ORIGINAL ARTICLE

Veterinary Research Forum. 2025; 16 (1): 43 - 50
doi: 10.30466/vrf.2024.2024674.4200
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Growth-promoting potential and immunostimulatory of poly-β-hydroxybutyrate in common carp (*Cyprinus carpio*) fingerlings culture

Rabeeh Ziaei[1], Hossein Ouraji[2], Ebrahim Najdegerami[3]*, Reza Akrami[4], Hossein Chitsaz[4]

[1] General Department of Fisheries of Golestan Province, Gorgan, Iran; [2] Department of Fisheries, Faculty of Animal Science and Fisheries, Sari Agricultural Sciences and Natural Resources University, Sari, Iran; [3] Department of Biology, Faculty of Science, Urmia University, Urmia, Iran; [4] Department of Fisheries, Azadshahr Branch, Islamic Azad University, Azadshahr, Iran.

Article Info

Article history:

Received: 16 March 2024
Accepted: 05 October 2024
Available online: 15 January 2025

Keywords:

Antioxidant
Common carp
Immunity
Poly-β-hydroxybutyrate
Short chain fatty acid

Abstract

The natural polymer poly-β-hydroxybutyrate (PHB) is converted to β-hydroxybutyric acid, which is similar to short-chain fatty acids, via microbial fermentation and host enzyme breakdown. This study investigated the impact of different PHB concentrations (Control, 1.00, 3.00, and 5.00% substitution) on growth performances and fish welfare in common carp fingerlings. After a 60-day trial, fish fed on diet containing 1.00% PHB exhibited significantly higher weight gain and improved feed conversion efficiency compared to the control group. Furthermore, analysis of enzymatic activity showed elevated levels of total protease and amylase in PHB-fed treatments compared to the control. Red blood cell counts, hemoglobin, and hematocrit levels remained unaffected and a significant increase in white blood cell count was observed in fish fed on diets containing 1.00 and 3.00% PHB compared to the control group. Furthermore, fish fed on diets containing 1.00 and 3.00% PHB demonstrated significantly higher total protein levels and lower glucose concentrations as well as reduced hepatic enzyme activities (aspartate aminotransferase and alanine aminotransferase) compared to both the control and 5.00% PHB groups. Assessment of antioxidant and immune parameters revealed significantly increased complement hemolytic activity and immunoglobulin M levels coupled with decreased malondialdehyde concentrations in the plasma of PHB-fed fish compared to the control group. In conclusion, dietary supplementation with PHB, especially at the 1.00% level, enhanced growth performance and improved nutritional and health indicators in the fingerlings. These findings suggested that PHB had the potential to be a valuable dietary additive for this species.

Introduction

In recent years, the intensive farming of common carp (*Cyprinus carpio*) has seen significant growth in the worlds resulting in an increase in bacterial disease outbreaks on farms. Historically, antibiotics served as a primary solution for managing bacterial diseases in both aquatic and terrestrial animal farming. However, with the ban on antibiotic use in these settings, researchers globally have embarked on exploring alternative methods for health maintenance and disease mitigation.[1] This quest has led to the investigation of various substitutes for antibiotics, including probiotics and dietary supplements like prebiotics, which are non-digestible components utilized by specific microorganisms.[2,3] The fermentation of dietary substrates by beneficial microorganisms within the gut leads to the production of short-chain fatty acids (SCFAs). These SCFAs play a multifaceted role in shaping the gut environment. They selectively promote the proliferation of commensal bacteria while exerting suppressive effects on pathogenic strains.[4,5] Additionally, SCFAs contribute to a reduction in luminal pH, thereby, enhancing the intestinal absorption of essential minerals.[6,7] Their influence extends beyond the gut, as SCFAs have been demonstrated to modulate lipid metabolism in both animal and human models.[8] Furthermore, in vitro studies have reported the inhibitory effects of SCFAs on fungal growth, including yeast and various enteric pathogens such as *Shigella flexneri*, *Escherichia coli*, and *Salmonella typhimurium*.[5]

Poly-β-hydroxybutyrate (PHB) is a key polymer within the polyhydroxyalkanoates group composed of β-hydroxy-butyrate, a SCFA produced by several bacteria species

***Correspondence:**

Ebrahim Najdegerami. PhD
Department of Biology, Faculty of Science, Urmia University, Urmia, Iran
E-mail: e.gerami@urmia.ac.ir

(e.g., *Alcaligenes eutrophus*, *Bacillus megaterium*) as a means of storing intracellular energy and carbon.[9] Bacterial extracellular enzymes can degrade PHB into β-hydroxybutyrate monomers.[10-14] Notably, β-hydroxybutyrate has been shown to safeguard *Artemia franciscana* against *Vibrio campbellii*.[5] Moreover, PHB has been associated with enhanced growth performance and modulation of the microbial community in sea bass intestines.[1] Similarly, positive effects of PHB on the immune system, growth performance and microbial community have been reported in Artemia,[5,14] sea bass[1] and freshwater prawn.[15] The growth-promoting properties of PHB in sea bass and giant freshwater shrimp are well-documented, however, the underlying mechanisms remain elusive.

