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Identification and expression profiling analysis of microRNAs in Nile tilapia (*Oreochromis niloticus*) in response to *Streptococcus agalactiae* infection

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ABSTRACT

MicroRNAs (miRNAs) play vital regulatory roles in various biological processes, including in immune responses. Nile tilapia (*Oreochromis niloticus*) is an important commercial fish species in China. To identify immune-related miRNAs of *O. niloticus*, 4 libraries from liver during *S. agalactiae* infection (0 h, 5 h, 50 h, and 7 d) were sequenced by high-throughput sequencing technology in tilapia. We obtained 10,703,531, 11,507,163, 11,180,179 and 13,408,414 clean reads per library, respectively. In our results, a total of 482 miRNAs were identified through bioinformatic analysis, including 220 conserved miRNAs and 262 putative novel miRNAs. Moreover, 21 (4.36%), 50 (10.37%), and 46 (9.54%) miRNAs were significantly differentially expressed at 5 h, 50 h and 7 d, respectively. In addition, 6939 target genes regulated by these differentially expressed miRNAs were predicted, and their functional annotations were predicted by Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, which revealed that a majority of differentially expressed miRNAs were involved in apoptotic process, metabolic process, and immune responses. Finally, Real-time quantitative PCR experiments were performed for 7 miRNAs by stem-loop RT-PCR, and a general agreement was confirmed between the sequencing and RT-qPCR data. To our understanding, this is the first report of comprehensive identification of *O. niloticus* miRNAs being differentially regulated in liver related to *S. agalactiae* infection. This work provides an opportunity for further understanding of the molecular mechanisms of miRNA regulation in *O. niloticus* host–pathogen interactions, and genetic resources for molecular assistant selection for disease resistant breeding program.

1. Introduction

MicroRNAs (miRNA), a class of endogenous short single-stranded non-coding RNAs discovered in early 1990s, are approximately 22 nucleotides (nt) in length, which can modulate gene expression in animals and plants through mRNA degradation or translational repression [1,2]. The primary miRNA transcripts are processed into two short mature miRNAs originating from the 5′ and the 3′ end of the hairpin precursor miRNA by endonucleases [3]. The miRNAs originating from the 5′ end of a precursor miRNA are labelled with the suffix -5p, and the miRNAs originating from the 3′ end were labelled with the suffix -3p [4,5]. Subsequently, pre-miRNAs are transported to the cytoplasm, and then further processed by the Dicer into a short double-strand RNA duplex. Finally, a helicase unwinds the duplex into mature miRNAs [6]. Mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) and bind to the complementary 3′-untranslated regions (3′-UTR) of their specific target mRNAs, leading to either mRNA degradation or inhibition of translation [7]. Typically in animals, there is a perfect complementarity between the seed sequence and the target site of the mRNA followed by some in complementarities (bulges) before some additional complementarity between the 3′ end of the miRNA and the target site [8]. It is estimated that more than 60% of mRNAs in mammals have conserved miRNA-binding sites [9,10]. As a class of gene regulatory molecules, miRNAs have been reported to be involved in development, immune responses to infection, and stress response in various organisms [11–15].

The innate immune system in fish plays major role in response to infection [16]. Thus, understanding of the immune regulation mechanism might contribute to better disease prevention and control measures. In vertebrates, miRNAs act as key *trans* acting factors that

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Table 1
Quality of sequencing data.

Sample	Total reads	N% > 10%	Low quality	5′ adapter contaminant	3′ adapter null or insert null	with ployA/T/G/C	Clean reads
0 h	10,848,428	0 (0.00%)	22972 (0.21%)	1416 (0.01%)	116590 (1.07%)	3919 (0.04%)	10703531 (98.66%)
5 h	11659828	0 (0.00%)	23625 (0.20%)	3335 (0.03%)	121384 (1.04%)	4321 (0.04%)	11507163 (98.69%)
50 h	11332003	188 (0.00%)	27099 (0.24%)	2764 (0.02%)	116471 (1.03%)	5302 (0.05%)	11180179 (98.66%)
7 d	13622349	191 (0.00%)	33937 (0.25%)	2630 (0.02%)	171678 (1.26%)	5499 (0.04%)	13408414 (98.43%)
Total	47,462,608	379	107,633	10,145	526,123	19,041	46,799,287(98.60%)

Table 2
Categorization of tilapia noncoding and organellar small RNAs.

Types of sRNA	0 h		5 h		50 h		7 d	
	Number of reads	Percent (%)	Number of reads	Percent (%)	Number of reads	Percent (%)	Number of reads	Percent (%)
Known_miRNA	5598264	57.82%	5673958	56.47%	4728851	54.59%	6981389	59.32%
Novel_miRNA	239660	2.48%	169309	1.69%	154355	1.78%	293049	2.49%
rRNA	11249	0.12%	30075	0.30%	21586	0.25%	20253	0.17%
tRNA	5	0.00%	20	0.00%	22	0.00%	17	0.00%
snRNA	4494	0.05%	6015	0.06%	4209	0.05%	5077	0.04%
snoRNA	42670	0.44%	46323	0.46%	29909	0.35%	43320	0.37%
Repeat	4690	0.05%	5632	0.06%	4296	0.05%	6875	0.06%
Exon: sense	153039	1.58%	216756	2.16%	348299	4.02%	374774	3.18%
Exon: antisense	42578	0.44%	52674	0.52%	49142	0.57%	56526	0.48%
Intron: sense	307754	3.18%	313731	3.12%	382504	4.42%	367323	3.12%
Intron: antisense	41175	0.43%	70792	0.70%	78443	0.91%	53354	0.45%
Unannotated	3237466	33.43%	3462357	34.46%	2860539	33.02%	3567957	30.31%
Total	9683044	100.00%	10047642	100.00%	8662155	100.00%	11769914	100.00%

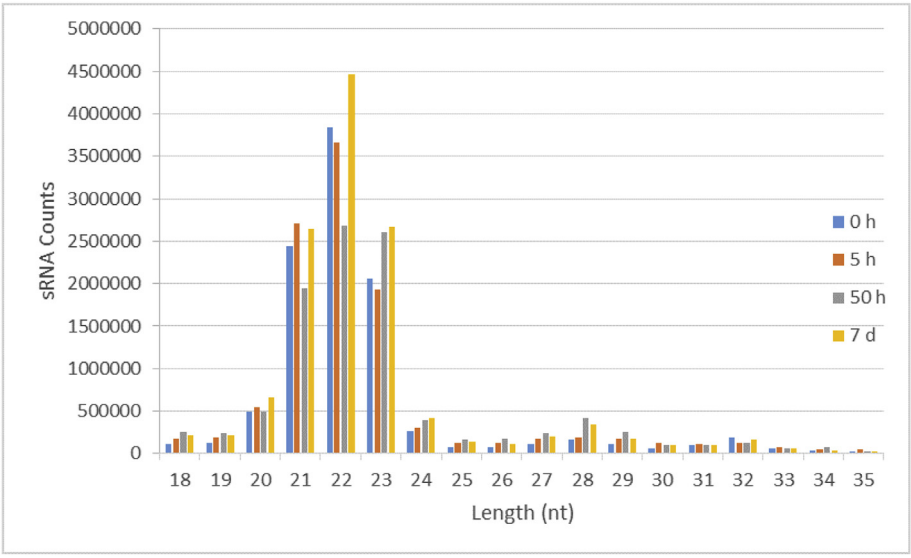

Fig. 1. Size distribution of unique sequences of sRNAs from 0 h, 5 h, 50 h and 7 d libraries.

regulate gene networks controlling fundamental biological processes such as growth, immune response, and tissue development [9,17–20]. In fish, a large amount of miRNAs have been characterized, and miRNA expression levels have been regulated in response to pathogen infection [21–27]. Consequently, investigations on miRNAs of immune relevance are indispensable for understanding of the host-pathogen interactions [28,29], which could aid the molecular-assistant disease resistant breeding program and the development of immune measures for disease control and prevention.

