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iTRAQ analysis of liver immune-related proteins from darkbarbel catfish (*Pelteobagrus vachelli*) infected with *Edwardsiella ictaluri*

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ABSTRACT

Edwardsiella ictaluri causes enteric septicemia of catfish (ESC), a major disease occurring in these siluriform fish. As the liver is an important organ for defending against bacterial pathogens in fish, this study aimed to determine the liver immune response at the protein level. The differential proteomes of the darkbarbel catfish liver in response to *E. ictaluri* infection were identified with isobaric tags for relative and absolute quantitation (iTRAQ) labeling followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Using a 1.2-fold change in expression as a physiologically significant benchmark, a total of 819 differentially expressed proteins were reliably quantified using iTRAQ analysis, including 6 up-regulated proteins and 813 down-regulated proteins. GO enrichment analysis indicated that the "complement activation, alternative pathway" and "complement activation, classical pathway" were significantly enriched. KEGG enrichment analysis indicated the "antigen processing and presentation" and "bacterial secretion system" were significantly enriched. We selected the 6 up-regulated proteins and 10 immune-related down-regulated proteins for validation using real-time PCR. The 10 immune-related proteins included complement component C1r, C3, C5, C7, and C9 and plasma protease C1 inhibitor (C1-INH), signal recognition particle 54 kDa protein (SRP54), SRP receptor, proteasome activator complex subunit 1 (PSME1) and major histocompatibility complex class I (MHC class I) were selected from the GO clusters and KEGG pathways. The variations in mRNA expression for these genes were similar to the results of iTRAQ. This is the first report detailing the proteome response in the darkbarbel catfish liver during *E. ictaluri* infection and markedly contributes to our understanding of the defense mechanisms in the livers of darkbarbel catfish.

1. Introduction

Edwardsiella ictaluri causes enteric septicemia of catfish (ESC) [1], which is considered the most economically important cause of disease in catfish farms in the USA and is responsible for the majority of disease-related mortality annually [2,3]. In May 2006, *E. ictaluri* first appeared in Liaoning Province and then spread rapidly to other culture regions in China [4]. There are two main symptoms of fish with ESC, either acutely or chronically. Acute disease caused by ESC is characterized grossly by cutaneous hemorrhage, ulceration, and enteritis, while the chronic disease is described as a soft, fluctuant red swelling on the dorsum of the head [4]. Siluriform fish are the main host of *E. ictaluri*, and in addition to channel catfish *Ictalurus punctatus*, *E. ictaluri* has been reported in the walking catfish *Clarias batrachus* [5], tadpole madtom *Noturus gyrinus* [6], brown bullhead *Ameieuris nebulosus* [7,8], white catfish *I. catus* [9], and yellow catfish *Pelteobagrus fulvidraco* [10–12]. Until now, however, there has been no report detailing the molecular immune mechanisms of darkbarbel catfish *Pelteobagrus vachelli* in response to *E. ictaluri* infection.

The darkbarbel catfish is an omnivorous freshwater fish with increasing cultural importance due to its high nutritional value and growing market demand in China. As it is only a small-scale species belonging to the order Siluriformes and is only distributed in large rivers in Asia, such as the Liaohe, Huaihe, Yangtze, Xiangjiang, Minjiang, and Pearl Rivers [13–15], there are fewer immune-related reports regarding the darkbarbel catfish when compared with the same genus of fish, such as yellow catfish or channel catfish. Qin et al. reported transcriptome analysis of the spleen of the darkbarbel catfish in response to *Aeromonas hydrophila* infection and then further revealed the molecular immune mechanisms of specific immune-related genes,

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such as toll-like receptor-5 [16], complement components 8α and 8β [17], and complement factor I [18]. However, these studies only revealed the immune mechanism of the darkbarbel catfish in response to bacterial infection at the transcriptional level. Although transcriptome analysis has provided a platform for identifying gene expression levels and new transcripts and is a powerful tool for species that lack a reference genome, comparisons of mRNA expression levels, protein amounts, and enzymatic activities have revealed low correlations between the metabolome and transcriptome, indicating that transcriptome analysis is insufficient for fully understanding protein dynamics or biochemical regulation [19,20]. Furthermore, proteins are the major determinants of biological functions and confer the actual phenotypes to an organism. As such, we can achieve an increased level of understanding using proteomic analyses [21].

Proteomics is a means of comprehensive interpretation that can be used to describe more direct molecular responses than conventional transcriptomics or genomics, which assess only the mRNA [22]. With the development of isobaric tags for relative and absolute quantitation (iTRAQ), large-scale comparisons and reliable quantitative measurements can be conducted for four or eight different samples at the same time, which provides more reliable quantitative measurements and comparisons among the samples then can be obtained with 2D-DIGE analysis [23,24]. In fish, this technique has been used to investigate protein alterations induced by bacterial and viral pathogens, including in zebrafish *Danio rerio* [25,26], brown-spotted grouper *Epinephelus tauvina* [27], rainbow trout *Oncorhynchus mykiss* [28], Japanese flounder *Paralichthys olivaceus* [29] and medaka fish *Oryzias latipes* [30].

In this study, we conducted a comparative quantitative proteomic analysis in the darkbarbel catfish livers using iTRAQ to reveal the immune mechanisms in response to *E. ictaluri* infection. The patterns of some differentially expressed proteins were further assessed by qRT-PCR during the course of *E. ictaluri* infection. These results offer substantial insights into the molecular mechanisms underlying bacterial infection in fish.

