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Digital gene expression analysis in the liver of ScpB-vaccinated and *Streptococcus agalactiae*-challenged Nile tilapia

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ABSTRACT

In recent years, streptococcal diseases have severely threatened the development of tilapia aquaculture, but effective prevention and control methods have not yet been established. To understand the immune responses of vaccinated Nile tilapia (*Oreochromis niloticus)*, digital gene expression (DGE) technology was applied in this study to detect the gene expression profile of the Nile tilapia (*O. niloticus)* liver in response to ScpB (Streptococcal C5a peptidase from group B Streptococcus, ScpB) vaccination and a *Streptococcus agalactiae*-challenge. The control and the ScpB-vaccinated Nile tilapia yielded a total of 25,788,734 and 27,088,598 clean reads, respectively. A total of 1234 significant differentially expressed unigenes were detected ($P < 0.05$), of which 236 were significantly up-regulated, and 269 were significantly down-regulated ($P < 0.05$, $|\text{fold}| > 2$, $\text{FDR} < 0.05$). Of the differentially expressed gene, the identified genes which were enriched using databases of GO and KEGG could be categorized into a total of 67 functional groups and were mapped to 153 signaling pathways including 15 immune-related pathways. The differentially expressed genes (*TLR1, TLR2, TLR3, TLR5, TLR9, MyD88, C3, IL-1β, IL-10*) were detected in the expression profiles, and this was subsequently verified via quantitative real-time PCR (qPCR). The results of this study can serve as a basis for future research not only on the molecular mechanism of *S. agalactiae* invasion, but also on the anti-*S. agalactiae* mechanism in targeted tissues of Nile tilapia.

1. Introduction

Streptococcus agalactiae, also known as group B streptococcus (GBS), is a gram-positive intracellular bacterium. It was first reported in 1939 as an important opportunistic agent [1]. Over the past few decades, the bacterium was recognized as a major cause of zoonosis, with a broad host range including humans, mice (*Mus musculus*), cattle (*Bos taurus*), cats (*Felis catus*), dogs (*Canis lupus familiaris*), camels (*Camelus bactrianus*), horses (*Equus caballus*), pigs (*Sus scrofa*), and fish [2]. Among fish species, tilapia (*Oreochromis niloticus*) is very sensitive to *S. agalactiae*, so continuous outbreaks of *S. agalactiae* infection have been reported in tilapia farms throughout the world. Tilapia is an important fish for the economy, especially in China. However, infectious diseases caused by *S. agalactiae* have been severe in recent years, resulting in great economic losses [3]. *S. agalactiae* mainly invade the tilapia brain, liver, spleen, and kidney, causing typical symptoms such as anorexia, erratic swimming, exophthalmia, corneal opacity, and hepatomegaly, leading to high mortalities in infected fish [4].

Vaccination is the most environmentally friendly disease control strategy and one of the most effective methods of combating threatening diseases in fish [5]. A number of vaccines are currently commercially available for use in the aquaculture industry, including the live attenuated *Edwardsiella ictaluri* vaccine for use in catfish (*Ictalurus punctatus*) [6] and the bacterin and polyvalent bacterin vaccines used against pathogenic vibriosis (*Vibrio anguillarum* and *Vibrio ordalii*) [7], furunculosis (*Aeromonas salmonicida*) [7,8], and enteric red mouth disease (*Yersinia ruckeri*), and new vaccines continue to be developed [9–11]. Although vaccines are expected to induce long-term,

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lymphocyte-mediated immunoprotection in fish, little is known about the basic protective mechanisms elicited by the immunization of fish. Moreover, studies on the fish antibacterial system and on the interactive mechanism or immune responses between host cells and *S. agalactiae* are still in their infancy.

Although many studies have investigated the molecular responses of tilapia against *S. agalactiae* infection, most of them focused on the characterization of the expression profiles of certain genes by qPCR [12–16]. Transcriptome profiling is a powerful method for analyzing gene products in cells or tissues. Newly developed deep sequencing methods, such as Solexa/Illumina RNA-seq and DGE, have dominated transcriptome studies. Research using these methods has already altered our view of the extent and complexity of eukaryotic transcriptomes, facilitated the discovery of novel genes, and stimulated comparative and integrative genomics. These transcriptomic methods have also been used on tilapia with the *S. agalactiae* infection [2,17,18]. Studies on these methods mainly focused on the gene transcript changes in the spleen and kidney of tilapia before and after *S. agalactiae* infection occurs. Many immune-related genes and signaling pathways were found, both of which represent an important anti-bacterial mechanism in tilapia at the early stage of *S. agalactiae* infection.

The aim of this study was to use deep-sequencing methods to further investigate the DGE profile in the liver of ScpB (Streptococcal C5a peptidase from group B Streptococcus, ScpB)-vaccinated and *S. agalactiae*-challenged Nile tilapia. Our findings may serve as a basis for future research not only on the molecular mechanism of *S. agalactiae* invasion, but also on the anti-*S. agalactiae* mechanism in the targeted tissues of Nile tilapia.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. agalactiae ZP-N was isolated from Nile tilapia (*O. niloticus*) and preserved in the Pearl River Fisheries Research Institute at the Chinese Academy of Fishery Science. *Escherichia coli* DH5α and BL21 (DE3) were purchased from Takara, Dalian, China. The *S. agalactiae* ZP-N strain was cultured in a brain-heart infusion broth (BHI, Huankai Co Ltd., Guangzhou, China) at 28 °C, whereas the *E. coli* strains were cultured in a Luria-Bertani (LB) broth medium at 37 °C.

