Fish & Shellfish Immunology 60 (2017) 520–528

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Effect of dietary poly-β-hydroxybutyrate (PHB) on growth performance, intestinal health status and body composition of Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931)

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ARTICLE INFO

Article history:
Received 3 August 2016
Received in revised form
15 October 2016
Accepted 7 November 2016
Available online 9 November 2016

Keywords:
Litopenaeus vannamei
Poly-β-hydroxybutyrate (PHB)
Short-chain fatty acids (SCFA)
Growth performance
Intestinal health
Body composition

ABSTRACT

In the present study, the effect of dietary supplementation of poly-β-hydroxybutyrate (PHB) on the growth performance, intestinal digestive and immune function, intestinal short-chain fatty acids (SCFA) content and body composition of Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931) was evaluated. The shrimp was fed for 35 days with four different diets: 0%, 1%, 3% and 5% PHB supplemented feed. The results indicated that supplementation of PHB significantly increased the growth performance of the shrimp, and the feed conversion rate (FCR) in 3%PHB treatment group was significantly lower than the control ($P < 0.05$). The intestinal amylase, lipase and trypsin activity in the three PHB treatment groups were all significantly higher than that of the control ($P < 0.05$), but the pepsin activity were only significantly affected by 3%PHB treatment ($P > 0.05$). The activities of intestinal immune enzymes such as total antioxidant capacity (T-AOC) and inducible nitric oxide synthase (iNOS) was significantly induced by 3%PHB treatment ($P < 0.05$), while lysozyme (LSZ) activity was significantly affected by 5%PHB treatment and nitric oxide (NO) content was significantly induced in three PHB treatments. Meanwhile, PHB induced significantly the expression level of intestinal heat shock protein 70 (HSP70), Toll and immune deficiency (Imd) gene. HE staining showed that PHB induced the intestinal health status of *L. vannamei*. Intestinal SCFA content analysis revealed that the content of propionic and butyric acid of 3% PHB treatment were significantly higher than that of the control ($P < 0.05$). Body composition analysis showed that the crude protein in 3% and 5%PHB treatments, and the crude lipid in 1% and 5%PHB treatments were all significantly higher than the control ($P < 0.05$). These results revealed that PHB could improve the growth performance, modulated intestinal digestive and immune function, increased intestinal SCFA content and body composition in *L. vannamei*, and the optimum dietary PHB requirement by *L. vannamei* was estimated at 3% (*w*/*w*) diet.

1. Introduction

Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931) is an important economical shrimp species worldwide especially in the Southeast Asian region [1]. *L. vannamei* possesses multiple merits, such as rapid growth, wide environmental adaptability and disease tolerance, which make it a good choice for extensive, intensive and semi-intensive strategies [2,3]. However, with the development of intensive culture and the environmental deterioration, frequent outbreaks of bacterial disease such as acute hepatopancreatic necrosis disease (AHPND) and/or early mortality syndrome (EMS) have caused large scale economic losses to *L. vannamei* aquaculture [4–7], and so far there have been no effective measure to control the spread of AHPND/EMS disease. Excessive and indiscriminate abuse of antibiotics not only make the multiple drug-resistant bacteria, but also can cause the immune function decline, intestinal microbial flora imbalance and drug residues in aquatic animal [8]. Therefore, a better understanding of the immunostimulants

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http://dx.doi.org/10.1016/j.fsi.2016.11.020
1050-4648/

regulatory mechanism of shrimp will be beneficial to the development of health management and disease control in shrimp aquaculture.

Short-chain fatty acids (SCFA) including acetic acid, propionic acid and butyric acid, which is the end-products of bacterial fermentation processes of nutrition such as carbohydrate and protein, and known to biological and ecofriendly approach of bacterial disease control in aquaculture [9–11]. For example, SCFA was regarded to inhibit the growth of pathogenic *Vibrio* spp [12]. However, SCFA is highly soluble in water which make it cannot uptake efficiency by aquatic animals and is not suitable for direct application in aquaculture [13]. Poly-β-hydroxybutyrate (PHB) is an intracellular energy and carbon storage compound accumulated by bacteria such as *Alcaligenes eutrophus* and *Bacillus megaterium* under physiological stress like nitrogen depletion and carbon excess conditions [9,14,15]. As a non-toxic and biodegradable polymer, PHB is insoluble in water and likely to be biologically degraded into antibacterial SCFA β-hydroxybutyric acid or PHB oligomers in the gastrointestinal of aquatic animals [9,16], which could slightly lower intestinal pH environment for benefiting the growth of probiotic bacteria [13]. It was reported that PHB-degrading bacteria have been isolated from the intestinal microbiota of aquatic animals, such as *Dicentrarchus labrax*, *Acipenser baerii* and *Macrobrachium rosenbergii*, and the PHB-degrading activity have been demonstrated that could increase the survival of *Artemia nauplii* larvae challenged with pathogenic *Vibrio campbellii* [16]. Previous studies have demonstrated that dietary PHB can benefit the growth and survival of aquatic animals [17–19], alter the intestinal microbial structure [13,15,17,20], increase the disease resistance [12,18,21–23] and inhibit the growth of pathogenic *Vibrio* strain [12].

Intestinal is an important organ for nutrients digestion and absorption of aquatic animals [24,25]. Meanwhile, intestinal health benefits the growth performance of aquatic animal [26,27]. Shrimp intestinal is continuously exposed to foreign substances including microbes, pathogens and other toxic substances from food [28], so intestinal immunity and structural integrity plays a pivotal role in maintaining intestinal health in aquatic animals [29–31]. However, the dietary role of PHB on growth performance and intestinal health regulatory in *L. vannamei* is uncertain and dearth the support information. The aim of this study was to: (1) investigate the growth performance and feed utilization of *L. vannamei*; (2) evaluate the intestinal microstructure changes of *L. vannamei*; (3) analyze the intestinal digestive capacity and immune status of *L. vannamei*; (4) compare the intestinal SCFA content and body composition of *L. vannamei* after different doses of PHB treatment. These results will be essential to better understand the role of PHB in intestinal health regulatory mechanism of *L. vannamei*.