Understanding the effects of PHB on common carp is mostly based on two previous research by Li *et al*.,[16,17] according to the author's literature review. These studies investigated the effects of PHB on this species (different with the current study) and under certain circumstances, including exposure to copper and lipopolysaccharide stress. As a result, the outcomes concerning the impacts of PHB in normal conditions are still obscure. The primary objective of this research was to explore how the consumption of PHB impacted the growth performance, digestive enzyme activity, body composition and immune response of common carp fingerlings.

Materials and Methods

Experimental set up and animals. The experiment was conducted at Gorgan University of Agricultural Sciences and Natural Resources, Golestan Province, Iran. Common carp fingerlings were procured from local hatcheries in Azadshahr, Golestan Province. The fingerlings were acclimated to the experimental conditions for 1 week during which their health and any abnormal behaviors were closely monitored. Following acclimation, fish with an average initial weight of 20.00 ± 1.50 g were randomly allocated to four experimental groups each housed in 60-L glass aquariums. The tanks were maintained under controlled conditions with a 30.00% daily water exchange and a stocking density of 15 fish *per* tank. Water was continuously aerated and temperature was kept constant at 25.00 ± 2.00 ˚C under a 12-hr light-dark photoperiod. Fish were fed thrice daily with uneaten feed and fecal matter removed daily. The experimental period lasted for 60 days. All experiments were performed following the protocol approved by the committee of ethics of the faculty of science or the University of Tehran (357/8 Nov/2021).

Experimental diets. Poly-β-hydroxybutyrate powder (98.00% PHB, 2.00% poly-β-hydroxyvalerate; Goodfellow, Huntingdon, UK) was used as the test substance in this experiment. A range of concentrations were employed: 0.00 (as control), 1.00, 3.00 and 5.00 %.[18] The PHB was incorporated as a substitute for carboxymethyl cellulose in the diet formulation. The mixture was pelletized using a cold palletization system with a die size corresponding to the fish's oral cavity. Individual ration components were homogenized using a household blender. Thereafter, tap water (500 mL kg^{-1}) was introduced to the blend and thoroughly incorporated. The mixture was then processed into elongated filaments (1.00 - 2.00 mm diameter) using a meat grinder equipped with a spring mechanism. The extruded strands were subjected to controlled airflow for optimal drying. The dried strands were manually chopped into smaller pellets resulting in a final product with a protein content of 32.00% and a fat content of 9.60%. The prepared pellets were stored at 4.00 ˚C until use.

Sampling and analysis. To monitor the health of the fish throughout the 60-day experiment, the authors conducted daily survival checks, removing any deceased fish from the tanks.[19] No mortality was observed in the fingerlings throughout the experiment. Fish from each tank were subjected to a 24-hr fasting period prior to weekly weighing to monitor growth performance. Specific growth rate (SGR) was determined based on the average final weight after 6 weeks, initial weight and the total experimental period of 60 days. To evaluate feed utilization efficiency, feed conversion ratio (FCR) was calculated as the ratio of dry feed consumed to the increase in wet fish weight.

Assessment of digestive enzymes. At the end of the experiment, three fish *per* tank were euthanized with clove powder (200 mg L^{-1}) and dissected for digestive tract collection.[18] Samples were mechanically homogenized in a cold buffer with protease inhibitors and detergent, then centrifuged to isolate the soluble fraction. The supernatant was stored frozen for further analyses. Protease activity was measured at room temperature (25.00 ˚C) using 1.00% (w/v) casein as a substrate (Sigma Aldrich, St. Louis, USA) in a buffered solution (pH = 7.00).[20] Amylase activity was evaluated as *per* the method described by Métais and Bieth [21] with a starch substrate (Sigma-Aldrich) in NaH_2PO_4 buffer (pH = 7.40; Sigma-Aldrich). Amylase activity was quantified based on the amount of substrate hydrolyzed under controlled conditions (37.00 ˚C, over a 30-min period). Lipase activity was determined using a p-nitrophenol myristate (Sigma-Aldrich) as a substrate in a Tris-based buffer (pH = 9.00; Sigma-Aldrich) and the unit of activity was defined based on the rate of substrate hydrolysis at 30.00 ˚C.[22] A colorimetric assay was used to quantify the total protein content within the homogenate (Infinite M200; TECAN, Grödig, Austria).[23] A commercially available reference protein standard like bovine serum albumin (Sigma-Aldrich) was employed for calibration. Enzyme activity for each measured enzyme was then normalized by dividing the activity units by the total protein content (U mg^{-1} protein).

Fish proximate composition analysis. Following the experimental period, the whole-body composition of the fish was evaluated using established protocols. Total nitrogen content, employed for estimating crude protein, was quantified using Kjeldahl method.[24] Crude protein content was then calculated by applying a pre-defined conversion factor. Ash content, representing the mineral residue, was determined by incineration in a muffle furnace at a specific temperature (550 ˚C) for 16 hr. Finally, lipid content was extracted from the fish tissues was measured according to the method outlined by Ways and Hanahan.[25]

Blood sample collection procedure. Following the 60-day trial, nine fish *per* treatment group were euthanized using a researcher-approved anesthetic protocol (clove oil solution, 200 mg L^{-1}; Momtaz, Tehran, Iran). Blood was then collected via caudal venipuncture employing a minimally invasive technique.[26] Each sample was subsequently divided into aliquots for designated analyses. The first aliquot was immediately processed and preserved at a chilled temperature (4.00 ˚C) in an anticoagulant solution (heparinized saline) for down-stream hematological evaluation. The second aliquot was allowed to undergo clot formation at ambient temperature. Following centrifugation at a predetermined force (1,500 *g*) for a specified duration (20 min), the resulting serum fraction was isolated and stored at sub-zero temperatures (– 20.00 ˚C) for future immunological and serological investigations.