Nile tilapia (*Oreochromis niloticus*) is one of the most important economical fishes and widely cultured throughout the world [30], especially in the Southern China. In recent years, infectious diseases caused by *Streptococcus*, *Saprolegnia* and *Ichthyophthirius sp* have been severe. Especially, *S. agalactiae* has become one of the most serious bacterial diseases in southern China, resulting in great economic loss and becoming a big obstacle to tilapia aquaculture [31]. *S. agalactiae*, a Gram-positive pathogenic bacterium, also known as group B streptococcus (GBS), was first reported in 1939 as an important opportunistic agent [32]. Over the past few decades, it was recognized as a serious causative agent of zoonosis with a broad host range. In addition, studies related to *Streptococcus* virulence factors, vaccines, host immune relevant genes and investigate transcriptomic changes of *S. agalactiae* infected tilapia have been reported [33–36]. These results showed that many immune-related genes in tilapia were significantly up-regulated after *S. agalactiae* infection [35,36]. Although mRNA-miRNA interactions have an abundant impact on the regulation of gene expression, limited information is available about the effects of *S. agalactiae* infection on tilapia miRNA as well as the possible regulatory pathways involved. In this regard, the Nile tilapia was utilized as a host model, we investigated the expression profiles of miRNAs in tilapia liver in

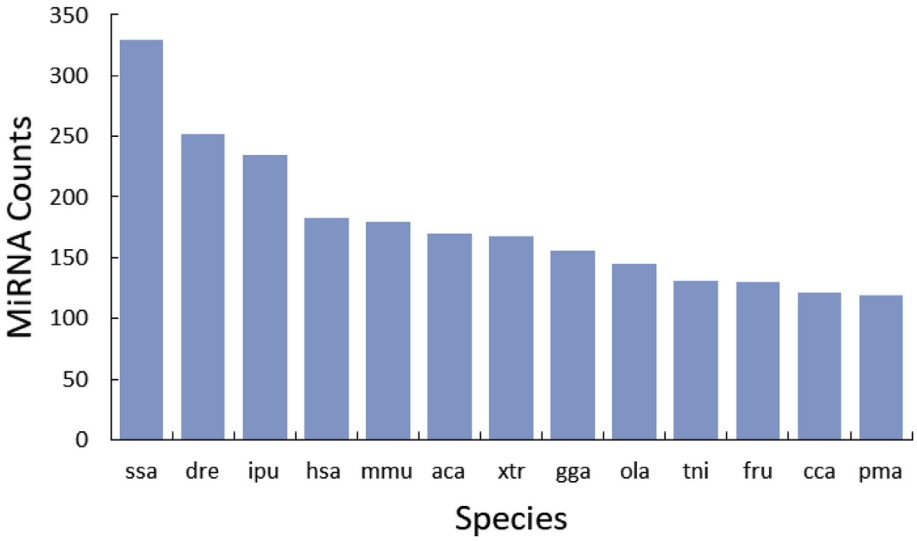

Fig. 2. Conservation profiles of the identified tilapia liver miRNAs with miRNAs from other fish species and higher vertebrates, including *Salmo salar* (ssa), *Danio rerio* (dre), *Ictalurus punctatus* (ipu), *Homo sapiens* (hsa), *Mus musculus* (mmu), *Anolis carolinensis* (aca), *Xenopus tropicalis* (xtr), *Gallus gallus* (gga), *Oryzias latipes* (ola), *Tetraodon nigroviridis* (tni), *Fugu rubripes* (fru), *Cyprinus carpio* (cca) and *Petromyzon marinus* (pma).

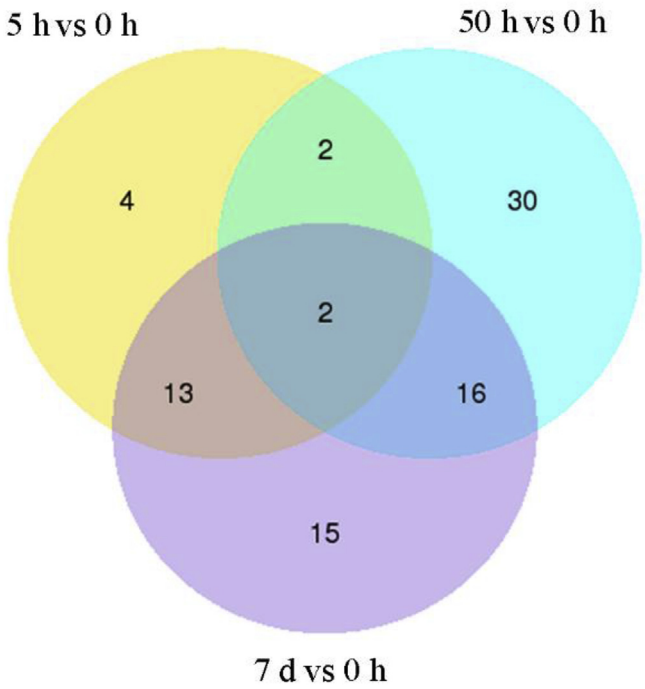

Fig. 3. Venn diagram of differentially expressed tilapia miRNAs.

response to *S. agalactiae* infection by high-throughput next-generation sequencing. Our results could expand the knowledge of the interactions between tilapia and *S. agalactiae*, and provide genetic resources for molecular assistant selection for disease resistant breeding program in tilapia.

2. Materials and methods

2.1. Experimental animals and tissue collection

Nile tilapia (average size 53 ± 0.45 g), obtained from Guangxi Academy of Fishery Sciences, were maintained at 24 ± 2 °C in a flow-through system and acclimated in the laboratory for two weeks before challenge and sampling. The bacterial isolate of *S. agalactiae* (HN016, serotype Ia, multilocus sequence type seven) was provided by the disease lab in Guangxi Academy of Fishery Sciences. After a pre-challenge, the bacteria was re-isolated from a symptomatic fish and biochemically confirmed before being inoculated in the shaker incubator overnight. Briefly, the bacteria were cultured in TSB broth in a shaker (180 rpm) incubator at 28 °C overnight. The concentration of the bacteria was determined using colony forming unit (CFU) per mL by plating 100 µl of 10-fold serial dilutions onto TSA agar plates. Fish were challenged in 30 L aquaria with three control and treatment groups. Aquaria were randomly divided into three sampling time-points: 5 h, 50 h, and 7 d post-infection. During the challenge, treatment fish were intraperitoneally injected with 0.3 ml bacterial culture at a concentration of 1.83×10^7 CFU/ml, while control fish were injected with the same volume of TSB broth. At each time-point, 15 fish were collected from the aquaria and anesthetized with MS-222 (200 mg/L). The liver tissue from 5 fish (3 replicates of 5 fish each) were pooled together, flash frozen in liquid nitrogen and store at −80 °C until RNA extraction.

2.2. Small RNA isolation, library construction and sequencing

Total RNA were extracted from the liver samples using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). The RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA) was used to assess RNA integrity. A total amount of 3 µg total RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA) following manufacturer's recommendations. Briefly, NEB 3′ SR Adaptor was directly and specifically ligated to 3′ end of miRNA. After the 3′ ligation reaction, the SR RT Primer hybridized to the excess of 3′ SR Adaptor (that remained free after the 3′ ligation reaction) and transformed the single-stranded DNA adaptor into a double-stranded DNA molecule. Then first strand cDNA was synthesized using M-M uLV Reverse Transcriptase (RNase H^-). PCR amplification was performed using LongAmp Taq 2X Master Mix, SR Primer for illumina and index (X) primer. PCR products were purified on an 8% polyacrylamide gel (100 V, 80 min). DNA fragments corresponding to 140–160 bp (the length of small noncoding RNA plus the 3′ and 5′ adaptors) were recovered and dissolved in 8 µL elution buffer. Library quality was assessed on the Agilent Bioanalyzer 2100 system using DNA High Sensitivity Chips. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq SR Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 2500 platform and 50 bp single-end reads were generated.

