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Effects of dietary poly-β-hydroxybutyrate supplementation on the growth, immune response and intestinal microbiota of soiny mullet (*Liza haematocheila*)

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ABSTRACT

Soiny mullet (*Liza haematocheila*) is an important economic fish species in China, but stress and diseases have seriously restricted its culture. There are no effective methods including vaccines to prevent or control these diseases. Alternative methods should be employed, such as using novel immunostimulant poly-β-hydroxybutyrate (PHB). The present study aimed to evaluate effects of dietary PHB supplementation on the growth, antioxidant enzymes activity, immune-related genes expression and intestinal microbiota in soiny mullet. The fish was fed for 30 or 60 days with six diets at different PHB supplementation of 0, 0.5, 1, 2, 4 and 8%, named as groups P0, P0.5, P1, P2, P4 and P8. The results showed that the weight gain and specific growth rate of fish in P2 and P0.5 groups were significantly higher than those in control P0 group at 30 and 60 days, respectively ($P < 0.05$). The antioxidant enzymes activity of catalase and superoxide dismutase in serum were significantly increased in P0.5/P1/P2 groups after 30 days. The transcriptional levels of penicillin-binding protein A and interleukin-8 analyzed by qRT-PCR were significantly upregulated in P2 and P4 groups compared to those in P0/P0.5/P1/P8 groups at 30 days. The transcriptional level of major histocompatibility complex class II in P2 group was significantly upregulated, and aldehyde oxidase downregulated compared to P0 group. Intestinal microbiota analysis by Illumina high-throughput sequencing showed that the microbiota diversity was not changed significantly, but the microbiota structure shifted significantly post PHB treatment. At the phyla level, Firmicutes and Proteobacteria were predominant in both P0 and P2 groups. At the genus level, the relative abundance of *Bacillus* spp. in P2 group increased significantly, and abundance of *Achromobacter* spp. decreased significantly. KEGG pathway analysis by PICRUSt showed that oral administration PHB significantly upregulated abundances of genes responsible for 10 pathways and downregulated genes involved in 17 pathways. In conclusion, soiny mullet fed with 2% PHB supplemental diets for 30 days showed better growth performance, higher antioxidant enzymes activity and immune-related genes expression. Their regulation of growth and immunity might be related with the intestinal microbiota change post PHB supplementation. It will provide very useful basic information to study the regulation mechanism of PHB in aquatic animals, and provide good green method to prevent disease in soiny mullet.

1. Introduction

Soiny mullet (*Liza haematocheila*) becomes an economically important fish species in China due to some of its excellent characteristics, such as rapid growth, resistance to disease, and tolerance to extreme environmental conditions [1–3]. In 2018, the culture production of soiny mullet in Jiangsu Province reached upto 60,000 tons. However, intensive aquaculture leads to more frequent or virulent outbreaks of diseases, especially the bacterial diseases [4,5]. Fish loss caused by diseases has seriously restricted the culture of soiny mullet [1,2]. Generally, antibiotics were used to treat bacterial pathogens in aquaculture. However, the overuse and misuse of antibiotics create antibiotic-resistant bacteria, cause the decline of fish immune function, and build up drug residue in aquatic animals with potential damage to human and environment [6,7]. Meanwhile, there are no effective methods such as vaccines to prevent or control these diseases [4]. The

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innate immune system of fish plays a vital role in resisting the invasion of pathogens [8]. Therefore, seeking alternative methods such as using novel immunostimulants to prevent the infectious disease and enhance fish stress-resistance is critically important. It is also needed to better understand the immunomodulatory mechanism to improve fish health and reduce disease outbreak in soiny mullet [7].

The short-chain fatty acids (SCFAs) are a product of bacterial metabolism from feed items usually not efficiently broken down by the host digestive system and therefore production of SCFAs is an important service provided by commensal bacteria. The SCFAs have been confirmed to inhibit the growth of several bacteria such as *Escherichia coli*, *Salmonella typhimurium* and *Shigella flexneri* [9–14]. Poly-β-hydroxybutyrate (PHB) is known as the polymer of SCFA, a compound that stores intracellular energy and carbon sources and is produced by various microorganisms [7,15]. PHB can be hydrolyzed into β-hydroxybutyric acid by the enzymes secreted in the intestinal tract of many aquatic animals and shows similar function as SCFAs, such as the antibacterial activity [16,17]. Recently, several studies have reported that feeding cultured animals with PHB could promote growth and increase their resistance to pathogens. For example, feeding giant river prawn (*Macrobrachium rosenbergii*) with PHB-enriched *Artemia* nauplii can reduce the number of pathogenic bacteria and improve larval survival [18]. The PHB addition of 2% and 5% significantly enhanced the growth of sea bass (*Dicentrarchus labrax*) juveniles [16].

To date, poly-β hydroxybutyrate-hydroxyvalerate (PHB-HV) from *Bacillus thuringiensis* has been reported to cause immunostimulation in tilapiine cichlid fish (*Oreochromis mossambicus*) [15]. It is unclear whether the supplementation of PHB could improve growth performance, increase the antioxidant enzymes activity and affect the expression of immune-related genes in soiny mullet. Thus, this study was conducted to illustrate the effects of dietary PHB supplementation on the growth, antioxidant enzymes activity, immune response and intestinal microbiota of soiny mullet since gut bacterial diversity can affect the health and immune system in animals [19]. This study will provide valuable information or potential immunostimulant for disease prevention and stress resistance in soiny mullet culture.

2. Materials and methods

2.1. Diet preparation

The composition of the basal diet is given in Table 1. Wheat flour and corn starch were used as sugar source. Fish meal, soybean meal and rapeseed meal were used as dietary protein source. Fish oil and soybean oil were used as lipid source. The basal diet without PHB supplementation was used as control diet and named as group P0. Each basal diet was supplemented with PHB (Ningbo Tianan Biological Material Co., Ltd, China) at concentration (w/w) of 0.5, 1, 2, 4 and 8%, and named as experimental groups P0.5, P1, P2, P4 and P8, respectively. All diets were grounded into powder and sieved through 80-mesh filter. All ingredients were then blended in a mixer thoroughly and fully mixed with fish oil and bean oil. A proper amount of water was added to ensure the uniformity of granulation. The 2-mm diameter granulated feed were wet-extruded by a pelletizer (F-26, South China University of Technology, Guangzhou, China) and dried in an electric thermostatic drying oven at 60 °C for 12 h 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

Soiny mullet (7.70 ± 0.85 g) were bought from Sheyang, Jiangsu. Prior to the experiment, the fish were fed with basal diet for 2 weeks for acclimation. Water qualities were measured throughout the experimental period and maintained at water temperature of 23–25 °C, pH 6.8–7.3, dissolved oxygen (DO) more than 6 mg L^{-1}, NH_4^+-N less than 0.1 mg L^{-1} and NO_2^--N less than 0.1 mg L^{-1}. Each tank was equipped with a 24 h continuous oxygen increasing machine. After acclimation, a total number of 540 normal fish with similar sizes were randomly divided into six groups (P0/P0.5/P1/P2/P4/P8), and each group included triplicate tanks (volume 400 L) with 30 fish. During the whole 60-days feeding trial, fish were fed with the experimental diets (Table 1) to satiation three times (7:00 to 7:30, 14:00 to 14:30, and 19:30 to 20:00) each day.

Table 1
Formulation and composition (g·kg^{-1}) of experimental diets.