2.2. Experimental fish

Healthy Nile tilapia (19.5 ± 3.2 g) were purchased from a local fish farm (Panyu, Guangzhou, PR China) and acclimatized in the laboratory for two weeks. The fish were maintained at 28 ± 2 °C in aerated freshwater and fed daily with commercial dry pellets. The fish were then anesthetized with tricaine methanesulfonate (Sigma, USA) prior to experimentation involving injection, blood collection, or sacrifice. Prior to experimentation, the fish were randomly sampled for relevant bacteria, and no bacteria were detected.

2.3. ScpB vaccination and bacterial challenge

The recombinant protein ScpB vaccine has been shown effective for tilapia *S. agalactiae* disease control. The methods of vaccine construction and tilapia vaccination were carried out as previous work [19]. Briefly, the prokaryotic expression vector pET32a (+) was used to construct a recombinant expression vector pET32a (+)-*ScpB*, which was then transformed into *Escherichia coli* BL21 (DE3). The recombinant strain could produce large amounts of ScpB protein, mainly in the form of an inclusion body. The purified fusion protein and Freund's adjuvant were mixed according to certain proportions to produce vaccines. Three groups of fish were injected intraperitoneally (i.p.) with 100 μL of the recombinant protein ScpB vaccine, and another three groups of fish sham were boosted with 100 μL PBS as a control (i.e., 30 fish in each group). The recombinant protein ScpB was diluted to 5 μg/g (ScpB weight/fish body weight).

On the 28th day post-boost, the fish were challenged with GBS ZP-N that had been cultured in a BHI medium (Huankai Co Ltd., Guangzhou, China) to the logarithmic phase, washed, and resuspended in PBS.

To challenge the fish with live bacteria, the cells were cultured in a BHI medium to an OD_{600} of ~0.8 and then resuspended in PBS to 8.4×10^7 CFU/mL. All of the experiments were approved by the Institutional Animal Care and Use Committee at the research facility.

2.4. RNA preparation

After challenged with live bacteria for 48 h, the total RNA was isolated from 10 mg of tilapia liver tissue using 1 mL of Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The RNA integrity was verified by electrophoresis on 0.7% agarose gels, and the RNA quantity and purity were measured using a Biophotometer (Eppendorf, Hamburg, Germany). Equivalent amounts of RNA, both from the six fish in the control groups (CG) and in the vaccinated groups (SG), were mixed and used for the DGE analysis. RNA yield and fragment size were measured using an Agilent Technologies 2100 Bioanalyzer (Agilent, USA).

2.5. Library construction and sequencing

In brief, poly (A) RNA was collected from 20 μg of total RNA using Sera-mag Magnetic Oligo (dT) Beads (Illumina). To avoid priming bias when synthesizing the cDNA, the purified mRNA was first fragmented into 200–700 bp via a fragmentation buffer. Then, the cleaved RNA fragments were transcribed into first-strand cDNA using reverse transcriptase and random hexamer primers (Illumina), followed by second-strand cDNA synthesis using DNA polymerase I and RNase H. The double-stranded cDNA was further subjected to end-pairing using T4 DNA polymerase and ligated with an adapter or an index adapter using T4 DNA ligase. A range of cDNA fragments was excised from the gel and then purified. Using these purified cDNA as templates, a paired-end library was constructed using the Genomic Sample Prep kit (Illumina) according to the manufacturer's instructions. The cDNA library was sequenced on a PE flow cell using Illumina HiSeq™ 2500 (Illumina Inc., San Diego, CA, USA) and subjected to 100 cycles of paired-end (2x100 bp) sequencing at the Shanghai Biotechnology Corporation (Shanghai, China).

2.6. Mapping tags to a reference database

The sequencing-generated raw image data were transformed into sequence data. The raw reads were filtered and then mapped to a reference database, which included 30 adaptor sequences, empty reads, low-quality tags with unknown sequences (Ns), tags too long or too short, and tags with a copy number of 1 (probably a sequencing error). The processing of fluorescent images into sequences, base-calling, and quality value calculations were performed using the illumina data processing pipeline (version 1.8). Prior to assembly, high-quality clean reads were generated using the FASTX toolkit pipeline (version 0.0.13), and the resulting high-quality reads were mapped to the UCSC (mm10) using Tophat (version: 2.0.6) [20]. Cufflink (version: 2.1.1) [21] was used to process the Tophat alignments. Additionally, transcript expression levels were estimated using Fragments Per Kilobase of exon model per Million mapped reads (FPKM) values. The differentially expressed genes were based upon the fold changes of the expression levels ($P < 0.05$).

2.7. Identification and annotation of differentially expressed genes

Following the method reported by Audic and Claverei [22], the differentially expressed genes were selected between the ScpB-

Table 1
Primers of tilapia used for qPCR in the study.

