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The effects of dietary poly-β-hydroxybutyrate on growth parameters, intestinal microflora, and histopathology of rainbow trout, *Oncorhynchus mykiss*, fingerlings

Tolga Şahin[1] | Sevdan Yılmaz[1] | İrfan Selçuk Yazıcı[2] | Selçuk Berber[2]

[1]Department of Aquaculture, Marine Sciences and Technology Faculty, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

[2]Department of Marine and Inland Water Sciences, Marine Sciences and Technology Faculty, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

Correspondence
Tolga Şahin, Marine Sciences and Technology Faculty, Çanakkale Onsekiz Mart University, Çanakkale 17020, Turkey.
Email: tolgasahin@comu.edu.tr

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Abstract

The present study aimed to evaluate the effects of poly-β-hydroxybutyrate (PHB)-supplemented diets for rainbow trout (*Oncorhynchus mykiss*) fingerlings. For this purpose, three experimental diets were prepared to contain 0, 2, and 5% PHB; and each diet was tested in a completely randomized design for 60 days in quadruple groups of 22 fish per tank. The results showed that the inclusion of PHB in the diets did not affect the growth parameters such as mean weight gain, specific growth rate, and feed conversion ratio among treatments. There was also no effect of the PHB on chemical composition, PCR-DGGE profile of the intestinal bacterial communities, total bacterial count, lactic acid bacteria, psychrophilic bacteria as well as the intestine and the liver histopathology of fish. However, the supplementation of 2 and 5% PHB in the diet significantly reduced total coliform counts, while 5% PHB diet additionally decreased Enterobacteriaceae counts. Both inclusion ratios resulted in significantly higher intestinal villus length and width at the end of the experiment. Considering the positive effects of dietary PHB on intestinal bacterial flora and villus sizes of

rainbow trout fingerlings, it can be used as an alternative microbial control agent.

Significance Statement: PHB is a biopolymer that has previously been reported to have positive effects on fish health, and no literature has been encountered investigating the effects of PHB on rainbow trout fry. The findings obtained in this study show that PHB has positive effects on the intestinal tissues of rainbow trout fry and provides an increase in the number of beneficial bacteria in the gut flora.

KEYWORDS

bacteria, denaturing gradient gel electrophoresis, histology, microbial community, polymerase chain reaction, prebiotic

1 | INTRODUCTION

The recent intensification of aquaculture systems leads to unfavorable water conditions or adverse social interactions such as induced stress and disease susceptibility, which may cause several problems on the cultured organisms (Pickering & Pottinger, 1989; Sapkota et al., 2008). These problems can result in substantial losses to the aquaculture sector (Hall & de la Vega, 2004; Vidal, Granja, Aranguren, Brock, & Salazar, 2001) that many researchers have been attempting to find solutions. One of the most effective tools to overcome such problems has been the use of antibiotics; however, it is not approved at present because they may promote the presence of antibiotic-resistant bacteria in the organism and its surrounding environment (Smith, Brown, & Hauton, 2003; Smith, Hiney, & Samuelsen, 1994). Therefore, this situation has canalized researchers to look for alternative biocontrol mechanisms and materials (Defoirdt, Sorgeloos, & Bossier, 2011; Karunasagar, Shivu, Girisha, Krohne, & Karunasagar, 2007).

Prebiotics, as non-digestible short-chain carbohydrate structures (Gibson & Roberfroid, 1995; Manning & Gibson, 2004; Najdegerami et al., 2012), are one of those biocontrol tools, which might stimulate the defense system of the host organism, thereby reduce the harmful effects caused by stressors (Akhtar, Kumar Pal, Sahu, Ciji, & Kumar, 2012). The inhibitory effect of prebiotics on pathogenic bacteria has been believed to depend on the ability to enhance the resistance of the organism by increasing the number of probiotic bacteria (Manning & Gibson, 2004).

