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Serum biochemistry, liver histology and transcriptome profiling of bighead carp *Aristichthys nobilis* following different dietary protein levels

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ABSTRACT

Dietary protein plays a major role in determining the rate of fish growth and overall health. Given that the liver is an important organ for metabolism and detoxification, we hypothesized that optimal dietary protein levels may benefit liver function. Herein, we investigated the effects of dietary protein level on serum biochemistry, liver histology and transcriptome profiling of juvenile bighead carp *Aristichthys nobilis* fed for 8 weeks on a diet supplemented with high protein (HP, 40%), low protein (LP, 24%) or optimal protein (OP, 32%; controls). The results revealed a significant change in liver morphology in LP and HP groups compared with the OP group, coupled with increased serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity. RNA sequencing (RNA-Seq) analysis of the liver transcriptome yielded 47 million high-quality reads using an Illumina platform, which were *de novo* assembled into 80,777 unique transcript fragments (unigenes) with an average length of 1021 bp. Subsequent bioinformatics analysis identified 878 and 733 differentially expressed unigenes (DEGs) in liver in response to LP and HP diets, respectively. KEGG enrichment analysis of DEGs identified immune and metabolism-related pathways, including Toll-like receptor signaling, PI3K-Akt signaling, NF-κB signaling, complement and coagulation, peroxisome, nitrogen metabolism, PPAR signaling, and glycolysis and gluconeogenesis pathways. Transcriptome profiling results were validated by quantitative real-time PCR for 16 selected DEGs. The findings expand our understanding of the molecular mechanisms underlying the effects of dietary protein level on liver function in bighead carp.

1. Introduction

Protein, the most expensive nutrient in feeds for fish and other animals, is a major factor affecting growth performance [1]. Increasing dietary protein generally improves fish production, but excessive amounts are metabolized to provide energy, which increases nitrogenous waste and may impair growth [2]. Undesirable links between dietary protein level and growth performance have been observed in various fish species including *Mystus nemurus* [3], *Bidyanus bidyanus* [4], *Puntius gonionotus* [5], *Diplodus vulgaris* [6], *Takifugu rubripes* [7] *Ctenopharyngodon idella* [8] and *Epinephelus akaara* [9]. To date, few studies have explored the relationships between dietary protein level and the physiological responses of fish, including related signaling pathways.

RNA sequencing (RNA-Seq) is a practical and efficient method for identifying genes and complex molecular pathways involved in physiological functions, and this approach has been applied to nutrient-sensitive pathways in aquatic animals [10–14]. Although a complex correlation between dietary protein level and oxidative damage is well documented in vertebrates such as pig, rat and goose [15–17], information on nutritive regulatory mechanisms in filter-feeding fish remains scarce.

As a filter-feeding fish, bighead carp (*Aristichthys nobilis*) is one of the four most important freshwater aquaculture fish species in China, where production exceeded 3.48 million tons in 2016 [18]. China is the largest producer and consumer of *A. nobilis*, which is widely cultured due to its rapid growth, high tolerance to stress conditions, and low cultivation costs. In recent years, more and more farmers have begun high-density cultivation of bighead carp in ponds using artificial feed in China. However, the development of formulated feed for this species is still in its infancy, and effort toward developing a well-balanced, cost-effective dietary formula suitable for intensive aquaculture in China has been limited [19,20]. Although one previous study reported an optimal dietary protein level for *A. nobilis* fry of 30% [21], further research is

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Table 1
Formulation and chemical composition of experimental diets.

Ingredients	Dietary protein level (%)		
	24%	32%	40%
Red fish meal	12	27	42
Soybean meal	21	10	1
Corn gluten meal	5	11	16
Cottonseed meal	2	2	2
Wheat starch	27	27	27
Fish oil	2	2	2
Soybean oil	4	3	2
Rice bran	20.5	11.5	1.5
Vitamin premix[1]	1	1	1
Mineral premix[2]	2	2	2
Soybean phospholipids	1	1	1
Cellulose microcrystalline	0	0	0
Sodium carboxymethyl cellulose	0	0	0
Choline chloride	0.15	0.15	0.15
$Ca(H_2PO_4)$	2.3	2.3	2.3
Ethoxyquin	0.05	0.05	0.05
Compositions (% in dry matter)			
Crude protein	24.2	32.5	39.6
Crude lipid	7.0	6.9	6.9
Ash	9.87	12	12.2
Moisture	9.88	10.04	11.66
Gross energy (kJ/g)[3]	14.86	14.46	14.15

Note: 1. Vitamin mix (IU or mg kg^{-1} of die): Vitamin A, 900,000 IU; Vitamin D, 250,000 IU; Vitamin C, 5000 mg; Vitamin E, 4500 mg; Vitamin B_2, 1090 mg; Vitamin K_3, 220 mg; Vitamin B_{12}, 0.02 mg; Vitamin B_1, 320 mg; Vitamin B_6, 5000 mg; Pantothenate, 1000 mg; Biotin, 50 mg; Folic acid, 165 mg; Niacin acid, 2500 mg; Choline, 60,000 mg; 2. Mineral mix (g kg^{-1} of diet): $CuSO_4$•$5H_2O$, 2.5 g; $FeSO_4$•$7H_2O$, 28 g; $ZnSO_4$•$7H_2O$, 22 g; $MnSO_4$•$4H_2O$, 9 g; Na_2SeO_3, 0.0 45 g; KI, 0.026 g; $CoCl_2$•$6H_2O$, 0.1 g; 3. Calculated gross energy (16.7, 37.6 and 16.7 kJ/g for proteins, lipids and carbohydrates, respectively).

necessary to explore links between dietary protein level and liver function in *A. nobilis*.