Genes	Forward primers (5′–3′)	Reverse primers (5′–3′)
TLR1	GAACCTCCTTGTGGACTTGAAT	TCCAGGTGTTTTCTAAGGTGAG
TLR2	GTATCTCAGTGCTCGTCGCTCA	TTTCATTATCGTCTCCAGTGCG
TLR3	GCCAAATGTCAGGGACGAGTAT	CGGCTGTGCTGCTCACTATGTA
TLR5	TTCCTGCCAGGTGAGGATCA	ACCTTTGGCTTCCCTACCAC
TLR9	TACTGAAGCACCTCTACGGC	CTCTGGACAGCACGAAAACT
MyD88	GGTATGTTGTGCTGTAGACTTCCGA	GTAGTTCTTTATTTCCAGGTAGTTG
IL-1β	CCTACACCCATCGCTGAGAC	GGGTAGCGGACAGACATGAG
IL-10	TTACCTGGACACGGTTCTGC	GTGACTTAAAGCAGCGCGAG
C3	CCGTCGCCCACTACAACA	CGGGAAGTCCACGCAATA
EF1α	CCCAGAAACACCGAAACTAAA	TGTCGATTCCTCCGCACT

vaccinated group and the control group. The P value corresponded to the differential gene expression test, and the threshold of the P value in multiple tests and analyses was determined by manipulating the FDR value [23]. A gene ontology functional enrichment analysis and a pathway enrichment analysis were performed by mapping all differentially expressed genes to terms in the GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases.

2.8. Verification by quantitative real-time PCR (qPCR)

To ensure the reliability of the RNA-Seq results, nine selected differentially expressed genes (*TLR1, TLR2, TLR3, TLR5, TLR9, MyD88, C3, IL-1β, IL-10*) involved in immune responses were chosen for validation using qPCR with SYBR Green dye (TaKaRa Biotechnology, Dalian, China), which was performed on an ABI 7300 Real-Time PCR system. The specific primers were shown in Table 1. The qPCR was performed in a total volume of 20 μL, and the thermal cycler program was run at 95 °C for 5 min, followed by 40 cycles at 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 30 s. All reactions were performed in triplicate. The expression level of each sample was described by the relative fold change to that of EF1α (elongation factor-1α) by using the $2^{-\triangle\triangle CT}$ method [24,25]. The statistical analysis of data was performed using the SPSS Statistics 17.0 with T-test, and the significant level was set as $P < 0.05$.

3. Results

3.1. ScpB vaccination and S. agalactiae challenge

Two days after the *S. agalactiae* challenge, mortalities were observed in the PBS-vaccinated control group without clinical signs. The mortalities in the control group continued until 14 days post-challenge. Clinical signs of *S. agalactiae* infection, which included anorexia, erratic swimming, exophthalmia, and corneal opacity, manifested on day 5 post-challenge. The cumulative mortality rate reached 62.82% in the control group challenged with four times LD_{50} (8.4×10^7 CFU/mL). In the ScpB-vaccinated group, mortalities were observed between 12- and 15-days post-challenge, with no additional deaths observed afterward. The mortality rate in the ScpB-vaccinated group was 7.44%. This result confirmed the effectiveness of the ScpB protein, and thus, it was used for further studies.

3.2. Analysis of RNA-seq data and read mapping

In the two RNA-seq libraries, the number of raw reads per library were 28,292,064 (SG) and 26,854,610 (CG). After filtering out the low-quality reads, we obtained 27,088,598 (SG) and 25,788,734 (CG) clean reads, which respectively account for 95.75% in SG group and 96.03% in CG group based their total raw reads. The *o. niloticus* genome (ensembl *Oreochromis_niloticus*.Orenil1.0.72) and gene model annotation files (NCBI *Oreochromis niloticus* Annotation Release 104) was used as reference. After directly mapping the clean reads from the two *O. niloticus* libraries to the reference unigenes, 72.94% (CG) and 70.79% (SG) of the clean reads from both library sequences were mapped to the genome ensemble *Oreochromis_niloticus*. Orenil 1.0.72. And the mapping ratio of the unique reads was 71.75% (CG) and 69.58% (SG). Raw sequencing reads data has been submitted to GEO (Gene Expression Omnibus) in NCBI, the bioproject ID is PRJNA531528.

3.3. Identification of differentially expressed genes

A total of 1234 significant differentially expressed unigenes were detected ($P < 0.05$) between the ScpB-vaccinated and the PBS sham-vaccinated control, of which 629 were up-regulated, and 605 were down-regulated. Of the 629 significant genes, 236 were up-regulated greater than 2 fold in the challenged vaccinated compared to that of the control ($P < 0.05$, fold > 2, FDR < 0.05). In the 236 remarkably up-regulated transcripts there were 103 genes were up-regulated 2–3 fold, 34 genes were up-regulated 3–4 fold, 26 genes were up-regulated 4–6 fold, and 44 genes were up-regulated 6–50 fold. There were also 29 transcripts that up-regulated greater than 100 fold, most of which were described as small nucleolar RNAs such as snoRNA SNORA3, SNORA5, SNORA7, SNORA13, SNORA23, SNORA53, SNORA74, SNORA75, SNORD15, and SNORD97. Some of other up-regulated genes were functionally known as immunity and defense related genes, including MHCII and interleukin-15 (IL-15).

Accordingly, of the 605 significantly down-regulated genes ($P < 0.05$), 269 were down-regulated greater than 2 fold in the challenged vaccinated compared to that of the control ($P < 0.05$, $|fold| > 2$, FDR < 0.05). Of the 269 remarkably down-regulated transcripts there were 91 genes were down-regulated 2–3 fold, 41 genes were down-regulated 3–4 fold, 93 genes were down-regulated 4–11 fold, and 31 genes were down-regulated 11–100 fold. There were also 13 transcripts that down-regulated more than 100 fold.