Ingredients	Experimental groups					
	P0	P0.5	P1	P2	P4	P8
Peruvian fish meal[a]	120	120	120	120	120	120
Soybean meal[a]	280	280	280	280	280	280
Rapeseed meal[a]	140	140	140	140	140	140
Wheat flour[a]	170	170	170	170	170	170
Corn starch[a]	50	50	50	50	50	50
Fish oil[a]	40	40	40	40	40	40
Bean oil[a]	40	40	40	40	40	40
$Ca(H_2PO_4)_2$[a]	20	20	20	20	20	20
MVP[a,d]	20	20	20	20	20	20
Carboxymethy cellulose[b]	120	115	110	100	80	40
PHB[c]	0	5	10	20	40	80
Total quantity	1000	1000	1000	1000	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 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 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.

2.3. Growth performance

At day 30 and 60 of the feeding trial, fish were weighed to evaluate the growth performance. The weight gain (WG) and specific growth rate (SGR) were calculated for fish fed with different diets using following formula:

Weight gain (WG, %) = 100 × (final body weight-initial body weight)/initial body weight; Specific growth rate (SGR, % day^{-1}) = 100 × (Ln final individual weight - Ln initial individual weight)/number of days.

2.4. Antioxidant enzymes activity analysis

To determine the effect of PHB supplementation on activity of antioxidant enzymes in serum, five fish from each tank at day 30 and 60 were sampled and anesthetized with tricaine methanesulfonate (MS-222) at 200 mg L^{-1}. The blood was collected and centrifuged at 3000 rpm for 6 min at 4 °C. The supernatants were stored at −80 °C for enzyme activity analysis. Antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD) and total antioxidant capacity (T-AOC) were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's protocol.

2.5. Immune-related genes expression analysis

2.5.1. Samples preparation

At day 30 and 60 in the feeding trial, fish were randomly sampled as mentioned in part 2.4. The spleen from each fish were collected, and the spleen from the same tank was pooled as one analytical sample. All

Table 2
Sequences of primers used for quantitative real-time PCR in this study.

Genes	Primers sequence (5′-3′)	Amplicon size (bp)
pbpA	Forward: GAGTTCTGTACCGTCTGCCTGATG Reverse: TTCACCAGCATCTTCAACCAGTCC	80
AOX	Forward: CACTGGCGAACCTGTCACTCAAG Reverse: ACGTCCAGTTCAGCAACAAGGC	130
IL-8	Forward: GAGACAGAGCGAGGCGAGGAG Reverse: GAGGCCACAGCACTTAGCACAG	136
MHC II	Forward: GCGTACCACATCTCTTCTCCATCC Reverse: CCTCTTCCTCTCCTCTGTCCTCTG	129
β-actin	Forward: CAGCCATACTGTGCCCATCT Reverse: TCCTTGATGTCACGCACGAT	200

pbpA, penicillin-binding protein A; AOX, aldehyde oxidase; IL-8, interleukin-8; MHC II, major histocompatibility complex class II.

samples were immediately placed in liquid nitrogen and stored at −80 °C until RNA extraction.

2.5.2. RNA extraction and cDNA synthesis

Each 50–100 mg tissue was crushed in 500 μL RNAiso Plus (Sigma) to disrupt cells and release RNA. Total RNA of each spleen sample was extracted as described by Panigrahi et al. [20]. The purity and concentration of RNA samples were determined using a Nanodrop ND-1000 spectrophotometer. The RNA quality was measured by electrophoresis on a 1.0% agarose gel and staining with 4S green plus nucleic acid stain (Takara Bio, Dalian, China). First-strand cDNA was synthesized using the Prime-script™ reagent kit with gDNA eraser (Takara Bio, Dalian, China) following the manufacturer's protocol. The synthesized cDNA was stored at −80 °C for mRNA expression analysis.

2.5.3. Quantitative real-time PCR (qRT-PCR)

Four immune-related genes including penicillin-binding protein A (pbpA), aldehyde oxidase (AOX), interleukin-8 (IL-8) and major histocompatibility complex class II (MHC II), were selected in this study. The gene-specific primers were designed with the aid of Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and listed in Table 2. The β-actin gene was used as an internal calibrator to normalize cDNA quantities. Primers for immune-related genes were synthesized by Sangon Biotech (Shanghai, China). The relative expression of the selected immune-related genes was determined using a CFX96 Real-Time PCR Detection System (Bio-Rad, USA) with SYBR® Premix Ex Taq™ kit (Takara Bio Co., Ltd., Dalian, China). The total volume of qRT-PCR 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 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 dissociation curve analysis at 65 °C for 5 s to verify the amplification of a single product. The relative fold changes of mRNA expression of target genes in fish from groups P0.5/P1/P2/P4/P8 were compared to P0 group using the $2^{-\Delta\Delta CT}$ method [21].

2.6. Intestinal microbiota analysis

2.6.1. 16S rRNA gene sequence analysis of intestinal microbiota

Based on the growth performance, immune-related enzymes activity and genes expression analysis, the fish fed with 2% PHB supplementation diet for 30 days were chosen to analyze the effect of PHB supplementation on intestinal microbiota of soiny mullet. The whole intestinal contents were sampled and pooled from four fish per tank with three tanks per treatment, immediately placed in liquid nitrogen and stored at −80 °C until DNA extraction. Microbial DNA was extracted from the intestinal samples using the E.Z.N.A.® Soil DNA kit (OMEGA, US) according to manufacturer's protocols [22]. The V4-V5 region of the bacteria 16S rRNA gene was amplified by PCR (95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min) using forward primer 515F (5′-barcode- GTGCCAGCMGCCGCGG-3′) and reverse primer 907R (5′-CCGTCAATTCMTTTR AGTTT-3′), where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate with 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu polymerase, and 10 ng of template DNA. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, US) according to the manufacturer's instructions.

The purified PCR products were quantified by Qubit®3.0 (Life Invitrogen) and every twenty-four amplicons with different barcodes were mixed equally. The pooled DNA product was used to construct Illumina Pair-End library following Illumina's genomic DNA library preparation procedure. Then, the amplicon library was paired-end sequenced (2 × 250) on a MiSeq sequencing platform (Illumina, USA) as described by Zhao et al. [23].

2.6.2. Bioinformatics analysis

Raw fastq files were de-multiplexed and quality-filtered using QIIME (version 1.17) with the following criteria: (i) The 250 bp reads were truncated at any site receiving an average quality score < 20 over a 10 bp sliding window, discarding the truncated reads that were shorter than 50 bp; (ii) exact barcode matching, two nucleotide mismatch in primer matching, reads containing ambiguous characters were removed; (iii) only sequences that overlap longer than 10 bp were assembled according to their overlap sequences [24]. Reads which could not be assembled were discarded. Bacterial operation taxonomic units (OUTs) were generated using the uclust function in QIIME (http://qiime.org/scripts/pick.outs.html). A Venn diagram was constructed to compare OUTs from control and PHB supplementation groups. Chao indices represent the total number of species. Simpson index and Shannon index in α-diversity are used to reflect microbial diversity. The ACE, Chao, Simpson and Shannon indices were calculated by mothur (version v.1.30.1) to evaluate α-diversity. Weighted UniFrac distance-based Nonmetric Multidimensional Scaling (NMDS) was used to assess β-diversity. Different taxa microbes were identified by Taxon-based analysis [25]. PICRUSt was used to predict microbial functions [26]. The OTUs were mapped to gg13.5 database at 97% similarity by QIIME's command “pick_closed_otus.” The OTUs' abundance was normalized automatically using 16S rRNA gene copy numbers from known bacterial genomes in Integrated Microbial Genomes. The predicted genes and their functions were aligned to Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and differences between groups were compared through software STAMP4 [27]. Two-side Welch's *t*-test and the Benjamini–Hochberg FDR ($P < 0.05$) correction were used to do statistical analysis between two groups.

2.7. Statistical analysis

All data were expressed as mean ± SD for three replicates, preliminarily arranged using Excel 2016 and then analyzed using ANOVA one-way analysis of variance followed by Duncan's multiple range tests with SPSS software (version 24.0). P-values of 0.05 or less were considered statistically significant.

