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Research Article

Dietary PHB Supplementation Strengthens the Gill Immune Barrier and Disease Resistance of *Carassius auratus gibelio* against CyHV-2 Early Infection

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Gibel carp (*Carassius auratus gibelio*) is an important economic fish species in China, but its development is seriously restricted by the crucian carp hematopoietic organ necrosis caused by Cyprinid herpesvirus 2 (CyHV-2). Gill is the key portal for CyHV-2 primary invasion, and the gill immune barrier is the first defense line against infection. Our previous studies showed that dietary poly-β-hydroxybutyrate (PHB) supplementation can enhance the disease resistance of gibel carps against CyHV-2 infection, and antioxidants play an important role against this infection. However, the effect of PHB supplementation on the gill immune barrier and primary proliferation of CyHV-2 in the gill is unclear. Herein, results showed that (1) PHB supplementation significantly increased the activities of SOD, GST, and other antioxidant-related enzymes in the gill and gill mucus; (2) PHB significantly upregulated the transcription of genes involved in the JAK/STAT signaling pathway, Th1 and Th2 cell differentiation, tryptophan metabolism, and MAPK signaling pathway; (3) PHB effectively reduced the cumulative mortality of fish post-CyHV-2 infection; (4) PHB significantly inhibited primary CyHV-2 proliferation in the gill; (5) at the early stage (6 hpi) of CyHV-2 infection, the transcription of *IFN-γ*, *IFN-γ*R, *TNF-α*, *JUN*, *TCR-β*, *IL*-4R, *STAT1*, and *AhR* in the gill was significantly upregulated by 14.15-folds, 6.55-folds, 9.32-folds, 6.77-folds, 9.84-folds, 5.55-folds, 9.11-folds and 13.06-folds, respectively; and (6) during the late stages (96 hpi and 168 hpi) of infection, some genes in the kidney and intestine were continuously upregulated. In conclusion, PHB can improve the antioxidant capacity of the gill, enhance the immune response, and inhibit the viral proliferation in the gill at the early infection stage. It is of great significance for us to develop novel prevention strategies to control CyHV-2 infection, considering immuno-modulation of the primary invasion organ, the gill, and host antioxidation.

1. Introduction

Gibel carp (*Carassius auratus gibelio*) is a major freshwater culture fish species due to the fast growth, delicious meat, and strong stress resistance [1]. In 2021, more than 2,700,000 tons of gibel carps with an economic value of 5 billion dollars were produced [2]. However, with the continuous expansion of the aquaculture scale and promotion of intensive breeding models, diseases caused by viruses, bacteria, and parasites have seriously threatened its culture-development [3–5]. Among them, crucian carp hematopoietic organ necrosis caused by cyprinid herpesvirus 2 (CyHV-2) has a mortality rate of almost 90%~100%, which results in huge economic losses for farmers and almost leads to the culture disappearance of this species [6]. Notwithstanding, the vaccine is the most appropriate and effective strategy to control viral

diseases, its development has not been enough to diminish the risk of disease in gibel carps [7, 8]. At present, the widely used methods to prevent and control diseases are to improve water quality, develop immuno-enhancements, and Chinese herbs and germ plasm resource [9–13].

Poly-β-hydroxybutyrate (PHB) is a compound that stores intracellular energy and carbon sources in microorganisms [14, 15]. PHB can be hydrolyzed into β-hydroxybutyrate (β-HB) by enzymes secreted by the intestinal tract of aquatic animals, which exhibits similar functions to short-chain fatty acids (SCFAs), such as antibacterial and antiviral activity [16, 17]. Growing studies illustrate that PHB in aquaculture water or diets can enhance the host immunity and disease resistance of aquatic animals [18]. PHB supplementation at a 1% level can improve the activities of antioxidant enzymes such as SOD, CAT, and POD in the serum of *Fenneropenaeus chinensis*, and enhance the body's antioxidant and antistress abilities [19, 20]. PHB addition at a 4% level can modulate the intestinal microflora of gibel carps, exert mucosal immunity, enhance the growth of probiotics, and inhibit the replication of CyHV-2 [21]. Nile tilapia (*Oreochromis niloticus*) larvae fed with PHB-enriched *Artemia nauplii* shows a higher survival rate of larvae post *Edwardsiella ictaluri* infection by 20% [22]. Recently, PHB has been reported to reduce total coliform counts and Enterobacteriaceae counts [23].

For aquatic animals, gills have been gradually realized to show four barrier functions, including microbial, chemical, physical, and immune barriers [24]. Gills are composed of the gill arch, gill filament, and gill rake, which show many important physiological processes, such as gas exchange, osmotic pressure regulation, ion transport, ammonia nitrogen excretion, and barrier function against external material invasion [24–26]. Aquaculture water contains numerous microorganisms, and gills are one of the fish organs which are directly exposed to pathogens and much easier infected by pathogens [27, 28]. To protect aquatic animals against pathogenic infection, the immunological and physical barriers of the gills are formed by the mucous layer, epithelial cells, and close connections between cells on the gill surface [29–31]. The immune barrier is tightly associated with antibacterial compounds, cytokines, and antibodies [32]. It was reported that antibody-secreting cells (ASC) in gills were increased in European sea bass (*Dicentrarchus labrax* L.) postinoculation with *Photobacterium damselae* [33]. The transcription of immunoglobulin light chain (Ig L) gene in the gill of *Takifugu rubripes* postexposure to external antigens was upregulated [34]. *Flavobacterium columnare* infection could significantly up-regulate the transcription of immune-related genes in gill of *Oncorhynchus mykiss*, such as *NF-κ*B and *MAPK* [35, 36]. Dietary pyridoxine (PN) supplementation impairs the immune barrier of the gill in grass carp (*Ctenopharyngodon idella*) after challenged with *F. columnare*, focusing on the improvement of antioxidant enzyme activities and mRNA expression of cytokines [37]. Dietary vitamin C, angelica, and glucan supplementation regulates the immune barrier of the gills in fish [10, 35, 38]. All these studies indicate that the immune barrier of the gill and gill mucus can be modulated by nutrition, and that it plays an important role against pathogenic invasion or infection.

It was reported that gills are the key portal for CyHV-2 invasion, and the immune barrier of gills is the first defense line against CyHV-2 invasion [39, 40]. Up to now, limited studies about the effect of gill barrier modulation on preventing CyHV-2 infection have been reported. In the present study, an effective prevention of PHB against CyHV-2 infection was chosen to investigate its effects on antioxidant enzyme activities and antiviral gene expression in the gill and early viral replication in the invasion portal. The results from this study will be expected to illustrate that nutrition can modulate the gill immune barrier, and this modulation might be helpful to protect hosts against CyHV-2 early infection. It will also provide valuable information or potential antioxidants to prevent this disease and thus promote the sustainable culture of gibel carps.

2. Materials and Methods

2.1. Diet Preparation. Diets were prepared following our previous research [18], and the composition is given in Table 1. Briefly, the basal diet was used as a control diet, and the diet with 4% (w/w, PHB weight/diet weight) PHB supplementation was used as the PHB experimental diet [18]. All the ingredients were ground into powder, sieved through a 80-mesh filter, blended in a mixer thoroughly, and then fully mixed with fish oil and bean oil. Finally, a proper amount of water was added to ensure the uniformity of granulation. The 2-mm-diameter granulated feed was wet-extruded by a pelletizer (F-26, South China University of Technology, Guangzhou, China) and dried at 60°C for 12 h in an oven until the moisture of diet was less than 10%. Then, all diets were sealed in plastic bags and stored at −20°C until used.