3.4. GO and KEGG pathway analysis

Of the differentially expressed gene, the identified genes were enriched using databases of GO and KEGG, which could be categorized into a total of 67 functional groups (Fig. 1). All of these functional groups could be divided into three ontologies: molecular function, cellular component, and biological process. The top 25 GO categories were shown in Fig. 2. In the KEGG pathway analysis, there were 153 signaling pathways were found, of which metabolic pathways represented the largest portion, whereas 15 pathways were related to immune or defence function (Table 2). The pathway enrichment analysis showed 280 differentially expressed genes that were annotated to 39 signaling pathways in KEGG (Fig. 3) ($P < 0.05$). Among them, three pathways (containing 42 differentially expressed genes) were immune-related. The significantly differentially expressed genes which assigned to these different immune related pathways were identified in Table 3.

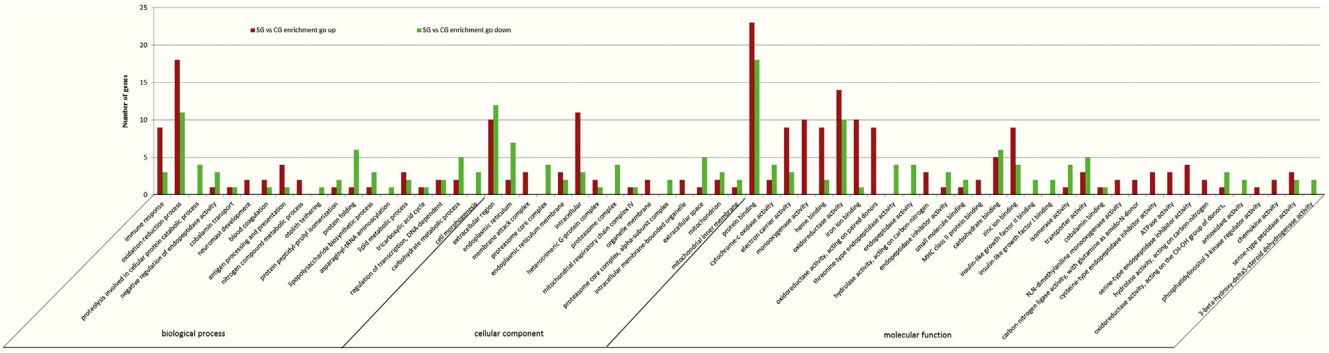

Fig. 1. GO enriched classification of differentially expressed unigenes in ScpB-vaccinated and *Streptococcus agalactiae*-challenged tilapia liver. All these annotated sequences were categorized into 67 functional groups which belong to three main big categories: biological process, cellular component, or molecular function.

3.5. Verification by qPCR

The results of the qPCR showed that the relative expression levels of the nine selected genes from tilapia liver have changed to different degrees after ScpB vaccination and *S. agalactiae*-challenged. The relative expression levels of *TLR1* ($p < 0.05$), *TLR2* ($p < 0.05$), *TLR3* ($p < 0.05$), *TLR9* ($p < 0.05$), and *C3* ($p < 0.01$) were up-regulated, and the relative expression levels of *TLR5* ($p < 0.01$), *MyD88* *($p < 0.05$), IL1B ($p < 0.01$)* and *IL10* ($p < 0.01$) were down-regulated. As shown in Fig. 4, the expression pattern of the selected genes was consistent with the high-throughput sequencing data in Table 3.

4. Discussion

Many studies have investigated gene and gene expression changes in naïve infected fish [26–28], in fish re-infected after survival from a