3. Results

3.1. Effect of dietary PHB supplementation on growth performance of soiny mullet

Dietary PHB supplementation and concentration affected growth performance of soiny mullet (Table 3). After 30-day experiment, WG and SGR in groups P2 and P4 were significantly higher ($P < 0.05$) than those in control group (P0). The highest values of WG and SGR were observed in P2 group, and increased by 90.47% and 74.32% compared

Table 3
Growth performance of soiny mullet (*Liza haematocheila*) fed with different concentrations of poly-β-hydroxybutyrate (PHB) supplementation for 30 and 60 days.

Groups (%)	IBW (g)	30 d			60 d		
		FBW (g)	WG (%)	SGR (%)	FBW (g)	WG (%)	SGR (%)
P0	7.73 ± 0.47	9.66 ± 0.19^{c}	24.87 ± 2.39^{c}	0.74 ± 0.06^{c}	13.90 ± 0.47^{bc}	79.72 ± 6.04^{bc}	0.98 ± 0.06^{bc}
P0.5		9.39 ± 0.12^{c}	21.37 ± 1.49^{c}	0.65 ± 0.04^{c}	15.10 ± 0.19^{a}	95.23 ± 2.39^{a}	1.12 ± 0.02^{a}
P1		10.10 ± 0.46^{bc}	30.60 ± 5.99^{bc}	0.89 ± 0.16^{bc}	13.89 ± 0.18^{bc}	79.63 ± 2.35^{bc}	0.98 ± 0.02^{bc}
P2		11.40 ± 0.83^{a}	47.37 ± 10.72^{a}	1.29 ± 0.24^{a}	14.19 ± 0.36^{b}	83.45 ± 4.70^{b}	1.01 ± 0.04^{b}
P4		10.74 ± 0.22^{ab}	38.83 ± 2.79^{ab}	1.09 ± 0.07^{ab}	13.41 ± 0.31^{c}	73.45 ± 4.01^{c}	0.92 ± 0.04^{c}
P8		10.09 ± 0.34^{bc}	30.43 ± 4.42^{bc}	0.88 ± 0.11^{bc}	12.45 ± 0.46^{d}	60.94 ± 5.96^{d}	0.79 ± 0.06^{d}

The data represent the mean ± standard deviation from triplicated tanks and were analyzed by Duncan's multiple range test. Values in the same column with different superscripts are significantly different ($P < 0.05$). IBW, initial body weight; FBW, final body weight; WG, weight gain; SGR, specific growth rate; P0, i.e. control group, soiny mullet fed with basal diet; P0.5, P1, P2, P4 and P8, soiny mullet fed with 0.5%, 1%, 2%, 4% and 8% PHB.

to P0 group, respectively. After 60-day treatment with PHB diets, WG and SGR in P0.5 group were significantly higher than those in P0 group ($P < 0.05$), and increased by 19.46% and 14.29%, respectively. In addition, WG and SGR in P8 group were significantly lower than any other groups including P0 group ($P < 0.05$).

3.2. Effect of dietary PHB supplementation on antioxidant enzymes activity of soiny mullet

Dietary PHB supplementation and concentration affected the antioxidant enzymes activity, including CAT, SOD and T-AOC (Fig. 1). After day 30 of feeding, PHB supplementation could significantly increase CAT and SOD activities in the serum of soiny mullet ($P < 0.05$). CAT activity in groups P0.5/P1/P2 was significantly higher than that in P0 group, but CAT activity in groups P4 and P8 was significantly lower than that in P0 group ($P < 0.05$). SOD activity in experimental groups except P8 was significantly higher than that in P0 group ($P < 0.05$). Low level of T-AOC activity was observed in soiny mullet from all groups. At the end of the 60-day experiment, the activity of CAT in groups P0.5 and P8 was significantly lower than that in P0 group ($P < 0.05$). There were no significant differences on CAT activity among P0/P1/P2/P4 groups ($P > 0.05$). The activity of SOD in groups P1/P4/P8 was significantly higher than that in P0 group ($P < 0.05$). There were no significant differences among groups P0/P0.5/P2 ($P > 0.05$). T-AOC activity of fish fed with experimental diets was significantly higher than that of fish fed with basal diet ($P < 0.05$), and the highest value was shown in P8 group.

3.3. Effect of dietary PHB supplementation on immune-related genes expression

Dietary PHB supplementation and concentration affected the immune-related genes expression in spleen of soiny mullet, including pbpA, IL8, AOX and MHC II (Fig. 2). At day 30 of feeding, the expression levels of pbpA and IL8 were significantly upregulated in groups P2 and P4 compared to those in groups P0/P0.5/P1/P8. Compared to P0 group, transcription of genes pbpA and IL8 were up-regulated highest by 14.17- and 4.33-folds in groups P2 and P4, respectively. There were no significant changes in the expression of AOX and MHC II in groups P0/P0.5/P1/P4/P8 except P2 group. In P2 group, the transcriptional levels of AOX and MHC II were significantly down-regulated by 0.15-folds and upregulated by 2.63-folds, respectively. After 60 days of feeding, no significant transcriptional levels of pbpA, AOX, IL8 and MHC II in most of groups were noted. The highest and lowest expression levels of pbpA were observed in groups P0.5 and P8, respectively. The highest and lowest expression levels of IL8 were observed in groups P8 and P1, respectively. The expression levels of AOX and MHC II were significantly upregulated in P2 group compared to those in P0 group, and upregulated by 1.82- and 3.35-folds, respectively.

3.4. Effect of dietary PHB supplementation on intestinal microbiota of soiny mullet

A total of 253,181 trimed sequences were obtained from all six samples, with an average of 42,197 sequences per sample (35,338–55,449 sequences) and 427 bp length. The OTUs rarefaction curve based on the similarity cutoff of 97% tended to reach a plateau (Fig. 3), indicating that the sequencing data was reasonable. Furthermore, Good's coverages for the treatments were close to 1 (Table 4), suggesting that the depth of sequencing had basically covered all species in the samples. Common OTUs analysis presented by Venn diagram indicated that there existed 306 unique OTUs in control (P0) group and 284 unique OTUs in PHB supplementation (P2) group, respectively, while 218 common OTUs were identified in both samples (Fig. 4).

α-diversity was explored to evaluate the microbiota community diversity. As shown in Table 4, Chao1 richness estimators in groups P0 and P2 were 207.67 and 224.00, respectively. The Shannon index in P2 group was lower than that in P0 group, but the Simpson index in P2 group was higher than that in P0 group. The larger of the Shannon index, the higher the community diversity, while the Simpson index is inverse. The present results indicated that the basal diet supplemented with 2% PHB could produce a higher level of biodiversity than basal diet, although there was no significant difference between them.

β-analysis including UniFrac NMDS was used to compare the similarity of overall community structure. As depicted in Fig. 5, the significant intergroup difference between groups P0 and P2 was noted.

For bacterial community structure, marked differences at both phylum and genus levels between groups P0 and P2 were observed. Overall, a total of 16 phyla were shared by all samples from two groups and some of them with relative abundance less than 1.0% were combined (S1). Of them, Firmicutes and Proteobacteria compromised over 90% of the total classified sequences. Relative abundances of Firmicutes and Proteobacteria displayed significant differences between groups P0 and P2. PHB supplementation significantly raised the relative abundances of Firmicutes from 67.44% to 82.55%, but reduced the abundances of Proteobacteria from 31.59% to 16.51% ($P < 0.01$) (Fig. 6). At the genus level, a total of 202 genera were identified and 12 genera were finally obtained after combining the genera with abundances of less than 1% (Fig. 7, Fig. S2). No genus was specific for any group. Five genera including *Bacillus*, *Lactococcus*, *Achromobacter*, *Delftia* and *Carnobacterium* were the predominant genera in both groups P0 and P2. Among them, six genera including *Bacillus*, *Carnobacterium*, *Paenibacillus*, *Enterococcus*, *Mesorhizobium* and *Shewanella* in P2 group were evidently higher than them in P0 group, while another three genera including *Achromobacter*, *Delftia* and *Halomonas* were significantly lower ($P < 0.01$). The most obvious increasement of relative abundances in P0 group were noted in genera *Bacillus* (29.12%–39.85%) and *Carnobacterium* (3.23%–4.08%), but the most obvious decrease was observed in genera *Achromobacter* (20.60%–7.31%) and *Delftia* (3.65%–2.32%).