2.2. Fish and Feeding Trial. Gibel carps (average body weight of 7.5 ± 0.5 g) were obtained from a fish farm located at the Sheyang, Jiangsu province. Prior to the experiment, the fish were acclimated for two weeks and fed with the basal diet. Water was exchanged daily by 1/4-1/3, and water qualities were maintained at a water temperature of 23–25°C, *pH* 6.8–7.3, dissolved oxygen (DO) higher than $5\,mg{\cdot}L^{-1}$, NH_4^+-N, and NO_2^--N less than $0.1\,mg{\cdot}L^{-1}$. Each tank was provided with 24-h continuous aeration. After acclimation, a total of 240 healthy fish with a similar size were randomly divided into two groups (Control and PHB), and each group included triplicate tanks (volume of 400 L) with 40 fish. Fish were fed the experimental diets (Table 1) to satiation three times (7 : 00 to 7 : 30, 13 : 00 to 13 : 30, and 19 : 30 to 20 : 00) each day within the 30-day feeding trial following the previous report [18].

2.3. Fish Sampling. At 30-day feeding, three fish from each tank were anesthetized with MS-222 at $100\,mg{\cdot}L^{-1}$. After dissection, gills were sampled, and continuous gill mucus was collected. All the samples were stored at −80°C until analysis.

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

Ingredients	Experimental groups	
	Control	PHB (4%, PHB weight/diet weight)
Peruvian fish meal[a]	120	120
Soybean meal[a]	280	280
Rapeseed meal[a]	140	140
Wheat flour[a]	170	170
Corn starch[a]	50	50
Fish oil[a]	40	40
Soybean oil[a]	40	40
Monocalcium phosphate[a]	20	20
MVP[a,d]	20	20
CMC[b]	120	80
PHB[c]	0	40
Total	1000	1000

[a]Provided by Hengxing Feed Co. Ltd., Yancheng, China. [b]CMC, carboxymethy cellulose, was purchased from Shanghai Jiande Industrial Co. Ltd., China. [c]PHB was purchased from Ningbo Tianan Biological Material Co., Ltd., China. [d]MVP, mineral and vitamin premixes (g, mg or IU kg^{-1}): 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 B_1, 50 mg; vitamin B_2, 20 mg; vitamin B_6, 50 mg; vitamin B_{12}, 0.1 mg; niacin, 250 IU; pantothenic acid, 50 mg; folic acid, 15 mg; vitamin A, 5000 IU; vitamin C, 400 mg; vitamin D_3, 2000 IU; vitamin E, 400 mg; vitamin K_3, 40 mg.

2.4. Antioxidant-Related Enzyme Activity Analysis. To determine the effect of PHB supplementation on antioxidant-related parameters in gills and gill mucus, the activities of SOD, OH^-, CAT, T-AOC, GR, GST, and GSH were measured using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturers' instructions. In brief, SOD was determined with a hydroxylamine method for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The inhibition activity of SOD was determined by measuring adsorption at 450 nm [41]. The hydroxyl radical was determined by the Fenton reaction, and its ability to inhibit the hydroxyl radical was determined at 550 nm. In the CAT assay, CAT activity was determined by measuring absorption at 405 nm [41]. 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 [41]. For the GR assay, HcTrxR3 (2 mG) was added to a mixture of assay buffer containing 0.1 mM NADPH and 0.05 mM GSSG. The decrease in absorbance at 340 nm was measured. The GST activity reaction mixture contained a suitable amount of the enzyme, KH_2PO_4 buffer (pH 7.4), CDNB (1 mM), and GSH (6 mM). The reaction was carried out at 37°C and monitored spectrophotometrically by the increase in absorbance of the conjugation of GSH with CDNB at 340 nm for 3 min [42]. GSH level, a precipitating solution containing 1 mM EDTA and 100 g/L TCA was added to 500 μL tissue supernatant to precipitate the protein contents and centrifuged at 15,000 rpm for 10 min. After centrifugation, DTNB solution was added to the supernatant, and the absorbance was measured at 412 nm using a microplate reader. A suitable amount of 0.3 M Na_2HPO_4 (disodium phosphate) and DTNB solution were added, and the GSH level was measured spectrophotometrically at 412 nm [42]. All enzyme activities were defined as the amount of enzyme unit per mg protein.

2.5. Immune-Related Gene Expression by RT-qPCR

2.5.1. RNA Extraction and cDNA Synthesis. The samples containing 200 μL RNAiso Plus (Sigma) were unfrozen, the total RNA was extracted as described previously [21]. Briefly, RNA was extracted with the RNeasy mini kit (Qiagen, Valencia, CA, USA) following the manufacturers' protocol. The isolated RNA was treated with DNase I (Qiagen) to minimize contamination of genomic DNA. The quantity of RNA samples was determined on a Nanodrop ND-1000 spectrophotometer. The purity of the extracted RNA was determined according to the OD_{260nm}/OD_{280nm} ratio, and the expected values were 1.8–2.0. First-strand cDNA was synthesized using the Prime-script™ reagent kit with a gDNA eraser (Takara Bio, Dalian, China) following the manufacturer's protocol. The synthesized cDNA was stored at −80°C until mRNA expression analysis.

2.5.2. Relative Expression Analysis of Immune-Related Genes by RT-qPCR. Based on our RNA-seq data (deposited in NCBI under accession numbers of SRR12596365/66/67/68/69/70) constructed from the spleen of gibel carps in PHB and control groups, 24 differentially expressed genes involved in the JAK/STAT signaling pathway, MAPK signaling pathway, tryptophan metabolism, and Th1 and Th2 cell differentiation were selected to study the effect of PHB on the immune barrier of gills in this study. Among them, genes *IFN-γ*, *IFNγ*R, *JAK*, *JAK1*, *STAT1*, and *STAT5* were involved in the JAK/STAT signaling pathway; genes *TNF-α* and *JUN* involved in MAPK signaling pathway; genes *AhR* and *CYP1A1* involved in tryptophan metabolism; genes T-*bet*, *Gata3*, *TCR-β*, *IL*-4, *IL*-4R, *IL*-2RB, *IL*-10, *IL*-12B, *IL*-12RB, *DLL*, *MHC*-II, cytokines; and *RUNX3* and *IL*-17RA involved in Th1 and Th2 cell differentiation.

The gene-specific primers were designed with the software Primer3 (https://biotools.umassmed.edu/bioapps/primer3_www.cgi). Sequences are listed in Table 2, and primers were synthesized by Sangon Biotech (Shanghai, China). The β-actin gene was used as an internal calibrator to normalize cDNA quantities. The relative expression of the target genes was determined using a CFX96 Real-Time PCR Detection System (Bio-Rad, USA) with the SYBR® Premix Ex Taq™ kit (Takara Bio Co., Ltd., Dalian, China). The total volume of RT-qPCR mixture was 25 μL, including 12.5 μL of SYBR® Premix Ex Taq™, 1.0 μL each of primers (10 μM), 2.0 μL of cDNA template (<100 ng), and 8.5 μL of nuclease-free water. The RT-qPCR thermal cycling parameters were 95°C for 30 s, 40 cycles of 95°C for 5 s, and 56.9–64.3°C for 30 s, followed by dissociation curve analysis at 65°C for 5 s to verify the amplification of a single product. The relative fold

Table 2: Sequences of primers used in this study for RT-qPCR.

