNAs at different times of coccidiosis infection in the jejunum and cecum tissues, along with their associated molecular mechanisms in chicken coccidiosis.

MATERIALS AND METHODS

Ethics Statement

All the experimental procedures in this study complied with animal welfare protocols. All efforts were adopted to minimize animal suffering following relevant guidelines and regulations of the Institute Animal Care the Use Committee (**IACUC**) of the International Livestock Research Institute (**ILRI**) poultry research facility in Addis Ababa, Ethiopia. The protocol was approved by the ILRI, IACUC committee number with the reference IACUC-RC2019-01.

Experimental Design and Sample Collection

Fertilized eggs from the Ethiopian Horro chicken breed were subjected to incubation in an automated incubator at 37.5°C and a relative humidity level of 78% a total of 48 one-day-old Horro chickens were divided randomly into two groups, namely the infected (**I**) and the control (**C**) groups, each consisting of 24 chickens, with four replicates per group (n = 6). The chickens were kept in starter brooder units at the ILRI poultry facility in Addis Ababa, Ethiopia. They were subjected to a temperature-controlled environment following a standard protocol and had access to starter diet feed and water ad libitum. *Eimeria maxima* oocysts (supplied by Foshan Standard Bio-Tech Co., Ltd.,Foshan, Guangdong, China) were administered to the challenge group of chickens via oral gavage on the 21-days-old, with each chicken receiving 2 mL containing 7×10^4 sporulated oocysts (Figure 1). The same volume of

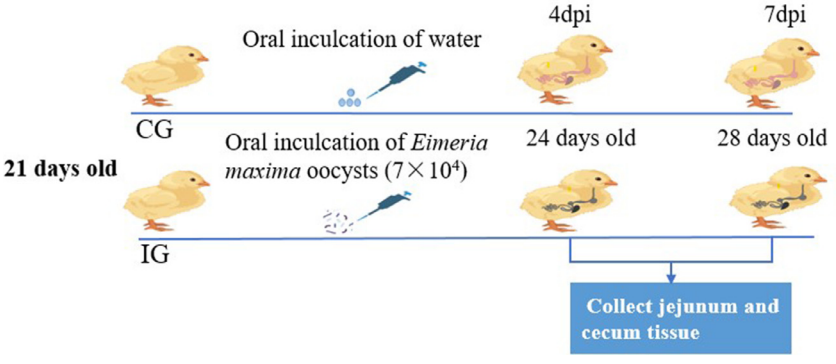

Figure 1. Experimental design and sample collection. CG, Control group; IG, Infection group; dpi; days postinfection.

distilled water was inoculated to each chicken in the control groups. To perform the impact of *Eimeria maxima* infection on the chicken intestinal tract, 32 chickens were chosen from the infected and control groups. All the experimental periods of the challenge and control groups of chicken were effectively followed up regarding physically change, feeding status, feces color, and ruffled feathers. In accordance with the life cycle of the *Eimeria* parasite within the chicken intestine, coccidia disease has an impact and leads to lesion formation at 4 and 7 d postinfection. At 4 and 7 d postinfection, 8 chickens in two groups were humanely killed, respectively. The chickens were humanely killed using cervical dislocation without pain, suffering or distress, followed by death. The gastrointestinal tract (**GIT**) of each selected chicken was dissected, and 200 mg of the jejunum and cecum were collected immediately postmortem from 8 chickens in each group at 4 and 7 d postinfection. Subsequently, the tubes containing the collected samples were frozen and stored at -80°C until the extraction of total RNA.

RNA Extraction and Quality Control

Total RNAs at 4 and 7 d postinfection of chicken jejunum and cecum tissue were isolated from three individual samples, each from infection and control groups, using the RNeasy Min Elute Cleanup Kit (Qiagen, Hilden, Germany) according to the manufacturer guidelines and stored at -80°C. All RNA samples were quickly tested for RNA degradation and potential contamination on a 1.5% agarose gel electrophoresis. The RNA purity was tested using a 2000 NanoDrop (ThermoFisher, Scientific), with all samples within the expected OD260/280 = 1.8−2.0 ratio, supporting optimal RNA purity. The assessment of RNA integrity involves running an aliquot of the RNA sample on an agarose gel stained with ethidium bromide. Total RNA integrity was determined using a 2,100 Bioanalyzer (Agilent, Palo Alto, CA). All samples with RNA integrity numbers (**RIN**) $\geq$ 7 were used for cDNA library construction using a TruSeq stranded messenger RNA sample preparation kit (Illumina, San Diego, CA) following the manufacturer's guidelines.

Library Preparation and Sequencing Analysis

mRNAs were purified from a total RNA using poly-T oligo-attached magnetic beads. Subsequently, fragmentation of the first strand cDNA was analyzed using random hexamer primers, followed by the second strand cDNA synthesis using either dUTP for the directional library or dTTP for a nondirectional library. The directional library was ready after a repair, A-tailing, adapter ligation, size selection, USER enzyme digestion, amplification and purification. The none-directional library was ready after end repair, A-tailing, adapter ligation, size selection, amplification, and purification. The index-coded samples were clustered according to the manufacturer's instructions. Afterwards, cluster generation, the library preparations, and all samples were sequenced using the Illumina NovaSeq 6,000 platform and paired-end reads were generated.

Sequence Reads Mapping to Reference Genome

Initially, raw data reads of fastq format were processed using in-house perl scripts. Subsequently, the clean data were obtained by eliminating reads containing adapter, low-quality reads, and poly-N from raw data. Simultaneously, the quality of the clean data was assessed for Q20, Q30, and GC content. Thus, all the further analyses were based on quality clean data. Reference genome and gene model annotation files downloaded from the genome website (ftp://ftp.ensembl.org/pub/release-75/fasta/gallus_gallus/dna/). The reference genome index was constructed using Hisat2 v2.0.5, and paired-end clean reads were aligned to the chicken genome (*Galgal* GRCg6a) using Hisat2 v2.0.5. Then selected Hisat2 as a mapping tool that Hisat2 can initiate a database of splice links based on the gene annotation file. Hence better mapping results than other nonsplice mapping tools.

Quantification and Differential Expression Analysis

FeatureCounts v1.5.0-p3 was used to count the reads numbers mapped to each gene. Then reads per kilobase of exon model per million mapped reads of each gene were calculated based on the gene length and read count mapped to the gene. FPKM, representing the predictable number of Fragments Per Kilobase of transcript sequence per Millions of base pairs sequenced, considers the effect of sequencing depth and gene length for the reads count simultaneously and, currently, the most commonly used method for estimating gene expression levels (Mortazavi et al., 2008). Differential expression analysis of 2 circumstances per group (three biological replicates per condition) was analyzed using the DESeq2 R package (1.20.0) (Anders and Huber, 2010). DESeq2 provides statistical procedures for determining differential expression in numerical gene expression data using a model based on the negative binomial delivery. The subsequent *P*-values were adjusted using Benjamini and Hochberg's (Benjamini and Hochberg, 1995) false discovery rate control. Genes with adjusted *P*-value $\leq$ 0.05 found by DESeq2 were allocated as differentially expressed. In the differential gene expression analysis, the edgeR program package adjusted the read counts for each sequenced library through 1 normalized scaling factor. Differential expression analysis for three conditions was analyzed using the edgeR R package (3.22.5). The *P* values were used in the Benjamini & Hochberg method. DEGs were set with a level of absolute log2fold-change

(FC) ≥0.5 and a corrected P-value ≤ 0.05 as the threshold for significantly differential expression.