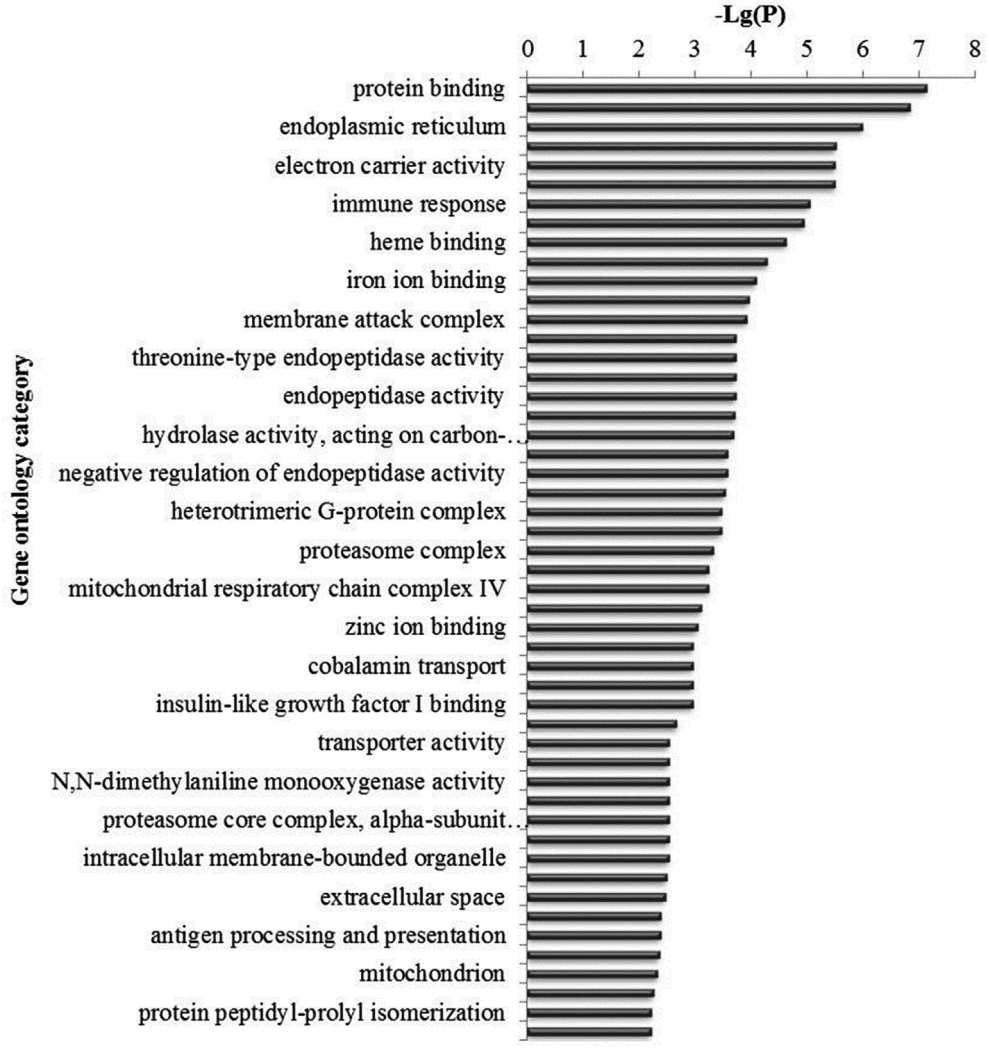

Fig. 2. GO category based on functional groups for enrichment differentially expressed genes. P-values < 0.05 and FDR < 0.05 were used as a threshold to select significant GO categories (Top 25). LgP is the logarithm of P-value.

Table 2
Immune related pathways enriched with differentially expressed genes in ScpB-vaccinated and *Streptococcus agalactiae*-challenged tilapia.

Pathways	DEGs genes with pathway annotation (354)	All genes with pathway annotation (4870)	Pathway ID
Phagosome	27 (7.63%)	186 (3.82%)	ko04145
Complement and coagulation cascades	18 (5.08%)	85 (1.75%)	ko04610
Endocytosis	14 (3.95%)	323 (6.63%)	ko04144
Cytokine-cytokine receptor interaction	13 (3.67%)	179 (3.68%)	ko04060
Jak-STAT signaling pathway	13 (3.67%)	123 (2.53%)	ko04630
Toll-like receptor signaling pathway	13 (3.67%)	96 (1.97%)	ko04620
Cell adhesion molecules (CAMs)	9 (2.54%)	167 (3.43%)	ko04514
NOD-like receptor signaling pathway	7 (1.98%)	60 (1.23%)	ko04621
Focal adhesion	7 (1.98%)	237 (4.87%)	ko04510
Lysosome	6 (1.69%)	140 (2.87%)	ko04142
Intestinal immune network for IgA production	5 (1.41%)	42 (0.86%)	ko04672
Adherens junction	3 (0.85%)	114 (2.34%)	ko04520
RIG-I-like receptor signaling pathway	2 (0.56%)	57 (1.17%)	ko04622
Chemokine signaling pathway	1 (0.28%)	2 (0.04%)	ko04062
TNF signaling pathway	1 (0.28%)	2 (0.04%)	ko04668

Note: The differentially expressed genes (DEGs) were annotated to 153 signaling pathways in KEGG. This table showed the 15 immune-related pathways and numbers of DEGs involved in each pathway.

previous infection [29], or in fish that differed in their inherent disease susceptibility. Some other studies also have looked at the effects of vaccination, with a primary focus on events occurring immediately after vaccination [30–33], but few studies have focused on the early events that occur post-challenge of vaccinated fish. The recombinant protein ScpB vaccine has been shown effective for tilapia *S. agalactiae* disease control. In the present study, two DGE libraries were created from tilapia liver of the ScpB-vaccinated (*S. agalactiae*-challenged after ScpB-vaccinated) and the control (*S. agalactiae*-challenged after PBS-vaccinated).

Tilapia is an important commercial fish and seriously affected by *S. agalactiae* disease. Tilapia liver is one of the main target tissues during *S. agalactiae* infection. In fact, the liver represents a major immune organ of vertebrates [34–36]. This function is related to the dual blood supply of the liver, which receives blood both via the portal vein containing dietary antigens, toxins as well as pathogens from the gastrointestinal tract, and via the hepatic artery containing antigens, pathogens and metastazing cells from systemic blood circulation [37]. In addition, the low velocity blood flow through the liver sinusoids with their fenestrated endothelium favours exchange of immunological information [38]. In this study, a total of 1234 significant differentially expressed unigenes were detected ($P < 0.05$) in the tilapia liver between the ScpB-vaccinated and the PBS sham-vaccinated control. The key tilapia genes that were differentially expressed following ScpB vaccine immunization and the *S. agalactiae* challenge included small nucleolar RNAs (snoRNAs), and many other immunity and defense related genes.

The most up-regulated small nucleolar RNAs (snoRNAs) in the present study were snoRNA SNORA3, SNORA5, SNORA7, SNORA13, SNORA23, SNORA53, SNORA74, SNORA75, SNORD15, and SNORD97. SnoRNAs are one of the most ancient and abundant families of non-coding RNAs (NcRNAs). NcRNAs play key roles in diverse cellular activities, and efficient ncRNA function requires extensive posttranscriptional nucleotide modifications. SnoRNAs are a group of ncRNAs that guide the modification of specific nucleotides in ribosomal RNAs (rRNAs) and small nuclear RNAs. Based on their structure, snoRNAs fall into two classes, box C/D snoRNAs and box H/ACA snoRNAs [39]. SnoRNAs can both participate in the regulation of methylation and pseudouridylation and regulate the expression pattern of their host genes. Many studies have suggested an important yet perhaps unappreciated functional role of snoRNAs in cellular physiology that, when deregulated, may directly contribute to disease [40,41]. In zebrafish, using a highly sensitive mass spectrometric analysis, it found that decreased snoRNA expression reduces the snoRNA-guided methylation of the target nucleotides, and impaired rRNA modification, even at a single site, led to severe morphological defects and embryonic lethality [42]. It is suggested that the high up-regulation of snoRNAs in ScpB-vaccinated tilapia may play some important positive function against streptococcus infection, although this remains unclear.