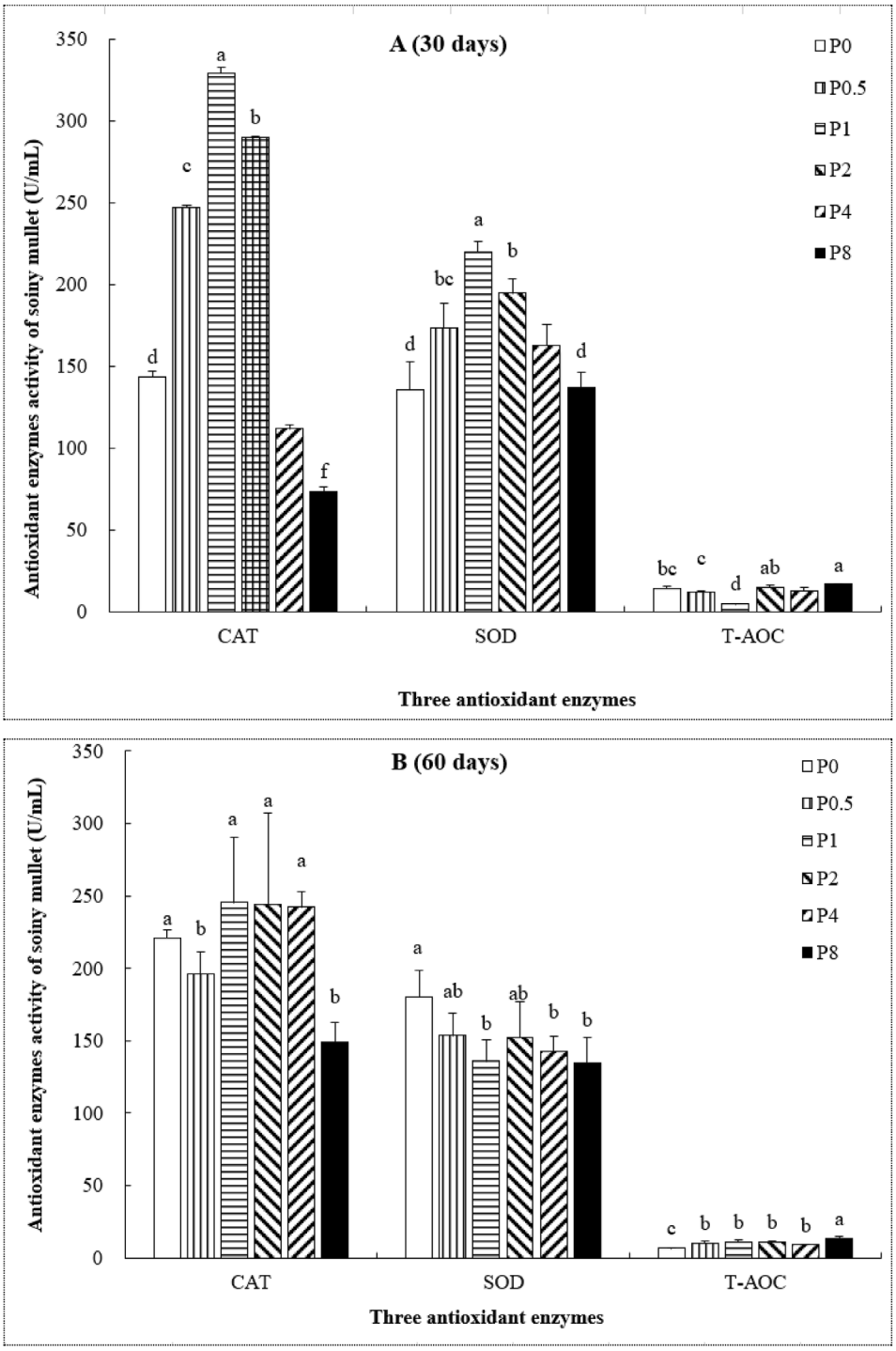

Fig. 1. Antioxidant enzymes activity in serum of soiny mullet (*Liza haematocheila*) fed with different concentrations of poly-β-hydroxybutyrate (PHB) supplementation at 30 (A) and 60 (B) days. Three antioxidant enzymes were analyzed, including catalase (CAT), superoxide dismutase (SOD) and total antioxidant capacity (T-AOC). The data represent the mean ± standard deviation from triplicated tanks and were analyzed by Duncan's multiple range test. Values for the same enzyme marked with a different superscript are significantly different ($P < 0.05$). P0, i.e. control group, soiny mullet fed with basal diet; P0.5, P1, P2, P4, P8, fish fed with 0.5%, 1%, 2%, 4% and 8% PHB.

Via comparing the sequencing data with those collected in KEGG pathway database by PICRUSt (Fig. 8, Fig. S3), it was found that PHB significantly upregulated abundances of genes that were responsible for 10 pathways, such as cellular processing and signaling, lipid metabolism, enzyme families, biosynthesis of other secondary metabolites, immune system diseases, immune system, replication and repair, and signaling molecules and interaction, but downregulated genes involved in 17 pathways, such as cancers, cell motility, energy metabolism, infectious diseases and signal transduction.

4. Discussion

In the past few decades, antibiotics have been widely used to prevent diseases and promote fish growth [24]. However, due to the adverse effects of overuse of antibiotics, the European Union and other countries have banned the use of antibiotics routinely to promote growth and prevent disease in animals, which poses a challenge to famers [16,28]. Researches have been conducted to look for alternatives to antibiotics for preventing diseases. Poly-β-hydroxybutyrate (PHB) has been reported in aquatic animals to promote growth and enhance their ability to resist pathogens, such as Siberian sturgeon (*Acipenser baerii*) [29], sea cucumber (*Apostichopus japonicus*) [30], black giant

