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Effect of dietary poly-β-hydroxybutyrate on growth performance, feed utilization, water quality, immunity and ammonia stress tolerance in *Penaeus vannamei* post-larvae

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Abstract

The effects of dietary poly-β-hydroxybutyrate (PHB) supplementation on growth, feed utilization, water quality parameters and ammonia stress resistance of post-larval Pacific white shrimp (*Penaeus vannamei*) were investigated in this study. A fish meal-based basal diet (P0.0) was formulated and another four diets were prepared by incorporating 0.25%, 0.50%, 1.00% and 2.00% PHB into the basal diet (P.25, P.50, P1.0 and P2.0, respectively). Triplicate groups of treatment, each containing 100 similar-sized post-larvae (2.5 ± 0.3 mg) per tank (96 L), were fed with the relevant test diet three times a day for five weeks. At the end of the feeding trial, shrimp were challenged against a 48 h-LD_{50} toxic ammonia concentration (0.150 ppm). Final body weight, weight gain, length gain, specific growth rate, feed conversion ratio and protein efficiency ratio of shrimp were not significantly affected. However, numerically higher final body weight and weight gain were shown by P.50 group, which was 31 mg higher in final body weight and 1,339% higher in weight gain compared to P0.0 treatment. P1.0 group showed a significantly higher survival than P0.0 and P2.0 groups. Prophenoloxidase (*proPO*) gene expression was significantly upregulated with dietary PHB supplementation at 0.25%–1.00% levels and the highest expression was observed in P1.0 group, which was 2.53 folds higher than P0.0 group. The water quality assessment test indicated that 2.00% PHB inclusion level significantly reduced water nitrite levels by 0.18 mg/L, but ammonia levels were not significantly affected. However, ammonia levels were reduced, showing a significant linear trend. At the end of the ammonia challenge, cumulative survivals in P0.0, P.25, P.50, P1.0 and P2.0 groups were 40.0%, 45.0%, 55.0%, 63.3% and 76.7%, respectively. *proPO* and lipopolysaccharide and β-1, 3-glucan-binding protein gene expressions in challenged shrimp were significantly upregulated in P1.0 and P2.0 groups compared to other groups. The results indicated that 1%–2% PHB in the diet can improve growth, health and ammonia stress resistance in Pacific white shrimp post-larvae.

Keywords: Ammonia stress tolerance, Immune gene expression, Pacific white shrimp post-larvae, Poly-β-hydroxybutyrate, Water quality management

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Introduction

Recent advancements in aquaculture nutrition have led to the development of sustainable feed ingredients that provide significant nutritional and health benefits for fish and shellfish. Poly-β-hydroxybutyrate (PHB) is a promising novel feed additive that is synthesized by a wide range of bacteria, including *Azotobacter*, *Bacillus*, *Rhizobium*, *Pseudomonas*, *Cupriavidus*, *Alcaligenes*, *Ralstonia* and *Nocardia* (Defoirdt et al., 2009). The synthesis process includes three enzymatic processes, including condensation of acetyl-CoA, reduction of acetoacetyl-CoA to hydroxybutyryl-CoA and polymerization of PHB during stress conditions (McAdam et al., 2020). Those microbes store PHB in the cell as carbon and energy source to utilize under adverse environmental conditions. PHB can be produced simply by controlling the environmental conditions of microbes, making it a sustainable and cost-effective ingredient for animal feeds. For instance, *Ralstonia eutropha* and *Azospirillum brasilense* can accumulate PHB up to 70%–90% dry weight of the cell (Defoirdt et al., 2009). PHB is degraded into β-hydroxybutyrate in the animal digestive tract and its nutritional value varies depending on production methods and microbial strain (McAdam et al., 2020).

Growth and health performance of farm animals are crucial factors for the productivity in aquaculture. Studies have demonstrated that dietary PHB supplementation improves the growth, innate immunity, digestive enzyme activities and robustness of various aquatic species (Kim et al., 2024; Liu et al., 2022). Previous studies observed significantly higher survival and growth performance in half-smooth tongue sole (*Cynoglossus semilaevis*) juveniles (Gao et al., 2020) and soiny mullet (*Liza hematochezia*) (Qiao et al., 2019). Additionally, dietary supplementation of gelatinized PHB has been shown to provide histopathological protection in Pacific white shrimp (*Penaeus vannamei*) against *Vibrio parahaemolyticus* (Kiran et al., 2020). Similar observations have been reported in gibel carp (*Carassius auratus gibelio*) and juvenile Chinese mitten crab (*Eriocheir sinensis*; Liu et al., 2022; Sui et al., 2016). Satoh (2023) highlighted that PHB is a promising prebiotic that releases β-hydroxybutyrate by gut microbial activity, promoting exogenous ketosis. Dietary PHB supplementation improved the survival percentages of the giant tiger prawn (*Penaeus monodon*) at ammonia challenges (Laranja et al., 2014).

