Fish and Shellfish Immunology 95 (2019) 314–327

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Antioxidant system of soiny mullet (*Liza haematocheila*) is responsive to dietary poly-β-hydroxybutyrate (PHB) supplementation based on immune-related enzyme activity and de novo transcriptome analysis

Guo Qiao, Qirui Sun, Mingming Zhang, Chen Xu, Tingli Lv, Zhitao Qi, Wenping Yang, Qiang Li*

Department of Marine Technology, School of Marine and Biological Engineering, Yancheng Institute of Technology, Yancheng, 224051, Province Jiangsu, China

ARTICLE INFO

Keywords:
Poly-β-hydroxybutyrate (PHB)
Liza haematocheila
Transcriptome analysis
Antioxidant system

ABSTRACT

As a dietary supplement, poly-β-hydroxybutyrate (PHB) has been reported to positively influence growth, boost the immune system and enhance disease resistance in fish and shellfish. However, the protective mechanism is little known. Thus, the present study was conducted to evaluate the effect of PHB supplementation on immune-related enzyme activity and transcriptome-based gene expression in soiny mullet (*Liza haematocheila*). Results showed that dietary PHB supplementation could increase antioxidant enzyme activity, including total antioxidant capacity, catalase and superoxide dismutase. A total of 7,082,094,175 and 7,650,341,357 raw reads with mean length of 757 bp were obtained from control and PHB (dietary PHB supplementation at 2%) groups, respectively. There were 46,106 differentially expressed genes (DEGs) between control and PHB groups, including 21,828 upregulated and 24,278 downregulated DEGs. All the DEGs were classified into three gene ontology categories, and 312 DEGs related with immune system process and 760 with the response to a stimulus. Additionally, all DEGs were allocated to 261 Kyoto Encyclopedia of Gene and Genome pathways, and major immune-related pathways were detected, including MAPK/PI3K-Akt/TNF/NF-κB/TCR/TLR signaling pathways. Moreover, the regulation of several observed immune-related genes was confirmed by qRT-PCR. Altogether, this study suggests that antioxidant system is more effective for dietary PHB supplementation and lays the foundation for further study on the precise immunostimulatory mechanism of PHB. Hopefully, it provides insights into exploring biomarker for assessment of immunostimulants in fish culture.

1. Introduction

Short-chain fatty acids (SCFA) are end-products of bacterial fermentation processes of nutrition, which known as a biological and eco-friendly approach of disease control in aquaculture [1–3]. However, SCFA cannot be uptaken efficiently by aquatic animals since they are highly soluble in water, and are not suitable to apply directly in aquaculture [4]. Poly-β-hydroxybutyrate (PHB) belongs to SCFA, but it is insoluble in water, and thus increases its uptake efficiency and application in aquaculture. PHB is accumulated by a wide variety of bacteria such as *Bacillus* spp. as an intracellular energy and carbon storage compound under conditions of nitrogen depletion and carbon excess [1,5,6]. PHB is known to have a wide range of applications in tissue engineering, pharmaceuticals, commodity packaging, agriculture and wastewater treatment due to some of its excellent characteristics, such as non-toxic, biocompatibility, hydrophobicity, low oxygen permeability and biodegradable [3]. As a feed supplementation in agriculture, PHB is biologically degraded into antibacterial SCFA β-

---

Abbreviations: PHB, poly-β-hydroxybutyrate; TLRs, Toll-like receptors; HSP70, heat shock protein 70; MHCs, myosin heavy chains; pbpA, penicillin-binding protein A; AOX, aldehyde oxidase; IL-8, interleukin-8; TGICL, TIGR Gene indices clustering tools; CDS, coding sequence; EGFR, epidermal growth factor receptor; FOS, proto-oncogene protein c-fos; MAPK, mitogen-activated protein kinase 8; TLR1, toll-like receptor 1; TLR5, toll-like receptor 5; IL12, interleukin-12; MAPKs, mitogen-activated protein kinase; SOS, son of sevenless; NF-κB, nuclear factor NF-kappa-B p105 subunit; TNFSF6, tumor necrosis factor ligand superfamily member 6; IL12A, interleukin 12A; TLR6, toll-like receptor 6; NF-κBIA, NF-kappa-B inhibitor alpha; PI3K-Akt, phosphatidylinositol 3-kinase/Protein Kinase-B; mTOR, mechanistic target of rapamycin; VWF, von Willebrand factor; ITGA2B, integrin alpha 2B; PTK2 (FAK), focal adhesion kinase 2; PKB/Akt, protein kinase B; I-κB, IkappaB; TNF, tumor necrosis factor; TAK, TAK1-binding protein 3; CEBPB, enhancer binding protein; PTGS, prostaglandin-endoperoxide synthase; RELA, transcription factor p65; TCR, T cell receptor; TIR, toll-interleukin 1 receptor; TLR14, toll-like receptor 14; TIRAP, toll-interleukin 1 receptor domain-containing; DEGs, differentially expressed genes; T-AOC, total antioxidant capacity; AKP, alkaline phosphatase; ACP, acyl carrier protein; SOD, superoxide dismutase

* Corresponding author.
E-mail address: fish790608@163.com (Q. Li).

https://doi.org/10.1016/j.fsi.2019.10.042
Received 15 July 2019; Received in revised form 20 October 2019; Accepted 22 October 2019
Available online 24 October 2019
1050-4648/ © 2019 Elsevier Ltd. All rights reserved.

hydroxybutyric acid or PHB oligomers by bacterial PHB depolymerases and/or digestive enzymes during gastrointestinal passage of aquatic animals [1,7]. The degradation products could slightly decrease intestinal pH environment for benefiting the growth of probiotics, and enhancing digestion and absorption of cultured species [5]. It was reported that dietary PHB has positive effects on the growth, survival and disease resistance of aquatic animals [4,7–15]. Studies demonstrated that PHB has immunostimulatory efficacy in some aquatic animals, such as soiny mullet (*Liza haematocheila*) [16], tilapia (*Oreochromis mossambicus*) [1], giant river water prawn (*Macrobrachium rosenbergii*) [10] and Pacific white shrimp (*Litopenaeus vannamei*) [17]. Dietary PHB supplementation could increase the immune-related activity of enzymes such as T-AOC, AKP, ACP, SOD, lysozyme in shrimp and mollusks, and up-regulated some immune-related gene expression such as TLRs, HSP70, hepcidin, MHCs, pbpA, AOX, IL-8 in fish [1,16,17]. The information on immunostimulatory mechanism is still limited in aquatic animals [1,10,16,17], so more studies on PHB application in aquaculture need to be performed after considering the effect of PHB on growth performance, immunity and disease resistance.

L. haematocheila has become an economic aquaculture mugilid species in China and other Asian countries due to its rapid growth, strong resistance to disease and improving water quality [18,19]. *L. haematocheila* can also serve as an ideal experimental model species or sensitive bioindicator in environmental biology and ecology studies since it possesses extreme environmental tolerance and is very common in coastal, short-distance migration as a native species [18]. Our previous study demonstrated that diet supplemented with 2% PHB could increase the growth performance, higher antioxidant enzyme activity and immune-related gene expression of *L. haematocheila* [16]. Recently, transcriptome analysis has become a useful tool for understanding the underlying pathways and mechanisms of the host in response to stimulation of immunostimulants [20]. The high-throughput transcriptome sequencing can provide accurate, digital gene expression profiles of sequenced transcripts for a model or non-model organism, particularly for the non-model organism which lacking reference genomes [21]. Thus, combined with enzymatical immune system, comparative transcriptome between fish fed with PHB (2%) supplementated diet and control without PHB supplementation was sequenced and assembled using Illumina sequencing technology. The differentially expressed genes (DEGs) with a focus on immune-related genes were analyzed to explore immunostimulatory mechanism of PHB. It was the first attempt to explore the molecular mechanism of PHB's immune regulation on *L. haematocheila*. This study will increase our understanding on the immunostimulatory mechanisms of PHB, and provide valuable information for fish disease prevention.

2. Materials and methods

2.1. Diet preparation

PHB was obtained from Ningbo Tianan Biological Material Co., Ltd, China. Wheat flour, corn starch, fish meal, soybean meal, rapeseed meal, fish oil and soybean oil were provided by Hengxing Feed Co. Ltd., Yancheng, China. Two experimental diets (0% and 2% PHB supplementation) were prepared as previously described [16], and the composition of diets is shown in Table 1. Briefly, all ingredients were ground into powder, sieved through 80-mesh, blended in a mixer thoroughly and mixed fully with fish oil and bean oil. A proper amount of water was added to ensure the uniformity of granulation, and granulated feeds (diameter of 2 mm) were wet-extruded by a pelletizer (F-26, South China University of Technology, Guangzhou, China) and then dried at 60 °C for 12 h. Then, the diets were kept at room temperature and air dried completely under ventilation condition. All diets were sealed in sample bags and stored at −20 °C until use.

Table 1
Formulation and approximate composition ($g \cdot kg^{-1}$) of experimental diets.

Items	Groups		Items	Groups	
	Control	PHB		Control	PHB
Peruvian fish meal[a]	120	120	Bean oil[a]	40	40
Soybean meal[a]	280	280	$Ca(H_2PO_4)_2$[a]	20	20
Rapeseed meal[a]	140	140	MVP[a,d]	20	20
Wheat flour[a]	170	170	Carboxymethy cellulose[b]	120	100
Corn starch[a]	50	50	PHB[c]	0	20
Fish oil[a]	40	40			
Total quantity	1000	1000			

[a] Provided by Hengxing Feed Co. Ltd., Yancheng, China.
[b] Purchased from Shanghai Jiande Industrial Co. Ltd., China.
[c] Purchased from Ningbo Tianan Biological Material Co., Ltd., China.
[d] MVP, mineral (mg or IU/kg feed) and vitamin premixes (mg or IU/kg feed): magnesium sulfate, 5500 IU; cobalt chloride, 4 g; manganese sulfate, 3 g; aluminum chloride, 8 g; potassium iodide, 7 g; zinc sulfate, 140 g; ferric citrate, 150 g; sodium selenite, 0.6 g; calcium dihydrogen phosphate, 15,000 IU; KCl, 4000 IU; copper sulfate, 8 g; biotin, 5 mg; inositol, 200 mg; vitamin B1, 50 mg; vitamin B2, 20 mg; vitamin B6, 50 mg; vitamin B12, 0.1 mg; niacin, 250 IU; pantothenic acid, 50 mg; folic acid, 15 mg; vitamin A, 5000 IU; vitamin C, 400 mg; vitamin D3, 2000 IU; vitamin E, 400 mg; vitamin K3, 40 mg.

2.2. Ethics statement

This study was carried out in accordance with the recommendation from the Management and Use of Laboratory Animals of Jiangsu Province. The study did not involve the endangered or protected species.

2.3. Experimental fish and feeding trial

Normal *L. haematocheila* (7.70 ± 0.85 g) was collected from a local hatchery at Yancheng City, Jiangsu province. Fish were acclimated in an indoor cement tank (3.0 m × 1.0 m × 0.8 m, L × W × H) at a water temperature of 24–26 °C, pH 6.8–7.3, dissolved oxygen (DO) greater than $5\ mg\ L^{-1}$, NH_4^+-N less than $0.1\ mg\ L^{-1}$ and NO_2^--N less than $0.1\ mg\ L^{-1}$ for two weeks prior to the experiment. During this period, fish were fed with commercial diets (Tongwei Feeding Company, China) three times (7:00 to 7:30, 14:00 to 14:30, and 19:30 to 20:00) daily at 3% of fish body weight under a 12L/12D light cycle. 30% of the tank water was exchanged daily.

After acclimatization, similar-sized fish were randomly divided into two groups, including control group without any PHB supplementation (0%) and PHB group with 2% supplementation. The fish were placed in 480 L recirculation tanks under a 12D/12L light cycle at 24 °C. There were 30 individuals each tank which was covered with a plastic mesh lid to prevent fish from jumping out of the tank. Each trial was conducted in quadruple. The fish were hand-fed with experimental diets to satiation three times per day for 4 weeks. All tanks were supplied with identical supplemental aeration and water flow (approximately 3.2 L/min). The other rearing conditions throughout the experimental period were similar to the acclimation conditions, including water temperature, pH, DO, NH_4^+-N and NO_2^--N concentration.

