Micro-RNA expression profile of chicken small intestines during *Eimeria necatrix* infection

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ABSTRACT *Eimeria necatrix* is a high pathogenic pathogen second to *Eimeria tenella* causing chicken coccidiosis. However, the precise underlying molecular mechanisms of interaction between *E. necatrix* and chickens are not fully understood. Accumulating evidences suggest that micro-RNAs (**miRNAs**) play pivotal regulatory roles in various diseases, including parasitic diseases. In the present study, the expression profile of miRNAs in Hy-line variety white chicken small intestines infected with *E. necatrix* was studied by using deep sequencing. A total of 35 miRNAs (including 16 significantly upregulated and 19 significantly downregulated miRNAs) were significantly differentially expressed (**DE**) in infected tissues at 108 h postinfection (pi). Real-time polymerase chain of 10 miRNAs (including 5 upregulated and 5 downregulated) randomly selected successfully confirmed the effectiveness of deep sequencing. Target prediction showed that 4,568 mRNAs could be regulated by 21 (including 12 upregulated and 9 downregulated) of 35 differentially expressed miRNAs. Functional analysis indicated that target genes of these differentially expressed miRNAs would be involved in pathways related to infection of *E. necatrix*, including cell differentiation, adhesion, proliferation, and apoptosis (e.g., MAPK signaling pathway and PPAR signaling pathway). To our best knowledge, this is the first study on the miRNA expression profile of small intestines during *E. necatrix* infection, and the findings in the present study suggested that these DE miRNAs would play important regulatory role in the interaction between *E. necatrix* and chicken intestines.

Key words: *E. necatrix*, miRNAs, expression profile, chicken, small intestine

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INTRODUCTION

Coccidiosis, caused by *Eimeria* spp., is a common disease in chickens of different ages, responsible for great economic losses of the poultry industry (Shirley et al., 2005; Dalloul and Lillehoj, 2006; Michels et al., 2011; Blake and Tomley, 2014). Over USD $3 billion has been spent for annual controlling chicken coccidiosis globally (Dalloul and Lillehoj, 2006). Of 7 recognized *Eimeria* species in chickens, *Eimeria necatrix*, a high pathogenic pathogen second to *Eimeria tenella* mainly colonized in the midsegments of the small intestines, causes the mucosal lesion and hemorrhage in parasitic sites, and affects metabolism and the absorption of nutrients, resulting in hemorrhagic diarrhea, weight loss, and even death of infected birds (Montes et al., 1998; Thebo et al., 1998; Morris and Gasser, 2006; Jadhav et al., 2011; Chen et al., 2016). In addition, *E. necatrix* is also a common risk factor of necrotic enteritis in chickens coinfecting with *Clostridium perfringens* (Dinh et al., 2014). However, the underlying molecular mechanisms of interaction between *E. necatrix* and chickens are still unclear.

Micro-RNAs (**miRNAs**), a group of small non-coding RNA with approximately 22 nucleotides in size, are conservatively found in animals and humans (Bartel, 2004; Kabekkodu et al., 2018). Increasing evidence indicated that miRNAs played pivotal regulatory roles involving into interaction between hosts and pathogens (e.g., bacteria, viruses, and parasites) through repressing or degrading target mRNAs at the posttranscriptional level (Holla and Balaji, 2015; Hu et al., 2018; Keshavarz et al., 2018). The profiles of miRNA expression have been investigated in chicken intestinal tissues after infections with some *Eimeria* species (e.g., *Eimeria papillata*, *Eimeria maxima*,

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Eimeria acervulina) separately or coinfection with *C. perfringens* (Al-Quraishy et al., 2011; Dinh et al., 2014; Hong et al., 2014; Sun et al., 2016). For example, 4 miRNAs (miR-1699, miR-7477-5p, miR-1451-5p, and miR-1608) were upregulated in chicken duodenums infected with *E. acervulina*, and the target gene CYP27A1 of miR-1699 and miR-1608 was downregulated at the protein level (Sun et al., 2016). Further studies showed that the decrease expression of the protein CYP27A1 was responsible for local cholesterol accumulation and contributed to the epithelial proliferation, suggesting the important regulatory role of both miRNA (miR-1699 and miR-1608) during infection of *E. acervulina* (Chiang, 1998; Li et al., 2007; Sun et al., 2016). However, there have been no public reports on miRNA expression in the intestines during *E. necatrix* infection before the present study. Herein, we investigated the profile of miRNAs expression in Hy-line variety white chicken midintestines infected with *E. necatrix*.