Table 3
The 15 most abundant miRNAs of 4 libraries.

0 h		5 h		50 h		7 d	
miRNA	expression	miRNA	expression	miRNA	expression	miRNA	expression
oni-miR-451	862079	oni-miR-451	893972	oni-miR-146a	1161697	oni-miR-146a	1248224
oni-miR-143	595989	oni-miR-143	865074	oni-miR-143	767894	oni-miR-21	1088110
oni-miR-146a	577151	oni-miR-146a	643638	oni-miR-21	717844	oni-miR-22a-3p	597981
oni-miR-21	352439	oni-let-7e	323572	oni-miR-451	328914	oni-miR-143	585817
oni-let-7e	303888	oni-miR-10c-5p	307720	oni-miR-10c-5p	249778	oni-miR-451	554744
oni-miR-126a-3p	287567	oni-miR-126a-3p	296612	oni-let-7e	178869	oni-miR-126a-3p	326907
oni-miR-462	281019	oni-miR-21	296113	oni-miR-22a-3p	158939	oni-let-7e	272996
oni-miR-10c-5p	275575	oni-miR-101a	182256	oni-miR-100-5p	132354	oni-miR-462	257117
oni-miR-22a-3p	191619	oni-miR-100-5p	171784	oni-miR-126a-3p	127538	oni-miR-100-5p	230283
oni-let-7a	180295	oni-miR-462	159633	oni-miR-462	102286	oni-miR-10c-5p	217502
oni-miR-101a	175796	oni-miR-22a-3p	150791	oni-miR-101a	76732	oni-let-7a	158816
oni-miR-100-5p	162482	oni-let-7a	142224	oni-let-7a	69539	oni-miR-101a	122943
oni-miR-26a-5p	125978	oni-miR-26a-5p	129692	oni-miR-26a-5p	60754	oni-miR-27b-3p	100622
oni-miR-92a-3p	96115	oni-miR-10a-5p	99607	oni-miR-10a-5p	59308	oni-miR-92a-3p	97646
oni-miR-10a-5p	81685	oni-miR-27b-3p	88318	oni-miR-27b-3p	43969	oni-miR-10a-5p	81756

2.3. Analysis of sequencing reads

The raw reads were firstly processed to remove low quality reads and adaptor sequences to obtain clean sequences. The filtered sequences between 18 nt and 30 nt in length were kept for downstream analyses. Subsequently, the clean reads were mapped to reference sequence using Bowtie-0.12.9 to analyze their expression patterns and distribution on the tilapia genome reference [37], allowing no more than one mismatch outside the seed region. The conserved and novel miRNAs were identified through BLAST searches against the known miRNAs of *O. niloticus* or other animal species in miRBase 21.0 [38], using miREvo [39] and mirdeep2 [40].

2.4. Differential expression analysis of miRNA

Expression levels of miRNA were estimated by transcripts per million (TPM) through the following formula: Normalized expression = mapped reads count/Total reads × 1000000 [41]. Then differential expression analysis was performed using the DESeq2 [42]. The P-values were adjusted using the Benjamini & Hochberg method. Corrected P-value of 0.05 was set as the threshold for significantly differential expression by default.

2.5. Target gene prediction and GO and KEGG enrichment analysis

The putative target genes of miRNA were predicted using miRanda for animals [43]. MiRanda was used to match the entire microRNA sequences. The parameter of microRnada was set as free energy < −20 kcal/mol. Gene Ontology (GO) enrichment analysis was used on the target gene candidates of differentially expressed miRNAs. GOseq based Wallenius non-central hyper-geometric distribution [44], which could adjust for gene length bias, was implemented for GO enrichment analysis of differentially expressed miRNAs. Kyoto Encyclopedia of Genes and Genomes (KEGG) is a database resource for understanding high-level functions and utilities of the biological system [45], such as cell, organism and ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). We used KOBAS software to test the statistical enrichment of the target gene candidates of differentially expressed miRNAs in KEGG pathways [46].

2.6. Validation of miRNA expression profiles

Seven differentially expressed (DE) miRNAs were selected for RT-qPCR to confirm the expression of miRNAs identified by the high-throughput sequencing. The small RNA (sRNA) of each groups in tilapia liver were extracted using RNAiso for small RNA Kit (Takara, Dalian, China) according to the manufacturer's protocol, then a Mir-X™ miRNA First-Strand Synthesis Kit (Takara, Dalian, China) was used to synthesize first-strand cDNA for mature miRNA expression analysis. Forward primers were designed based on mature miRNA sequences. If the melting point of a mature miRNA was < 60 °C, it was adjusted by adding G's or C's to the 5′ end and/or A's to the 3′ end of the miRNA sequence. The RT-qPCRs were performed using a miRNA SYBR Green RT-qPCR Kit (Takara, Dalian, China) with the provided miRNA reference gene (U6) on a CFX96 realtime PCR detection system (Bio-Rad Laboratories, Hercules, CA) under the following thermal cycling conditions: 94 °C for 2 min, 40 cycles of 94 °C for 20 s, 60 °C for 34 s. The 25 μL PCR mixture contained the RT product (template) 2.0 μL, 2× SYBR Advantage Premix 12.5 μL, ddH_2O 9 μL, 50× ROX Dye 0.5 μL, miRNA-specific primer (10 μM) 0.5 μL, and mRQ 3′primer 0.5 μL. Dissociation curve analysis of the amplified products was performed after each PCR reaction to confirm that only one PCR product was amplified and detected. For each cDNA, three-well replicates were used. Each threshold cycle (Ct) value used in the calculation was the mean obtained from each cDNA in triplicate. Expression differences were analyzed and assessed for statistical significance using a randomization test in the Relative Expression Software Tool (REST©) (version 2009) [47]. Data of RT-qPCR are expressed as mean ± standard error (SE). Statistical significance of differences between groups was reached at $p < 0.01$ and $|\text{fold-change}| \geq 2$. RT-qPCR analysis was repeated in triplicate runs (technical replicates) to confirm expression patterns.

3. Results

3.1. Overview of sequencing data

In order to identify the miRNAs involved in bacterial infection and host immune response, tilapia were infected with *S. agalactiae* for 0 h (control group), 5 h, 50 h and 7 d. Four small-fragment RNA libraries from tilapia liver, which representing those 4 time points were constructed and subjected to sequence analysis using a Solexa platform (Illumina). As shown in Table 1, a total of 47,462,608 raw sequences were generated. After filtering the low-quality tags and removing adapter sequences, polyA sequences, and sequences < 18 nt, 10,703,531 (98.66% of the raw reads), 11,507,163 (98.69% of the raw reads), 11,180,179 (98.66% of the raw reads) and 13,408,414 (98.43% of the raw reads) clean reads were obtained from 0 h, 5 h, 50 h and 7 d, respectively (Table 1). These represented 9,683,044, 10,047,642, 8,662,155 and 11,769,914 unique sequences in those 4 libraries, respectively (Table 2). Of the unique reads, 5,598,264 (57.82% of unique

Table 4
The differentially expressed miRNAs in tilapia following *Streptococcus agalactiae* challenge. Bold values indicate timepoints where the miRNA was significantly changed relative to the control.