Therefore, we conducted the present study to (1) evaluate the effects of dietary protein level on serum biochemical parameters in juvenile bighead carp; (2) analyze liver histology and transcriptome profiles of juveniles fed different dietary protein levels; (3) quantitatively explore the effects of dietary protein level and identify differentially expressed genes (DEGs) in the liver of juveniles. These findings expand our understanding of correlations between dietary protein and fish liver function.

2. Materials and methods

2.1. Experimental fish and feeding trials

Three isoenergetic experimental diets were formulated to contain 24, 32 and 40% crude protein, the ingredients of which are listed in Table 1. This equated to high protein (HP, 40%), low protein (LP, 24%), and optimal protein (OP, 32%; control) diets, respectively. *A. nobilis* juveniles were obtained from the Freshwater Fisheries Research Center of the Chinese Academy of Fishery Sciences, Yixing, China. Prior to beginning experiments, juveniles were acclimated to the experimental conditions and fed a commercial diet for 2 weeks. To initiate the trial, 15 fish of uniform size (initial body weight = 175.25 ± 10.33 g) were randomly distributed into 18 re-circulating fiberglass tanks (500 L) with equal supplemental aeration. Throughout experiments, water temperature was maintained at 25.5 ± 2.5 °C, dissolved oxygen was kept above 6 mg/L, ammonia-N was consistently below 0.05 mg/L, and pH varied between 7.8 and 8.0. Fish were hand-fed the experimental diet to apparent satiation over three equal feedings per day at 8:00, 12:00, and 16:00, according to the method described in our previous study [22].

2.2. Sample collection and chemical analysis

After exsanguination, livers were excised and stored at −80 °C, and blood samples were immediately collected from the caudal vein using heparinized syringes and stored at −80 °C to provide serum samples for analysis of aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (GLU) and total protein (TP) using an automatic biochemical analyzer (Mindray BS-400; Mindray Bio-Medical Electronics, Shenzhen, China).

2.3. RNA extraction, cDNA library construction and Illumina sequencing

2.3.1. Total liver tissue RNA from three biological replicates per condition

Total RNA from LP, OP and HP samples was extracted using TRIzol Reagent (Life Technologies, USA) according to the manufacturer's instructions, and possible RIN numbers were inspected for RNA integration using an Agilent Bioanalyzer 2100 (Agilent Technologies, USA). A total of 12 libraries were synthesized using a Genomic Sample Prep Kit (Illumina, USA) following the manufacturer's instructions. The cDNA library was subsequently sequenced on an Illumina HiSeq 2500 platform using the single-end paired-end approach in a single run by Biozeron Technologies Co. LTD, Shanghai, China.

2.4. De novo assembly and functional annotation

To exclude poor-quality reads prior to assembly, clean reads were *de novo* assembled using Trinity software without a reference genome [23]. Raw data have been submitted to the NCBI Short Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/Traces/sra/) under accession number SRP132686. For homology annotation, non-redundant sequences were searched against public databases including NCBI (http://www.ncbi.nlm.nih.gov/), non-redundant protein (Nr), and non-redundant nucleotide (Nt), Swiss-Prot (http://www.ebi.ac.uk/uniprot/), Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/), Gene Ontology (GO, http://www.geneontology.org/), and Eukaryotic Orthologous Groups (KOG, http://www.ncbi.nlm.nih.gov/kOG/), using the BLASTx algorithm (E-value < 10^{-5}).

2.5. Read mapping and analysis of differentially expressed genes (DEG)

Cleaned reads were mapped to the assembled reference transcriptome [24], and ~80% could be mapped to the corresponding reference for each sample (Table 1). RSEM was then used to quantify gene and isoform abundance based on the transcriptome assembled by Trinity. The edgeR program was then used to normalize expression levels for each sample and identify differentially expressed transcripts via pairwise comparisons [25]. GO term enrichment and KEGG pathway analysis of DEGs was performed using KOBAS [26].

2.6. Real-time fluorescence-based quantitative PCR (qRT-PCR) validation of RNA-Seq data

qRT-PCR was carried out to validate the expression of 18 randomly selected DEGs. Sequences of primers are listed in Table S1. For qRT-PCR, a Bio-Rad iCycler iQ5 Real Time System (Bio-Rad Inc., Berkeley, CA, USA) was employed with the β-actin gene as an internal control [27]. Each sample was analyzed in triplicate accompanied by the internal control gene. To confirm that only one PCR product was amplified and detected, dissociation curve analysis was performed at the end of each amplification, and relative mRNA expression levels were calculated according to the $2^{-\Delta\Delta CT}$ comparative CT method [28]. Pearson correlations between qPCR data (average expression levels for each experimental group) and Illumina data obtained by RNA-Seq were calculated for each candidate gene.