MATERIALS AND METHODS

Ethics Approval and Consent to Participate

All the protocols of this study were approved by the ethics committee of Northwest A&F University and were in compliance with the animal ethics requirements of the People's Republic of China. All effort was made to minimize suffering and damage of experimental chickens.

Oocyst and Animal

E. necatrix oocysts were gifted from Foshan Standard Biological Technology Co., Ltd., China, and passaged in 10-day-old Hy-line variety white chickens under the specific pathogen-free condition. The sporulated oocysts were purified by using the saturated saline floatation and treated with 5% bleach for 20 min on ice before infection.

One-day-old Hy-line variety white male chickens used in the present study were purchased from Giant Long Company (Shaanxi, China) and reared in a pathogen-free laboratory. The chickens were divided into 2 groups at 10-day-old, namely experimental (S) and control (N) groups, with 20 chickens in each group. The group S was orally infected with 8,000 oocysts, while the group N was orally administered with the same volume of PBS. The chickens of 2 groups were separately reared with feeds free of anticoccidial drugs and adequate lukewarm boiled water. To monitor the infection of chickens, the stool of each chicken was examined, and the midintestinal mucosal scrapings of 2 chickens were microscopically observed at the 2 D interval.

Preparation of Samples and Extraction of Total RNA

At 108 h post-infection (**pi**), 3 chickens from each group were anesthetized with the anhydrous ether through inhalation anesthesia, and the midsegments of each chicken small intestine in both groups were separately collected. After discarding intestinal contents, the tissues were washed with the nuclease-free PBS solution 3 times, and then 600 μL lysis/binding buffer and 30 μL miRNA homogenate additive were added into each washed tissue. The total RNA for miRNA sequencing was extracted using mirVana miRNA ISOlation Kit (Ambion-1561) according to the manufacturer instructions and stored at −70°C. The RNA integrity was assessed by using the Bioanalyzer 2100 RNA-6000 Nano Kit (Agilent Technologies, CA), and RNA samples with the RNA integrity number value >7.0 were used for miRNA sequencing. To remove RNase, all the reagents and instruments were treated with the 1/1,000 diethyl pyrocarbonate solution.

Small RNA Library Construction and Sequencing

The qualified RNA samples were separated in 6% PAGE and the RNAs with the length of 22 to 30 nt were recovered and purified. A total of 5 μL purified RNAs was used to construct the small RNA (**sRNA**) sequencing library by using the TruSeq Small RNA Library Prep Kit (Illumina, San Diego, CA) according to the manufacturer's instructions. The sRNA library was validated by using an Agilent Technologies 2100 Bioanalyzer to check its size and purity. Subsequently, the qualified sRNA library was sequenced on the Illumina HiSeq 2500 sequencing platform (OE biotech, Shanghai, China).

Bioinformatic Analysis

The raw FASTA data obtained by Illumina Hiseq sequencing was checked and processed according to the methods of Zhao et al. (2019). The raw data were deposited into Sequence Read Archive within NCBI, with the accession number of SRR9213835-SRR9213840. The clean reads were mapped to the reference genomic sequences of *Gallus gallus* (http://www.mirbase.org/cgi-bin/browse.pl?org=gga). The known miRNAs were identified by alignment with miRBase (http://www.mirbase.org/) using Bowtie (https://sourceforge.net/projects/bowtie-bio/files/bowtie2/2.3.5.1/), whereas the unannotated sequences were analyzed by using miRdeep2 (Friedländer et al., 2012) to predict novel miRNAs.

Analysis of Differentially Expressed miRNAs

To analyze the expression level of differentially expressed (**DE**) miRNAs during *E. necatrix* infection, the raw expression data were normalized using the transcript per million method (Sun et al., 2014), and the differential expression was calculated by using the DESeq2 R package, with the P-value < 0.05 and fold change (**FC**) > 1.3 considered as significant difference.

Table 1. Statistics of small RNA sequences of the small RNA libraries.