One of the substances, which have some prebiotic effects used for that purpose, is Poly-β-hydroxybutyrate (PHB), which is a prominent member of the polyhydroxyalkanoate family (PHAs) (Anderson & Dawes, 1990). As a natural polymer, PHB can be depolymerized into water-soluble short-chain fatty acid monomers, which might serve as microbial control agents (Najdegerami et al., 2012). Such activity of PHB has been explained in the literature as lowering gut pH, thus inhibiting the growth of pathogenic bacteria (De Schryver et al., 2010; Najdegerami et al., 2015), as well as contributing to the growth of beneficial gastrointestinal bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. (Cotter & Hill, 2003; Franke et al., 2017).

Considering its beneficial effects on the intestinal microflora of the organism, PHB has been found to increase weight gain, survival, specific growth rate, and short-chain fatty acid concentration in the gastrointestinal tract of certain aquaculture species such as European sea bass (*Dicentrarchus labrax*), Siberian sturgeon (*Acipenser baerii*), and giant river prawn larvae (*Macrobrachium rosenbergii*) (De Schryver et al., 2010; Najdegerami et al., 2012; Nhan et al., 2010). It also has protective efficacy on *Artemia franciscana* nauplii against vibriosis (Baruah et al., 2015; Defoirdt et al., 2007; Halet et al., 2007; Van Cam et al., 2009) and rainbow trout fry (*Oncorhynchus mykiss*) against

Yersinia ruckeri (Najdegerami, Tokmachi, & Bakhshi, 2017). Moreover, bio-encapsulated PHB into Rotifer and *Artemia* has also been shown to be effective on increased survival, development rate, and osmotic tolerance of Chinese mitten crab (*Eriocheir sinensis)* zoea larvae (Sui et al., 2014). As one of the most important freshwater fish species of major economic interest worldwide (Olsen, Hjortaas, Tengs, Hellberg, & Johansen, 2015), the present data of PHB on rainbow trout is limited based on the reviewed literature.

Despite the results reported on mineral uptake, lipid metabolism, and digestive enzyme activity, well-documented information on growth and intestinal microbial flora of rainbow trout fed with PHB supplemented diets are lacking. Consequently, this study was performed to document the dietary effects of PHB on growth parameters, intestinal microflora, and histopathological condition of rainbow trout fry.

2 | MATERIALS AND METHODS

2.1 | Experimental set-up and animals

A 60-day experiment was conducted at the Fish Breeding Laboratory belonging to Aquaculture Department of Çanakkale Onsekiz Mart University, Çanakkale, Turkey. Rainbow trout obtained from the same faculty were adapted to the experimental conditions for 2 weeks before the experiment and fed three times per day with a control diet at apparent satiation. After the acclimation, 264 fish (2.6 ± 0.38 g; mean ± *SD*) were randomly distributed into 12 experimental tanks at an initial density of 22 individuals per tank, and each treatment was run in four replicates. The experiment was conducted in a recirculating system equipped with mechanical, biological, and ultraviolet filters. Water was replaced 10% of the total volume daily by using a reservoir tank and aerated continuously. The adequate temperature was provided by chillers and maintained at 14 ± 1°C throughout the experiment. Temperature and pH were recorded by using "YSI Ecosense pH100," and dissolved oxygen was monitored by a multi-probe "YSI 85." The results (mean ± *SD*) were recorded as 7.9 ± 0.1 for pH and 7.2 ± 0.3 mg/L for dissolved oxygen. A natural light/dark regime was applied in the trial. The experiment was carried out by the principles of the animal experimentation ethics committee of Çanakkale Onsekiz Mart University.

2.2 | Experimental diets and feeding protocol

Three experimental diets were formulated to contain 50% crude protein and 15% crude lipid by using commercial ingredients (Table 1).

All dry ingredients were finely ground and mixed using a laboratory mixer. The mixtures were then primed with deionized water to yield a suitable pulp. Subsequently, wet diets were homogenized and formed into 2 mm pellets by a meat mincer; and finally dried at 40 ± 1°C in a circulating air dryer until achieving adequate moisture. The pellets were then crushed into suitable particle sizes and sieved. Following this, the PHB (Sigma-Aldrich® 363,502) was administered in feeds, as described by Najdegerami et al. (2012). Briefly, the PHB was dissolved in chloroform:water solution (80:20, v:v) and mixed with feeds at levels of 0 (control), 2, and 5%. The feeds were then kept in room temperature for evaporation of the chloroform and stored at +4°C until used. Among the trial, fish were fed three times a day at apparent satiation, and tanks were siphoned daily to get rid of feces.