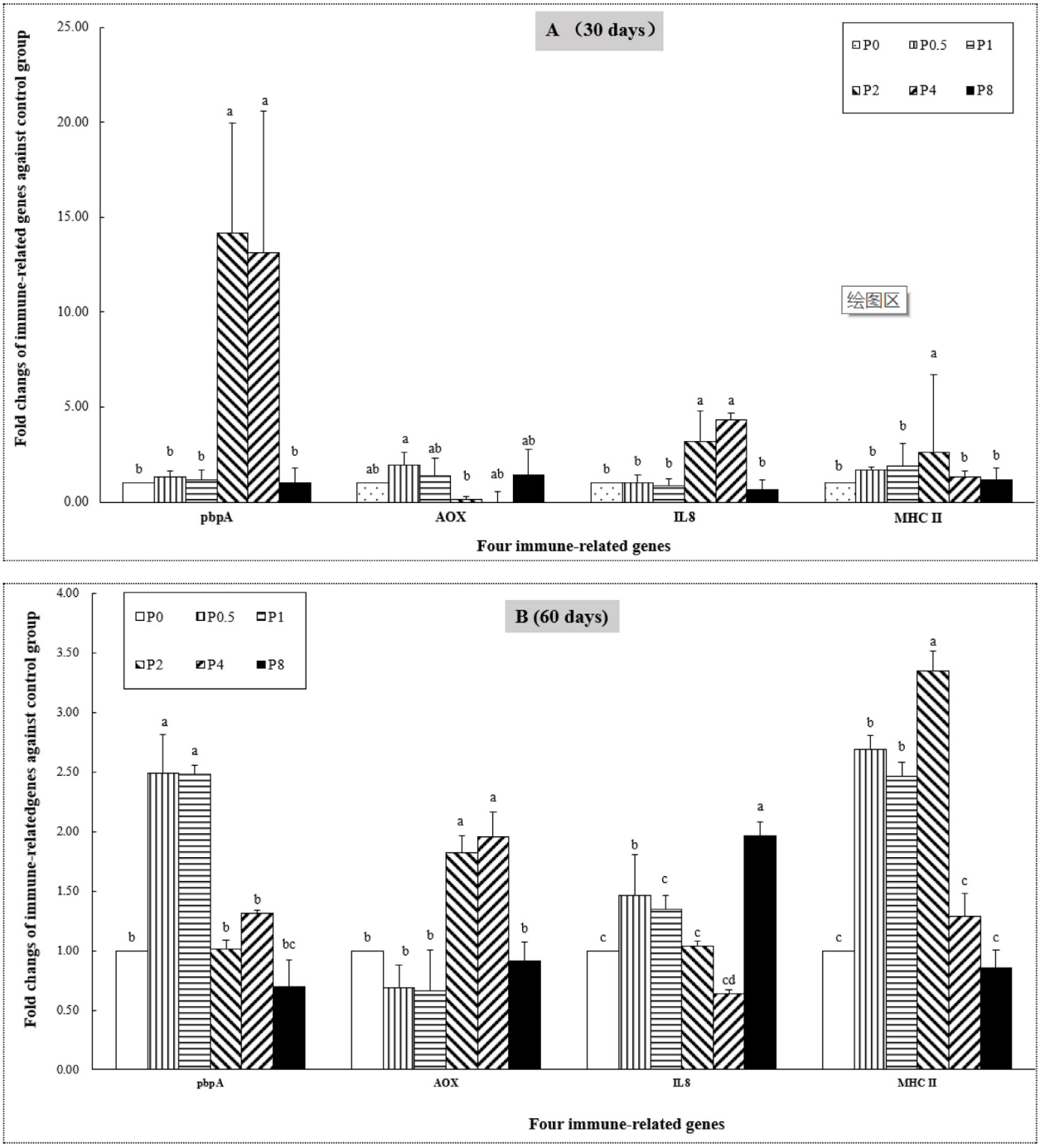

Fig. 2. Relative expression of immune-related genes in spleen of soiny mullet fed with different concentrations of PHB supplementation at 30 (A) and 60 (B) days. Four immune-related genes including penicillin-binding protein A (pbpA), aldehyde oxidase (AOX), interleukin-8 (IL-8), and major histocompatibility complex class II (MHC II), were analyzed by quantitative real time PCR. The data (mean relative expression ± standard deviation) are presented as relative fold changes of target genes in fish from groups P0.5/P1/P2/P4/P8 compared to control (P0) group. The data were derived from triplicated samples from three tanks, and each sample in one tank was pooled from five fish. All the data were analyzed by Duncan's multiple range tests. Values for the same gene marked with a different superscript are significantly different ($P \leq 0.05$). P0, i.e. control group, soiny mullet fed with basal diet; P0.5, P1, P2, P4 and P8, soiny mullet fed with 0.5%, 1%, 2%, 4% and 8% PHB.

tiger shrimp (*Penaeus monodon*) [31], Chinese mitten crab (*Eriocheir sinensis*) [32,33], Nile tilapia (*Oreochromis niloticus*) [34] and *Oreochromis mossambicus* [35]. The present research on PHB was mainly focused on growth, antioxidant enzymes activity and immune-related genes expression of soiny mullet. Weight gain and special growth rate were significantly higher in groups P2/P4 than that in the control group (P0) after 30 days, which was in accordance with previous report by Najdegerami et al. [29]. However, the lowest values of WG and SGR were obtained in P8 group after 60-day feeding of PHB. It suggested that long-term feeding of high concentration of PHB would have a negative impact on the growth of soiny mullet, which might be related with the insoluble characteristics of PHB.

PHB supplementation can improve the antioxidant capacity of soiny mullet. The activities of CAT and SOD were significantly increased in P0.5/P1/P2 groups after 30 days. CAT, SOD and T-AOC are the important antioxidant enzymes of first line defense system [36,37]. CAT is

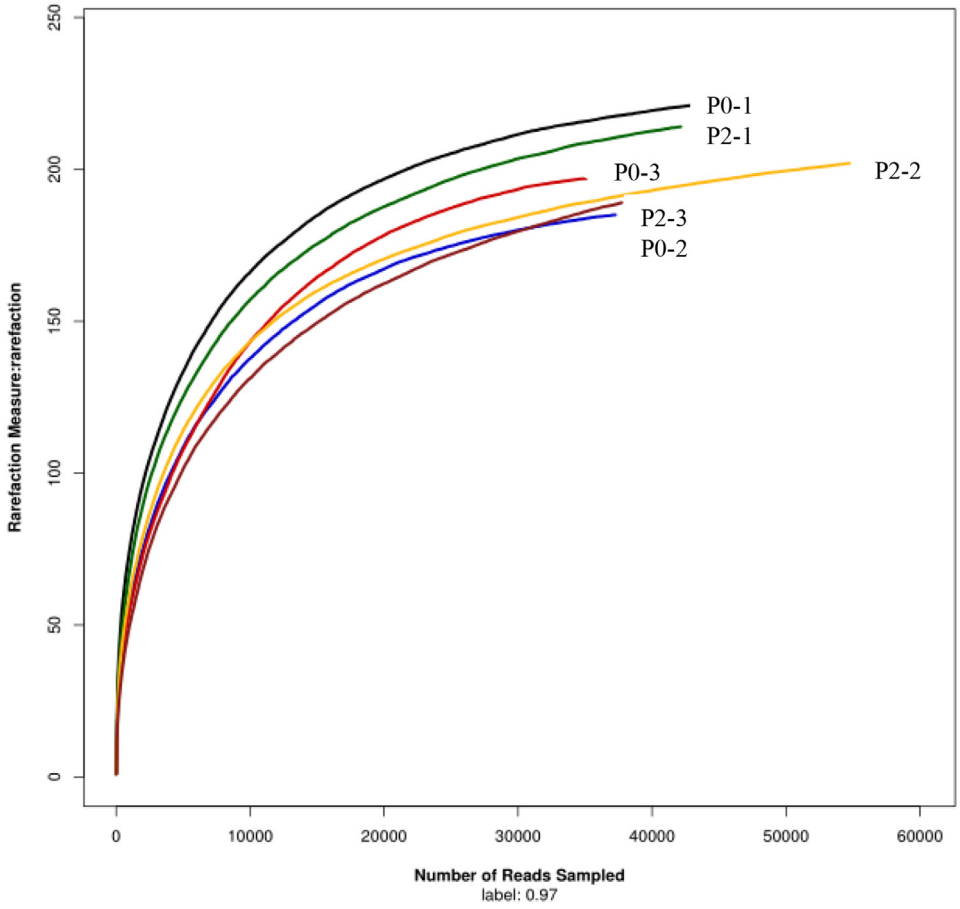

Fig. 3. Operation taxonomic units (OUTs) rarefaction curve. P0-1/2/3, soiny mullet fed with basal diet in triplicate; P2-1/2/3, soiny mullet fed with 2% PHB supplementation diet in triplicate.