Sample	Raw reads	Low quality	Clean reads	Clean reads unique	Aligned reads	Aligned (%)
N1	23,162,567	1,006,082	22,156,485	882,657	20,968,264	94.64
N2	20,868,174	958,083	19,910,091	729,795	18,910,028	94.98
N3	18,150,667	1,002,476	17,148,191	534,218	16,335,233	95.26
S1	29,958,303	1,054,031	28,904,272	804,930	27,372,901	94.7
S2	20,809,713	834,132	19,975,581	519,646	19,070,337	95.47
S3	23,221,673	711,436	22,510,237	555,719	21,502,677	95.52

Verification of miRNA Expression by Using Quantitative Real-Time PCR

Ten (including 5 upregulated and downregulated miRNAs) significantly DE miRNAs were randomly selected for quantitative real-time PCR (qRT-PCR) to validate the deep sequencing data. The midsegments of chicken small intestines from 3 samples in both N and S groups were collected at 108 h pi, and the total RNA sample from each sample was extracted by using the Trizol reagent (Invitrogen, Carlsbad, CA) and chloroform-isopropyl alcohol (Liu et al., 2018). cDNA samples were synthesized by using the Mir-XTM miRNA First-Strand Synthesis Kit (Clontech, Mountain View, CA). Each qRT-PCR reaction was performed in a 25 μL mixture containing 2 μL cDNA, 0.5 μL ROX Dye (50X), 0.5 μL miRNA-specific forward primer (Supplementary Table 1), 0.5 μL mRQ 3′primer, and 12.5 μL SYBR Advantage Premix (2X) (Takara, Shiga-ken, Japan) under the following conditions: 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 30 s, and 95°C for 15 s. Each reaction was performed with 3 biological repeats, and the expression level was normalized to *U6* small nuclear RNA (**snRNA**), and expressed as $2^{-\Delta\Delta Ct}$ according to our previous study (Liu et al., 2018).

Function Prediction of DE miRNAs

To identify the functions of these DE miRNAs, the possible target genes of them were predicted by using the miRanda algorithm to scan 3′ untranslated regions from *Gallus gallus* reference genome (ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/002/315/GCF_000002315.5_GRCg6a/GCF_000002315.5_GRCg6a_genomic.fna.gz), with the parameters of single-residue-pair match scores ≥ 150, ΔG ≤ −30 kcal/mol and demand strict 5′ seed pairing according to the previous study (John et al., 2004). Then these miRNA targets were subjected to GO enrichment analysis by calculating the *P*-value using R based on the hypergeometric distribution. Three terms in the GO database were mapped, including biological process (**BP**), cellular component (**CC**), and molecular function (**MF**). In addition, the Pathway database within KEGG database was used to