GO and KEGG Enrichment Analysis of Differentially Expressed Genes

Gene ontology (GO) terms were widely used to define cellular components, molecular function, and biological processes of genes. The GO term with a P-value < 0.05 was considered as significantly enriched for considered differentially expressed genes. KEGG is a database source for thoughtful high-level functions of the biological system, from molecular-level information, particularly large-scale molecular datasets generated by genome sequencing technologies. The cluster Profiler R package was used to analyze differential expression genes' statistical enrichment. This study used the top 20 most significantly enriched pathways analysis chosen in the KEGG database for visualizing KEGG enrichment.

RESULTS

Sequence Data Analysis

Sequencing was performed on 24 mRNA samples from the chicken jejunum and cecum of both infected and non-infected chickens with *Eimeria maxima*. The samples were collected at 4 and 7 d postinfection, with 3 biological replicates used for each infected and control group. The number of raw reads generated for each sample ranged from 40,499,966 to 50,067,496 upon *Eimeria maxima* infection. After trimming for low quality and adapter removal, total clean reads ranging from 38,660,522 to 47,315,148 were obtained in jejunum and cecum libraries for downstream analyses (Supplementary Table S1). The jejunum and cecum tissue RNA sequence data analysis of clean reads mapped percentage showed over 83.71% with the chicken reference genome (GRCg6a_94). The Q20 and Q30 percentages were greater than 96.56% and 91.52%, respectively. The GC content of samples ranged from 51.46% to 53.53%, indicating the overall reliability of the GC content in each sample

Reads Distribution in Reference Genome and Principal Component Analysis

Mapped regions are classified as exons, introns, and intergenic regions based on the reference genome. Exon-mapped reads were the most abundant type when annotated with the reference genome, while intron reads derived from pre-mRNA contamination (Supplementary Table S2). The predominant reason for reads mapping to intergenic regions was the presence of less detailed annotations in the reference genome. The distribution of sequencing reads of all samples in the pie chart mapped on different gene regions was generated (Supplementary Table S1). Principal component analysis (**PCA**) assessed variations between different groups and detects potential sample duplications within the same group. In this study, PCA was conducted using the FPKM values for each sample (Figure 2A). In an ideal scenario, samples between groups should be significantly dispersed, while samples within each group should be closely clustered. The correlation of the gene expression levels between samples plays an important role in verifying the reliability and sample selection and estimating the differential gene expression analysis. The gene expression levels (FPKM) for each sample were used to calculate correlation coefficients between groups, visually represented as heat maps (Figure 2B). A higher correlation coefficient indicates a closer similarity in expression patterns between samples. A correlation coefficient closer to one signifies higher similarity among the samples. Comparison of gene expression levels among different samples is visualized using boxplots. The box plot examines the dispersion in gene expression within a single sample and enables the comparison of overall expression levels across different samples (Figure 2C).

Overview of Gene Expression Upon Chicken Eimeria Maxima Infection

Analyzing gene expression levels under varying experimental conditions is fundamental in RNA-seq technology. Therefore, this study has employed chicken jejunum and cecum samples infected with *Eimeria maxima* at two different time points (4 and 7 d postinfection) to investigate mRNA expression. The gene expression in jejunum and cecum after *Eimeria maxima* infection and the control group were analyzed in different time point infections. Gene expression levels were estimated by the abundance of the transcript mapped to exon, and the Venn diagram presents the number of genes uniquely and co-expressed in the infection and control groups (Figure 3). In the gene expression analysis, 5,504 mRNAs were consistently detected in both the 4-d postinfection and the control group of jejunum samples (Figure 3A). In contrast, 5,494 mRNAs were identified in both the 4-d postinfection and the control group of cecum samples (Figure 3B). Likewise, 5,602 mRNAs were found to exhibit common expression in both the 7-d postinfection and the control group of jejunum samples (Figure 3C), while 5,515 mRNAs were shared between the 7-d postinfection samples and the control group of cecum samples (Figure 3D). Similarly, 5286 mRNA expressions were shared between the jejunum and cecum samples at both 4 and 7 d postinfection (Figure 3E).

Differential Gene Expression Analysis

The expression of a gene can be influenced by both internal environment and external stimuli, which are highly temporal and tissue specific. Genes exhibited significant differences in expression levels under various conditions, including treatment versus control at different time points and tissues. Consequently, this research