2. Materials and methods

2.1. E. ictaluri infection in darkbarbel catfish

Darkbarbel catfish (20 ± 1.84 g weight, 14 ± 1.65 cm length) were cultured at the Nanjing Fisheries Research Institute, China. After acclimation at 25 ± 1 °C, pH 7.2–7.4 for 2 weeks and sequential feed restriction for 2 days in a bio filtered water recirculation system (equipped with cooling and heating functions, a volume of 200 L, and flow rate of 5 L/min), the fish were randomly classified into two groups: control and experimental (100 individuals/group). To determine the 50% lethal concentration (LC_{50}) of *E. ictaluri* at 72 h, a total of 30 fish were intraperitoneally administered with 0.1 mL of 10^3, 10^4, 10^5, 10^6, or 10^7 CFU/mL of *E. ictaluri*. Fish mortality was monitored every 2 h. The LC_{50} was determined to be 2.0×10^4 CFU/mL. The experimental group was intraperitoneally injected with 0.1 mL *E. ictaluri* (Zhejiang Institute of Freshwater Fisheries, Huzhou, China) at a density of 2.0×10^3 CFU/mL (1/10th LC_{50}). The control group was injected with an equal volume of phosphate buffer solution (PBS). At 0, 6, 12, 24, 36, 48, and 72 h after bacterial challenge, liver tissues were quickly dissected, frozen in liquid nitrogen, and stored at −80 °C prior to further analyses. Sampling of the control and experimental groups included three biological replicates, with each sample comprised of a pool of four different individual liver tissues of equal size. We selected the experimental and control groups at 24 h for iTRAQ sequencing (Fig. S1, Supporting Information). This sampling time point post-injection was chosen based on preliminary experiments. In the experimental and control groups, fish livers were collected at the seven time points to assay for ROS (reactive oxygen species), SOD (superoxide dismutase), ACP (acid phosphatase), and AKP (alkaline phosphatase) using available test kits (Jiancheng Bioengineering, Nanjing, China). The results showed that the oxidative stress was most severe after 24 h (Table S1, Supporting Information). Moreover, the swimming frequency of darkbarbel catfish decreased significantly at 24 h post injection.

2.2. Protein extraction

Liver samples were ground into powder in liquid nitrogen, extracted with lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris-HCl, pH 8.5) containing 1 mM PMSF and 2 mM EDTA (final concentration). After 5 min, 10 mM DTT (final concentration) was added to the samples. The suspension was sonicated at 200 W for 15 min and then centrifuged at 4 °C, 30000 g for 15 min. The supernatant was mixed with a 5-fold volume of chilled acetone containing 10% (v/v) TCA and incubated at −20 °C overnight. After centrifugation at 4 °C, 30000 g, the supernatant was discarded. The precipitate was washed three times with chilled acetone. The pellet was air-dried and dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% NP40, 20 mM Tris-HCl, pH 8.0–8.5). The suspension was sonicated at 200 W for 15 min and centrifuged at 4 °C, 30000 g for 15 min, and the supernatant was then transferred to another tube. To reduce disulfide bonds in the proteins of the supernatant, 10 mM DTT (final concentration) was added, and the solution was incubated at 56 °C for 1 h. Subsequently, 55 mM 2-iodoacetamide (IAM) (final concentration) was added to block the cysteines from forming disulfide bonds, followed by incubation for 1 h in the dark. The supernatant was mixed with a 5-fold volume of chilled acetone for 2 h at −20 °C to precipitate the proteins. After centrifugation at 4 °C, 30000 g, the supernatant was discarded, and the pellet was air-dried for 5 min, dissolved in 500 μL 0.5 M TEAB (Applied Biosystems, Milan, Italy), and sonicated at 200 W for 15 min. Finally, samples were centrifuged at 4 °C, 30000 g for 15 min. The supernatant was then transferred to a new tube and quantified using SDS-PAGE. The proteins in the supernatant were kept at −80 °C for further analyses.

2.3. iTRAQ labeling and SCX fractionation

Total protein (100 μg) was taken from each sample solution and then digested with Trypsin Gold (Promega, Madison, WI, USA) at a ratio of protein: trypsin = 30:1 at 37 °C for 16 h. After trypsin digestion, peptides were dried by vacuum centrifugation. Peptides were reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for the 8-plex iTRAQ reagent (Applied Biosystems). Briefly, one unit of iTRAQ reagent was thawed and reconstituted in 24 μL of isopropanol. Samples were labeled with the iTRAQ tags as follow: control samples (P0 a, P0 b, P0 c tags) and infected samples (P24 a, P24 b, P24 c tags). The peptides were labeled with the isobaric tags and then incubated at room temperature for 2 h. The labeled peptide mixtures were then pooled and dried by vacuum centrifugation.

SCX chromatography was performed using a LC-20AB HPLC pump system (Shimadzu, Kyoto, Japan). The iTRAQ-labeled peptide mixtures were reconstituted with 4 mL buffer A (25 mM NaH2PO4 in 25% ACN, pH 2.7) and loaded onto a 4.6 × 250 mm Ultremex SCX column containing 5 μm particles (Phenomenex). The peptides were then eluted at a flow rate of 1 mL/min with a gradient of buffer A for 10 min, 5–60% buffer B (25 mM NaH2PO4, 1 M KCl in 25% ACN, pH 2.7) for 27 min, and 60–100% buffer B for 1 min. The system was then maintained at 100% buffer B for 1 min before equilibrating with buffer A for 10 min prior to the next injection. Elution was monitored by measuring the absorbance at 214 nm, and fractions were collected every 1 min. The eluted peptides were pooled into 20 fractions, desalted with a Strata X C18 column (Phenomenex), and vacuum-dried.

2.4. LC-ESI-MS/MS analysis based on triple TOF 5600

Each fraction was resuspended in buffer A (5% ACN, 0.1%FA) and centrifuged at 20000 g for 10 min. The final concentration of peptides

was about 0.5 g/L on average. A total of 10 μL of supernatant was loaded in a LC-20AD nanoHPLC (Shimadzu, Kyoto, Japan) via the autosampler on a 2 cm C18 trap column. Then, the peptides were eluted onto a 10 cm analytical C18 column (inner diameter 75 μm) packed in-house. The samples were loaded at 8 μL/min for 4 min, then a 35 min gradient was run at 300 nL/min starting from 2% to 35% B (95% ACN, 0.1% FA), followed by a 5 min linear gradient to 60%, 2 min linear gradient to 80%, maintained at 80% B for 4 min, and finally return to 5% for 1 min.