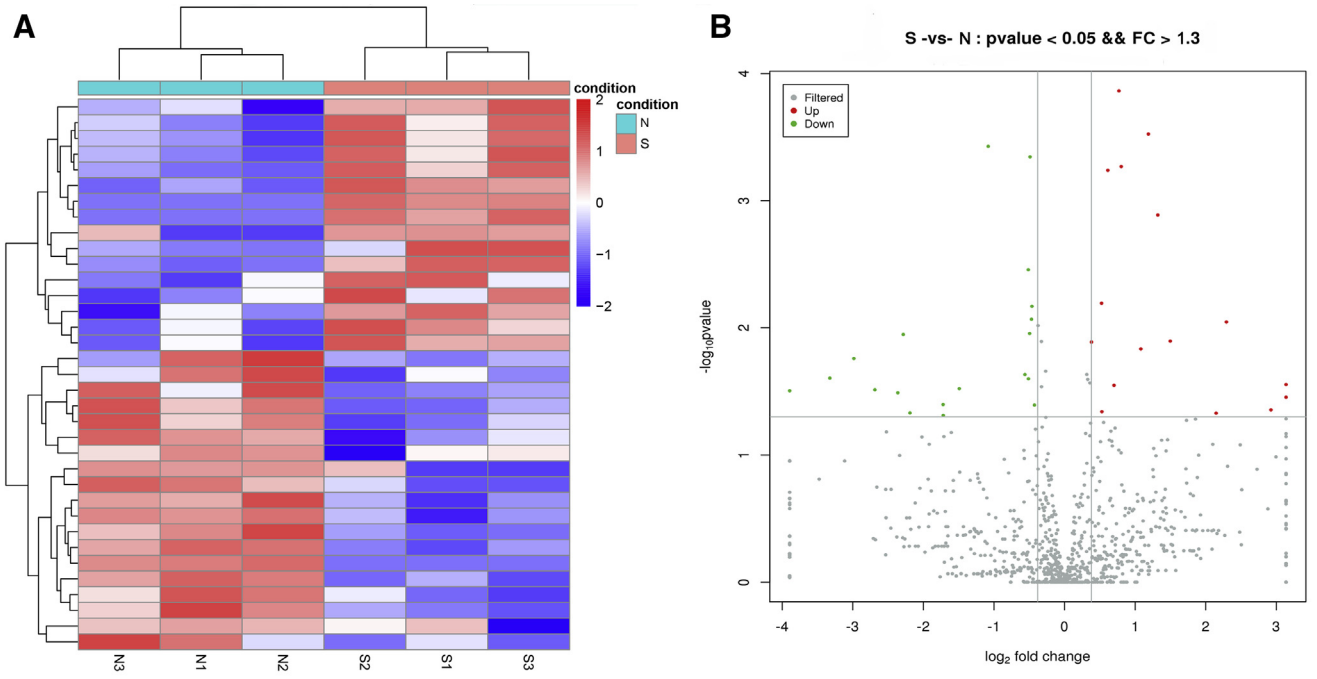

Figure 1. The expression pattern of differentially expressed miRNAs in chicken midsegments of small intestines infected with *E. necatrix* oocysts. (A) The hierarchical clustering plot shows the expression profiles of miRNAs. The S1-3 represents samples infected with *E. necatrix* oocysts and the N1-3 represents samples without infection. (B) The volcano plot shows the distributions of miRNAs. The significantly upregulated and downregulated miRNAs are presented as red and green dots, respectively, and the expression of miRNAs not significantly differentially expressed is presented as black dots (FC > 1.3 and *P*-value < 0.05).

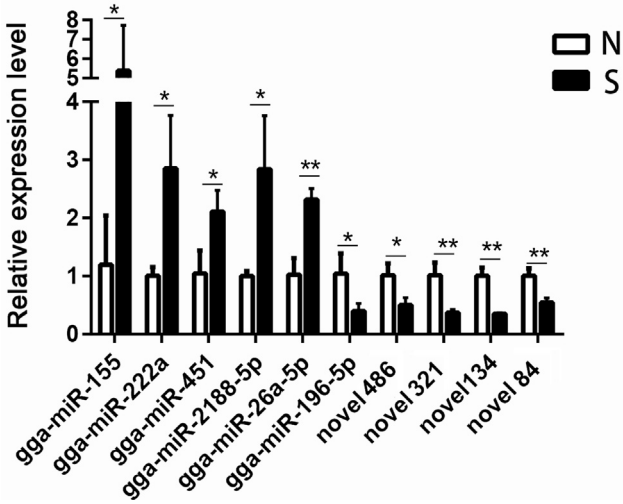

Figure 2. Validation of the differentially expressed miRNAs using qRT-PCR, with 3 biological repeats included for each miRNA. $^{*}P < 0.05$, $^{**}P < 0.01$.

identify the pathways participated in for the DE miRNAs to understand biological functions of these genes.

Statistical Analysis

The Student's *t*-test within the software GraphPad Prism 7.0 (http://www.graphpad.com) was used to determine statistical differences between the groups S and N, with *P*-value < 0.05 considered as statistically significant. All data were presented as mean ± SD from 3 independent experiments.

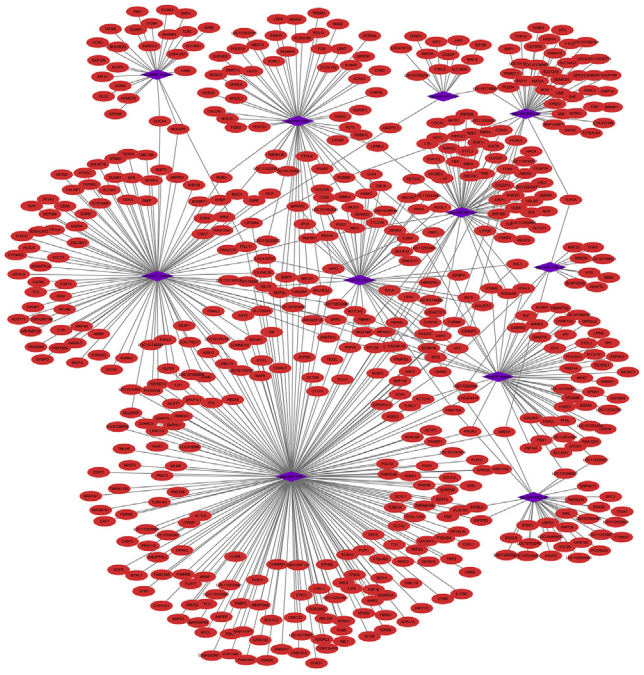

Figure 3. Coexpression network of the representative miRNAs and their partial target mRNAs. Different colors were used to show different genes, with purple for miRNAs and red for mRNAs, and the black solid line indicates the correction of miRNAs and mRNAs.

