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Transcriptome analysis of liver, gill and intestine in rainbow trout *(Oncorhynchus mykiss)* symptomatically or asymptomatically infected with *Vibrio anguillarum*

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ABSTRACT

Rainbow trout (*Oncorhynchus mykiss*), an important economic cold-water fish worldwide, is severely threatened by viruses and bacteria in the farming industry. The vibriosis outbreak has caused a significant setback to aquaculture. *Vibrio anguillarum*, one of the common disease-causing vibriosis associated with severe lethal vibriosis in aquaculture, infects fish mainly by adsorption and invasion of the skin, gills, lateral line and intestine. To investigate the defense mechanism of rainbow trout against the pathogen after infection with *Vibrio anguillarum*, trout were intraperitoneally injected by *Vibrio anguillarum* and divided into symptomatic group (SG) and asymptomatic group (AG) according to the phenotype. RNA-Seq technology was used to evaluate the transcriptional signatures of liver, gill and intestine of trout injected with *Vibrio anguillarum* (SG and AG) and corresponding control groups (CG(A) and CG(B)). The GO and KEGG enrichment analyses were used to investigate the mechanisms underlying the differences in susceptibility to *Vibrio anguillarum*. Results showed that in SG, immunomodulatory genes in the cytokine network were activated and tissue function-related genes were down-regulated, while apoptosis mechanisms were activated. However, AG responded to *Vibrio anguillarum* infection by activating complement related immune defenses, while metabolism and function related genes were up-regulated. Conclusively, a rapid and effective immune and inflammatory response can successfully defend *Vibrio anguillarum* infection. However, a sustained inflammatory response can lead to tissue and organ damage and cause death. Our results may provide a theoretical basis for breeding rainbow trout for disease resistance.

1. Introduction

Transcriptome sequencing (RNA-Seq) analyses all RNAs, mainly mRNA and non-coding RNA, transcribed by different cells or tissues under specific conditions at the overall transcriptional level to reveal biological pathways and molecular regulatory mechanisms [1–4]. In recent years, there has been an increasing number of studies on immunological aspects of fish using transcriptomic techniques, eg: interleukin-1 beta (*il1β*) [5], tumour necrosis factor α (*tnfα*) [6], C-X-C motif chemokine receptor 1 (*cxcr1*), C-X-C motif chemokine receptor 1 (*cxcr2*) [7,8], interleukin-8 (*il-8*) [9].

Vibrio anguillarum (*V. anguillarum*) belongs to Vibrionaceae, *Vibrio*, and is a Gram-negative pathogen with typical bacterial characteristics of the genus *Vibrio*. Over 50 teleost species have been reported to be susceptive to *V. anguillarum*, including rainbow trout (*Oncorhynchus mykiss*), causing serious economic losses in aquaculture [10,11]. In salmon trout culture in China, *V. anguallanim* is one of the four common pathogenic *Vibrio* associated with severe lethal vibriosis in aquaculture [11] and infects fish mainly through adsorption and invasion of the skin, gills, lateral line and intestine [12]. Fish infected with *V. anguallanim* will show symptoms of progressive disease, some fish infected with *V. anguallanim* will initially show local discoloration, anal redness and swelling, followed by diffuse or punctate bleeding of muscle and blackening of the body surface and autopsy will reveal a clear yellow viscous fluid in the abdominal cavity, decay and detachment of intestinal mucosa tissue, and liver necrosis in some fish [11]. In addition, *Vibrio* in water can infect humans through surface trauma or the food chain [13]. Therefore, our research on *V. anguallanim* is important for identifying the pathogenic mechanism and breeding disease-resistant species.

Liver of fish is often referred as a metabolic organ, but it also contains

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Abbreviations

CG	control group
SG	symptomatic group
AG	asymptomatic group
HSP90	Heat Shock Protein 90
PDIA3	Protein Disulfide Isomerase Family A Member 3
PCK2	Phosphoenolpyruvate Carboxykinase 2, Mitochondrial
GLUD1b	Glutamate Dehydrogenase 1 beta
ENO3	Enolase 3
GADPH	Glyceraldehyde-3-Phosphate Dehydrogenase
ANGPT	Angiopoietin
POLD1	DNA Polymerase Delta 1, Catalytic Subunit
RPA3	Replication Protein A3
COL	Collagen Type
TNNT2	Troponin T2, Cardiac Type
ACTB	Actin, cytoplasmic 1
FOS	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit
JUN	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit
PFKFB3	6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3
HK1	Hexokinase 1
SLC2A1	Solute Carrier Family 2 Member 1
PTGS2	Prostaglandin-Endoperoxide Synthase 2
VEGFAA	Vascular Endothelial Growth Factor A
SERPINE1	Serpin Family E Member 1
FGG	Fibrinogen Gamma Chain
C3	Complement C3
FGA	Fibrinogen Alpha Chain
PDGFC	Platelet Derived Growth Factor C
DGAT	Diacylglycerol *O*-Acyltransferase
MTTP	Microsomal Triglyceride Transfer Protein

natural immune cells and it plays a key role in maintaining normal metabolism and innate immune function [14–17]. Liver bleeding and blackened edges in rainbow trout infected with *V. anguallanim* [18]. Furthermore, it was shown that rainbow trout livers exposed to *V. anguallanim* showed higher levels of genes such as IL-1β, IL-6, IL-8, TNFα and the gene encoding IgM [19]. Thus, both its dual role in immune function and metabolism makes the liver to be an interesting candidate for studying the innate immune response and metabolic adjustments in *V. anguallanim* attacking rainbow trout [17,20].

The aquatic environment is conducive to microbial growth compared to air environment. These conditions may increase additional challenges to the mucosal immune system of aquatic vertebrates [21]. which plays a key role in defense mechanisms against pathogens [22]. The intestine, skin and gill of fish share immune features with the type I mucosal surfaces of mammals [21]. The gill and intestine of fish are important physical and immune barriers [23,24]. Gill is one of the ways that teleost contact their aquatic environment. As the main organ responsible for the exchange and excretion of gases and electrolytes, the gills are also considered to be the main organ of infection for pathogens [25,26]. Previous studies have shown that fish gill appears to be able to absorb fish pathogens [26]. In rainbow trout (*Salmo gairdneri*), Germon (1987) suggested that gill was the initial site of *V. anguallanim* infection [27]. Ane Rebecca Engelsen et al. considered the skin, gill, and intestine were three potential sites for *V. anguallanim* to enter the fish [28]. Zebrafish intraperitoneal injected with *V. anguallanim* for 8 h observed that *V. anguallanim* was mainly observed in the ventral regions and in the gill [29].

The intestine plays an important role in health maintenance through the associated innate and adaptive immune system [30]. Teleost intestine with diffuse gut associated lymphoid tissue (GALT) consisting of individual and scattered lamina propria (LP) and intraepithelial (IE) leukocytes [31]. Furthermore, studies have shown that after *V. anguallanim* passed through the stomach, the intestine becomes a site of adhesion, colonization and proliferation. *V. anguillarum* uses flagellum-mediated motility to infect and colonise the surface and intestine of fish hosts [32] and used intestine mucus as an important source of nutrition [11]. The intestine may swell and fill with clear, viscous fluid after infection *V. anguallanim* in teleost [11]. Flagellin stimulates intestine inflammation through activation of the Toll-like receptor 5 (TLR5) signaling pathway [33,34] and *V. anguillarum* also crosses the intestine epithelium by endocytosis and the pathogen enters the bloodstream, causing blood poisoning (septicaemia) or infection of various internal organs, such as the liver, spleen and kidneys [11].