miRNA	5h fold change	50h fold change	7d fold change
oni-let-7i	1.22	**2.72**	1.96
oni-miR-1	1.24	**2.35**	−1.19
oni-miR-107a-3p	−1.08	1.26	**3.71**
oni-miR-10d-5p	**−6.03**	**2.43**	−1.57
oni-miR-122	**−20.44**	−1.12	**−11.69**
oni-miR-125a	−1.07	**−2.25**	−1.22
oni-miR-128-3p	1.09	**−3.06**	−1.20
oni-miR-135b-5p	−1.42	**−2.21**	**−2.04**
oni-miR-1388-3p	1.17	**−3.96**	**−3.24**
oni-miR-1388-5p	1.34	**−2.25**	**−2.07**
oni-miR-139-5p	1.02	**−2.72**	**−3.50**
oni-miR-141-3p	−1.09	**2.94**	1.03
oni-miR-142a-5p	−1.00	**−2.11**	−1.10
oni-miR-143	1.65	**2.10**	−1.03
oni-miR-145-5p	−1.03	**−2.63**	**−2.17**
oni-miR-146a	1.27	**3.28**	**2.13**
oni-miR-148	**−3.06**	1.04	**−2.33**
oni-miR-153a-3p	−1.21	1.40	**−3.33**
oni-miR-155	−1.27	**−2.63**	**−2.32**
oni-miR-16a	−1.02	**−2.52**	−1.17
oni-miR-16b	−1.03	**−2.51**	−1.37
oni-miR-181a-2-3p	1.03	1.72	**2.01**
oni-miR-184	−1.87	**38.84**	**6.15**
oni-miR-187	**3.00**	1.37	**4.83**
oni-miR-192	**−4.48**	1.65	**−5.08**
oni-miR-194a	**−5.31**	−1.58	**−7.94**
oni-miR-199-3-3p	1.13	**−2.82**	−1.15
oni-miR-199-3p	1.02	**−2.30**	−1.43
oni-miR-19b-3p	1.37	**−2.12**	−1.37
oni-miR-19c-3p	1.62	**−2.20**	−1.91
oni-miR-19d-3p	**2.05**	**−2.12**	−1.39
oni-miR-200a-3p	−1.27	**2.36**	−1.14
oni-miR-203a-3p	−1.46	−1.12	**2.88**
oni-miR-204-5p	**2.20**	**−2.16**	**−2.39**
oni-miR-205-5p	−5.11	1.97	**5.88**
oni-miR-206-3p	−1.44	**5.19**	2.30
oni-miR-21	−1.05	**3.32**	**3.05**
oni-miR-210-3p	−1.01	**−2.54**	**−2.68**
oni-miR-210-5p	1.03	**−3.26**	−1.95
oni-miR-216b	**−8.03**	−1.21	**−9.48**
oni-miR-217	**−9.60**	−1.39	**−10.74**
oni-miR-2188-5p	−1.14	**−2.42**	−1.75
oni-miR-223	**2.20**	−1.19	**2.06**
oni-miR-22a-3p	−1.12	1.35	**3.08**
oni-miR-22a-5p	−1.32	**2.14**	**2.59**
oni-miR-29a	−1.13	**−2.44**	−1.53
oni-miR-29b	1.09	**−2.32**	−1.59
oni-miR-30c-5p	1.11	**−2.44**	−1.24
oni-miR-338	1.28	**−2.19**	**−3.47**
oni-miR-365	−1.03	**−4.17**	**−5.64**
oni-miR-375	−1.80	−1.38	**−4.05**
oni-miR-460-5p	1.21	1.61	**−2.92**
oni-miR-7147	1.75	**−2.17**	−1.06
oni-miR-722	**−3.82**	−1.18	−1.21
oni-miR-730	**−3.26**	1.23	**−3.22**
oni-miR-731	−1.65	**−3.48**	−1.01
oni-miR-7a	−1.14	**3.01**	**3.05**
oni-miR-92b-3p	**−99.11**	−1.94	1.53

sequences in 0 h), 5,673,958 (56.47% of unique sequences in 5 h), 4,728,851 (54.59% of unique sequences in 50 h) and 6,981,389 (59.32% of unique sequences in 7 d) were identified as known miRNAs. The remaining sequences were other types of RNA, including tRNA, rRNA, small nuclear RNA, and small nucleolar RNA (Table 2).

As small RNAs (sRNAs) with known functions are typically 20–24 nucleotides (nt) long [48], the length distribution analysis of the unique sRNA sequences was conducted after removing the other RNA types. As shown in Fig. 1, the majority of the unique sRNAs from the 4 libraries ranged from 20 to 24 nt, the peak distribution of sequences was 22 nt long, followed by those that were 21, 23, 20, and 24 nt in length, which was consistent with the typical sizes of dicer processing products.

3.2. Expression patterns of miRNAs during the bacterial infection

Genome Analyzer Pipeline software (Illumina), Bowtie-0.12.9 and modified mirdeep2 program were used to process the sequencing data, not only to explore the expression profiling of host miRNAs at different infection time points, but also to examine whether the miRNAs identified in tilapia were evolutionarily conserved across species, which is a key feature of miRNAs. A total of 240 miRNA precursors and 222 miRNAs matures were identified. In detail, 230, 225, 229 and 233 pre-miRNAs were identified at 0 h, 5 h, 50 h and 7 d, while 207, 203, 195 and 205 miRNAs matures were discovered at 0 h, 5 h, 50 h and 7 d, respectively. Subsequently, we detected a total of 262 novel miRNAs and 482 conserved miRNAs belonging to 94 miRNA families (supplementary Table 1). In addition, those conserved miRNAs mapped to a large proportion of the miRNA precursors from other fish species and higher vertebrate species including salmon (ssa), zebrafish (dre), catfish (ipu), human (hsa), mouse (mmu), lizard (aca), frog (xtr), chicken (gga) and tilapia (ola) (Fig. 2). In detail, the highest confidence tilapia miRNA orthologs were found in three teleost species including salmon (329), zebrafish (252) and catfish (235). Meanwhile, 183, 179, 170, 168 and 156 tilapia miRNA orthologs were also detected from higher vertebrate species with inclusion of human, mouse, lizard, frog and chicken, respectively (Fig. 2).

3.3. Differentially expressed miRNAs induced by bacterial infection

DE analysis was conducted to identify host miRNAs involved in immune activities in *O. niloticus*. As shown in Fig. 3, a total of 82 miRNAs were differentially expressed compared to the expression levels at 0 h (Table 4, Fig. 3). Among which, there were 21, 50 and 46 miRNAs differentially expressed at 5 h, 50 h, and 7 d, respectively (Figs. 3 and 4). In detail, 4, 30 and 15 miRNAs were specifically expressed at 5 h, 50 h, and 7 d, respectively, while only 2 miRNAs (oni-miR-204-5p, oni-novel_168) were differentially expressed at all three time points (Fig. 3). Specifically, as shown in the Volcano plot, there were 6, 16 and 22 DE miRNAs up-regulated, while 15, 34 and 24 DE miRNAs down-regulated at 5 h, 50 h and 7 d, respectively (Fig. 4).

For 82 DE miRNAs, in order to observe their expression patterns along with the infection process, a heatmap was generated, and clustering analysis was conducted based on their similar expression patterns. As shown in Figs. 5 and 4 major clusters were observed, which included 11, 19, 31 and 21 DE miRNAs, respectively. As the infection progressed, the expression patterns of the miRNAs exhibited dynamic changes and formed various patterns, including sustained up-regulation/down-regulation during most infection period, up-regulation/down-regulation followed by down-regulation/up-regulation, and diphasic expression patterns (Fig. 5).

In addition, 335 miRNAs were expressed at all examined time points, while 9, 6, 7 and 20 miRNAs were expressed specifically at 0 h, 5 h, 50 h and 7 d, respectively. The read counts of the miRNAs were ranged from 1 to > 1 million, indicating the sequencing data has a wide range spanning seven orders of magnitude. Several members of the let-7 and miR-10 family, miR-451, miR-143 and miR-146a were of the highly abundant miRNAs in all 4 libraries (Table 3). We selected the top 15 most abundant miRNAs, composed 78.26%, 81.75%, 87.44% and 82.68% of the total miRNA counts at 0 h, 5 h, 50 h and 7 d, respectively. Among these, 13 miRNAs were universally expressed in all 4 libraries. The abundance of 163, 167, 147 and 207 miRNAs were lower than 10 at 0 h, 5 h, 50 h and 7 d, respectively. In addition, 60, 74, 91 and 50 miRNAs were not expressed in 4 corresponding libraries.