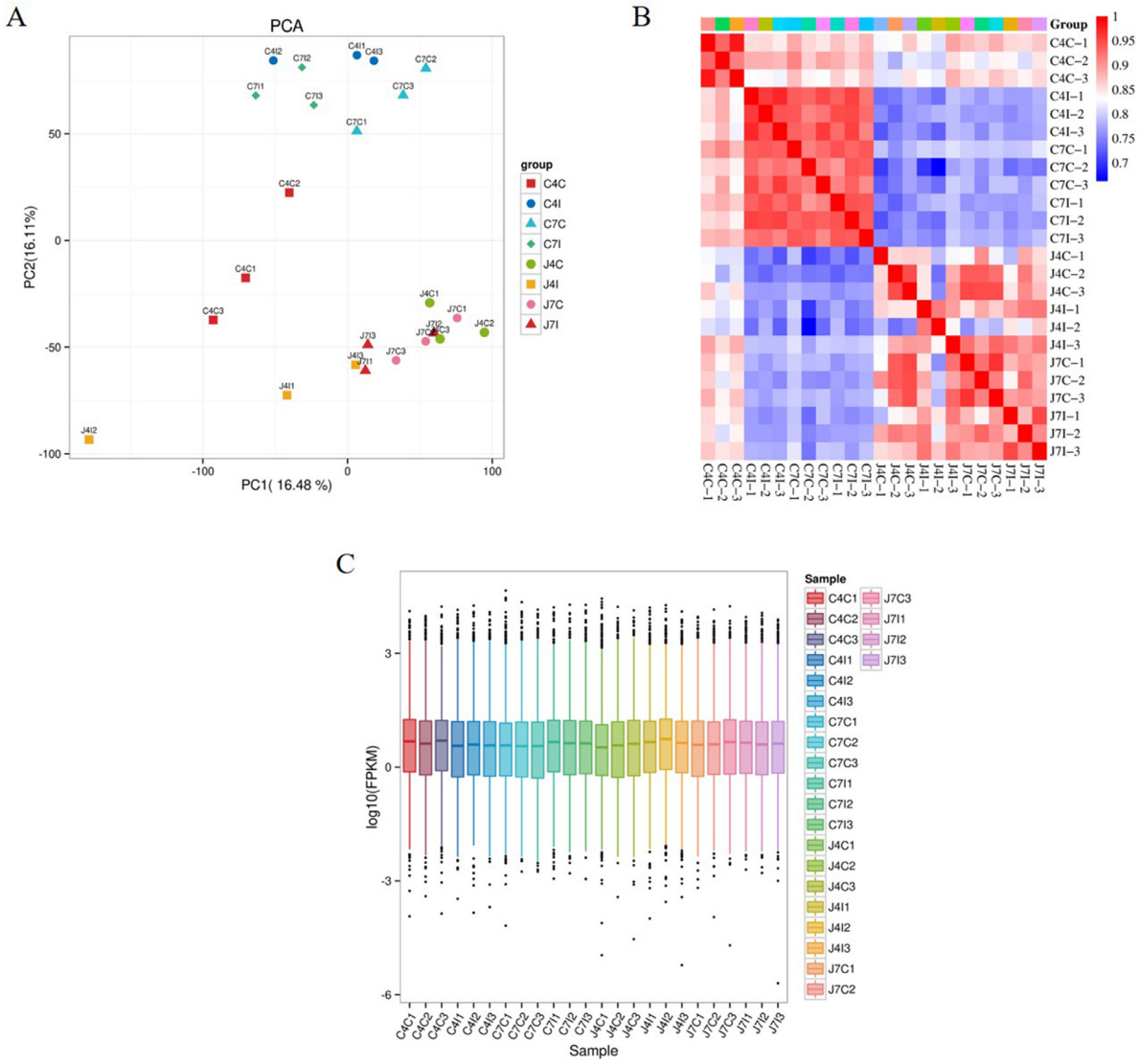

Figure 2. Distribution of gene expression. (A) Principal Component analysis of sample group replicate; (B) Correlation heat map between samples; (C) Box plot sample gene expression distribution.

utilized chicken jejunum and cecum tissues infected with *Eimeria maxima* at both 4 and 7 d postinfection to identify the differentially expressed genes in both infected and non-infected tissues (Figure 4). The collection of genes acquired in differential expression analysis represented the control group versus the treatment group. Fold change (**FC**) represents the gene expression ratio between 2 samples, and genes with significant differences in expression were identified using criteria of |log2Fold Change|≥ 0.5 and a *P*-value < 0.05. Using a threshold | Log2Fold change|≥0.5, this study analyzed differential expression to categorize genes as up-regulated and down-regulated in samples collected 4 and 7 d postinfection compared to the control group. Thus, the study pinpointed 823 up-regulated and 737 down-regulated DEmRNAs in the comparison between the J4C_vs_J4I groups, while in the J7C_vs_J7I contrast groups there were 710 up-regulated genes and 368 down-regulated genes (Figure 4A). Likewise, within the chicken jejunum, a total of 1112 DEmRNAs exhibited significant differences in the J4I_vs_J7I comparison group, including 572 genes were up-regulated, while 540 genes were down-regulated, as observed in the samples collected at 4 and 7 d postinfection (Figure 4A).

In another way, the analysis of DEmRNAs in the chicken cecum was conducted for samples collected at 4 and 7 d postinfection with *Eimeria maxima* and in the control group samples. In the C4C_vs_C4I comparison, 1424 and 1930 genes were up-regulated and down-regulated, while in the C7C_vs_C7I comparison, 77 genes were up-regulated and 191 genes were down-regulated (Figure 4A). Furthermore, a total of 623 DEmRNAs were detected in the C4I_vs_C7I comparison, comprising 290 up-regulated and 333 down-regulated genes (Figure 4A). The reading fraction varied significantly among chicken jejunum and cecum samples infected with *Eimeria maxima* on different postinfection days.

The analysis was made between the differentially expressed genes in the jejunum and cecum regarding

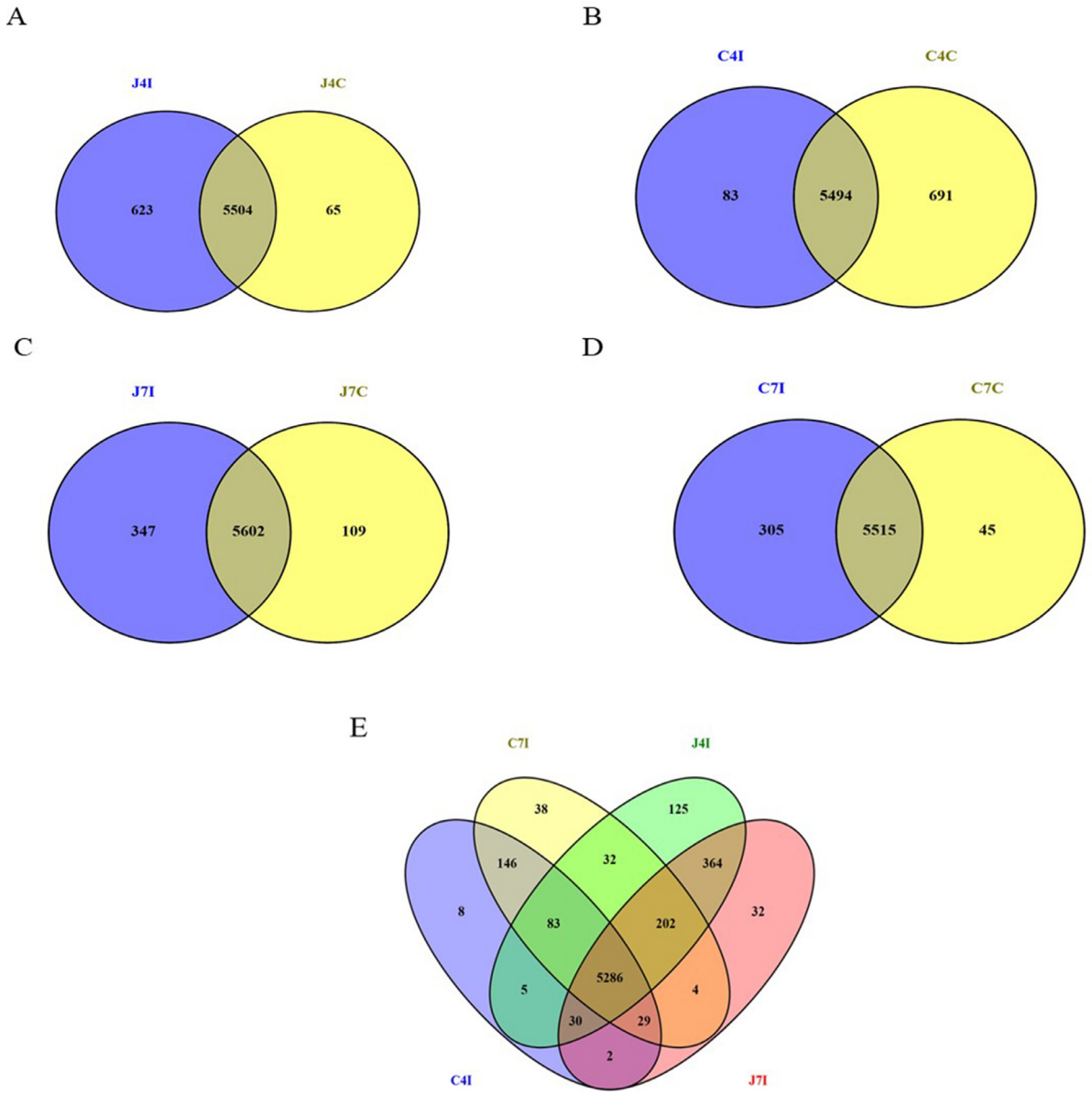

Figure 3. Venn diagram of gene expression at 4 and 7 d postinfection and control group samples. (A) J4I and J4C; (B) J7I and J7C; (C) C4I and C4C; (D) C7I and C7C; (E) J4I, J7I, C4I and C7I.

unique genes and those shared across various comparison groups. Thus, the Venn diagram showed that 40 DEmRNAs were shared in J4I_vs_J4C, J7I_vs_J7C and J4I_vs_J7I comparison groups (Figure 4B). The 33 DEmRNAs were shared equally among the comparison groups of the chicken cecum for both 4 and 7 d postinfection and the control group (Figure 4C).