Genes	Sequence of forward primer ($5' \longrightarrow 3'$)	Sequence of reverse primer ($5' \longrightarrow 3'$)
β-actin	GATGATGAAATTGCCGCACTG	ACCGACCATGACGCCCTGATGT
IFN-γ	CCCAGTCGTCCAGCACAAAGC	TTTGTCGTCTCCTGCGCTCTTTG
*IFNγ*R	AGCCCCTCCGCCTGGAAATC	GGACTTGGACATGCTTGCAGAGG
TNF-α	CATTCCTACGGATGGCATTTACTT	CCTCAGGAATGTCAGTCTTGCAT
T-*bet*	CTTCACGAGAACCGATTCCGATGG	TCTGACAGATTCACAGCGTTTGGG
JAK	CGCTCTCCGTGTAGACCTGATCC	GTGGCAGCGGTGAGCAAGTG
JAK1	CCATCCAGGACAGCAACATAACCC	AGATCCCTCGCTCTCACCAGATAC
JUN	CGCCCTCGCTCTCTCCCATC	AGCACCTTGACCTTCTCCTCCAG
Gata3	ACACAATATCAACCGACCGCTCAC	TTGCTCTTCTTGGACTTGCTGGAC
TCR-β	GAAGCCCTCTGAAATCGAGACTGG	GCTCACATGGTCTGGGTAGAAGTC
IL-4	GTGAGTCTCCTGAACAGCCTGAAC	AGTGTGATGAGTTGTGGCGTCTTC
IL-4R	ATCACCATTCCCTGCCAACAATCC	GCACGGCTGTGTTCTCTTGAGG
STAT1	CAGTGGTGGCAGAGTGTCTTCAAC	CTGGTCATCTAGCTGGCTCTTGC
STAT5	GCTTGCTGGTCGGAGGGAAAC	ACTGCTGTCATTGCGGGTGTTC
IL-2RB	ACAGCCCATGAGCCCAGACTC	TCCTTCAGCCGCACATCTCTCC
IL-10	GTCCAGAAGAACCACTTGCACTCC	TTTAGGGTGGCGAACTCAAAGGG
IL-12B	AGCCCCCTGAAACATGGAGT	ACTTCAACACGCCTTCCTGTG
IL-12RB	TGGGGCAATAAGTGGAGGAGAGG	GGAAAGGGCTGTGAGTGCTTGG
AhR	TGCTCTCCGCCCTCTTCTTCTAC	TGCTCTCCGCCCTCTTCTTCTAC
CYP1A1	GATCCGTCAGTGGTGGTCTCAAAG	GCAGAGCGAAGGCACAGAGAAC
DLL	ATTTCTGCCGTTCACGAGATGACC	AATCGCCATTCCATCCAGGTAAGC
MHC-II	ACGCCTGCTGAAGGAGACATTTAC	TCAGACCCACTCCGCAGAACAC
Cytokines	ACGGTGTCGGGTCCATAGTCATC	GGAAAGGAATCATGGCTGGAGAGG
RUNX3	AGCACTGGAATCGGCGGTCTC	TACGGTGAGGAGTTGGTCTGGAAG
IL-17RA	GTGATATGCTGACTCTCGCCCTTC	ACATCGCCTTCGCTGCTTACATC
CyHV-2	TTAGCGTCAGGTCCATAG	GGCGTGTAGAAATCAAACT

changes of mRNA expression of target genes in fish from the PHB group were compared to the control group using the $2^{-\Delta\Delta Ct}$ method [43].

2.6. Effect of PHB on the Disease Resistance of Gibel Carps

2.6.1. CyHV-2 Solution Preparation and Viral Quantification. CyHV-2 solution was prepared as previous description by Qiao et al. [21]. In brief, the kidney from a naturally CyHV-2-infected gibel carp with typical signs of gill hemorrhage was sampled, homogenized by mixing with 10 times volume of PBS (0.01 M, pH7.2), centrifuged at $12{,}000 \times g$ for 30 min, and filtered to remove bacteria.

DNA from the kidney was extracted using the viral DNA extraction kit (TaKaRa, China), and the extracted DNA assessed and adjusted to $50\ \text{ng}\cdot\mu\text{L}^{-1}$ by Biophotometer Plus (Eppendorf). The primers (CyHV-2-RT-F and CyHV-2-RT-R) for quantitative real-time PCR (qPCR) are listed in Table 2. The qPCR reactions were performed using the SYBR® Premix ExTaq™ kit (Takara Bio Co., Ltd., Dalian, China) in a total volume of 25 μL, containing 12.5 μL SYBR Premix Ex Taq II (TaKaRa, Japan), 2 μL template DNA, 1 μL each primer, and 8.5 μL ddH_2O. Distilled water replaced the extracted DNA as negative control for each new run. The thermal cycling condition was as follows: one cycle of 95°C for 3 min, followed by 40 cycles at 95°C for 5 s and 55°C for 30 s, then by dissociation curve analysis (65–95°C: increment 0.5°C for 5 s) [21]. After amplification, melting curve analysis was performed, and Ct values were obtained. The standard curve of viral DNA copy number is shown in Figure S1. Plasmid DNA containing the sequence of the CyHV-2 helicase gene was selected to serve as the standard for virus quantification. The amplified DNA fragment was gel-purified using a Fermentas Gel Extraction Kit (Thermo). The resulting DNA fragment was inserted into the pMD19-T plasmid to produce pMD-CyHV-2. A ten-fold series dilution of pMD-CyHV-2 was used as the standard template in qPCR. Finally, a viral stock solution with a concentration of 1.5×10^5 copies/μL to 1.5×10^7 copies/μL was prepared.

2.6.2. Effect of PHB on the Cumulative Mortality of Fish Post CyHV-2 Infection. At 30 days of feeding, fish from both PHB and control groups were treated with PBS and CyHV-2 solutions. Each group included six tanks (volume of 100 L), and each tank had eight individuals. All fish were treated with PBS or CyHV-2 solution through "per-gill" method, mocking natural infection. This method makes only the gills directly exposed to viruses, and it has been used to infect carps like KHV and CHNV [40, 44], since the gill is the first organ of aquatic animals exposed to pathogens and a key invasion portal for CyHV-2 [39, 45, 46]. Briefly, fish were anesthetized with MS-222 at 100 mg/L, and inoculated with CyHV-2 solution (1.5×10^5 copies/μL to 1.5×10^7 copies/μL) at 10 μL/fish^{-1} into both sides of the gills in air. An equal volume of PBS was inoculated into the gills as the control group. After gill exposure to viral solution or PBS, fish were wrapped with wet papers and kept in air for 5–7 min at 25°C to allow the virus to adsorb into the gill tissue [40, 44]. Then, fish were returned to the tank and maintained at 25°C with

24-h continuous aeration circulation. During the challenge test, fish were not fed, the water temperature was maintained at 23–25°C, and continuous aeration provided. Mortality was recorded daily for 14 days postinjection (dpi).

2.6.3. Effect of PHB on the CyHV-2 Replication in Gills In Vivo. Challenge tests were conducted as described in the above 2.6.2. Fish were infected by CyHV-2 solution (3.0×10^7 copies/fish) through gills, and three individuals from both control and PHB groups were sampled at 0, 6 hpi, 24 hpi, 72 hpi, 120 hpi, 168 hpi, 216 hpi, 288 hpi, and 360 hpi to analyze the effect of PHB supplementation on early replication of CyHV-2 in gills *in vivo*. Gills were taken, and CyHV-2 load detected by qPCR as described in part 2.6.1. All samples were tested in triplicate.

2.6.4. Effect of PHB on the Immune Response of Fish against CyHV-2 Infection. Challenge tests were conducted as described in the above 2.6.2. At 6 hpi, 24 hpi, 96 hpi, and 168 hpi, tissues including gill, kidney, and intestine of fish from both PHB and control groups were sampled, dissected, and stored in 200 μL RNAiso Plus (Sigma) at −80°C. RNA extraction, cDNA synthesis, and RT-qPCR were conducted as described in part 2.5. The relative expression of genes in PHB-CyHV-2-infection group against the control-CyHV-2-infection group was calculated using the $2^{-\Delta\Delta Ct}$ method [43].