Management practices, high stocking densities, artificial environment conditions and formulated feed usage in intensive shrimp culture often led to poor water quality, chronic stressful conditions and weakened immunity in shrimp. These conditions create a favorable environment for pathogenic bacteria to harbor in the intestines and hepatopancreas of shrimp, resulting in disease outbreaks, mass mortalities and significant economic losses (Walker & Mohan, 2009). Therefore, farmers tend to use antibiotics in aquaculture feed targeting short-term benefits. However, such practices can be harmful due to the bioaccumulation of antibiotics, the emergence of antibiotic-resistant pathogenic bacteria and potential adverse effects on consumer health. As an alternative, PHB can be used as a prebiotic to colonize beneficial bifidobacteria species in the intestine and suppress the growth of pathogenic bacteria (Das et al., 2017). During this symbiosis, bifidobacteria breaks down PHB into its oligomers and short-chain fatty acids. These short-chain fatty acids exert antimicrobial effects by lowering gut pH, disrupting bacterial metabolism through proton accumulation, decreasing the toxicity of enterotoxins, suppressing the expression of pathogenic genes and inhibiting the virulent factor of harmful bacteria (Defoirdt et al., 2009). Additionally, the resulting short-chain fatty acids serve as an energy source for enterocytes, enhance mucus secretion, support the mucosal immune system, and increase nutrient absorption (Xiong et al., 2022). Studies have shown that the antimicrobial activity of PHB improved immune parameters and enhanced the resistance against *Vibrio*sis in Pacific white shrimp and kuruma shrimp (*Marsupenaeus japonicus*; Fukami et al., 2021; Kim et al., 2024; Kiran et al., 2020).

High-density aquaculture systems often face water quality-related challenges, particularly the accumulation of ammonia, nitrite and nitrates, which negatively affect the physiological processes of fish and shrimp. Prolonged exposure to ammonia damages hepatopancreas and gill tissue, suppresses chitinase expression and molting, nutrient metabolism and compromises immune functions, decreasing resistance against pathogens (Zhao et al., 2020). Dietary PHB supplementation may mitigate these adverse effects by enhancing gut microbial activity and diet digestibility, as well as reducing nitrogenous waste excretion and organic matter accumulation (Luo et al., 2019). Pacific white shrimp is the most widely farmed aquacultured shrimp species, accounting for a total global production of approximately 6.8 million metric tons in 2022 (FAO, 2024), owing to numerous favorable characteristics suitable for high-density aquaculture systems. Considering the improved performance of shrimp species fed with PHB at 0.40%–5.00% levels in previous studies (Duan et al., 2017; Laranja et al., 2014) and pretest conducted in the laboratory, this study was designed to evaluate the effect of dietary supplementation of graded levels of

PHB at 0.25%–2.00% on growth, feed utilization, water quality management, ammonia stress tolerance and gene expressions of prophenoloxidase (*proPO*) and lipopolysaccharide and β-1, 3-glucan-binding protein (*LGBP*) in post-larval Pacific white shrimp.

Materials and Methods

Experimental diets

The basal diet (P0.0) was formulated using fish meal (26%) and cod liver oil (3%) to contain 47% crude protein and 7.5% crude lipid. The diet formulation and proximate compositions are shown in Table 1. Another four diets were formulated, incorporating 0.25%, 0.50%, 1.00% and 2.00% PHB into the P0.0 at the expense of starch. PHB (98%) was provided by CJ Cheiljedang BIO, Protein Solution Dept (Seoul, Korea). These diets were named P.25, P.50, P1.0 and P2.0, respectively. According to the formulation, all ingredients were measured and mixed using a dough mixer, adding distilled water (15%) and cod liver oil. The wet dough was pelleted using a pellet machine (SP-50, Kum-Kang Engineering, Daegu, Korea) and air-dried using an electric air-dryer (SI-2400, Shinil General Drier, Daegu, Korea) at 24℃ for 8 hours. Then, the dried diets were crumbled and separated into three different sizes using standard test sieves of 500, 710 and 850 μm to feed post-larvae with the growth. Diets were stored at −20℃ until use. Moisture, ash and crude protein levels were determined according to AOAC (2005) guidelines. Crude lipid content was analyzed following the method of Folch et al. (1957).

