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RNA-seq analysis of local tissue of *Carassius auratus gibelio* with pharyngeal myxobolosis: Insights into the pharyngeal mucosal immune response in a fish-parasite dialogue

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ABSTRACT

The lack of practical control measures for pharyngeal myxobolosis is becoming an important limiting factor for the sustainable development of the gibel carp (*Carassius auratus gibelio*) culture industry in China. *Myxobolus honghuensis* has been identified as the causative agent of this pandemic disease, which exclusively infects the pharynx of gibel carp, a potential important mucosal lymphoid-associated tissue (MLAT). Myxozoa generally initiate invasion through the mucosal tissues of fish, where some of them also complete their sporogonial stages. However, the pharynx-associated immune responses of teleost against myxosporeans infection remain unknown. Here, a *de novo* transcriptome assembly of the pharynx of gibel carp naturally infected with *M. honghuensis* was performed for the first time, using RNA-seq. Comparative analysis of severely infected and mildly infected pharyngeal tissues (SI group and MI group) from the same fish individuals and control pharyngeal tissues (C group) from the uninfected fish was carried out to investigate the potential mucosal immune function of the fish pharynx, and characterize the panoramic picture of pharynx local mucosal immune responses of gibel carp against the *M. honghuensis* infection. A total of 242,341 unigenes were obtained and pairwise comparison resulted in 13,009 differentially-expressed genes (DEGs) in the SI/C group comparison, 6014 DEGs in the MI/C group comparison, and 9031 DEGs in the SI/MI group comparison. Comprehensive analysis showed that *M. honghuensis* infection elicited a significant parasite load-dependent alteration of the expression of numerous innate and adaptive immune-related genes in the local lesion tissue. Innate immune molecules, including mucins, toll-like receptors, C-type lectin, serum amyloid A, cathepsins and complement components were significantly up-regulated in the SI group compared with the C group. Up-regulation of genes involved in apoptosis signaling pathway and the IFN-mediated immune system were found in the SI group, suggesting these two pathways played a crucial role in innate immune response to *M. honghuensis* infection. Up-regulation of chemokines and chemokine receptors and the induction of the leukocyte trans-endothelial migration pathways in the severely and mildly infected pharynx suggested that many leucocytes were recruited to the local infected sites to mount a strong mucosal immune responses against the myxosporean infection. Up-regulation of CD3D, CD22, CD276, IL4/13A, GATA3, arginase 2, IgM, IgT and pIgR transcripts provided strong evidences for the presence of T/B cells and specific mucosal immune responses at local sites with *M. honghuensis* infection. Our results firstly demonstrated the mucosal function of the teleost pharynx and provided evidences of intensive local immune defense responses against this mucosa-infecting myxosporean in the gibel carp pharynx. Pharyngeal myxobolosis was shaped by a prevailing anti-inflammatory response pattern during the advanced infection stages. Further understanding of the functional roles of fish immune molecules involved in the initial invasion and/or final sporogony site may facilitate future development of control strategies for this myxobolosis.

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1. Introduction

The allogynogenetic gibel carp, *Carassius auratus gibelio* (Bloch) is one of the most important freshwater aquaculture species in China, owing to its rapid growth and high market demand since the early 1980s [1]. The annual production of gibel carp was above three million tons in 2018. However, epizootic diseases have serevely limited the sustainable development of gibel carp culture, and also caused environmental and aquatic food safety-related issues. During the past 10 years, myxosporidiosis has been one of the most devastating diseases of gibel carp, which can affect the whole culture cycle [2–6]. Among myxosporidiosis, pharyngeal myxobolosis caused by *Myxobolus honghuensis* (Myxozoa), is responsible for the biggest economic losses for gibel carp culture in China [4]. The development of practical prophylaxis and treatment strategies for pharyngeal myxobolosis is thought to be a high priority to support the sustainable development of gibel carp industry in China. The pathogenesis of this myxobolosis and the host-parasite interactions, however, are still far from being fully understood.

Myxozoa is a group of diverse endoparasitic metazoans, generally with a biphasic life cycle involving invertebrates (mainly aquatic annelids) and vertebrate host (mainly fish) as definitive and intermediate hosts, respectively [7]. Currently, more than 2500 myxozoan species have been recorded worldwide, and some of them severely threaten fishery and aquaculture industries [8]. Although the invasion processes and intra-piscine migration routes of most fish myxozoans are not fully understood, it is widely accepted that fish mucosal surfaces, generally gills and skin, are exploited by actinospores, the infectious stages of myxozoans, as portals for entry. After invasion, their extra-sporogonic stages further develop and apply the vascular system to travel to the final infection sites, where sporogony occurs to produce mature myxospores and plasmodia or pseudocysts [9]. A series of effector immune cells and molecules has been identified in fish, and have been suggested to be involved in the interplay between the fish host and different developmental stages of myxozoans, from the initial attachment of actinospores on mucosal surfaces to their establishment in the final infection sites [10]. Differences in the intrinsic immunity of fish directly determine the host specificity, and susceptibility to a certain myxozoan parasite. Since the milestone work of Kallert et al. [11] demonstrated that actinospore behavior during the initial invasion is fairly non-specific, it has been proposed that the mounting of systematic and local immune responses to eliminate the myxozoan parasites are the main mechanisms underlining differences in host susceptibility and parasite pathogenicity [9,10,12]. Recently, local inflammatory responses involving pro-inflammatory cytokines and Ig^+ cells were shown to play important roles in the prevention of the proliferation of *Ceratonova shasta* in the intestines and subsequent successful regulation by decreasing the extensive inflammatory responses to avoid potential immunopathology and excessive host tissue damage [12]. Cellular effectors such as antigen-presenting cells (monocyte/marcrophage and dendritic cells) and B and T cells were also shown to play important roles in the local immune response of Atlantic salmon against *Kudao thyrsites*, an intracellular histozoic myxosporean [13]. The importance of local immune responses have thus been shown in several mucosal tissue-infecting myxosporean-fish host interactions [14,15]. Regarding the adaptive mucosal immune responses to the parasite infection, IgT were identified to be a specialized fish mucosal immunoglobulin and crucial in the responses of rainbow trout to the infection of *C. shasta* and *Ichthyophtirius multifiliis* [16–19].

Mucosal tissue harbors epithelial cells, which are continuously exposed to a wide variety of pathogenic and non-pathogenic organisms, and which consequently suffer from the invasion of these external microbes within a complex aquatic environment. The main mucosa-associated lymphoid tissues (MALT) of teleost comprise gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT) and the recently discovered nasopharynx-associated lymphoid tissue (NALT) [16,20]. NALT in mammals and birds have been demonstrated to be typical MALT and nasal-associated lymphoid tissue has also been recently shown to contain diffuse B cells and T cells in rainbow trout [16,21]. Little attention, however, has been paid to the fish pharynx, a possible MALT which warrants further investigation. Previous histological analysis showed that the pharynx of gibel carp was also covered with a mucus layer and contained an epithelial layer, a lamina propria and a muscular layer [6]. However, an abundance of taste buds distributed among the mucosal epithelium of gibel carp pharynx suggest that it is not exclusively an olfactory organ. Furthermore, the gibel carp nasal cavity is found to be not connected to the pharynx (authors' observation), indicating that the teleost pharynx is possibly structurally and physiologically different from those of terrestrial vertebrates and birds. No studies have so far been performed on teleost pharynx to discover alterations in local immune genes expression in response to parasitic infections. *M. honghuensis* exclusively infects the gibel carp pharynx and produces severe disease, so it will be an excellent model for exploring teleost pharyngeal mucosal immunity.

Tools for transcriptomic analysis, in particular RNA-seq, have been widely applied to elucidate the molecular processes behind fish-parasite interactions. Many previous RNA-seq studies have documented expression level changes in various immune-related genes in different fish-parasite dialogues, including Atlantic salmon *Salmo salar* and Coho salmon *Oncorhynchus kisutch* infected with the sea louse *Caligus rogercresseyi* [22], the naked carp *Gymnocypris przewalskii* infected by the ciliate *I. multifiliis* [23], the large yellow croaker *Larimichthys crocea* infected with the ciliate *Cryptocaryon irritans* [24], the gilthead sea bream *Sparus aurata* infected with the myxosporean *Enteromyxum leei* [25], and the turbot *Scophthalmus maximus* infected with the *E. scophthalmi* [14,15].

We conducted here a comparative transcriptomic analysis of severely infected and mildly infected pharynx tissues of naturally infected gibel carp and control gibel carp to reveal alteration in gene expression related to the local immune response of the pharynx to *M. honghugensis* infection. This study will disclose the primary landscape of mucosal immune system of teleost pharynx, improve our understanding of the molecular mechanisms of host-parasite relationships of mucosa-infecting myxosporeans, and finally contribute to the development of immunotherapeutic agents and the design of vaccines targeted against fish myxosporidiosis.