2.7. Statistical Analysis. All data are expressed as the mean ± SD for triplicates, preliminarily arranged using Excel 2017, and then analyzed by Students' *t*-test with the SPSS software (version 24.0). GraphPad Prism 8.0 was used for graphics. A statistically significant difference was considered at $P \leq 0.05$.

3. Results

3.1. Antioxidant-Related Enzyme Activities in the Gill. In the gill tissue, the activities of OH^- and CAT in the PHB group were higher than that in the control group, while the activity of T-AOC was significantly lower than the control group ($P \leq 0.05$). In the gill mucus, activities of SOD, T-AOC, GSH, and GST in the PHB group were significantly increased, while the activities of OH^-, CAT, and GR were significantly decreased ($P \leq 0.05$) (Table 3).

3.2. Effects of PHB on the Transcription of Some Immune-Related Genes in the Gill. In the JAK/STAT signaling pathway, the expression levels of *IFN* and *JAK* in the PHB group were significantly upregulated by 2.24-folds and 4.61-folds, respectively; the expression of *IFNγR*, *JAK1*, *STAT1*, and *STAT5* between two groups was not significantly different (Figure 1(a)). In the Th1/Th2 cell differentiation, the transcription of T-*bet*, *Gata3*, *TCR-β*, *IL*-4R, *IL*-10, *DLL* and *RUNX3* in the PHB group was significantly upregulated by 2.40-folds, 3.03-folds, 2.42-folds, 2.55-folds, 2.38-folds, 3.75-folds, and 3.60-folds, respectively; the expression of *IL*-4, *IL*-2RB, *IL*-12B, *IL*-12RB, *MHC*-II, *IL*-17RA between two groups was not significantly different (Figure 1(b)). In the tryptophan metabolism, the transcription of *AhR* was significantly upregulated by 3.19-folds, and there was no significant difference in *CYP1A1* transcription (Figure 1(c)). In the MAPK signaling pathway, the expression level of *TNF-α* was significantly up-regulated by 3.19-folds, and the transcription of *JUN* was not significantly different (Figure 1(d)).

3.3. Cumulative Mortality. The cumulative mortality of fish infected by CyHV-2 at a concentration of $1.5\,\mu \times 10^8$ copies/fish was 100% in both PHB and control groups. Cumulative mortality (33.3%) at 1.5×10^7 copies/fish in PHB group was significantly lower than that (100%) in the control group. The relative protection rate of PHB supplementation was 66.7%. There were no deaths in both PHB and control groups while infected at the viral concentration of 1.5×10^6 copies/fish (Figure 2).

3.4. Dynamic Changes of CyHV-2 in the Gill. The time-course viral load in the gill from control and PHB groups is shown in Figure 3. At 6 hpi, the viral load in the gill from the two groups was not significantly different; the values were close to 10^4 copies/mg · gill. After 24 hpi, the viral load in the gill of fish from the PHB group was significantly lower than that of control group. The viral load in the control group at 72 hpi was highest with the value of 5.39×10^4 copies/mg · gill, and it was 6.39×10^3 copies/mg · gill in the PHB group. After 120 hpi, viral load in the gill was reduced in both PHB and control groups, but it decreased more in the PHB group ($P > 0.05$) (Figure 3).

3.5. Effect of PHB on the Immune Response of Fish against CyHV-2 Infection

3.5.1. Transcription of Immune-Related Genes in the Gill. Compared to the fish treated with PBS, genes *IFN-γ*, *IFN-γ*R, and *JAK*1 involved in JAK/STAT signaling pathway (Supplementary Figure 2a), genes T-*bet*, *TCR-β*, *IL*-12RB, *DLL*, *MHC*-II, and *RUNX3* involved in Th1 and Th2 cell differentiation (Supplementary Figure 2b), genes *AhR* and *CYP1A1* involved in tryptophan metabolism (Supplementary Figure 2c), and genes *TNF-α* and *JUN* involved in the MAPK signaling pathway (Supplementary Figure 2d) in the gill responded to CyHV-2 infection significantly.

Dietary PHB supplementation strengthened the immune response of gill against CyHV-2 early infection. Within 6 hpi, the expression of *IFN-γ*, *IFN-γ*R, *JAK*, *JAK1*, and *STAT1* involved in the JAK/STAT signaling pathway was upregulated highest by 14.15-folds, 6.55-folds, 3.66-folds, 3.55-folds, and 9.11-folds, respectively (Figure 4(a)). The transcription of most genes involved in Th1 and Th2 cell differentiation, including T-*bet*, *TCR-β*, *IL*-4R, *IL*-10, *IL*-12RB, and *DLL*, were upregulated. They were upregulated by 3.35-folds, 9.84-folds, 5.55-folds, 7.56-folds, 6.04-folds, and 6.88-folds, respectively (Figure 4(b)). The transcription of *AhR* involved in tryptophan metabolism was upregulated highest by 13.06-folds (Figure 4(c)). The transcription of

Table 3: Antioxidant-related enzyme activities.

Organs	Groups	Antioxidant-related enzymes (U/mg prot)						
		SOD	OH^-	T-AOC	CAT	GR	GSH	GST
Gill	PHB	0.29 ± 0.15	5.16 ± 1.512217	$40.20 \pm 6.58^*$	$6.79 \pm 1.79^*$	407.36 ± 1.91	25.11 ± 4.91	0.0095 ± 0.0109
	Control	0.20 ± 0.13	3.21 ± 1.24	161.21 ± 13.77	2.98 ± 0.79	401.29 ± 1.12	32.50 ± 3.85	0.0113 ± 0.0079
Gill mucus	PHB	$59.61 \pm 5.29^*$	$9149.42 \pm 73.01^*$	$113.88 \pm 18.24^*$	$358.32 \pm 29.74^*$	$0.34 \pm 0.13^*$	$7066.67 \pm 146.46^*$	$0.0027 \pm 0.0003^*$
	Control	31.09 ± 0.17	9468.59 ± 60.35	69.89 ± 11.72	438.12 ± 22.95	1.67 ± 0.47	5680.00 ± 120.77	0.0010 ± 0.0001

The data represent the mean ± standard deviation from triplicates and were analyzed by Students' *t*-test. $^*P \leq 0.05$.