Experimental setting and feeding

Shrimp post-larvae were obtained from a private hatchery (Daedong Fisheries, Seoul, Korea) and acclimatized to the experimental facilities and conditions for 5 days supplying the P0.0 diet at the Kidang Marine Science Institute, Jeju National University, South Korea. Following the acclimation, groups of 100 average-sized post-larvae (initial mean body weight, 2.5 ± 0.3 mg; PL-10 to 12 stages) were assigned to each aquarium (96 L). Each dietary treatment was tested in triplicate (a total of 15 tanks). Shrimp were fed three times daily (08:00, 13:00 and 18:00) with an experimental diet for five weeks and tank biomasses were checked in the third week. The feeding rate (5%–10% tank biomass) was adjusted to avoid feed wastage by observing the feed intake and the leftover feed deposited, if any, on the tank floor (Hasanthi & Lee, 2023). Aquarium heaters were used to maintain the water temperature between 30.0℃ ± 1.0℃. Dissolved oxygen level was maintained at 5.88 ± 0.13 mg/L through continuous aeration and average salinity and pH levels were 32.5 ± 0.5 ppt and 7.88 ± 0.25, respectively. Rearing water was exchanged every four days with preheated seawater.

Table 1. Dietary formulation and proximate composition of the basal diet for Pacific white shrimp (*Penaeus vannamei*) post-larvae

Ingredients	g/kg
Fish meal, sardine[1)]	130.0
Fish meal, tuna[2)]	130.0
Soybean meal	380.0
Soybean protein concentrate[3)]	50.0
Tankage meal	50.0
Squid liver powder	50.0
Wheat flour[4)]	38.0
Starch	50.0
Cod liver oil[5)]	30.0
Vit/Min premix[6)]	30.0
Choline chloride (50%)	20.0
Lecithin[7)]	10.0
Cholesterol	2.0
Monocalcium phosphate	30.0
Proximate composition (%)	
Moisture	8.94
Crude protein	47.6
Crude lipid	7.51
Ash	14.0

Five experimental diets were prepared by incorporating 0.00%, 0.25%, 0.50%, 1.00% and 2.00% poly-β-hydroxybutyrate into the basal diet at the expense of starch.
[1), 2)] Orizon S. A., Santiago, Chile (crude protein: 75% and 60%; crude lipid: 9.5% and 17.1%).
[3)] Solae LLC, Saint Luis, MO, USA.
[4)] Daehan Flour, Incheon, Korea.
[5)] E-wha oil & fat Industry, Busan, Korea.
[6)] Vitamin/Mineral premix (g kg^{-1} of mixture): retinol, 3.0; cholecalciferol, 1.0; ascorbic acid, 20.0; tocopherol, 20.0; menadione, 2.0; thiamine, 4.0; riboflavin, 6.0; pyridoxine, 5.0; cobalamin, 6.0; inositol, 54.0; pantothenic acid, 12.0; biotin, 0.2; niacin amide, 40.0; folic acid, 2.0; ferrous sulfate, 10.0; copper sulfate, 1.0; zinc sulfate, 30; manganous sulfate, 2.0; cobalt chloride, 10; potassium iodide, 1.0; potassium, 6.0; sodium selenite.
[7)] Lysoforte™ Dry, KEMIN Korea, Seongnam, Korea.

Sample collection

At the end of the feeding trial, individual shrimp were weighed and their rostrocaudal lengths were measured. Final mean body weight (FBW), weight gain percentage (WG), length gain (LG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and survival were evaluated. A total of 5 average-sized shrimp per tank (15 per treatment) were randomly selected for gene expression analysis and euthanized by

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dipping in ice-cold water. Then, hepatopancreas were separated under sterile conditions and samples were immediately frozen in liquid nitrogen.

Ammonia and nitrite excretion evaluation

Ammonia and nitrite levels in the rearing water were analyzed. In line with the feeding trial, 15 additional tanks of similar size (120 L capacity) were arranged and each tank was filled with 100 L of preheated seawater. Water temperature, dissolved oxygen, salinity and pH values were 30.2℃ ± 1.4℃, 6.04 ± 1.83 mg/L, 32.8 ± 0.7 ppt and 7.67 ± 0.31, respectively. Initial concentrations of water ammonia and nitrite were determined according to the Strickland & Parsons (1972) and APHA (1995) methods and respective values were 0.015 ± 0.003 mg/L and 0.01 ± 0.002 mg/L.

A group of 100 post larvae with an average initial body weight of 0.153 ± 0.025 g were stocked in each tank and fed their respective diet at 5% of body weight. Shrimp were acclimatized for the first 4 days. Rearing water was completely replaced with preheated seawater at 4-day intervals and feed residues and fecal matter were removed. Triplicate water samples from each tank were collected to monitor water ammonia and nitrite levels just before each of the following 3 consecutive water changes. Samples were stored in airtight plastic sampling bottles and immediately analyzed for ammonia and nitrite levels.