2. Materials and methods

2.1. Experimental design and sample preparation

All gibel carp used for the present work were sourced from a hatchery of Wuhan Branch of Charoen Pokphand Group in Huanggang city of Hubei province, China. For the full life cycle of *M. honghuensis* has not been disclosed, experimental infection of gibel carp can't be performed. Here, 1000 gibel carp larvae of 10 days post-hatch from a broodstock were exposed to infective water on Apr. 25 of 2018 in a 2^3 tuck net installed in a pond with recurrent pharyngeal myxobolosis. Routine cultivation was performed by splashing soybean milk to produce zooplankton at early stage and then feeding gibel carp commercial diet of small particle size. After presence of typical symptoms of some fish (Fig. S1) by daily routine inspection [6,26], about 50 infected and uninfected gibel carp juvenile were sampled simultaneously by hand net on Jun. 6 of 2018. The body length and weight of all sampled fish were individually measured. The infection severity of all fish samples was determined and quantified by the following developed PCR and QPCR, respectively. Briefly, genomic DNA was extracted from three groups using a DNA Blood and Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. A same primer pair targeting the ITS loci of *M. honghuensis* (Table S1) was designed for these two assays. The PCR program was as follows: an initial denaturation step at 95 °C for 3 min, followed by 30 cycles at 95 °C for 30s, 56.2 °C for 30s, 72 °C for 30s, and a final extension step at 72 °C for 10 min. The PCR products

were then excised from 1% agarose gels. The thermal profile of QPCR was 95 °C for 3 min, followed by 40 cycles of 95 °C for 12s, 56.2 °C for 20s, and 72 °C for 20s. The gene copy number was determined using the standard curve method with plasmid DNA containing a copy of the target as a control.

Finally, three infected fish with similar infection intensity and 3 uninfected fish with similar size with the sampled infected fish were randomly selected for tissue isolation. These three groups of tissues were designated as Group SI, Group MI and Group C, respectively. Group SI represents lesion tissue in pharynx of infected fish, where severe infection can be found and severe inflammatory responses are obvious. Group MI represents the mildly infected pharynx tissue of the same fish individual of SI group, where inflammatory responses are mild and low parasite load occurs. Group C represents the pharynx tissue from clinically healthy fish without *M. honghuensis* infection. All pharynx tissue samples were preserved quickly in cold RNA later (OMEGA), kept at 4 °C overnight and then transferred to −80 °C for the following RNA extraction.

2.2. Total RNA extraction, library construction and sequencing

Total RNA from nine samples (3 samples per group) were extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions and then treated with RNase-free DNase I (Thermo Scientific, USA) to remove genomic DNA contaminants. Subsequently, the quantity, purity and integrity of RNA were evaluated in a 1.2% (w/v) agarose gel electrophoresis, NanoDropND-2000 spectrophotometer (Thermo, Waltham, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Richardson, USA), respectively. RNAs with a RNA Integrity Number (RIN) > 8, 28S/18S > 0.7, and A260/280 values of about 2.0 were subjected to the RNA-Seq library construction using the TruSeq™ RNA Sample Prep Kit (Illumina, San Diego, CA, USA). The nine libraries were sequenced using an Illumina Hiseq2500 instrument, with 150bp pair-end reads produced.

2.3. De novo assembly and annotation

The clean reads were obtained by trimming adaptor sequences and filtering short reads with lengths of less than 10bp and low-quality reads (i.e. the percentage of bases of quality value less than 5 exceeds 50% in the read). High-quality reads were assembled using Trinity software with the default parameters [27] and then were mapped back to the Thelohanellus kitauei databases to remove myxosporean contamination using the soft Bowtie2 [28]. The longest transcript in each cluster was designated as a unigene. The obtained unigenes were annotated by five public databases, including National Center for Biotechnology Information (NCBI) non-redundant protein sequences (Nr), Swiss-Prot, the evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) [29], Gene Ontology (GO) [30], and Kyoto of Encyclopedia of Genes and Genomes (KEGG) [31].

2.4. Differentially expressed genes analysis and enrichment analysis

All clean reads from each of the nine transcriptome libraries were, respectively, mapped back to the transcriptome assembly using Bowtie2 software [28]. The number of reads aligned to each unigene in the alignment file was counted for each sample using RSEM software [32]. These read counts were normalized as FPKM (expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced) values [33]. The differential analysis was performed using DESeq R package (1.18.0), and p-values were adjusted using Benjamini-Hochberg's approach to control false discovery rate (FDR). Finally, genes with adjusted p-value less than 0.05 and the absolute value of log2 (Fold change) more than 1 were seen as differentially expressed genes (DEGs) [34]. The clustering analysis was conducted using Pheatmap package in R based on FPKM of DEGs. Then DGEs were enriched and analyzed with GO [35] and KEGG pathway [36]. Common and unique immune-related DEGs between groups were visualized using Circos software [37].

2.5. Experimental validation using QPCR

Eight immune related DEGs were selected to validate the RNA-Seq results using QPCR. Reversed cDNA from the total RNA used for transcriptome sequencing was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Shanghai, China). A SYBR Premix Ex Taq Kit (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instructions. Primers pairs (Table S3) were designed using primer 5.0 software. All QPCR reactions were done in three biological experiments and target specificity was determined by the dissociation curve analysis. The relative expression ratio of the target genes versus 18s rRNA was calculated using 2-ΔΔCT method. The obtained data were subjected to statistical analysis using SPSS 19.0 software.

3. Results

3.1. Transcriptome sequencing, de novo assembly and annotation

The infection intensity of Group SI, MI and C were measured to be 10^9, 10^7 and 0 copies/mg by QPCR, respectively (Fig. S2, Table S2). By using Illumina HiSeq™ 2500 platform, a total of 392 million raw reads were obtained from nine samples from the three groups. All raw reads have been deposited at the NCBI SRA repository under the accession number PRJNA534336. After removing reads with low quality or adaptors, 387 million clean reads were obtained and *de novo* assembled into a total of 242,341 unigenes with an average length of 510 bp and a N50 length of 591 bp (Table S4). The length distribution of the unigenes was shown in Fig. S3. Subsequently, these unigenes were annotated using information from databases, including Nr, Swiss-Prot, eggNOG, GO, and KEGG (Fig. 1a).

We found that 47.04% of unigenes were annotated in at least one public database. Of these, 37,957 unigenes were classified into 62 GO terms (Fig. 1a and b). “Cellular process”, “Cell” and “Binding” were dominant in the categories “Biological process”, “Cellular component”, and “Molecular function”, respectively. Of the sequences categorized as “Biological process”, 2022 unigenes were related to the term “immune system process”. To identify the possible pathways involved in physiological function of pharyngeal tissues, 12,947 unigenes were classified into 210 KEGG pathways (Fig. 1a, c). The pathway with most annotated unigenes was “Signal transduction” (1846 unigenes), followed by “Infectious diseases” (1178 unigenes) and “Cancers” (1097 unigenes). In addition, 901 unigenes were annotated to pathways related to the immune system.

3.2. Identification and enrichment analysis of differentially expressed genes (DEGs)

Pairwise transcriptome comparison identified 13,009 DEGs in the SI/C group (7929 up-regulated and 5080 down-regulated), 6014 DEGs in the MI/C group (2698 up-regulated and 3316 down-regulated), and 9031 DEGs in the SI/MI group (5471 up-regulated and 3560 down-regulated) (Fig. 2a). A group of 446 DEGs were shared between the three pairwise comparisons, and a total of 3,471, 2366 and 1119 DEGs were specifically identified between SI/C, MI/C and SI/MI, respectively (Fig. 2b). Based on the expression level of DEGs, a hierarchical clustering analysis suggested that intra-group samples of each group formed an independent cluster (Fig. 2c), suggesting the homogeneity of intra-groups samples. Moreover, fold changes of gene with alterative expression in the SI group were significantly higher than those in the MI group. All of the DEGs were selected for further enrichment analysis of GO term and KEGG pathways.

a

Database-Annotated	Number of annotated unigenes	Percentage of annotated unigenes (%)
Annotated in Nr	106235	43.84
Annotated in Swiss-prot	83426	34.43
Annotated in eggNOG	92322	38.1
Annotated in GO	37957	15.66
Annotated in KEGG	12947	5.34
Annotated in at least one database	113997	47.04