Hematology and serum biochemical analysis. Red blood cell (RBC) and white blood cell (WBC) counts were manually determined using a hemocytometer. Hematocrit levels (%) were measured utilizing the microcentrifuge technique and the average of triplicate microhematocrits (7,000 *g* for 10 min) were recorded. Commercial kits were employed to estimate total serum proteins, albumin, glucose, triglyceride and cholesterol levels (Pars Azmun, Tehran, Iran). Serum globulin levels were calculated subtracting the albumin concentration from the total serum protein concentration.

Assessment of immunological parameters and antioxidant status. To evaluate the immunological health of the fish following the 60-day experiment, blood samples were collected and subsequently processed for various analyses. Serum total immunoglobulin levels were determined using a modified protein precipitation technique, providing insight into the overall antibody production capacity.[27] In summary, the total protein concentration was determined using the Bradford method[23] before and after precipitating the immuno-globulins with a 12.00% polyethylene glycol solution (Sigma-Aldrich). The difference between the two measurements indicated the total immunoglobulin content. Additionally, lysozyme activity, a key enzyme component of the innate immune system, was measured using a commercially available bacterial substrate.[28] In summary, *Micrococcus lysodeikticus* was used as a substrate in 0.01 M phosphate buffered saline buffer (pH = 6.40). Serum (50.00 µL) was added to 3.00 mL of the suspension in an ice bath. Absorbance at 570 nm was recorded initially (A1) and subsequently after a 30-minute incubation period at 37.00 ˚C (A2) to evaluate the reaction's progression. The difference between these two readings reflects the reaction's intensity under the given conditions, offering a reliable quantification for subsequent analyses. Lysozyme activity was calculated as:

$$U = (A1 - A2) / A1$$

Furthermore, the authors investigated the alternative complement pathway, a critical arm of the humoral immune response, by determining the alternative complement activity (ACH50). The volume required to achieve 50.00% hemolysis was utilized to calculate the complement activity in the sample using the following formula:

$$ACH50\ (U\ mL^{-1}) = K \times (\text{reciprocal of the serum dilution}) \times 0.50$$

where, *K* represents the volume (mL) of serum causing 50.00% lysis, *0.50* is the correction factor and the assay was conducted at half the scale of the original method.[29]

The activity of antioxidant enzymes in the fish serum was evaluated. A commercially available kit (Arsam Farazist kits, Urmia, Iran) was employed to determine serum glutathione peroxidase (GPx) activity, with results expressed in mmol L^{-1} of serum. Superoxide dismutase (SOD) activity was measured using the method described previously.[30,31] The results were expressed in U mg^{-1} protein. Lipid peroxidation, an indicator of oxidative stress, was assessed in the serum using the thiobarbituric acid test. The concentration of malondialdehyde (MDA), a byproduct of lipid peroxidation, was quantified as mmol L^{-1} of serum.[32]

Statistical analysis. Data were analyzed using a commercially available statistical software package (version 21; SPSS, Armonk, USA). One-way analysis of variance was employed to assess the influence of treatment on the measured variables. Post-hoc analysis for pairwise comparisons among treatment means was conducted using a multiple comparison test (Duncan). A significance level of $p < 0.05$ was applied.

Results

Growth performance. The study investigated the impact of varying concentrations of PHB on the growth performance and nutritional parameters of common carp fingerlings with detailed findings provided in Table 1. Notably, fingerlings fed on 1.00 and 3.00% PHB exhibited the highest weight gain compared to the control group ($p < 0.05$). Conversely, the utilization of different dietary

rations did not yield significant effects on SGR ($p > 0.05$). However, a noteworthy reduction in FCR was observed in fingerlings consuming the 1.00% PHB diet, significantly differing from other treatments ($p < 0.05$). In contrast, the highest FCR was evident in the 5.00% PHB diet and control group displaying significant disparities from other experimental groups ($p < 0.05$).

Body composition. The study examined the impact of various percentage of PHB on the body composition of common carp fingerlings as outlined in Table 2. Notably, the utilization of different PHB concentrations did not affect significant alterations in the fish protein content ($p > 0.05$). However, there was a significant increase in the lipid content of fingerlings bodies when PHB was introduced compared to the control group ($p < 0.05$). Additionally, the ash content in fingerlings subjected to 1.00 and 3.00% PHB showed a marked increase compared to other treatments with statistically significant differences observed ($p < 0.05$).

Digestive enzymes activity. The study investigated the impact of varying levels of PHB on the digestive enzyme activity in common carp fingerlings as outlined in Table 3. Notably, the utilization of different PHB concentrations demonstrated significant effects on the activity of total protease and amylase enzymes ($p < 0.05$) and no significant impact was observed on lipase activity ($p > 0.05$). Specifically, the 1.00% PHB treatment exhibited the highest total protease activity significantly differing from the control treatment ($p < 0.05$). Furthermore, there were no significant disparities in total protease enzyme activity among the PHB treatments ($p > 0.05$). Additionally, PHB feeding significantly influenced amylase activity with fingerlings fed on PHB treatments displaying markedly higher amylase activity compared to the control treatment ($p < 0.05$).