Ammonia challenge test

After the feeding trial, an ammonia challenge experiment was conducted. Prior to starting the ammonia challenge test, a preliminary test was conducted to evaluate 48-hour LD_{50} ammonia concentration by exposing another set of shrimp to 0.10, 0.15 and 0.30 mg/L ammonia concentrations in triplicate tanks containing 20 shrimp (0.25 ± 0.07 g) in each tank. Based on the preliminary test, 0.15 mg/L concentration was identified as the 48 h LD_{50} toxic ammonia level. The required amount of ammonium chloride was calculated following Bower & Bidwell (1978) and Erickson (1985) ammonia levels were confirmed with Strickland & Parsons (1972) method.

To evaluate the ammonia resistance of PHB fed shrimp, post-larvae in each treatment were pooled and sixty average-sized shrimp per dietary treatment were randomly selected and redistributed among three replicate tanks with 20 shrimp per tank (96 L, total of 15 tanks). Initial water ammonia concentration was 0.152 ± 0.002 mg/L and the shrimp were not fed during the stress test. Mortality was recorded at 1-hour intervals for 48 hours. At the end of challenge tests, hepatopancreas samples were collected from five shrimp per tank following the above-explained procedure to evaluate the expression of *proPO* and *LGBP* genes.

Real-time quantitative reverse transcription polymerase chain reaction analysis

Total RNA was extracted from the hepatopancreas samples using TRI-zol® reagent (Sigma-Aldrich, St. Louis, MO, USA) and RNA concentration and purity (OD_{260}/OD_{280}) were measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized using the PrimeScript™ First-Strand cDNA Synthesis Kit (TaKaRa, Shiga, Japan). *β-actin* was used as the reference gene. NCBI GenBank® acquisition numbers and forward (F) and reverse (R) primer sequences are as follows: β-*actin* (AF300705.2), β-*actin*-F: CCACGAGACCACCTACAAC and β-*actin*-R: AGCGAGGGCACTGATTTC; *proPO* (AY723296), *proPO*-F: CGGTGACAAAGTTCCTCTTC and *proPO*-R: GCAGGTCGCCGTAGTAAG; *LGBP* (EU102286.1), *LGBP*-F: CAGGGGCAACGACAACTTTG and *LGBP*-R: GTGTGGGGATCTACTGCTCG. Gene expression levels were evaluated using the real-time quantitative reverse transcription polymerase chain reaction analysis (RT-qPCR) method, as described in Ko et al. (2024) and the relative gene expression was calculated following the method explained in Pfaffl (2001).

Calculations and statistical analysis

Growth performance and feed utilization were calculated using the equations below.

WG (%) = [(final body weight − initial body weight) / initial body weight] × 100%

SGR (%/d) = {[ln (final body weight) − ln (initial body weight)] / days} × 100%

FCR = dry feed fed / wet weight gain

PER = wet weight gain / total protein given

LG (%) = [(final body length − initial body length) / initial body length] × 100%

Treatments for the feeding trial and challenge test were arranged in a completely randomized design to control the variabilities and isolate the treatment effect. Arcsine transformation was applied to percentage data and all the data were normalized. Data were presented as mean ± SD. One-way analysis of variance was conducted using SPSS (version 18.0, IBM, Armonk, NY, USA) to determine significant differences among treatments, assuming normally distributed residuals, homogeneity of variances across groups and independence of observations. Tukey's honestly significant difference (HSD) post hoc multiple comparison test ($p < 0.05$) was used to compare mean differences between treatments. The orthogonal polynomial contrast test assessed whether the effect of increasing PHB levels followed a linear and/or quadratic trend. The adjusted R^2 values obtained from univariate analysis of variance (ANOVA) were used to evaluate the strength of the relationship between treatments and outcomes.

Results

Growth performance, feed utilization and survival

FBW, WG, LG and SGR did not differ significantly ($p > 0.05$) among dietary treatments (Table 2). However, LG showed a significant positive linear trend ($p < 0.05$). Feed utilization parameters, including FCR and PER, also showed no significant differences among treatments ($p > 0.05$). A significantly higher survival was observed in P1.0 group compared to P0.0 group and showed a significant quadratic trend with increasing PHB levels ($p < 0.05$).

Water quality parameters

PHB supplementation improved water quality parameters (Table 3). Water ammonia levels did not differ significantly among treatments ($p > 0.05$); however, a significant linear reduction in ammonia levels was observed with increasing dietary PHB levels in diets. A significantly lower nitrite level was observed in P2.0 treatment compared to P.25 group ($p < 0.05$). Water nitrite levels showed no significant linear or quadratic trends ($p > 0.05$).