2. Materials and methods

2.1. Animal materials

Healthy juvenile L. *vannamei*, averaging weight 5.66 ± 0.14 g, were collected from a local hatchery and reared in a semi-intensive culture pond at Shenzhen Base, South China Sea Fisheries Research Institute of Chinese Academy of Fishery Sciences (Shenzhen, China). They were cultured in filtered aerated seawater (salinity 30‰, pH 8.5) at 25 ± 0.5 °C for one week before processing, and fed daily with a ratio of 5% of body weight using the commercial formulated pellet feed (Haida Feed, Jieyang, China), which composition was crude protein ≥40%, crude fat ≥ 4%, crude fiber ≤ 5%, crude ash ≤ 15%, moisture ≤ 12%, lysine ≥ 1.9% and total phosphorus 0.9%–1.45%. One-third of the water was renewed once daily.

2.2. Diet preparation

PHB was obtained from Ningbo Tianna Biological Material CO., LTD, China. Four experimental diets (control, 1%PHB, 3%PHB, 5% PHB) were prepared containing 0% (control), 1%, 3% and 5% (*w*/*w*) supplementation of the same commercial formulated pellet feed (Haida Feed, Jieyang, China) described above with PHB on a weight basis. The control diet was sprayed with equivalent sterile purified water. PHB solution prepared according to De Schryver et al. [13]. PHB was dissolved in 80% chloroform solution and then sprayed homogeneously with formulated feed pellets at the above ratio. The diets were kept at room temperature and air dried under ventilation condition for evaporating the chloroform 2 days.

2.3. Experimental design and feeding trial

The experiments were divided into four groups and each group had three replicate tanks. The experiment consisted of 12 fiberglass tanks (300 L, 0.5 m^2 round bottom area) and each tank contained 200 L of filtered aerated seawater. There were 30 shrimp per tank which covered with a plastic mesh lid to prevent shrimp from jumping out of the tank. The water was continuously aerated with two air stones in each tank. Before starting the experiment, the shrimp were examined health status and with no sign of infection. The shrimp of four groups were fed with one of these four diets above with a ratio of 5% of body weight, and feeds were given three times per day (08:00, 12:00 and 18:00). The light regime was set at a fixed 14 h light and 10 h dark. During the feeding trial, shrimp were fed closely to satiation and the uneaten feed particles after feeding 1 h were collected, dried and weighed for correction of feed intake. The feeding trial lasted for 35 days. Intestinal tissue without faeces of shrimp from each tank were randomly sampled at the end of experiment, respectively, snap frozen in liquid nitrogen and stored at −80 °C for determination of digestive and immune enzymes activity, immune gene expression and SCFA content. Additionally, another three shrimp from each tank were randomly sampled for proximate composition analysis of the whole body.

2.4. Growth performance

At the end of experiment, shrimp were fasted for 24 h and then weighed to determine the growth performance. The growth performance and survival of *L. vannamei* for all groups were calculated with the following equations:

$$\text{Weight gain (WG, \%)} = (\text{Wt} - \text{Wi})/\text{Wi} \times 100$$

$$\text{Specific growth rate } \left(\%\text{day}^{-1}\right) = 100 \times [\ln\text{Wt} - \ln\text{Wi}]/\text{t}$$

$$\text{Feed conversion ratio (FCR)} = \text{W}_\text{f} \Big/ (\text{Wt} - \text{Wi})$$

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

where, W_t represents the final body weight (g) at feeding time t (days), W_i is the initial body weight (g), W_f is the total feed consumption as dry matter (g).

2.5. HE stain of the intestinal tissue

Intestinal of three shrimp from each group were randomly

sampled at 35 d, respectively, and stored in 4% paraformaldehyde for 2 h. After rinsed with flow water 8 h, the tissues were dehydrated in series of ethanol (70%, 80%, 90% and 100%), transparented with xylene, embedded in paraffin and cut in a microtome (Leica, RM2016, Germany) at 4 μm thickness. After HE dye, stained sections were examined and photographed under an inverted phase-contrast microscope (Hitachi, Japan). The intestinal epithelial cells height was quantified within three different fields which randomly selected visual from triplicates in each group.

2.6. Determination of intestinal digestive and immune enzymes

Intestinal of three shrimp were homogenized by adding sterile 0.9% saline solution to prepare 10% (W:V) homogenates. Homogenates were centrifuged at 1000 × g for 10 min at 4 °C. After removing precipitates, supernatants were immediately used for digestive and immune enzyme activities analyze, which were carried out using a spectrophotometer (Bio-RAD, USA). Total protein content in tissue homogenates was measured by Coomassie brilliant blue protein assay kit (Jiancheng, Ltd, Nanjing, China) following the manufacturer's protocol. Assays were all run in three replicate samples.

Digestive enzymes (e.g. amylase, lipase, trypsin and pepsin) activity in the intestinal of *L. vannamei* were analyzed with the amylase, lipase, trypsin and pepsin kits respectively (Jiancheng, Ltd, Nanjing, China) following the manufacturer's instruction and read on a spectrophotometer (Bio-RAD, USA). One unit of amylase activity was defined as the amount of enzyme per mg tissue protein every 30 min hydrolyzes 10 mg starch at 37 °C. One unit of lipase activity was defined as the amount of enzyme per mg tissue protein every minute catalyzes 1 μmol substrate at 37 °C. One unit of trypsin activity was defined as the amount of enzyme per mg tissue protein every minute make the absorbance change 0.003 at 37 °C under the pH 8.0 assay condition. One unit of pepsin activity was defined as the amount of enzyme per mg tissue protein every minute generate 1 μg amino acid by hydrolyzing protein at 37 °C.