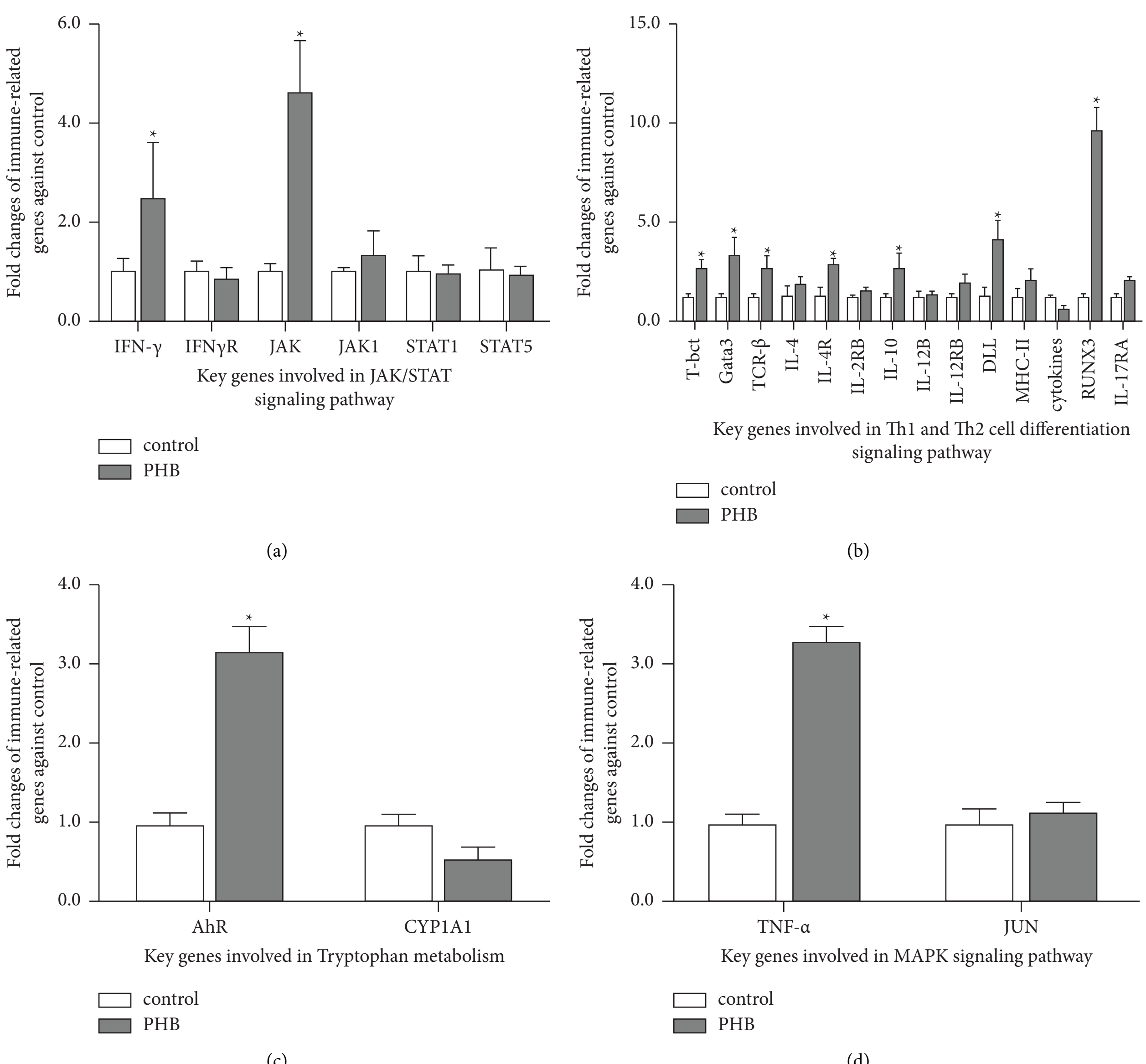

Figure 1: Effects of PHB on the transcription of some genes in the gill. The relative expression of these genes involved in the JAK/STAT signaling pathway (a), Th1 and Th2 cell differentiation signaling pathway (b), tryptophan metabolism (c), and MAPK signaling pathway (d).

JUN involved in MAPK signaling pathway was upregulated by 6.77-folds (Figure 4(d)). The transcription of *STAT5* from the JAK/STAT signaling pathway, *CYP1A1* from tryptophan metabolism, and cytokines from Th1 and Th2 cell differentiation during the whole infection were not significantly different in the gill ($P > 0.05$). After 6 hpi, the transcription of most genes in the gill was not significantly different. However, these differences could be observed in the kidney and intestine (Figures 5 and 6).

3.5.2. Transcription of Immune-Related Genes in the Kidney and Intestine. Compared to the organ gill, transcription of genes in the kidney and intestine showed more difference after 6 hpi. The mRNA expression of *IFN-γ*, *JAK*, and *STAT1* at 96 hpi and 168 hpi in the kidney was extremely significant and upregulated most by 8.07-folds and 2.81-folds, 3.80-folds and 2.02-folds, 4.66-folds, and 4.23-folds, respectively (Figure 5(a)). In Th1 and Th2 cell differentiation, mRNA expression of genes *IL*-4, *IL*-10, and *DLL* was the most significant and upregulated by 2.87-folds and 6.51-folds, 3.74-folds, and 3.60-folds, 2.11-folds and 2.06-folds, respectively (Figure 5(b). In the tryptophan metabolism, *AhR* was up-regulated most by 96 hpi-folds and 168 hpi-folds (Figure 5(c)). In the MAPK signaling pathway, *JUN* was upregulated most by 96 hpi and 168 hpi, respectively (Figure 5(d)). The transcription of some of these genes was also different at 6 hpi and 24 hpi, such as *JAK1*, *STAT5*, *GATA3*, and *RUNX3*. However, the most difference in the kidney was observed at 96 hpi and 168 hpi, especially at 96 hpi (Figure 5).

The transcription of genes in the intestine is shown in Figure 6. In the JAK/STAT signaling pathway (Figure 6(a)) and Th1 and Th2 cell differentiation (Figure 6(b)), the obvious transcriptin difference of most genes was observed at

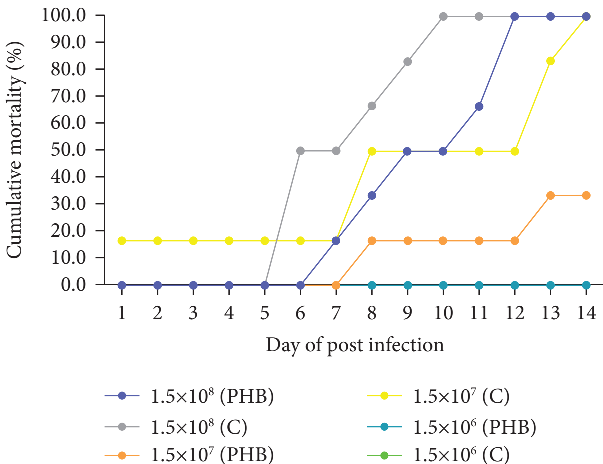

Figure 2: Effect of PHB supplementation on the cumulative mortality of fish postinfected by CyHV-2 at different concentrations.

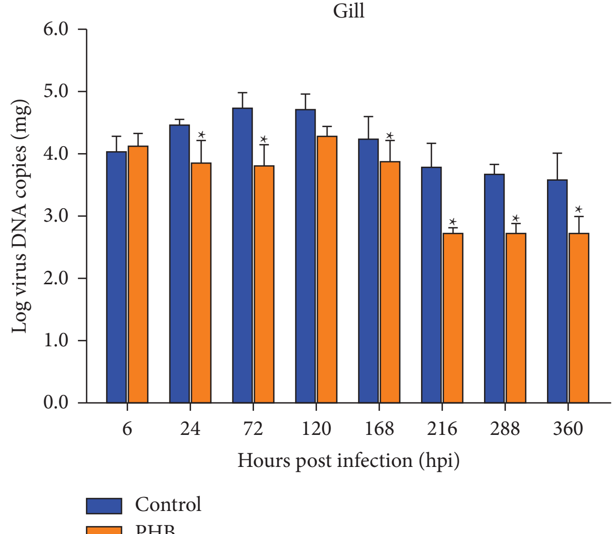

Figure 3: Dynamic changes of CyHV-2 in the gill. Asterisk means significant difference between the PHB and control group at the same time point. $^{*}P \leq 0.05$.

6 hpi. For tryptophan metabolism (Figure 6(c)) and the MAPK signaling pathway (Figure 6(d)), the obvious transcription difference was shown after 24 hpi. At the later stages of infection (96 hpi and 168 hpi), there were significant differences in the transcription of some genes involved in the above four important pathways. It suggested that the intestine plays a role in the entire antiviral process.