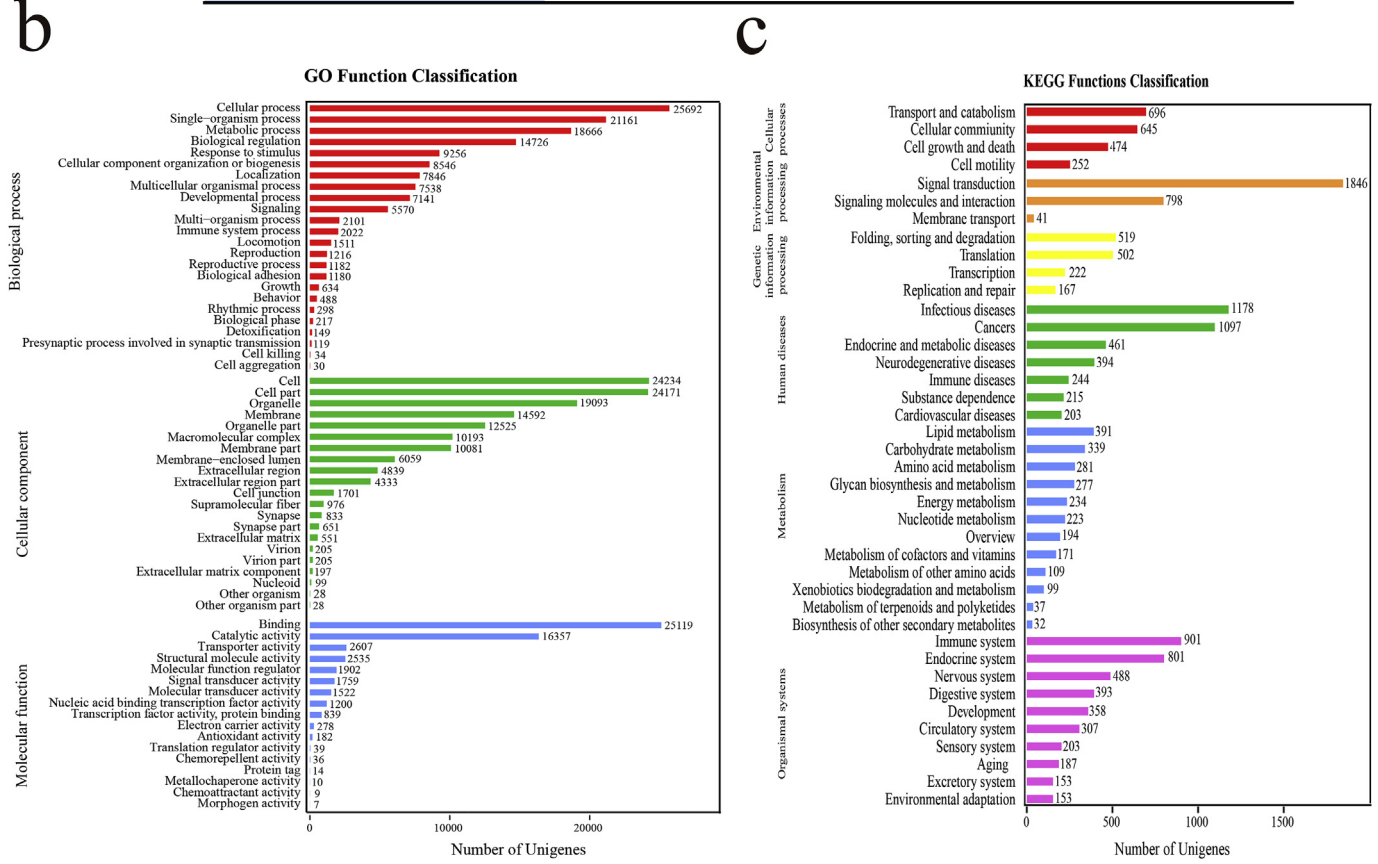

Fig. 1. Annotation summary and unigene classification of allogynogenetic gibel carp pharyngeal tissue transcriptome. (a) Overview of unigene annotation. (b) Histogram of GO classification. 37,957 unigenes were assigned 62 terms grouped into three main categories: biological process (red bars), cellular component (green bars) and molecular function (blue bars). (c) Histogram of KEGG classification. 12,947 unigenes were mapped to 42 terms grouped into six main categories: Cellular processes (red bars), Environmental Information Processing (orange bars), Genetic Information Processing (yellow bars), Human Diseases (green bars), Metabolism (blue bars) and Organismal systems (purple bars). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

GO analysis was conducted to investigate the DEGs involved in gibel carp responses to *M. honghuensis* infection amongst the three groups. The top 30 most significant GO terms from each pairwise comparison were identified and shown in Fig. S4. Two immune-related terms in the SI/C comparison (GO: 0042571 immunoglobulin complex, circulating and GO: 0008009 chemokine activity) were enriched, while no immune-related term was enriched in either the MI/C or the SI/MI comparison, suggesting that the expression of immune-related genes was more intensively regulated in the severe infection.

KEGG pathway enrichment analysis showed that 41 of the 50 top pathways enriched in the SI/C, MI/C, and SI/MI pairwise comparisons were immune- and disease-related pathways (Fig. 3), indicating that among the whole transcriptome, immune responses played a crucial role in responses to this parasitic infection. Infectious disease-related pathways were enriched in the SI/C and MI/C comparisons but not in the SI/MI comparisons. Among the twelve identified infectious disease pathways, five were protozoan parasite disease pathways including toxoplasmosis, leishmaniasis, malaria, Chagas disease, and amoebiasis, suggesting that the responses of gibel carp to *M. honghuensis* infection might have a similar pattern to the mammalian responses against protozoan pathogens. Of the sixteen immune-related KEGG pathways, a total of 440, 206 and 189 immune-related DEGs were identified in the SI/C, MI/C and SI/MI comparisons, respectively. The five most differentially expressed immune-related genes enriched pathways were as follows: Leukocyte trans-endothelial migration, Platelet activation, Chemokine signaling pathway, Complement and coagulation cascades, and Fc gamma R-mediated phagocytosis (Table S5).

3.3. Analysis of key DEGs related to immune responses

Based on the DEGs annotated in the above mentioned five databases and KEGG pathway analysis, 111 representative genes involved in innate and adaptive immunity were identified. They were further divided into five subgroups, including “Innate immune molecules”, “Complement activation”, “Apoptosis-related molecules”, “Cytokines”, and “Adaptive immune molecules” (Table 1). The number of up-regulated genes far exceeded the number of down-regulated ones. A circos map was applied to show that representative immune-related genes in

a

Group	Total DEGs	Up	Down
SI vs C	13009	7929	5080
MI vs C	6014	2698	3316
SI vs MI	9031	5471	3560

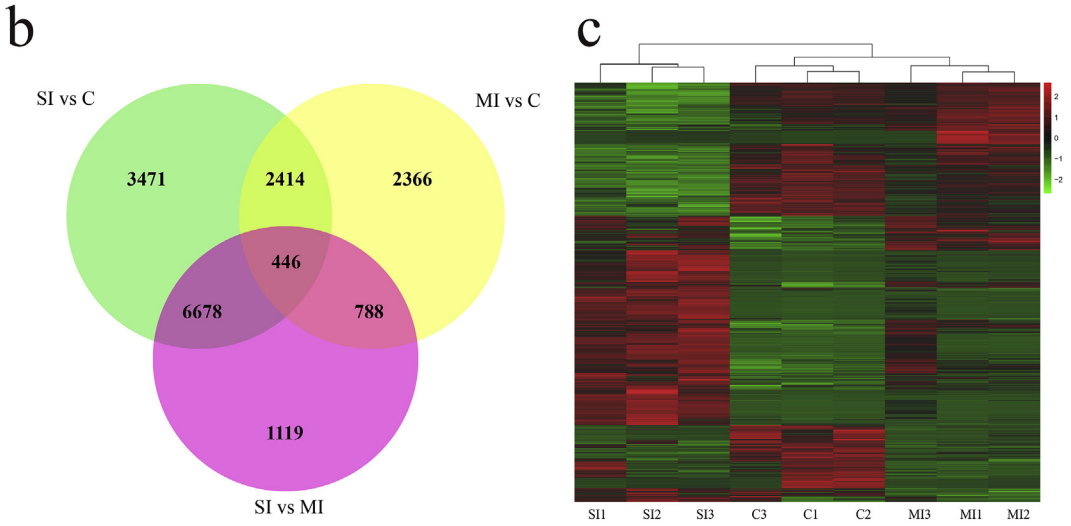

Fig. 2. (a) Summary of differently expressed genes (DEGs) among three groups with a cutoff of FDR corrected *P*-value < 0.05 and fold-change > 2. SI: severe infection; MI: mild infection; Con: control pharynx. (b) Venn diagram of DEGs among three groups. (c) Hierarchical clustering analysis divided the individual samples of each group based on FPKM of DEGs. Red and green indicated that the gene expression level was up-regulation and down-regulation, respectively. The scale of the color bar represented fold changes of gene expression. SI: 1–3 and MI: 1–3 represent tissue samples with severe infection and mild infection from the same challenged fish, respectively. C: 1–3 represent control pharynx tissue from healthy fish without *M. honghuensis* infection. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