Most studies of the immune response in teleost fish have typically focused on changes in expression of the head kidney and spleen. They represented the major hematopoiesis and the major secondary lymphoid organ of the fish, respectively. However, the liver also plays an important role in fish immunity and, as fish live in an aquatic environment where the gills and intestines are in direct contact with pathogenic bacteria, they are also the first organs to respond to pathogens and provide an important safeguard for the overall health of the fish. To investigate the molecular response of the liver and mucosal surfaces of rainbow trout in response to *V. anguallanim*, and to obtain the key genes and pathways of immunity to *V. anguallanim*, we performed transcriptional signature of the liver, intestine and gill of rainbow trout infected with *V. anguallanim* and investigated the reasons for the different phenotypes of rainbow trout in response to *V. anguallanim*.

2. Materials and methods

2.1. Ethics statement

The fish used throughout the experiments were rainbow trout and did not involve any endangered or protected species. All animal experiments were conducted in accordance with the guidelines and approval of the Animal Research and Ethics Committee of Ocean University of China (Permit number: 20141201) and the National Research Council's Guide for the Care and Use of Laboratory Animals. The fish used in the experiments were juveniles and not yet sexually mature, so the effect of sex was not considered during the experiments.

2.2. V. anguillarum challenge experiment and fish sample

The samples used in this experiment are the same as those used in Yang et al. (2022) for *mkk* gene family study [35]. The previous study only focused on identification and expression of *mkk* gene family [35], while this study exerted a comprehensive analysis of the defense mechanism of rainbow trout against *V. anguillarum* infection with transcriptomic data. The rainbow trout (9 ± 0.75 g, 8 ± 0.5 cm) used in this experiment were provided by Linqu Salmon and Trout Aquatic Breeding LLC (Weifang, Shandong, China) and were acclimatized in the Experimental Fish Facility in Key Laboratory of Mariculture, Ministry of Education (KLMME), Ocean University of China for 14 days. The water temperature and dissolved oxygen levels were adjusted to ~16 °C and ~7 mg/L, respectively, with 12 h of light and 12 h of darkness to simulate the natural light cycle. Feed twice a day (8:30 and 17:30) with commercial feed (about 7% of body weight), sucking up excrement and residual bait from the bottom of the tank after 30 min of each feeding and appropriate water replenishment. The fish are healthy and unharmed during feeding. The size of tanks is 30*40*45 cm. The *V. anguillarum* strain was obtained from the Laboratory of Aquatic Animal Pathology and Immunology, Ocean University of China [36]. Using

the aid of a Mackenzie turbidimeter, it was prepared in PBS as a 10^7 CFU/ml suspension for subsequent attack experiments. Using the same method described previously, rainbow trout infected with *V. anguillarum* were divided into symptomatic group (SG) and asymptomatic group (AG) [35], and the corresponding control group (CG). The control group was named CG(A) for SG and CG(B) for AG. The fish were anaesthetized with MS-222 (35–45 mg/L (ppm)) before sampling, and after the fish were free from struggle, they were immediately dissected and the gills, livers and intestines were separated into RNA-free enzyme tubes, rapidly frozen in liquid nitrogen and stored at −80 °C for use. RNA from the samples was extracted and RNA from the same tissue from the same group of three fish in the same tank was mixed into one tube and so on to obtain a total of 36 RNA samples (Table S1) [35].

2.3. RNA extraction, library construction

Total RNA was isolated from gill, liver and intestine using Total RNA Extraction Reagent (Vazyme Biotech, China) kit. RNA concentration and purity were measured using a biophotometer (OSTC, China) and RNA quality was measured by agarose gel electrophoresis. The integrity of total RNA was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). In order to minimize the variation among individuals, RNA from the same tissue from the same group of three fish in the same tank was mixed into one tube and finally we obtain 36 RNA samples (Table S1). A total of 36 sequencing libraries [3 tissues (gill, liver, intestine) × 3 replicated samples (each sample contained three pooled individuals) × 4 groups (SG, AG and 2 CGs)] were generated using TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Then, samples were sequenced on the Illumina sequencing platform (Illumina HiSeqTM 2500 or Illumina HiSeq XTen) and 125bp/150bp paired-ended reads were generated. The transcriptome sequencing and analysis were conducted by OE Biotech Co, Ltd. (Shanghai, China).

2.4. Transcriptome data analysis

Raw reads in fastq format were processed using Trimmomatic [4] to remove poor-quality data and Q20 to obtain clean data. Comparison of clean data with the reference genome (GCF_013265735.2) of rainbow trout using hisat2 [3]. Clean reads in each sample mapped to the protein-coding genes were obtained by htseq-count [2], and cufflinks [1] was used to calculate the expression of protein-coding genes FPKM (Fragments Per kb Per Million Reads) [37]. The DESeq (2012) R package was used for differentially expressed genes (DEGs) analysis [38]. Genes with "*p*-value" < 0.05 and $|\log2 \text{(fold change)}| > 1$ were considered as DEGs. These DEGs were analyzed for GO and KEGG enrichment [39]. The DEGs were also subjected to unsupervised hierarchical clustering, and heatmaps were used to show the expression patterns of the DEGs across the samples. The RNA sequence data were deposited at the GenBank SRA database under the accession numbers PRJNA867038, PRJNA866872, PRJNA866205, PRJNA667799 [35], PRJNA865462 [40].

2.5. Quantitative real-time PCR validation of DEGs

The quantitative real-time PCR (qRT-PCR) analysis was performed to validate the candidate DEGs under the following conditions: 2 min at 95 °C, followed by 40 cycles of 30 s at 94 °C, 10 s at 60 °C and 30 s at 75 °C. All reactions were performed in triplicate in a 96 well plate. Those DEGs for validation include chemokine CXCF1b (*cxcf1b*), leukocyte cell-derived chemotaxin 2 (*lect2*), microfibril associated protein 2 (*mfap2*), CEA cell adhesion molecule 6 (*ceacam6*), 4-aminobutyrate aminotransferase (*abat*), phospholipid phosphatase 1a (*plpp1a*), acyl-CoA dehydrogenase long chain (*acadl*) (Table 1). The house keeping gene *β-actin* was used for normalizing cDNA amounts. Three replicates were performed for each sample, and the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative level of gene expression. The correlation coefficient between the fold changes in RNA-Seq group and qRT-PCR group was determined by SPSS25.0, one-way ANOVA followed by Duncan's multiple range tests and differences were accepted as statistically significance when $p < 0.05$.

Table 1
Primer sequences used for qPCR validation.