2.4. Sample collection and processing

At the end of the experiment, the fish were starved for 24 h and anesthetized with tricaine methanesulfonate (MS222) at $200\ mg\ L^{-1}$ before handling. The survival was expressed as the percentage of live fish at the end of experiment relative to the total initially stocked fish. Ten fish from each tank were sampled to calculate weight gain (WG) and specific growth rate (SGR):

$$WG(\%) = (W_t) - (W_i)/(W_i) \times 100.$$

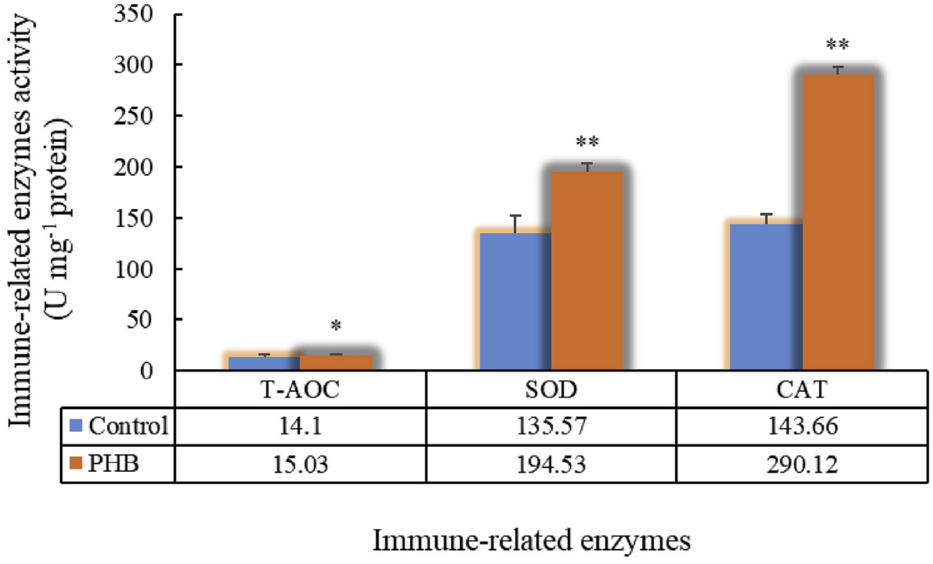

Fig. 1. Immune-related enzyme activity in serum of soiny mullet (*Liza haematocheila*) fed with 2% poly-β-hydroxybutyrate (PHB) supplementation diets for 30 days. Three antioxidant enzymes were analyzed, including catalase (CAT), superoxide dismutase (SOD) and total antioxidant capacity (T-AOC). The data represent the mean ± standard deviation from triplicated tanks and were analyzed by *t*-test. Asterisk means significant difference between control and PHB groups. *, $P < 0.05$; **, $P < 0.01$.

Table 2
Statistics of transcriptomic sequences from six libraries.

Groups	Total raw reads	Total clean reads	Clean reads ration (%)	Q20 (%)	Q30 (%)
Control-1	8,243,423,100	7,942,464,024	96.35	98.26	94.59
Control-2	6,834,243,000	6,498,124,726	95.08	98.27	94.65
Control-3	7,115,705,400	6,805,693,774	95.64	98.28	94.69
Mean	7,397,790,500	7,082,094,175	95.96	98.27	94.64
SE	745,738,655	760,806,735	0.64	0.01	0.05
PHB-1	7,623,321,300	7,254,682,508	95.16	98.29	94.72
PHB-2	8,077,702,200	7,801,207,776	96.58	98.31	94.69
PHB-3	8,211,849,600	7,895,133,788	96.14	98.28	94.67
Mean	7,970,957,700	7,650,341,357	95.96	98.29	94.69
SE	308,443,157	345,853,969	0.73	0.02	0.03

$$\mathrm{SGR}(\%\ \mathrm{day}^{-1}) = 100[1\mathrm{n}\ W_t - 1\mathrm{n}\ W_i]/t$$

$$\mathrm{Survival}(\%) = \text{ final number of fish/initial number of fish} \times 100$$

where, W_t represents the final body weight (g) at feeding time t (days), W_i is the initial body weight (g).

Then, fish blood and spleen were collected from another five fish in each tank and pooled as one analytical sample to evaluate the effect of PHB supplementation on immune responses of *L. haematocheila*. All samples were stored at −80 °C until use, and no samples were frozen more than once.

2.5. Determination of immune-related enzymes

Immune-related enzymes, including SOD, CAT and T-AOC in the serum, were analyzed by assay kits (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturers' instruction and read on a spectrophotometer (Bio-RAD, USA) [17,22,23]. In brief, SOD was determined with hydroxylamine method for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The inhibition activity of SOD was determined by measuring adsorption at 450 nm [22]. In CAT assay, CAT first decompose hydrogen peroxide (H_2O_2) into water and oxygen, and then the unconverted H_2O_2 formed a complex with ammonium molybdate to produce a light yellow solution. CAT activity was determined by measuring absorption at 405 nm [23]. One unit of T-AOC activity was defined as the amount of enzyme per mg tissue protein every minute to increase 0.01 absorbance at 37 °C [17]. All enzyme activities were defined as the amount of enzyme unit per mg protein.

Table 3
Statistics of unigenes assembled from six libraries.

Total unigenes number	223,055
Total unigenes length (bp)	168,717,141
Average unigenes length (bp)	757
Maximum unigene length (bp)	19,986
Minimum unigene length (bp)	201
N50	1225
N90	301
GC percentage (%)	43.92

2.6. Total RNA extraction and cDNA library construction

Total RNA was extracted from spleen samples using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions and treated with RNase free DNase I (Qiagen, Valencia, CA, USA). The purity of RNA was examined by NanoDrop™ and quality was assessed with Agilent RNA 6000 Nano Kit on Agilent 2100 Bio-analyzer (Agilent, USA). Only high quality ($OD_{260}/OD_{280} > 1.9$) of RNA was used for further cDNA library construction. For each group, equal amounts of RNA from three *L. haematocheila* individuals per tank were pooled as one spleen sample and three samples per group were collected to provide templates for de novo transcriptome assembly. Same RNA samples were used in quantitative RT-PCR for the gene expression, and transcriptome analysis.

Illumina sequencing was performed at the Nanjing Aurora Gene Technology Co., Ltd. (Nanjing, China; http://jiguanggene.bioon.com.cn). Total RNA was digested by DNase I (NEB, USA), purified by Dynabeads™ mRNA purification kit (Invitrogen, USA), and then the poly (A)-containing mRNA was enriched with fragment buffer (Ambion, USA). The fragmented RNA was transcribed into the first strand cDNA using N6 primers and SuperScript II reverse transcriptase (Invitrogen, USA), followed by synthesis of the second strand cDNA with second strand master mix (Invitrogen, USA). Then, the double-stranded cDNA was subjected to end repair, A-tailing and adapter ligation. The suitable adapter-ligated fragments were enriched by PCR amplification with PCR primer cocktail and PCR master mix (Thermo Fisher Scientific, USA). PCR products were purified using 2% agarose gel electrophoresis and quantified by Qubit (Invitrogen, USA). The cDNA libraries were prepared and subsequently sequenced using an Illumina HiSeq™ 3000 platform.

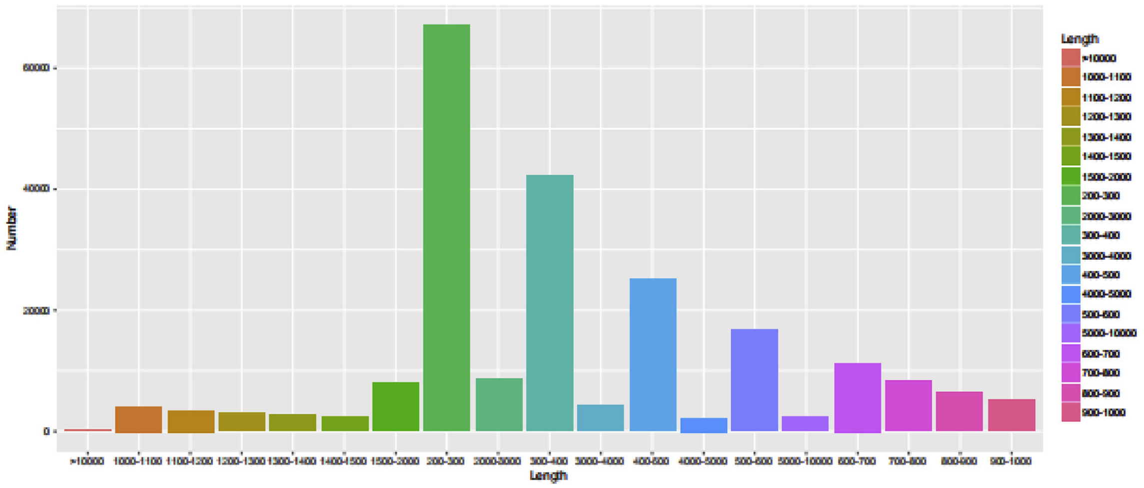

Fig. 2. Length distribution of all unigenes assembled from six libraries.

Table 4
Statistics of annotation results for soiny mullet (*Liza haematocheila*) spleen transcriptome unigenes.

Database	Number of unigenes	Percentage (%)
Annotated in Nr	11,239	5.04
Annotated in Uniprot	11,304	5.07
Annotated in GO	8787	3.94
Annotated in COG	8672	3.89
Annotated in KEGG	9619	4.31
Total annotated unigenes	11,309	5.07
Total unigenes	223,055	100.00

2.7. Transcriptome sequencing and de novo assembly of transcripts

The raw sequencing reads were filtered to discard the reads with adapters, reads with unknown nucleotides (bases N) higher than 5%, and reads with more than 20% of low-quality bases (base quality ≤ 10). After filtering, clean reads were obtained and stored in FASTQ format [24]. The clean reads were de novo assembled using Trinity (http://trinityrnaseq.sf.net) with default parameters to get the unique transcript fragments (unigenes). Then, non-redundant unigenes as long as possible were clustered using TIGR Gene Indices Clustering Tools (TGICL) software [25].

2.8. Unigenes functional annotation and classification

The assembled unigenes were aligned by BLASTX search (cut-off E-value of 1e^{-6}) in known protein databases to identify the coding sequence (CDS), including NCBI non-redundant protein database (Nr: http://www.ncbi.nlm.nih.gov), Swiss-Prot database (http://www.uniprot.org), Clusters of Orthologous Groups of Proteins database (COG: http://www.ncbi.nlm.nih.gov/COG/), and Kyoto Encyclopedia of Genes and Genomes (KEGG: http://www.genome.jp/kegg) by Blastx with E-values of less than 10^{-5}. Unigenes which cannot be aligned to any protein database were also scanned using ESTScan [26], producing predict coding region and direction. If results from the databases conflicted, the priority order of alignment was Nr, KEGG, Swiss-Prot and COG databases. The Blas2GO software was used to obtain gene ontology (GO) (http://www.geneontology.org/) annotation of the unigenes based on BLASTX hits against Nr annotation, and then WEGO software was used to perform GO functional classification [27]. Pathway assignments were generated using the KEGG database (http://www.genome.jp/kegg), and KEGG Orthology (KO) of unigenes were

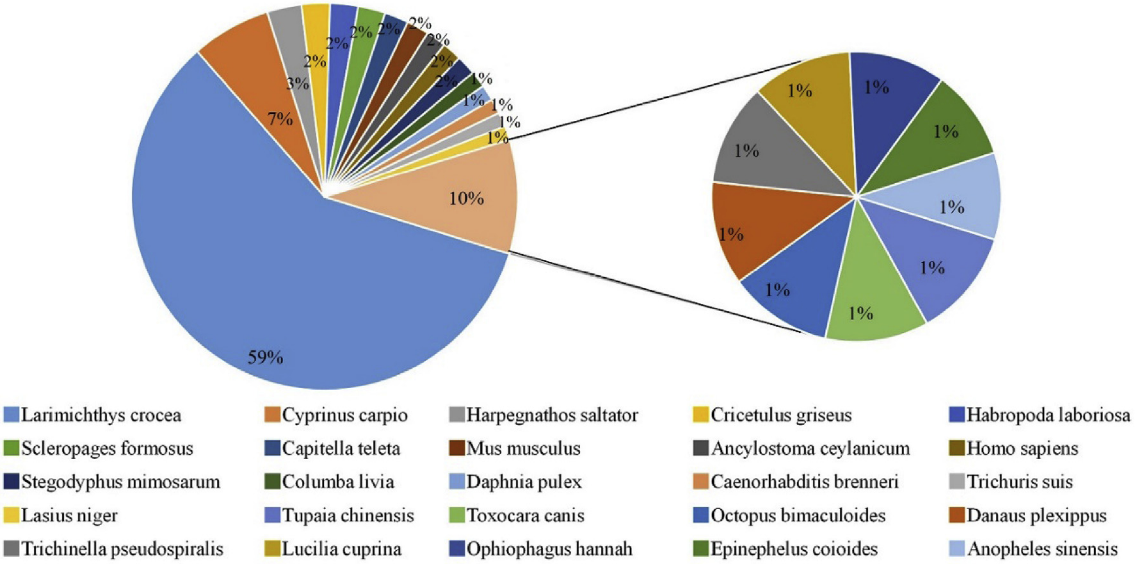

Fig. 3. Species distribution using Blastx against NCBI Nr database.