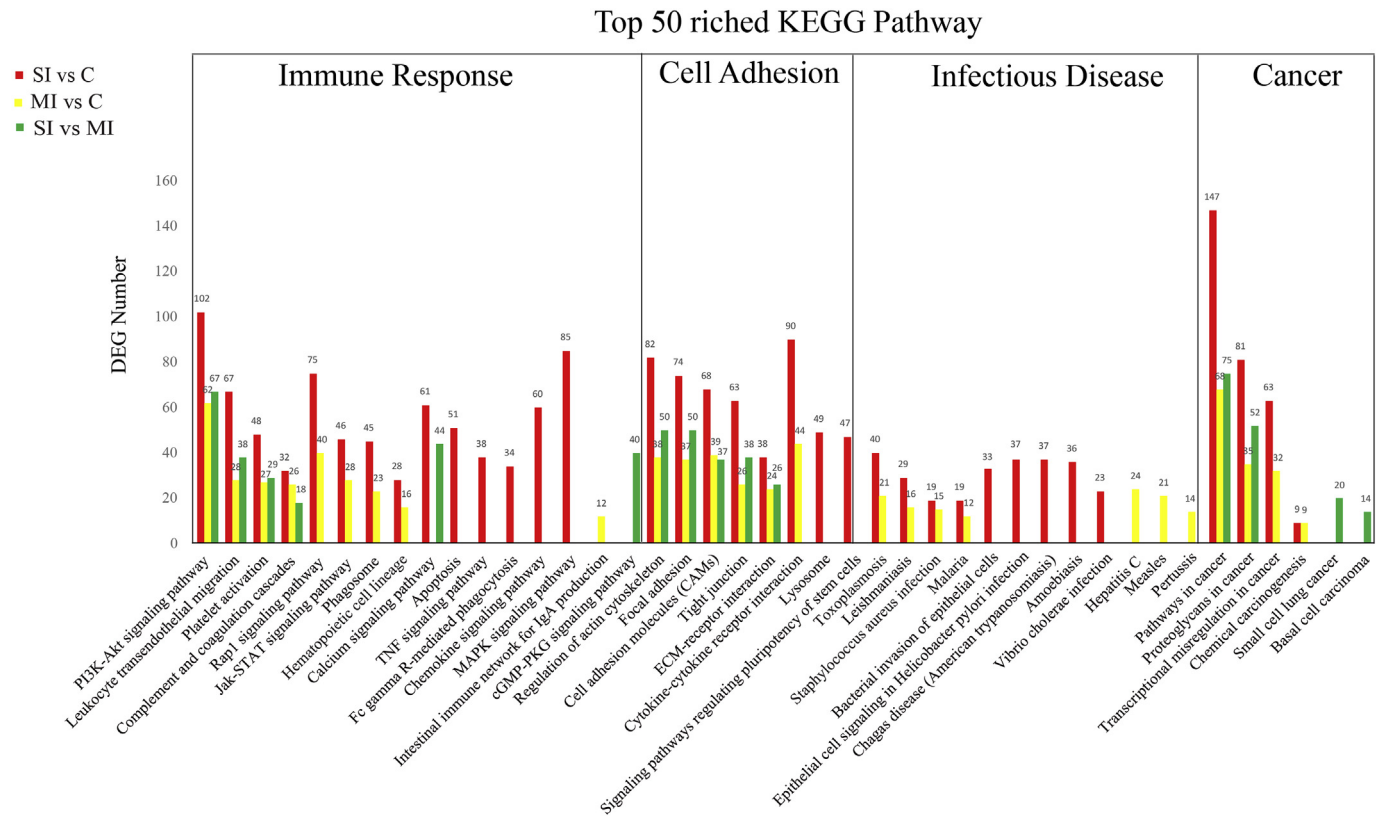

Fig. 3. The number of DEGs in the top 50 enriched KEGG pathway among three group. SI: severely infected pharynx tissue; MI: mildly infected pharynx tissue; Con: control pharynx tissue. The x-axis was KEGG pathway classification and y-axis was the number of DEGs.

Table 1
Representative differentially expressed immune genes among the severely (SI), mildly (MI) and control (C) pharyngeal tissue against *M. honghuensis* infection. Statistically significant fold changes are shown for 111 relevant genes associated with this pharyngeal myxobolosis. Non-significant differences have been marked as "-".

Gene name	Description	SI/C	MI/C	SI/MI
		Log2(Fold)	Log2(Fold)	Log2(Fold)
Innate immune molecules				
CTL	C-type lectin	3.38	2.47	–
TLR1	Toll-like receptor 1	3.83	3.19	–
TLR2	Toll-like receptor 2	1.99	–	1.64
TLR13	Toll-like receptor 13	5.62	–	–
SAA	Serum amyloid A protein	7.25	–	–
LYZC	Lysozyme C	−2.90	−1.42	−1.50
CTSA	Cathepsin A	2.06	–	1.27
CTSB	Cathepsin B	2.35	1.08	1.27
CTSD	Cathepsin D	2.76	1.08	1.63
CTSK	Cathepsin K	4.27	2.56	–
CTSL	Cathepsin L	3.75	1.86	1.89
CTSS	Cathepsin S	3.02	1.83	–
CTSX	Cathepsin X	2.59	1.03	1.55
TI	Trypsin inhibitor	3.30	–	2.09
MUC2	Mucin-2	5.03	–	4.33
MUC5	Mucin-5AC-like	2.55	0.35	1.55
MUC13	Mucin-13-like isoform X2	6.22	–	4.96
Complement activation				
C1	Complement component 1	2.05	1.65	–
C1QA	Complement C1q subcomponent subunit A	2.48	1.71	–
C1QB	Complement C1q subcomponent subunit B	2.39	1.60	–
C1QC	Complement C1q subcomponent subunit C	1.98	1.37	–
C1QR	Complement component C1q receptor-like	1.44	1.15	–
C3	Complement component 3	4.07	2.08	2.01
C4	Complement component 4	2.49	3.45	–
C6	Complement component 6	3.78	3.53	–
C7	Complement component 7	5.04	4.52	–
CFB	Component factor B	6.55	5.40	–
CFH	Complement factor H	4.43	–	–
CFI	Complement factor I	4.76	3.56	1.23
CFP	Complement factor properdin	2.21	1.82	–
CD59	CD59 antigen	2.25	–	–
Apoptosis-related molecules				
TNFα1	Tumor necrosis factor alpha 1	5.48	–	–
TNFR1	Tumor necrosis factor receptor superfamily member 1A	2.28	1.09	1.18
CYTC	Cytochrome *c*	11.03	–	5.64
CASP3	Caspase-3	1.87	–	–
CASP7	Caspase-7	5.56	1.53	4.02
CASP8	Caspase-8	1.02	–	–
FADD	FAS-associated death domain protein	1.30	–	–
BID	BH3 interacting domain death agonist	1.75	–	–
BAX	Apoptosis regulator BAX	1.08	–	–
BCL2L1	Bcl-2-like 1 (apoptosis regulator Bcl-X)	1.60	–	–
MCL1	Myeloid leukemia cell differentiation protein MCL-1	2.28	1.70	–
GZMB	Granzyme B	3.11	2.26	–
PRF1	Perforin-1	3.16	2.60	–
Cytokine-chemokines and chemokine receptors				
CCL19	C-C motif chemokine 19	2.40	1.79	–
CCL20	C-C motif chemokine 20	3.15	2.33	–
CXCL8/IL-8	C-X-C motif chemokine 8/Interleukin-8	7.61	–	–
CXCL9	C-X-C motif chemokine 9	5.11	4.21	–
CXCL12	C-X-C motif chemokine 12	2.67	2.07	–
CMKLR1	Chemokine-like receptor 1	3.15	–	2.70
CCR5	C-C chemokine receptor type 5	5.33	3.23	2.10
CXCR3	C-X-C chemokine receptor type 3	1.76	–	–
CXCR4	C-X-C chemokine receptor type 4	2.15	2.11	–
CXCR5	C-X-C chemokine receptor type 5	2.55	–	1.69
CXCR7	C-X-C chemokine receptor type 7	2.14	1.44	–
XCR1	Chemokine XC receptor 1	1.78	1.36	–
IL8RB	Interleukin 8 receptor beta	2.84	–	3.39
Cytokine-interferon				
IFNα4	Interferon alpha-4	1.59	–	1.87
IFNα13	Interferon alpha-13	7.40	–	6.40
IFNγ	Interferon gamma	3.56	1.88	–
IRF4	Interferon regulatory factor 4	4.87	3.98	–
IRF8	Interferon regulatory factor 8	2.40	1.42	–
MXE	Interferon-induced GTP-binding protein MxE	3.28	–	2.10
Cytokine-interleukin				
IL1β	Interleukin 1 beta	6.16	–	–

(*continued on next page*)

Table 1 (*continued*)