Table 2
Effects of dietary protein level on serum biochemical indices in *Aristichthys nobilis*.

Ingredients	Dietary protein level %		
	24%	32%	40%
GLU (mg/L)	1050.31 ± 0.30[a]	702.49 ± 1.12[b]	682.01 ± 0.18[b]
TP (g/L)	10.87 ± 0.11[a]	11.97 ± 0.08[a]	11.35 ± 0.06[a]
ALT (U/L)[a]	5.96 ± 0.88[a]	5.07 ± 0.58[a]	8.08 ± 0.89[b]
AST (U/L)[b]	31.96 ± 9.78[a]	25.60 ± 1.56[b]	32.32 ± 2.29[a]

Data are means of three replicate groups; values in the same row with different superscripts are significantly different ($p < 0.05$).

[a] ALT, alanine aminotransferase, units per litre (U/L). One unit is the amount of enzyme oxidizing 1 μmol/L of NADH per min.

[b] AST, aspartate aminotransferase, units per litre (U/L). One unit is the amount of enzyme oxidizing 1 μmol/L of NADH per min.

2.7. Histopathology

Herein, nine fish were used to investigate histopathological effects in liver following consumption of different dietary protein levels as previously described [29]. Sections (5 μm thickness) were stained with hematoxylin and eosin, and examined by light microscopy.

3. Results

3.1. Physiobiochemical responses

No significant differences in serum total protein concentration were observed among fish groups ($p > 0.05$; Table 2), but serum GLU levels were significantly decreased with increasing dietary protein up to the OP diet, then decreased thereafter ($p < 0.05$). Additionally, the lowest serum AST and ALT concentrations were observed in the OP group ($p < 0.05$), and hepatocytes from the OP group displayed normal histology (Fig. 1). By contrast, hepatic tissue in the LP group was disordered, cell outlines were indistinguishable, and some nuclei were fractured and/or completely absent. The HP group also exhibited these phenotypes, along with fat droplets in cells.

3.2. Transcriptome sequencing and unigene analysis

In total, 43,970,983, 44,887,579 and 48,832,224 clean reads were obtained for LP, OP and HP groups, respectively (Table S2). *De novo* assembly yielded 80,777 unique transcript fragments (unigenes) with an average length of 1021 bp and an N50 value of 1323 bp (Table S3). Length distributions of unigenes are shown in Fig. S1. Among the 80,777 unigenes, 34,243 (51.5%) were successfully annotated by searching with BLASTx, BLASTn against Nr, KOG, KEGG, and GO public databases (Fig. S2).

3.3. Functional annotation and classification of unigenes

All identified carp liver unigenes were used for functional enrichment and classifications analyses. Clusters of Orthologous Groups (COG) is a classification system based on orthologous genes (genes sharing the same function and a common ancestor). Herein, 11,118 (13.76%) unigenes were annotated by KOG, the eukaryote-specific version of COG, and grouped into 25 KOG categories (Fig. S3). General function prediction only was the largest cluster, followed by signal transduction mechanisms and transcription, posttranslational modification, protein turnover, and chaperones. Subsequent searching against the GO database resulted in 20,441 (65.37%) unigenes annotated to 51 GO terms (Fig. 2). Binding (6155 unigenes) and catalytic activity (4356 unigenes) were the dominant terms in the molecular function category, while cell part (5768 unigenes), cell (5790 unigenes), organelle (4030 unigenes) and membrane part (2387 unigenes) were the major terms in the cellular component category. Cellular process (7019 unigenes), single-organism process (6247 unigenes) and metabolic process (5699 unigenes) were the most abundant terms in the biological process category.

3.4. Identification and functional analysis of differentially expressed genes

Based on the criteria $|\log(\text{fold change})| \geq 1$ and false discovery rate (FDR) ≤ 0.05, significantly altered genes were analyzed using MA and Volcano plots. Under these criteria, 878 and 733 DEGs were identified in the two pairwise comparisons (Fig. 3). As shown in Fig. 4, KEGG pathway enrichment analysis of the identified DEGs revealed strong links to protein metabolism and immunity response-related pathways following consumption of feed containing different levels of dietary protein. The most closely linked metabolic pathways included glycolysis/gluconeogenesis (15 unigenes, 8.7% of all unigenes with KEGG annotation in this pathway), followed by pyruvate metabolism (10 unigenes, 10.3%), the citrate cycle (eight unigenes, 12.5%), and nitrogen metabolism (four unigenes, 13.8%). Some pathways associated with the immune system were identified, including cell adhesion molecules (17 unigenes, 5.8%), the PI3K-Akt signaling pathway (16 unigenes, 2.31%), complement and coagulation cascades (10 unigenes, 8.40%), the Toll-like receptor signaling pathway (eight unigenes, 3.8%) and the NF-κB signaling pathway (six unigenes, 13.0%; Table S4). Many genes associated with the biosynthesis of immunity-related signaling pathways were detected in the LP vs. OP and HP vs. OP comparisons, including immune responses (lysozyme, immunoglobulin, alkaline phosphatase, etc.), inflammatory reactions (nuclear factor kappa B,