Primers for qPCR	Primer sequence (5′ to 3′)
β-actin-F	GATGGGCCAGAAAGACAGCTA
β-actin-R	TCGTCCCAGTTGGTGACGAT
cxcf1b-F	ACACGTTCCAGGTGCCAGAT
cxcf1b-R	GCTTGTCGTCCTTGTTTATT
lect2-F	GCTGTTCTTTTGTTTACTGTGGTG
lect2-R	TGTCCTTGTCCCCATCTGTC
mfap2-F	CCTGGAGGACTATGACCCT
mfap2-R	CTGCTCTTCTCGGCAATCT
ceacam6-F	TGGTGGCTTCTATCAGTGCG
ceacam6-R	ACCGACGGGATCCATTTCAG
abat-F	TGGGAGCAGTCCATTTCTT
abat-R	ATGAGTGAGGGATGGTTGTAT
plpp1a-F	TTCTGCTTGACATCGCCTGT
plpp1a-R	AAGTGAAGCCCACACCGTAG
acadl-F	GGGAGAAGGCAGGTATGGT
acadl-R	TCTCAGAGTGGAGGGCAAA

3. Result

3.1. Differential gene expression analysis

Comparing the liver transcriptional signature of control and infected rainbow trout, we found 7160 and 761 DEGs in SG and AG respectively. In SG liver, 3375 genes were up-regulated and 3785 genes were down-regulated. In AG liver, 488 genes were up-regulated and 273 genes were down-regulated. In gill, we found 1977 and 621 DEGs in SG and AG, respectively. In SG gill, 1217 genes were up-regulated and 760 genes were down-regulated. In AG gill, 322 genes were up-regulated and 299 genes were down-regulated. There were 2589 and 1438 DEGs in SG intestine and AG intestine respectively. In SG intestine, 1798 genes were up-regulated and 791 genes were down-regulated and 808 genes were up-regulated and 630 genes were down-regulated in AG intestine (Table 2).

The GO functional annotation and functional classification showed that these DEGs were assigned to three components: cellular components, biological processes and molecular functions, mainly to "immune processes" (e.g. Bcl3/NF-kappaB2 complex and proteasome complexes) and "metabolic processes" (e.g. retinol metabolic process).

Metabolic pathway analysis of DEGs identified several immune-related classes of pathways, including Cytokine-cytokine receptor interaction, Complement and coagulation cascades, NOD-like receptor signaling pathway, NF-κB signaling pathway, the Toll-like receptor signaling pathway, and the Antigen processing and presentation. More detailed analysis of these signaling pathways showed that several genes were significantly increased in infected fish, including the *cxc*, *cc chemokine subfamilies*, the *il1r* family and the *tnf* family (Fig. 1). Most of these genes are up-regulated in infected fish.

3.2. Function analysis of liver transcripts

GO functional annotation of the 7160 DEGs screened from SG liver showed that in the classification of molecular function, The majority of DEGs in "binding" and "catalytic activity" were significantly down-regulated. In the classification of cellular component, DEGs on "proteasome complex" and "Bcl3/NF-kappaB2 complex" were significantly up-regulated (Fig. 2). KEGG pathway analysis was performed on DEGs to further elucidate the functional status of transcripts assembled in a possible functional state (Fig. S1). The results showed the SG liver

Table 2
Transcriptional signature obtained by RNA-Seq analys.

Sample	DEGs	Up-regulated gene counts	Down-regulated gene counts	Log_2(Fold change)
SG-Liver	7160	3375	3785	−11.68–14.72
AG-Liver	761	488	273	−6.68–8.01
SG-Gill	1977	1217	760	−6.31–10.20
AG-Gill	621	322	299	−5.18–13.53
SG-Intestine	2589	1798	791	−10.55–9.24
AG-Intestine	1438	808	630	−10.01–15.15

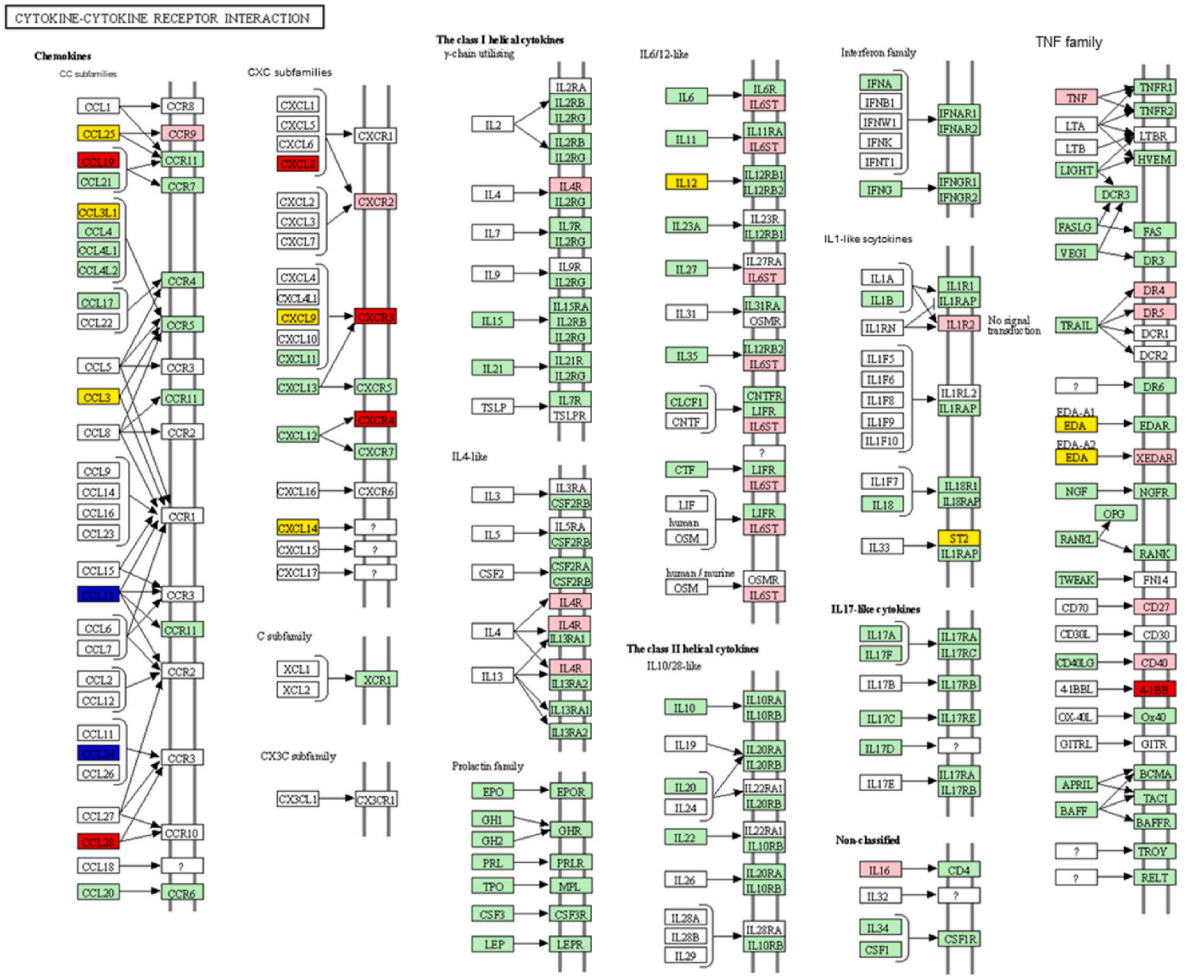

Fig. 1. KEGG enrichment pathway map (Part of Cytokine-cytokine receptor interaction). Red box: log2(Fold Change) > 4; Pink box:2< log2(Fold Change) < 4; Yellow box: 0.0625 < log2(Fold Change) < 0.5; Blue box: log2(Fold Change) < 0.0625.

exhibited a high level of inflammatory response. The pathways associated with the "immune response" include: Proteasome, NOD-like receptor signaling pathway, NF-κB signaling pathway, T cell receptor signaling pathway, toll-like receptor signaling pathway, etc. coagulation cascades. Most immune-related signaling pathways were significantly up-regulated. There are also pathways related to "metabolism" and "liver function": Retinol metabolism, Fat digestion and absorption, Starch and sucrose metabolism, and Fat digestion and absorption. These pathways were mostly significantly down-regulated (Table 3).