Gene name	Description	SI/C	MI/C	SI/MI
		Log2(Fold)	Log2(Fold)	Log2(Fold)
IL3NF	Interleukin 3 regulated nuclear factor	1.74	–	–
IL4/13A	Interleukin 4/13A precursor	5.72	5.00	–
IL6	Interleukin 6	2.52	1.62	–
IL6ST	Interleukin 6 signal transducer	2.76	2.44	–
IL10	Interleukin 10	4.75	–	3.74
IL11	Interleukin-11	5.56	–	–
IL17B	Interleukin 17B	−2.02	–	−1.63
IL1RL2	Interleukin-1 receptor-like 2	1.73	–	1.20
IL2Rβ	Interleukin-2 receptor subunit beta	1.69	1.34	–
IL2Rγ	Interleukin 2 receptor subunit gamma	2.53	1.45	–
IL4R	Interleukin 4 receptor	2.55	–	1.54
IL6Rβ	Interleukin-6 receptor subunit beta	2.17	1.71	–
IL8Rβ	Interleukin 8 receptor subunit beta	5.19	–	–
IL10Rβ	Interleukin-10 receptor subunit beta	2.18	2.11	–
IL13RA1	Interleukin-13 receptor subunit alpha-1-like isoform X1	2.30	1.32	–
IL17RC	Interleukin-17 receptor C	−1.05	–	–
IL21R	Interleukin 21 receptor	1.83	–	1.37
IL22RA2	Interleukin 22 receptor subunit alpha 2	1.96	–	1.92
Cytokine-others				
TGFβ1	Transforming growth factor beta-1	1.94	1.45	
TGFβR3	Transforming growth factor beta receptor type 3	3.25		1.50
ATF3	Activating transcription factor 3	2.61	–	–
GATA3	Transcription factor GATA-3	2.37	–	1.89
STAT1	Signal transducer and activator of transcription 1	2.41	1.47	–
STAT3	Signal transducer and activator of transcription 3	1.25	1.22	–
SOCS1	Suppressor of cytokine signaling 1	2.45	–	1.31
SOCS3	Suppressor of cytokine signaling 3	3.21	2.29	–
SOCS7	Suppressor of cytokine signaling 7	2.06	1.58	–
TBX2b	T-box transcription factor TBX2b	−1.55	−0.84	–
TNFAIP	Tumor necrosis factor alpha-induced protein 8	6.64	–	5.59
LITAF	Lipopolysaccharide-induced tumor necrosis factor-alpha factor homolog	3.46	1.91	1.58
TNFSF6	Tumor necrosis factor ligand superfamily member 6	1.65	–	–
TNFSF11	Tumor necrosis factor ligand superfamily member 11	1.53	–	–
Adaptive immune molecules				
CD3D	T-cell surface glycoprotein CD3 delta chain-like	1.12	1.08	–
CD22	CD22 antigen	4.71	–	–
Cd209c	CD209 antigen-like protein C	4.08	3.06	–
Cd209d	CD209 antigen-like protein D	2.59	1.64	–
CD276	CD276 antigen homolog	3.59	1.43	2.17
IGL	Immunoglobulin light chain	3.01	–	–
IGMH	Immunoglobulin mu heavy chain	6.04	4.27	–
IGZH	Immunoglobulin zeta heavy chain	7.80	6.33	–
MHCII	Major histocompatibility complex, class II	1.43	–	–
PIGR	Polymeric immunoglobulin receptor	7.97	5.89	–
IGSF2	Immunoglobulin superfamily member 2	3.99	–	–
ILDR2	Immunoglobulin-like domain-containing receptor 2	2.99	1.40	1.60
LYN	Tyrosine-protein kinase Lyn	2.15	1.49	–
PSTPIP1	Proline-serine-threonine phosphatase interacting protein 1	1.55	0.89	–
ARG2	Arginase-2	2.22	1.66	–

the MI/C and SI/MI groups were covered in the SI/C group (Fig. 4a). Following hierarchical clustering, a heatmap was generated to illustrate the differential gene expression patterns (Fig. 4b). These results demonstrated that the SI group had different gene expression pattern than the MI group from the same challenge fish and the C group from the healthy fish. Fold changes of up-regulated genes in the SI group were higher than those in the MI group, indicating an intense activation of local immune response in severely infected pharynx. Twenty-three immune-related genes were significantly expressed only in the SI group, while fifty immune-related genes were significantly expressed in both the SI and MI groups compared with the C group.

3.4. Validation of DEGs using QPCR

To confirm the RNA-seq results, eight immune-related DEGs were chosen for analysis by QPCR. Results from QPCR were almost consistent with those of the transcriptomic profile analysis, suggesting the accuracy and the reliability of RNA-seq results (Fig. 5).

4. Discussion

The present study was performed to investigate the local immune response of gibel carp against *M. honghuensis* infections using comparative transcriptomic analysis of the pharyngeal tissue samples with three different parasite load. Our discussion mainly focuses on the pharynx-associated molecular immune mechanism involved in this fish-myxosporean dialogue.

4.1. Innate immunity component molecules

When any mucosal barrier in fish senses a danger signal, an immediate innate immune response is triggered. Fish mucus exerts a protective effects by immobilizing or inhibiting pathogen binding, and also by acting as a vehicle for mucins (MUCs) and innate immune factors [38]. Among the innate immunity component molecules, toll-like receptors (TLRs), C-type lectin (CTL), serum amyloid A (SAA), lysozyme C (LYZC), proteases such as cathepsins (CTSs), and proteases inhibitor such as trypsin inhibitor (TI) were found to have significant

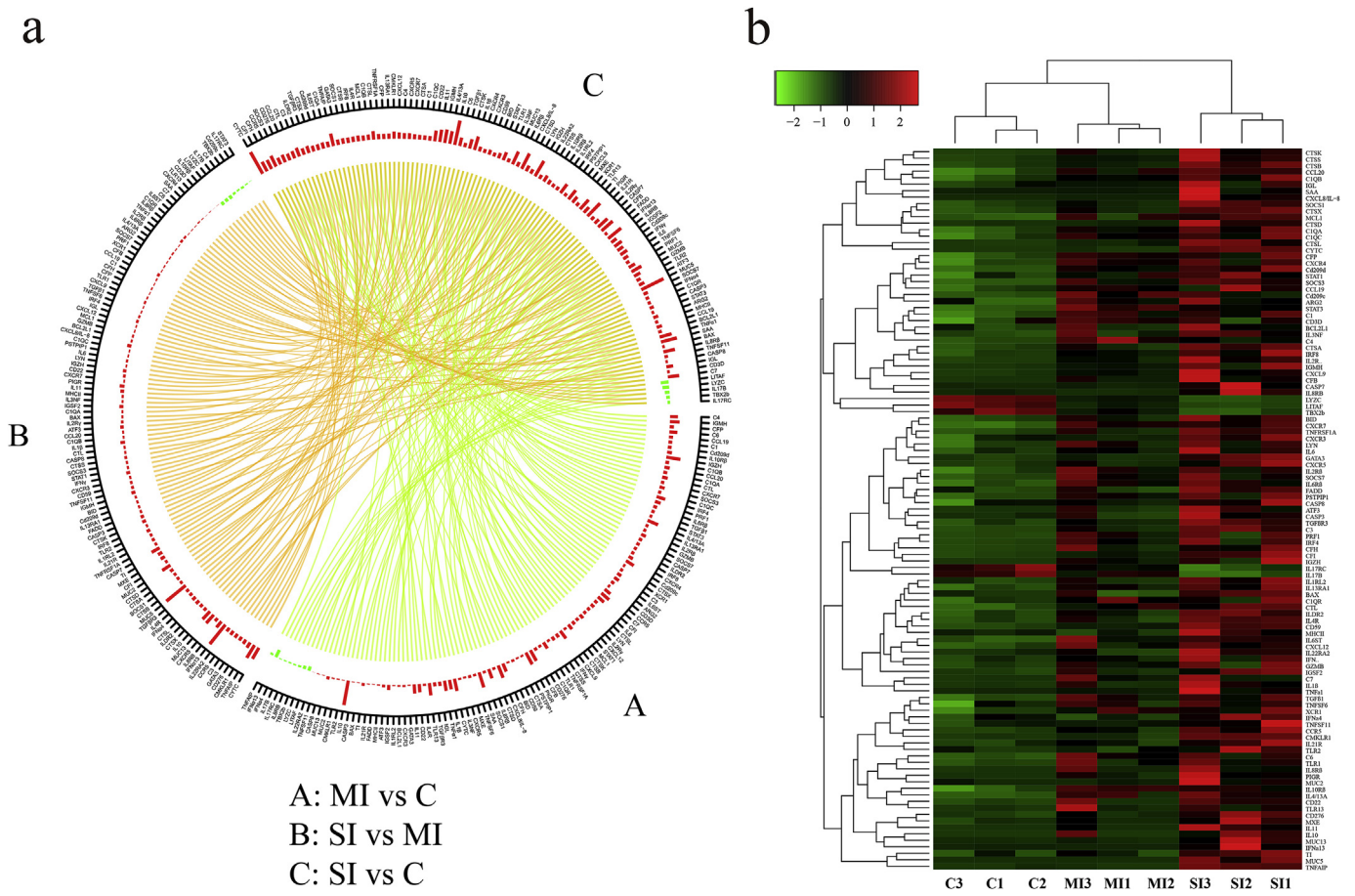

Fig. 4. (a) Circular representation of immune-related DEGs among three groups. The inner circular diagram showed that the gene expression level was up-regulated (Red) or down-regulated (Green). The outer circular diagram showed the gene name of DEGs. (b) Heatmap of immune-related DEGs. Red and green indicated the up-regulated or down-regulated gene, respectively. The scale of the color bar represented fold changes of gene expression. SI: 1–3 and MI: 1–3 represented severely infected pharynx tissue and mildly infected pharynx tissue from the same challenged fish, respectively. C: 1–3 represented control pharynx tissue from healthy fish without *M. honghuensis* infection. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