RESULTS

Identification of miRNAs in Midsegments of Chicken Small Intestines

A total of 136,171,097 raw reads were produced from the small RNA library by using the Illumina HiSeq 2500 sequencing platform. After discarding the low-quality reads (the junction sequence, N base sequence, and the sequence with too low Q20 ratio), we obtained 130,604,857 clean reads, responding to 4,026,965 unique tags, and 95.06% (124,159,440) of these clean reads were matched to the reference genome of *Gallus gallus* (Table 1). The lengths of them were 15 to 41 nt, with 22 nt as the most abundant size in both S and N groups, consistent with other studies in chickens (Burnside et al., 2008; Zhao et al., 2017) (Supplementary Figure 1).

miRNA Expression Patterns Associated With E. necatrix Infection

In the present study, a total of 663 known miRNAs and 603 novel miRNAs were identified (Supplementary Table 2). Comparison analysis of miRNAs between the groups S and N showed that 23 known (13 upregulated and 10 downregulated) and 12 novel (3 upregulated and 9 downregulated) miRNAs were significantly DE during *E. necatrix* infection according to the criterion of *P*-value < 0.05 and FC > 1.3 (Figure 1A, Supplementary Table 3), including 16 significantly upregulated and 19 significantly downregulated miRNAs (Figure 1B). The lengths of these known and novel miRNAs were 20 to 24 nt and 18 to 25 nt, respectively, and the size of 22 nt was also the most abundant group in both of them (Supplementary Figure 2).

Validation of DE miRNAs by Using qRT-PCR

To validate the deep-sequencing results, 5 upregulated (gga-miR-155, gga-miR-222a, gga-miR-451, gga-miR-2188-5p, and gga-miR-26a-5p) and 5 downregulated miRNAs (gga-miR-196-5p, novel486, novel321, novel134, and novel84) were randomly selected for qRT-PCR analysis. The expression results of qRT-PCR were consistent with miRNA sequencing, confirming the facticity of miRNAs expression profiles sequenced (Figure 2, Supplementary Figure 3).

Target Prediction and Functional Annotation of DE miRNAs

To understand the biological function of miRNAs during *E. necatrix* infection, the target genes of all DE miRNAs were predicted by constructing miRNA-target networks (Figure 3) using the miRanda algorithm (John et al., 2004). Of 35 miRNAs, possible target genes were identified for 21 miRNAs (12 upregulated and 9 downregulated miRNAs), corresponding to 4,568 mRNAs (Supplementary Table 4). These target genes were enriched into 4,644 GO terms (Supplementary