GO functional annotation of 761 DEGs in AG liver (Fig. 2) showed that DEGs were mainly enriched in "metabolism" and "liver function" related functions, such as, positive regulation of fatty acid beta-oxidation, triglyceride metabolic process and retinol metabolic process were all significantly up-regulated. Others were enriched in immune processes such as regulation of defense response to virus, innate immune response, antigen processing and presentation, and the DEGs associated with these processes tend to be down-regulated. KEGG pathway annotation of DEGs in AG liver transcriptional signature showed a significant enrichment of DEGs between AG and CG(B) to 30 pathways (Fig. S1). Most of the pathways related to immune and inflammatory responses (e.g. cytochrome P450 metabolism of xenobiotics, NF-κB signaling pathway) were significantly down-regulated, but complement and coagulation cascades were significantly up-regulated. In contrast, most of the pathways related to metabolism and liver function showed up-regulated (Table 4). The GO analysis of AG liver showed a significant up-regulation of a large number of GO Terms related to liver function, while some of the DEGs related to the innate immune response were down-regulated in SG liver (Fig. 2).

3.3. Function analysis of gill transcripts

GO functional annotation of 1977 DEGs screened from SG gill

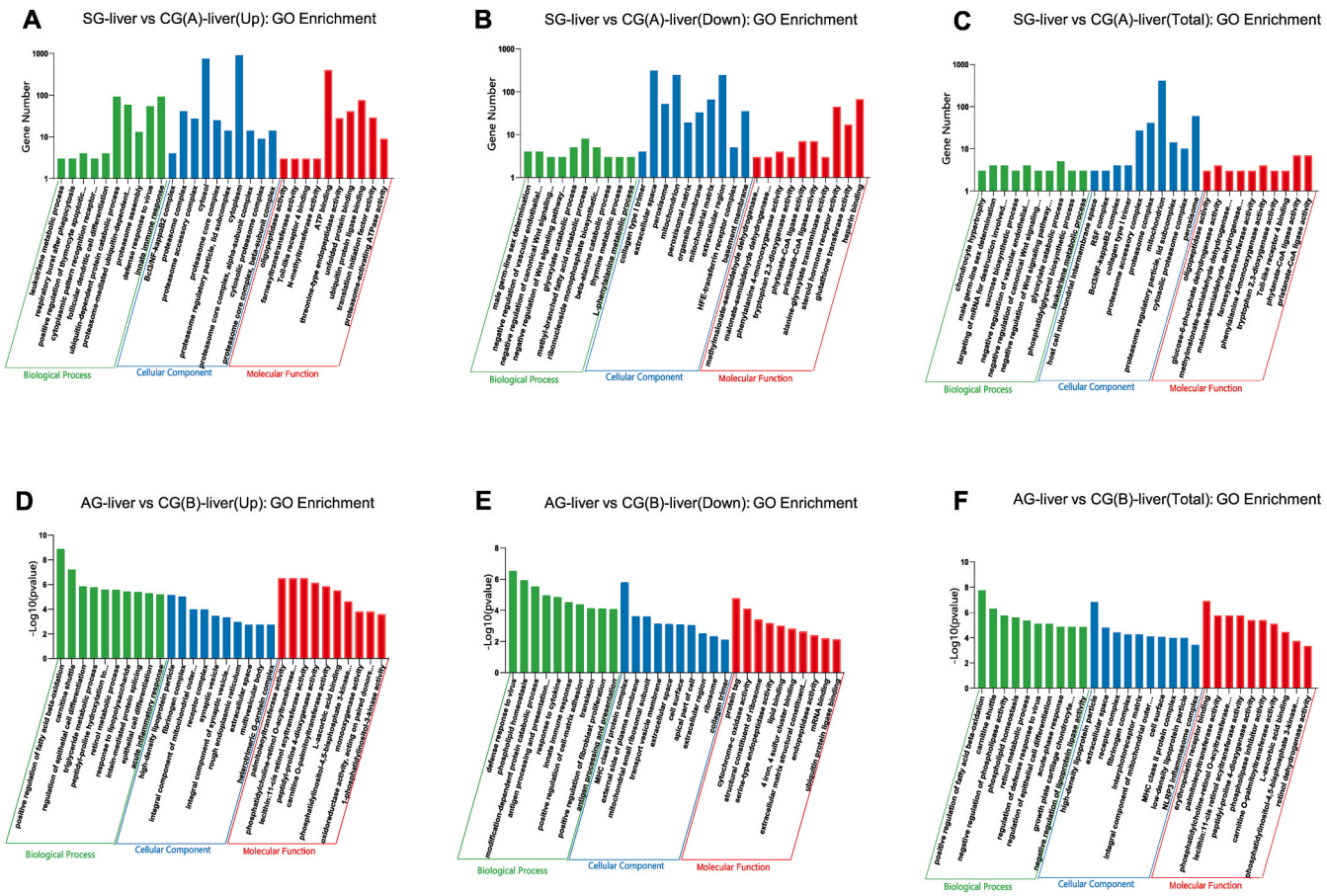

Fig. 2. GO enrichment results of DEGs in liver. (**A**) Down-regulated GO enrichment in liver transcriptional signature of SG and CG(A). (**B**) Up-regulated GO enrichment in liver transcriptional signature of SG and CG(A). (**C**) Total of DEGs in GO enrichment in liver transcriptional signature of SG and CG(A). (**D**) Down-regulated GO enrichment in liver transcriptional signature of AG and CG(B). (**E**) Up-regulated GO enrichment in liver transcriptional signature of AG and CG(B). (**F**) Total of DEGs in GO enrichment in liver transcriptional signature of AG and CG(B).

Table 3
List of KEGG for DEGs in liver transcriptional signature of SG and CG(A) ($p < 0.05$).

KEGG Pathway	ListHits	Trend	*p*-value	KEGG Pathway	ListHits	Trend	*p*-value
NOD-like receptor signaling pathway (ko04621)	42	Up	1.39E-08	Apoptosis (ko04210)	66	Up	5.20E-07
TNF signaling pathway (ko04668)	64	Up	1.39E-11	HIF-1 signaling pathway (ko04066)	44	Up	2.04E-04
Protein processing in endoplasmic reticulum (ko04141)	77	Up	1.80E-09	Retinol metabolism (ko00830)	27	Down	6.54E-08
NF-κB signaling pathway (ko04064)	46	Up	3.10E-08	PPAR signaling pathway (ko03320)	48	Down	1.01E-13
Toll-like receptor signaling pathway (ko04620)	48	Up	2.60E-05	Carbon metabolism (ko01200)	69	Down	4.12E-13
Proteasome (ko03050)	62	Up	1.12E-34	Starch and sucrose metabolism (ko00500)	31	Down	1.22E-09
T cell receptor signaling pathway (ko04660)	48	Up	1.90E-05	Fat digestion and absorption (ko04975)	33	Down	2.61E-09

Table 4
List of KEGG for DEGs in liver transcriptional signature of AG and CG(B) ($p < 0.05$).