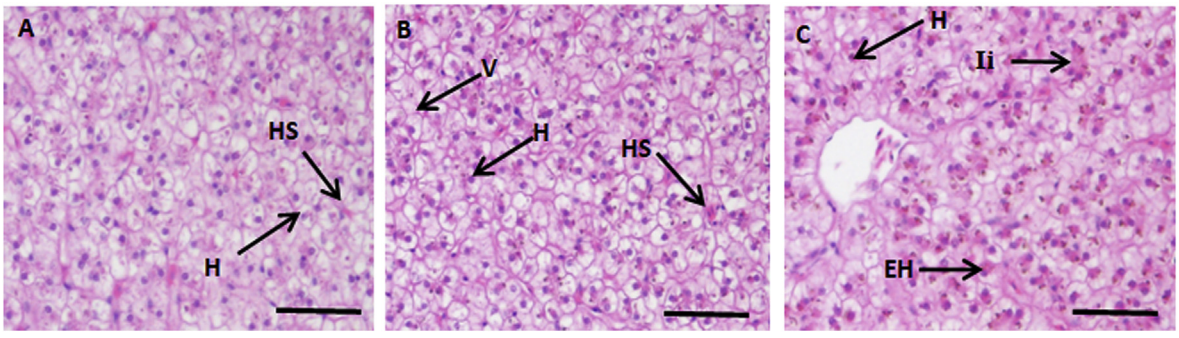

Fig. 1. Histological analysis of liver sections from bighead carp (bighead carp *Aristichthys nobilis*) fed different amounts of dietary protein. (A) Sections from the optimal protein (OP) group reveal normal-shaped hepatocytes with regular gross morphology and clearly located cell nuclei. (B) Hepatocyte vacuolization is visible in the low protein (LP) group. (C) The high protein (HP) group exhibit enlarged hepatocytes and apparent hepatic steatosis with lipid droplets apparent. Staining was performed with hematoxylin and eosin (H&E). Scale bar = 75 μm. H, hepatocyte; V, vacuolization; EH, enlarged hepatocyte; Ii, inflammatory cell infiltration.

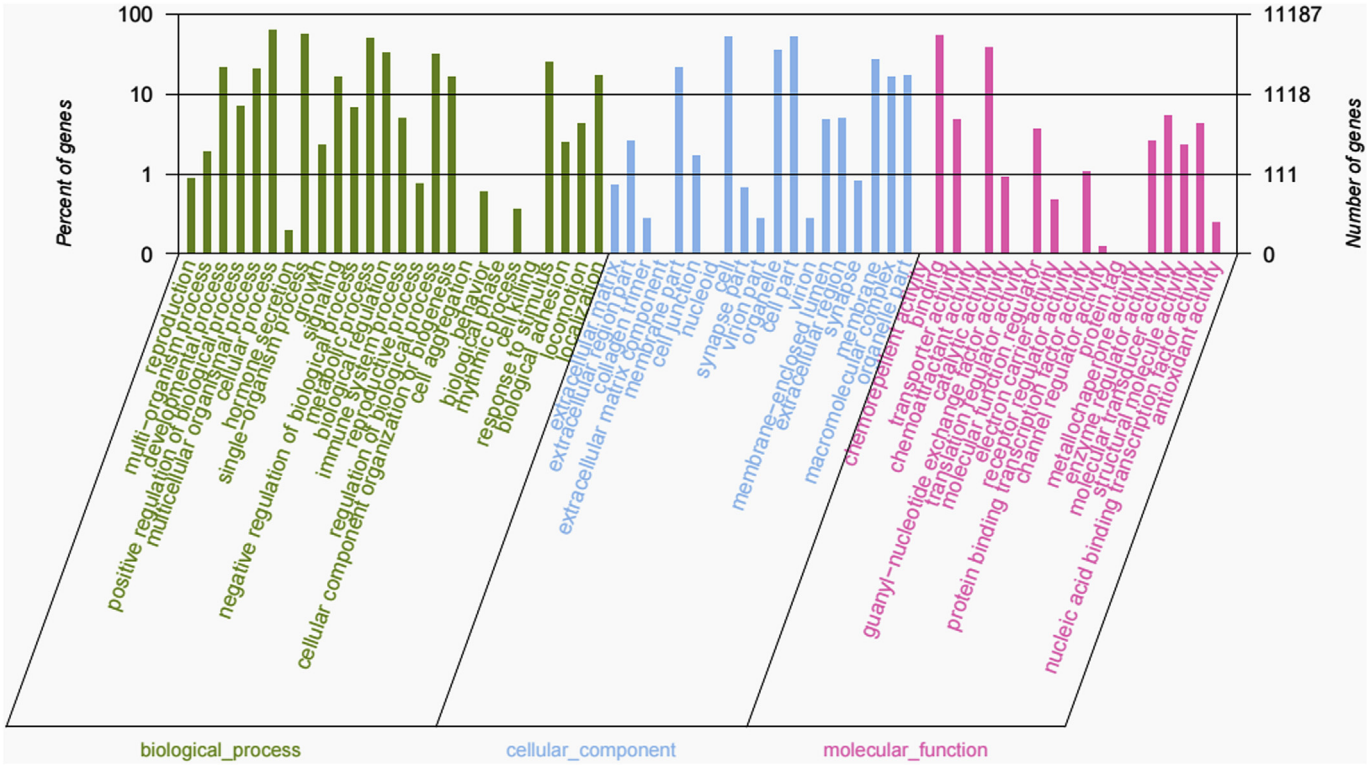

Fig. 2. Gene Ontology (GO) classification of *A. nobilis* liver unigenes.