Clustering Genes and Volcano Plot Analysis

Differential expression genes in comparison groups were analyzed during infection with *Eimeria maxima* at different time points. The potential function of mRNA, differentially expressed genes were identified by mRNA sequence analysis (|log2fold change|>1.5, P<0.05) and clustered based on their expression profiles. The DEmRNAs from the comparison sample groups were combined to form the differential gene set. This allows cluster analysis across multiple experimental groups using distinct gene sets. The FPKM values of genes used for hierarchical heat map clustering genes and similar expression patterns in the heat map are gathered together (Figures 5A−5B). DEmRNAs of the jejunum group samples from both 4 and 7 d postinfection and the control group are shown (Figure 5A), and these genes that exhibited similar differential expression patterns were clustered together. In another way, the cecum sample group at 4 and 7 d postinfection and control groups performed DEmRNAs with the same expression mode clustered together (Figure 5B).

Volcano plots can directly present the overall distribution of DEmRNAs between 2 comparison samples. This study used fold change and P value to analyze and evaluate DEmRNAs. Fold change |log2FC|≥ 1.5 and significant level P<0.05 were thresholds to construct volcano plots. The volcano plots indicated that DEmRNAs analysis of jejunum samples in 2 groups revealed 426 up-regulated and 265 down-regulated genes in J4I_vs_J4C

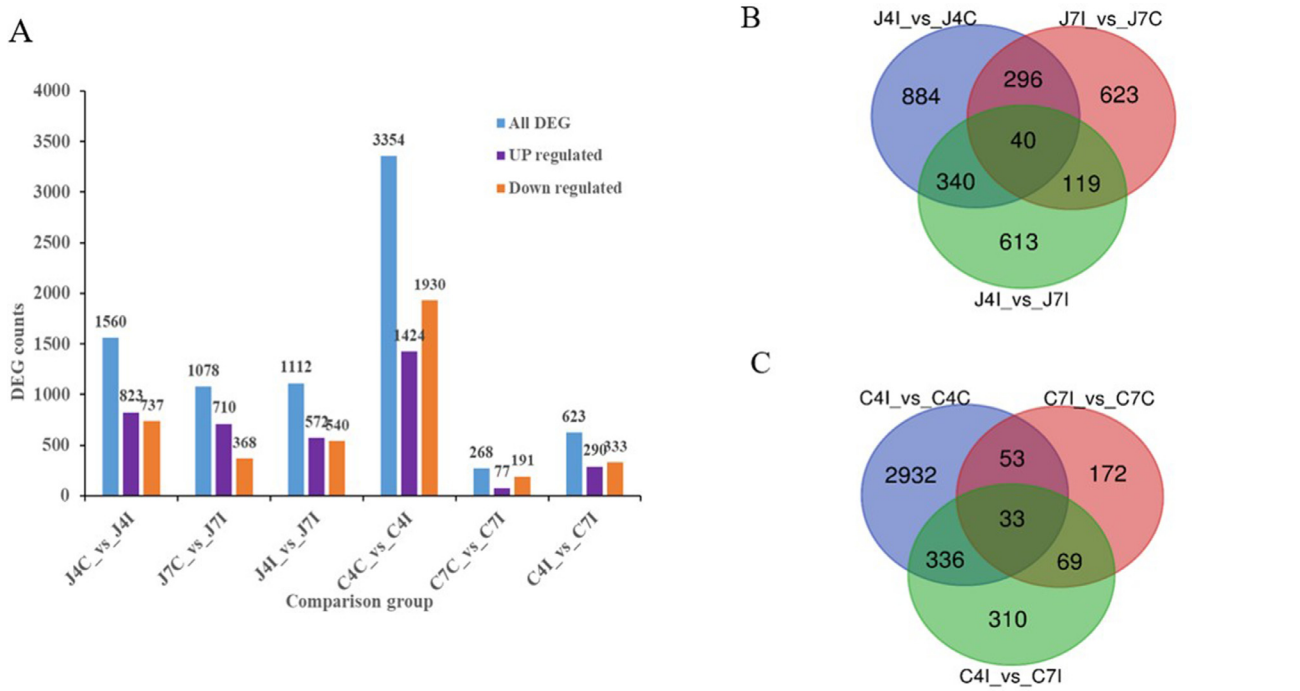

Figure 4. Deferentially expressed gene statistics of chicken jejunum and cecum comparison group samples infection with *Eimeria maxima*. (A) Differential expression gene up and down-regulated genes. Purple and red color represents differential expression gene for up and down-regulated respectively while, the Blue represents all DEmRNAs counts; (B) Venn diagram of DEmRNAs in jejunum sample group; (C) Venn diagram of DEmRNAs in cecum sample group.

(Figure 5C). Similarly, the DEmRNAs, 285 up-regulated genes and 70 down-regulated genes were identified in the J7I_vs_J7C comparison group (Figure 5D). From DEmRNAs in the jejunum, the 426 up-regulated gene and 285 up-regulated gene were identified in the J4I and J7I comparison group, respectively, indicating that the number of up-regulated DEmRNAs were relatively increased at 4 d postinfection with *Eimeria maxima*. These mRNA sequence results confirmed the differential expression of several genes at 4 and 7 d postinfection of chicken jejunum with *Eimeria maxima*, such as *SLC7A5, IL1R2, GLDC, ITGB6, ADAMTS4, IL1RAP, TNFRSF11B, IMPG2, WNT9A,* and *FOXF1.*

Furthermore, volcano plots analysis in cecum samples in two-time points postinfection 233 up-regulated and 847 down-regulated genes in C4I_vs_C4C, whereas DEmRNAs 19 and 42 up and down- regulated genes were observed in C7I_vs_C7C comparison group (Figures 5E−5F). Sequencing analyzed in high throughput technology showed that differentially expressed genes related to host immune response were observed in 4 and 7 d postinfection of the cecum sample group, for example, *FSTL3, RBP7, CCL20, DPP4, PRKG2, TFPI2, and CDKN1A.* The result showed that immune-related differentially expressed gene varies in chicken jejunum and cecum due to site specifically infection of the host with *Eimeria maxima* coccidian.

Immune Responses and Gene Co-Expression Network Analysis

The analysis of regulatory networks for mRNA-miRNA interaction revealed that the DEmRNAs played essential roles in the host's inflammatory and immune responses during *Eimeria* parasite infection. In order to gain deeper insights into the functional mechanisms of immune-related differentially expressed mRNAs integrated with miRNAs, this study utilized the miRDB website to predict potential mRNA-miRNA network interaction pairs characterized by negative expression patterns during *Eimeria maxima* infection of chicken intestinal tract (Figure 6). Host immune response DEmRNAs, *SLC7A5, IL1R2, GLDC, ITGB6, ADAMTS4, IL1RAP, TNFRSF11B, IMPG2, WNT9A, FOXF1* were analyzed with miRNA predicted regulatory network interaction between 4 and 7 d postinfection of chicken jejunum (Figure 6A). In addition, DEmRNAs related with host immune response, *FSTL3, RBP7, CCL20, DPP4, PRKG2, TFPI2*, and *CDKN1A* were analyzed to predict the mRNA-miRNA network interaction between 4 and 7 d postinfection of chicken cecum (Figure 6B). The *Eimeira maxima* infection response of chicken jejunum and cecum examined the DEmRNAs with the most significant mean difference, with Log2FC>1.5, during *Eimeria maxima* infection at 4 and 7 d. Subsequently, we selected the top 50 genes and visualized them in a heat map (Figure 6C−6D).