Ammonia exposure challenge

Within the first 24 hours of the ammonia challenge, no mortality was reported (Fig. 1). However, after 48 h of challenge, the survival percentages in P0.0, P.25, P.50, P1.0 and P2.0 groups were 40.0%, 45.0%, 55.0%, 63.3% and 76.7%, respectively.

Relative gene expression levels

According to the gene expression data presented in Table 4, *proPO* gene expression was significantly upregulated in P.25 and P1.0 groups compared to other treatments, showing a significant quadratic trend ($p < 0.05$). Additionally, P.50 group showed

Table 2. Growth performance, feed utilization and survival of Pacific white shrimp (*Penaeus vannamei*) post-larvae fed different levels of poly-β-hydroxybutyrate (PHB) incorporated experimental diets for five weeks

	FBW[1)]	WG[2)]	LG[3)]	SGR[4)]	FCR[5)]	PER[6)]	Survival[7)]
P0.0	272 ± 28.8	10,789 ± 1,153	278 ± 12.2	13.4 ± 0.31	1.39 ± 0.20	1.53 ± 0.20	81.7 ± 3.21[b]
P.25	293 ± 15.9	11,618 ± 638	291 ± 11.2	13.6 ± 0.16	1.27 ± 0.15	1.55 ± 0.05	79.0 ± 10.1[ab]
P.50	308 ± 15.8	12,128 ± 633	297 ± 10.0	13.7 ± 0.15	1.39 ± 0.07	1.57 ± 0.04	83.7 ± 6.66[ab]
P1.0	288 ± 17.6	11,417 ± 704	293 ± 12.4	13.6 ± 0.17	1.28 ± 0.06	1.58 ± 0.08	89.0 ± 4.57[a]
P2.0	303 ± 16.9	12,028 ± 680	303 ± 16.4	13.7 ± 0.16	1.50 ± 0.09	1.43 ± 0.01	78.7 ± 2.89[b]
Pr > *F*[*]							
ANOVA	0.304	0.304	0.229	0.302	0.470	0.483	0.034
Linear	0.144	0.144	0.044	0.140	0.552	0.401	0.084
Quadratic	0.353	0.353	0.551	0.337	0.119	0.158	0.035
Regression							
Model	NS	NS	L	NS	NS	NS	Q
Adj R^2	0.102	0.102	0.164	0.103	–0.012	–0.019	0.092

P0.0, P.25, P.50, P1.0 and P2.0 are 0.00%, 0.25%, 0.50%, 1.00% and 2% PHB incorporated treatments.
The initial mean body weight of post-larvae was 2.5 ± 0.3 mg.
If statistical significance ($p < 0.05$) was detected, the model that best fit the data was chosen.
Values are the mean of triplicate (n = 3) and presented as mean ± SD.
[1)] Final body weight (mg).
[2)] Weight gain (%).
[3)] Length gain (%).
[4)] Specific growth rate (%/d).
[5)] Feed conversion ratio.
[6)] Protein efficiency ratio.
[7)] Survival (%).
[a,b] Values with different superscript letters in the same column are significantly different ($p < 0.05$).
[*] Significance probability associated with the *F*-statistic.
L, linear model; Q, quadratic model; NS, no structure; Adj R^2, adjusted R^2.

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Table 3. Water quality parameters in the rearing tanks of Pacific white shrimp (*Penaeus vannamei*) post-larvae fed different levels of poly-β-hydroxybutyrate (PHB) incorporated experimental diets for five weeks

	Ammonia[1)]	Nitrite[2)]
P0.0	0.038 ± 0.002	0.98 ± 0.10[ab]
P.25	0.038 ± 0.003	1.03 ± 0.07[a]
P.50	0.038 ± 0.001	0.95 ± 0.05[ab]
P1.0	0.035 ± 0.003	0.97 ± 0.10[ab]
P2.0	0.032 ± 0.004	0.85 ± 0.10[b]
Pr > F[*]		
ANOVA	0.098	0.042
Linear	0.018	0.086
Quadratic	0.146	0.220
Regression		
Model	L	NS
Adj R^2	0.318	0.152

P0.0, P.25, P.50, P1.0 and P2.0 are 0.00%, 0.25%, 0.50%, 1.00% and 2.00% PHB incorporated diet treatments.
If statistical significance ($p < 0.05$) was detected, the model that best fit the data was chosen.
Values are the mean of triplicate (n = 3) and presented as mean ± SD.
[1)] Ammonia NH_3 (mg/L).
[2)] Nitrite NO_2-N (mg/L).
[a,b] Values with different superscript letters in the same column are significantly different ($p < 0.05$).
[*] Significance probability associated with the *F*-statistic.
L, linear model; Q, quadratic model; NS, no structure; Adj R^2, adjusted R^2.