well known as an antioxidant enzyme that catalyzes hydrogen peroxide to water and oxygen, and plays an important role against hydroxyl radical toxicity [36,38,39]. SOD is an important antioxidant enzyme to remove excessive reactive oxygen species (ROS) and avoid adverse reactions [40,41]. T-AOC can reflect the total antioxidant capacity of an organism [37,42]. Unfortunately, the measurement of T-AOC in serum of this study was not well correlated with SOD and CAT. However, this is the first time that the antioxidant enzymes have been analyzed in soiny mullet after fed with PHB. The tested enzymes in groups P0.5/P1/P2 were significantly higher than those in control P0 group, suggesting that PHB could enhance the antioxidant capacity of soiny mullet within a suitable supplemental concentration and feeding period. In studies for other aquatic animals, PHB-enriched feed can trigger antioxidant function and antioxidant defense system of white Pacific shrimp (*Litopeneaus vannamei*) [7]. Meanwhile, mrigal (*Cirrhinus mrigala*) juveniles showed higher level of CAT and SOD after fed with dietary symbiotics [43]. The activity of SOD was also increased in both roho labeo (*Labeo rohita*) and *O. mossambicus* after fed with dietary supplementation *Lactobacillus plantarum* VSG3 and *Bacillus licheniformis* Dahb1 [44,45]. In the current study, the activity of SOD in fish fed PHB diets for 60 days was similar to or lower compared to P0 control fish. This result showed that long-term feeding of PHB (60 days) had no advantage compared to short term feeding (30 days).

Fig. 4. Venn diagram based on intestinal microbiota of soiny mullet from 2% PHB supplementation group (P2) and control (P0).

PHB can enhance the innate immunity of soiny mullet. Expression of immune-related genes pbpA, IL-8 and MHC II in soiny mullet was significantly upregulated in groups P2/P4, and expression of AOX was downregulated in P2 group at 30 days of feeding. Genes pbpA and IL-8

Table 4
α-diversity of intestinal microbiota of soiny mullet fed with 2% PHB (P2) and 0 (P0) supplementation diets.

Groups	Chao1	Simpson	Shannon	Coverage
P0	207.67 ± 22.30	0.12 ± 0.02	2.80 ± 0.25	0.999509 ± 0.000057
P2	224.00 ± 7.21	0.17 ± 0.07	2.58 ± 0.37	0.999263 ± 0.000339

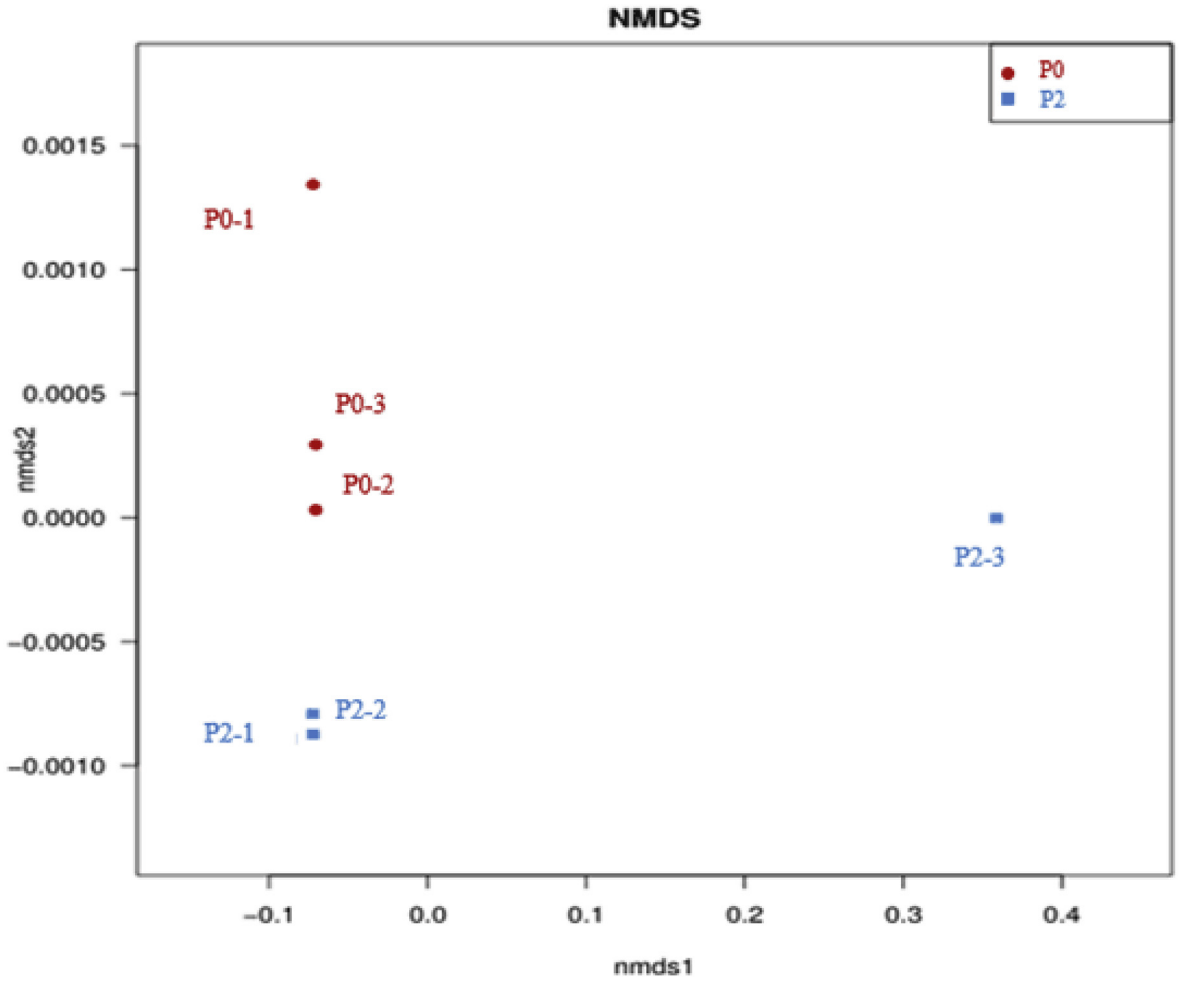

Fig. 5. UniFrac distance-based Nonmetric Multidimensional Scaling (NMDS) analysis based on intestinal microbiota of soiny mullet from groups P2 and P0. P0-1/2/3, soiny mullet fed with basal diet in triplicate; P2-1/2/3, soiny mullet fed with 2% PHB supplementation diet in triplicate.

were reported to play an important role on the immune response against infection [46–49]. PbpA belongs to the family of high molecular weight penicillin-binding proteins (PBPs), which acts on the growth and crosslinking of peptidoglycan. Peptidoglycan is an immunopotentiator of the immune system [46,47]. IL-8 is a chemotactic factor produced by pathogen recognition receptor-expressing cells, which has been widely studied due to their ability to attract and activate white blood cells and their potential role as inflammatory mediators [48,49]. AOX is a member of the xanthine oxidase (XO) family and involved in the pathophysiological process of organism [50]. MHC II plays an important role in activating fish innate immunity, and is essential for antigen presentation and recognition of invading pathogens [51,52]. The MHC II expression was significantly upregulated in groups P0.5/P1/P2 after 60 days in this study. In other studies, the expression of MHC II was upregulated in *D. labrax* juveniles after 60-day feeding with mannan oligosaccharides [53]. After 60-day feeding, the fold changes of immune-related genes were much smaller than those in 30-day groups, it is possible that prolonged feeding of PHB causes immune tolerance in soiny mullet. The above results on the growth performance, antioxidant enzymes activity and immune-related genes expression in our study suggested that the optimum dose of dietary PHB was 2% and fed for 30 days in soiny mullet. The optimum doses of dietary PHB supplementation for other aquatic animal species were recommended as: 3% PHB in *L. vannamei*, 2%-5% PHB in *D. labrax*, and 5% PHB in *O. mossambicus* [7,15,16].