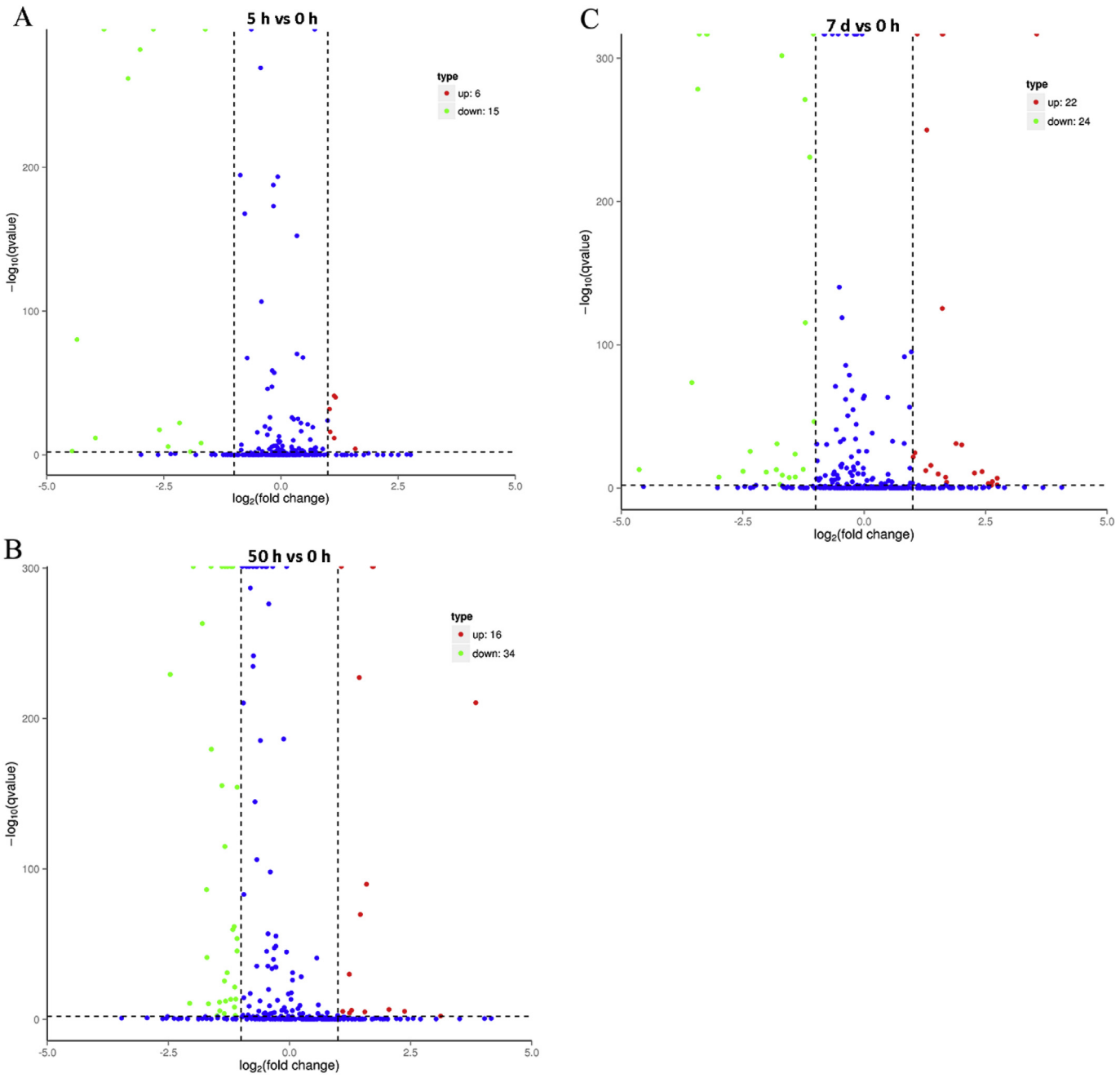

Fig. 4. Volcano plot of the differential expression of miRNAs in tilapia liver (A–C). The horizontal coordinate represents the fold change of the expression of miRNAs at 5 h, 50 h and 7 d post-bacterial infection in comparison with that at 0 d. The vertical coordinate represents the statistical significance of the fold change of miRNAs expression. Each dot represents an individual miRNA. Red dots show apparently up-regulated miRNAs and green dots show apparently down-regulated miRNAs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.4. Target prediction for differently expressed miRNAs and enrichment analysis

Potential targets of the 82 differentially expressed miRNAs were predicted using miRanda algorithms. The results showed that for all those 82 miRNAs, 85,247 putative target sites in 6939 tilapia genes were predicted (supplementary Table 2). In order to identify the specific functions of the differently expressed miRNAs, the predicted miRNA targets were annotated by GO enrichment and KEGG pathway analysis. The GO analyses of these predicted target genes revealed that some of them might play crucial roles in tilapia following the *S. agalactiae* challenge. Specific GO terms of the target genes were mainly involved in biological processes (e.g., metabolic process, biological process, and organic substance metabolic process), cell components (e.g., cellular component, signal peptidase complex, and cytoplasmic vesicle part), and molecular function (e.g., organic cyclic compound binding, heterocyclic compound binding, and catalytic activity) (Fig. 6).

Similar to GO analysis, KEGG pathway analysis showed that the putative target genes predicted in tilapia were grouped into 154 pathways. 15 common pathways were predicted from the top 20 enriched pathways of each four libraries, with inclusion of Glycolysis/Gluconeogenesis, Carbon metabolism, Citrate cycle (TCA cycle), Focal adhesion, Biosynthesis of amino acids, VEGF signaling pathway, ECM-receptor interaction, NOD-like receptor signaling pathway, Butirosin and neomycin biosynthesis, Pyruvate metabolism, Adrenergic signaling