change in transcript levels in severely infected tissues, but less in mildly infected tissues. MUCs, the most abundant components of mucus, are high molecular weight glycoproteins secreted by goblet cells and have been documented to play important roles in the defense of the mucosa against pathogens [39,40]. Three transcripts exhibiting homology to mammalian mucins (MUC2, MUC5, and MUC13) were found to be significantly up-regulated in the severely-infected pharynx compared with mildly-infected and control pharynx, which possibly represented a strategy of gibel carp to prevent the local adhesion of *M. honghuensis* to the pharyngeal mucosa surface. TLR and CTL are both pattern recognition receptors (PRRs) which interact with the pathogen-associated molecular patterns (PAMPs) that result in activation of innate immunity [41]. TLRs, the best-characterized PRRs were previously reported to be activated in many fish species against parasitic infection [42]. Significant up-regulation of TLR1 and TLR2 were found here, consistent with previous reports of channel catfish skin of post-immunization with *I. multifills* [42] and *Epinephelus coioides* skin infected by the parasitic ciliate *C. irritans* [43]. CTL is well known for their ability to recognize specific pathogen-associated carbohydrate structures. Such specific lectin-carbohydrate interaction at the parasite membrane and host–parasite interface have been detected in several myxozoans, such as *Tetracapsuloides bryosalmonae*, *M. cerebralis* and *E. scophthalmi* [44–47]. So, it can be supposed that the up-regulated CTL helped *M. honghuensis* actinospores to attach and adhere to the mucosal epithelial cells of the host to complete its successful invasion. Up-regulation of CTL was also found in pyloric caeca of turbot with severe enteromyxosis caused by the intestinal myxozoan parasites *E. scophthalmi* [14]. SAA is a major acute phase protein and generally plays an effector function immediately after infection [48]. Increased expression of SAA have been previously reported in skin mucus after *E. coioides* challenged with *C. irritans* [43] and in rainbow trout gills infected by *I. multifiliis* [49]. CTSs constitute a group of lysosomal proteolytic enzymes, and are classified into three groups: serine proteases (CTSA), aspartic proteases (CTSD), and cysteine proteases (CTSB, CTSK, CTSL, CTSS, CTSX) [50]. CTSB, CTSL, and CTSS have also been shown to be up-regulated in *E. coioides* skin during *C. irritans* invasion [43]. Lysozyme is an important antibacterial enzyme produced by leucocytes, especially monocytes, macrophages and neutrophils [51], and has been described as active effector against some fish parasites. Previous reports showed contradictory results regarding the role of lysozyme against infection with myxosporean parasites [52–54]. Increased expression of lysozyme was reported in European sea bass infected with *Sphaerospora dicentrarchi* [52], but significantly down-regulated expression was found in gilthead sea bream infected by *E. leei* [53] and in turbot exposed to *E. scophthalmi* [54], which was in line with the result of the present study. TI is a type of serine protease inhibitor that is the largest and most diverse family of protease inhibitors [55]. The up-regulation of the TI transcript in severely infected tissue rather than mildly infected tissue suggested that gibel carp produced locally TI to inhibit possibly host or parasite-derived proteases to further reduce tissue damage at the advanced stage of gibel carp pharyngeal myxobolosis [8,56].

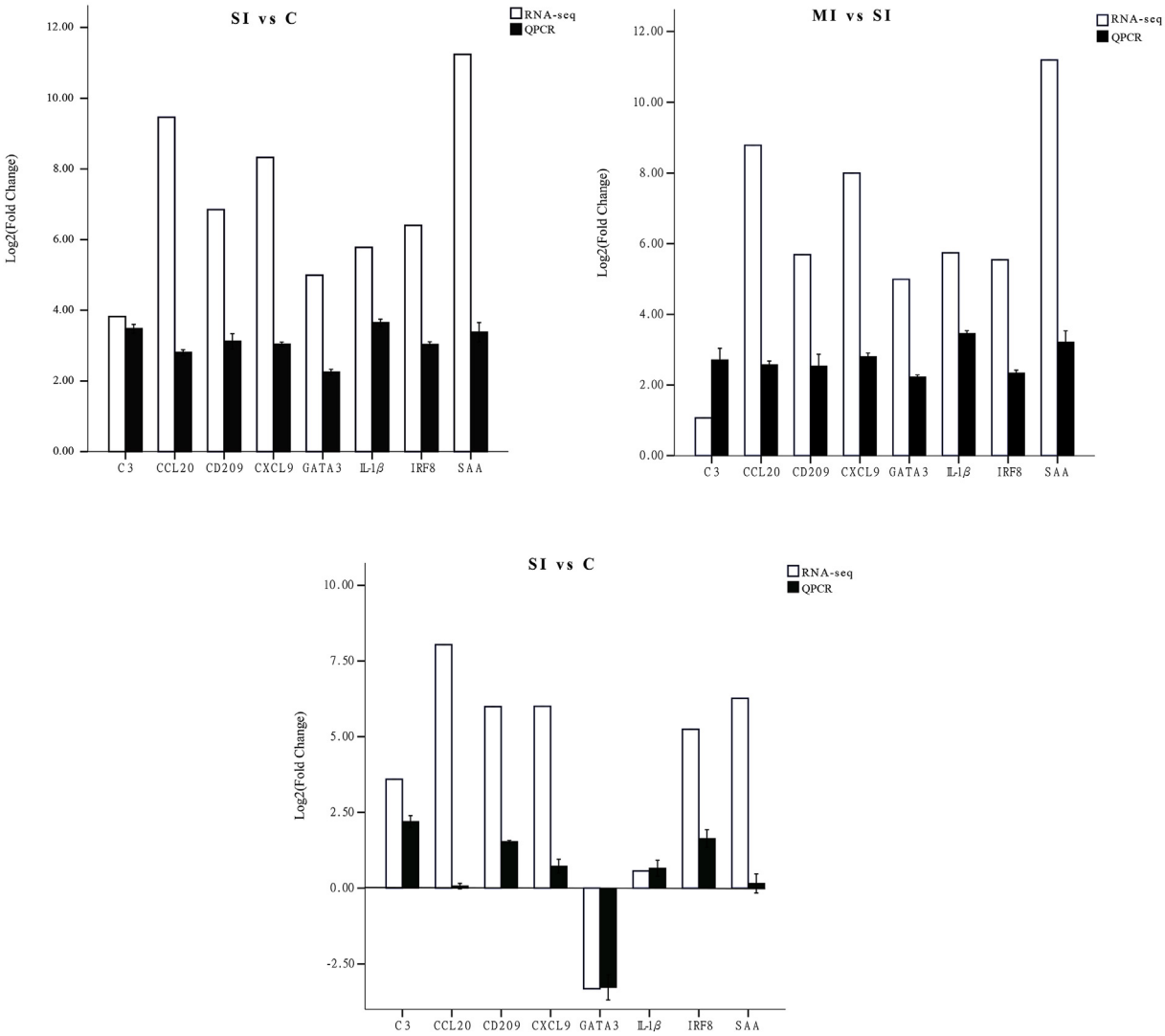

Fig. 5. Validation of RNA-seq data by using real-time RT-QPCR. The expression of C3, CCL20, CD209, CXCL9, GATA3, IL-1β, IRF8 and SAA was detected by RNA-seq (white column) and QPCR (black column).

4.2. Complement activation

Complement is a group of important effectors involved in the initiation of the innate response and subsequent mounting of an adaptive response in fish. The complement cascade is a part of phylogenetically ancient innate immune response, which functions in opsonization of pathogens, recruitment of local inflammatory and immune competent cells, and the direct killing of pathogens [57,58]. In the current study, a total of thirteen complement-related genes (C1, C1QA, C1QB, C1QC, C1QR, C3, C4, C6, C7, CFB, CFH, CFI, CFP) were almost significantly up-regulated in severely and mildly infected tissues, indicating that all three pathways of complement (classical, lectin and alternative pathways) were activated in the interaction of gibel carp pharyngeal mucosa with the infection of *M. honghuensis*. C1q, consisting of C1QA, C1QB, and C1QC, plays an essential role in the initiation of the classical complement pathway [59]. C3 is the central factor in all three complement activation pathways [58]. C3 has also been shown to be up-regulated in pyloric caeca during the late stages of turbot enteromyxosis [14]. C4 plays a central role during the early stage of lectin and classical pathway activation, however, C6, as well as C7 are critical at the end stage of the complement activation cascade to form the membrane attack complex (MAC), which disrupts the membrane of the targeted pathogen cells [60]. Previous reports have shown that the complement lectin pathway was probably activated by surface-associated carbohydrate moieties such as CTL, in several fish parasitic diseases models [14,61]. The alternative complement activity was also increased in the serum of gilthead sea bream exposed to *E. leei* [62]. Considering the similarity in the surface components of myxosporean parasites, similar activation of the complement-induced cascade could occur in the defense processes of gibel carp against *M. honghuensis* infection. Significant activation of complements play a crucial role in host defense against infection, however, excessive activation potentially cause serious damage to host tissue. The significantly up-regulated expression of CFH, CFI, and host cell membrane surface expression CD59 to limit MAC assembly suggested that the pharynx of gibel carp had evolved successful strategies to maintain locally the homeostasis during the interactions with *M. honghuensis* [63,64].