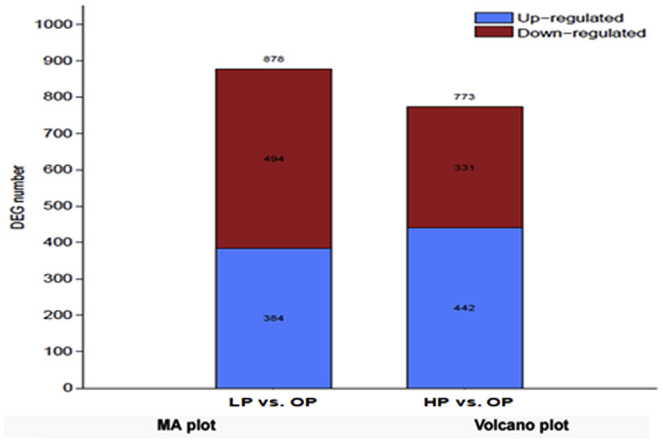

Fig. 3. Differentially expressed genes (DEGs) identified in low protein vs. optimal protein and high protein vs. optimal protein comparisons.

interleukin-8, interleukin-10, etc.) and antioxidant ability (catalase, glutathione S-transferase, glutathione peroxidase, etc.). The discovery of immune-related pathways and unigenes could provide a theoretical basis for understanding the molecular mechanisms of dietary protein regulation of liver function.

3.5. Validation of RNA-Seq results by qRT-PCR

qRT-PCR was performed on 16 selected genes identified in the LP vs. OP and HP vs. OP comparisons, including toll-like receptor 4 (TLR4), glucokinase (GK), pyruvate kinase (PK), MHC class I antigen (MHCI), lysozyme (LZM), immunoglobulin heavy chain (IgM), alkaline phosphatase (AKP), cAMP-dependent protein kinase catalytic subunit alpha (AMPKα), catalase (CAT), glutathione-S− transferase (GST), glutathione peroxidase (GPX), nuclear factor-kappa B p65 (NF-κB), interleukin 10 (IL-10), IL-6, IL-8, and heat stress protein 90β (Hsp90β). The expression patterns revealed by qPCR analysis were similar to those revealed by RNA-Seq for the same genes (Fig. 5), confirming the accuracy and reliability of the procedure.

4. Discussion

Bighead carp is primarily believed to be a filter feeder that consumes zooplankton and phytoplankton. Recently, our research group showed that this species also consumes small pieces of artificial feed, depending on the diameter of branchial rakers (Fig. S4). The health status of fish liver tissue is closely correlated with detoxification and biosynthetic proteins, and histological changes in liver are considered a useful indicator for evaluating nutritional condition [30,31]. Thus, investigated the effects of dietary protein level on liver histology in juvenile bighead carp, and found that consumption of the HP diet caused abnormalities, including inflammatory cell infiltration. Serum biochemical parameters are often measured to investigate overall health and nutritional metabolism, and this approach has been applied to fish fed different dietary supplements [32–34]. In the present study, a significant increase in serum AST and ALT activities indicated hepatic damage following consumption of the HP diet, consistent with the observed histopathological damage in liver sections. One reasonable explanation is that AST and ALT may be released into plasma following tissue damage and dysfunction [35].

Evidence suggests that dietary exogenous protein not only reduces the growth performance, but is also detrimental to proper liver function. We therefore investigated the effects of dietary protein level on gene expression in the liver of juvenile bighead carp. Transcriptomic analysis using Illumina RNA-Seq data yielded 52 million raw reads, 45 million clean reads, and ultimately 80,777 unigenes. To identify dysregulated genes and pathways, GO and KEGG analyses were performed. A subset of enriched DEGs in the LP vs. OP comparison were found to be associated with immune responses and glycolipid metabolic processes. Interestingly, a subset of enriched DEGs in the HP vs. OP comparison indicated that consumption of the HP diet activated liver inflammation