Immune enzymes such as lysozyme (LSZ), total antioxidant capacity (T-AOC) and inducible nitric oxide synthase (iNOS) activity and nitric oxide (NO) content in the intestinal of *L. vannamei* were analyzed with the LSZ, T-AOC, iNOS and NO kits respectively (Jiancheng, Ltd, Nanjing, China) following the manufacturer's instruction and read on a spectrophotometer (Bio-RAD, USA). One unit of T-AOC activity was defined as the amount of enzyme per mg tissue protein every minute make the absorbance increase 0.01 at 37 °C. One unit of LSZ activity was defined as the amount of sample causing decrease in absorbance of 0.01 per min at 530 nm. NO was determined by nitric acid deduction method using nitrate chromogenic agent generation red azoic compound to measure indirectly. iNOS activity was measured by catalyzing reaction of L-arginine and O_2 at 530 nm.

2.7. Immune gene expression analysis

Total RNA was extracted from intestinal of three shrimp in each tank using Trizol Reagent (Invitrogen, USA) following the manufacturer's protocol. The RNA samples were analyzed in 1.0% agarose electrophoresis and quantitated at 260 nm, all OD_{260}/OD_{280} were between 1.8 and 2.0. Total RNA (5 mg) was reverse transcribed using the PrimeScript™ Real time PCR Kit (TaKaRa, Japan) for real-time quantitative RT-qPCR analysis.

Real time quantitative RT-qPCR was performed on an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA) to investigate the expression of heat shock protein 70 (HSP70), Toll and immune deficiency (Imd) gene. The β-actin gene of *L. vannamei* was used as an internal control to verify the successful reverse transcription and to calibrate the cDNA template. The RT-qPCR was carried out in a total volume of 20 μL, containing 10 μL SYBR® Premix Ex Taq™ II (2 ×) (TaKaRa), 2 μL of the 1:5 diluted cDNA, 0.8 μL each of 10 μmol/L forward and reverse primers (or β-actin-F and β-actin-R to amplify the β-actin gene) (Table 1), 0.4 μL ROX Reference Dye II (50 ×)*3 and 6 μL DEPC-treated water. The PCR program was 95 °C for 30 s, then 40 cycles of 95 °C for 5 s and 60 °C for 34 s, followed by 1 cycle of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s. DEPC-treated water for the replacement of template was used as negative control. RT-qPCR data from three replicate samples were analyzed with the ABI 7300 system SDS Software (Applied Biosystems, USA), for estimating transcript copy numbers for each sample. The expression level of relative gene was calculated by the $2^{-\triangle\triangle CT}$ comparative C_T method.

2.8. Determination of intestinal SCFA content and body composition

Intestinal SCFA content including acetic, propionic and butyric acid of three shrimp in each tank were measured by gas chromatography, according the method of Weitkunat et al. [11]. Body composition of crude protein, crude lipid and ash contents of three shrimp in each tank were analyzed according to the standard methods of AOAC (1995) [32]. Moisture was determined by oven drying at 105 °C for 24 h. Crude protein was determined indirectly (nitrogen × 6.25) using the Kjeldahl method (Buchi, Flawil, Switzerland). Crude lipid was determined by the Soxtec System HT (Soxtec System HT6, Tecator, Sweden). Ash was determined by combustion in a muffle furnace at 550 °C for 8 h.

2.9. Statistical analysis

The value of each variable was expressed as mean ± SE. Statistical analysis was performed using SPSS software (Ver 17.0). Statistical significance was determined using one-way ANOVA and post hoc Duncan multiple range tests. Significance was set at $P < 0.05$. All the data were tested for normality, homogeneity and independence before ANOVA. When the homogeneity of variances was violated, a non-parametric test (the Welch test) was performed in this study.

3. Results

3.1. Growth performance

Growth performance was evaluated through the final weight, weight gain, and specific growth rate of shrimp after feeding for 35 days. The growth of the shrimp in three PHB treatment groups was significantly better than that of the control ($P < 0.05$). The FCR in 3% PHB treatment group was significantly lower than the control ($P < 0.05$). No significant difference was observed in the survival

Table 1
Primer sequence used in this study.

Primer name	Sequence (5′-3′)	GenBank accession number
HSP70-F	AGGAGACCGCTGAGGCTTAC	AY645906
HSP70-R	AGCACATTCAGACCCGAGAT	
Toll-F	CCAGCTTAGAAGACCGGCAA	DQ923424
Toll-R	GTTGTCCGAGCAGAAGTCCA	
Imd-F	TGGGTCCGTGTCCAGTGATT	FJ592176
Imd-R	AGAGCCGCCGGTTATGTTGT	
β-actin-F	GCCCTGTTCCAGCCCTCATT	AF300705
β-actin-R	ACGGATGTCCACGTCGCACT	

Table 2
Growth performance of *L. vannamei* fed the control diet and three PHB-containing diets for 35 days.