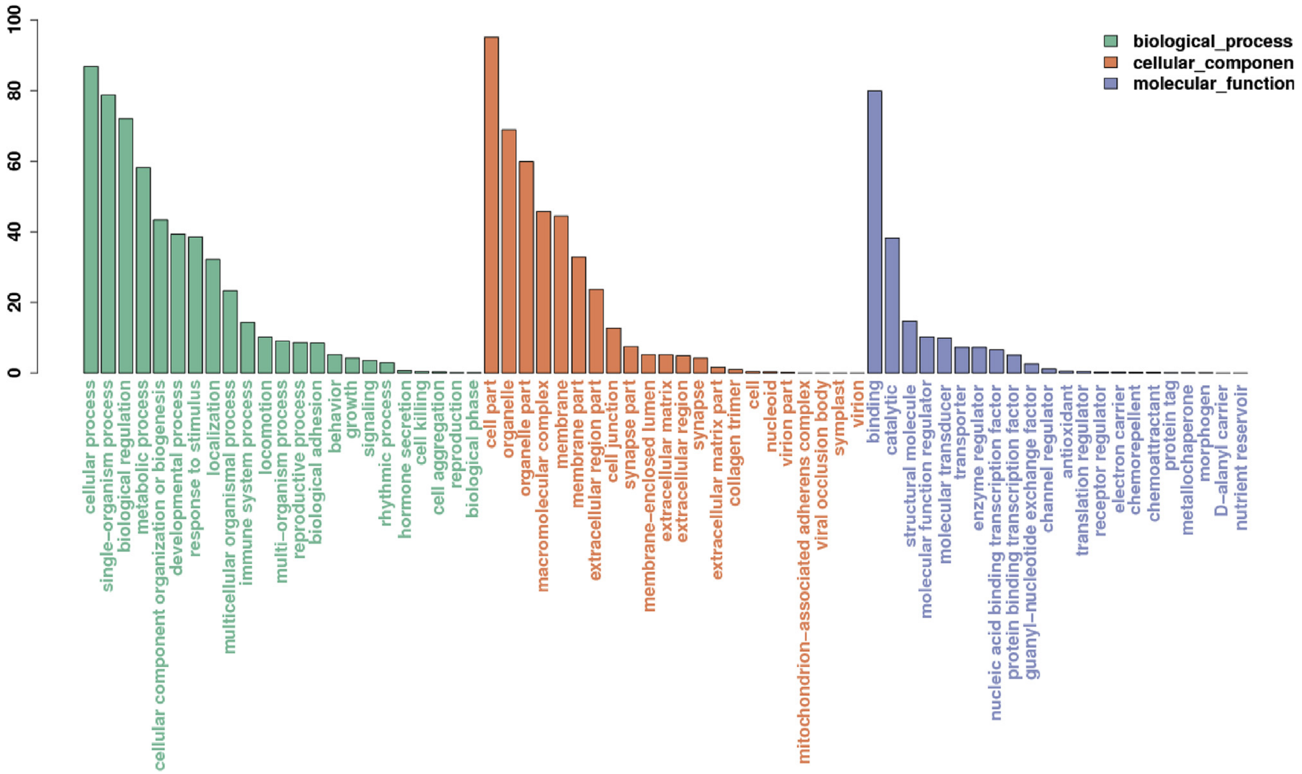

Fig. 4. Gene ontology (GO) analysis of the assembled unigenes. A total of 8787 unigenes were classified into three GO categories containing 67 sub-categories.

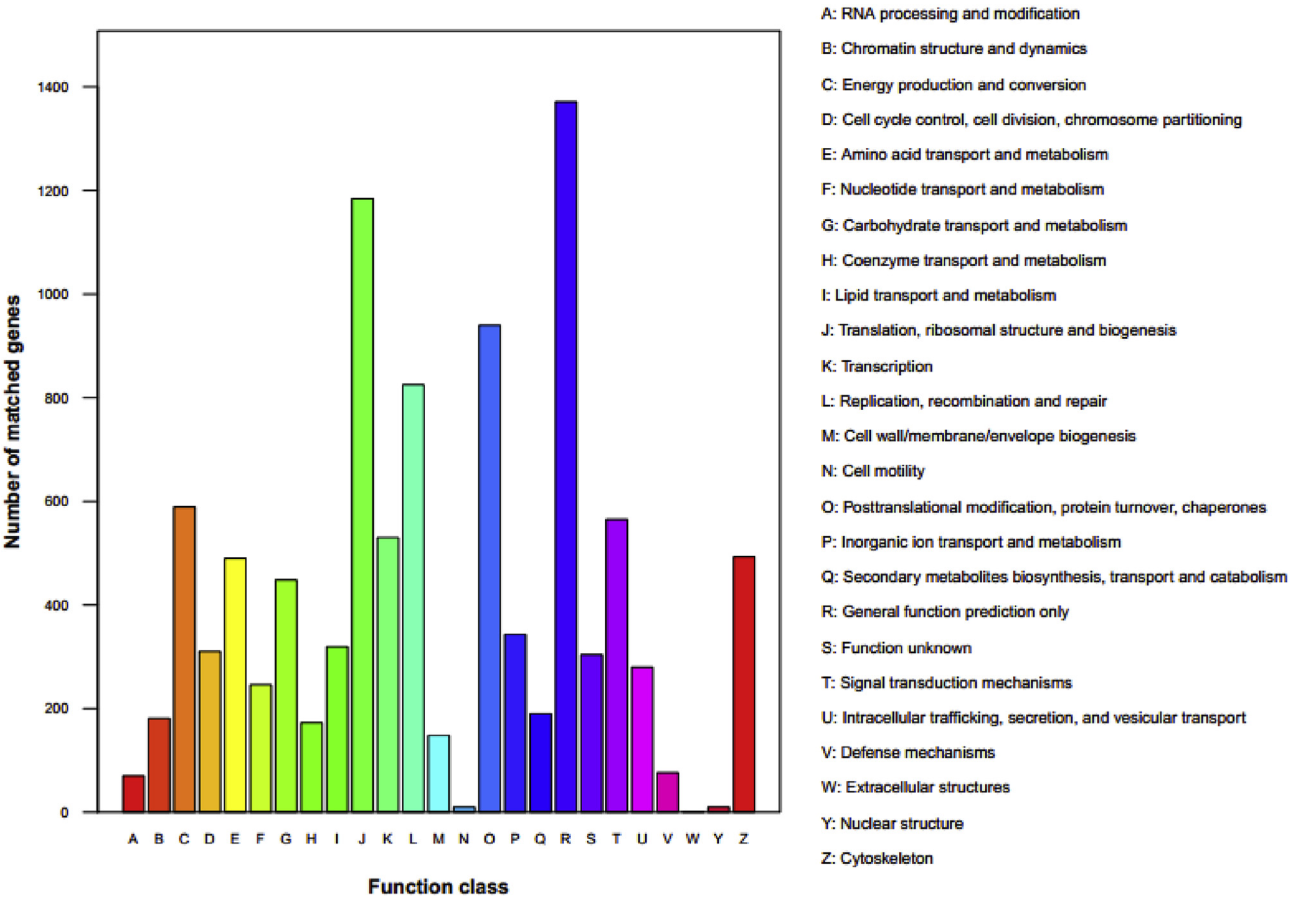

Fig. 5. Cluster of Orthologous Group (COG) classification analysis of unigenes.

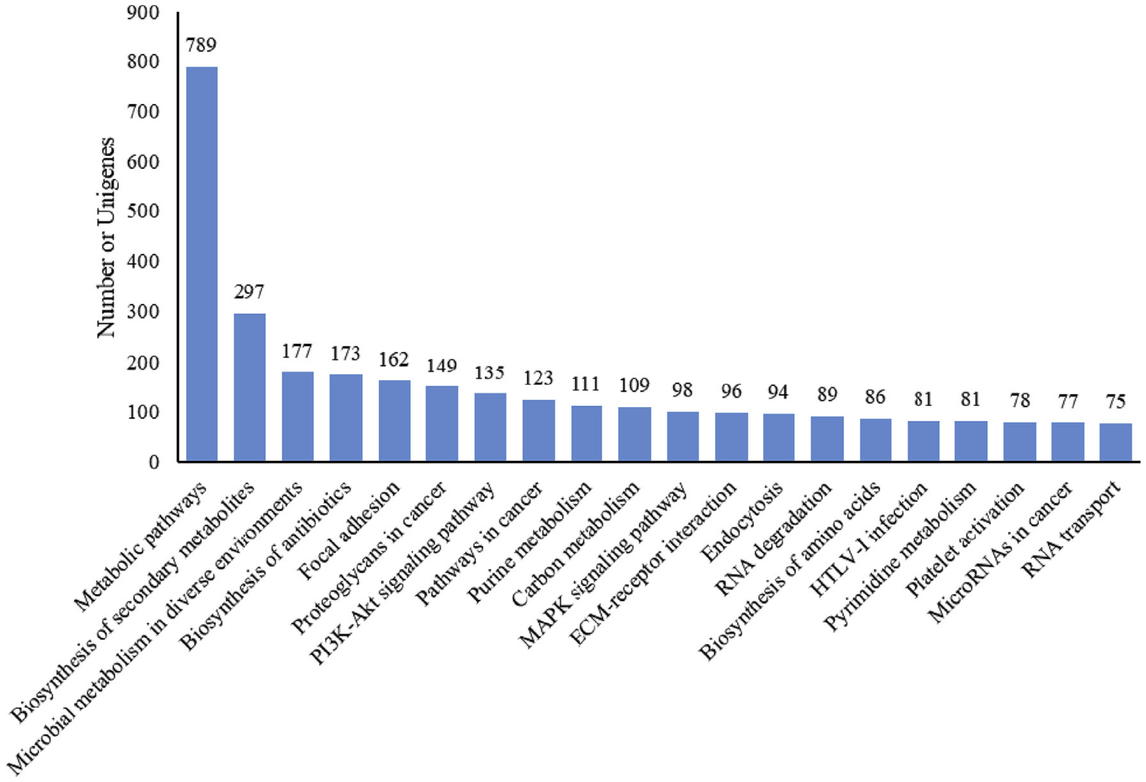

Fig. 6. Top 20 pathways with the largest numbers of unigenes based on KEGG annotation analysis.

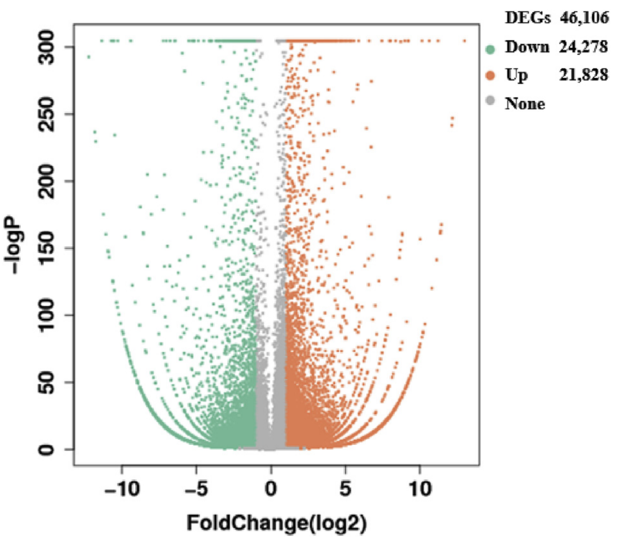

Fig. 7. Volcano analysis of different expression genes (DEGs) in spleen of soiny mullet by comparing PHB group to control. The expression level for each unigene was included in the volcano plot. Non, no differential expression; Up, upregulation; Down, downregulation. The X-axis means fold change of the gene between control and PHB groups. Y-axis shows LogP of unigene. The significant DEGs were considered as FDR ≤0.001 and fold change ≥1. PHB, soiny mullet fed with 2% PHB supplementation diet; CON, soiny mullet fed with basal diet without PHB supplementation.

analyzed based on blastx hits against the KEGG database.

2.9. Differential expression and enrichment analysis

Gene expression levels were first estimated by mapping clean reads to Unigenes using Bowtie 2 (version v2.2.5), and then calculated for each sample with RSEM (version v1.2.12) [28]. The abundance of all genes was normalized and calculated using uniquely mapped reads by the RPKM (reads per kilobase of transcript per million reads mapped) method [29]. Differentially expressed genes between control and PHB groups were analyzed according to method of Audic and Claverie [30]. To be biologically meaningful for single replicate experiments, stringent criteria with a false discovery rate (FDR) ≤ 0.001 and the absolute value of log2 ratios (fold change between control/PHB samples) ≥ 1 was used as the threshold to judge the significance of the gene expression differences [31]. In addition, please note that for single replicate experiments, though DEGs analysis could still provide P-values for gene expression, these P-values had no real statistical power and were for exploratory purposes only. A Venn Diagram was generated using R software (version 3.2.0). All DEGs were then carried out into GO functional enrichment analysis and KEGG pathway analysis with Goatools software (http://github.com/tanghaibao/GOatools) and KOBAS software (http://kobas.cbi.pku. edu.cn/home.do).