Data acquisition was performed with a TripleTOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA). Data were acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 psi, nebulizer gas of 15 psi, and an interface heater temperature of 150 °C. The MS was operated with an RP greater than or equal to 30000 FWHM for TOF MS scans. For IDA, survey scans were acquired in 250 ms, and as many as 30 product ion scans were collected if exceeding a threshold of 120 counts per second (counts/s). Total cycle time was fixed to 3.3 s. The Q2 transmission window was 100 Da for 100%. Four time bins were summed for each scan at a pulse frequency value of 11 kHz by way of monitoring of the 40 GHz multichannel TDC detector with four-anode channel detection. A sweeping collision energy setting of 35 ± 5 eV coupled with iTRAQ adjusted rolling collision energy was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for 1/2 of the peak width (15 s), and the precursor was then refreshed off the exclusion list.

2.5. Data analysis

Raw data files acquired from the Orbitrap were converted into MGF files using Proteome Discoverer 1.2 (PD 1.2, Thermo), [5600 ms converter], and the MGF files were searched. Protein identification was performed by using the Mascot search engine (Matrix Science, London, UK; version 2.3.02). Gln- > pyro-Glu (N-term Q), Oxidation (M), and Deamidated (NQ) were considered the potential variable modifications, and Carbamidomethyl (C), iTRAQ8plex (N-term), and iTRAQ8plex (K) as the fixed modifications. The charge states of the peptides were set to +2 and + 3. Specifically, an automatic decoy database search was performed in Mascot by choosing the decoy checkbox, by which a random sequence from the database is generated and tested for raw spectra. To reduce the probability of false peptide identification, only peptides with significance scores ≥20 at the 99% confidence interval by a Mascot probability analysis greater than "identity" were counted as identified. Additionally, each confident protein identification involved at least one unique peptide.

For protein quantitation, it was required that a protein contained at least two unique peptides. The quantitative protein ratios were weighted and normalized by the median ratio in Mascot. We only used ratios with p-values < 0.05, and only fold changes > 1.2 were considered significant.

2.6. RNA extraction and real-time PCR

Total RNA was extracted from all samplings using High Purity RNA Fast Extract Reagent (Bioteke, Beijing, China). First strand cDNA was synthesized using HiScript™ QRT SuperMix (Vazyme, NJ, USA). The primers for qRT-PCR were listed in Table 1. The qRT-PCR was performed on an ABI StepOnePlus™ (Applied Biosystems, Foster City, CA, USA) using SYBR Green Master (Vazyme Biotech Co., Ltd, China). Expression levels of the target genes were analyzed using the comparative threshold cycle method ($2^{-\triangle\triangle CT}$) with β-actin as the reference gene, and three biological replicates under similar conditions were performed for each experiment. All data were tested for meeting the requirements for ANOVA application, and the different data were analyzed via a one-way ANOVA test, followed by the honestly significant difference test (Tukey's HSD test). All data were expressed as the mean ± standard deviation. All statistical analyses were carried out using SPSS statistical software 22.0. Differences were considered to be significant at $p < 0.05$.

Table 1
The primers used for Real-time PCR.

Primer	Sequence (5′-3′)
C1r-F	TGGAGGAAGAGGAGGAGCG
C1r-R	TAGAGACTCGACAGGAAGTGGGT
C3-F	CCTTCGTTCCGCTTCGTG
C3-R	GTGTCAGGCGGCTCTTGCT
C5-F	GACTAGCTTGCCCAGAGGTTCT
C5-R	ACTGTTCTCCTGCTTAGACTTGAATG
C7-F	CGGTGGGAGTGTTTGCACAG
C7-R	TGCTCATCTAAACCATCCTCCTG
C9-F	GATTATGGGACTCATTACACCAGG
C9-R	TGTGTCACCCGTTATCAGTTCTGT
C1-INH-F	TCCACTTTGCTCCACTCGGT
C1-INH-R	TATCTCCTGTATCTTTGACCTTGTACC
SRP54-F	GGCTGAATAAGCGGCGAATG
SRP54-R	GTTTTCCATCCTTTTCTTTGGTAGT
SRP receptor-F	TATGAAGGGTGACCTGCGAGAT
SRP receptor-R	TCTTCTCCAAAACAGGTTCCATAT
PSME1-F	GGAAATCCCGATACCAGACCC
PSME1-R	GAATGTGGGGTTTTATGACTTTAAT
MHC class I-F	GGAAGAACTACCTGGAGAACATCT
MHC class I-R	AGCTTCCATCCTGGTTGAGTAG
β-actin-F	CACTGCTGCCTCTTCCTC
β-actin-R	ATCCACATCGCACTTCAT

2.7. GO and KEGG pathway enrichment analysis

Gene ontology (GO) enrichment analysis showed differential proteins that were enriched for GO terms compared to the background identified proteins, which connected the differential proteins to GO databases (ftp://ftp.ncbi.nih.gov/gene/DATA/gene2go.gz). The GO enrichment term of differential proteins in the background of the identified proteins was determined by hypergeometric test ($p < 0.05$).

Similar to GO function enrichment analysis, pathway enrichment analysis of the significant proteins was carried out using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg). Significantly enriched pathways ($p < 0.05$, FDR < 0.05) were used as thresholds to select significant KEGG pathways compared to all hypergeometrically tested proteins.

3. Results

3.1. Protein profiling

All MS/MS spectra were processed using ProteinPilot software. iTRAQ analysis of the darkbarbel catfish liver proteome showed 55786 queries in this database (a total of 198659 spectra) and resulted in 2608 proteins hits (Fig. 1). Using the GO classification system, the 2608 proteins could be categorized into three major functional categories: cellular component, biological process, and molecular function. Proteins involved in the "cytoplasm" (17%) and "integral to membrane" (12%) groups were notably represented in the cellular components category (Fig. 2A). Among the biological processes, "translation" (10%) was the most commonly represented, followed by "protein transport" (9%) and "protein folding" (7%) (Fig. 2B). In the category of molecular function, a significant proportion of clusters were assigned to "ATP binding" (17%) (Fig. 2C).