Hematological, immunological and serum biochemical parameters. The results of dietary treatments on blood parameters are presented in Table 4. Analysis of the data indicated that different percentage of PHB did not affect RBC count, hematocrit, and hemoglobin (Hb) levels and no significant different was observed ($p > 0.05$). Conversely, a notable increase in WBC count was observed in treatments employing 1.00 and 3.00% PHB, exhibiting statistical significance when compared to the control group ($p < 0.05$). The findings of serum biochemical parameters are outlined in Table 4. Analysis revealed that concentrations of 1.00 and 3.00% PHB elicited a significant elevation in total protein levels in blood serum ($p < 0.05$), whereas, 5.00% PHB resulted in a significant reduction in protein concentration compared to the control group ($p < 0.05$). No significant difference was observed in triglyceride, cholesterol and serum albumin levels among the fish fed on diets containing various levels of PHB compared to the control group ($p > 0.05$). The highest and lowest concentrations of glucose were recorded in the fish fed on 5.00 and 1.00% PHB diets, respectively ($p < 0.05$). Additionally, analysis of liver metabolic enzymes indicated that the fingerlings fed on 1.00 and 3.00% PHB diets exhibited lower levels of alanine aminotransferase and aspartate aminotransferase compared to both control and 5.00% groups ($p < 0.05$).

The results of immunity and antioxidant parameters are presented in the Table 4. There was no statistically significant difference observed in total serum antibody and lysozyme levels between the experimental treatments and the control group ($p > 0.05$). The ACH50 and immunoglobulin M values were measured in the blood serum of the fingerlings revealing significantly higher levels in 1.00 and 3.00% PHB compared to the control treatment ($p < 0.05$).

Table 1. Mean ± SD of growth performances for common carp fingerlings fed on different diets containing poly-β-hydroxybutyrate (PHB).

Parameters	Control	1.00% PHB	3.00% PHB	5.00% PHB
Initial weight (g)	19.30 ± 0.20^{a}	19.90 ± 0.40^{a}	19.60 ± 0.90^{a}	19.50 ± 0.60^{a}
Final weight (g)	33.00 ± 1.00^{b}	37.90 ± 2.90^{a}	37.98 ± 1.20^{a}	34.80 ± 0.70^{ab}
Weight gain (g)	13.70 ± 0.90^{b}	18.00 ± 2.90^{a}	18.30 ± 1.40^{a}	16.39 ± 1.30^{ab}
Specific growth rate (% per day)	0.89 ± 0.04^{a}	1.06 ± 0.13^{a}	1.09 ± 0.08^{a}	1.05 ± 0.09^{a}
Feed conversion ratio	1.90 ± 0.03^{b}	1.79 ± 0.03^{a}	1.93 ± 0.05^{b}	2.14 ± 0.11^{c}

^{abc} Different letters within a row denote significant differences ($p < 0.05$).

Table 2. The impacts of dietary treatments containing poly-β-hydroxybutyrate (PHB) on fingerlings whole body compositions.

Parameters	Control	1.00% PHB	3.00% PHB	5.00% PHB
Protein (%)	67.00 ± 0.40^{a}	66.30 ± 0.40^{a}	67.10 ± 1.20^{a}	67.00 ± 1.10^{a}
Lipid (%)	19.10 ± 0.60^{b}	21.60 ± 1.80^{a}	21.80 ± 0.90^{a}	22.30 ± 1.40^{a}
Ash (%)	7.90 ± 0.30^{b}	8.40 ± 0.70^{a}	8.90 ± 0.50^{a}	7.00 ± 0.60^{b}

^{ab} Different letters within a row denote significant differences ($p < 0.05$).

Table 3. Digestive enzymes activity in the common carp fingerlings fed on different concentration of poly-β-hydroxybutyrate (PHB).

Parameters	Control	1.00% PHB	3.00% PHB	5.00% PHB
Protease (U L^{-1})	65.50 ± 3.10^{b}	75.00 ± 3.60^{a}	70.50 ± 2.30^{ab}	70.00 ± 1.70^{ab}
Lipase (U L^{-1})	1.20 ± 0.10^{a}	1.30 ± 0.00^{a}	1.40 ± 0.10^{a}	1.30 ± 0.08^{a}
Amylase (U L^{-1})	2.85 ± 0.30^{b}	3.80 ± 0.30^{a}	3.90 ± 0.20^{a}	3.40 ± 0.20^{a}

^{ab} Different letters within a row denote significant differences ($p < 0.05$).

Table 4. Serum parameters in the fish fed on different diets containing poly-β-hydroxybutyrate (PHB).