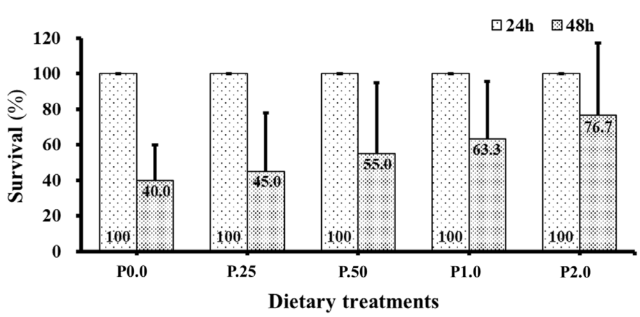

Fig. 1. Survival of Pacific white shrimp (*Penaeus vannamei*) fed the five experimental diets after 24 and 48 h ammonia exposure challenge. P0.0, P.25, P.50, P1.0 and P2.0 are 0.00%, 0.25%, 0.50%, 1.00% and 2.00% poly-β-hydroxybutyrate (PHB) incorporated diet treatments.

significantly higher *proPO* gene expression compared to P0.0 and P2.0 groups ($p < 0.05$). However, *LGBP* gene expression did not differ significantly among treatments ($p > 0.05$). In challenged shrimp, higher PHB inclusion levels than 0.50% significantly upregulated the *proPO* gene expression ($p < 0.05$) and the highest expression levels was observed in P1.0 group and P2.0. *LGBP* gene expression was significantly upregulated in post-larvae fed with diets containing PHB levels higher than 1.0% ($p < 0.05$). Both *proPO* and *LGBP* gene expressions showed significant linear trends ($p < 0.05$).

Discussion

Dietary supplementation of PHB at a 2.00% level increased WG by 1,239% compared to the control treatment. However, the overall growth performance of post-larval Pacific white shrimp was not significantly different among treatments. LG was also increased in a significant linear trend with increasing PHB supplementation, suggesting that supplementing even higher levels might significantly improve growth performance in post larvae. Previous studies have reported that PHB is a multifunctional feed additive in aquaculture (Defoirdt et al., 2009; Satoh, 2023). Primarily, PHB serves as an energy source by providing β-hydroxybutyrate, a short-chain fatty acid or ketone body that is readily absorbed and metabolized, and acts as a signaling molecule. Dietary PHB is a prebiotic that modifies the gut microbiome, promotes butyrate-producing bacteria and has potential therapeutic effects against chronic gut inflammation and metabolic diseases (Defoirdt et al., 2009). In contrast to traditional fiber-based prebiotics, PHB functions as a ketone-donating prebiotic, preventing gut diseases and chronic inflammation (Satoh, 2023). Further, Satoh (2023) reported that the anti-inflammatory effect of PHB is exerted by enhancing the differentiation of regulatory T cells. However, the relevant mechanism in crustaceans is yet to be investigated. The ketone bodies, β-hydroxybutyrate, generated during the microbial degradation of PHB are an alternative energy source that is more efficient than simple sugars and undergoes β-oxidation to produce ATP in mitochondria, similar to long-chain fatty acids, but at a faster rate. Colonocytes prefer butyrate over glucose as an energy source during cell growth and their growth is essential to maintaining gut integrity (Liu et al., 2022). The renewal of intestinal stem cells induced by PHB is regulated via the Notch signaling pathway through the inhibition of histone deacetylase (Satoh, 2023). This enhances gut barrier function by enhancing mucin production and tight junction proteins, preventing pathogen entry. Accordingly, PHB might have positively affected growth performance by enhancing nutrient absorption, improving intestinal integrity, reducing inflammation and providing an alternative fast-metabolizing energy source for shrimp post-larvae in this study. Moreover, improved water quality and immune-related gene expressions might have indirectly contributed to the enhanced growth performance of post-larvae. Previous studies also showed dietary PHB supplementation improved growth performance and feed utilization in Giant river prawn (*Macrobrachium rosenbergii*; Nhan et al., 2010), European bass (*Dicentrarchus labrax*; de Schryver et al., 2010), Giant tiger prawn (Laranja et al., 2014) and Nile tilapia (*Oreochromis niloticus*; Rodriguez-Estrada et al., 2021). However, growth performance was not affected by PHB in juvenile Pacific white shrimp and European sea bass yolk-sack larvae, suggesting that the growth-promoting effect of PHB is species-specific (Franke et al., 2017). Several previous studies reported that dietary supplementation of PHB significantly enhanced digestive enzyme activities, including pepsin, trypsin, lipase and amylase, in various aquatic species such as gibel carp, Chinese mitten crab and Pacific white shrimp, suggesting potential growth-promoting mechanisms (Duan et al., 2017; Liu et al., 2022; Sui et al., 2016). The efficacy of PHB is mainly dependent on its digestibility within the intestine. PHB degradation in animals heavily relies on bacterial fermentation since animals do not produce PHB depolymerase (Paloyan et al., 2025). However, dietary PHB significantly increases the butyrate-producing bacteria population in the gut, which also synthesizes exogenous PHB depolymerase, as PHB serves as a metabolic substrate for them (Satoh, 2023). Moreover, the efficiency of PHB utilization is influenced by factors such as gut microbiome composition, the presence of PHB-dependent probiotics, feeding habits, gut pH and the availability of fermentation sites within the digestive tract (Liu et al., 2022; Rodriguez-Estrada et al., 2021).