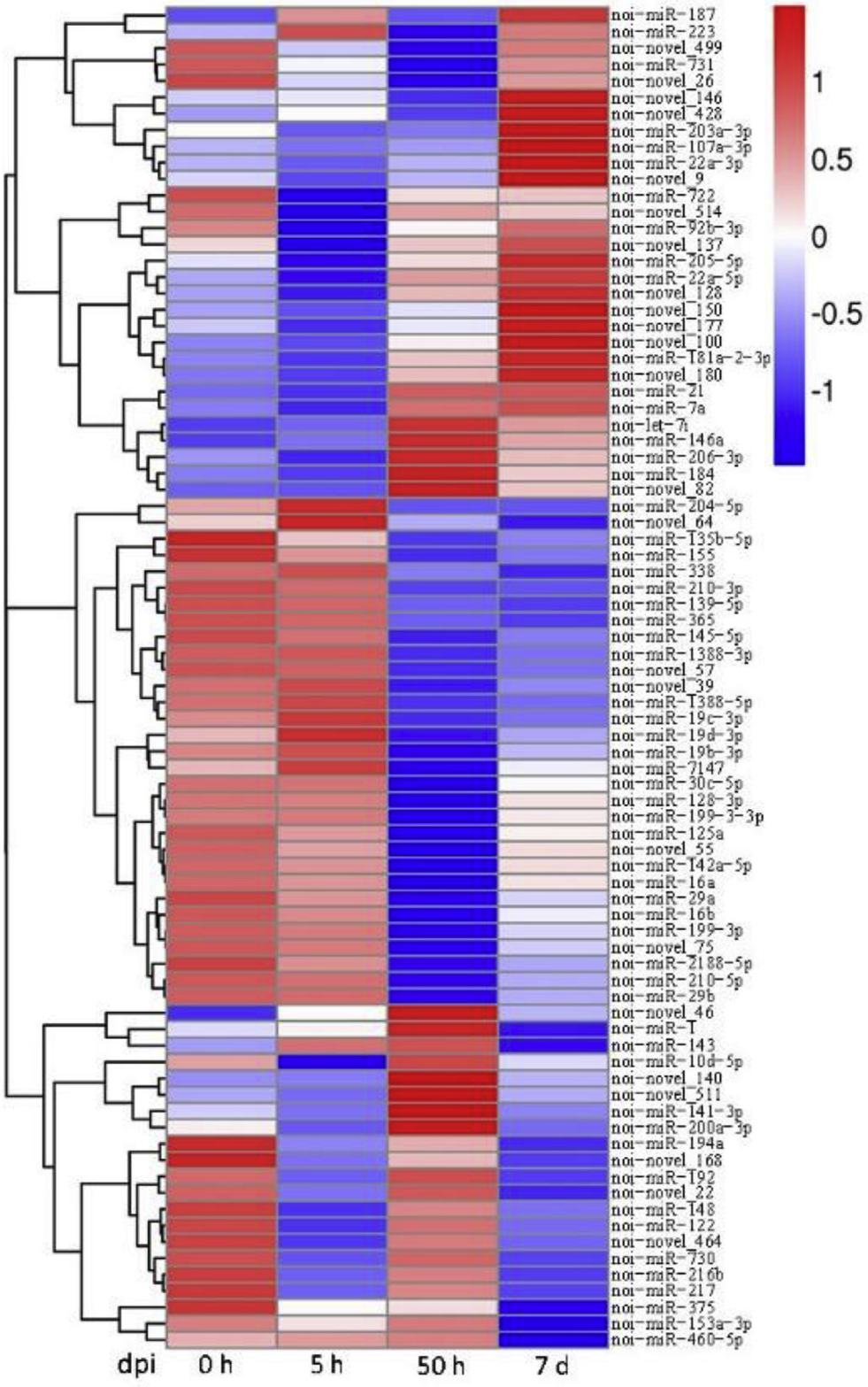

Fig. 5. Clustering of the expression patterns of 82 host miRNAs differentially expressed during bacterial infection. The expression levels of the 82 miRNAs at 0 h to 7 d post-infection (dpi) are shown in different colors. Each horizontal color bar represents one miRNA, with the name of the miRNA indicated on the right of the bar. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

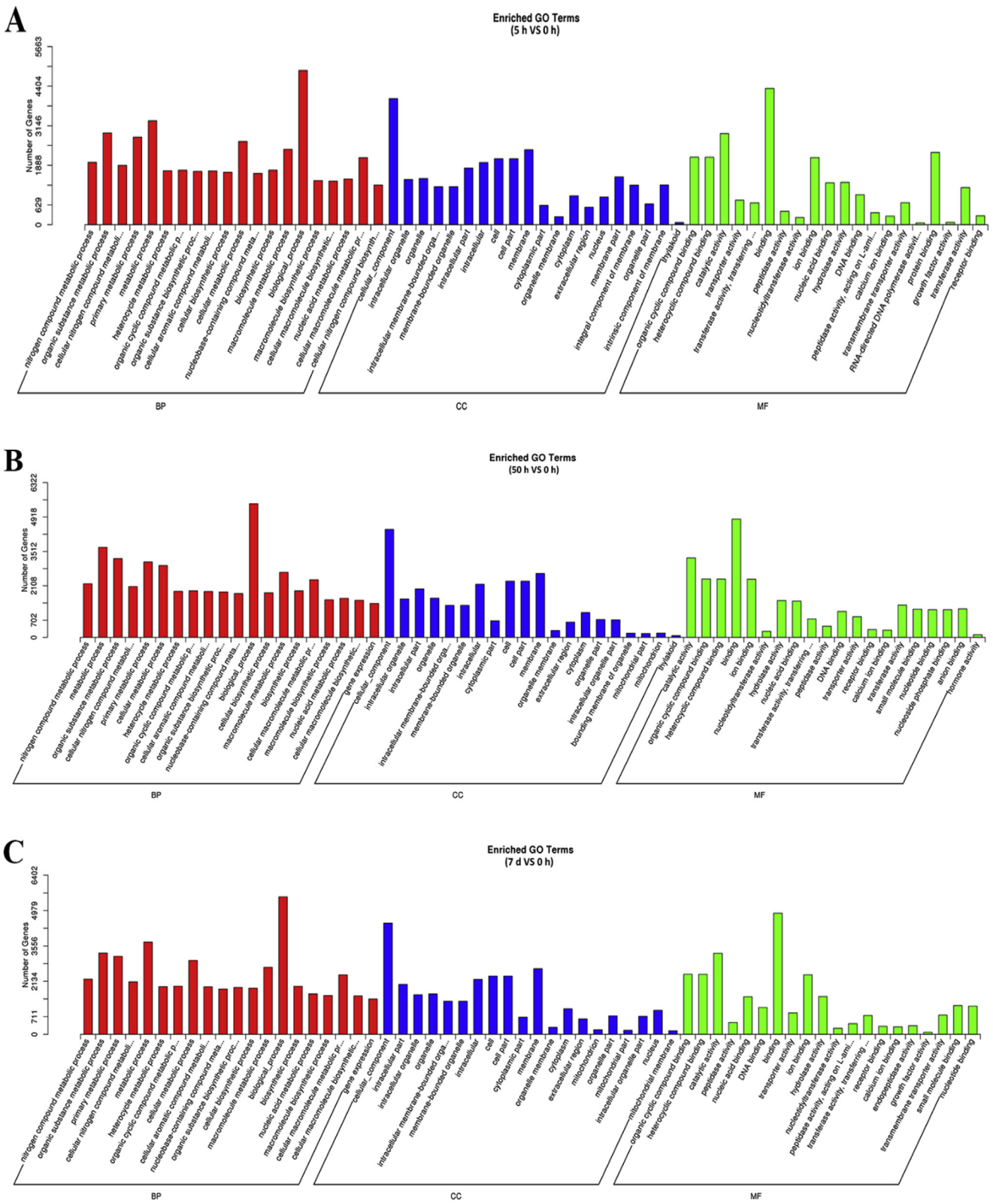

Fig. 6. Classification of the GO enrichment for the negatively correlated miRNA–mRNA pairs of tilapia exposed to *Streptococcus agalactiae* infection at three time-point, 5h (A), 50 h (B) and 7 d (C), respectively. Negative correlation is defined as having opposite expression patterns between miRNAs and their target mRNAs. BP, CC and MF represent biological process, cellular component and molecular function, respectively.