KEGG Pathway	ListHits	Trend	*p*-value	KEGG Pathway	ListHits	Trend	*p*-value
NF-κB signaling pathway (ko04064)	10	Down	8.11E-05	Protein digestion and absorption (ko04974)	20	Up	3.82E-08
Arachidonic acid metabolism (ko00590)	14	Down	4.62E-06	Mineral absorption (ko04978)	14	Up	2.05E-08
Bile secretion (ko04976)	18	Down	9.86E-04	Sphingolipid metabolism (ko00600)	11	Up	9.20E-05
Metabolism of xenobiotics by cytochrome P450 (ko00980)	4	Down	2.32E-03	Linoleic acid metabolism (ko00591)	7	Up	7.94E-05
Vitamin digestion and absorption (ko04977)	10	Up	3.44E-07	Fat digestion and absorption (ko04975)	19	Up	5.79E-12
Complement and coagulation cascades (ko04610)	38	Up	3.52E-24	Steroid biosynthesis (ko00100)	7	Up	4.92E-07

(Fig. 3). Under the classification of biological processes, most DEGs were enriched in GO terms related to "cardiac function" (e.g. cardiac muscle contraction, cardiac muscle fiber development) and inflammatory responses. In the molecular function classification, most genes were annotated to GO terms such as chemokine activity, C-X-C chemokine receptor activity, complement binding and oxygen binding. KEGG pathway annotation of DEGs in SG gill transcriptional signature showed a significant enrichment of DEGs between SG and CG(A) to 61 pathways (Fig. S2). The majority of these pathways were enriched for immune and inflammatory responses (e.g. cytokine-cytokine receptor interaction,

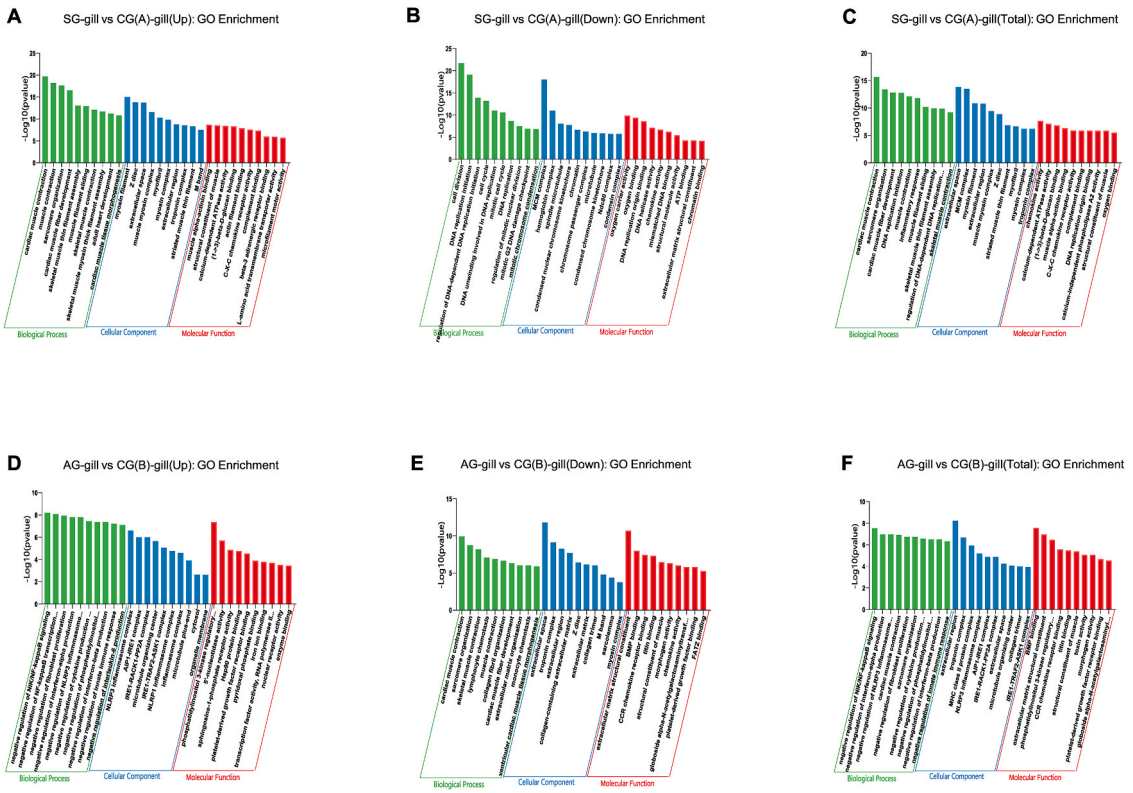

Fig. 3. GO enrichment results of DEGs in gill. (**A**) Up-regulated GO enrichment in gill transcriptional signature of SG and CG(A). (**B**) Down-regulated GO enrichment in gill transcriptional signature of SG and CG(A). (**C**) Total of DEGs in GO enrichment in gill transcriptional signature of SG and CG(A). (**D**) Up-regulated GO enrichment in gill transcriptional signature of AG and CG(B). (**E**) Down-regulated GO enrichment in gill transcriptional signature of AG and CG(B). (**F**) Total of DEGs in GO enrichment in gill transcriptional signature of AG and CG(B).

mismatch repair), as well as for cell cycle and cardiac function pathways (Table 5).

Processing of 621 DEGs screened from AG gill and GO functional annotation of these DEGs revealed (Fig. 3) that most DEGs were enriched for negative regulation of the immune response (e.g. negative regulation of NIK/NF-κB signaling, negative regulation of interferon production, negative regulation of the innate immune response, etc). They were significantly up-regulated, indicating that the immune response was suppressed in AG gill. In addition, GO terms such as cardiac muscle contraction and myotomy were also enriched, and most of the DEGs associated with them were down-regulated in AG gill. KEGG pathway annotation of DEGs in the AG and CG(B) gill transcriptional signature showed that the 20 most significant pathways were enriched (Fig. S2). Most of the enrichment to pathways related to immune response and cardiac function was accompanied by a partial down-regulation of pathways related to stress and immunity (Table 6).

3.4. Function analysis of intestine transcripts

The 2589 DEGs from SG intestine were GO functionally annotated and mostly enriched for GO terms such as inflammatory response, defence response to bacteria, cellular response to interleukin-1, lymphocyte chemotaxis, DNA damage response, Toll-like receptor binding, complement binding, chemokine activity, etc (Fig. 4). Further KEGG pathway annotation of DEGs (Fig. S3) showed significant enrichment for pathways including cytokine-cytokine receptor interactions, antigen processing and presentation, complement and coagulation cascades, and most DEGs associated with these pathways were significantly up-regulated in SG intestine, which showed serious immune and inflammatory responses, in addition to down-regulated DNA replication and cell cycle in SG intestine (Table 7).

The 1438 DEGs screened from AG intestine were annotated for GO function, and the significantly enriched GO terms (Fig. 4) included mainly complement activation, alternative pathways, establishment of skin barrier, extracellular matrix, serine-type endopeptidase inhibitor activity, endopeptidase inhibitor activity , neutral amino acid transmembrane transport activity, chloride channel activity, and extracellular matrix structural constituent. KEGG was enriched for the 20 most significant pathways (Fig. S3), including complement and coagulation cascades, cytokine-cytokine receptor interaction, NF-κB signaling pathway, vitamin digestion and absorption, and PI3K-Akt signaling pathway (Table 8).

Table 5
List of KEGG for DEGs in gill transcriptional signature of SG and CG(A) ($p < 0.05$).