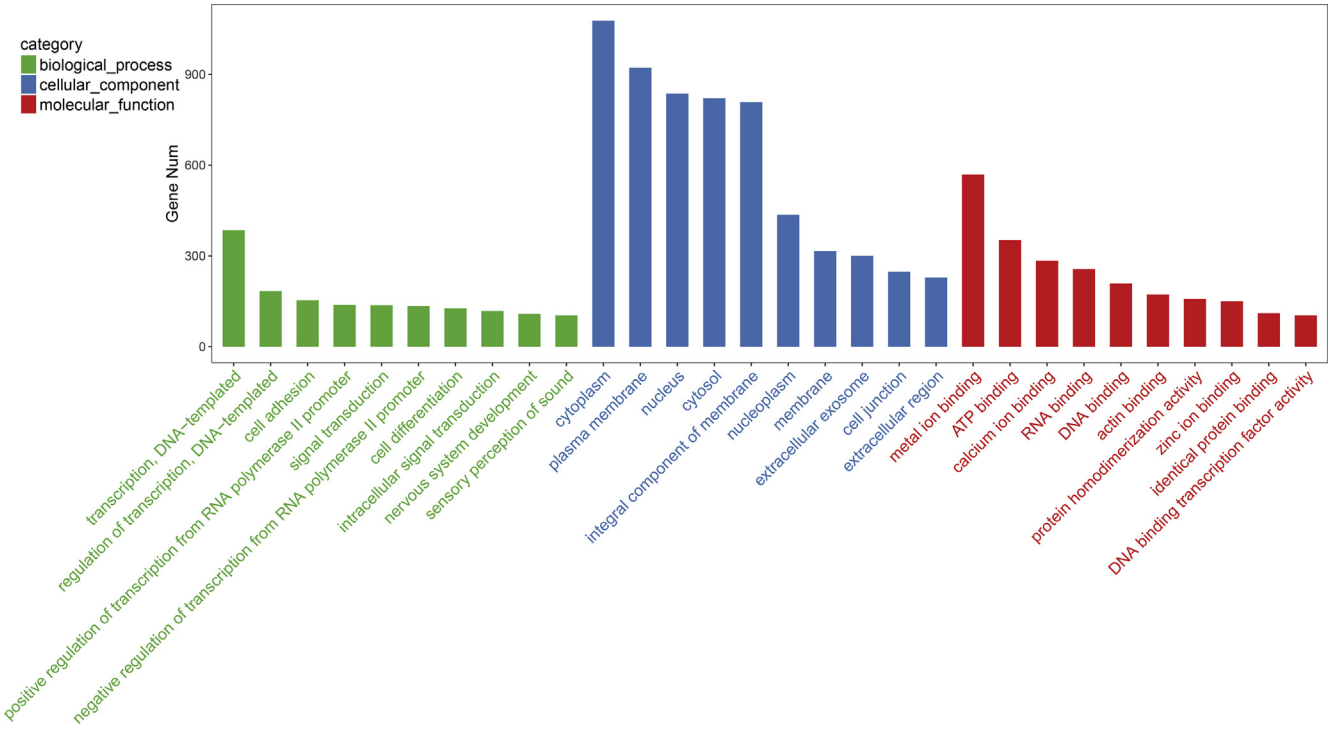

Figure 4. Predicted GO terms targeted by miRNAs differentially expressed in chicken mid-small intestines infected with *E. necatrix* oocysts ($P < 0.05$).

Table 5), including BP component (e.g., transcription, regulation of transcription, cell adhesion, positive regulation of transcription from RNA, signal transduction, and negative regulation of transcription from RNA), CC (e.g., cytoplasm, plasma membrane, nucleus, and cytosol), and MF (e.g., metal ion binding, ATP binding, calcium ion binding, RNA binding, DNA binding, and actin binding) (Figure 4). Among these GO annotations, gga-miR-1453, gga-miR-1464, gga-miR-34a-5p, novel100-mature, and novel78-mature were involved in the cell differentiation and cell adhesion, implicating these DE miRNAs playing important regulatory roles in cell development.

In addition, pathway analysis of these target genes of DE miRNAs identified 54 terms ($P < 0.05$) within KEGG database (Supplementary Table 6). The top 20 terms were shown in Figure 5. Among them, we identified some pathways associated with cell proliferation and apoptosis (e.g., MAPK signaling pathway and PPAR signaling pathway).

DISCUSSION

Chicken coccidiosis causes dramatic economic losses in poultry industry worldwide (Shirley et al., 2005; Dalloul and Lillehoj, 2006; Michels et al., 2011; Blake and Tomley, 2014). However, currently, the control of *Eimeria* infection is challenged because of the resistance and residue for coccidiostats and the risk of excreting and diffusing unwanted *Eimeria* spp. for live attenuated vaccines (Peek and Landman, 2011; Djemai et al., 2016). Therefore, to develop novel strategies for effectively combating this disease, the interplay between *Eimeria* spp. and chicken tissues should be deeply understood. miRNA has been evidenced as an important posttranscriptional regulator in a variety of biological processes, including the infectious diseases (Holla and Balaji, 2015; Hu et al., 2018; Keshavarz et al., 2018). Expression profiles of miRNA have already been identified in chicken duodenums infected with *E. acervulina* (Sun et al., 2016), and spleens and intestines coinfected with *E. maxima* and *C. perfringens* (Al-Quraishy et al., 2011; Dinh et al., 2014; Hong et al., 2014). In the present study, we first investigated the miRNA profile in small intestines at the early infective phase of the highly pathogenic *E. necatrix*.