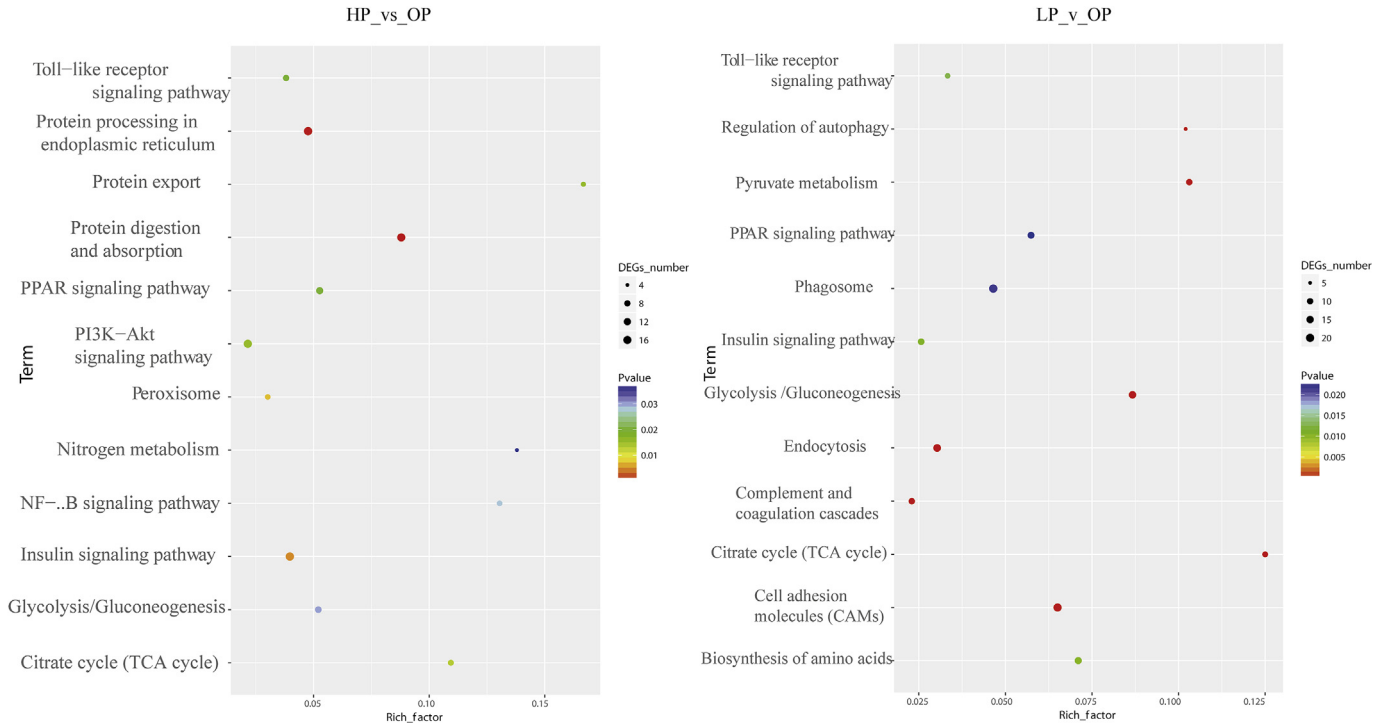

Fig. 4. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification of DEGs. Significant KEGG pathway classifications (corrected *p*-value < 0.05) of DEGs are shown for (A) LP vs. OP and (B) HP vs. OP comparisons.

responses and antioxidant signaling pathways, triggering abnormal metabolism.

NF-kB is crucial for mediating expression of inflammatory cytokine genes in humans [36]. Surprisingly, we found that the HP diet up-regulated the pro-inflammatory cytokines NFκB and IL-8 in bighead carp, but down-regulated the anti-inflammatory cytokines IL-10 and IL-6, potentially inducing inflammation [37,38]. These results imply that an optimal level of dietary protein could attenuate liver inflammation in fish. NF-kB can be activated via two mechanisms; the classical NF-kB pathway (by NF-kB P65), and the alternative pathway (by NF-kB P52). In the present study, the OP diet down-regulated mRNA levels of NF-kB P65 (rather than NF-kB P52) juvenile bighead carp liver tissue, indicating that dietary protein level may at least partially regulate inflammation through the classical NF-kB P65 pathway, consistent with a previous study on *Pelteobagrus fulvidraco* [39] and *C. idella* [40,41]. Additionally, IL-15 was overexpressed in liver in the HP group, which could promote inflammatory responses since this protein is critical for the functions of T cells and the trafficking of memory $CD8^+$ T cells [42,43]. Dietary protein level also had a significant effect on IL-6 transcription in the liver of carp. Interestingly, NF-kB P52 regulates IL-6 expression in mice [44], suggesting a conserved mechanism in fish and mammals.

In our previous study, an optimal dietary protein level decreased the production of reactive oxygen species (ROS) and malondialdehyde in the liver of bighead carp (unpublished), suggesting that an OP diet may protect against oxidative damage in the liver. The antioxidant ability of fish is dependent on both enzymatic and non-enzymatic mechanisms [45]. In the present study, the OP diet up-regulated the mRNA levels of antioxidant enzymes including catalase, glutathione-S-transferase, and glutathione peroxidase in the liver of bighead carp, compared with the HP diet. It is known that antioxidant enzyme activities are tightly correlated with their mRNA levels in fish [46], suggesting that an OP diet can enhance the liver antioxidant capacity.