2.10. Validation of RNA-Seq results with qRT-PCR

To validate our RNA-Seq sequencing data, seven putative DEGs of control vs PHB were randomly selected for qRT-PCR analysis, including EGFR, FOS, MAPK, p38, TLR1, TLR5, IL8. The same RNA samples were used for qRT-PCR amplification and RNA-Seq profiling mentioned above. Reverse transcription with random hexamer primers was performed with the MMLV reverse transcriptase kit (TaKaRa, Dalian, China). Gene-specific primers were designed based on the assembled gene sequences using Primer Premier 6 software (Premier Biosoft International, CA, USA) (Table S1). β-actin was used as a reference gene for calculating the relative expression level of these seven DEGs. The qRT-PCR was performed in a 25 μL reaction volume containing 12.5 μL of SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China), 1.0 μL of each primer (10 μM), 2.0 μL of cDNA template (< 100 ng), and 8.5 μL of nuclease-free water with a CFX96 Real-Time PCR detection system (Bio-Rad, USA). The qRT-PCR thermal cycling parameters were 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 56.9–64.3 °C for 32 s, followed by

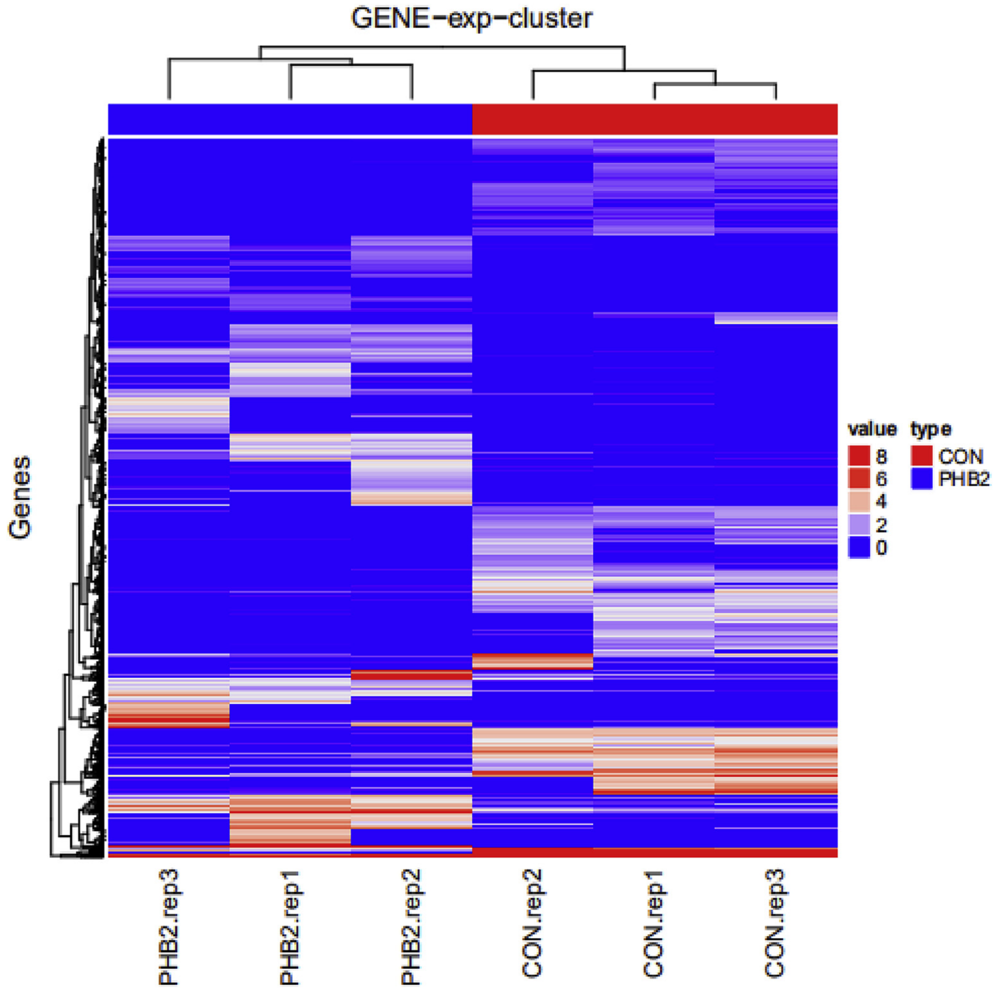

Fig. 8. Hierarchical clustering of DGEs in soiny mullet fed with PHB supplementation diets. PHB2. rep3/2/1, soiny mullet fed with 2% PHB supplementation diet in triplicate; CON. rep3/2/1, soiny mullet fed with basal diet without PHB supplementation.

dissociation curve analysis at 65 °C for 5 s to verify the amplification of a single product. Three biological replicates were analyzed for each treatment and each sample was analyzed in technical triplicates. Reverse transcriptase negative controls and no template controls were included on all plates. Melting curve analysis was performed at the end of each PCR assay to determine target specificity. The relative expression changes of each gene were calculated according to $2^{-\Delta\Delta Ct}$ method and normalized to expression of the endogenous control gene β-actin [32].

2.11. Statistical analysis

All data were expressed as the mean ± SD (standard deviation). Significant differences between control and PHB group were determined using Dunnett's *t*-test by a statistical analysis system software (SPSS 17.0). Statistical significance was set at the level of $P < 0.05$.

3. Results and discussion

3.1. Growth performance

Significant differences were observed in the growth parameters between control and PHB groups ($P < 0.05$). Final body weight (FBW), weight gain (WG) and specific growth rate (SGR) in the PHB group were 11.40 ± 0.83 g, 47.37 ± 10.72% and 1.29 ± 0.24% day^{-1}, respectively. In control group, FBW, WG and SGR were 9.66 ± 0.19 g, 24.87 ± 2.39% and 0.74 ± 0.06% day^{-1}, respectively. No significant difference was observed in the survival of *L. haematocheila* between control (100%) and PHB (100%) groups ($P > 0.05$) (Table S2). In other studies, PHB has been reported to promote the growth of tilapia (*Oreochromis niloticus*) [33], European sea bass (*Dicentrarchus labrax*) [4], *M. rosenbergii* larvae [24], Siberian sturgeon (*Acipenser baerii*) [6], Chinese mitten crab (*Eriocheir sinensis*) larvae [10,34] and sea cucumber (*Apostichopus japonicus*) [35]. Our previous study also demonstrated that 2% PHB-supplemented diet for 4 weeks or 0.5% PHB-supplemented diet for 8 weeks could increase *L. haematocheila* growth [16]. Thus, feeding 2% PHB supplemented level for 4 weeks was conducted in the present study. The growth-promoting effect might be mainly ascribed to intestinal pH slight reduction, gastrointestinal health status increment, or stimulation of the intestinal microbiota [2,15–17,36]. Dietary PHB supplementation could increase the relative abundance of *Bacillus* [16,17].

3.2. Immune-related enzyme activity

SOD, CAT and T-AOC play important roles in the antioxidant defense system [37–43]. Compared with the control group, T-AOC, SOD and CAT activities in the PHB group were significantly induced. SOD and CAT activities in the PHB group increased from 135.57 to 143.66 to 194.53 and 290.12 U mg^{-1} protein, respectively ($P < 0.01$) (Fig. 1). In other studies, T-AOC, SOD and CAT activities were enhanced significantly in *O. niloticus* and shrimp fed diets supplemented with PHB

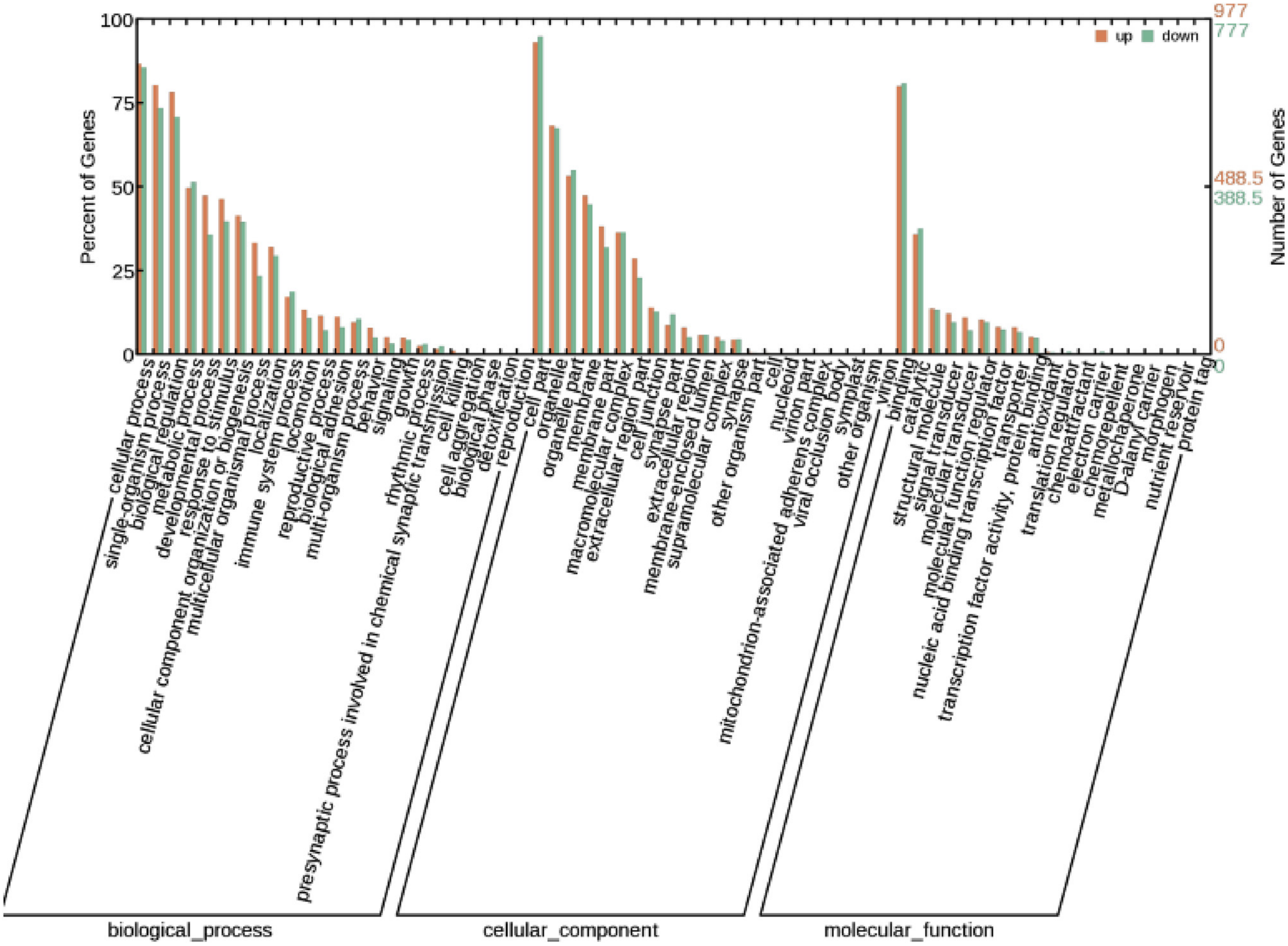

Fig. 9. Gene Ontology (GO) classification analysis of DEGs.

[1,17]. Thus, de novo transcriptome analysis in *L. haematocheila* spleen was further conducted to exploit the immune response pathway and genes in response to this prebiotic, PHB. It will provide very useful information to develop ecofriendly high efficient immune-strategies to prevent diseases. The spleen was chosen to do transcriptome analysis in this study for three reasons: 1) spleen and head kidney are often used to investigate immune responses of organisms against prebiotics, probiotics and pathogenic infection [19,20,44]; 2) many immune-related genes are highly expressed in head kidney or spleen [19,20]; 3) transcriptome of *L. haematocheila* spleen had been constructed, and 11,461 differentially expressed unigenes were identified post *Streptococcus dysgalactiae.* Fifteen pathways annotated by KEGG were identified to be related with the immune system [19].

3.3. Transcriptome sequencing and assembly of transcripts

After filtering dirty reads from the raw reads, 7,082,094,175 ± 760,806,735 clean reads were obtained from spleen in the control group, and 7,650,341,357 ± 345,853,969 clean reads were achieved in the PHB group, respectively. Both groups showed similar Q20% (the rate of bases which quality is greater than 20) over 98%, Q30% (the rate of bases which quality is greater than 20) over 94% (Table 2), and GC percentage of 43.92% (Table 3). These clean reads were further assembled into 223,055 unigenes with a N50 of 1225 bp. The mean length of assembled unigenes was 757 bp, with a total length of 168,717,141 bp (Table 3). Length distribution of all the unigenes showed that the most abundant unigenes were clustered in a group with 200–400 bp (Fig. 2). These data were in agreement with previous studies using Illumina sequencing [19,45,46], which revealed that transcriptomes of *L. haematocheila* spleens were effectively constructed and facilitated a systematic genetic information resource for further analysis. The raw data from this study were deposited in the NCBI Sequence Read Archive database under accession numbers SRR9662972, SRR9662973, SRR9662974, SRR9662975, SRR9662976 and SRR9662977. Assembled unigenes and annotation information are available from the authors upon request.