3.2. iTRAQ quantification

Using a 1.2-fold increase or decrease in protein expression as a benchmark for physiologically significant changes following *E. ictaluri* infection, 819 differentially expressed proteins were reliably quantified

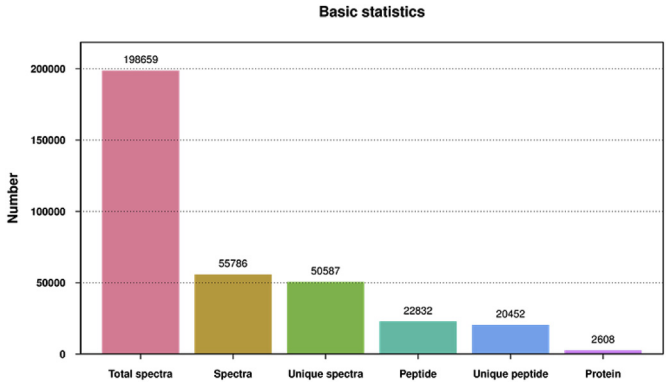

Fig. 1. Analysis of the darkbarbel catfish liver proteome profile via iTRAQ. "Total spectra" is the number of the secondary mass spectra, and "spectra" is the number of secondary mass spectra after quality control. "Unique peptide" is the number identified peptides that belong only to a group of proteins, while "Protein" is the number of proteins identified by the Mascot 2.3.02 software.

using iTRAQ analysis, including 6 up-regulated proteins (ATP-citrate synthase [ACS], fatty acid-binding protein liver-type [L-FABP], trypsinogen, protein disulfide-isomerase [PDI], glycogenin-1, unnamed protein) and 813 down-regulated proteins.

3.3. GO and KEGG analysis of the differentially expressed proteins

By way of GO enrichment analysis, a total of 56 GO clusters were found to change significantly after *E. ictaluri* infection ($p < 0.05$) (Fig. 3). Among these, 16 GO clusters belonged to the molecular function category, mainly related to the regulation of enzyme activity (e.g., "protein disulfide isomerase activity," "retinol dehydrogenase activity," and "protein disulfide oxidoreductase activity"). A total of 23 GO clusters belonged to biological processes related to cellular immunity (e.g., "complement activation, alternative pathway" and "complement activation, classical pathway"), protein synthesis (e.g., "translational elongation," "translational termination," and "negative regulation of translation"), and material transport (e.g., "mRNA export from nucleus mRNA" and "transmembrane transport"). In total, 17 GO clusters belonged to cellular components, mainly related to the ribosome (e.g., "ribosome," "large ribosomal subunit," and "small ribosomal subunit") and bio-membrane systems (e.g., "endoplasmic reticulum membrane," "mitochondrial inner membrane," and "Golgi apparatus"). Significantly enriched ($p < 0.05$) immune-related GO clusters and KEGG pathways were listed in Table 2.

The KEGG pathway analysis for the identified proteins showed that these pathways were mostly related to protein synthesis and transport, biosynthesis, and immune-related pathways. Moreover, an immune-related pathway ("antigen processing and presentation") was significantly enriched (Fig. 4).

3.4. Real-time PCR

To further validate the iTRAQ results, we selected 10 down-regulated immune-related genes (C1r, C3, C5, C7, C9, C1-INH, SRP54, SRP receptor, PSME1, MHC class I) and 6 up-regulated proteins (ACS, L-FABP, trypsinogen, PDI, glycogenin-1, unnamed protein) to perform qRT-PCR verification.

The PSME1 (Fig. 5A) and MHC class I (Fig. 5B) mRNA expressions peaked at 6 and 12 h after challenge with *E. ictaluri*, were sharply down-regulated at 24 h, and then steadily expressed at a low level thereafter. The mRNA expressions of C9 (Fig. 5C), SRP54 (Fig. 5D), and SRP receptor (Fig. 5E) were sharply up-regulated and reached the maximum at 12 h and demonstrated low expression levels at other time periods. The C5 (Fig. 5F) mRNA expression peaked at 12, 36, and 72 h and showed lower expression levels at 24 and 48 h. The C3 (Fig. 5G) mRNA expression was first up-regulated and then down-regulated from 0 to 12 h, reaching a maximum at 6 h, and was then up-regulated and then down-regulated from 12 to 72 h, peaking at 36 h. The C1r (Fig. 5H) mRNA expression was first increased, then decreased, and then increased again from 0 to 72 h, with peak expression at 12 and 72 h and the lowest expression at 36 h. The C7 (Fig. 5I) mRNA expression was first up-regulated and then down-regulated from 0 to 24 h, peaking at 12 h; subsequently, the expression level remained at a high level after 24 h. The C1-INH (Fig. 5J) mRNA expression was first up-regulated and then down-regulated from 0 to 24 h, peaking at 12 h, and then was up-regulated and then down-regulated from 24 to 72 h with a peak at 36 h. The mRNA expression levels of the 6 up-regulated genes (Fig. 6) were all significantly up-regulated relative to the control group at 24 h after infection.

4. Discussion

Enteric septicemia of catfish (ESC) and "hole-in-head" are common diseases caused by *E. ictaluri* in siluriform fish, which has resulted in great economic losses to fish farmers [4,5]. Darkbarbel catfish belong to the order Siluriformes and is one of the hosts of *E. ictaluri*. iTRAQ profiling is a newly developed method for the determination of proteins and plays an important role in quantifying changes in fish immune-response proteins [25,26]. As the liver is an important organ for bacterial pathogen attacks in fish, we conducted a comparative quantitative proteomic analysis of the liver using iTRAQ to reveal the immune mechanisms of darkbarbel catfish. To the best of our knowledge, this is the first report to explore the molecular immune mechanisms of darkbarbel catfish in response to *E. ictaluri* infection using the iTRAQ method.

The iTRAQ results indicated that complement pathway might play an important role in darkbarbel catfish in response to *E. ictaluri* infection. The complement system is known as an essential humoral system of innate immunity that serves as a link between the innate and adaptive immune responses. The complement system is comprised of more than 35 distinct soluble and membrane-bound proteins [31,32]. In addition, the activation of the complement system also significantly contributes to adaptive immune responses [33]. In vertebrates, three major pathways can activate the complement system: the classical, alternative, and lectin pathways [34]. In this study, the alternative pathway and the classical pathway of complement activation were significantly enriched in GO analysis, including the differentially expressed proteins C1r, C3, C5, C7, C9, and C1-INH. In mammals, these genes are interrelated in the complement pathway [35]. C1r combines with C1q and C1s to initiate the classical pathway of complement activation, and then the downstream genes C3, C5, C7 and C9 are activated step by step [36]. As the only known inhibitor of C1s and C1r, the C1-INH plays an important role in the classical pathway [37,38], it either binds reversibly to C1r and C1s within C1 complex to prevent self-activation or binds to activated C1r and C1s or dissociates them from C1q [39,40]. Although the complement system has been studied extensively in mammals, considerably less is known about complement in teleost fish [41–43], in particular darkbarbel catfish [17,18]. This study will help to reveal the molecular function and mechanism of darkbarbel catfish complement system.