In this study, dietary PHB supplementation increased the survival percentage of post-larvae during the feeding trial. In addition to its role as an energy source, PHB is known for its antimicrobial properties (Defoirdt et al., 2009). It modulates the physicochemical properties of the environment and disrupts the physiological conditions in pathogens by altering membrane structure, interfering with macromolecule synthesis, downregulating the expression of virulence factor gene, reducing intestinal colonization and adhesion and inhibiting quorum sensing of pathogenic bacteria (Galán, 1996; Ricke, 2003). When β-hydroxybutyrate diffuses into the bacterial cell, it dissociates into ions, lowering cytoplasmic pH. To maintain homeostasis, microbes should continuously spend energy to export excess protons. Constant energy expenditure eventually retards their growth (Ricke, 2003). Additionally, the intestinal pathophysiological protection effect of monomer units of PHB may have contributed to the increased survival observed in this study (Liu et al., 2022). Previous studies exhibited that dietary PHB and its monomer units modify the gut microbiome, increasing beneficial bacteria populations such as *Lactococcus* spp., *Lactobacillus* spp. and *Bacillus* spp. while inhibiting pathogenic *Vibrio* spp. (Duan et al., 2017; Gao et al., 2020). These effects could explain improved survival during the feeding trial of this study.

The ammonia stress test revealed that dietary supplementation of PHB linearly increased the resistance of post-larvae against toxic water ammonia exposure. Ammonia and nitrites are unavoidable stressors in aquaculture, negatively affecting the growth and productivity of shrimp. The toxicity of ammonia in crustaceans occurs through three primary mechanisms: (1) ammonia accumulation in hemolymph and body fluids disrupts osmoregulation as NH_4^+ competes with Na^+ for passive cation diffusion in the gills, (2) ammonia stress induces gill damage, including lamellar fusion and epithelial detachment, impairing gas exchange and (3) finally ammonia stress induces oxidative and endoplasmic reticulum stress, leading to cell membrane damage, apoptosis and eventual cell death (Zhao et al., 2020). To resist ammonia toxicity and secondary pathogenic infections, shrimp require a well-functioning immune system and sufficient energy reserves. In this scenario, the role of PHB is significant as an alternative energy source to aid in tissue repair and immune defense. When depleting glucose energy reserves while combating secondary pathogenic infections during the ammonia stress test, PHB-derived ketone bodies can act as an alternative energy source for shrimp to increase the survival percentage (Satoh, 2023). Several studies have shown that PHB supplementation enhances stress tolerance against a wide range of stressors. Increasing dietary PHB levels increases the tolerance against osmotic stress, ammonia toxicity and *Vibrio*sis caused by *Vibrio penaeicida*, *V. parahaemolyticus* and *Vibrio harveyi* in Kuruma shrimp, Pacific white shrimp, Giant tiger prawn, Giant river prawn and artemia (*Artemia franciscana*; Fukami et al., 2021; Kim et al., 2024; Laranja et al., 2014).

The *LGBP* gene, encoding lipopolysaccharide and β-1,3-glucan-binding protein, plays a crucial role in the innate immune system of crustaceans by recognizing pathogen-associated molecular patterns (Chen et al., 2016). This binding activates the *proPO* cascade, which triggers immune responses such as degranulation of immune cells, releasing phenoloxidase, melanization and encapsulation of pathogens (Betancourt et al., 2024). In this study, dietary PHB significantly upregulated *proPO* gene expression, following a quadratic trend. Although *LGBP* gene expression showed no significant changes with di-

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Table 4. Relative gene expression levels of *proPO* and *LGBP* in Pacific white shrimp (*Penaeus vannamei*) post-larvae fed with different levels of poly-β-hydroxybutyrate (PHB) incorporated experimental diets for five weeks and challenged with toxic ammonia concentrations