KEGG Pathway	ListHits	Trend	*p*-valual	KEGG Pathway	ListHits	Trend	*p*-valual
Cell cycle (ko04110)	28	Down	1.14E-13	NOD-like receptor signaling pathway (ko04621)	10	Up	9.33E-04
Mismatch repair (ko03430)	8	Down	1.06E-08	NF-κB signaling pathway (ko04064)	13	Up	4.80E-03
Nucleotide excision repair (ko03420)	7	Down	4.06E-05	Complement and coagulation cascades (ko04610)	30	Up	4.92E-16
Homologous recombination (ko03440)	8	Down	9.82E-08	Adrenergic signaling in cardiomyocytes (ko04261)	25	Up	6.15E-04
TNF signaling pathway (ko04668)	21	Up	4.43E-05	HIF-1 signaling pathway (ko04066)	18	Up	5.74E-04

Table 6
List of KEGG for DEGs in gill transcriptional signature of AG and CG(B) ($p < 0.05$).

KEGG Pathway	ListHits	Trend	*p*-valual	KEGG Pathway	ListHits	Trend	*p*-valual
Complement and coagulation cascades (ko04610)	3	Down	3.46E-02	Cardiac muscle contraction (ko04260)	6	Down	5.41E-04
Cytokine-cytokine receptor interaction (ko04060)	9	Down	4.37E-03	Adrenergic signaling in cardiomyocytes (ko04261)	7	Down	1.22E-02
PI3K-Akt signaling pathway (ko04151)	12	Down	1.72E-02	Platelet activation (ko04611)	4	Down	9.57E-02
Protein digestion and absorption (ko04974)	3	Down	1.09E-05	Focal adhesion (ko04510)	14	Down	1.30E-05
Antigen processing and presentation (ko04612)	5	Down	4.93E-04	HIF-1 signaling pathway (ko04066)	4	Down	4.56E-02

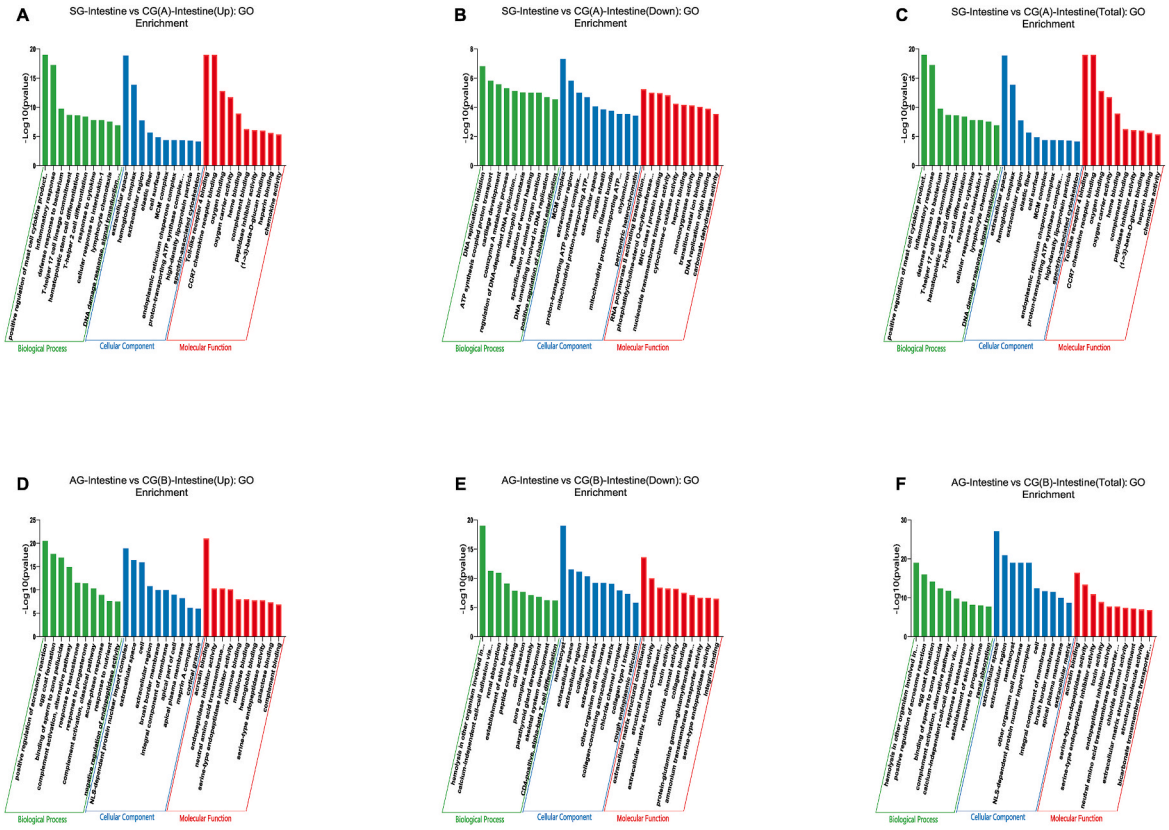

Fig. 4. GO enrichment results of DEGs in intestine. (**A**) Up-regulated GO enrichment in intestine transcriptional signature of SG and CG(A). (**B**) Down-regulated GO enrichment in intestine transcriptional signature of SG and CG(A). (**C**) Total of DEGs in GO enrichment in intestine transcriptional signature of SG and CG(A). (**D**) Up-regulated GO enrichment in intestine transcriptional signature of AG and CG(B). (**E**) Down-regulated GO enrichment in intestine transcriptional signature of AG and CG(B). (**F**) Total of DEGs in GO enrichment in intestine transcriptional signature of AG and CG(B).

Table 7
List of KEGG for DEGs in intestine transcriptional signature of SG and CG(A) ($p < 0.05$).

KEGG Pathway	ListHits	Trend	*p*-valual	KEGG Pathway	ListHits	Trend	*p*-valual
Meiosis – yeast (ko04113)	9	Down	1.24E-04	Cytokine-cytokine receptor interaction (ko04060)	65	Up	3.82E-13
DNA replication (ko03030)	6	Down	2.15E-04	NF-κB signaling pathway (ko04064)	21	Up	4.98E-04
Cell cycle – yeast (ko04111)	8	Down	4.72E-03	Complement and coagulation cascades (ko04610)	26	Up	3.89E-08
Cell cycle (ko04110)	12	Down	6.90E-03	TNF signaling pathway (ko04668)	41	Up	4.31E-11
Cardiac muscle contraction (ko04260)	8	Down	1.84E-02	HIF-1 signaling pathway (ko04066)	30	Up	6.12E-06
NOD-like receptor signaling pathway (ko04621)	16	Up	5.03E-05	Toll-like receptor signaling pathway (ko04620)	23	Up	4.13E-03

Table 8
List of KEGG for DEGs in intestine transcriptional signature of AG and CG(B) ($p < 0.05$).