The SRP54 is a key component of the ribonucleoprotein complex that mediates the co-translational targeting and the translocation of secretory and membrane proteins to the endoplasmic reticulum (ER) by interacting with the SRP receptor [44,45]. This protein is involved in the differentiation of neutrophil granulocytes [46], which are major inflammatory cells and key players in the infection process [47]. SRP54 is specifically up-regulated during neutrophil differentiation *in vitro*, and the mutation or knockout of SRP54 results in a significant decrease in neutrophil proliferation [46]. Down-regulation of SRP54 and the SRP receptor may lead to a decrease in neutrophils and further inhibition of the body's immune response.

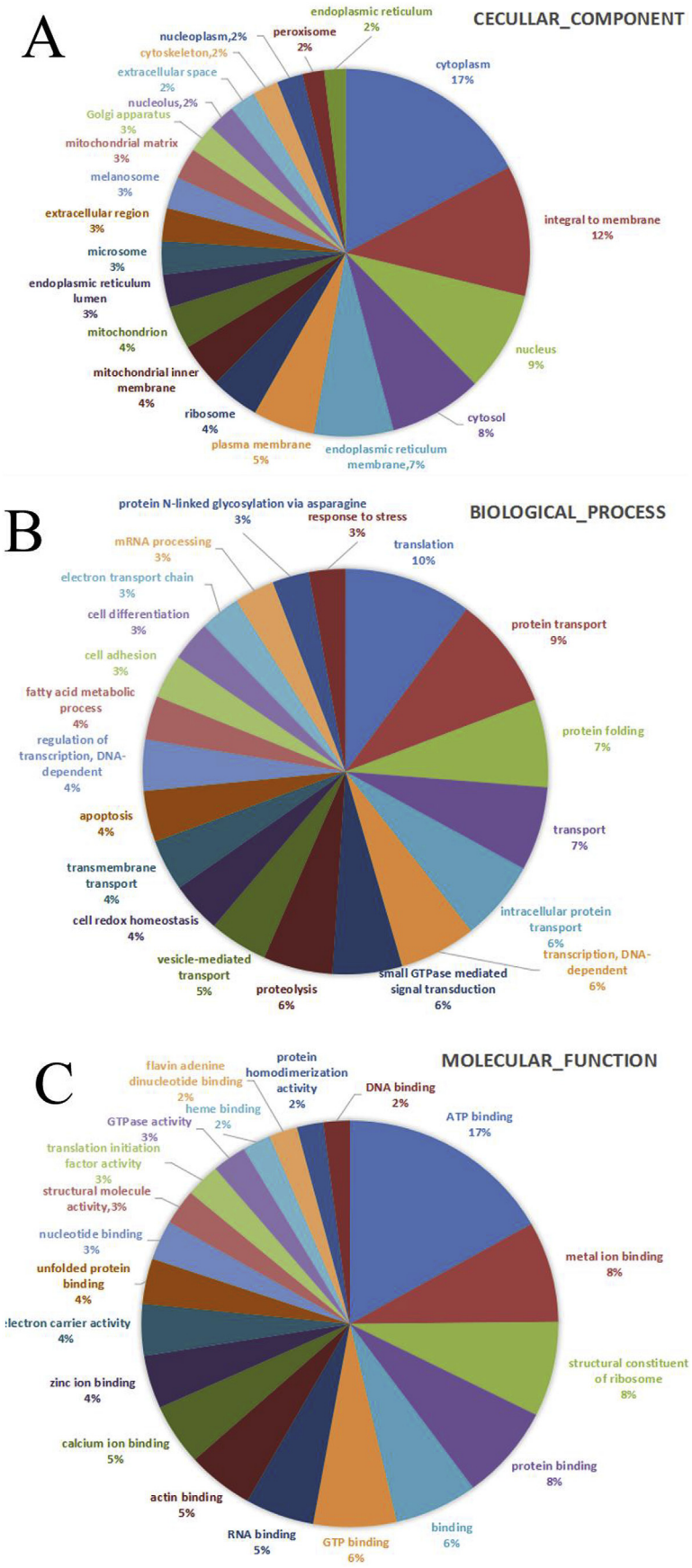

Fig. 2. Gene ontology (GO) analysis of total proteins based on cellular component (A), biological process (B), and molecular function (C). The top 20 in each section are shown.