GO Enrichment Annotation Analysis for DEG

Gene set enrichment analysis (**GSEA**) was conducted using the gene ontology (**GO**) database to delve into functional analysis during chicken *Eimeria maxima* infection at 4 and 7 d postinfection. The DEmRNAs were grouped into three GO categories: molecular

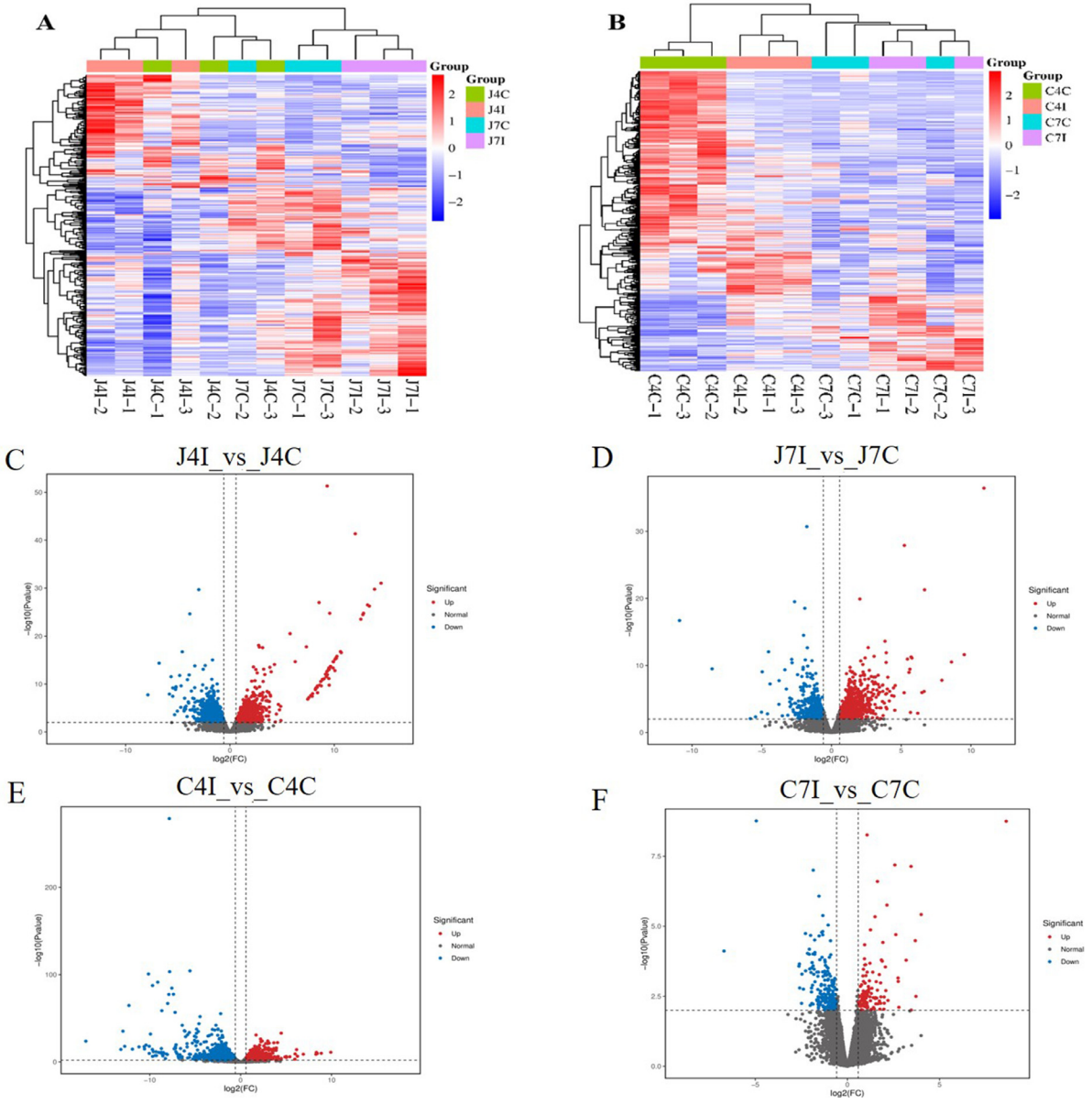

Figure 5. Hierarchical clustering heat map and volcano plot of differentially expressed genes. (A) Heat map clustering of jejunum DEG at 4 and 7 d postinfection and control group with *Eimeria maxim*, (B) Heat map of cecum DEmRNAs at 4 and 7 d postinfection and control group with *Eimeria maxima*, (C) Volcano plot on differential expression genes in J4I_vs_J4C; (D) Volcano plot on differential expression genes in J7I_vs_J7C; (E−F) Volcano plot on differential expression genes in cecum sample group C4I_vs_C4C (E) and C7I_vs_C7C (F). (In volcano plot, each dot represents a gene. X-axis: log2Fold change of expression; Y-axis: -log10 (*P* value), Blue dotes down-regulated while red dots up-regulated genes.

function, biological process, and cellular components, for both the jejunum and cecum sample groups (Figure 7).

The GO term analysis the jejunum at 4 d postinfection revealed various biological processes, including the host's immune response and other functions. These processes encompass the inactivation of MAPK activity, cellular responses to cytokine stimuli, interleukin-1 responses, inflammatory responses, toll-like receptor 10 signaling pathways, cytokine-mediated signaling pathways, immune response functions, organic acid metabolic processes, and drug metabolic processes (Figure 7A). Likewise, the analysis of biological processes at 7 d postinfection revealed the regulation of cell population proliferation, inflammatory responses, extracellular matrix organization, immune responses, positive regulation of the ERK1 and ERK2 cascades, and the Wnt signaling pathway (Figure 7B).

Also, the GO enrichment of DEGs was applied at the 4 d postinfection stage in the cecum. This analysis identified biological processes (**BP**) related to the inflammatory response, immune response, immune system processes, carbohydrate derivative metabolic processes, responses to oxidative stress, and wound healing (Figure 7C). Furthermore, at the 7 d postinfection stage in the cecum sample group, various biological processes (BP) were identified, including the Wnt signaling pathway, response to bacteria, defense against viruses, negative regulation of angiogenesis, and cellular responses to tumor necrosis factor (Figure 7D).