Combining with GO and KEGG analysis, many of significantly differentially expressed immunity and defense related genes were assigned to different immune-related categories or pathways (Table 3). Among these genes, interleukin-15 (IL-15) was an important differentially up-regulated one. Furthermore, the transcripts IL-1β and IL-10 were both down-regulated. The major advances of fish cytokine genes have been carried out by "comparative genomics" through the genome projects of pufferfish (*Fugu rubripes* and *Tetraodon nigroviridis*) and zebrafish (*Danio rerio*) [43]. It was known that ILs signalled not only between leucocytes, but also produced by and targeted a wide variety of cells and comprised a complex system of cell-signalling in the immune system [44]. In general, cytokines are produced at the site of entry of a pathogen and drive inflammatory signals that regulate the capacity of resident and newly arrived phagocytes to destroy the invading pathogen. In mammals they also regulate antigen presentation function in dendritic cells, and their migration to lymph nodes to initiate the adaptive immune response [45]. IL-15 or its homologues have been reported in several fish [46]. Functionally IL-15 has similar biological activities as IL-2 and IL-21 like stimulating macrophages, $CD8^+$ epithelial T cells and memory T cells to develop, proliferate, secrete cytokines and exhibit increased cytotoxicity and production of antibodies [47–49]. These results suggested that IL-15, IL-1β and IL-10 may represent an important anti-bacterial mechanism in tilapia liver at the early stage of *S. agalactiae* infection.

The fold of the up-regulated genes SSX2IP (Synovial sarcoma, an X breakpoint 2 interacting protein) were 11.4. SSX2IP could be attached to the pathway of adherens junction (adhere and colonize). Klinger et al. (2014) [50] showed that SSX2IP localized to the basal body of primary cilia in human and murine ciliated cells and it is important for efficient recruitment of the ciliopathy-associated satellite protein to both satellites and the basal body. The loss of SSX2IP drastically reduces the entry of membrane proteins to primary cilia and interferes with efficient accumulation of the key regulator of ciliary membrane protein. The primary cilium is an ideal cellular location for sensing and transducing signals [51]. Defects in ciliogenesis have been found to lead to a wide range of human diseases [52–54]. In this study, whether the up-regulated SSX2IP had some relationship with the treatment of *S. agalactiae*-infection in the liver of tilapia needs to be further studied.

The other differently up-regulated genes were the major histocompatibility complex (MHC) class I and class II proteins or peptides, such as H-2 class II histocompatibility antigen, E-S beta chain, HLA class II histocompatibility antigen, DRB1-8 beta chain-like, major histocompatibility complex, class I. MHC class I and class II proteins play a