Parameters	Control	1.00% PHB	3.00% PHB	5.00% PHB
WBC (× 10^3 µL^{-1})	200.20 ± 8.90^{b}	227.20 ± 3.60^{a}	221.50 ± 7.20^{ab}	204.30 ± 14.30^{b}
RBC (× 10^6 µL^{-1})	1.44 ± 0.20^{a}	1.40 ± 0.08^{a}	1.30 ± 0.02^{a}	1.30 ± 0.01^{a}
Hemoglobin (g dL^{-1})	11.60 ± 0.90^{a}	10.80 ± 0.40^{a}	10.20 ± 0.10^{a}	11.00 ± 0.60^{a}
Hematocrit (%)	25.50 ± 2.30^{a}	27.00 ± 1.60^{a}	25.30 ± 0.60^{a}	25.90 ± 0.30^{a}
Total protein (g dL^{-1})	16.10 ± 0.10^{b}	16.70 ± 0.10^{a}	16.90 ± 0.20^{a}	15.60 ± 0.20^{c}
Glucose (mg dL^{-1})	126.00 ± 4.50^{b}	105.30 ± 2.50^{c}	115.30 ± 3.50^{bc}	155.60 ± 9.50^{a}
Triglyceride (mg dL^{-1})	643.00 ± 40.50^{a}	600.30 ± 20.50^{a}	588.00 ± 39.60^{a}	634.00 ± 48.80^{a}
Cholesterol (mg dL^{-1})	405.00 ± 18.00^{a}	408.00 ± 16.10^{a}	408.10 ± 33.80^{a}	448.00 ± 18.00^{a}
Albumin (g dL^{-1})	11.20 ± 0.10^{a}	11.40 ± 0.30^{a}	11.60 ± 0.40^{a}	11.60 ± 0.30^{a}
AST (U L^{-1})	74.00 ± 4.50^{a}	61.30 ± 12.10^{b}	43.00 ± 4.50^{c}	52.00 ± 6.00^{ab}
ALT (U L^{-1})	58.60 ± 3.50^{ab}	48.00 ± 3.60^{b}	51.60 ± 4.10^{ab}	60.00 ± 4.50^{a}
Total antibody (g dL^{-1})	4.90 ± 0.10^{a}	5.30 ± 0.30^{a}	5.30 ± 0.30^{a}	4.00 ± 0.50^{a}
Lysozyme (µg mL^{-1})	41.50 ± 3.50^{a}	43.80 ± 2.60^{a}	44.10 ± 4.30^{a}	42.30 ± 3.70^{a}
ACH50 (U mL^{-1})	134.40 ± 3.90^{b}	141.20 ± 0.20^{a}	141.30 ± 1.90^{a}	136.30 ± 4.10^{ab}
SOD (U mg^{-1})	53.00 ± 1.30^{ab}	57.40 ± 3.50^{a}	55.80 ± 1.30^{ab}	52.80 ± 1.40^{b}
MDA (µg mL^{-1})	5.50 ± 0.30^{a}	4.70 ± 0.20^{b}	4.70 ± 0.70^{b}	5.00 ± 0.10^{ab}
GPX (U mL^{-1})	0.14 ± 0.00^{a}	0.15 ± 0.00^{a}	0.15 ± 0.00^{a}	0.15 ± 0.00^{a}

WBC: White blood cells; RBC: Red blood cell; AST: Aspartate aminotransferase; ALT: Alanine transaminase; ACH50: Alternative complement activity; SOD: Superoxide dismutase; MDA: Malondialdehyde; GPX: Glutathione peroxidase.
^{ab} Different letters within a row denote significant differences ($p < 0.05$).

Antioxidant indices were assessed in fish blood serum indicating no significant difference in GPX enzyme activity compared to the control treatment ($p > 0.05$). However, the highest levels of MDA were detected in the 5.00% PHB and control treatments which were significantly different from the 1.00 and 3.00% PHB treatments ($p < 0.05$). Furthermore, SOD enzyme activity exhibited a significant increase in the 1.00% PHB compared to the 5.00% PHB treatment as indicated by the obtained results.

Discussion

This study investigated the effects of dietary PHB supplementation on common carp fingerlings. Parameters assessed included growth performance, body composition, digestive enzyme activity, hematological as well as serum biochemical profiles, immunity and antioxidant status. Results indicated significant enhancements in specific aspects of fish health and welfare suggesting the potential benefits of PHB incorporation into common carp diets.

PHB is an emerging prebiotic in aquaculture with demonstrated potential to enhance the health, disease resistance and growth performance of cultured aquatic organisms.[1,15,18,33] Based on these studies, PHB effects on growth performance ended up the change in gut microbiota composition[34] and improved nutrient utilization through increased digestive enzyme activity.[26,35,36] In this study, dietary PHB was found to have a beneficial effect on the fingerling's growth performance. Interestingly, fingerlings fed diets supplemented with 1.00 and 3.00% PHB exhibited significantly higher weight gain compared to the control group. These results suggested that PHB supplementation might enhance nutrient utilization and promote accelerated growth in fingerlings. The SGR was not changed, though, suggesting that the observed weight gain might have been mostly caused by an increase in feed consumption. Notably, the 1.00% PHB diet yielded the most substantial improvement in FCR compared to other treatments suggesting enhanced nutrient digestibility and absorption. Conversely, the 5.00% PHB group and the control group exhibited the highest FCR values indicating suboptimal feed utilization.