4. Discussion

Dietary PHB supplementation increases activities of immune-related enzymes in the gill of gibel carps, focusing on antioxidant capability. Under normal physiological conditions, excessive free radicals will be cleared by the endogenous antioxidant defense through either enzymatic or nonenzymatic pathways [47]. The primary antioxidant enzymes include SOD, CAT, T-AOC, GST, and GSH, while the major nonenzymatic antioxidants include bilirubin, a-tocopherol, carotene, albumin, and uric acid [48]. SOD can remove excessive ROS to avoid adverse reactions [49, 50]. CAT plays an important role in the toxicity of hydroxyl radicals [51], and T-AOC can reflect the total antioxidant competency of organisms [52, 53]. GST is a multifunctional enzyme associated with cellular detoxification and antioxidant activity [54]. GSH is an important antioxidant and free radical scavenger in the body [55, 56]. GR can affect the expression of GSH and ROS and regulate oxidative damage [57, 58]. In this study, PHB addition can improve the activities of SOD, OH^{-}, T-AOC, CAT, GR, GSH, and GST in the gill or/and gill mucus (Table 3). Accordingly, PHB has been reported to enhance the antioxidant capability of the hepatopancreas in *L. vannamei* [15] and the activities of antioxidant-related enzymes such as PO, SOD, and T-AOC [59, 60]. Dietary PHB addition significantly increased activities of SOD, ALP, ACP, CAT, and lysozyme in coelomocytes in sea cucumber (*Apostichopus japonicus*) [61] and the antioxidant capability of soiny mullet (*Liza haematocheila*) [62].

In the present study, PHB addition can upregulate the transcription of immune-related genes in the gill of gibel carps. Twenty-four genes involved in four important signaling pathways were selected to study the effect of PHB on the transcription of immune-related genes in the gill based on the comparative transcriptome analysis of spleen from PHB and control groups (RNA-seq data was deposited in NCBI under accession numbers SRR12596365/66/67/68/69/70). The differentially expressed genes (DEGs) were annotated into these four signaling pathways mainly, and these signaling pathways play a key role in the body's resistance to external infection [63–68]. The JAK/STAT signaling mediates a variety of innate immune processes, including activation of neutrophils and macrophages, regulation of inflammatory responses, and wound repair [69, 70]. Th1 and Th2 cell differentiation, modulating $CD4^{+}$ T cells, play an indispensable role in virus-induced inflammation [71, 72]. The imbalance of Th1/Th2 cells may be a key factor for the progression and severity of virus-related diseases, such as respiratory syncytial virus [67, 73, 74]. Tryptophan metabolism can modulate immunologic tolerance in primitive vertebrate lamprey via IDO-kynurenine-AHR pathway. Tryptophan is not only able to reduce the expression of proinflammatory factors such as *TNF-α*, *IL1β* and *NF-κ*B, but also upregulate the expression of anti-inflammatory factor such as *TGF-β* in *Lampetra japonicum* [75]. The MAPK signaling pathway is involved in cellular processes [76]. Yaun et al. (2020) studied the effects of different concentrations of choline added to diets on the gill cell apoptosis of juvenile *Ctenopharyngodon idella* and its potential mechanism. The results showed that appropriate choline could downregulate the mRNA levels of apoptosis genes such as caspase-3, caspase-8, and caspase-9, and regulate the p38MAPK and JAK1(TYK2)/STAT3 signaling pathways in the gill, which reduce the incidence of gill rot and effectively enhance the physical and immune barrier of the gill [77, 78]. It has also shown that pyridoxine, protein,

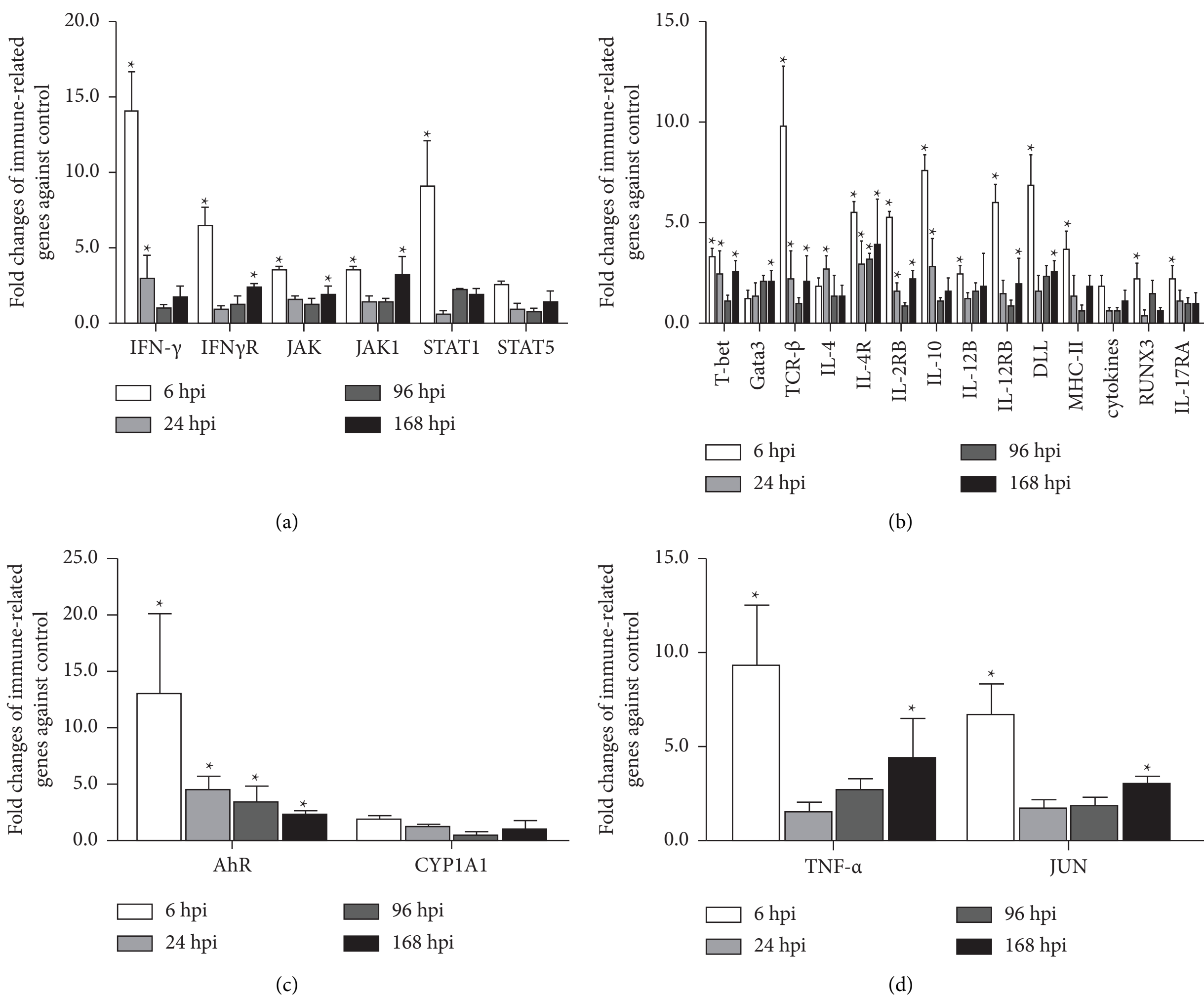

Figure 4: Effect of PHB supplementation on the immune response of the fish gill, against CyHV-2 infection. $^*P \leq 0.05$. (a) Key genes involved in the JAK/STAT signaling pathway. (b) Key genes involved in Th1 and Th2 cell differentiation signaling pathway. (c) Key genes involved in tryptophan metabolism. (d) Key genes involved in the MAPK signaling pathway.

and other deficiencies can damage the physical barrier function of the gill, and then lead to the impairment of gill barrier functions related to immunity, apoptosis, antioxidant, and tight connection [37, 79].