4.3. Apoptosis-related molecules

Apoptosis is an essential biological process an important part of the fish innate immune response which is generally activated to respond extrinsic stimuli such as tissue injury [65,66]. In this study, thirteen apoptosis-associated genes (TNFα1, TNFR1, CYTC, CASP3, CASP7, CASP8, FADD, BID, BAX, BCL2L1, MCL1, GZMB, PRF1) were significantly up-regulated in severely-infected pharynx, suggesting the

activation of intrinsic and extrinsic pathways of apoptotic processes in the local pharyngeal tissue of gibel carp after *M. honghuensis* infection. TNFα1, binding to a specific cell surface receptor TNFR1, was an indicator of the initiation of the extrinsic signaling pathway [67] during the late stage of *M. honghuensis* infection. CYTC is an important molecule indicative of the activation of the intrinsic apoptotic pathway, as it damages the integrity of the mitochondrial outer membrane [44]. We observed a considerable up-regulation of CYTC transcript in the SI/C and SI/MI group comparison, but there was no significant difference in the MI/C group comparison, indicating that severe *M. honghuensis* infection in the pharynx of gibel carp produced serious tissue damage. Differences in the fold changes of CYTC in the SI/C and SI/MI group comparisons suggested that tissue damage caused by this myxosporean was parasite load dependent. CASP3 and CASP8, essential players in the execution phase of apoptosis, were found to be significantly up-regulated in severely affected pharyngeal mucosa, but not in mildly affected tissue, compared with the control group. These results indicated that apoptosis occurred primarily in the late stage of the pharyngeal myxobolosis, consistent with the previous report of significant up-regulation of CASP3 and CYTC in pyloric caeca during the advanced stage of enteromyxosis in turbot [14].

4.4. Cytokines

Several groups of cytokines, including chemokines (CKs), interleukins (ILs), interferons (IFNs), transforming growth factors (TGFs), and others involved in innate immune responses, generation of cytotoxic T cells and the production of antibodies [68] were identified in the present study.

4.4.1. Chemokines

Chemotactic cytokines orchestrate the migration of immune cells to the site of infection, and are critical elements of the innate immune system and cross-talk between innate and adaptive immune responses [69]. In this study, we identified five CKs (CCL19, CCL20, CXCL8, CXCL9 and CXCL12), and eight CK receptors (CMKLR1, CCR5, CXCR3, CXCR4, CXCR5, CXCL7, XCR1, and IL8RB). Four of the CKs including CCL19, CCL20, CXCL9, and CXCL12 were significantly up-regulated in the SI/C and MI/C groups, but no significant difference was found in the SI/MI group. There were higher-fold changes of expression in the SI/C group than in the MI/C group, suggesting that the recruitment of immune cell to the pharynx tissues was parasite load dependent. CCL19 and CCL20 play vital roles in trafficking T, B cell and mature dendritic cells migration to specific sites within lymphoid tissues. Previous studies have shown an increase in CCL19 and CCL20 production in turbot intestine against the myxozoan parasite *E. scophthalmi* [15], in orange-spotted grouper skin post *C. irritans* infection [43], and in the liver of the large yellow croaker in response to *C. irritans* [24]. CXCL9 is an interferon-inducible chemoattractants which recruits activated $CD4^+$ Th1 cells, $CD8^+$ T cells and NK cells [70]. CXCL12 can induce the migration of dendritic cells, which orchestrate the positioning of effector cells in a timely manner for optimizing the elimination of malaria infection [71]. Overexpression of CXCL9 and CXCL12 were found in orange-spotted grouper skin infected with *C. irritans* [43]. IL-8 (also known as CXCL8), an attractant of neutrophil, was significantly up-regulated only in severe-infected pharynx, but not in mild-infected pharynx from the same challenged fish, demonstrating that intensive immune responses occurred at the severe infection sites. The expression of IL-8 was previously reported to elevate in salmon skin responding to *Lepeophtheirus salmonis* [72]. Together with activation of the leukocyte trans-endothelial migration pathway, these results suggested that significant numbers of leucocytes were recruited to the infected sites to mount a local immune defense and response degree depended highly on the parasite load. These CKs and CK receptors identified in this study could not be confirmed to be specific to the pharyngeal mucosal system, but it can be induced that they did regulate the immune cells of the pharyngeal mucosa migration in response to *M. honghuensis* infection.

4.4.2. Interferons

IFNs play diverse roles in coordinating the innate and adaptive immune responses to pathogens [73]. There are multiple lines of evidences to suggest a vital role of the IFN-mediated immune response against the infection of myxozoan parasites in teleosts [12,14,15,56,74]. In this study, we observed elevated expression of IFN-related genes such as IRF4, IRF8, IFNα4, IFNα13, IFNγ, and MXE in the infected pharynx compared with the control pharynx. Among these, up-regulation of IRF4, IRF8, and IFNγ expression was found not only in severely affected but also in mildly affected pharyngeal tissues from gibel carp infected with *M. honghuensis*, although the extent of expression change was almost double in the former than in the latter. The IRF4 and IRF8 genes contribute to B-lymphocyte development and play a major role in defense against bacteria and viruses in fish [75,76]. Our findings provided further evidence for their involvement in the immune response against parasites. IFNγ, produced by natural killer cells, can activate T lymphocytes and mediate a diverse range of host immune responses, including inflammation, antigen processing, cell death, and other anti-pathogen responses [77]. Up-regulation of the IFNγ gene in local immune responses of the olive flounder against a myxozoan parasite *Kudoa septempunctata* has been observed [74]. IFNγ has also been hypothesized as a potential candidate marker for *E. leei* resistance due to its up-regulation in exposed but not parasitized gilthead sea bream after chronically exposure to *E. leei* [56]. The expression of IFNγ was markedly increased during the early stage of turbot enteromyxosis after exposure to *E. scophthalmi* [15], as well as in the early stage of trout whirling disease, caused by *M. cerebralis* [77] while in the late stage of pharyngeal myxobolosis, the expression of IFNγ was also up-regulated. So, it can be speculated that IFNγ is a key effector of immune defenses in fish against the myxosporean infection at both early and advanced disease stages.

4.4.3. Interleukins and others

ILs are a group of cytokines to play a major regulatory role in the immune system during infection [78]. In this study, we found 21 transcripts encompassing 9 ILs (IL1β, IL3NF, IL4/13A, IL6, IL6ST, IL10, IL11, IL12β, IL17B), 11 IL receptors (IL1RL2, IL2Rβ, IL2Rγ, IL4R, IL6Rβ, IL8Rβ, IL10Rβ, IL13RA1, IL17RC, IL21R, IL22RA2), and several other immune-relevant cytokines (TNFα, TGFβ, GATA3, SOCS3). Previous report has indicated that IL-related genes in the gilthead sea bream infected with *E. leei* play both pro- and anti-inflammatory roles [78]. It could be suspected that a partial shift in the type of immune response from pro-inflammatory to anti-inflammatory occurred in the pharyngeal mucosal system of gibel carp at the advanced stage of this myxobolosis, based on the changes of gene expression profiles of signature cytokines produced by Th subsets (Th1, Th2, Th17, Treg cells). The immune control of parasites was previously suggested to involve temporal switches in the activation of different Th subsets [79]. For example, Th17 cells promote tissue inflammation, while Treg cells have been identified as immune suppressors to maintain peripheral tolerance. Th17 cells produce IL17 cytokine to be involved in the inflammatory and neutrophil responses and play a critical role in mucosal and epithelial host defense against extracellular pathogens [80,81]. Our RNA-seq analysis found a significant down-regulation of IL17B and IL17RC in the SI/C group, but no significant changes in the MI/C group, suggesting that intensive anti-inflammatory responses occurred in the severely infected pharynx. The expression of these two genes presents the same pattern in turbot pyloric caeca with severe entermyxosis. SOCS3, an inhibitor of IL-17 expression, was also up-regulated in the turbot spleen [14]. IL-10 and TGF-β are of two important anti-inflammatory cytokines, secrected by Treg cells. IL-10 can inhibit the expression of many pro-inflammatory cytokines, including Th1 pathway-related to cytokines (IL-1β, TNFα and IFNγ), drives proliferation of memory T cells, and regulates B cell differentiation and