Despite the higher total protease activity in the fingerlings gut in lower concentration of PHB, it might not have a significant impact on protein deposition due to various factors. Susilo *et al.*,[37] found that protease activity in fish could vary with body size while Kopple,[38] suggested that changes in proteolytic enzymes might not necessarily reflect in vivo activity. Holm[39] observed a decrease in endogenous digestive protease activity in Atlantic salmon fingerlings given a diet supplemented with zooplankton which could also affect protein deposition. In addition, Zhang *et al.*[40] reported that the specific activity of proteinase and amylase in the intestine of carps can be influenced by probiotics. These findings highlight the complexity of factors influencing protein deposition in fish and the need for further research to understand the disparity in protease activity and its impact. Although lipase activity was not altered in our investigation, the fingerlings fed PHB diets showed a significant increase in lipid content in comparison to the control, possibility that other mechanisms might be in operation. This could be explained by PHB ability to be converted into energy stores or by its possible ability to promote lipogenesis.[18,35] Moreover, a number of variables including altered gut microbiota and increased mucosal surface area as well as species-specific modifications promote lipogenesis via non-lipase pathways and decrease lipid catabolism. These contribute to improved dietary lipid absorption.[41] To explore these options and learn more about the observed

event, more study is required. Also, in the fingerlings fed on 1.00 and 3.00% PHB diets, the ash content, a measure of mineral deposition-rose dramatically, indicating possible advantages for bone health and general mineral status. Najdegerami *et al*.,[26,35] observed a decrease in gut pH following dietary supplementation with PHB in both Siberian sturgeon larvae and rainbow trout fingerlings. Our findings are consistent with the established notion that lower gut pH, often induced by SCFAs released during microbial degradation of PHB, is associated with increased mineral bioavailability.[42]

The present study investigated the effect of PHB supplementation on digestive enzymes in common carp fingerlings. Total protease and amylase activity were significantly increased in fish fed on diets containing 1.00% PHB and lipase activity remained unchanged compared to the control group. These findings partially were in agreement with previous research, showing PHB ability to enhance pancreatic enzyme secretion in some fish species such as rainbow trout and gibel carp.[26,43] However, other studies have reported suppression or no significant effect of PHB on digestive enzyme activity in fish like Persian sturgeon larvae and Siberian sturgeon fingerlings.[18,44] These conflicting results across studies suggest species-specific effects and the potential role of gut microbiota modulation by PHB in influencing digestive enzyme activity. Several studies have demonstrated that PHB can alter gut microbial composition which might contribute to the observed variations in enzyme activity.[1,18] Further research is needed to elucidate the underlying mechanisms and their dependence on fish species and dietary factors.

The results indicated that different PHB concentrations had no significant effects on Hb levels, hematocrit, or RBC count. This implied that PHB supplementation within the examined range had no negative effect on the fish ability to produce RBCs or transport oxygen. Our results were in agreement with those of Rodriguez-Estrada *et al*.,[45] who reported that the use of pure polyhydroxyalkanoate did not affect Nile tilapia RBC and Hb. In contrast to the control, the fingerlings fed on 1.00% PHB diets showed a noticeable rise in WBC count. Since WBCs are essential to the immune system, an increase in them may be a sign that PHB has immunomodulatory effects.

The results regarding the biochemical characteristics of serum showed a complicated correlation between PHB content and other components of blood. In comparison to the control, 1.00% and 3.00% PHB significantly increased total protein levels indicating either enhanced protein synthesis or decreased protein breakdown. However, 5.00% PHB significantly decreased total protein levels. To establish the ideal PHB level for preserving protein homeostasis and comprehend the underlying processes causing this disparity, more research is necessary. Also, no statistically significant variations were noted in the levels of albumin, cholesterol or triglycerides indicating that PHB had no effect on these particular parameters. According to the data from the literature, stress in the fish environment often causes catecholamines to produce glucose which in turn stimulates the liver glycogenolysis.[46] Interestingly, fish given 1.00 and 5.00% PHB diets had the lowest and the highest glucose levels, respectively, indicating possible impacts on glucose metabolism suggesting 5.00% PHB concentration might induce stress in the fingerlings. This finding was in agreement with observations from liver enzyme results where the fingerlings fed on 5.00% PHB exhibited elevated levels of aspartate aminotransferase and alanine aminotransferase enzymes, potentially indicating a stress response and cellular damage.

The PHB supplementation did not significantly alter overall serum antibody or lysozyme levels, however, it did significantly raise ACH50 and immunoglobulin M levels in fingerlings given 1.00 and 3.00% PHB in comparison to the control. These results implied that PHB supplementation might improve certain immunological pathways especially those connected to complement function and humoral immunity. The control and 5.00% PHB treatments showed the greatest amounts of MDA, a sign of oxidative stress, however, GPX activity remained unchanged. This showed that 1.00 and 3.00% PHB might provide some oxidative stress protection which might enhance fish resistance and overall health. Additionally, compared to the 5.00% PHB group, SOD activity showed a substantial increase in the 1.00% PHB treatment, providing additional evidence for the possible antioxidant advantages of PHB supplementation at particular dosages. Our results were in agreement with previous results who indicated that PHB in specific concentration improved immune response in different aquatic animals.[5,26,34,35,43,45]

In conclusion, dietary supplementation with 1.00 and 3.00% PHB in common carp fingerlings improved the growth and safety indicators. However, the absence of statistically significant differences between these concentrations combined with cost considerations in global markets suggested that 1.00% PHB merited further investigation and research as the preferred dietary inclusion level.