Parameters	Control	1%PHB	3%PHB	5%PHB
Initial weight (g)	5.65 ± 0.14^{a}	5.66 ± 0.18^{a}	5.68 ± 0.12^{a}	5.67 ± 0.13^{a}
Final weight (g)	8.64 ± 0.18^{a}	9.79 ± 0.19^{c}	9.82 ± 0.16^{c}	9.47 ± 0.11^{b}
Weight gain (%)	52.81 ± 2.16^{a}	72.69 ± 2.23^{c}	72.64 ± 4.82^{c}	67.05 ± 4.24^{b}
Specific growth rate (%day^{-1})	1.21 ± 0.08^{a}	1.54 ± 0.12^{b}	1.55 ± 0.09^{b}	1.47 ± 0.06^{b}
Feed conversion rate (FCR)	1.54 ± 0.05^{b}	1.47 ± 0.05^{ab}	1.43 ± 0.03^{a}	1.51 ± 0.04^{b}
Survival (%)	96.18 ± 1.36^{a}	97.31 ± 2.23^{a}	97.04 ± 1.82^{a}	98.81 ± 2.85^{a}

Vertical bars represented the mean ± SE ($N = 3$). Data indicated with different letters were significantly different ($P < 0.05$) among treatments.

rate of shrimp of the four groups, and they were all above 96% ($P > 0.05$) (Table 2).

3.2. Intestinal tissue structure

After the intestinal tissue of *L. vannamei* stained with HE dye, compared with the control group (Fig. 1A), the tissue of the three PHB group showed intestinal epithelium connection closely and height increased, microvilli neat rows and dense, nuclei numbers increased significantly, and no sign of necrotic enterocytes or cell damage observed (Fig. 1B, C, D).

3.3. Intestinal digestive enzyme activity

Compared with the control group, amylase, lipase and trypsin activities in the three PHB treatment groups were all significantly higher than that of the control ($P < 0.05$). Amylase activity in 1%PHB groups was the highest, but there were no significant difference between the 3%PHB and 5%PHB group ($P > 0.05$) (Fig. 2A). Lipase activity of three PHB treatment groups were statistically the same with each other ($P > 0.05$) (Fig. 2B). Trypsin and pepsin activity in 3%PHB group was significantly higher than 1%PHB and 5%PHB group ($P < 0.05$) (Fig. 2C and D). However, no significant difference were observed in pepsin activity of the control, 1%PHB and 5%PHB treatment group ($P > 0.05$) (Fig. 2D).

3.4. Intestinal immune enzyme activity

Compared with the control group, LSZ activity was significantly induced in 5%PHB group ($P < 0.05$), but there were no significant changes in 1%PHB and 3%PHB group ($P > 0.05$) (Fig. 3A). T-AOC and iNOS activity of 3%PHB group were significantly higher than 1%PHB and 5%PHB group ($P < 0.05$) (Fig. 3B and C). NO content in the three PHB treatment groups were all significantly higher than that of the control ($P < 0.05$), and NO content of 3%PHB group was the highest, but no significant difference were observed between 1%PHB and 5% PHB group (Fig. 3D).

3.5. Intestinal immune gene expression analysis

Compared with the control group, the relative expression level of HSP70 gene was induced significantly in the three PHB treatment groups, and the highest level was in 3%PHB group ($P < 0.05$) (Fig. 4A). The relative expression level of Toll gene increased gradually after PHB treatment and reached the highest level in 5% PHB group ($P < 0.05$) (Fig. 4B). The relative expression level of Imd gene was induced significantly in the 1%PHB group ($P < 0.05$), but no significant change was observed in the 3%PHB and 5%PHB group

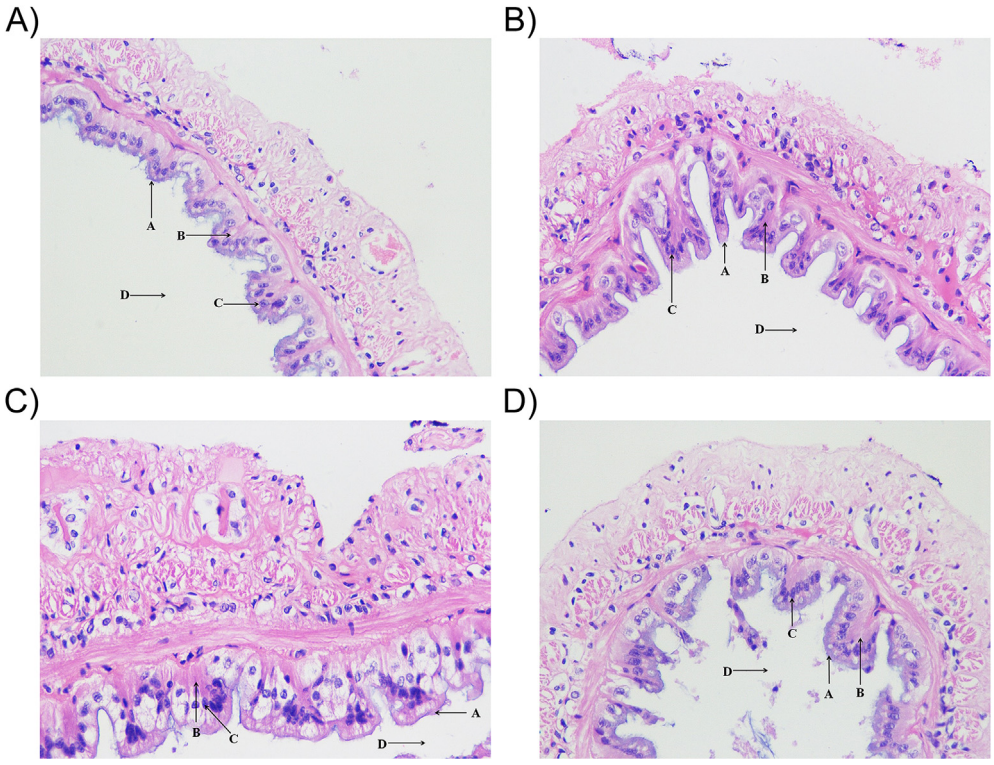

Fig. 1. Intestinal tissue of *L. vannamei* stained with HE stain after fed the control diet and three PHB-containing diets for 35 days (×400 magnifications). A: the control group, B: the 1%PHB group, C: the 3%PHB group, D: the 5%PHB group. The letters in the figure indicated that: A (brush border), B (epithelium), C (nuclei), D (lumen).