	Feeding trial		Challenge test	
	proPO[1)]	*LGBP*[2)]	*proPO*	*LGBP*
P0.0	1.00 ± 0.00[c]	1.00 ± 0.00	1.00 ± 0.00[d]	1.00 ± 0.00[b]
P.25	3.27 ± 0.13[a]	1.04 ± 0.22	0.97 ± 0.29[d]	1.01 ± 0.65[b]
P.50	1.93 ± 0.30[b]	1.86 ± 0.21	2.10 ± 0.18[c]	1.44 ± 0.28[b]
P1.0	3.53 ± 0.09[a]	1.65 ± 0.48	5.25 ± 0.28[a]	2.35 ± 0.04[a]
P2.0	1.19 ± 0.13[c]	1.48 ± 0.47	3.78 ± 0.86[b]	1.94 ± 0.28[a]
Pr > *F*[*]				
ANOVA	0.000	0.129	0.000	0.000
Linear	0.071	0.074	0.000	0.000
Quadratic	0.000	0.148	0.550	0.107
Regression				
Model	Q	NS	L	L
Adj R^2	0.975	0.272	0.878	0.970

P0.0, P.25, P.50, P1.0 and P2.0 are 0.00%, 0.25%, 0.50%, 1.00% and 2.00% PHB incorporated diet treatments.
If statistical significance ($p < 0.05$) was detected, the model that best fit the data was chosen. Values are the mean of triplicate (fold differences compared to the control group; n = 3) and presented as mean ± SD.
1) Prophenoloxidase.
2) Lipopolysaccharide and β-1, 3-glucan-binding protein.
a–d Values with different superscript letters in the same column are significantly different ($p < 0.05$).
* Significance probability associated with the *F*-statistic.
L, linear model; Q, quadratic model; NS, no structure; Adj R^2, adjusted R^2.

etary PHB, a slight upregulation was observed at 0.5% or higher inclusion levels. Both *LGBP* and *proPO* gene expressions were significantly upregulated following the ammonia challenge, likely by enhancing shrimp immunity and contributing to the observed increase in survival. However, whether *LGBP* gene expression was upregulated in response to PHB or secondary pathogenic exposure remains unclear. Previous studies suggest that immune mechanisms, including increased hemocyte counts and activation of the *proPO* cascades, can be triggered when dietary prebiotic supplementation enhances intestinal probiotic populations and immunogenic compounds such as lipopolysaccharides and β-1,3-glucan (Hardy et al., 2013). Qiao et al. (2019) reported that PHB supplementation significantly upregulated the penicillin-binding protein-A gene in soiny mullet. In contrast, Franke et al. (2017) found no significant upregulation of pathogen recognition-related genes. Many studies have shown that short-chain fatty acids, including β-hydroxy butyrate, enhanced immune-related gene expressions such as *proPO*, crustin and penaeidin and antioxidant-related genes in fish and shellfish species (Franke et al., 2017).

Polyhydroxyalkanoates, including PHB, serve as a biodegradable carbon source for denitrifying bacteria such as *Comamonas*, *Acidovorax* and *Dechloromonas*, which use the electrons from PHB degradation to reduce nitrate (NO_3^-) into nitrogen gas (N_2), completing the denitrification process (Xu et al., 2018; Zhu et al., 2023). Unlike conventional carbon sources, PHB releases carbon slowly to sustain those bacteria without drastically depleting dissolved oxygen levels in aquaculture systems such as bio-biofloc systems (Zhu et al., 2023). This study showed that dietary supplementation of PHB reduced the water nitrite and ammonia levels during the water quality assessment test. Though there is no previous study evaluated the water quality parameters providing dietary PHB for aquatic species, some studies that treated PHB for bio-flock systems for tilapia and Pacific white shrimp demonstrated that NH_4^+-, NO_2^-- and NO_3^--nitrogen levels were maintained at lower levels with minimal water exchanges (Luo et al., 2017, 2019). Therefore, PHB can be considered a novel and sustainable feed additive that enhances the productivity of the aquaculture industry while reducing water pollution.

Conclusion

Supplementation of PHB in aquaculture species can enhance growth, immune-related gene expressions and resistance against toxic water ammonia levels. Dietary supplementation of PHB is a promising strategy to remove ammonia from the aquaculture systems. The inclusion of PHB at 1%–2% in the diets of Pacific white shrimp post-larvae would maximize the performance of shrimp and reduce water pollution.

Competing interests

No potential conflict of interest relevant to this article was reported.

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Availability of data and materials

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Ethics approval and consent to participate

The protocols of this feeding trial were evaluated and approved by the Institutional Animal Care and Use Committee of Jeju National University.

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