Soiny mullet fed PHB diets at the concentration of 2% showed better growth performance, higher antioxidant enzymes activity and immune-related genes expression. Thus, fish in groups P2 and P0 (control) were used to analyze the effect of PHB supplementation on intestinal microbiota.

In fish, the intestinal microbiota is known to affect degree of nutrient harvest, epithelial proliferation, physiological development, health status and intestinal immune responses [54,55]. The intestinal mucosal immunity is the first barrier against pathogen invasion, and plays an important role in the immune system of fish [56–58]. The intestinal microbiota is related with surrounding environment, feeding, developmental stage of aquatic animals, etc. [59–63]. Effects of dietary probiotics or immunoenhancer on intestinal microbiota of aquatic animals have been frequently reported [64–66]. However, there is no information available on intestinal microbiota in fish after fed with PHB supplementary diets. Recently, advanced next-generation sequencing techniques of bacterial DNA is often used to study the entire bacterial community [67]. α-diversity analysis including Chao 1, ACE, Shannoon and Simpson based on sequencing was conducted to study the microbiota community diversity. Chao1 and ACE indices are estimators for community richness [68,69]. Shannon and Simpson indices represent community diversity and uniformity [70]. As depicted by the α-diversity analysis data in this study (Table 4), the overall diversity was not affected by dietary PHB supplementation. However, the present β-diversity analysis showed that the intergroup overall community structure was significantly different. At the phyla level, Firmicutes and Proteobacteria were the predominant phyla in both P0 and P2 groups. Accordingly, these two phyla are also dominant in the intestine of *Oncorhynchus mykiss* and *Scophthalmus maximus* [59,71,72]. To our

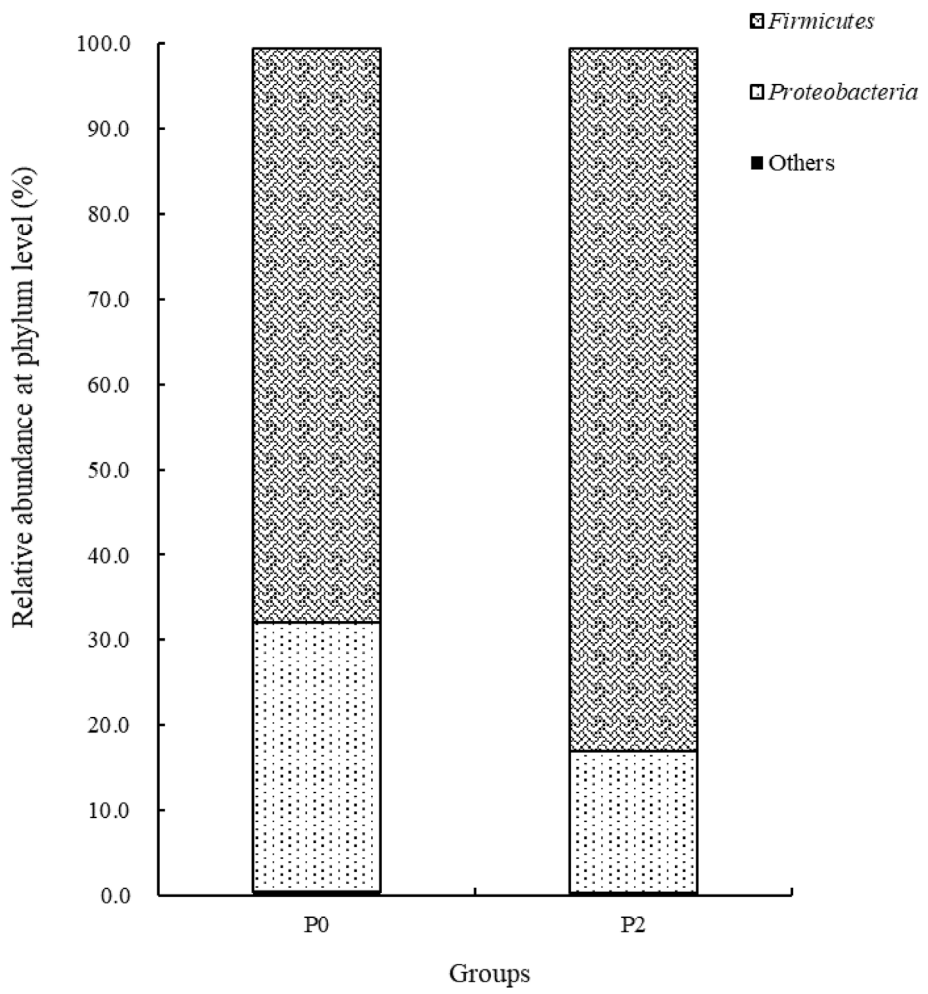

Fig. 6. Taxonomy analysis on phyla level from groups P0 and P2. P0, soiny mullet fed with basal diet; P2, soiny mullet fed with 2% PHB supplementation diet.

knowledge, this is the first study to examine gut microbiota of soiny mullet using high-throughput sequencing [73]. The relative abundance of Firmicutes in P2 group was significantly higher than that in P0 group, which was in accordance with previous reports in probiotics [74]. Moreover, along with the development of *Paralichthys olivaceus*, the relative abundance of Firmicutes increased and abundance of Proteobacteria decreased [75]. In our study, the familiar shift was noted in P2 group compared to P0 group, and growth performance of soiny mullet in P2 group was better than those in P0 group. However, whether the intestinal flora changes are caused by fish developmental stage or PHB addition, and bot of them still needs to be further studied. At genus level, *Bacillus*, *Lactococcus*, *Achromobacter*, *Delftia* and *Carnobacterium* were the commonly predominant genera. The relative abundance of *Bacillus* spp. in P2 group was significantly higher than that in P0 group, and abundance of *Achromobacter* spp. decreased significantly. *Bacillus* spp. are considered probiotics and widely used in Aquaculture. It can increase the host digestion, absorption and immunity [76–78]. *Achromobacter* spp. are reported as one of dominant bacterial species in fish intestine [79] and considered as a pathogen related with human species and shaggy-mane (*Coprinus comatus*) [80,81]. It suggested that dietary PHB supplementation changed the intestinal microbiota structure, increased probiotics growth and abundance, maybe inhibited opportunistic bacterial pathogen growth. It will benefit for host growth and immunity. Additionally, KEGG pathway analysis proved that up-regulated genes involved in some metabolism pathway, biosynthesis of other secondary metabolites, immune system, replication and repair, and signaling molecules and interaction, and some down-regulated genes involved in diseases pathway. So it is probable that oral administration PHB was able to directly affect the grow homeostasis within the microbiota *via* regulating its metabolism mode and host immune system, and possibly, serves as selective growth and immune-related promoters to soiny mullet. However, the relationship between metabolism regulation of gut microbiota and host response against these changes caused by gut microbiota need further study.

5. Conclusion

This study evaluated the effects of dietary PHB supplementation on the growth, antioxidant enzymes activity, immune-related genes expression and intestinal microbiota in soiny mullet. Soiny mullet fed PHB diets at the concentration of 2% for 30 days showed better growth performance, higher antioxidant enzymes activity and immune-related genes expression. Excessive PHB concentration and prolonged feeding time have a negative effect on the growth and antioxidant enzymes activity of soiny mullet. Oral administration PHB could not affect overall intestine microbiota diversity, but change the microbiota structure. The relative abundance of *Bacillus* spp. increased, and *Achromobacter* spp. decreased significantly. Further studies are needed to find the better feeding strategy to increase economic benefit for continuous feeding or intermittent feeding. It is also needed further studies on the relationship among the intestinal bacterial community changes post PHB treatment, growth affected by gut pH decrease caused by production of short-chain fatty acid and metabolism, bacterial pathogens inhibition, and intestinal immune function.