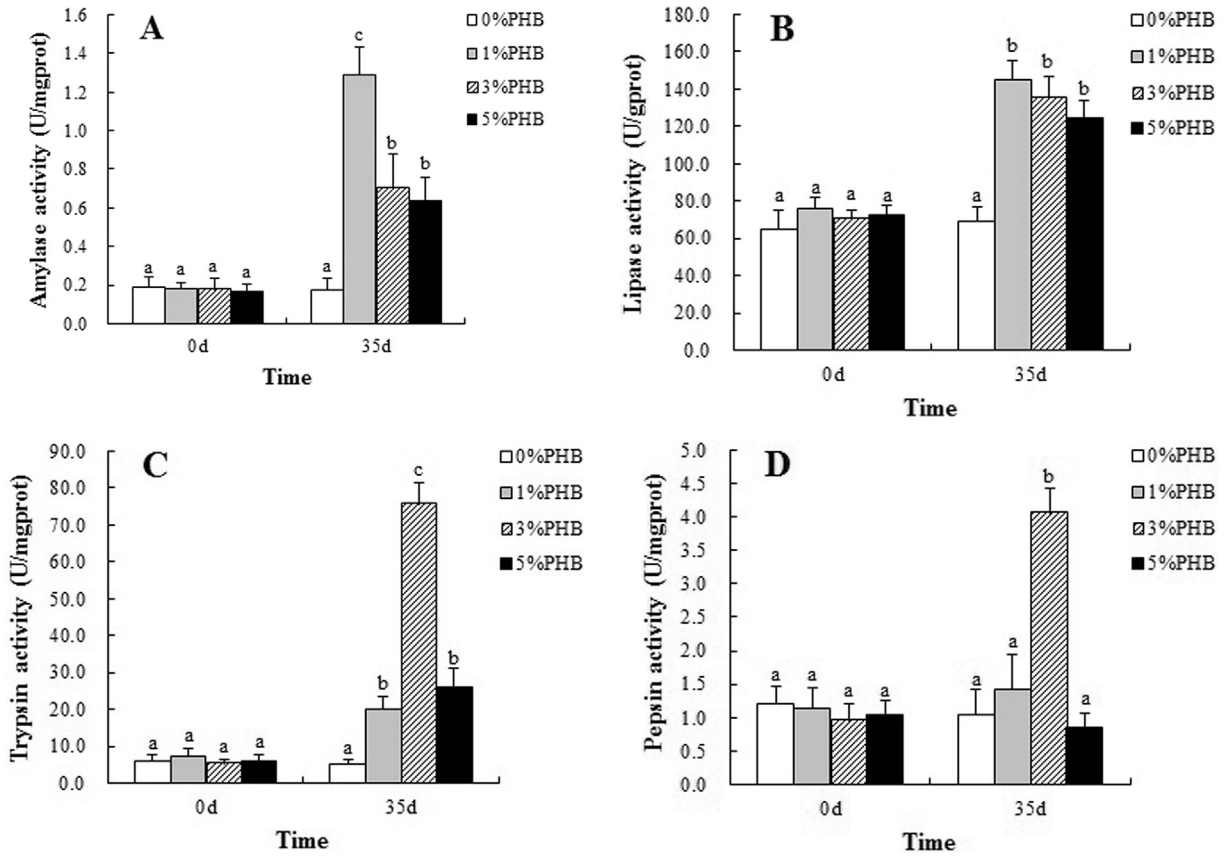

Fig. 2. Amylase (A), lipase (B), trypsin (C) and pepsin (D) activity in intestinal of *L. vannamei* fed the control diet and three PHB-containing diets for 35 days. Vertical bars represented the mean ± SE ($N = 3$). Data indicated with different letters were significantly different ($P < 0.05$) among treatments.

(Fig. 4C).

3.6. Intestinal SCFA content and body composition

Compared with the control group, the content of acetic acid in three PHB treatment groups were all significantly higher than the control, and the highest was the 5%PHB treatment group ($P < 0.05$). The content of propionic acid in 1% and 3%PHB treatment group was significantly higher than the control, but 5%PHB treatment group was lower than the control ($P < 0.05$). Butyric acid content of the 3%PHB treatment group was the highest, and the 1% and 5%PHB treatment groups were both significantly lower than the control ($P < 0.05$) (Table 3).

Compared with the control group, no significant difference was observed in the moisture and ash content between three PHB treatments and the control group ($P > 0.05$). The content of crude protein in 3% and 5%PHB treatment group were both significantly higher than the control ($P < 0.05$), and no significant difference was existed between the 3% and 5%PHB treatment group ($P > 0.05$). The content of crude lipid in 5%PHB treatment group increased significantly and higher than other three groups ($P < 0.05$), but there were no significant difference in the control, 1%PHB and 3%PHB treatment group ($P > 0.05$) (Table 4).