3.4. Unigene functional annotation

A total of 223,055 unigenes were subjected to annotation analysis by at least one of the reference databases (Nr, Uniprot, GO, COG and KEGG). The number of unigenes with significant similarity to sequences in Nr, Uniprot, GO, COG and KEGG databases were 11,239 (5.04%), 11,304 (5.07%), 8787 (3.94%), 8672 (3.89%) and 9619 (4.31%), respectively (Table 4). The higher BLASTx hits were observed in KEGG and Nr databases. The BLASTx top-hit species rankings of gene annotations showed that the highest sequence similarity was with large yellow croaker (*Larimichthys crocea*) (59% hit ration), followed by common carp (*Cyprinus carpio*) (7% hit ration) (Fig. 3). As demonstrated by Qi et al. [19], BLASTx top-hit species rankings of gene annotations of *L. haematocheila* showed 57.9% sequence similarity with *O. niloticus*. Actually, both *L. crocea* and *O. niloticus* belong to Percoidei in taxonomy, and they show high similarity in its habit and morphology. These high species sequence similarities suggested that the phylogeny is conserved between *L. haematocheila* and other fish species.

The GO is a standardized system for categorizing genes and gene products across species [47]. GO functional analysis based on Nr annotation from NCBI showed that 8787 unigenes were classified into 67

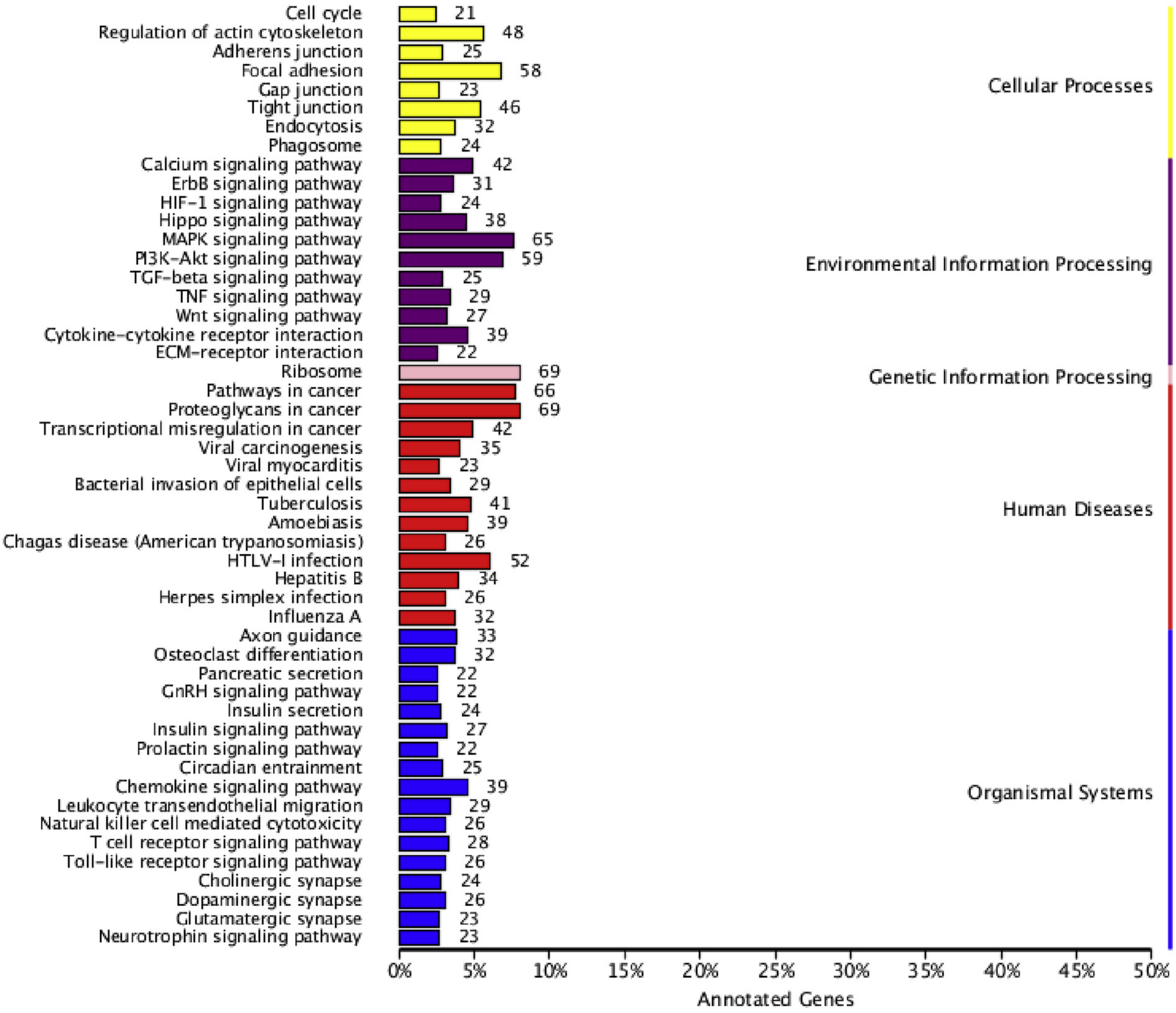

Fig. 10. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of DEGs between control and PHB groups.

subcategories of three major categories: the biological process, cellular component and molecular function (Fig. 4). In the biological process category, cell part and organelle were two most abundant subcategories (Fig. 4). Within the cellular component category, the most common groups were cellular process, followed by a single-organism process and biological regulation. In the molecular function category, the most dominant groups were binding and catalytic activity.

The COG database was used to further align the assembled unigenes to classify phylogenetically the proteins encoded by unigenes [19,48]. In total, 8672 unigenes were classified into 25 functional categories. The predominant categories were the cluster of general function prediction (1371 unigenes, 15.80%), post translational modification, protein turnover and chaperones (940, 10.84%), replication, recombination and repair (825, 9.51%). The least abundant categories were cell motility (10, 0.12%), nuclear structure (10, 0.12%) and extracellular structures (1, 0.01%). A number of 76 unigenes (0.88%) were donated into defense mechanism, indicating these unigenes might be involved in the immune defense of *L. haematocheila* (Fig. 5). These results were similar to those obtained from *L. haematocheila*, *C. carpio* and *Onchidium struma* [19,49].

The KEGG database (http://www.genome.jp/kegg/) as a bioinformatics base was used to analyze the gene functions [50]. A total of 9619 unigenes were grouped into 348 known pathways (Fig. 6). Among 348 known pathways, the five top pathways were metabolic pathways (789, 8.2%), biosynthesis of secondary metabolites (297, 3.1%), microbial metabolism in diverse environments (177, 1.84%), biosynthesis of antibiotics (173, 1.8%) and focal adhesion (162, 1.68%) (Fig. 6). It was worth mentioning that four out of 20 top pathways were related to the immune system, involving 491 unigenes. The most-represented immune system pathway and immune signal transduction pathway were: focal adhesion (162 unigenes), PI3K-Akt signaling pathway (135 unigenes), MAPK signaling pathway (98 unigenes) and ECM-receptor interaction (96 unigenes), respectively (Fig. 6). Vast majority of genes in these immune-relative pathways were also annotated. The identification of large numbers of immune-relative pathways/genes in *L. haematocheila* transcriptome supported the idea that the immune-relative pathways were well conserved between fish and mammals.

3.5. Identification of differently expressed immune-related genes and pathways

Differently expressed genes in the spleen of *L. haematocheila* were analyzed between control and PHB groups by calculating the reads per kilobase per million reads value [51]. The 46,106 unigenes showed different expression, including 21,828 up-regulated unigenes and 24,278 down-regulated unigenes in the PHB group (Figs. 7 and 8). To identify the function and pathways of DEGs, we performed GO and KEGG enrichment analysis. A total of 1754 DEGs were assigned into three categories including 65 GO terms. Among them, 312 DEGs were involved in immune system process, and 760 unigenes in response to a

Table 5
Immune-related differentially expressed genes (DEGs) regulated in spleen of soiny mullet fed with 2% poly-β-hydroxybutyrate (PHB) supplementation diets for 30 days.

Category/Gene name	Description	Fold change	p-value	Up/down
MAPK signaling pathway				
EGFR	epidermal growth factor receptor	1.84	2.46E-20	up
SOS	son of sevenless	13.91	1.42E-02	up
FOS	proto-oncogene protein c-fos	36.41	0.00E+00	up
MAPK	mitogen-activated protein kinase 5	5.53	0.00E+00	up
NF-κB	nuclear factor NF-kappa-B p105 subunit	2.12	5.71E-05	up
p38	p38 MAP kinase	0.06	3.26E-05	down
PI3K-Akt signaling pathway				
VWF	von Willebrand factor	2.47	2.09E-04	up
ITGα2B	integrin alpha 2B	1.65	3.74E-45	up
PTK2, FAK	focal adhesion kinase 2	4.06	5.51E-02	up
PIK3CA_B_D	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha/beta/delta	0.19	9.97E-03	down
TNFSF6	tumor necrosis factor ligand superfamily member 6	2.90	4.26E-126	up
TNF signaling pathway				
TAK	TAK1-binding protein 3	0.50	6.62E-02	down
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	15.78	0.00E+00	up
PIK3CA_B_D	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha/beta/delta	0.19	9.97E-03	down
RELA	transcription factor p65	0.47	1.60E-02	down
NF-κBIA	NF-kappa-B inhibitor alpha	2.99	0.00E+00	up
T cell receptor signaling pathway				
PIK3CA_B_D	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha/beta/delta	0.19	9.97E-03	down
MAP3K8	mitogen-activated protein kinase 8	5.53	0.00E+00	up
NF-κB	nuclear factor NF-kappa-B p105 subunit	2.12	5.71E-05	up
NF-κBIA	NF-kappa-B inhibitor alpha	2.99	0.00E+00	up
Toll-like receptor signaling pathway				
TLR1	toll-like receptor 1	0.38	2.20E-17	down
TLR5	toll-like receptor 5	24.53	1.84E-94	up
TLR6	toll-like receptor 6	0.48	2.75E-05	down
TIRAP	toll-interleukin 1 receptor (TIR) domain-containing	0.40	2.07E-09	down
RAC1	Ras-related C3 botulinum toxin substrate 1	2.55	1.77E-03	up
To be continued				
NF-κB	nuclear factor NF-kappa-B p105 subunit	2.12	5.71E-05	up
IL8	interleukin 8	6.68	8.55E-18	up
NF-κBIA	NF-kappa-B inhibitor alpha	2.99	0.00E+00	up
MAP3K8	mitogen-activated protein kinase 8	5.53	0.00E+00	up
FOS	proto-oncogene protein c-fos	36.41	0.00E+00	up
MAP2K4	mitogen-activated protein kinase 4	0.33	5.54E-14	down
NF-kappa B signaling pathway				
TNFRSF5	tumor necrosis factor receptor superfamily	2.46	0.00E+00	up
TAK	TAK1-binding protein 3	0.50	6.62E-02	down
NF-κB	nuclear factor NF-kappa-B p105 subunit	2.12	5.71E-05	up
NF-κBIA	NF-kappa-B inhibitor alpha	2.99	0.00E+00	up
RELA	transcription factor p65	0.47	1.60E-02	down
PTGS2	prostaglandin-endoperoxide synthase 2	4.58	6.91E-158	up

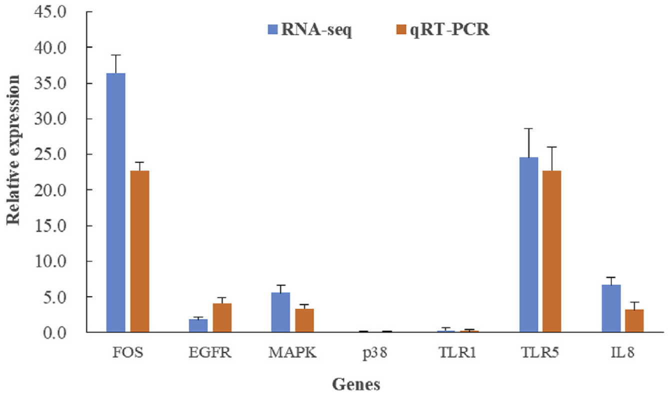

Fig. 11. Comparison of relative fold changes between RNA-seq and qRT-PCR in spleen of soiny mullet. Fold changes are expressed as the ration of gene expression after PHB supplementation to the control group as normalized to β-actin. See abbreviations in the text.

stimulus (Fig. 9). These results were similar to previous reports in the spleen of *L. haematocheila* post *Str. dysgalactiae* challenge [19]. 388 DEGs were involved in the immune system process, and 1441 DEGs were in response to a stimulus. Further KEGG analysis annotated 3339 DEGs to 261 signaling pathways. Six pathways associated with the immune system were identified: MAPK signaling pathway, PI3K-Akt signaling pathway, TNF signaling pathway, TCR signaling pathway, TLR signaling pathway and NF-κB signaling pathway (Fig. 10, Table 5). Most of the pathways are responsible for the antioxidant system and inflammatory response [52].