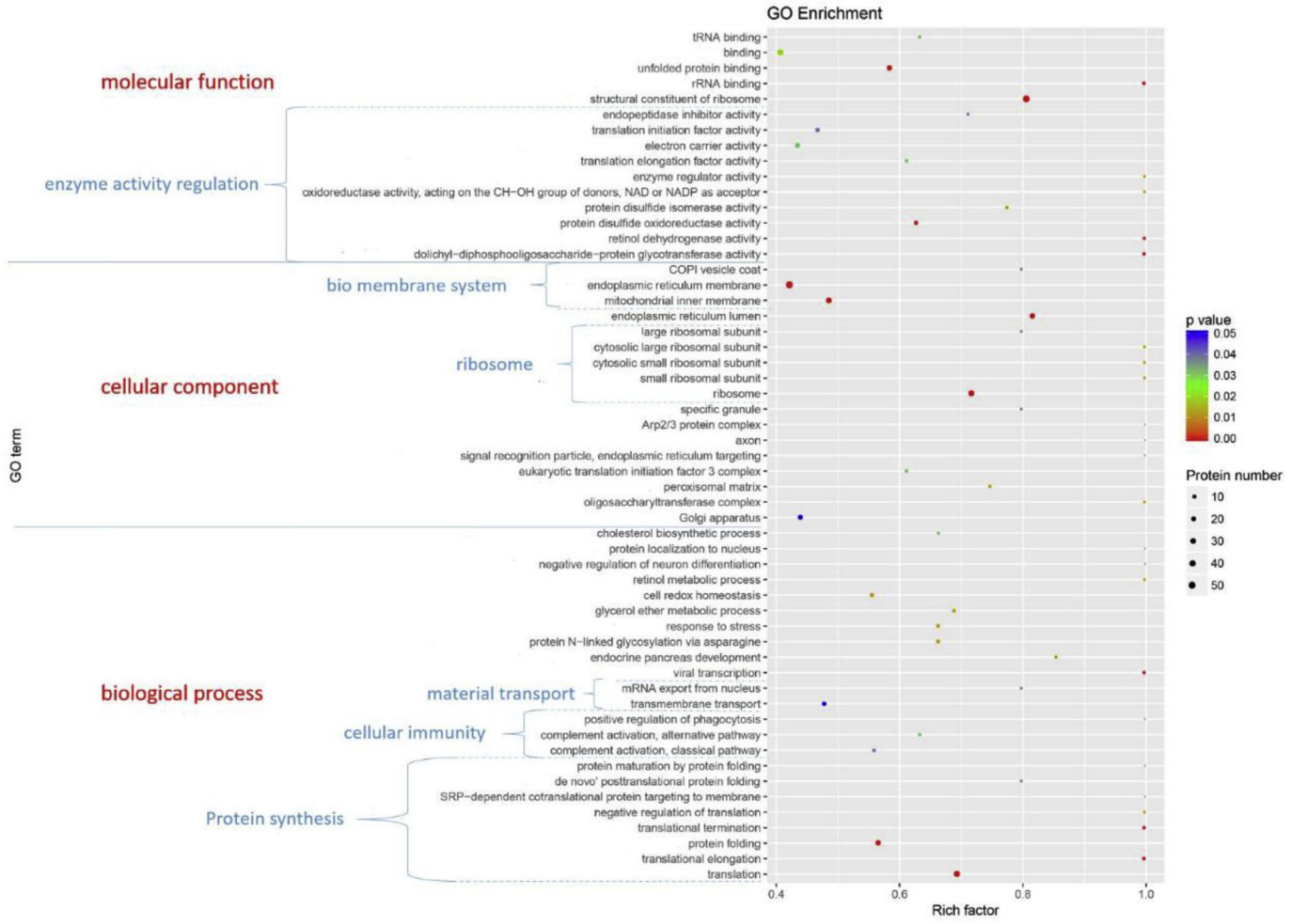

Fig. 3. Gene ontology (GO) enrichment analyses of all differentially expressed proteins (55 gene ontology clusters, *p*-value of Fisher's exact test < 0.05).

The MHC is expressed on the cell surface as a component of the antigen-presenting pathway, which mediates the immune response of the corresponding T cells by recognizing different antigens, thereby achieving pathogen clearance and an autoimmune response [48]. MHC gene-encoded protein products can be divided into two categories in fish: MHC class I molecules and MHC class II molecules, and both class I and class II molecules are heterodimers composed of type 1 transmembrane α and β proteins [49]. In the MHC class I pathway, the antigenic peptide (AP) produced by proteasome degradation and the antigen processing pathway is recognized by MHC class I and presented to cytotoxic T lymphocytes (CLTs), which is key to the immune process [50]. Consequently, the repertoire of peptides displayed by MHC class I molecules at the cell surface depends on proteasome activity, which may vary according to the presence of proteasome subtypes and regulators, such as PSME1 [51]. Therefore, the expression level of PSME1 should positively correlate with MHC class I. Both PSME1 and MHC class I were down-regulated in the iTRAQ results representing the infected fish.

The iTRAQ results showed that the majority of the differentially expressed proteins were down-regulated except for 6 up-regulated proteins (ATP-citrate synthase [ACS], liver-type fatty acid-binding protein [L-FABP], trypsinogen, protein disulfide-isomerase [PDI], glycogenin-1, unnamed protein). However, these proteins are not closely related to the immunity. The ATP-citrate synthase and glycogenin-1 are mainly involved in glucose metabolism [52]. The L-FABP is involved in the formation and metabolism of fatty acids [53]. The primary function of trypsinogen is about proteolysis [54], while protein disulfide-isomerase assists in the folding of protein spatial structures by catalyzing the formation and isomerization of disulfide bonds [55]. To further support our iTRAQ analyses, we selected ten immune-related proteins from GO clusters ("complement activation, alternative pathway" and "complement activation, classical pathway") and KEGG pathways ("antigen processing and presentation" and "bacterial secretion system") to perform qRT-PCR in the experimental and control groups at 0, 6, 12, 24, 36, 48, and 72 h. These GO clusters and KEGG pathways are both immune-related and significantly enriched. We found that these immune-related genes were highly expressed at 6 h (PSME1, MHC class I, C3, C7, and C1-INH) or 12 h (PSME1, MHC class I, C9, SRP54, SRP receptor, C5, C1r, C7, and C1-INH), low expression level at 24 h. A similar situation has been observed in channel catfish, Liu et al. reported that TLR3 and TIR-domain containing adapter molecules (TICAMs) were down-regulated in the head kidney of channel catfish at 24 h after *E. ictaluri* infection [56]. Therefore, 24 h may be a critical time point in catfish after *E. ictaluri* infection. In addition, Wang et al. reported that C3 mRNA expression in the large yellow croaker is influenced by bacterial stress and C3 might play an important role in immunity mechanisms [42]. Zhong et al. found that C1r and C1s were likely to get involved in the immune response of Nile tilapia against bacterial infection [43]. These studies suggest that the complement pathway plays an important role in response to bacterial infection in teleost fishes.

It is worth noting that the results of qRT-PCR showed that the expression levels of mRNA and protein are not always linear correlation. For example, the mRNA expression levels of the 10 immune-related

Table 2
Significantly enriched ($p < 0.05$) immune-related GO clusters and KEGG pathways among the differentially expressed proteins.