4. Discussion

Many alternative biocontrol compounds studies have been performed to find alternative ways to promote growth of aquaculture species [33,34]. Evidence indicates that PHB can beneficially influence the growth performance of aquatic animals such as European sea bass (*D. labrax*) [13], giant river water prawn (*M. rosenbergii*) larvae [17], Siberian sturgeon (*A. baerii*) [15] and Chinese mitten crab (*Eriocheir sinensis*) larvae [14]. In this study, the final weight, weight gain and specific growth rate of *L. vannamei* in three PHB treatments was significantly higher than that of the control, while only the feed conversion rate of was 3%PHB treatment significantly lower than that of the control, indicating that diet supplementation of PHB especially 3%PHB dose could improve the growth performance and feed utilization in shrimp, and had the similar effects as prebiotic.

Intestinal was regarded as the digestion and absorption center and the most sensitive tissue response to environmental stress in crustacean. PHB polymers are thought to be degraded into SCFA β-hydroxybutyric acid monomer in the intestinal of aquatic animals affecting the intestinal pH [35], and the pH decreased gradually as levels of PHB progressed [13]. Moreover, the intestinal pH reduction might improve the digestive enzymes activity, thus inducing the better nutrients digestive and absorption ability from the feed [36,37]. The activities of digestive enzyme (e.g. amylase, trypsin, lipase and pepsin) are recognized as an important indicator of digestive function [38]. In this study, the amylase, lipase and trypsin activity of the three PHB treatments and the pepsin activity of 3% PHB treatment in intestinal of *L. vannamei* were all significantly higher than that of the control. However, PHB did not affect the lipase and trypsin activity and decreased amylase activity in intestinal of Siberian sturgeon *A. baerii* [19]. The different effect of PHB on the same digestive enzyme activity might be associated with the difference of fish and crustacean species and development

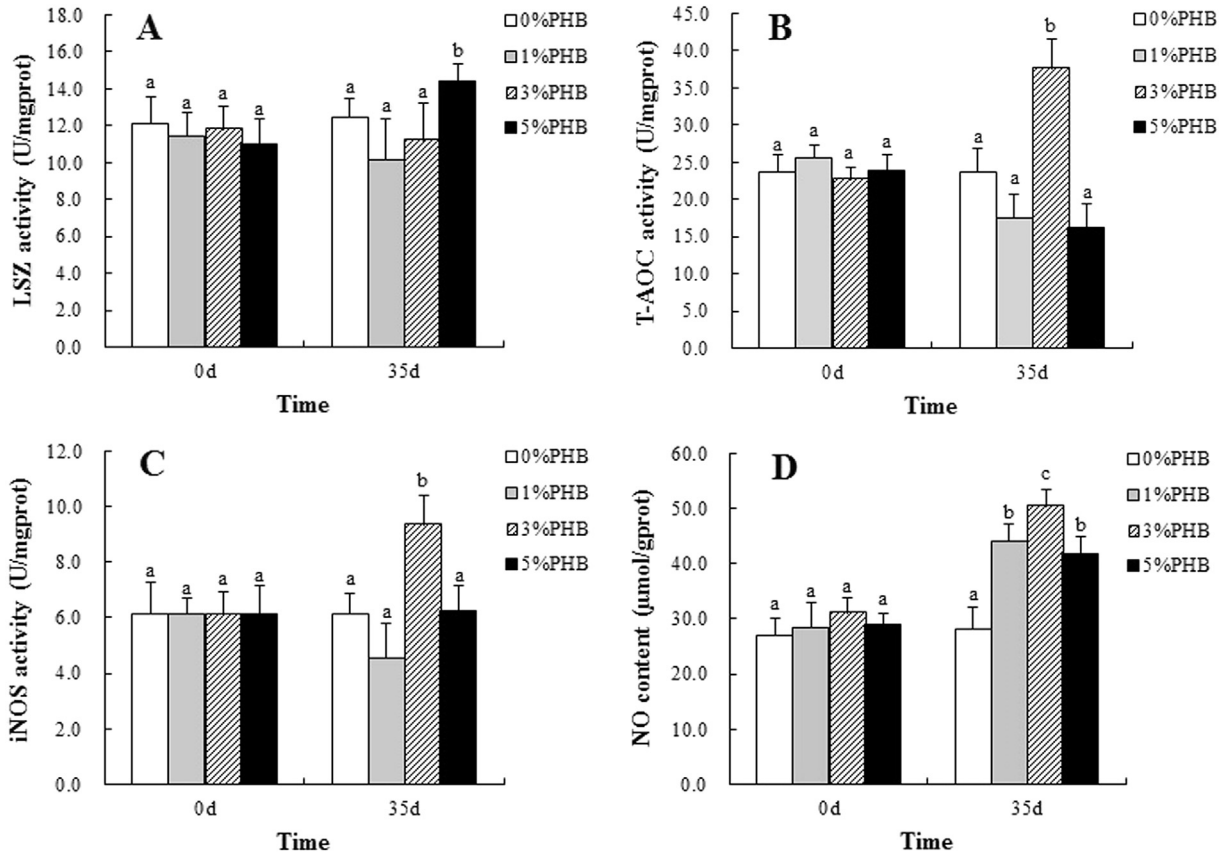

Fig. 3. LSZ (A), T-AOC (B), iNOS (C) activity and NO (D) content in intestinal of *L. vannamei* fed the control diet and three PHB-containing diets for 35 days. Vertical bars represented the mean ± SE ($N = 3$). Data indicated with different letters were significantly different ($P < 0.05$) among treatments.

stages. The increase of intestinal epithelial cell microvilli and enterocytes height and/or density has been demonstrated that they could provide a vast absorptive surface area and nutrient absorptive ability [39,40]. In this study, the intestinal well and enterocytes height of PHB treatments increased significantly which indicated that PHB could benefit the nutrient absorptive function.