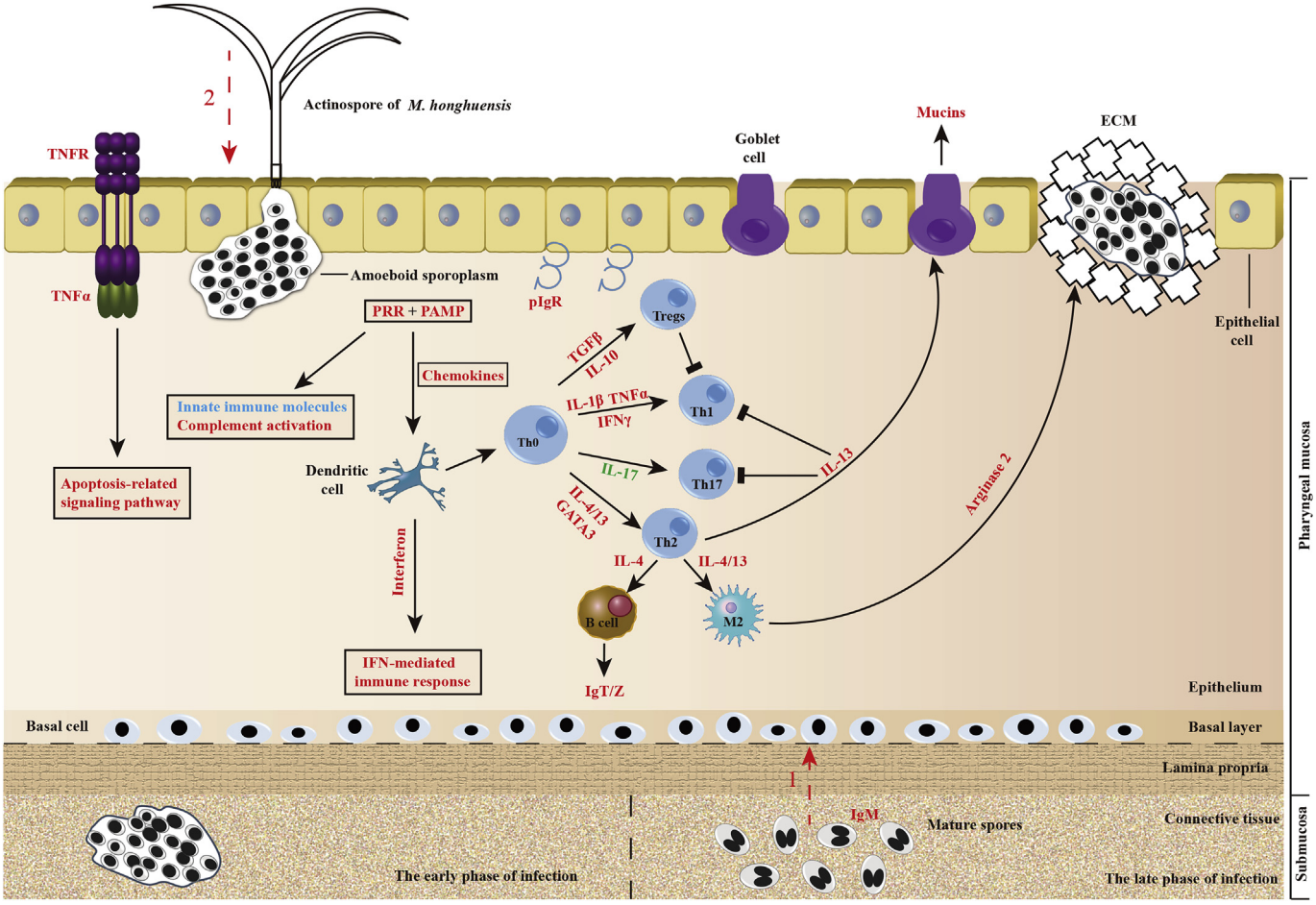

Fig. 6. Schematic diagram showing the main immune-related events involved in pharynx myxobolosis in allogynogenetic gibel carp (*Carassius auratus gibelio*) inferred from the results of this study. Upon actinospore of *M. honghuensis* attachment to the pharyngeal mucosa, the valve cells open to release the infective sporoplasm covered by the membranous endospore sheath. Then the sporoplasm actively moves by amoeboid movement to penetrate into the pharyngeal mucosa. Host pattern recognition receptors (PRRs) interact with the pathogen-associated molecular patterns (PAMPs) that result in activation of innate immune molecules, apoptosis, IFN-mediated immune response, T and B cell-mediated immune responses. Arginase 2 may promote extracellular matrix (ECM) deposition that can either repair damage or encapsulate parasites. Red words represent up-regulation; green words represent down-regulation; blue words represent not only up-regulation, but also down-regulation. The number 1 and 2 of dotted red arrow represents two suspected invasion and migration routes of *M. honghuensis*, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

antibody secretion [82,83]. TGF-β plays pleiotropic effects on the immune system, including inhibition of T cell proliferation and macrophage activation, and promoting the development of Th17 cells and tissue repair after a local immune and inflammatory reaction [82]. The involvement of a Treg subset, as implied by the up-regulation of IL-10 and TGF-β may mitigate the deleterious effects of pharyngeal inflammation caused by the *M. honghuensis* infection. Similarly, the involvement of this Treg subset was also reported in gilthead sea bream intestine infected with *E. leei* [78] and Atlantic salmon skin infected with salmon louse at the late infection [84].

IL-4/13A and GATA3, generally related to the Th2 pathway, were found both to be significantly up-regulated in the SI/C group, which was an indicative of an intense Th2 response to *M. honghuensis* infection at the late stage. IL-4 promotes Th2 cells development, and suppresses the development of Th1 and Th17 cells [85,86]. The secretion of IL-4 can induce an alternative pathway of macrophage (M2) activation or be involved in the production of antibodies, especially in parasitic infections [87,88]. The synergistic up-regulation of IL-4/13A, transcription factor GATA3, and arginase 2 (ARG2), a typical marker of M2 here strongly suggested that the pronounced activation of M2 and a partial shift towards a Th2 response occurred in the local pharyngeal responses to *M. honghuensis* infection. This type of Th2 shift was also found in the skin of rainbow trout infected with the parasitic flagellate *Ichthyobodo necator* [89] and gills of Atlantic salmon in response to infection with the amoeba *Paramoeba perurans* [90]. Immune regulation and responses to parasitic infection commonly result from the synergistic effects of different T-cells [84]. In this study, the down-regulation of the Th17 pathway and the up-regulation of the Th2 and Treg pathway indicated that a pro-inflammatory IL expression profile was over-shadowed by a prevailing anti-inflammatory pattern in the advanced stages of *M. honghuensis* infection.

4.5. Adaptive immunity component molecules

The above results indicated that innate immunity and cytokines related to adaptive immunity played an important role in the late stage of *M. honghuensis* infection. In addition, molecules associated with antigen presentation and the production of antibodies were also identified. Antigen processing and presentation are essential elements of adaptive immunity in teleost fishes. In this study, Cd209c, Cd209d and MHC-II, which were involved in antigen processing and presentation, were all significantly up-regulated in the SI/C group, but not significantly expressed in the SI/MI group. Cd209c and Cd209d, the markers of dendritic cells, were also up-regulated in turbot pyloric caeca suffering severe enteromyxosis and in orange-spotted grouper skin infected with the parasite *C. irritans* [14,15,43]. MHC-II, expressed

on the surface of antigen-presenting cells, is responsible for the presentation of exogenous antigens to $CD4^+$ T cells, which are critical for controlling parasite infections in fish [44,49]. The MHC-II gene was also up-regulated in the intestine of gilthead sea bream exposed but non-infected with *E. leei* [8]. The T-cell markers CD3D and CD276, which participated in the regulation of the T-cell-mediated immune response [91] were both observed to be significantly up-regulated in severely-infected and mildly-infected pharynx compared with control pharynx. The B-cell receptor CD22, which mediated B cell migration to intestinal mucosal tissue [92], was significantly up-regulated only in the SI group. Up-regulation of CD3D, CD22 and CD276 possibly suggested the presence of B and T cells at locally-infected sites, indicative of the activation of a local specific immune response. Synergetic significant up-regulation of heavy chain transcripts encoding IgM, IgT and polymeric immunoglobulin receptor PIGR were found in SI and MI group, however, their magnitude of expression changes was higher in the former than in the latter. This result suggested that pIgR was required for the transformation of IgM and IgT to the pharynx mucosal surface of gibel carp to function the local adaptive immunity to against the *M. honghensis* infection and this requirement was also parasite load-dependent [93]. The specific mucosal immune functions of IgT in the skin, gut, gills, and olfactory organs of teleost against parasite have intensively been demonstrated [16–19]. Our results provided the first evidence that IgT production occurred in the pharynx of teleost fish, although it remained unknown if it was *M. honghuensis*-specific. IgT was present in higher concentrations and titers in the mucosa, while IgM mainly functioned in the systemic immune organs [42]. Higher mRNA expression of heavy chain genes of IgT than that of IgM in the severely infected gibel carp pharyngeal mucosa suggested that IgT played a dominant role to locally respond to the *M. honghuensis* infection and IgM was possibly induced in systematic immune organs during the development of this myxosporean in vascular system and then transferred to the local site.

5. Conclusion

In this work, a combination of DEGs, GO, KEGG enrichment analysis and immune-related gene differential expression analysis showed that more significant changes of gene expression occurred in locally severely-infected tissues compared with mildly-infected tissues of the same challenged fish and the local immune response of pharyngeal mucosa of gibel carp was remarkably activated to defend from *M. honghuensis* infection. The gibel carp immune response during the advanced stage of *M. honghuensis* infection was chiefly characterized by effective innate and adaptive immune responses, including complement cascades, apoptosis signaling pathway, the IFN-mediated immune system, and a mixture of Th subsets effector responses. A prevailing anti-inflammatory pattern and Th2 effector responses to up-regulate the expression of IgT and IgM were also remarkable in this fish-myxosporean dialogue. This knowledge is essential for the investigation of the pathogenic mechanisms of fish-myxozoan interactions in this pharyngeal myxobolosis and will be useful for the further development of new therapeutic strategies. And, we found here evidences that teleost pharynx had a similar immune functions to those of other MALTs such as GALT, SALT, GIALT, and NALT. Finally, we suspected that *M. honghuensis* and other mucosa-infecting tissue myxosporeans has two invasion routes based on the identified DEGs and involved pathway here (Fig. 6). For the first invasion route, amoeboid sporoplasm of *M. honghuensis* actinospores applies MALTs as entry loci and then migrate through the vascular system to colonize in pharynx, which could lead to the production of IgM. For the second invasion route, infective actinospores sporoplasm of *M. honghuensis* can directly invade and colonize the pharyngeal mucosa to complete sporogony.

Conflicts of interest statement

The authors have no conflicting commercial or financial interest in publishing this paper.

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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.2019.08.076.

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