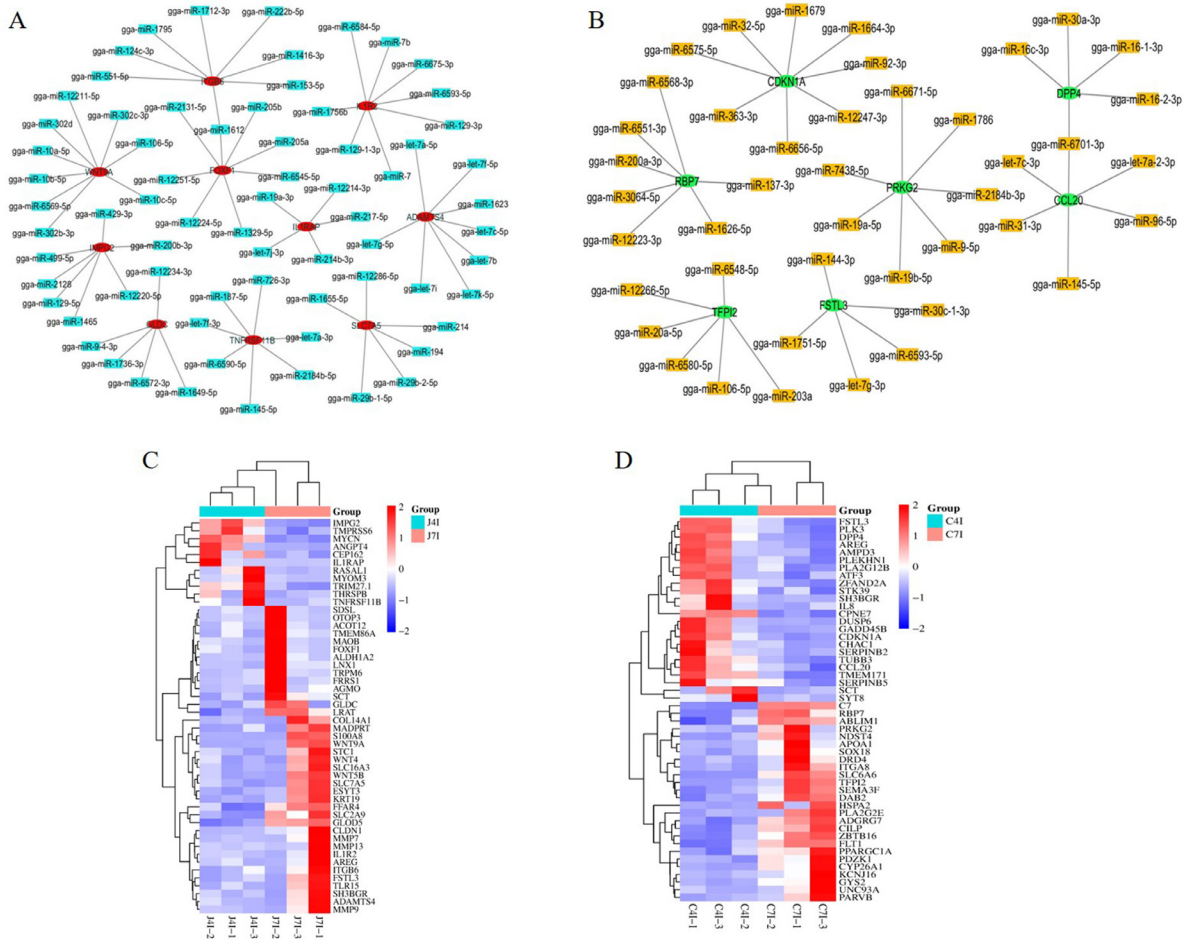

Figure 6. Networks of DEmRNAs-DEmiRNAs involved in the immune response to *Eimeria maxima* infection predicted by miRDB. (A) J4I_vs_J7I network of DEmRNAs-DEmiRNAs generated using Cytoscape; (B) C4I_vs_C7I network of DEmRNAs-DEmiRNAs generated using Cytoscape, (C−D) Heat map of DEG in J4I_vs_J7I (C) and C4I_vs_C7I (D).

Top KEGG Pathway Enrichment Analysis on DEGs

Enrichment factors and the Fisher test were applied to determine the enrichment degree and significance of the pathway. Among the KEGG gene sets that exhibited up-regulation in the comparison between J4I_vs_J7I, the most significantly enriched pathways included steroid biosynthesis, drug metabolism via cytochrome P450, metabolism of xenobiotics by cytochrome P450, ascorbate and aldarate metabolism, retinol metabolism, porphyrin, and chlorophyll metabolism, and the *PPAR* signaling pathway (Figure 8A). Whereas the KEGG pathway in the down-regulated the gene set, the fincani anemia pathway and homologous recombination were analyzed in J4I_vs_J7I (Figure 8A). Contrarily, in the comparison between C4I_vs_C7I, KEGG enrichment analysis revealed that up-regulated genes were related with starch and sucrose metabolism while the down-regulated genes were related to ribosome biogenesis in eukaryotes (Figure 8B). The number of up-regulated genes in the KEGG pathway enrichment analysis for the J4I_vs_J7I comparison group was greater than that observed in the C4I_vs_C7I comparison group during *Eimeria maxima* infection. This suggests that the KEGG functional enrichment pathways were more prominently detected up-regulated genes in the jejunum compared to the cecum in the 4 and 7 d postinfection with *Eimeria maxima*. This observation is consistent with the well-known fact that *Eimeria* parasites exhibit site-specificity in their damage to the chicken mucosal epithelium of chickens.

DISCUSSION

Coccidiosis of the chicken is an enteric severe parasitic disease that causes high economic loss in the commercial poultry industry. Understanding the basis of host resistance to *Eimeria maxima* is vital for poultry production. *Eimeria* parasites have a complex developmental life cycle with two stages: the exogenous and the endogenous phases (Allen and Fetterer, 2002; López-Osorio et al., 2020). The initial infective unit of the *Eimeria* parasite is the sporozoite stage (Geetha and Palanivel, 2018). The sporozoite is the beginning and the end of any coccidian's life cycle, and the infective forms found in sporulated oocysts are the result of protoplasm segmentation (Augustine, 2001; López-Osorio et al., 2020).