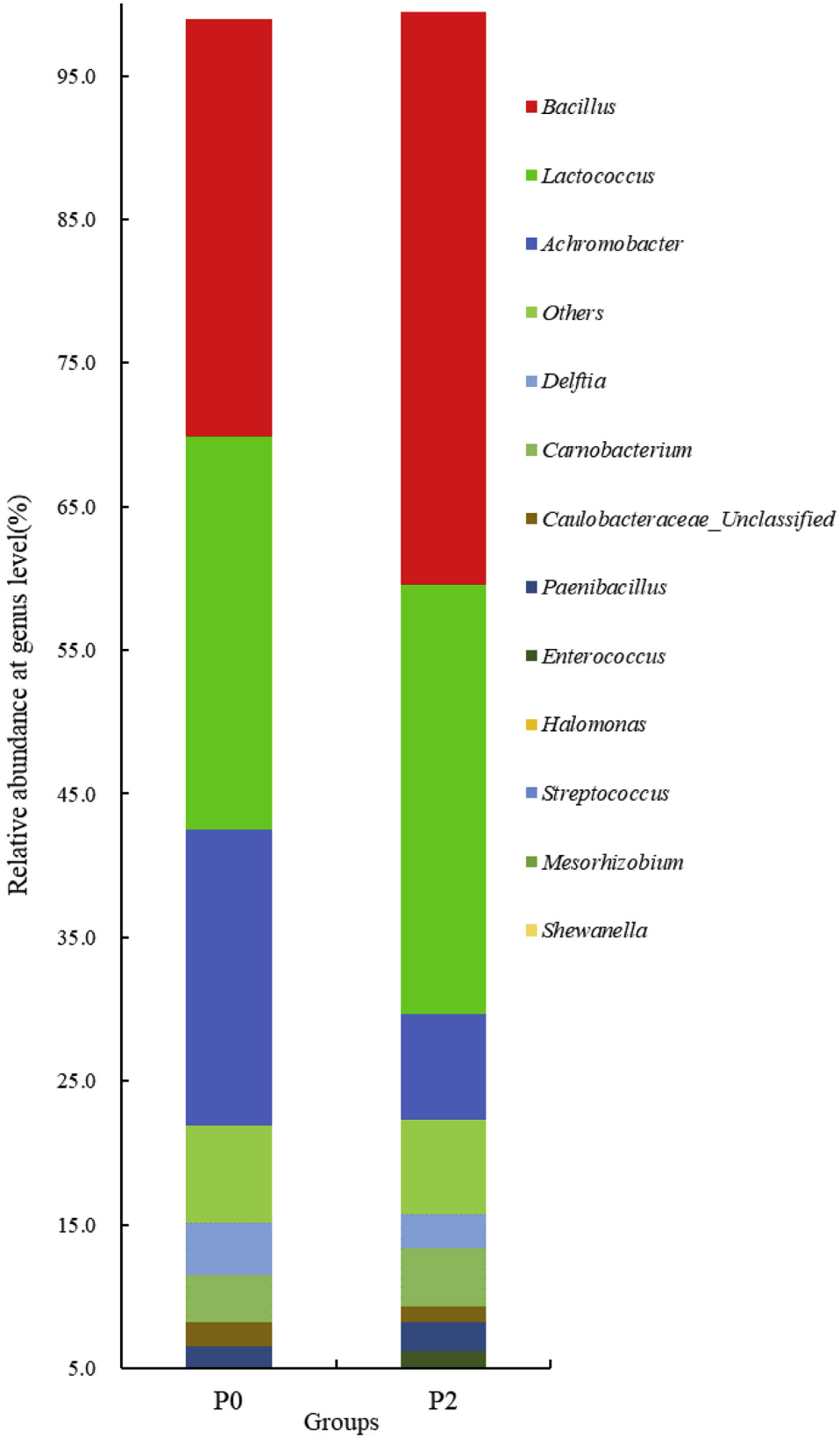

Fig. 7. Taxonomy analysis on genus level from groups P0 and P2. P0, soiny mullet fed with basal diet; P2, soiny mullet fed with 2% PHB supplementation diet.

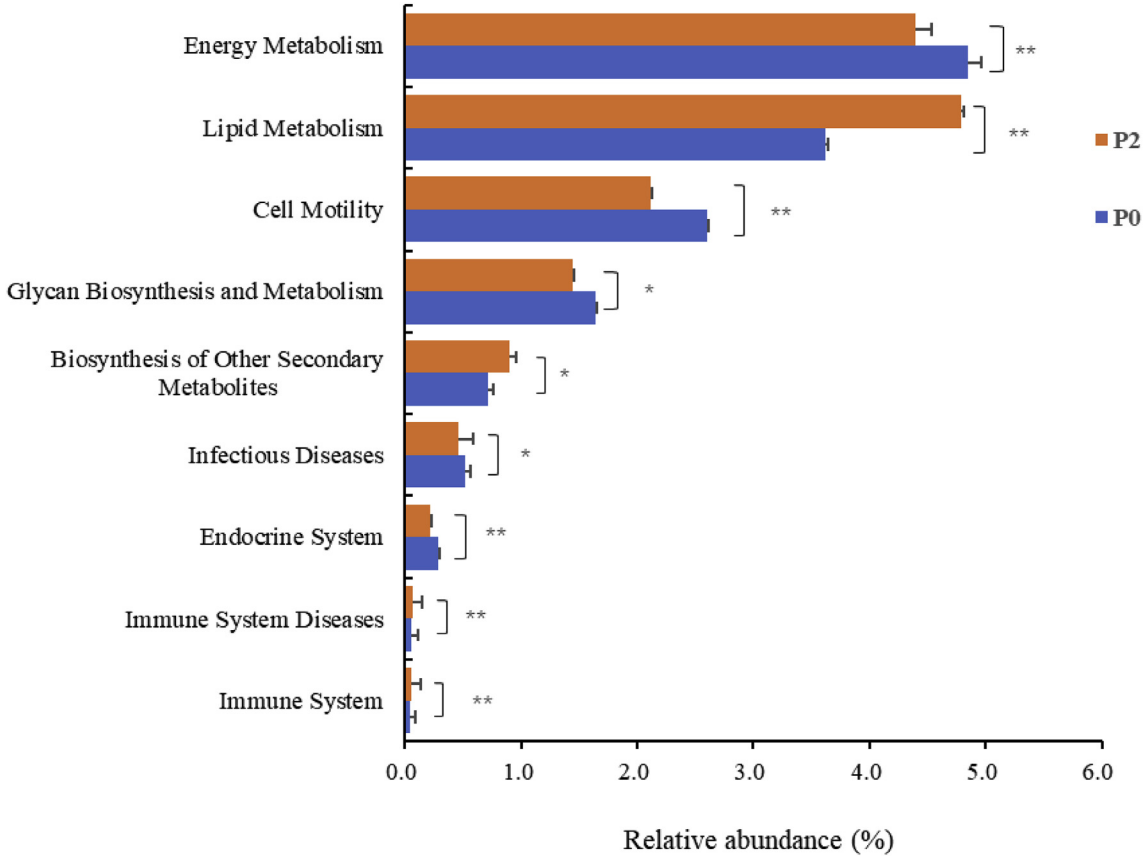

Fig. 8. Microbiome function prediction according to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. Values are presented the means ± SE. * $P < 0.05$ and ** $P < 0.01$ versus control group. P0, soiny mullet fed with basal diet; P2, soiny mullet fed with 2% PHB supplementation diet.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.05.038.

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