Infection of *E. necatrix* begins with oral uptake of sporulated oocysts (Su et al., 2017). After being released from oocysts in the intestines, the infectious sporozoites invade epithelial cells and undergo 2 generations of schizogony in midsegments of small intestines (Su et al., 2017). Increasing evidence suggested that the pathogenic effect of *E. necatrix* mainly relies on the size and inhabit location (tunica propria of crypts) of the second-generation meronts (McDonald and Shirley, 1987), which has been concerned to be necessary for inducing the protective immune response of host against *Eimeria* spp. (Pierce et al., 1963; Rose and Hesketh, 1976). In our study, the scrapings of midsegments in chicken small intestines infected with *E. necatrix* were collected from 96 h to 120 h and observed by light microscopy (400×). The most abundant of the second-generation meronts was detected at 108 h pi (Supplementary Figure 4). Thus, the midsegments of small intestines in chickens of both S and N groups were subjected to deep sequencing.

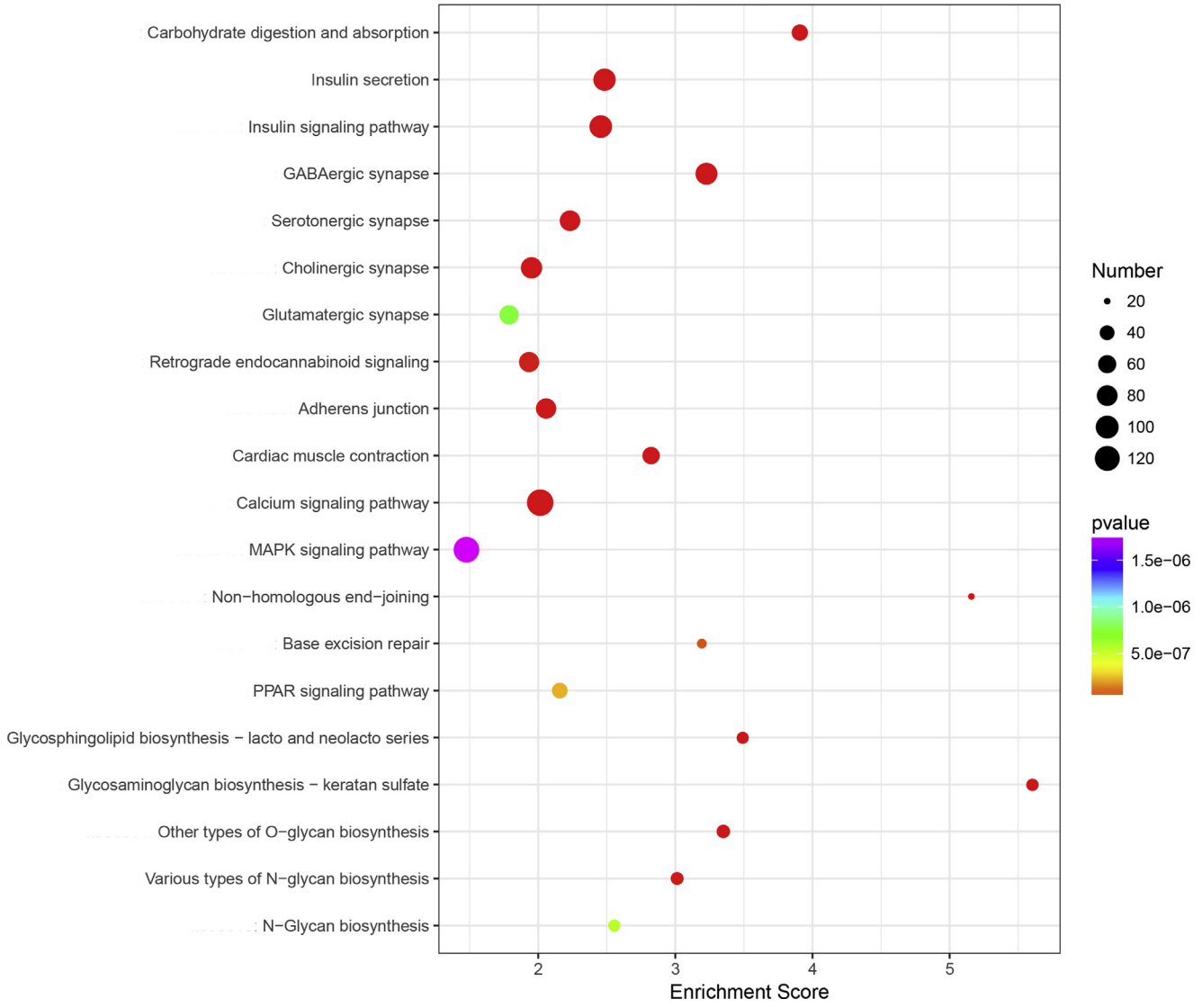

Figure 5. Predicted pathways targeted by miRNAs differentially expressed in chicken mid-small intestines infected with *E. necatrix* oocysts ($P < 0.05$).