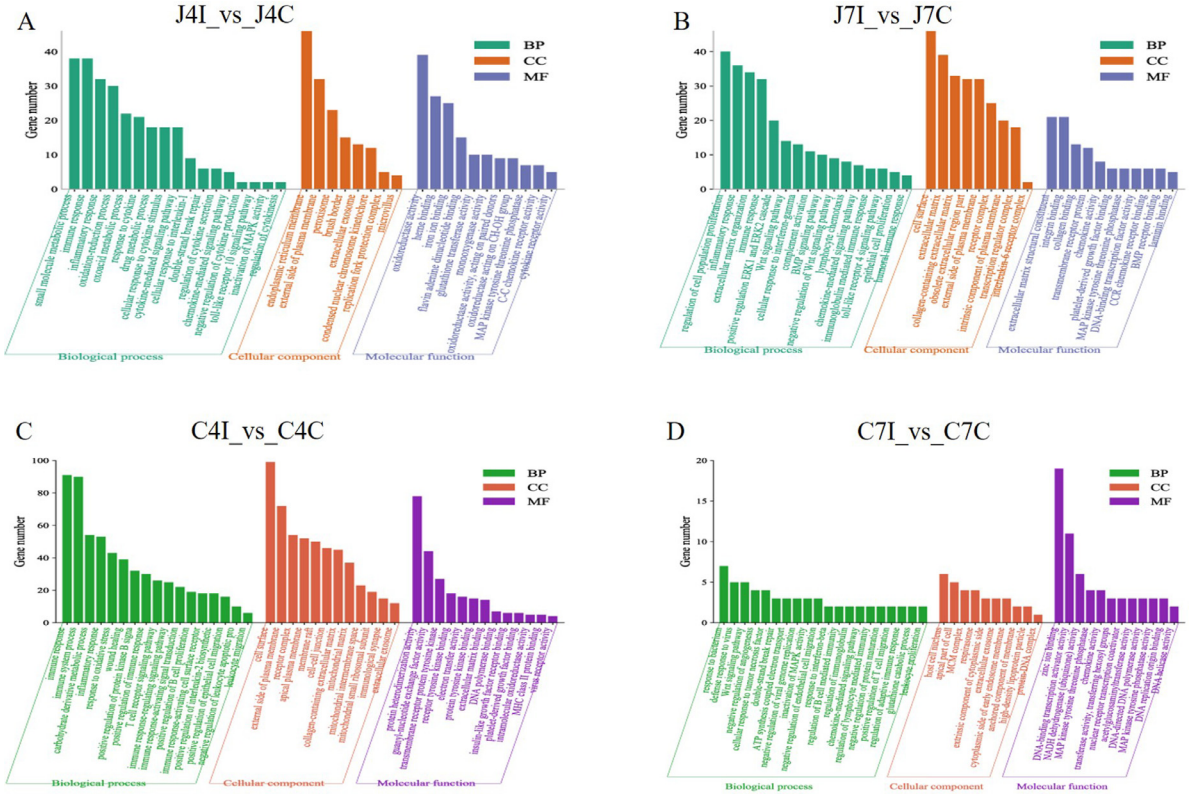

Figure 7. The most gene ontology functional enrichment analysis of DEmRNAs in chicken jejunum and cecum infected and none infected comparison group with *Eimeria maxima*. (A) GO classification in J4I_vs_J4C differential expression genes; (B) GO classification in J7I_vs_J7C differential gene expression. (C) GO classification in C4I_vs_C4C differential expressed genes; (D) GO classification in C7I_vs_C7C differential expressed genes. The X-axis is GO term and the Y-axis is GO term level of significance enrichment number of genes.

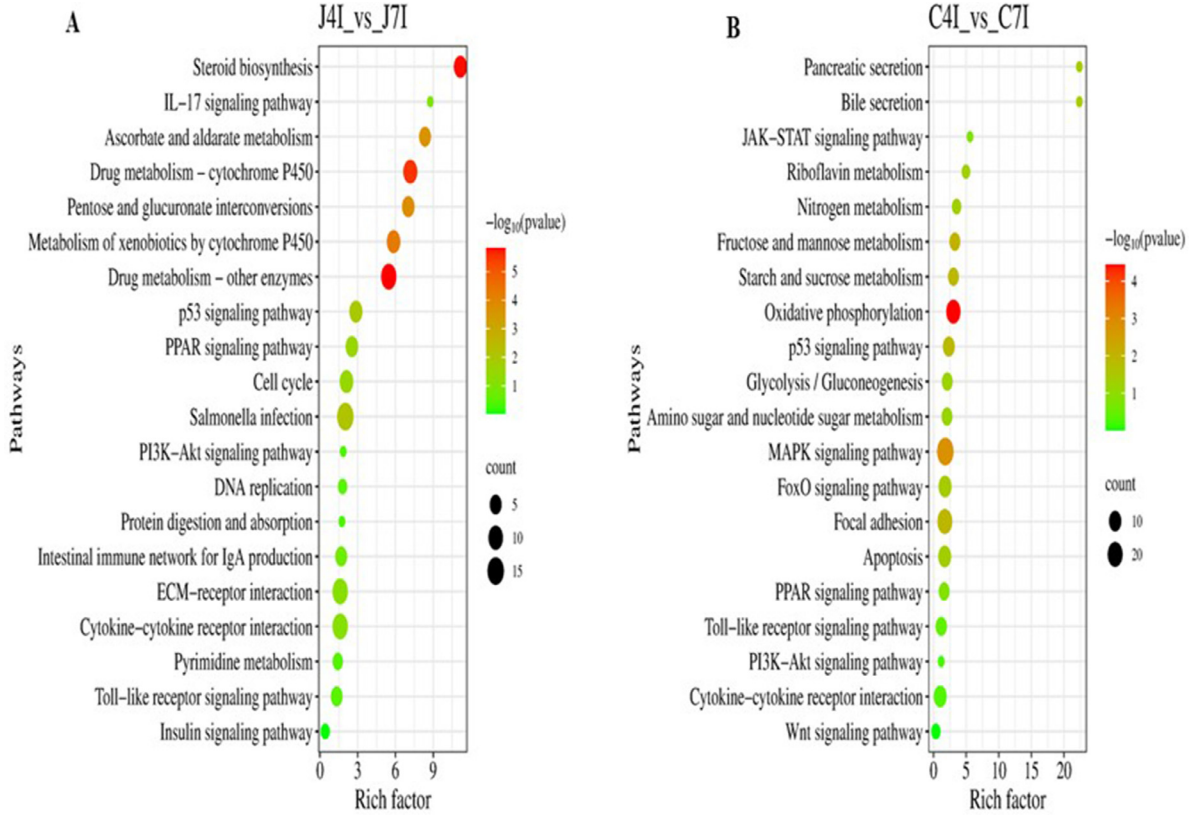

Figure 8. Gene statistics enrichment pathway of up and down-regulation (A) KEGG pathway of J4I_vs_J7I (B) KEGG pathway of C4I_vs_C7I.

Eimeria maxima coccidian infection causes significant chicken gut damage, including inflammation, hemorrhage, and diarrhea, resulting in high mortality (Yin et al., 2014). Herein, this study used 4 and 7 d postinfection at the early age of Ethiopian Horro chicken to investigate host response immunity gene against *Eimeria maxima* infection. The mRNA sequencing technology (mRNA-Seq) contributes to enhancing our comprehension of host-parasite interactions in both jejunum and cecum tissues, shedding light on their underlying immunological functions. Our study utilized a comparative and integrated analysis of DEmRNAs in the transcriptome at 4 and 7 d after chicken jejunum and cecum infections. The mRNA sequencing technology (**mRNA-Seq**) advances an understanding of the host-parasite interactions in the jejunum and cecum tissue and further reveals the fundamental immunological function. DEmRNAs transcriptome analysis was conducted, comparing and integrating analyzed tissues from chicken jejunum and cecum at 4 and 7 d postinfection. The number of DEmRNAs in 4 d postinfection was relatively more observed than 7 d postinfection of jejunum and cecum tissues. Thus, the result suggested that the timing of *Eimeria maxima* infection plays a crucial role in shaping the host's immune gene response to coccidiosis. The study by (Kim et al., 2022) showed that after 7 d postinfection of chicken with *Eimeria* parasite, the intestinal damage alleviated and, the host response gene decreased; finally, at 21 d postinfection less significant difference was observed. Although, previous study reported that before 8 d postinfection, chickens with damaged intestinal mucosa caused by *Eimeria* coccidian exhibit inflammatory symptoms while the host's intestinal inflammation response following infection showed decreases after 8 d (Morris et al., 2007). Similarly, the general analysis observation of the immune-related gene in this study was the large number of up-regulated genes found at 4 d postinfection, compared with 7 d postinfection.