KEGG Pathway	ListHits	Trend	*p*-valual	KEGG Pathway	ListHits	Trend	*p*-valual
Complement and coagulation cascades (ko04610)	33	Up	3.52E-24	Focal adhesion (ko04510)	22	Down	9.56E-07
Protein digestion and absorption (ko04974)	20	Up	3.82E-08	NF-κB signaling pathway (ko04064)	10	Down	8.11E-05
Fat digestion and absorption (ko04975)	17	Up	5.79E-12	PI3K-Akt signaling pathway (ko04151)	28	Down	7.98E-06

3.5. Protein-protein interaction analysis

A putative protein-protein interaction (PPI) network was analyzed and a visual representation created using STRING (Fig. 5). This analysis revealed a very significant enrichment ($P < 1.0e-16$) of PPIs among the groups. Analysis of SG livers showed that the majority of the PPIs were centered on three main node clusters, representing the immunity (e.g. CASPS8, IL10), and molecular chaperone systems (e.g. HSP90B1, HSP90a1.2, PDIA3), and metabolism-related proteins (e.g. PCK2, GLUD1b, ENO3) linked to molecular chaperones (Fig. 5A). In the AG liver, proteins associated with immunity are linked to proteins associated with protein hydrolysis (Fig. 5B). Analysis of SG gills showed that proteins associated with the HIF-1 signaling pathway (e.g. GADPH, ANGPT1, ANGPT2) were linked to apoptosis-related proteins. Proteins associated with the cell cycle (e.g. MCM7, POLD1, RPA3) formed separate protein clusters (Fig. 5C). Analysis of AG gill showed that the majority of the PPIs were centered on two main node clusters. It represents immune (COL1A1, COL1A2, etc.), and myocardial-related proteins (e.g. TNNT2A, ACTB2), and is directly linked to both (Fig. 5D). Analysis of SG intestine revealed that DNA replication and cell cycle related proteins (e.g., FOS, JUN, PFKFB3) and metabolism related proteins (e.g. HK1, SLC2A1B) were all associated with immune related proteins (e.g., PTGS2, VEGFAA, SERPINE1) (Fig. 5E). Analysis of AG intestine showed that most of the PPI were concentrated on 3 main protein clusters representing immunity (e.g. FGG, C3A, FGA, etc.), angiogenesis (e.g. ANGTP1, ANGTP2, PDGFC), and metabolism-related proteins (e.g. DGAT1a, DGAT2, MTTP) (Fig. 5F).

3.6. Confirmation of differently expressed genes by qRT-PCR

We confirmed the expression of 7 candidate DEGs by qRT-PCR in the liver, gill, intestine samples. The expression of all the genes was in

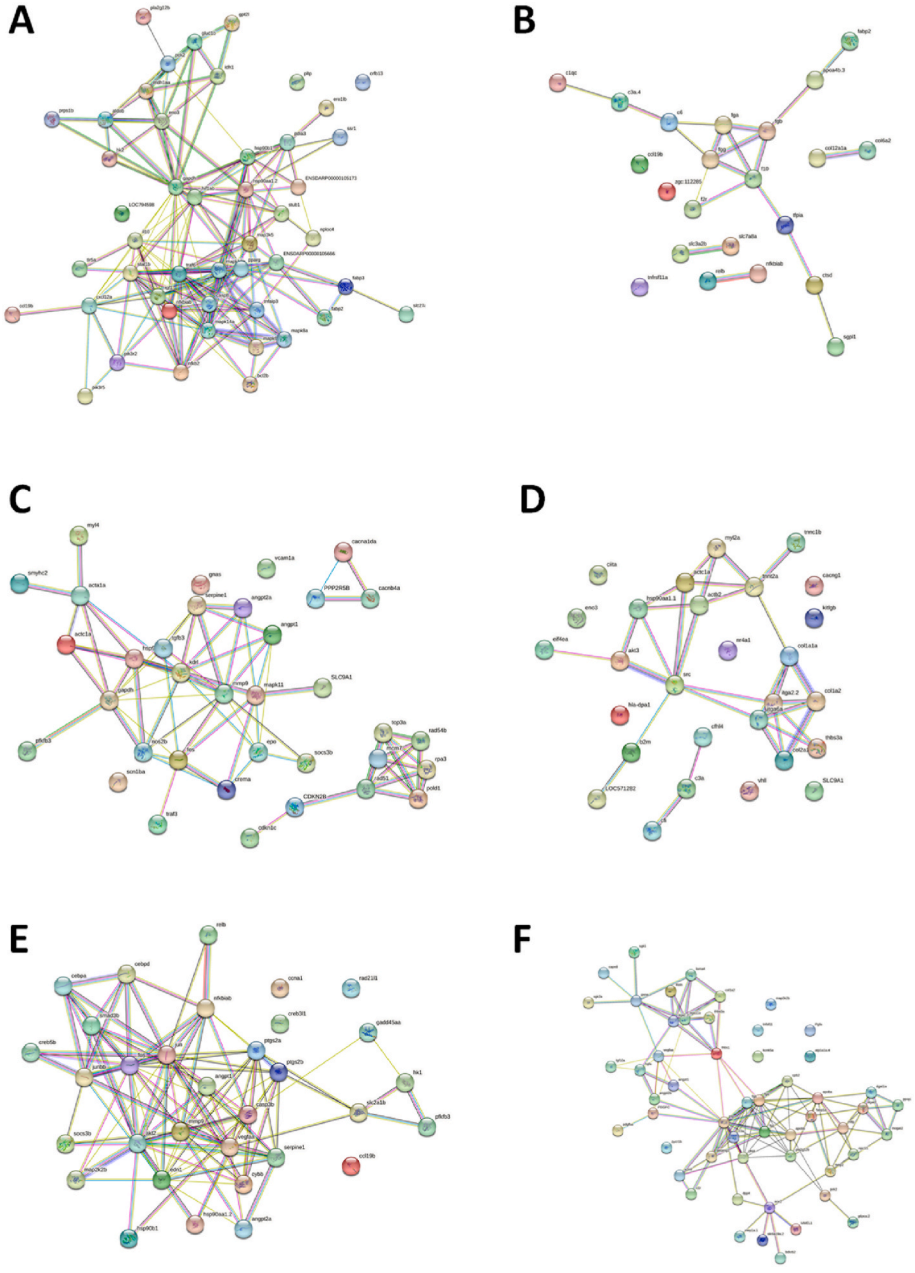

Fig. 5. STRING protein-protein interaction network (Fig. 5A is PPI of SG liver. Fig. 5B is PPI of AG liver. Fig. 5C is PPI of SG gill. Fig. 5D is PPI of AG gill. Fig. 5E is PPI of SG intestine. Fig. 5A is PPI of AG intestine).

agreement with their transcript abundance changes determined by RNA-seq (Fig. 6).

4. Discussion

In this study, we analyzed transcriptional signatures of liver, gill and intestine of SG and AG after *V. anguallanim* infection. GO and KEGG enrichment showed that the most significantly enriched pathways including inflammatory responses, metabolic responses and apoptotic signaling. Pathways associated with immune response were up-regulated in SG, with a down-regulated in AG.

Liver is one of the main target organs for metabolism. Nutrients are absorbed into the body through intestine and then transported to the liver. Recent studies have confirmed the interaction between immunity and metabolism in the liver [14,41]. It has been shown that LPS-induced inflammation affected systemic energy balance, including carbohydrate, lipid and amino acid metabolism [42–45]. In this study, lipid-metabolism-related pathways were down-regulated in SG liver, while inflammation-related pathways, such as TNF signaling pathway [46,47], NF-κB signaling pathway [48], Toll-like receptor signaling pathway [49,50], were up-regulated. In addition, a down-regulated in carbohydrate, lipid and acid metabolism pathways was observed in SG livers [43,45,51]. We hypothesize that down-regulation of metabolism-related pathways is associated with inflammatory responses in SG rainbow trout livers. *Il1β* was up-regulated 118.03-fold and peroxisome proliferator activated receptor alpha (*pparα*) was down-regulated 9.71-fold in SG livers. These results suggested a steady activation of inflammasome in the liver after *V. anguillarum* stimulation. It caused a sustained increase in *il1β* in liver. *Il1β* could directly down-regulated lipid metabolism through down-regulation of peroxisome proliferator-activated *pparα*. However, lipid metabolism is positively regulated in AG liver [44,45]. It has been suggested that lipid synthesis and accumulation have a positive effect on inflammation, while lipolysis exerts a negative feedback regulation on inflammation [14]. Therefore, our results indicated that the abilities of trout against to *V. anguillarum* infection were associated with liver metabolism function. A better liver metabolism function was conducive to the high resistance to *V. anguillarum*, resulting in a low inflammatory response.