Previous studies demonstrated that consumption of low dietary protein can suppress immune responses and disease resistance in fish [47,48]. In the present study, the LP diet had a significant effect on toll-like receptor (TLR) signaling pathways related to immunity. TLRs play crucial roles, and 12 TLRs recognizing specific microbial components have been identified [49,50]. Interestingly, only TLR4 functions in both MyD88-dependent and Trif-dependent pathways and thereby helps to achieve optimal innate immune responses [51]. In the present study, TLR4 expression was significantly decreased following consumption of the LP diet, which might decrease virus recognition and suppress other immune responses. Dietary long-chain (n = 3) polyunsaturated fatty acids (PUFAs) can alleviate *Escherichia coli* lipopolysaccharide (LPS)-induced tissue injury via modulation of TLR signaling pathways in animals [52–54], consistent with the link between TLR signaling and dietary FA composition in our present work. In addition, lysozyme (LZM), secreted immunoglobulin (IgM) and alkaline phosphatase (AKP) were significantly down-regulated in the LP group, suggesting that low dietary protein may decrease the production of antibacterial compounds.

Herein, serum glucose was elevated following consumption of the LP diet, which had a high carbohydrate content. Glucose is completely catabolized by glycolysis, the citrate cycle, and the respiratory chain during ATP production, and excess glucose can be stored as glycogen or converted into lipids [55]. Our results revealed higher expression of hepatic glucokinase in bighead carp fed an LP diet, similar to an earlier study in Gilthead sea bream fed a high carbohydrate/low protein diet [56,57]. We also observed a significant increase in the expression of peroxisome proliferator receptors (PPARs) in the LP group, suggesting that lipid accumulation occurred due to consuming higher levels of carbohydrate, consistent with previous research on tilapia (*Oreochromis niloticus*) [58].

5. Conclusions

In summary (Fig. 6), we found that consumption of an optimal amount of dietary protein improved liver immune responses (LZM, IgM, AKP, etc.) in juvenile bighead carp compared with low and high protein groups. Transcriptome analysis identified DEGs in the liver of juvenile bighead carp in response to different dietary protein levels, and