3.6. Validation of seven DEGs by RT-qPCR

Following RNA-seq transcriptomic profiling of pooled data, expression of seven DEGs were selected to re-assess by qRT-PCR using individual biological replicates (n = 6 per treatment) and normalized to β-actin. The results are shown in Fig. 11. The expression results obtained by qRT-PCR and RNA-Seq showed a high similarity, further supporting the reliability of the gene identified by RNA-seq.

3.7. MAPK signaling pathway

Mitogen-activated protein kinase (MAPKs) signaling pathway plays an essential role in modulating other intracellular proteins and regulating vital functions like cell differentiation, apoptosis, proliferation and immune responses [53]. In this study, some of the genes such as

EGFR, SOS, FOS, MAPK and NF-κB in the PHB group were up-regulated by 1.84-, 13.91-, 36.41-, 5.53- and 2.12-folds, respectively (Table 5). EGFR is a membrane receptor and activated to regulate many cellular actions, such as cell growth, differentiation, apoptosis, proliferation and vasculogenesis [54,55]. MAPK is an activated factor in MAPK signaling pathway, which regulates the expression of NF-κB and further participate in the cell proliferation, apoptosis and inflammation [56]. Upregulation of EGFR, SOS, FOS, MAP3K8 and NF-κB could increase cellular processes, especially inflammation, to protect themselves or adapt to the environment [57]. Inflammation plays an important role in host defense reaction in response to the invasion of pathogens, tissue damage, or environmental assaults [58]. A balance of injury and restoration during an inflammatory response must be achieved to keep a good health of animals. If the balance is broken due to excessive pro-inflammatory response, chronic tissue damage will occur [57]. Meanwhile, the initiation and functionality of the cellular processes involved in a pro-inflammatory response can also be developed as potential targets for anti-inflammatory therapeutics [58]. In the current study, the transcription levels of some pro-inflammatory cytokines such as TNFSF6 and IL8 were upregulated, and some of them such as TLR1 and TLR6 were downregulated. Simultaneously, the transcription of some anti-inflammatory factors such as NF-κB and NF-κBIA in different signaling pathways was upregulated. These dynamic or diverse regulation of both pro-inflammatory cytokines and anti-inflammatory factors will be beneficial to achieve the balance and enhance the host defense. Accordingly, in the antioxidant defense system, enzymatical T-AOC, SOD and CAT activities which represented by inflammatory cytokines and gene regulation [52] were increased in this study. However, excessive nitric oxide (NO) production was not detected (data not shown), suggesting that no excessive pro-inflammatory response was occurred in the present study. Excessive NO production can lead to cytotoxicity and autoimmune disorders [59]. Astaxanthin has been proved to protect *Channa argus* against lipopolysaccharide (LPS) induction through NF-κB and MAPKs signaling pathways [59]. More protection mechanism of PHB in this signaling pathway *in vivo* and *ex vivo* should be studied.

3.8. PI3K-Akt signaling pathway

The phosphatidylinositol 3-kinase/protein kinase-B (PI3K-Akt)/mTOR (mechanistic target of rapamycin) signaling pathway is known in regulating adaptive immune cell activation, and also plays critically physiological and pathological roles in the innate immune response against pathogens and environmental pollutants [60–64]. In the current study, the expressions of VWF, ITGα2B, PTK2 (FAK) and TNFSF6 were upregulated by 2.47-, 1.65-, 4.06- and 2.90-folds, respectively (Table 5). The expression of PI3K was downregulated. PI3K is a crucial coordinator of intracellular signaling in response to the extracellular stimulators. Hyperactivation of PI3K could have a beneficial clinical effect such as in cancer therapy and inhibition of viral replication [62]. Lots of drugs play roles in disease treatment through inhibition of various components in PI3K-Akt pathway. De-regulation of the PI3K communication pathway can reduce the oxidative stress [52]. Then, PI3K activates protein kinase B (PKB, also termed as Akt) and triggers a series of signal transductions [63]. Akt is one of the most important kinases involved in regulating broad cellular functions including immunity, growth and survival. Meanwhile, Akt is known as a phosphorylation factor on IkappaB (I-κB) with the function of activation of NF-κB and mTOR [64,65]. NF-κB and mTOR plays a pivotal role in the development of immune responses, and regulation of the gene expression involved in cell survival, cell death, inflammation, proliferation and cell differentiation [54,61]. However, different Akt expression between PHB group and control was not detected at this time point. More studies on Akt expression at different time point post PHB stimulation should be conducted in future researches. Although different Akt transcription was not detected, the expressions of TNF in both PI3K-Akt and NF-κB signaling pathways were upregulated in the current study (Table 5). As we know, TNF is also one of physiological inducers for the nuclear transcription factor NF-κB [66]. It suggested that NF-κB signaling in fish might be well-related with PHB stimulation in cell growth and proliferation, but not with mTOR signaling. The relative expression levels of the mTOR signaling-related genes in shrimp were reported to increase post PHB stimulation [67]. The above results implicated that signaling pathway might be somehow different between fish and shrimp species.

3.9. TNF and NF-κB signaling pathway

TNF and NF-κB signalings control peripheral immune activation and protective inflammatory responses, and develop as a therapeutic target in disease treatment, such as multifactorial autoimmune inflammatory diseases [68]. In this study, the transcription of TAK was downregulated by 0.5-fold, but CEBPB was upregulated by 15.78-folds for TNF signaling pathway (Table 5). For NF-κB signaling pathway, the transcriptions of some inducers such as TNF, NF-κB and PTGS were upregulated, and the transcriptions of some factors controlled excessive expression such as NF-κBIA, RELA and TAK were different between PHB group and control. These different expressions of targeting genes activate TNF and NF-κB signaling pathways, and control the balance not inflammation to take protective effect of PHB supplementation in *L. haematocheila*. As previous studies demonstrated that the NF-κB/PI3K-Akt/TCR signaling are involved in response to dietary lipid supplementation [52]. After all, PHB is familiar to lipid considering their common characteristics that both of PHB and lipid are insoluble and can be depolymerized into organic acids [16,52]. The function of targeting genes post PHB stimulation in different pathways should be studied further to explore the critical regulation mechanism for developing a more effective prevention method in sustainable *L. haematocheila* culture.

3.10. TCR and TLR signaling pathway

Signaling from T cell receptor (TCR) and Toll-like receptor (TLR) are also critical for mounting the innate and adaptive immune responses [69]. T cells are important lymphoid cells involved in cellular immunity [70]. TLRs are the first characterized innate immune receptors [71], and play important roles in innate and adaptive immunity by activating antigen-producing cells in fish [71]. In the present study, some genes in TLR signaling such as TLR5, IL8 and FOS were upregulated significantly by 24.53-, 6.68-, and 36.41-folds, respectively (Table 5). The others such as TLR1 and TIRAP were down-regulated by 0.38-, 0.40-folds. The expression level of NF-κBIA was also increased to inhibit excessive inflammation (Table 5). TLR1 molecules do not have LRRNT modules in the N-terminal and are important for dimerization with TLR2 which recognizes various ligands from bacteria [72]. TLR1 plays an important role in anti-microbial immunity through possible recognition of LPS, poly (I:C) and lipopeptides [71]. TLR5 is responsible for the detection of flagellin, and specially recognizes the constant domain relatively conserved among different bacteria species through close physical interaction between TLR5 and flagellin [73–75]. Then, NF-κB signaling is activated to fight for infection. There is a two-step flagellin response predicted to occur for host defense against bacterial infection: 1) flagellin first induces the basal activation of NF-κB via TLR5; 2) the inducible TLR5 amplifies TLR5-mediated cellular responses in a positive feedback profile [76]. In the current study, the expression level of TLR5 post PHB stimulation was increased by 24.53-folds (Table 5), which would raise the initial flagellin recognition via TLR5 and an exaggerated inflammatory response would be avoided against the bacterial infection because of the binding of TLR5 to flagellin [77]. As reported in rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (*Sparus aurata*), TLR5 could bind flagellin and enhance innate immune response [77]. In this study, TLR6 was annotated and the expression level were decreased (Table 5). Actually, TLR6 has not yet reported in fish, but some closed genes such as TLR14 were found in fish [78–80]. This gene has functions in generating immune response against various pathogens in water [72]. The present result need to be explained more in future, including whether it is resulted from wrong annotation or due to TLR6 exists in *L. haematocheila*, such as molecular cloning, cDNA sequence and bioinformatics analysis. Based on above results, dietary PHB supplementation could enhance anti-bacterial infection through possible TLR5 binding to flagellin. However, the precise mechanism needs further studies. After the recognition of the corresponding ligands via interactions with LRRs, TLRs are activated and the related adaptor proteins are recruited with the cytoplasm, and different signaling cascades such as NF-κB are triggered [71,74,81,82].

4. Conclusions

In this study, we evaluated the immunostimulatory mechanism of dietary PHB supplementation on *L. haematocheila* by antioxidant enzyme activity and transcriptome-based MAPK/PI3K-Akt/TNF/NF-κB/TCR/TLR signaling pathways. The antioxidant enzyme activity including T-AOC, SOD and CAT increased, and MAPK/PI3K-Akt/TNF/NF-κB/TCR/TLR signaling were activated in response to PHB stimulation. These signaling pathways are well-related with both innate and adaptive immune response, especially antioxidant system. In each signaling pathway, the expression levels of some inducers such as MAPK and NF-κB were increased, and some inhibitors such as NF-κB inhibitor alpha were also increased. These interaction among different genes would be beneficial to alleviate overactive innate response, but enhance immunity and disease resistance of aquatic animals as reported in fish, shrimp, and oyster species. Overall, immuno-enhancement of PHB might be more related to the antioxidant system based on both enzyme activities and transcriptome-based signaling. That information provides a useful and valuable evaluation of PHB immunity enhancement in *L. haematocheila*. Further studies on the detailed/precise mechanisms between intestine microbiota such as relative abundance increasement of *Bacillus* sp. and immune-related signaling pathway induced by PHB should be conducted. PHB has a high potential to develop as novel prebiotics in Aquaculture. Hopefully, biomarkers can be developed for evaluation of effects of immunostimulants in the future study based on the findings from this study.

Declaration of competing interest

The research was conducted in the absence of any commercial or financial relationships. We declare no conflict of interest.

Acknowledgment

This research was financially supported by the National Natural Science Foundation of China (Grant no. 31602179), Talent Introduction Program at Yancheng Institute of Technology (Grant no. xj201513), National Nature Science Foundation of Jiangsu Province (Grant no. BK20171274), and partially by the Subei Special Project (Grant no. SZ-YC2017006, SZ-YC2018050) and Projects on Natural Science Research in Colleges and Universities of Jiangsu Province (No. 17KJB240002).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.10.042.

References

[1] P. Suguna, C. Binuramesh, P. Abirami, V. Saranya, K. Poornima, V. Rajeswari, R. Shenbagarathai, Immunostimulation by poly-β-hydroxybutyrate-hydroxyvalerate (PHB-HV) from *Bacillus thuringiensis* in *Oreochromis mossambicus*, Fish Shellfish Immunol. 36 (2014) 90–97, https://doi.org/10.1016/j.fsi.2013.10.012.

[2] J.D. Iwanek, P. Zagrodzki, M. Wozniakiewicz, A. Wozniakiewicz, M.Z. Wcisło, D. Winnicka, P. Pasko, Procedure optimization for extracting short-chain fatty acids from human faces, J. Pharm. Biomed. 124 (2016) 337–340, https://doi.org/10.1016/j.jpba.2016.02.042.

[3] K. Weitkunat, S. Schumann, K.J. Petzke, M. Blaut, G. Loh, S. Klaus, Effects of dietary inulin on bacterial growth, short-chain fatty acid production and hepatic lipid metabolism in gnotobiotic mice, J. Nutr. Biochem. 26 (2015) 929–937, https://doi.org/10.1016/j.jnutbio.2015.03.010.

[4] P. De Schryver, A.K. Sinha, P.S. Kunwar, K. Baruah, W. Verstraete, N. Boon, G. DeBoeck, P. Bossier, Poly-β-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass (*Dicentrarchus labrax*), Appl. Microbiol. Biotechnol. 86 (2010) 1535–1541, https://doi.org/10.1007/s00253-009-2414-9.

[5] L.Y. Sui, Y. Liu, H.X. Sun, M. Wille, P. Bossier, P. De Schryver, The effect of poly-β-hydroxybutyrate on the performance of Chinese mitten crab (*Eriocheir Sinensis*) (Milne-Edwards) zoea larva, Aquacult. Res. 45 (2014) 558–565, https://doi.org/10.1111/are.12077.