Bacterial infections lead to the release of multiple cytokines, including TNF-α, IL-1β and IL-8, thus inducing inflammation and the release of reactive oxygen species (ROS) to treat oxidative stress [52–54]. TNF signaling pathway is closely related to apoptosis and *tnfα* upregulation directly leads to apoptosis of hepatocytes, causing liver injury [52,55,56]. The *tnfα* in SG liver was up-regulated 15.76-fold. Apoptosis is a response of cells to multiple triggers, including cell contraction, chromatin condensation, DNA fragmentation and the formation of apoptosome [52,57,58]. In SG livers was enriched for apoptotic pathways, proteasomes and protein processing pathways in the endoplasmic reticulum. *Caspase* family is a key protein in the execution of apoptosis program, among which *casp8* is the initiator of apoptosis, *casp3* and *casp6* are the executioners of apoptosis [59]. We observed that *casp8* was up-regulated 6.82-fold in SG liver, the two transcripts of *casp3* were up-regulated by 3.30-fold and 64.01-fold, and *casp6* was up-regulated by 7.77-fold, which indicated that inflammatory and apoptotic processes coexisted in liver and that ultimately an excessive inflammatory response will lead to liver dysfunction and even liver necrosis [60]. These results indicated that the SG liver was not only affected by pathogens in terms of metabolic function, but also interfered with protein synthesis in hepatocytes, and also initiated apoptosis.

The mucosal immune system of the gill consists of innate immunity and adaptive immunity that interact to fight infection by pathogenic bacteria [21]. The gill transcriptomic studies showed that proteases and antiviral peptides are activated, such as cathepsin 12 (*cts12*) up-regulated in SG gills, as well as cytokine and chemokine production, including up-regulation of the pro-inflammatory factor *il1β*, *tnfα* and *cxcr1* in SG gills. Therefore, we presumed that immunity was activated in gill transcriptomic, and a rapid and effective immune and inflammatory responses can successfully defend against *V. anguallanim*. However, a sustained inflammatory response can cause tissue and organ damage with a cytokine storm [10,19,61,62]. For example, damaged or even lost breathing function of the gills causes death of rainbow trout and showed signs of susceptibility. In addition, down-regulated DNA replication, homologous recombination and cell cycle pathways were significantly enriched in SG gills, showed that cell cycle was disrupted. The down-regulated minichromosome maintenance complex component 5 (*mcm5*) and minichromosome maintenance complex component 7 (*mcm7*) played an important role in the initiation and expansion of DNA replication and mediating DNA repair and chromatin remodeling [63,64], even directly threatening cell survival [65].

In this experiment, the gills may cause immune damage to tissues due to excessive cytokine accumulation induced by *V. anguallanim* [66]. The blood vessel wall becomes more permeable, and the capillaries begin to leak blood and plasma, therefore the gills begin to bleed, and then fail with local necrosis. Another study confirmed that the gill tissue of fish has a unique function of gill circulation [67,68]. It was hypothesized that the SG gill was severely damaged, causing hypoxia and up-regulation of genes associated with cardiac muscle contraction in SG gill and causing abnormal heart rhythms through the gill circulation.

Immunity activity in intestine is mediated through GALT, including many innate and adaptive immune cells [69,70]. The complement system is an important mechanism of innate immunity in fish, and previous studies have reported up-regulated genes related to the complement system in fish following various types of bacterial infections [71–74]. The complement and coagulation cascades pathway were significantly up-regulated in SG intestine. This pathway was also observed to be activated in AG intestine. Its activity protects intestine from damage. The intestine relies on T cells in the mucosa for adaptive immunity [30]. We found in our transcriptional signature resulted that the T cell subtype signature cytokines *il22* and *il17* were up-regulated in SG intestine, indicating a strong inflammatory response. Compared with SG rainbow trout, a large number of immune and inflammation-related negative regulators were enriched in AG intestine. It suggested that there was a

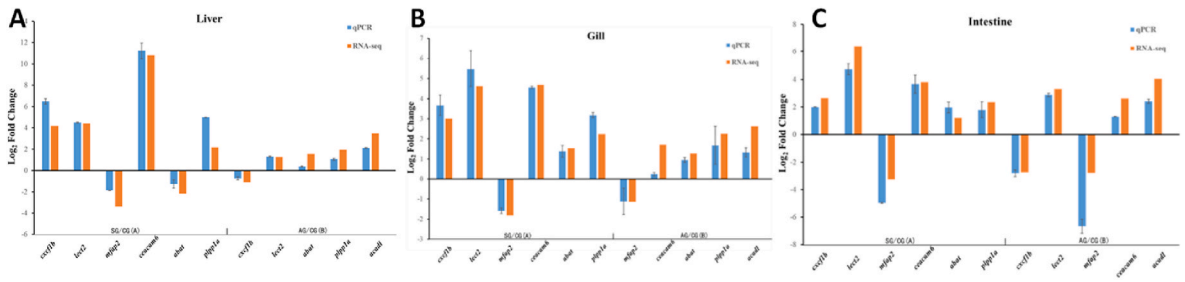

Fig. 6. Comparison of relative fold changes between qPCR and RNA-Seq results. (**A**) The expression of DEGs and their transcript abundance in the liver, (**B**) the expression of DEGs and their transcript abundance in the gill, (**C**) the expression of DEGs and their transcript abundance in the intestine.

rapid and effective inflammatory response in AG and that the inflammation was well controlled. We also found that transferrin (*tfα*) correlated with susceptibility to *V. anguallanim* in rainbow trout and *tfα* up-regulated in AG intestine.

Adipocytokine signaling pathway is an important pathway in intestine, which can be activated by the recognition of adiponectin (*adpn*), leptin (*lep*) or *tnfα*. And *tnfα* can disrupt the early stages of insulin signaling to accelerate lipid catabolism [75]. In SG intestine, we observed activity of the adipocytokine signaling pathway with significant up-regulated of *tnfα*, but no activity in AG intestine. However, the reason behind this change is not known, but may be related to reduced food intake after infection [30].

5. Conclusion

The reduced metabolic function of the liver may be related to the sustained acute-phase response (APR) in the body. Although the inflammatory response can help the body clear pathogens, persistent and systemic APR has the potential to cause metabolic disturbances that can fatten the liver and lead to organ failure. A short APR may up-regulate metabolic capacity and thus facilitate resistance to *V. anguillarum* infection. The mechanism of severe infection after infection of *V. anguallanim* may be related to a sustained and excessive immune response in the gills and intestines of trout. The continuous immune response will lead to tissue damage and organ failure, and eventually lead to the death of rainbow trout. Our research provided great significance to the immune mechanism of trout against *V. anguallanim* infection.

Declarations of competing interest

None.

CRediT authorship contribution statement

Qian Yang: Project administration, Methodology, Writing – original draft. **Xiao-Dong Yang:** Formal analysis, Project administration. **Meng-Qun Liu:** Project administration. **Chu Zeng:** Project administration. **Hong-Kui Zhao:** Project administration. **Kai-Wen Xiang:** Project administration. **Zhi-Shuai Hou:** Formal analysis, Writing – review & editing. **Hai-Shen Wen:** Project administration. **Ji-Fang Li:** Conceptualization, Supervision.

Data availability

Data will be made available on request.

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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.2023.108643.

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