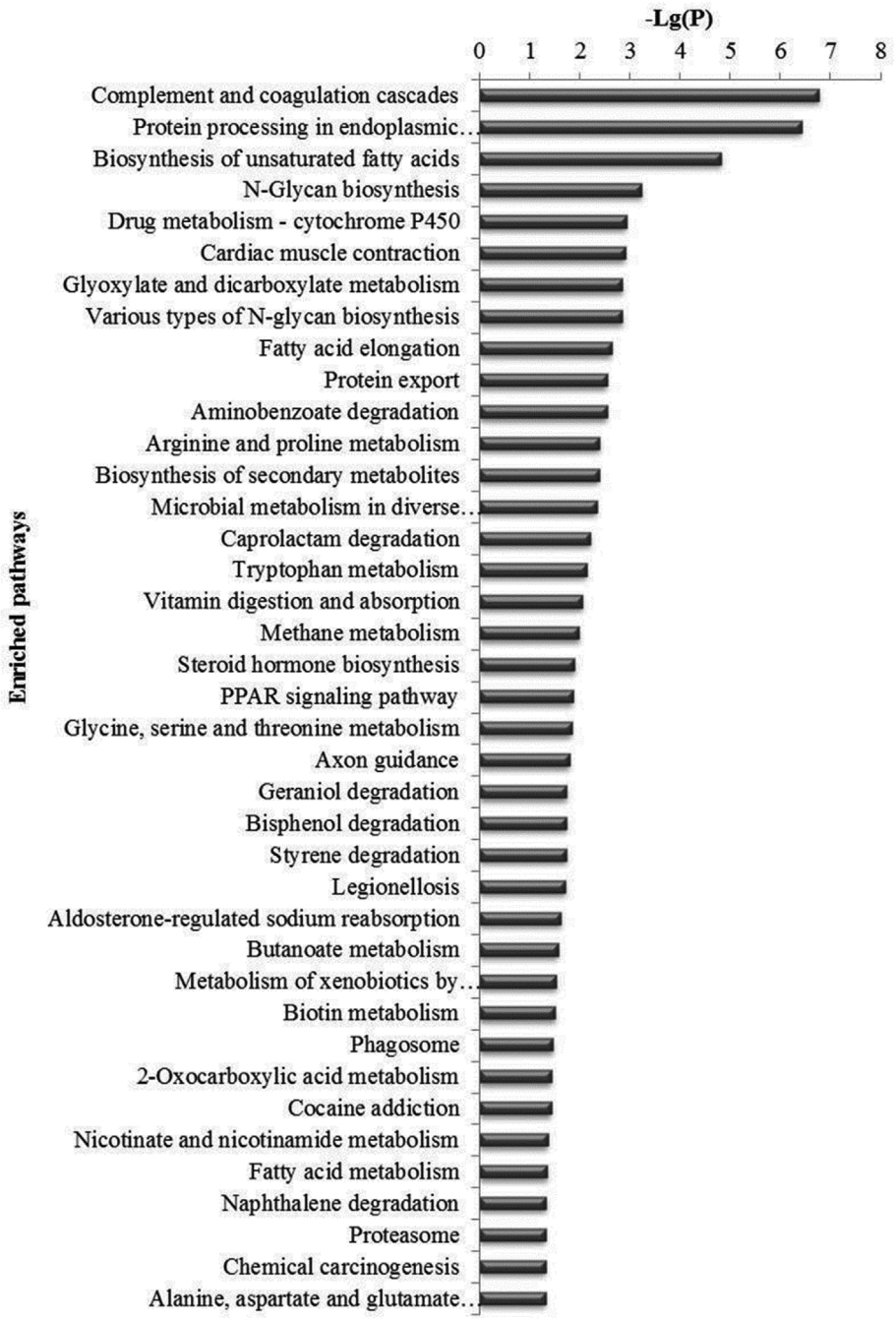

Fig. 3. Pathway enrichment analysis showed 280 differentially-expressed genes that were annotated to 39 signaling pathways in KEGG (P-values $<$ 0.05). LgP is the logarithm of P-value.

pivotal role in the adaptive branch of the immune system. Both classes of proteins share the task of presenting peptides on the cell surface for recognition by T cells. Immunogenic peptide–MHC class I (pMHCI) complexes are presented on nucleated cells and are recognized by cytotoxic $CD8^+$ T cells. The presentation of pMHCII by antigen-presenting cells (e.g., dendritic cells), macrophages, or B cells) can activate $CD4^+$ T cells, leading to the coordination and regulation of effector cells [55]. These results were similar as those of the gene transcript changes of tilapia in spleen and kidney after *S. agalactiae* infected [17], which suggested that MHCI and MHCII also play an important anti-bacterial mechanism in liver of the ScpB-vaccinated tilapia at the early stage of *S. agalactiae* infection.

Besides, the expression of significantly regulated genes in rainbow trout at 8 weeks post-vaccination showed the complement component 3 (C3) was significantly up-regulated in the liver of vaccinated fish [56]. In this study, we found the gene encoding complement component C3 was significantly up-regulated, and iC3b, iC3, C7, C3, C6, and C9 were also significantly up-regulated. C3 plays a central role in the complement system and contributes to innate immunity. Its activation is required for both classical and alternative complement activation pathways. Antibodies are activators of the classical complement pathway and the elevation of C3 expression and antibody levels in this study may reflect their joint involvement in development of immunity in the vaccinated fish. IC3b is a proteolytically inactive product of the complement cleavage fragment C3b [57] that still opsonizes microbes, but it cannot associate with factor B, and thus, it prevents the amplification of the complement cascade or activation through the alternative pathway. Once the alternative C3 convertase enzyme is formed on a

Table 3
Immune related significantly differentially expressed genes following ScpB-vaccinated and *Streptococcus agalactiae*-challenged in tilapia liver.

Genes	Gene annotations	Fold-change	P-value
MHC II	HLA class II histocompatibility antigen, DRB1-8 beta chain-like	> +100	< 0.01
IL-15	Interleukin-15	> +100	< 0.05
SSX2I	Synovial sarcoma, X breakpoint 2 interacting protein	+11.4	< 0.01
TLR2	Toll-like receptor 2	+5.64	< 0.05
MHCII	H-2 class II histocompatibility antigen, E-S beta chain	+4.6	< 0.01
MHCI	Major histocompatibility complex, class I	+4.5	< 0.01
G3S1A18	Class I histocompatibility antigen, F10 alpha chain	+4.5	< 0.01
TRIM25	Tripartite motif containing 25	+3.3	< 0.01
TLR1	Toll-like receptor 1	+3.14	< 0.05
CCL2	C–C motif chemokine 2	+2.7	< 0.01
iC3b	Complement component 3	+2.6	< 0.01
C7	Complement component 7	+2.6	< 0.01
ARPC1B	Actin related protein 2/3 complex, subunit 1A/1B	+2.5	< 0.01
CD74	CD74 molecule	+2.4	< 0.01
iC3	Bcomplement component 3	+2.3	< 0.01
CADM1	Cell adhesion molecule 1	+2.2	< 0.05
DAB2	Disabled homolog 2	+2.2	< 0.01
C3	Complement component 3	+2.1	< 0.01
C6	Complement C6	+2.1	< 0.01
C9	Complement C9	+2.1	< 0.01
TLR3	Toll-like receptor 3	+2.13	< 0.05
F13B	Coagulation factor XIII, B polypeptide	+2	< 0.01
TLR9	Toll-like receptor 9	+1.91	< 0.05
hsp90b1	HSP90B1	−9.2	= 0
IL10	Interleukin 10	−12	< 0.01
IL-1β	Interleukin 1, beta	−11.4	< 0.01
TLR5	Toll-like receptor 5	−7.06	< 0.01
IL18R1	Interleukin 18 receptor 1	−7.04	< 0.01
SOCS3	Suppressor of cytokine signaling 1	−4.3	< 0.01
irak1	Interleukin 1 receptor associated kinase 1	−3.6	< 0.05
HSP90AA1	Heat shock protein 90 kDa alpha (cytosolic), class A member 1	−3.3	< 0.05
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	−3.2	< 0.01
TNFAIP3	TNF alpha induced protein 3	−3.2	< 0.01
MYD88	Myeloid differentiation primary response 88	−2.3	< 0.01