Acknowledgments

The authors extend their gratitude to the Department of Aquaculture at Gorgan University of Agricultural Science and Natural Resources, Gorgan, Iran, for their generous provision of resources and support for this study. Additionally, special thanks to Dr. Ali Jafarnoudeh for his invaluable assistance in conducting the research.

Conflict of interest

The authors declare no conflicts of interest.

References

1. De Schryver P, Sinha AK, Kunwar PS, et al. Poly-beta-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, *Dicentrarchus labrax*. Appl Microbiol Biotechnol 2010; 86(5):1535-1541.
2. Ashaolu TJ, Ashaolu JO, Adeyeye SAO. Fermentation of prebiotics by human colonic microbiota in vitro and short-chain fatty acids production: a critical review. J Appl Microbiol 2021; 130(3): 677-687.
3. Wee W, Hamid NKA, Mat K, et al. The effects of mixed prebiotics in aquaculture: a review. Aquac Fish 2024; 9(1): 28-34.
4. Van Immerseel F, De Buck J, Pasmans F, et al. Invasion of Salmonella enteritidis in avian intestinal epithelial cells in vitro is influenced by short-chain fatty acids. Int J Food Microbiol 2003; 85(3): 237-248.
5. Sui L, Liu X, Pan N, et al. Halomonas-PHB protects gnotobiotic Artemia against Vibrio and modifies Artemia gut microbiota in xenic culture conditions. J Ocean Limnol 2023; 41: 1292-1299.
6. Li S, Heng X, Guo L, et al. SCFAs improve disease resistance via modulate gut microbiota, enhance immune response and increase antioxidative capacity in the host. Fish Shellfish Immunol 2022; 120: 560-568.
7. Zhou L, Han F, Lu K, et al. Comparative study on prebiotic effects of different types of prebiotics in an in vitro fermentation by gut microbiota of shrimp (Litopenaeus vannamei). Aquaculture. 2023; 574: 739687. doi: 10.1016/j.aquaculture.2023.739687.
8. Mussatto SI, Mancilha IM. Non-digestible oligo-saccharides: a review. Carbohydr Polym 2007; 68(3): 587-597.
9. Müller-Santos M, Koskimäki JJ, Alves LPS, et al. The protective role of PHB and its degradation products against stress situations in bacteria. FEMS microbiol Rev 2021; 45(3): fuaa058. doi: 10.1093/ femsre/fuaa058.
10. Mukai K, Doi Y, Sema Y, et al. Substrate specificities in hydrolysis of polyhydroxyalkanoates by microbial esterases. Biotechnol Lett. 1993;15: 601-604.
11. Freier T, Kunze C, Nischan C. In vitro and in vivo degradation studies for development of a bio-degradable patch based on poly(3-hydroxybutyrate). Biomaterials 2002; 23(13): 2649-2657.
12. Kato N, Konishi H, Shimao M, et al. Production of 3-hydroxybutyric acid trimer by *Bacillus megaterium* B-124. J Ferment Bioeng 1992; 73(3): 246-247.
13. Patnaik PR. Perspectives in the modeling and optimization of PHB production by pure and mixed cultures. Crit Rev Biotechnol 2005; 25(3): 153-171.
14. Laranja JLQ, Bossier P. Poly-beta-hydroxybutyrate (PHB) and infection reduction in farmed aquatic animals. In: Goldfine H (Ed). Health consequences of microbial interactions with hydrocarbons, oils, and lipids. Cham, Switzerland: Springer 2020; 457-482.
15. Nhan DT, Wille M, De Schryver P, et al. The effect of poly β-hydroxybutyrate on larviculture of the giant freshwater prawn (*Macrobrachium rosenbergii*). Aquaculture 2010; 302(1-2): 76-81.
16. Li L, Wei XF, Yang ZY, et al. Alleviative effect of poly-β-hydroxybutyrate on lipopolysaccharide-induced oxidative stress, inflammation and cell apoptosis in *Cyprinus carpio*. Int J Biol Macromol 2023; 253(Pt 2): 126784. doi: 10.1016/j.ijbiomac.2023.126784.
17. Li L, Wang YT, Meng ST, et al. Effects of poly-β-hydroxybutyrate on growth, antioxidant capacity, and lipid metabolism of common carp (*Cyprinus carpio*) under waterborne copper exposure. Aquaculture 2024; 580(Part 1): 740300. doi: 10.1016/ j.aquaculture.2023.740300.
18. Najdegerami EH, Tran TN, Defoirdt T, et al. Effects of poly-β-hydroxybutyrate (PHB) on Siberian Sturgeon (*Acipenser baerii*) fingerlings performance and its GI tract microbial community. FEMS Microbol Ecol 2012; 79(1): 25-33
19. Chatzifotis S, Papadaki M, Despoti S, et al. Effect of starvation and re-feeding on reproductive indices, body weight, plasma metabolites and oxidative enzymes of sea bass (*Dicentrarchus labrax*). Aquaculture 2011; 316(1-4): 53-59.
20. Walter H. Proteinases: methods with hemoglobin, casein and azocoll as substrates. 5th ed. Weinheim, Germany 1994; 270-277
21. Métais P. Bieth J. Determination of alfa-amylase by microtechnic [French]. Ann Biol Clin (Paris) 1968; 26(1): 133-142.
22. Iijima N, Tanaka S, Ota Y. Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish Physiol Biochem 1998; 18: 59-69.
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem.1976; 72: 248-254.
24. AOAC International, In: Horwitz W (Ed). Official methods of analysis of AOAC international. 17th ed. Virginia, USA: Association of Official Analytical Chemist. Inc. 2000; 239-243
25. Ways P, Hanahan DJ. Characterizations and quantification of red cell lipids in normal man. J Lipid Res 1964; 5(3): 318-328.
26. Najdegerami EH, Tokmachi A, Bakhshi F. Evaluating the effects of dietary prebiotic mixture of mannan oligosaccharide and poly-β-hydroxybutyrate on the growth performance, immunity, and survival of Rainbow Trout, *Oncorhynchus mykiss* (Walbaum 1792), fingerlings. J World Aquac Soc 2016; 48(3): 415-425.
27. Siwicki AK, Anderson DP, Rumsey GL. Dietary intake of

immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. Vet Immunol Immunopathol 1994; 41(1-2): 125-139.
28. Hultmark D, Steiner H, Rasmuson T, et al. Insect immunity: purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of Hyalophora cecropia. Eur J Biochem 1980; 106(1): 7-16.
29. Amar EC, Kiron V, Satoh S, et al. Effects of dietary β-carotene on the immune response of rainbow trout *Oncorhynchus mykiss*. Fish Sci 2000; 66(6): 1068-1075.
30. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974; 47(3): 469-474.
31. Tianyu J, Xiaoyu Z. The improved pyrogallol method by using terminating agent for superoxide dismutate measurement [Japanese]. Prog Biochem Biophys. 1995; 22(1): 84-86
32. Satoh K. Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. Clin Chim Acta. 1978; 90(1): 37-43.
33. Li D, Lv L, Chen JC, et al. Controlling microbial PHB synthesis via CRISPRi. Appl Microbiol Biotechnol 2017; 101(14): 5861-5867.
34. Qiao G, Chen P, Sun Q, et al. Poly-β-hydroxybutyrate (PHB) in bioflocs alters intestinal microbial community structure, immune-related gene expression and early Cyprinid herpesvirus 2 replication in gibel carp (*Carassius auratus gibelio*). Fish Shellfish Immunol 2020; 97: 72-82.
35. Najdegerami EH, Baruah K, Shiri A, et al. Siberian sturgeon (*Acipenser baerii*) larvae fed Artemia nauplii enriched with poly-β-hydroxybutyrate (PHB): effect on growth performance, body composition, digestive enzymes, gut microbial community, gut histology and stress tests. Aquac Res 2015; 46(4): 801-812.
36. Wu YW, Yang SH, Hwangbo M, et al. Analysis of Zobellella denitrificans ZD1 draft genome: genes and gene clusters responsible for high polyhydroxy-butyrate (PHB) production from glycerol under saline conditions and its CRISPR-Cas system. PLoS One 2019; 14(9): e0222143. doi: 10.1371/journal.pone.0222143.
37. Susilo U, Rachmawati FN, Wibowo ES. Protease and amylase activities of Javaen barb (*Systomus rubripinnis* Val.). J Biodjati 2022; 7(1): 45-55.
38. Kopple JD. Nutrition and protease activity. Adv Exp Med Biol 1988; 240: 411-419.
39. Holm JC, Torrissen KR. Growth depression and acclimatization of protease activity in Atlantic salmon first-feeding fry responding to a diet supplemented with zooplankton. Aquaculture 1987; 65(2): 171-174.
40. Zhang JH, Ni EQ, He HJ, et al. The effects of different probiotics on proteinase activity and amylase activity in the intestine of carps [Chinese]. Jiangxi Nongye Daxue Xuebao. 2005; 27(4): 513-516.
41. Martinez-Guryn K, Hubert N, Frazier K, et al. Small intestine microbiota regulates host digestive and absorptive adaptive responses to dietary lipids. Cell Host Microbe 2018; 23(4): 458-469e5.
42. Bongers A, van den Heuvel EGHM. Prebiotics and the bioavailability of minerals and trace elements. Food Rev Int 2003; 19(4): 397-422.
43. Liu Y, Chen P, Wang Z, et al. Dietary poly-β-hydroxy-butyrate supplementation can effectively improve growth, digestive enzyme activities, immune-related gene expression, disease resistance, and intestinal mucosal barrier of gibel carp, *Carassius auratus gibelio*. J World Aquac Soc 2022; 53(4): 816-835.
44. Najdegerami E, Jafari A, Bakhshi F,et al. Effect of poly-β-hydroxybutyrate on growth performance, body composition, digestive enzymes activity, gut microbial community of the hatchling Persian sturgeon (*Acipenser persicus*) [Persian]. Fish Sci Technol 2015; 4(2): 103-115.
45. Rodriguez-Estrada U, Tachibana L, de Carla Dias D, et al. Dietary pure polyhydroxyalkanoate effects on growth, nutrient utilization, apparent digestibility, and hematology in Nile tilapia. N Am J Aquac 2021; 83(4): 240-254.
46. Mommsen TP, Vijayan MM, Moon TW. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev Fish Biol Fish 1999; 9: 211-268.