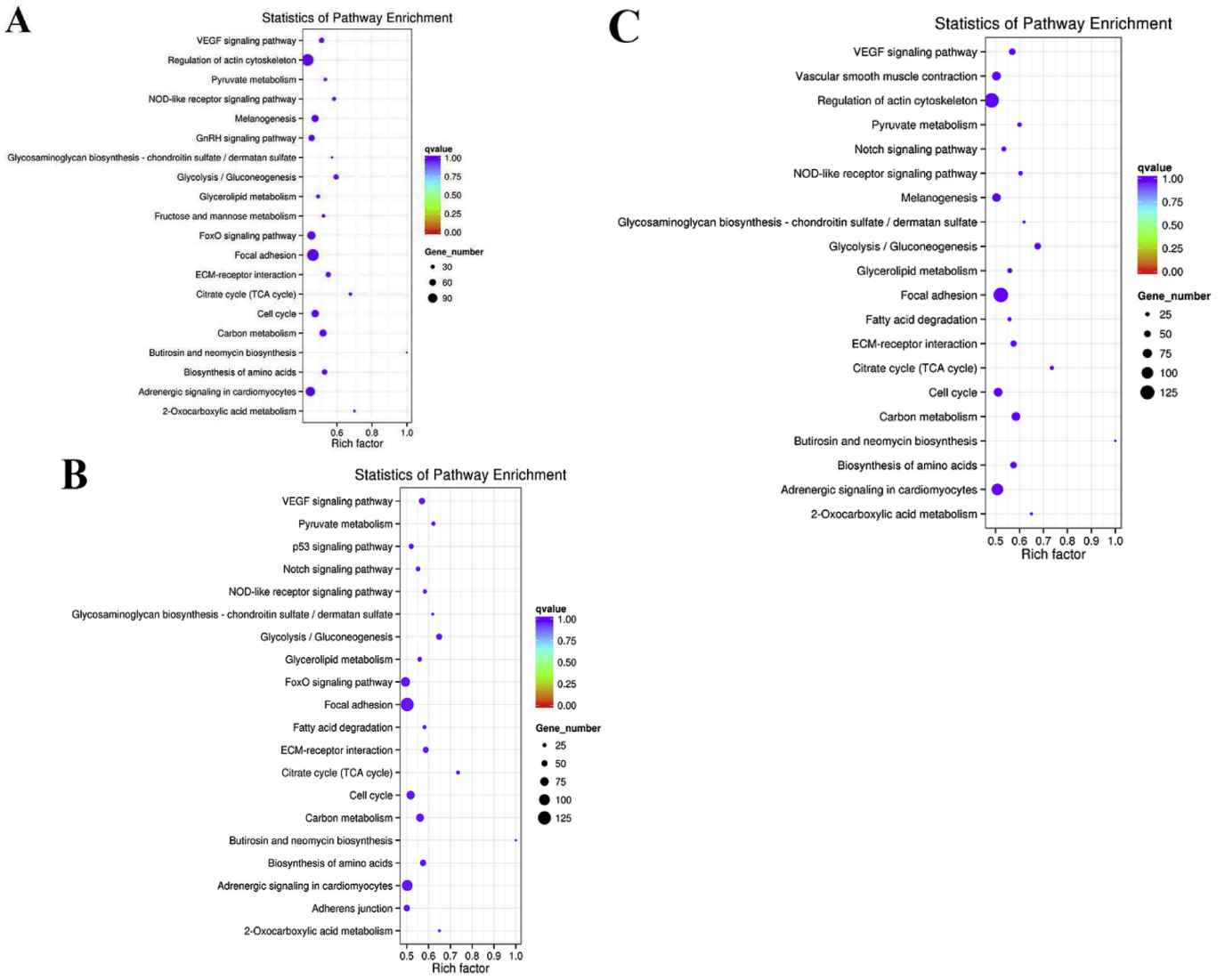

Fig. 7. KEGG pathway enrichment analysis of the differentially expressed mRNAs from the liver of tilapia exposed to *Streptococcus agalactiae* infection at 5 h (A), 50 h (B) and 7 d (C), respectively.

in cardiomyocytes, Glycerolipid metabolism, Cell cycle, 2-Oxocarboxylic acid metabolism, and Glycosaminoglycan biosynthesis - chondroitin sulfate/dermatan sulfate (Fig. 7).

3.5. Validation of selected DE miRNAs by RT-qPCR

Seeking to validate the differentially expressed miRNAs identified by high-throughput sequencing, 7 miRNAs with different expression patterns were selected for RT-qPCR confirmation. Fold changes from RT-qPCR were compared with the high-throughput sequencing expression analysis results. As shown in Fig. 8, there was similarity between the quantitative assay and high-throughput sequencing analysis of the 7 miRNAs in terms of fold-change and significance of differential expression. Although there was difference between fold changes in the two methods, the variation trend was identical mostly. The discrepancy between the two techniques might due to incorrect assignment of high-throughput sequence reads to paralogous miRNAs. In general, the high-throughput sequencing results were confirmed by the RT-qPCR results, indicated the reliability and accuracy of high-throughput sequence expression analysis.

4. Discussion

In the present study, a high-throughput sequencing approach was used to sequence 4 sRNA libraries from liver of tilapia to investigate the expression patterns of tilapia miRNAs following *S. agalactiae* infection. The length distribution of unique sequences across the 4 libraries was the highest for 22 nt sequences. Previous studies have revealed the sRNA length distribution pattern of many species exhibited a major peak at 24 nt, such as thale cress (*Arabidopsis thaliana*), barrel clover (*Medicago truncatula*), and Chinese fir (*Cunninghamia lanceolata*), which is the typical length of plant repeat-associated small interfering RNAs [49–51]. In consistent with current study, the major peak of sRNA length distribution in Japanese flounder and half-smooth tongue-sole is at 22 nt, which is the typical length of animal miRNAs [15,52].

In tilapia, several studies have characterized miRNAs in different biological processes, including skeletal muscle development [53], skin color differentiation [54], and immune responses to infection [55]. In current study, 482 miRNAs were identified in tilapia through high-throughput sequencing, of which 220 were known miRNAs while 262 were discovered for the first time. The reads number of these miRNA sequences in different libraries was ranged from 0 to 1,248,224, indicating that the miRNAs had a wide range of expression levels. In addition, several miRNAs showed high expression levels in multiple