Differently, the study examined the number of DEmRNAs that were either up-regulated or down-regulated in response to *Eimeria maxima* infection in the cecum. Interestingly, a higher number of down-regulated genes was observed compared to up-regulated genes, in the cecum both at 4 and 7 d following *Eimeria maxima* infection. The gene expression profiles in the jejunum and cecum samples exhibit variations in response to *Eimeria maxima* infection at different time points. Consequently, the temporal progression of infections in both the jejunum and cecum by *Eimeria maxima* substantially influences the expression of immune-related genes in the host. The host immune response to *Eimeria* coccidiosis was appears to vary due to time point infection (Bremner et al., 2021). The research revealed the impact of the regulatory functional network interaction between immune-related mRNA and miRNAs on *Eimeria maxima* infection in the chicken jejunum. Our previous study revealed that the expression of miRNAs can altered the response to *Eimeria maxima* infection, and they modulate the expression of their target genes in jejunum infection (Jebessa et al., 2023). Subsequently, the study investigated the effect of ten selected representative DEmRNAs namely; *SLC7A5, IL1R2, GLDC, ITGB6, ADAMTS4, IL1RAP, TNFRSF11B, IMPG2, WNT9A, and FOXF1*. Different tissues types within the chicken may respond differently to coccidian infections. mRNA-miRNA regulatory networks can vary between tissues, influencing the local immune response and tissue-specific changes the infection induces (Fan et al., 2022; Gebert and MacRae, 2019; Guo et al., 2014). Hence, from this study, DEmRNA-miRNA regulatory network interaction is involved in the immune response during *Eimeria maxima* infection of the chicken cecum tissue (*FSTL3, RBP7, CCL20, DPP4, PRKG2, TFPI2,* and *CDKN1A*). mRNA-miRNA interactions are involved in modulating these pathways, potentially affecting the progression of the infection and the host capacity to mount an effective response (Andrés-León et al., 2017).

The study revealed that multiple up and down-regulated DEmRNAs were associated with the significantly enriched GO category and KEGG pathway. The immune system detects the presence of coccidia parasites and triggers an inflammatory response. Immune cells release signaling molecules, such as cytokines and chemokines, which attract other immune cells to the infected site (Györffy et al., 2016). The GO category biological process showed DEmRNAs functional enrichment in several processes during a 4-d postinfection period in the jejunum. These processes included small molecule metabolic processes, immune response, inflammatory response, and oxidative reduction processes. At 7 d postinfection, the jejunum exhibited regulation of cell population proliferation, inflammatory response, extracellular matrix organization, and immune response in biological process. Previous studies have revealed that the host cells could endoplasmic reticulum stress because of *Eimeria* parasite infection of the intestinal tract, leading to an unfolded protein response (Galluzzi et al., 2017). Coccidian infections can affect various cellular pathways, including those related to cell proliferation, apoptosis, and inflammation (Zhu et al., 2021). The pathway gene analysis from intestinal following *Eimeria maxima* infection in chicken showed that many modulated genes were related to host immune response, *IL10, IRF8, TGFBR3, TNFSF8*, and small molecule metabolic process, *SAT1, GALT, DCK, GCSH, AHCYL2* genes were involved. The pathway analysis of intraepithelial lymphocytes following *Eimeria maxima* infection in chicken indicated that apoptosis-related genes, *MAPK, JAK/STAT*, interleukin and TLR signaling pathways involving innate and adaptive immune response (Kim et al., 2008).

Furthermore, the GO categories analysis related to biological processes identified significant DEmRNAs in the cecum at 4 and 7 d postinfection with *Eimeria maxima*. These DEmRNAs were functionally associated with host pathways, including the immune system process, cellular response to tumor necrosis factor, Wnt signaling pathway, carbohydrate derivative metabolic

process, toll-like receptor signaling pathway and inflammatory response. The study reported that the related *TLRs* revealed new approaches to understanding the fundamental mechanisms of the immune system, the pattern of resistance against disease, and the key roles they play in the defense mechanism against infectious diseases (Brownlie and Allan, 2011). The data on *TLRs* such as *TLR4*, *TLR5*, and *TLR7* are widely available, however, very little information has been published on TLR10 (Balachandran et al., 2015). Most of the *TLR10* expression data have been obtained in cultured cells by using recombinant expression proteins (Kim et al., 2010; Balachandran et al., 2015; Behzadi et al., 2021). Previous studies revealed that the pathogens provoke local inflammatory responses, including the production of the pro-inflammatory cytokine; for example, lipopolysaccharide-induced *TNF* factor (*LITAF*), *IL-1β, IL*-6 and several *β*-defensins were known as up-regulated during inflammation (Van Dijk et al., 2007; Su et al., 2017).

According to the data from target prediction, the KEGG pathway analysis revealed significant enrichment of specific pathways for these targets. These pathways include focal adhesion, cytokine-cytokine receptor interaction, intestinal immune network for IgA production, arginine biosynthesis, and DNA replication. KEGG enrichment pathways are crucial in host immune-related gene response and metabolic activities upon coccidia infection. During *Eimeria maxima* infection of the cecum, the most gene category represented by the highly differentiated host genes suggested enrichment in immune responses, MARK signaling pathway, vascular smooth muscle contraction, and protein processing in the endoplasmic reticulum (Li et al., 2019). The KEGG pathway enrichment gene up-regulated set in J4I_vs_J7I is higher than KEGG pathway enrichment in C4I_vs_C7I comparison group under *Eimeria maxima* infection. It suggested that the KEGG functional enrichment pathway was revealed more at the up-regulated gene in the jejunum than the cecum during *Eimeria maxima* infection. The *Eimeria* parasite is recognized for its site-specific damage to the mucosal epithelium in chickens. Specifically, *Eimeria maxima* coccidia invade the middle jejunum and ileum, resulting in the destruction of the enteric mucosa and the occurrence of severe lesions in chickens (Witcombe and Smith, 2014; Ghareeb et al., 2022).

CONCLUSIONS

The study identified DEmRNAs related to the immune system in response to *Eimeria* coccidian infection of chickens jejunum and cecum, aiming to enhance our understanding of transcriptomic functional enrichment in host genes. Upon closer investigation, the immune-related up and down-regulated DEGs in chicken jejunum and cecum were observed at 4 and 7 d postinfection. The functional pathway and expression pattern of host immune-related DEmRNAs varied at 4 and 7 d postinfection under *Eimeria* coccidian. Significant enrichment of DEmRNAs between the chicken jejunum and cecum was observed in KEGG and GO functional pathways. These pathways may have a role in the chicken's response to *Eimeria maxima* infection. Altogether, the comprehensive analysis of DEmRNA profiles includes examining expression patterns, functional annotations, and regulatory networks of potential immune-related mRNAs, along with the prediction of target miRNAs. This analysis involved the host's immune response to *Eimeria maxima* infection. The study might enhance the understanding of host-parasite interaction molecular mechanisms.

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Author Contributions: EJ conducted the data analysis for the experiment and prepared the manuscript draft. QN and OH were responsible for the study's design and manuscript editing. YX and BC contributed to conducting part of the experiments. SFB, MDT, MG and FB reviewed the manuscript. All authors have read and approved the final manuscript's publication.

DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.103716.

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