Uniprot_Swissprot Accession	Uniprot_Swissprot Description	Score[a]	Coverage[b]	Peptide[c]	Fold change (mean ± SD)[d]
complement activation, alternative pathway (GO:0006957)					
P98093	Complement C3, partial	1486	50.10	73	0.62
P06684	Complement C5	124	14.20	27	0.77
P06682	Complement component C9, partial	107	32.70	12	0.67
P01027	Complement C3	103	18.40	13	0.71
Q29RQ1	Complement component C7	59	29.50	13	0.58
complement activation, classical pathway (GO:0006958)					
P98093	Complement C3, partial	1486	50.10	73	0.62
P06684	Complement C5	124	14.20	27	0.77
P06682	Complement component C9, partial	107	32.70	12	0.67
P01027	Complement C3	103	18.40	13	0.71
Q6P734	Plasma protease C1 inhibitor	72	7.60	6	0.71
Q29RQ1	Complement component C7	59	29.50	13	0.58
P00736	Complement C1r subcomponent	33	23.50	3	0.77
positive regulation of phagocytosis (GO:0050766)					
P27797	Calreticulin	312	45.20	19	0.54
P01027	Complement C3	103	18.40	13	0.71
Antigen processing and presentation (ko04612)					
O57521	Heat shock protein HSP 90-beta	1086	39.00	36	0.61
P08108	Heat shock cognate 70 kDa protein	1047	51.20	25	0.58
P38657	Protein disulfide-isomerase A3	885	58.80	24	0.64
P19120	Heat shock cognate 71 kDa protein	823	29.20	24	0.55
P11501	Heat shock protein HSP 90-alpha	585	35.20	31	0.64
P27797	Calreticulin	312	45.20	19	0.54
Q61316	Heat shock 70 kDa protein 4	232	39.80	31	0.68
P13284	Gamma-interferon-inducible lysosomal thiol reductase	126	22.30	3	0.69
P24643	Calnexin	96	27.50	14	0.68
P97371	Proteasome activator complex subunit 1	80	33.30	7	0.78
P16391	MHC class I antigen	57	31.70	5	0.74
Q9GL24	Cathepsin L1	52	14.80	5	0.75
Bacterial secretion system (ko03070)					
Q6NWG1	Leucine-rich repeat-containing protein 59	70	27.40	10	0.66
P14576	Signal recognition particle 54 kDa protein	66	32.50	21	0.66
P06625	Signal recognition particle receptor	55	28.80	12	0.64

[a] Score indicates identification score of proteins.
[b] Peptide indicates peptide sequence number matching a protein.
[c] Coverage indicates the coverage of protein sequence.
[d] The values are calculated as the ratio of experimental group to control group.

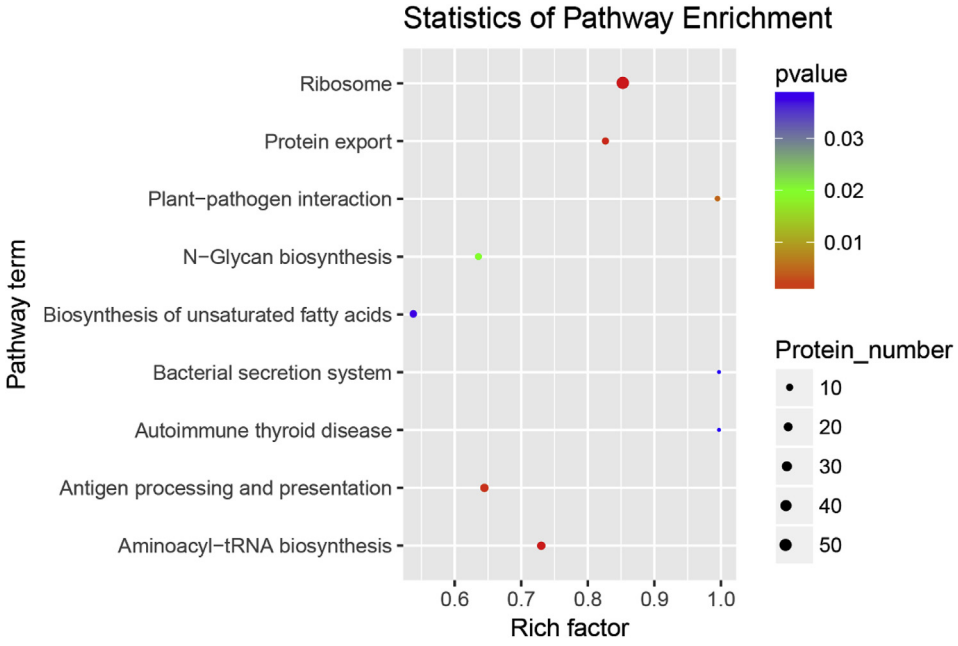

Fig. 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of all differentially expressed proteins (nine pathways, *p*-value of Fisher's exact test < 0.05).