[6] E.H. Najdegerami, T.N. Tran, T. Defoirdt, M. Marzorati, P. Sorgeloos, N. Boon, P. Bossier, Effects of poly-β-hydroxybutyrate (PHB) on Siberian sturgeon (*Acipenser baerii*) fingerlings performance and its gastrointestinal tract microbial community, FEMS Microbiol. Ecol. 79 (2012) 25–33, https://doi.org/10.1111/j.1574-6941.2011.01194.x.

[7] Y. Liu, P. De Schryver, B.V. Delsen, L. Maignien, N. Boon, P. Sorgeloos, W. Verstraete, P. Bossier, T. Defoirdt, PHB-degrading bacteria isolated from the gastrointestinal tract of aquatic animals as protective actors against luminescent Vibriosis, FEMS Microbiol. Ecol. 74 (2010) 196–204, https://doi.org/10.1111/j.1574-6941.2010.00926.x.

[8] T. Defoirdt, D. Halet, H. Vervaeren, N. Boon, T.V. Wiele, P. Sorgeloos, P. Bossier, W. Verstraete, The bacterial storage compound poly-β-hydroxybutyrate protects *Artemia franciscana* from pathogenic *Vibrio campbellii*, Environ. Microbiol. 9 (2007) 445–452, https://doi.org/10.1111/j.1462-2920.2006.01161.x.

[9] D.T. Nhan, M. Wille, P. De Schryver, T. Defoirdt, P. Bossier, P. Sorgeloos, The effect of poly-β-hydroxybutyrate on larvi culture of the giant freshwater prawn (*Macrobrachium rosenbergii*), Aquaculture 302 (2010) 76–81, https://doi.org/10.1016/j.aquaculture.2010.02.011.

[10] L.Y. Sui, J. Cai, H.X. Sun, M. Wille, P. Bossier, Effect of poly-β-hydroxybutyrate on Chinese mitten crab (*Eriocheir sinensis*) larvae challenged with pathogenic *Vibrio anguillarum*, J. Fish Dis. 35 (2012) 359–364, https://doi.org/10.1111/j.1365-2761.2012.01351.x.

[11] E.H. Najdegerami, K. Baruah, A. Shiri, A. Rekecki, W. Van den Broeck, P. Sorgeloos, N. Boon, P. Bossier, P. De Schryver, Siberian sturgeon (*Acipenser baerii*) larvae fed Artemia nauplii enriched with poly-β-hydroxybutyrate (PHB): effect on growth performance, body composition, digestive enzymes, gut microbial community, gut histology and stress tests, Aquacult. Res. 46 (2015) 801–812, https://doi.org/10.1111/are.12231.

[12] P. De Schryver, K. Dierckens, Q.Q. Bahn Thi, R. Amalia, M. Marzorati, P. Bossier, N. Boon, W. Verstraete, Convergent dynamics of the juvenile European sea bass gut microbiota induced by poly-β-hydroxybutyrate, Environ. Microbiol. 13 (2011) 1042–1051, https://doi.org/10.1111/j.1462-2920.2010.02410.x.

[13] D. Halet, T. Defoirdt, P. Van Damme, H. Vervaeren, I. Forrez, T. Van de Wiele, N. Boon, P. Sorgeloos, P. Bossier, W. Verstraete, Poly-β-hydroxybutyrate accumulating bacteria protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*, FEMS Microbiol. Ecol. 60 (2007) 363–369, https://doi.org/10.1111/j.1574-6941.2007.00305.x.

[14] T.V.C. Dang, V.H. Nguyen, K. Dierckens, T. Defoirdt, N. Boon, P. Sorgeloos, P. Bossier, Novel approach of using homoserine lactone-degrading and poly-β-hydroxybutyrate-accumulating bacteria to protect Artemia from the pathogenic effects of *Vibrio harveyi*, Aquaculture 291 (2009) 23–30, https://doi.org/10.1016/j.aquaculture.2009.03.009.

[15] K. Baruah, T.T. Huy, P. Norouzitallab, Y.F. Niu, S.K. Gupta, P. De Schryver, P. Bossier, Probing the protective mechanism of poly-β-hydroxybutyrate against Vibriosis by using gnotobiotic *Artemia franciscana* and *Vibrio campbellii* as host-pathogen model, Sci. Rep. 5 (2015) 9427, https://doi.org/10.1038/srep09427.

[16] G. Qiao, C. Xu, Q.R. Sun, D.H. Xu, M.M. Zhang, P. Chen, Q. Li, Effects of dietary poly-β-hydroxybutyrate supplementation on the growth, immune response and intestinal microbiota of soiny mullet *(Liza haematocheila*), Fish Shellfish Immunol. 91 (2019) 251–263, https://doi.org/10.1016/j.fsi.2019.05.038.

[17] Y.F. Duan, Y. Zhang, H.B. Dong, X.T. Zheng, Y. Wang, H. Li, Q.S. Liu, J.S. Zhang, Effect of dietary poly-β-hydroxybutyrate (PHB) on growth performance, intestinal health status and body composition of Pacific white shrimp (*Litopenaeus vannamei)* (Boone, 1931), Fish Shellfish Immunol. 60 (2017) 520–528, https://doi.org/10.1016/j.fsi.2016.11.020.

[18] L.H. An, J.Y. Hu, M. Yang, B.H. Zheng, A. Wei, J.J. Shang, X.R. Zhao, CYP1A mRNA expression in redeye mullets (*Liza haematocheila*) from Bohai Bay, China, Mar. Pollut. Bull. 62 (2011) 718–725, https://doi.org/10.1016/j.marpolbul.2011.01.019.

[19] Z.T. Qi, P. Wu, Q.H. Zhang, Y.C. Wei, Z.S. Wang, M. Qiu, R. Shao, Y. Li, Q. Gao, Transcriptome analysis of soiny mullet (*Liza haematocheila*) spleen in response to *Streptococcus dysgalactiae*, Fish Shellfish Immunol. 49 (2016) 194–204, https://doi.org/10.1016/j.fsi.2015.12.029.

[20] Y. Cai, S. Wang, W. Guo, Z. Xie, Y. Zheng, Z. Cao, Y. Zhou, Transcriptome analysis provides insights into the immune responsive pathways and genes in the head kidney of tiger grouper (*Epinephelus fuscoguttatus*) fed with spatholobus suberectus, phellodendron amurense, or eclipta prostrata, Fish Shellfish Immunol. 73 (2018) 100–111, https://doi.org/10.1016/j.fsi.2017.12.004.

[21] Z. Wang, M. Gerstein, M. Snyder, RNA-Seq: a revolutionary tool for transcriptomics, Nat. Rev. Genet. 10 (2009) 57–63, https://doi.org/10.1038/nrg2484.

[22] X.T. Hong, L.X. Xiang, J.Z. Shao, The immune-stimulating effect of bacterial genomic DNA on the innate immune responses of bivalve mussel (*Hyriopsis cumingii* Lea), Fish Shellfish Immunol. 21 (2006) 357–364, https://doi.org/10.1016/j.fsi.2005.12.013.

[23] B. Yang, C. Wang, H. Hu, Y. Tu, D. Han, X. Zhu, J. Jin, Y. Yang, S. Xie, Repeated handling compromises the immune suppression and improves the disease resistance in overwintering channel catfish (*Ictalurus punctatus*), Fish Shellfish Immunol. 47 (2015) 418–428, https://doi.org/10.1016/j.fsi.2015.09.010.

[24] P.J.A. Cock, C.J. Fields, N. Goto, M.L. Heuer, P.M. Rice, The Sanger FASTQ file format for sequences with quality scores, and the Solexa/Illumina FASTQ variants, Nucleic Acids Res. 38 (2010) 1767–1771, https://doi.org/10.1093/nar/gkp1137.

[25] G. Pertea, X. Huang, F. Liang, V. Antonescu, R. Sultana, S. Karamycheva, Y. Lee, J. White, F. Cheng, B. Parvizi, TIGR Gene Indices clustering tools (TGICL): a software system for fast clustering of large EST datasets, Bioinformatics 19 (2003) 651–652, https://doi.org/10.1093/bioinformatics/btg034.

[26] C. Iseli, C.V. Jongeneel, P. Bucher, ESTScan: a program for detecting, evaluating, and reconstructing potential coding regions in EST sequences, ISMB (1999) 138–148, https://doi.org/10.1002/9780470999455.ch1.

[27] J. Ye, L. Fang, H. Zheng, Y. Zhang, J. Chen, Z. Zhang, J. Wang, S. Li, R. Li, L. Bolund, J. Wang, WEGO: a web tool for plotting GO annotations, Nucleic Acids Res. 24 (2006) W293–W297, https://doi.org/10.1093/nar/gkl031.

[28] B. Langmead, S.L. Salzberg, Fast gapped-read alignment with Bowtie 2, Br. J. Pharmacol. 9 (4) (2012) 357–359, https://doi.org/10.1038/nmeth.1923.

[29] B. Li, C.N. Dewey, RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome, BMC Bioinf. 12 (1) (2011) 323, https://doi.org/10.1186/1471-2105-12-323.

[30] S. Audic, J.M. Claverie, The significance of digital gene expression profiles, Genome Res. 7 (1997) 986–995, https://doi.org/10.1101/gr.7.10.986.

[31] Y. Benjamini, D. Yekutieli, The control of the false discovery rate in multiple testing under dependency, Ann. Stat. 29 (2001) 1165–1188, https://doi.org/10.1214/aos/1013699998.

[32] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method, Methods 25 (2001) 402–408, https://doi.org/10.1016/meth.2001.1262.

[33] M.L. Situmorang, P. De Schryver, K. Dierckens, P. Bossier, Effect of poly-β-hydroxybutyrate on growth and disease resistance of Nile tilapia (*Oreochromis niloticus*) juveniles, Vet. Microbiol. 182 (2016) 44–49, https://doi.org/10.1016/j.vetmic.2015.10.024.

[34] L. Sui, G. Ma, W. Lu, Y. Deng, P. Bossier, P. De Schryver, Effect of poly-β-hydroxybutyrate on growth, enzyme activity and intestinal microbial community of Chinese mitten crab (*Eriocheir sinensis*) (Milne-Edwards) juveniles, Aquacult. Res. 47 (2016) 3644–3652, https://doi.org/10.1111/are.12817.

[35] Y. Yamazak, P.M. Meirelles, S. Mino, W. Suda, K. Oshima, M. Hattori, F.L. Thompson, Y. Sakai, T. Sawabe, Individual *Apostichopus japonicus* fecal microbiome reveals a link with polyhydroxybutyrate producers in host growth gaps, Sci. Rep. 6 (2016) 21631, https://doi.org/10.1038/srep21631.

[36] A. Koh, V.F. De, P. Kovatcheva-Datchary, F. Backhed, From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites, Cell 165 (2016) 1332–1345, https://doi.org/10.1016/j.cell.2016.05.041.

[37] O.M. Ighodaro, O.A. Akinloye, First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defense grid, Alexandria Med. J. 54 (2017) 287–293, https://doi.org/10.1016/j.ajme.2017.09.001.

[38] X.J. Wu, W. Cao, G. Jia, H. Zhao, X.L. Chen, C.M. Wu, J.Y. Tang, J. Wang, G.M. Liu, New insights into the role of spermine in enhancing the antioxidant capacity of rat spleen and liver under oxidative stress, Anim. Nutr. 3 (2017) 85–90, https://doi.org/10.1016/j.aninu.2016.11.005.

[39] T.V. Bagnyukova, O.Y. Vasylkiv, K.B. Storey, V.I. Lushchak, Catalase inhibition by amino triazole induces oxidative stress in goldfish brain, Brain Res. 1052 (2005) 180–186, https://doi.org/10.1016/j.brainres.2005.06.002.

[40] P. Chelikani, I. Fita, P.C. Loewen, Diversity of structures and properties among catalases, Cell. Mol. Life Sci. 61 (2004) 192–208, https://doi.org/10.1007/s00018-003-3206-5.

[41] Q.G. Meng, J. Chen, C.C. Xu, Y.Q. Huang, Y. Wang, T.T. Wang, X.T. Zhai, W. Gu, W. Wang, The characterization, expression and activity analysis of superoxide dismutases (SODs) from *Procambarus clarkii*, Aquaculture 406–407 (2013) 131–140, https://doi.org/10.1016/j.aquaculture.2013.05.008.

[42] L. Wang, Z.Q. Wu, X.L. Wang, Q. Ren, G.S. Zhang, F.F. Liang, S.W. Yin, Immune responses of two superoxide dismutases (SODs) after lipopolysaccharide or *Aeromonas hydrophila* challenge in pufferfish (*Takifugu obscurus*), Aquaculture 459 (2016) 1–7, https://doi.org/10.1016/j.aquaculture.2016.03.016.

[43] W.K. Ren, Y.L. Yin, G. Liu, X.L. Yu, Y.H. Li, G. Yang, T.J. Li, G.Y. Wu, Effect of dietary arginine supplementation on reproductive performance of mice with porcine circovirus type 2 infection, Amino Acids 42 (2012) 2089–2094.