Intestinal immunity status plays a vital role in protecting shrimp from various pathogens and environmental stresses. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are two of the most important immune systems in aquatic animals [41,42]. T-AOC is the important body of antioxidant defense system, which can prevent the deleterious effects of ROS and is recognized as an important indicator of total intracellular antioxidant status [43]. iNOS pathway is an important antimicrobial system of phagocytic cells, which is responsible for the generation of NO radicals, and is collectively known as RNS [44,45]. In this study, 3%PHB treatment significantly increased the intestinal T-AOC and iNOS activity of *L. vannamei*, and three PHB treatments all significantly induced the intestinal NO content of *L. vannamei*, suggesting that the protective effect of PHB-enriched feed might be partly associated with its antioxidant function and antioxidant defense system triggered following the SCFA degraded from PHB administration. LSZ was also called muramidase, an effective antibacterial agent, which could destroy peptidoglycan support and cause bacterial splitting under osmotic pressure within bacteria and plays an important role in the crustacean immune defense [46]. In this study, 5%PHB treatment had a significant effect on the activity of the intestinal LSZ activity of *L. vannamei*, which indicated that adding a proper amount of PHB in the diet could improve non-specific immunity of shrimp. HSP70 play an important role in maintaining protein homeostasis and signaling pathways and protecting organisms from the detrimental effects by environmental stressors [47]. Toll and Imd pathways are the two major components of the shrimp immune system which actively against gram-positive and gram-negative bacteria respectively through controlling different kinds of antimicrobial peptides [48,49]. In this study, the up-regulation of HSP70, Toll and Imd gene expression suggested that additional PHB in diet could improve the enhance the intestinal health status of *L. vannamei* for resisting different pathogens and environmental stressors.

SCFAs play an important role in the lipid metabolism of the digestive tract and maintaining the intestinal health of the animals [50,51]. Previous studies have demonstrated that un-dissociated form of the acid such as SCFA could penetrate through the bacterial cell wall, released its protons ($H+$) in the neutral cytoplasm and lowered the intracellular pH. The bacterium redirects its efforts towards the efflux of the excess protons, thereby exhausting the cell metabolism and leading to lower cell growth and even cell death [13,15,52]. In this study, the content of acetic, propionic and butyric acid of 3%PHB treatment were significantly higher than that of the control, revealing that it might be used as source of energy or for lipid biosynthesis in shrimp. The increasing trend of SCFA content induced by PHB has also been observed in *D. labrax* and *M. rosenbergii* [12,17]. In addition, the present study showed that the crude protein and lipid content in 5%PHB group were all increased significantly, indicating that PHB could improve the body nutrition composition.