PHB can enhance the disease resistance of gibel carps against CyHV-2 infection. Like other aquatic animals, dietary PHB and/or mannooligosaccharides (MOS) addition can significantly improve the resistance of juvenile *Oncorhynchus mykiss* against *Yersinia lathani* infection and of pacific white shrimp (*Litopenaeus vannamei*) against *Vibrio harveyi* and *Vibrio parahaemolyticus* infection [19, 60, 80–83]. Dietary PHB addition reduced the rate of renal tubular epithelial cell lesions in the hepatopancreas and protected *L. vannamei* from *V. harveyi* infection [82]. In this study, the cumulative mortality rate in the PHB group was significantly lower than that in the control group when the viral concentration was 1.5×10^7 copies/fish, which was consistent with the previous report by Liu et al. [18].

PHB addition inhibits CyHV-2 early replication in the gill, which might be related to the enhancement of the gill immune barrier. Among them, the antioxidant system plays a pivotal role in the immune barrier. Fish gills are not only respiratory organs but also the first line of defense against pathogenic infections [84]. Gills are the key portal for the CyHV-2 invasion. Normally, viruses generally stimulate the host's immune system response during the infection process and destroy the homeostasis of the cell. The balance of prooxidation/antioxidation in the internal environment is very important for maintaining the integrity of cellular structure and function. An excess of bad radical species, including ROS and RNS, is often associated with important diseases such as cancer and neurodegeneration [48]. In this study, the antioxidant capability of the gills in the PHB group was improved significantly. Accordingly, recent studies demonstrated that induction of ROS is necessary for efficient onset of CyHV-2 replication, and developing antioxidants might be a novel antiviral strategy in gibel carps [13]. Other researches also show that pathogen infections or diets can affect the immune barrier of the gill. The expression levels of *plgR* and *IgM* genes in the gill and intestine of flounder

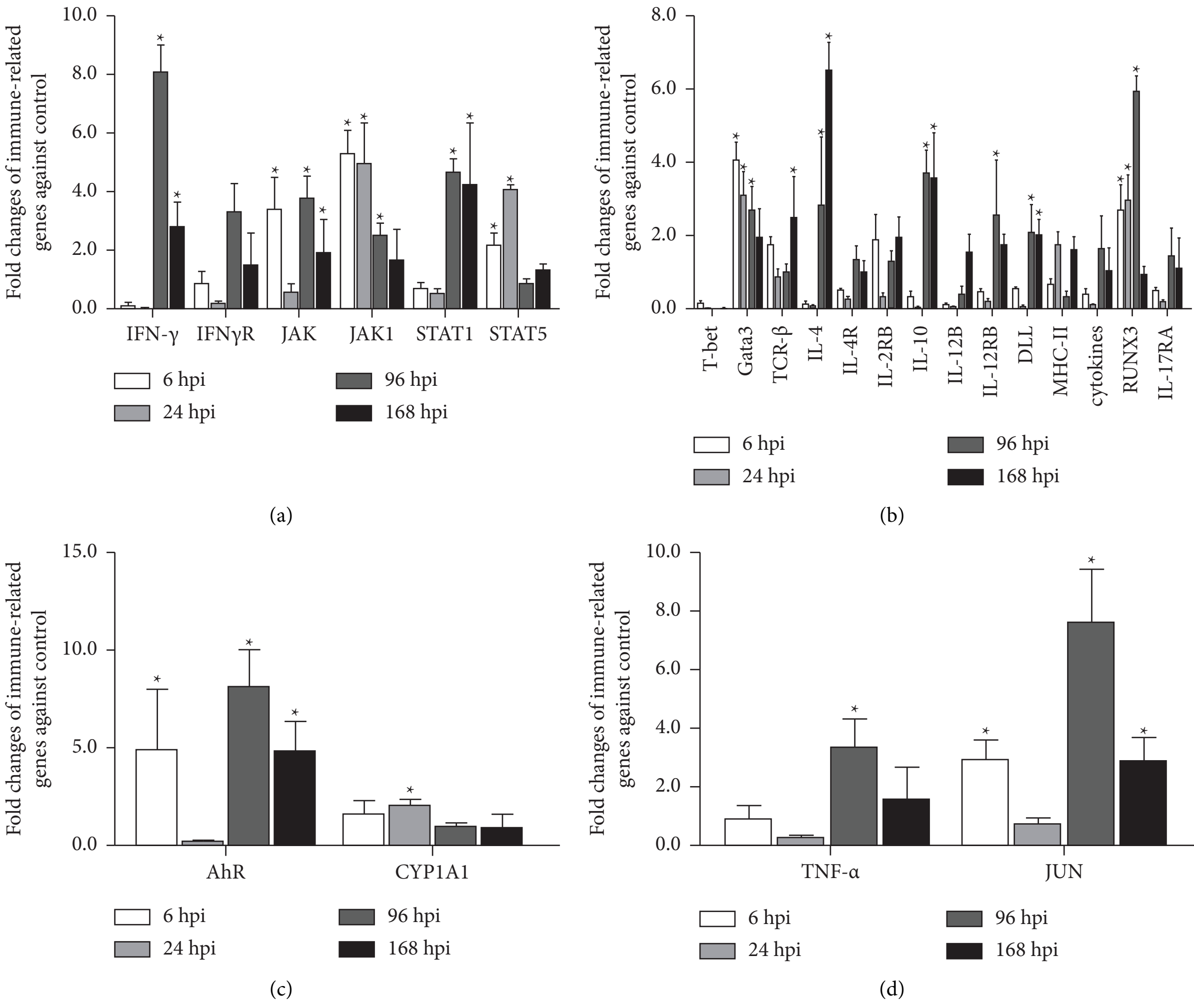

Figure 5: Effect of PHB supplementation on the immune response of the kidney against CyHV-2 infection. $^*P \leq 0.05$. (a) Key genes involved in the JAK/STAT signaling pathway. (b) Key genes involved in Th1 and Th2 cell differentiation signaling pathway. (c) Key genes involved in tryptophan metabolism. (d) Key genes involved in the MAPK signaling pathway.

(*Paralichthys olivaceus*), were upregulated post-immunization with the inactivated *Vibrio anguillarum*; the peak value of *plgR* appeared in the gill within 24 hpi, and the peak value was earlier than that in the intestinal tract [85, 86]. The higher expression of *IFN* and innate antiviral genes in the gill in the hypoxic group was observed in *Atlantic salmon* challenged with *Moritela viscosa* [87]. Salmon Branchella virus infection induced the innate antiviral responses in the gills of *Atlantic salmon* during the early stage, and some immuneeffectors involved in mucosal protection were downregulated at the advanced stage, suggesting that salmon gill poxvirus (SGPV) infection may impair mucosal defense [88]. In addition, Nectin-4 (an established NNV receptor) and GHSC70 were upregulated in the gill of seven-band grouper (*Epinephelus septemfasciatus thunberg*) infected with neuronecrosis virus (NNV) throughout the initial challenge period [89]. In this study, at the early stage of CyHV-2 infection, the expression of genes involved in the JAK/TAT signaling pathway, Th1 and Th2 cells differentiation, tryptophan metabolism, and MAPK signaling pathway in the gill was upregulated more, suggesting the gill plays a key role against CyHV-2 early infection. After 24 hpi, the kidney plays a more important role at the late infection stage.