In the present study, a total of 1,266 miRNAs were identified in both infected and control groups. Compared with miRNA data published previously, 603 novel miRNAs were determined, extending the miRNA database of chickens. Further analysis revealed 35 miRNAs (including 23 known and 12 novel miRNAs) were significantly DE during *E. necatrix* infection, including 16 upregulated and 19 downregulated miRNAs. Among them, 4 miRNAs (gga-miR-1454, gga-miR-199-3p, gga-miR-1a-3p, and gga-miR-30a-5p) were also respectively downregulated during infections of bronchitis virus (Yang et al., 2017), Marek's disease virus (**MDV**) (Lian et al., 2015), and *Campylobacter jejuni* (Wang et al., 2018b), and 2 miRNAs (gga-miR-155 and gga-miR-451) were also upregulated after infections of infectious bursal disease virus and *Mycoplasma gallisepticum* (**MG**) (Wang et al., 2018a; Zhao et al., 2018), respectively. Furthermore, the downregulated gga-miR-199-3p could inhibit the proliferation of MDV-transformed lymphoid cell line (**MSB1**) cells and suppress Marek's disease tumor tumorigenesis. The upregulated gga-miR-451 could inhibit the proliferation of MG-infected DF-1 cells and promote apoptosis. The upregulated gga-miR-26a-5p could promote cell proliferation of chicken theca cells through inhibiting cell apoptosis (Kang et al., 2017). These results suggested that these miRNAs would be involved in the interplay between *E. necatrix* and chicken intestines through affecting the cell progression.

Host anti-infectious strategies (including immune response) will be elicited by the infection of parasites (Zhou et al., 2013). Functional analysis of predicted targets of DE gga-miR-1453, gga-miR-1464, novel188_mature, novel271_mature, and novel78_mature showed that these genes were involved in toll-like receptor signaling pathway and TNF signaling pathway, which have been identified to be important host responses against *Eimeria* infection (Zhou et al., 2013; Pérez et al., 2015). On the other hand, to successfully invade and colonize in hosts, *Eimeria* spp. will inhibit host immune response through negatively regulating the production of inflammatory cytokines (Zhao et al., 2018). Of 16

miRNAs upregulated during *E. necatrix* infection in our study, gga-miR-451 could decrease inflammatory cytokine production induced by MG infection, including tumor necrosis factor-α, IL-1β, and IL-6, through directly targeting tyrosine 3-monooxygenase/tryptophan5-monooxygenase activation protein zeta (**YWHAZ**) (Zhao et al., 2018), suggesting the possible negative role of this miRNA in regulating chicken immunity during *E. necatrix* infection.

Thus, it can be seen from findings in the present study, *E. necatrix* infection induced the significant alteration of the miRNA expression, and these DE miRNAs would participate into host strategies anti-*E. necatrix* infection and affect host cell progression through interaction with their targets. Associations of miRNAs and candidate target genes in this study may be recommended for the development of methods for early diagnosis of coccidiosis. Therefore, the present study will be helpful in elucidating the infection mechanism of *E. necatrix* as well as the host response in relation to miRNA expression, which opens a new window to unveil the mystery of the interplay between host and *E. necatrix*. However, further research is essential to investigate the role of these DE miRNAs in *E. necatrix* infection.

CONCLUSION

The present study first determined the miRNA expression profile in the mid-segments of chicken small intestines infected with *E. necatrix*. Deep sequencing identified 1,266 miRNAs, including 663 known and 603 novel miRNAs. Of them, *E. necatrix* infection altered the expression of 35 miRNAs. Functional analysis demonstrated that targets of these dysregulated miRNAs would be involved in biological processes during *E. necatrix* infection. These findings provided novel insights into the regulatory mechanisms that mediate the interaction between *E. necatrix* and hosts.

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SUPPLEMENTARY DATA

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