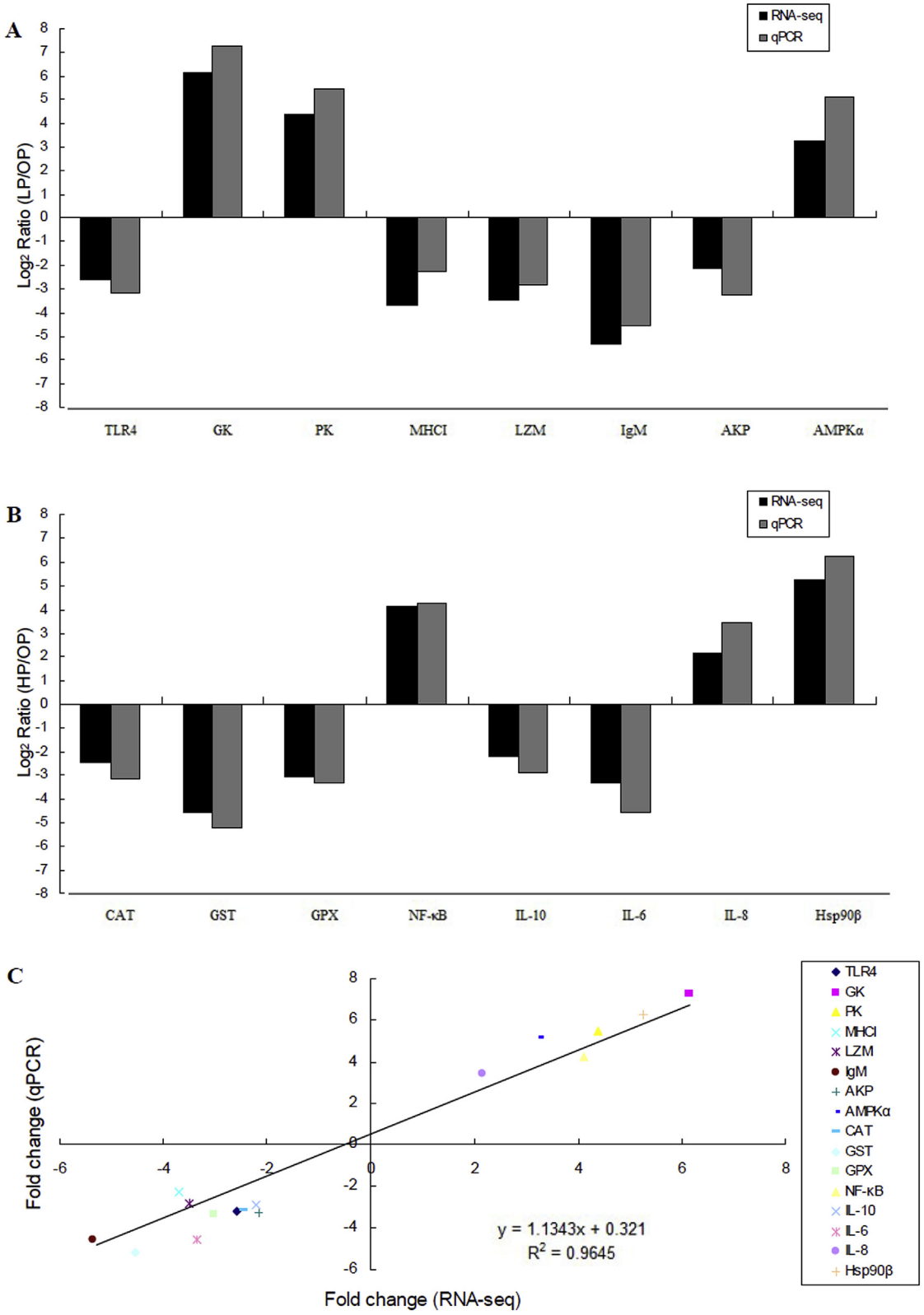

Fig. 5. Comparison of gene expression patterns obtained using RNA-Seq and qRT-PCR. Log (fold change) values are expressed as gene expression ratios after normalisation against the β-actin internal reference gene. (A) *A. nobilis* juveniles fed an LP vs. OP diet, and (B) an HP vs. OP diet. (C) Relationship between relative changes in gene expression measured by qPCR and RNA-seq.

bioinformatics analysis linked many DEGs to immune responses, inflammatory responses and energy metabolism. Moreover, abnormal serum biochemical indices were apparent in the HP group, suggesting that consuming large quantities of protein in excess of requirements may aggravate liver metabolic burden. These findings expand our understanding of protein utilization mechanisms in bighead carp.

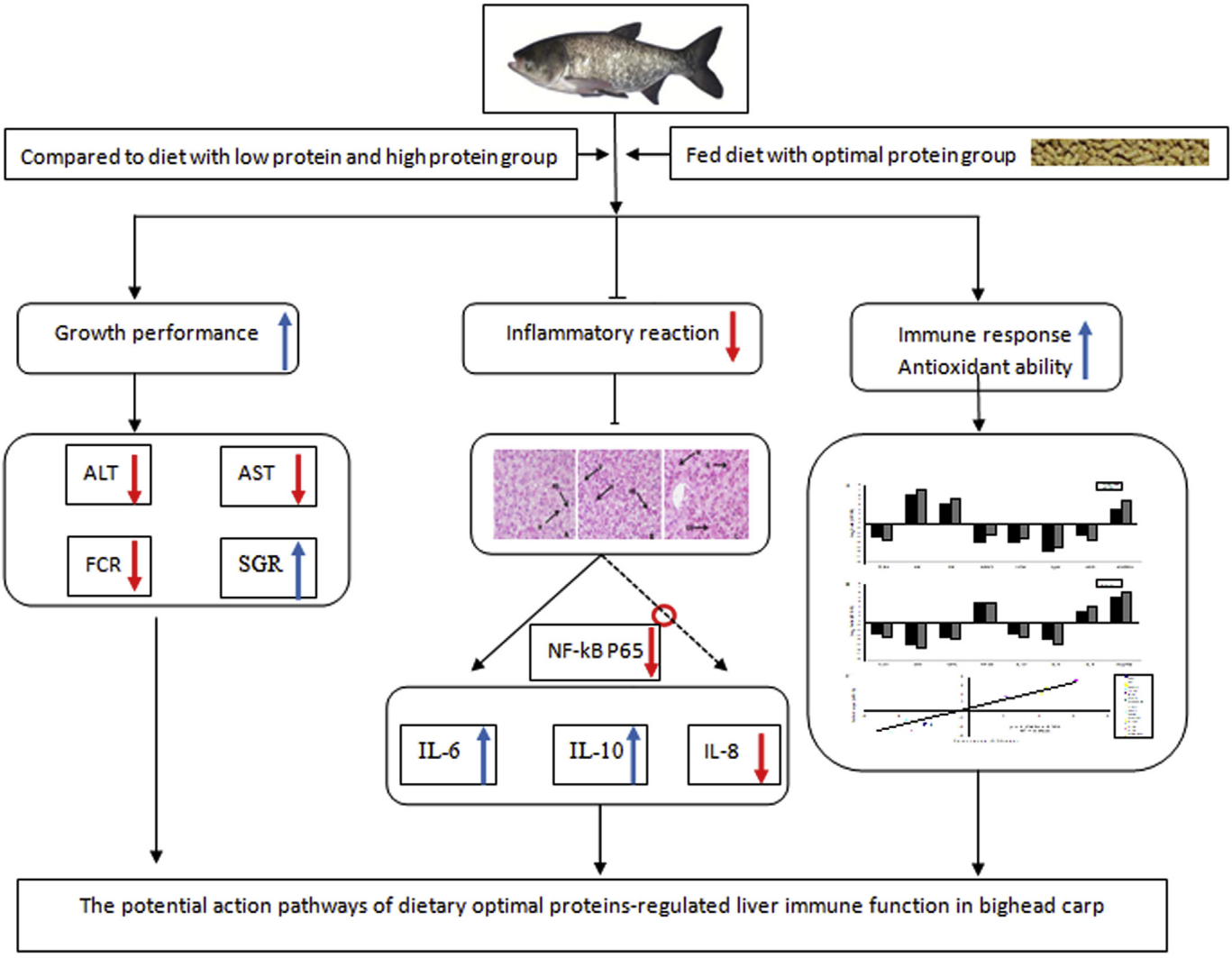

Fig. 6. Potential dietary protein-regulated pathways related to liver immune functions in juvenile bighead carp. Blue: up-regulated; Red: down-regulated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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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.2018.12.028.

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