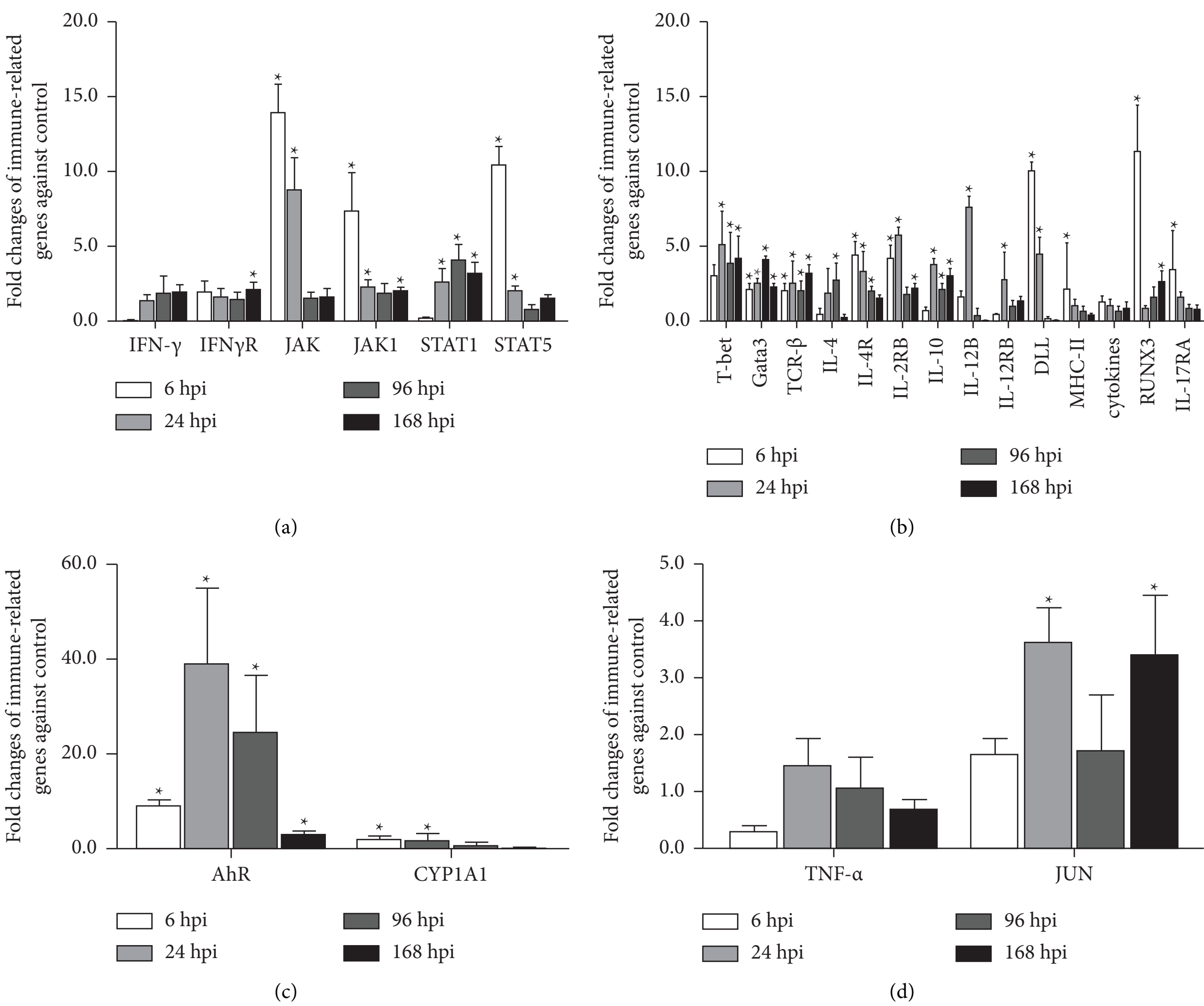

Figure 6: Effect of PHB supplementation on the immune response of the intestine against CyHV-2 infection. $^*P \leq 0.05$. (a) Key genes involved in the JAK/STAT signaling pathway. (b) Key genes involved in Th1 and Th2 cell differentiation signaling pathway. (c) Key genes involved in tryptophan metabolism. (d) Key genes involved in the MAPK signaling pathway.

In conclusion, dietary PHB supplementation affected the immune barrier of the gill in gibel carps and enhanced the disease resistance against CyHV-2 infection. The antioxidant capability of the gill was improved significantly, and gill plays a crucial role in the primary infection of CyHV-2, the kidney is more involved at the late infection stage. Besides, it was reported that the induction of ROS is necessary for the efficient onset of CyHV-2 replication [13]. Taken together, these findings shed light on developing novel prevention and control strategies for viral diseases, preventing viral adhesion in the gill from primary infection, and further inhibiting the occurrence of diseases.

Abbreviations

PHB: Poly-β-hydroxybutyrate
MS-222: Tricaine methane sulfonate
RT-qPCR: Real-time quantitative PCR
PBS: Phosphate buffer solution
ROS: Reactive oxygen species
RNS: Reactive nitrogen species
SOD: Superoxide dismutase
OH: Hydroxyl radical
T-AOC: Total antioxidant capacity

CAT: Catalase
GR: Glutathione reductase
GSH: Glutathione
GST: GSH-S-transferase
PO: Propofol oxidase
ALP: Alkaline phosphatase
ACP: Acid phosphatase
IFN-γ: Interferon-gamma
IFNγR: Interferon-gamma receptor
TNF-α: Tumor necrosis factor superfamily, member 2
T-bet: T-box expressed in T cells
JAK: Janus kinase
JAK1: Janus kinase 1
JUN: AP-1 transcription factor subunit
GATA3: Transcription factor GATA-3 isoform X2
TCR-β: T cell receptor beta variable region
IL-4: Interleukin-4
IL-4R: Interleukin-4 receptor
STAT1: Signal transducer and activator of transcription 1-alpha/beta-like isoform X1
STAT5: Signal transducer and activator of transcription 5-alpha/beta-like isoform X1
IL-2RB: Interleukin-2 receptor subunit beta
IL-10: Interleukin-10
IL-12B: Natural killer cell stimulatory factor 2
IL-12RB: Interleukin-12 receptor subunit beta
AhR: Aryl hydrocarbon receptor-like
CYP1A1: Cytochrome P450 family 1 subfamily A polypeptide 1
DLL: Delta-like ligand protein
MHC-II: Major histocompatibility complex II
NF-κB: Nuclear factor kappa-B
RUNX3: Runt-related transcription factor 3
IL-17RA: Interleukin-17 receptor A
MAPK8: Mitogen-activated protein kinase 8.

Data Availability

The data that support the findings of this study are available upon reasonable request to the corresponding author.

Ethical Approval

This study was conducted in accordance with the regulations for the administration of laboratory animals in Jiangsu province, China.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

G. Qiao conceived the project and was involved in conceptualization and manuscript revision. Y. C. Zhang conducted the experiment and wrote the manuscript. J. W. Zhang and W. Chen conducted the experiment. C. Wei, P. Chen, and W. P. Yang collected the samples. M. M. Zhang conceived the project and performed the experiment analysis. Z. G. Zhao, Z. Zhu, and X. H. Wan conceived the project. All authors read and approved the final manuscript. Guo Qiao and Yichan Zhang have contributed equally to this work and are the co-first authors.

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Supplementary Materials

Supplementary data to this article can be found in a file called "Supplementary Figures." (*Supplementary Materials*)

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