Note: "+"represents up-regulated, "-" represents down-regulated.

pathogen or cell surface, it may bind covalently with another C3b, to form C3bBbC3bP, the C5 convertase. This enzyme then cleaves C5 to C5a, a potent anaphylatoxin, and C5b. The C5b then recruits and assembles C6, C7, C8, and multiple C9 molecules to assemble the membrane attack complex, which can kill or damage the pathogen or cell. In this study, the highly upregulated iC3b, iC3, C7, C3, C6, and C9 mRNA that may reveal, after the challenge of the vaccinated tilapia, complement system played an important role in combating the tilapia *S. agalactiae* infection at 48 h. Other differentially expressed immunity and defense related genes also played their unique roles in resistant to streptococcal infection in the liver of tilapia which vaccinated with ScpB.

Of the nine differentially expressed genes confirmed by QPCR, many of them have been previously reported in tilapia spleen and kidney after infected by *S. agalactiae* [17]. Most of them could be attached to TLR signaling pathway. Toll-like receptors (TLRs) are a group of evolutionarily conserved receptors that function in innate immunity through recognition of the conserved pathogen-associated molecular patterns (PAMPs) of an invading pathogen and eliciting inflammatory immune responses [58]. The best characterized ligands that TLRs recognize include: lipoproteins by TLR2, dsRNA by TLR3, lipopolysaccharide (LPS) by TLR4, and bacterial flagellin by TLR5 [59]. In mammals, inflammation will result in a cytokine cascade whereby tumor necrosis factor a is released, followed by IL-1b and IL-6. After the release of these cytokines, chemokines are released to serve as potent chemoattractants to induce migration of neutrophils and macrophages to the site of infection [56–61].

5. Conclusions

In summary, a total of 1234 significant differentially expressed unigenes were detected ($P < 0.05$) in the tilapia liver between the ScpB-vaccinated and the PBS sham-vaccinated control at 48 h after *S. agalactiae* challenged, of which 236 were significantly up-regulated, and 269 were significantly down-regulated ($P < 0.05$, $|\text{fold}| > 2$, $FDR < 0.05$). Of the differentially expressed gene, the identified genes could be categorized into a total of 67 functional groups and were annotated to 153 signaling pathways including 15 immune-related pathways. Combining with GO and KEGG analysis, many of significantly differentially expressed immunity and defense related genes were identified. These results revealed an overall and a very complex network including immune and various related metabolic pathways in the targeted liver of tilapia under the ScpB-vaccinated and *S. agalactiae*-

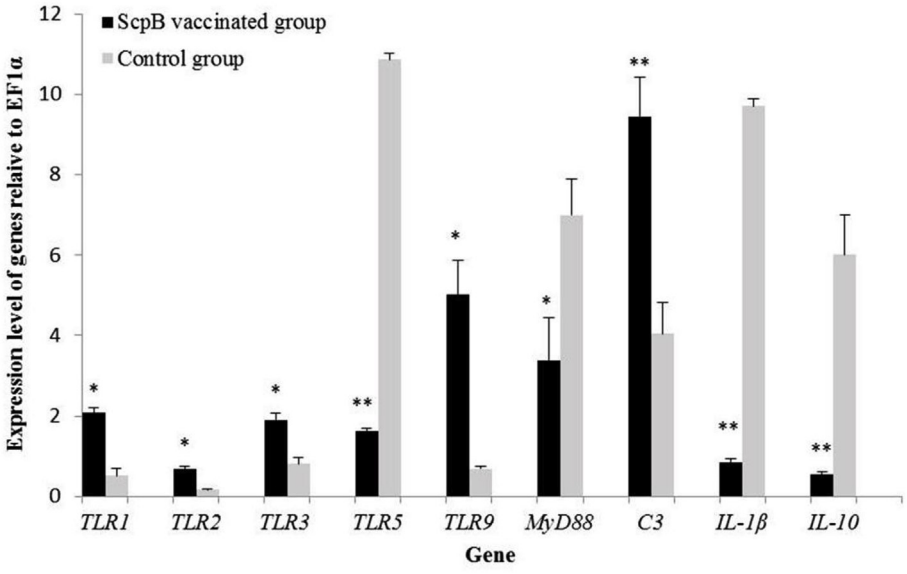

Fig. 4. Expression analyses of nine genes (*TLR1, TLR2, TLR3, TLR5, TLR9, MyD88, C3, IL-1β, IL-10*) by quantitative real-time PCR. The black column indicates the gene expression level in ScpB-vaccinated and *Streptococcus agalactiae-challenged* tilapia liver, and the gray column indicates the gene expression level of the control group. The statistical significance of the relative expression level is presented * $P < 0.05$, ** $P < 0.01$.

challenged, which also provided a theoretical basis on the anti-*S. agalactiae* mechanism in the targeted tissues and disease resistance breeding of Nile tilapia.

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