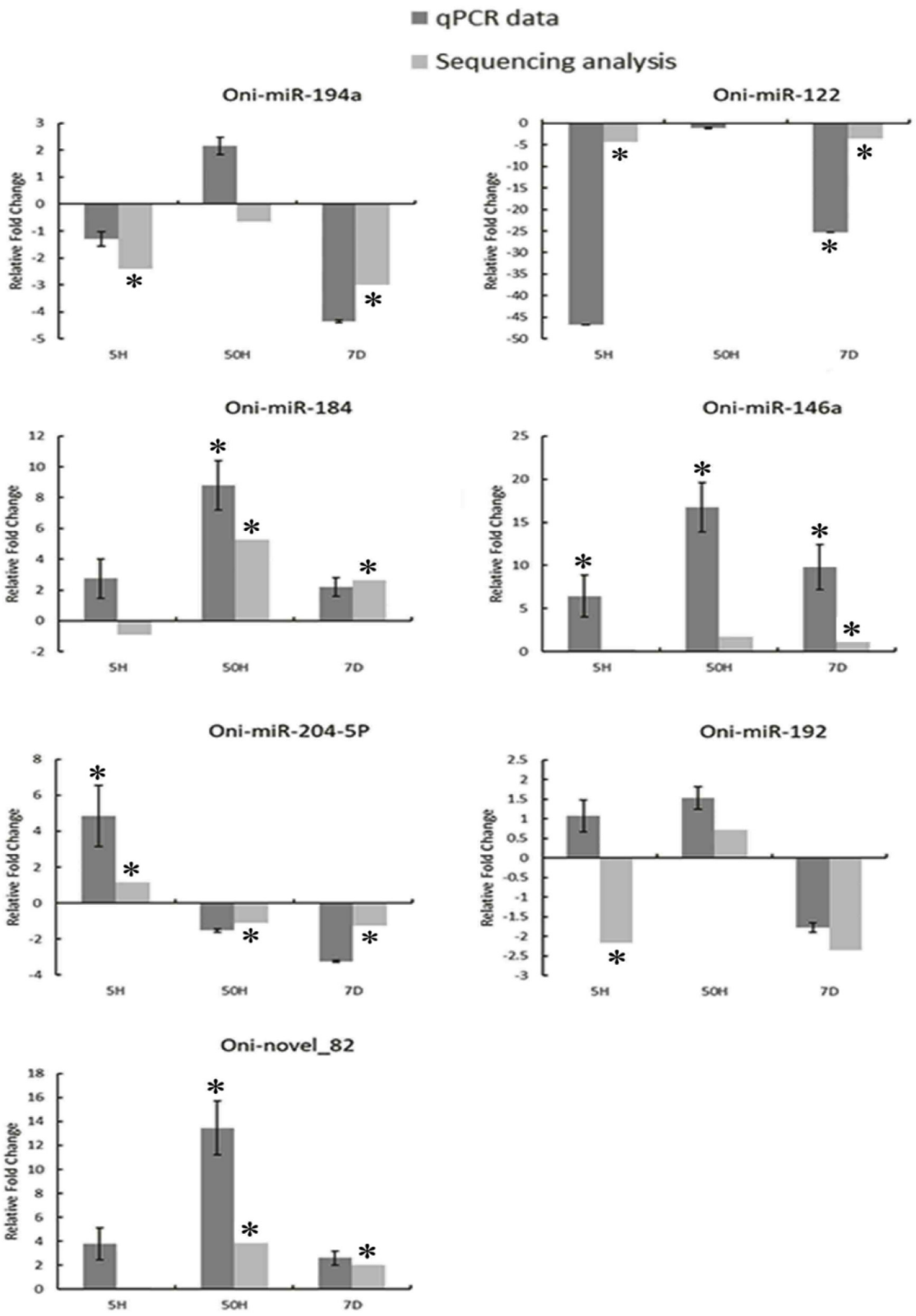

Fig. 8. Relative expression levels of 7 miRNAs in 5h, 50h, and 7d. Fold-changes were calculated by the change in expression at a given time point relative to the untreated control and normalized by change in the U6 housekeeping miRNA. The results were presented as mean ± SE of fold changes and * indicated statistical significance at $P < 0.05$. The relative expression levels by high-throughput sequencing analysis are represented by $2^{\log2(\text{treatment/control})}$. The expression level of miRNAs was analyzed by one-way ANOVA followed by Duncan's test. Groups marked with the same letters are not statistically different.

timepoints were previously reported to participate in various pathophysiological conditions in liver. For instance, miR-143 (highly expressed in all the time points), which could induce apoptosis of liver carcinoma cells through regulation of the NF-κB pathway [56]. In addition, the target genes of miR-143 including cathepsin K, cathepsin S, which were highly expressed in *S. agalactiae* resistant tilapia [57]. Moreover, miR-146a (highly expressed in all the time points), was reported to be involved in liver injury in mammals [58], and could promote viral infection by restraining anti-viral cellular responses in fathead minnow [59].

Additionally, several evolutionarily conserved miRNAs (let-7a and miR-21) that demonstrated by previous studies [60,61], were among the most abundantly expressed miRNAs (Table 3). Let-7e, miR-122, and miR-192 were all more abundant miRNAs in 4 time points, with deep sequencing revealing 10^5-10^6 expression levels. Other members of the let-7 family, such as let-7a–e, g, h, and i, were also expressed abundantly in this 4 time points during bacterial infection. Previous studies have shown that the let-7 family can regulate the expression of major cytokine-inducible proteins in response to microbial challenge in mammals [62,63]. Furthermore, 220 conserved miRNAs belonging to 94 miRNA families were identical in sequence to that from other fish species listed in miRBase 20.0, including *Salmo salar* (ssa), *Danio rerio* (dre), *Ictalurus punctatus* (ipu), *Rattus norvegicus* (rno) and *Oryzias latipes* (ola). Evolutionary conservation is one key feature of miRNAs and accumulating evidence reveals that miRNAs might be excellent phylogenetic markers [64]. Here, tilapia miRNAs are phylogenetically conserved with other identified fish miRNAs, which is in consistent with previous research that even distantly related species shared a large number of homologous miRNAs.

Detailed analysis of the expression profiles of tilapia miRNAs at different time points revealed that dynamic changes were associated with the course of bacterial infection. The identification and characterization of miRNAs involved in the immune response are now essential for the elucidation of host–pathogen interactions [65]. In recent years, an increasing number of miRNAs involved in the innate and adaptive immune responses have been identified, such as miR-155, -150, the miR-17-92 cluster; the let-7 family and the let-7 gene family et al. [65–68]. In present study, a total of 82 differentially expressed miRNAs were identified, associated with 85,248 putative target sites in tilapia genes. Notably, miR-92b-3p was the most down-regulated at 5 h post infection. Interestingly, the miR-92 family was highly expressed in tilapia head-kidney [69], and the inhibition of miR-92 family resulted in the enhanced inflammation responses in tilapia in response to *S. iniae* infection [70]. In addition, cathepsin F was one of the target gene of miR-92b-3p, which showed higher expression level in *S. agalactiae* resistant tilapia [71]. Following miR-92b-3p, miR-122 was down-regulated with −20.43 fold at 5 h post infection, and showed the most down-regulation at 7 d post infection. In miiuy croaker, miR-122 was participated in regulating RIG-I signaling pathway after poly(I:C) stimulation [72], and involved in the regulation of toll-like receptor signaling pathway after *Vibrio anguillarum* infection by targeting TLR14 [73]. In tilapia liver, inhibition of miR-122 caused a significant increase of metallothionein expression, which could promote promotes hepatic antioxidant defense exposed to cadmium [74]. Notably, miR-184 showed the most up-regulation at 50 h post infection. In orange-spotted grouper (*Epinephelus coioides*), the over-expression of miR-184 could promote the replication of red-spotted grouper nervous necrosis virus [24], further studies are needed to characterized the detailed mechanisms of miR-184 in bacterial infection.

The putative target genes of miRNA were predicted using miRanda for animals. MiRanda is the first microRNAs target gene prediction software developed in May 2003 [75]. It has a wide range of applications and is not subject to species limitations. The previous study of tilapia miRNAs was also utilized miRanda for target prediction [76]. GO and KEGG pathway analysis showed that the predicted target genes are involved in diverse biological processes ranging from fundamental cellular operations to stress response, suggesting that miRNAs may have a widely effect on the host system. It is of note that the majority of the differentially expressed miRNAs were down-regulated at early infection time. This indicated that the genes associated with many crucial cellular pathways are probably up-regulated in response to bacterial infection. In order to protect against attaching and invading pathogens, organ-specific and systemic immunological host responses are both activated by the pathogen-associated molecular-pattern pathway via membrane-associated Toll-like receptors (TLR) and cytoplasmic Nod-like receptors [77]. In our results, the NOD-like receptor signaling pathway was significantly enriched at all the time points following infection, suggested that tilapia miRNAs were induced to promote host defense, might contribute to trigger intracellular signaling pathways that lead to effector mechanisms in innate immunity and inflammation for pathogen clearance [78].

Previous studies reported that miRNAs were commonly affected by various bacterial infections and involved in the regulation of host immune response [79]. In mammals, miRNAs significantly regulated the sentinel capacity of mammary epithelial cells to mobilize the innate immune system, following the infection of the gram-positive bacteria, *S. uberis* [80]. In plants, miR-393 contributed to resistance against the extracellular pathogen *Pseudomonas syringa* in *Arabidopsis e*, presumably by repressing auxin signaling [81]. Similarly in lower vertebrates, *Vibrio anguillarum* infection regulated the expression of miRNAs in the immune tissues of half-smooth tongue-sole [15]. In this study, we found that 82 tilapia miRNAs were significantly differentially expressed during *S. agalactiae* infection in tilapia liver. The targets of host miRNAs were grouped into a wide range of functional categories, in particular those associated with immune defense/evasion and NOD-like receptor single pathway. These results suggested that in teleost, as in higher vertebrates, miRNAs prominently contribute to immune responses, protecting the organism against overwhelmed inflammation upon infection. Furthermore, comparing to a previous miRNA study has been conducted in tilapia spleen following *S. agalactiae* infection, we detected 262 novel miRNAs in tilapia, several of them were significantly expressed following challenge [76]. Moreover, their analysis was performed at a timescale of 72 h divided into six different time points, while our study was conducted until 7 d post infection. With the different tissues, our study could provide novel reference for further characterize tilapia miRNAs in response to *S. agalactiae*. Collectively, this study provides an opportunity for further understanding of the molecular mechanisms of miRNA regulation in *O. niloticus* host–pathogen interactions, and gene resources for molecular assistant selection for disease resistant breeding program.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.01.018.

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