[44] M.E. Kotas, R. Medzhitov, Homeostasis, inflammation, and disease susceptibility, Cell 160 (2015) 816–827, https://doi.org/10.1016/j.cell.2015.02.010.

[45] Y. Mu, M. Li, F. Ding, Y. Ding, J. Ao, S. Hu, X. Chen, De novo characterization of the spleen transcriptome of the large yellow croaker (*Pseudosciaena crocea*) and analysis of the immune relevant genes and pathways involved in the antiviral response, PLoS One 9 (5) (2014) e97471, , https://doi.org/10.1371/journal.pone.0097471.

[46] J.H. Xu, Z. Jing, Y.Q. Li, Y.Y. Zou, B.J. Lu, Y.Y. Chen, Y.Z. Ma, H.S. Xu, Evaluation of differentially expressed immune-related genes in intestine of *Pelodiscus sinensis*, after intragastric challenge with lipopolysaccharide based on transcriptome analysis, Fish Shellfish Immunol. 56 (2016) 417–426, https://doi.org/10.1016/j.fsi.2016.07.032.

[47] M. Ashburner, C.A. Ball, J.A. Blake, D. Botstein, H. Butler, J.M. Cherry, A.P. Davis, K. Dolinski, S.S. Dwight, J.T. Eppig, M.A. Harris, D.P. Hill, L. Issel-Tarver, A. Kasarskis, S. Lewis, J.C. Matese, J.E. Richardson, M. Ringwald, G.M. Rubin, G. Sherlock, Gene ontology: tool for the unification of biology. The Gene Ontology Consortium, Nat. Genet. 25 (1) (2000) 25–29, https://doi.org/10.1038/75556.

[48] R.L. Tatusov, E.V. Koonin, D.J. Lipman, A genomic perspective on protein families, Science 278 (5338) (1997) 631–637, https://doi.org/10.1126/science.28.5338.631.

[49] M.M. Zhang, G. Qiao, Q. Li, D.H. Xu, Z.T. Qi, A.M. Wang, M.Y. Xu, J.T. Huang, Transcriptome analysis and discovery of genes involved in immune pathways from coelomocytes of *Onchidium struma* after bacterial challenge, Fish Shellfish Immunol. 72 (2018) 528–543, https://doi.org/10.1016/j.fsi.2017.11.024.

[50] M. Kanehisa, Y. Sato, M. Kawashima, M. Furumichi, M. Tanabe, KEGG as a reference source for gene and protein annotation, Nucleic Acids Res. 44 (D1) (2015) D457–D462, https://doi.org/10.1093/nar/gkv1070.

[51] J. Chen, Y. Li, W. Wang, L. Xia, Z. Wang, S. Hou, J. Huang, Y. Lu, Transcriptome analysis of immune-related gene expression in hybrid snakehead (*Channa maculata* ♀ × *Channa argus* ♂) after challenge with *Nocardia seriolae*, Fish Shellfish Immun. 81 (2018) 476–484, https://doi.org/10.1016/j.fsi.2018.07.039.

[52] S. Changyou, L. Bo, X. Pao, J. Xie, X.P. Ge, Q.L. Zhou, C.X. Sun, H.M. Zhang, F. Shan, Z.F. Yang, Oxidized fish oil injury stress in *Megalobrama amblycephala*: evaluated by growth, intestinal physiology, and transcriptome-based PI3K-Akt/NF-κB/TCR inflammatory signaling, Fish Shellfish Immunol. 81 (2018) 446–455, https://doi.org/10.1016/j.fsi.2018.07.049.

[53] Y. Yang, S.C. Kim, T. Yu, Y.S. Yi, M.H. Rhee, G.H. Sung, B.C. Yoo, J.Y. Cho, Functional roles of p38 mitogen-activated protein kinase in macrophage-mediated inflammatory responses, Mediat. Inflamm. (2014) 352–371, https://doi.org/10.1155/2014/352371.

[54] B.C. Liu, D.M. Han, T.T. Zhang, G.H. Cheng, Y.L. Lu, J.B. Wang, H.F. Zhao, Z.P. Zhao, Hypoxia-induced autophagy promotes EGFR loss in specific cell contexts, which leads to cell death and enhanced radio sensitivity, Int. J. Biochem. Cell Biol. 111 (2019) 12–18, https://doi.org/10.1016/j.biocel.2018.09.013.

[55] X.T. Wang, Y. Chen, X.T. Zuo, N. Ding, H. Zeng, X. Zou, X. Han, Microcystin (-LR) induced testicular cell apoptosis via up-regulating apoptosis-related genes *in vivo*, Food Chem. Toxicol. 60 (2013) 309–317, https://doi.org/10.1016/j.fct.2013.07.039.

[56] J.R. Glossop, S.H. Cartmell, Effect of fluid flow-induced shear stress on human mesenchymal stem cells: differential gene expression of IL1B and MAP3K8 in MAPK signaling, Gene Expr. Patterns 9 (2009) 381–388, https://doi.org/10.1016/j.gep.2009.01.001.

[57] Y.A. Ivanenkov, K.V. Balakin, Y. Lavrovsky, Small molecule inhibitors of NF-κB and JAK/STAT signal transduction pathways as promising anti-inflammatory therapeutics, Mini Rev. Med. Chem. 11 (2011) 55–78, https://doi.org/10.2174/138895511793564079.

[58] H.Y. Chung, M. Cesari, S. Anton, E. Marzetti, S. Giovannini, A.Y. Seo, C. Carter, B.P. Yu, C. Leeuwenburgh, Molecular inflammation: underpinnings of aging and age-related diseases, Ageing Res. Rev. 8 (2009) 18–30, https://doi.org/10.1016/j.arr.2008.07.002.

[59] M.Y. Li, L. Sun, X.T. Niu, X.M. Chen, J.X. Tian, Y.D. Kong, G.Q. Wang, Astaxanthin protects lipopolysaccharide-induced inflammatory response in *Channa argus* through inhibiting NF-κB and MAPKs signaling pathways, Fish Shellfish Immunol. 86 (2019) 280–286, https://doi.org/10.1016/j.fsi.2018.11.011.

[60] S.Y. Choi, J.H. Hwang, S.Y. Park, Y.J. Jin, H.C. Ko, S.W. Moon, S.J. Kim, Fermented guava leaf extract inhibits LPS induced COX-2 and i-NOS expression in mouse macrophage cells by inhibition of transcription factor NF-κB, Phytother Res. 22 (2008) 1030–1034, https://doi.org/10.1002/ptr.2419.

[61] S. Goldar, M.S. Khaniani, S.M. Derakhshan, B. Baradaran, Molecular mechanisms of apoptosis and roles in cancer development and treatment, Asian Pac. J. Cancer Prev. APJCP 16 (6) (2015) 2129–2144, https://doi.org/10.7314/APJCP.2015.16.6.2129.

[62] S. Noorolyai, N. Shajari, E. Baghbani, S. Sadreddini, B. Baradaran, The relation between PI3K/Akt signaling pathway and cancer, Fish Shellfish Immunol. 698 (2019) 120–128, https://doi.org/10.1016/j.gene.2019.02.076.

[63] T.F. Franke, D.R. Kaplan, L.C. Cantley, A. Toker, Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3, 4-bisphosphate, Science 275 (1997) 665–668, https://doi.org/10.1126/science.275.5300.665.

[64] O.N. Ozes, L.D. Mayo, J.A. Gustin, S.R. Pfeffer, L.M. Pfeffer, D.B. Donner, NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase, Nature 401 (6748) (1999) 82–85, https://doi.org/10.1038/43466.

[65] L.P. Kane, V.S. Shapiro, D. Stokoe, A. Weiss, Induction of NF-kappaB by the Akt/PKB kinase, Curr. Biol. 9 (11) (1999) 601–604, https://doi.org/10.1016/S0960-9822(99)80265-6.

[66] S. Schutze, K. Wiegmann, T. Machleidt, M. Kronke, TNF-induced activation of NF-κB, Immunobiology 193 (2–4) (1995) 193–203, https://doi.org/10.1016/s0171-2985(11)80543-7.

[67] Y.F. Duan, Y. Zhang, H.B. Dong, Y. Wang, J.S. Zhang, Effects of dietary (PHB) on microbiota composition and the mTOR signaling pathway in the intestines *of Litopenaeus vannamei*, J. Microbiol. 55 (12) (2017) 946–954, https://doi.org/10.1007/s12275-017-7273-y.

[68] C.M. Guire, M. Prinz, R. Beyaert, G.V. Loo, Nuclear factor kappa B (NF-κB) in multiple sclerosis pathology, Trends Mol. Med. 19 (10) (2013) 604–613, https://doi.org/10.1016/j.molmed.2013.08.001.

[69] Y. Palti, Toll-like receptors in bony fish: from genomics to function, Dev. Comp. Immunol. 35 (12) (2011) 0–1272, https://doi.org/10.1016/j.dci.2011.03.006.

[70] J.E. Smith-Garvin, G.A. Koretzky, M.S. Jordan, T cell activation, Annu. Rev.

Immunol. 27 (1) (2009) 591–691, https://doi.org/10.1146/annurev.immumol.021908.132706.

[71] J. Zhang, X.H. Kong, C.J. Zhou, L. Li, G.X. Nie, X.J. Li, Toll-like receptor recognition of bacteria in fish: ligand specificity and signal pathways, Fish Shellfish Immun. 41 (4) (2014) 380–388, https://doi.org/10.1016/j.fsi.2014.09.022.

[72] Y. Palti, M.F. Rodriguez, S.A. Gahr, M.K. Purcell, C.E. Rexroad, G.D. Wiens, Identification, characterization and genetic mapping of TLR1 loci in rainbow trout (*Oncorhynchus mykiss*), Fish Shellfish Immunol. 28 (5) (2010) 918–926, https://doi.org/10.1016/j.fsi.2010.02.002.

[73] S. Akira, S. Uematsu, O. Takeuchi, Pathogen recognition and innate immunity, Cell 124 (4) (2006) 783–801, https://doi.org/10.1016/j.cell.2006.02.015.

[74] A. Rebl, T. Goldammer, H.M. Seyfert, Toll-like receptor signaling in bony fish, Vet. Immunol. Immunopathol. 134 (3) (2010) 0–150, https://doi.org/10.1016/j.vetimm.2009.09.021.

[75] K.D. Smith, E. Andersen-Nissen, F. Hayashi, K. Strobe, M.A. Bergman, S.L.R. Barrett, B.T. Cookson, A. Aderem, Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility, Nat. Immunol. 4 (12) (2003) 1247–1253, https://doi.org/10.1038/ni1011.

[76] T. Tsujita, H. Tsukada, M. Nakao, H. Oshiumi, M. Matsumoto, T. Seya, Sensing bacterial flagellin by membrane and soluble orthologs of Toll-like receptor 5 in rainbow trout (*Onchorhyn*chus *mikiss*), J. Biol. Chem. 279 (47) (2004) 48588, https://doi.org/10.1074/jbc.M407634200.

[77] I. Munoz, M.P. Sepulcre, J. Meseguer, V. Mulero, Molecular cloning, phylogenetic analysis and functional characterization of soluble Toll-like receptor 5 in gilthead seabream (*Sparus aurata*), Fish Shellfish Immunol. 35 (1) (2013) 36–45, https://doi.org/10.1016/j.fsi.2013.03.374.

[78] J.C. Roach, G. Glusman, L. Rowen, A. Kaur, M.K. Purcell, K.D. Smith, L.E. Hood, A. Adeerem, The evolution of vertebrate Toll-like receptors, Proc. Natl. Acad. Sci. U.S.A. 102 (27) (2005) 9577–9582, https://doi.org/10.1073/pnas.0502272102.

[79] A. Ishii, A. Matsuo, H. Sawa, T. Tsujita, K. Shida, M. Matsumoto, T. Seya, Lamprey TLRs with properties distinct from those of the variable lymphocyte receptors, J. Immunol. 178 (1) (2007) 397–406, https://doi.org/10.4049/jimmunol.178.1.397.

[80] S.D. Hwang, H. Kondo, I. Hirono, T. Aoki, Molecular cloning and characterization of Toll-like receptor 14 in Japanese flounder (*Paralichthys olivaceus*), Fish Shellfish Immun. 30 (1) (2011) 425–429, https://doi.org/10.1016/j.fsi.2010.08.005.

[81] P.R. Rauta, M. Samanta, H.R. Dash, B. Nayaka, S. Das, Toll-like receptors (TLRs) in aquatic animals: signaling pathways, expressions and immune responses, Immunol. Lett. 158 (1) (2014) 14–24, https://doi.org/10.1016/j.imlet.2013.11.013.

[82] R. Madzhitov, Toll-like receptors and innate immunity, Nat. Rev. Immunol. 1 (2) (2001) 135–145, https://doi.org/10.1038/35100529.