In conclusion, dietary supplementation of PHB significantly

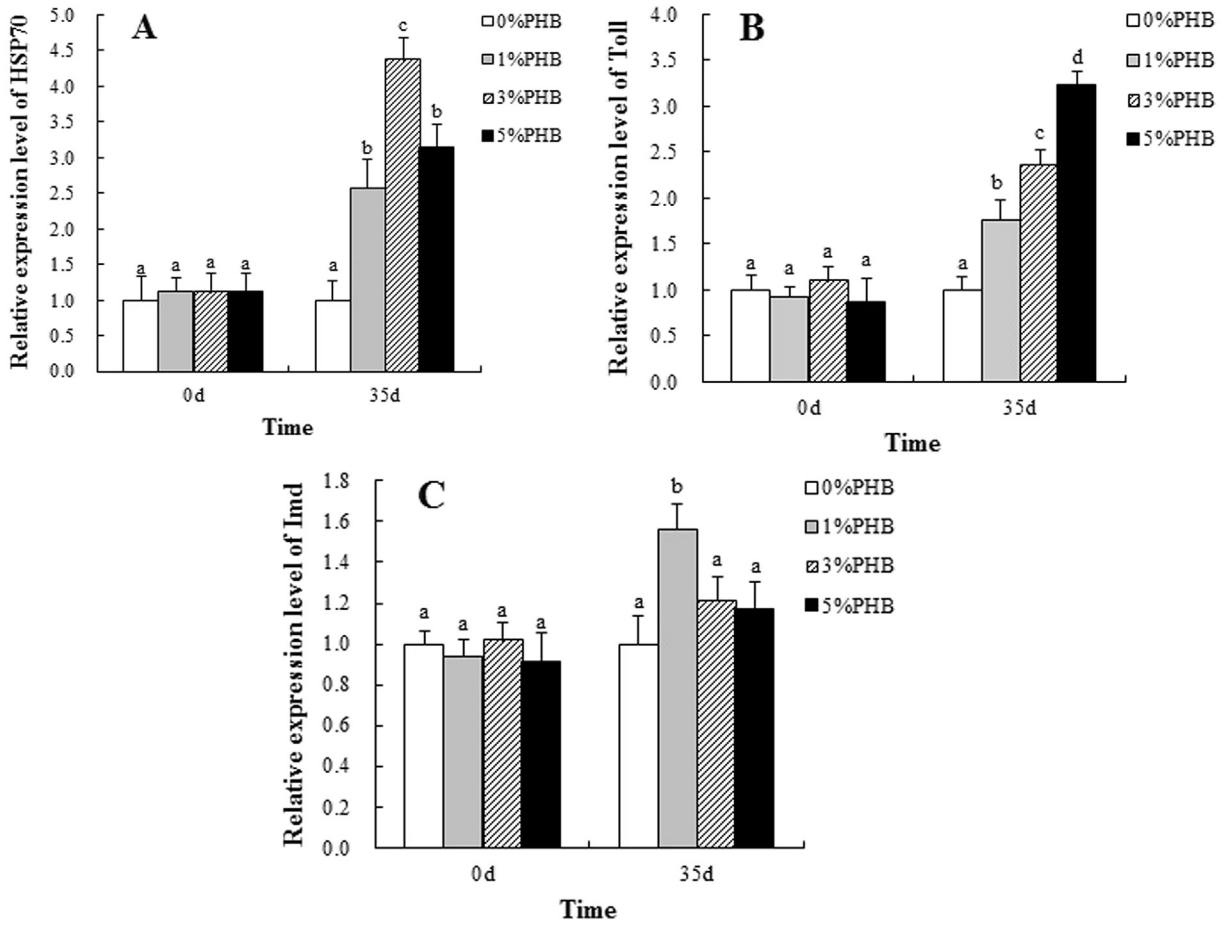

Fig. 4. HSP70 (A), Toll (B) and Imd (C) gene expression level in intestinal of *L. vannamei* fed the control diet and three PHB-containing diets for 35 days. The reference gene is β-actin. Vertical bars represented the mean ± SE ($N = 3$). Data indicated with different letters were significantly different ($P < 0.05$) among treatments.

improved the growth performance and feed utilization efficiency of *L. vannamei*. The intestinal digestive and immune enzymes activity and immune gene expression level were induced by PHB treatment and contributed to the enhanced feed utilization and intestinal digestive and immune function. In addition, PHB affected the intestinal SCFA content and body composition of *L. vannamei*. Therefore, PHB is future candidate for use in shrimp aquaculture and the optimum dose of dietary PHB was 3% (*w*/*w*) at our study condition. These results can provide the valuable data about the intestinal health regulatory mechanism of PHB in *L. vannamei*. Further studies will be focus on the effect of PHB on the relevance of intestinal microbial changes with the host metabolism in shrimp.

Table 3
Intestinal SCFA content (μg/g) of *L. vannamei* fed the control diet and three PHB-containing diets for 35 days.

Parameters	Group	At the beginning of feeding trial	At the end of feeding trial
		0 d	35 d
Acetic acid	Control	15642.90 ± 13.18^{a}	15639.90 ± 15.23^{a}
	1%PHB	15651.90 ± 10.21^{a}	17474.66 ± 17.62^{b}
	3%PHB	15649.90 ± 12.10^{a}	17875.34 ± 20.23^{c}
	5%PHB	15645.90 ± 17.13^{a}	19913.74 ± 19.14^{d}
Propionic acid	Control	826.31 ± 4.68^{a}	825.44 ± 7.15^{b}
	1%PHB	829.26 ± 5.12^{a}	840.79 ± 6.25^{c}
	3%PHB	831.19 ± 5.45^{a}	1179.45 ± 8.52^{d}
	5%PHB	827.24 ± 8.15^{a}	570.70 ± 10.32^{a}
Butyric acid	Control	414.23 ± 4.15^{a}	413.50 ± 3.20^{c}
	1%PHB	415.18 ± 3.13^{a}	39.18 ± 1.56^{a}
	3%PHB	413.15 ± 4.26^{a}	1713.88 ± 4.31^{d}
	5%PHB	414.05 ± 3.22^{a}	72.33 ± 2.15^{b}

Vertical bars represented the mean ± SE ($N = 3$). Data indicated with different letters were significantly different ($P < 0.05$) among treatments at the same sampling time.

Table 4
Body composition (% wet weight basis) of the whole body of *L. vannamei* fed the control diet and three PHB-containing diets for 35 days.

Parameters	Group	At the beginning of feeding trial	At the end of feeding trial
		0 d	35 d
Moisture	Control	76.42 ± 0.13^{a}	76.12 ± 0.35^{a}
	1%PHB	76.41 ± 0.16^{a}	76.36 ± 0.18^{a}
	3%PHB	76.41 ± 0.18^{a}	75.69 ± 0.24^{a}
	5%PHB	76.41 ± 0.14^{a}	76.25 ± 0.23^{a}
Crude protein	Control	17.62 ± 0.19^{a}	17.64 ± 0.15^{a}
	1%PHB	17.64 ± 0.16^{a}	17.89 ± 0.21^{ac}
	3%PHB	17.62 ± 0.20^{a}	18.32 ± 0.19^{b}
	5%PHB	17.63 ± 0.18^{a}	18.17 ± 0.22^{bc}
Crude lipid	Control	1.28 ± 0.10^{a}	1.25 ± 0.08^{a}
	1%PHB	1.28 ± 0.15^{a}	1.16 ± 0.12^{a}
	3%PHB	1.27 ± 0.09^{a}	1.33 ± 0.06^{a}
	5%PHB	1.29 ± 0.11^{a}	1.58 ± 0.03^{b}
Ash	Control	2.25 ± 0.10^{a}	2.29 ± 0.11^{a}
	1%PHB	2.23 ± 0.07^{a}	2.21 ± 0.09^{a}
	3%PHB	2.22 ± 0.08^{a}	2.19 ± 0.05^{a}
	5%PHB	2.19 ± 0.13^{a}	2.15 ± 0.04^{a}

Vertical bars represented the mean ± SE ($N = 3$). Data indicated with different letters were significantly different ($P < 0.05$) among treatments at the same sampling time.

Acknowledgments

The authors were grateful to all the laboratory members for experimental material preparation and technical assistance. This study was supported by the earmarked fund for Special Scientific Research Funds for Central Non-profit Institutes, South China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (2016TS07), Guangdong Marine Economy Innovation Development Demonstration Project (GD2013B03005), Guangdong Provincial Special Fund for Marine Fisheries Technology (A201501B15, A201508B05), National Natural Science Foundation of China (31502183), Guangdong Natural Science Foundation (2014A030310116, 2015A030310393), and Guangdong Science and Technology Planning Project (2013B021100014).

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