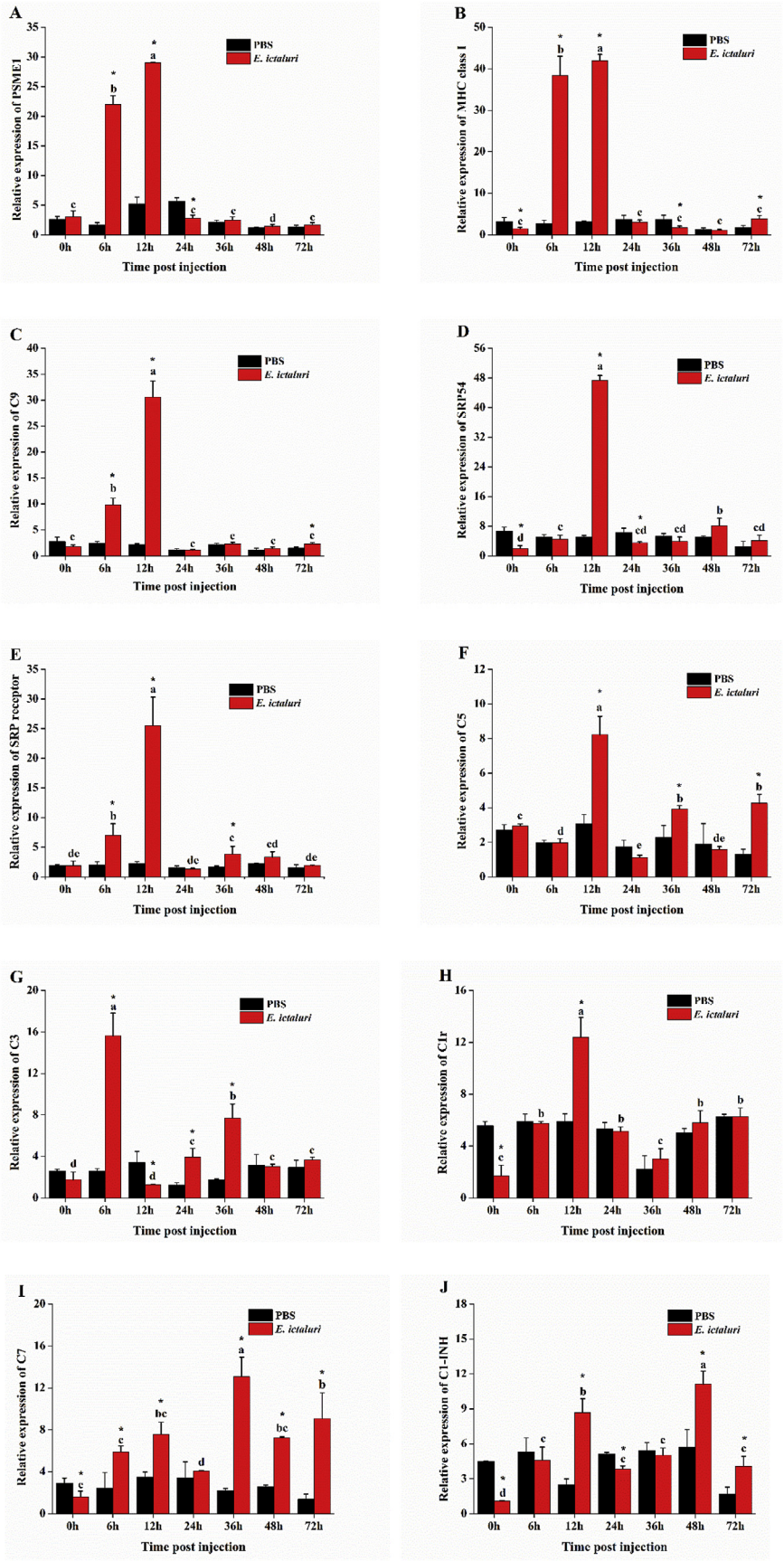

Fig. 5. The mRNA expression of down-regulated immune-related genes in darkbarbel catfish liver after *E. ictaluri* infection, as based on qRT-PCR, at 0, 6, 12, 24, 48 and 72 h. According to the LSD post-hoc test results: (1) letters (a, b, c, d, and e) indicate significant differences ($P < 0.05$) in the experimental group between different time points. (2) Significant differences ($p < 0.05$) between the control and experimental groups at the same time point are indicated with an asterisk (*). Fig. 5A: PSME1 mRNA expression. Fig. 5B: MHC class I mRNA expression. Fig. 5C: C9 mRNA expression. Fig. 5D: SRP54 mRNA expression. Fig. 5E: SRP receptor mRNA expression. Fig. 5F: C5 mRNA expression. Fig. 5G: C3 mRNA expression. Fig. 5H: C1r mRNA expression. Fig. 5I: C7 mRNA expression. Fig. 5J: C1-INH mRNA expression.

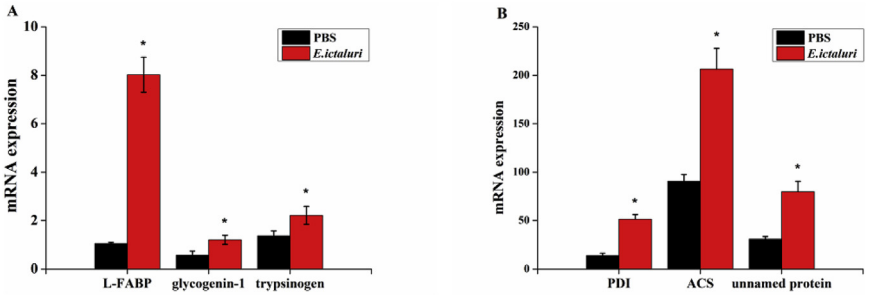

Fig. 6. The mRNA expression of down-regulated immune-related genes in darkbarbel catfish liver after *E. ictaluri* infection, as based on qRT-PCR at 24 h. A: The mRNA expression of trypsinogen, L-FABP and glycogenin-1. B: The mRNA expression of PDI, ACS and unnamed protein.

genes at 24 h were conforms to iTRAQ results, except for C3 and C7. On top of that, the expression of MHC class I, C9, SRP receptor, C1r and C7 at 24 h have no significant difference comparing with the control groups. The phenomenon of nonlinear relationship between transcription and protein levels may be due to the existing of post transcriptional regulation, pretrans lational events such as alternative splicing or transcription initiation and termination, as well as posttranslational modifications such as phosphorylation, acetylation, methylation, ubiquitination, cysteine oxidation, and nitrosylation [57]. The similar situations that genes are inconsistent in expression at the transcriptional and protein levels have been reported in other articles. Long et al. reported the transcriptomic and proteomic analyses of splenic immune mechanisms of rainbow trout infected by *Aeromonas salmonicida* subsp. *Salmonicida*, in which part of the validation genes were not significantly different from the control group at the transcriptional level [28]. Meng et al. reported the iTRAQ-based proteomic study of the effects of *Spiroplasma eriocheiris* on Chinese mitten crab *Eriocheir sinensis* hemocytes, some of significantly different proteins in the iTRAQ results also showed no significant differences in transcriptional levels during the validation process [58].

In conclusion, we explored the immune relationship between darkbarbel catfish and *E. ictaluri* at the transcriptional and protein levels. Our results will help to further our understanding regarding the molecular immune mechanisms of darkbarbel catfish in response to *E. ictaluri* infection as well as provide a good case study for analysis of protein expression and profiling of nonmodel fish species.

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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.2019.01.036.

Data availability

The raw data of proteomics were uploaded into public database after this paper accepted.

Conflicts of interest

The authors declare that they have no conflict of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

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