2.3 | Calculations and analytical determination

The survival rates were calculated based on the daily observations of dead fish seen in the tanks. The fish were weighed individually at the beginning and at the end of the experiment for each treatment. Evaluations of the growth

TABLE 1 Formulation and chemical composition of the experimental diets

	0% PHB (control)	2% PHB	5% PHB
Ingredients (% dry matter)			
Fish meal	44.0	45.0	45.0
PHB	0.0	2.0	5.0
Soybean meal	30.0	30.0	30.0
Wheat meal	11.499	8.499	5.499
Mineral premix	2.0	2.0	2.0
Vitamin premix	1.0	1.0	1.0
Butylated hydroxytoluene (BHT)	0.001	0.001	0.001
Fish oil	11.5	11.5	11.5
Nitrogen free extract (NFE)	17.8	17.1	17.4
Gross energy (GE) (kJ/g)	21.0	21.0	20.9
Protein/energy ratio (P/E)	2.4	2.4	2.4
Chemical composition			
Dry matter (%)	92.9	92.8	92.7
Crude protein[a]	50.0	50.4	50.1
Crude lipid[a]	15.5	15.6	15.5
Ash[a]	7.6	7.7	7.7

[a]% dry basis.

performances and feed utilization were performed considering the equations given below (Silva-Carrillo, Hernández, Hardy, González-Rodríguez, & Castillo-Vargasmachuca, 2012). The abbreviations in the equations indicate, WG = Weight gain, FW=Final weight, IW = Initial weight, SGR = Specific growth rate, FCR = Feed conversion ratio, T = Number of experimental days, FI = Feed intake.

$$\mathrm{WG}\,(\%) = 100\,(\mathrm{FW} - \mathrm{IW}) \times \mathrm{IW}^{-1}$$

$$\mathrm{SGR}\left(\%\mathrm{day}^{-1}\right) = 100\,[\mathrm{Ln}\,(\mathrm{FW}) - \mathrm{Ln}\,(\mathrm{IW})] \times \mathrm{T}^{-1}$$

$$\mathrm{FCR} = \mathrm{FI} \times \mathrm{WG}^{-1}$$

Crude protein, moisture, and ash of the experimental diets were determined using standard methods of the Association of Official Analytical Chemists methods (AOAC, 1998). Crude lipids were extracted after 12 hr of homogenization with chloroform-methanol (2:1, v:v) followed by separation and vacuum drying in 2 ml of chloroform aliquot, and quantified gravimetrically (Folch, Lees, & Stanley, 1957).

2.4 | Histological experiments

After dissection of the fish, the liver and foregut samples were fixed using Bouin's solution. Samples were then rinsed in water, subjected to graded series of alcohol, cleared in xylene, and embedded in paraffin. Transverse sections (5 μm) were obtained using a rotary microtome (Leica, Olympus Corporation, Tokyo Japan), then stained with hematoxylin and eosin (H&E). Stained sections were observed under a microscope (Olympus CX21) and photographed by

a camera (Olympus BX51). The histomorphometric analysis was performed using the Olympus Analysis LS software (Liu et al., 2017).

2.5 | Bacterial community analysis and DGGE

2.5.1 | Classical identification

One gram of foregut sample was taken by sterile pens under aseptic conditions and homogenized with sterile glass homogenizer in 9 ml PBS to determine the bacterial activity. Required dilutions were prepared from the homogenates, and bacterial counts were performed. The following mediums were used for bacterial countings: the Man-Rogosa Sharp Agar medium (MRS) for lactic acid bacteria (Andani, Tukmechi, Meshkini, & Sheikhzadeh, 2012), the Tryptone Soy Agar medium (TSA) for psychrotrophic (Lyhs et al., 2001) and aerobic bacteria (Merrifield, Dimitroglou, Bradley, Baker, & Davies, 2010), the Violet Red Bile Dextrose Agar medium (VRBD) for Enterobacteriaceae (Giannenas et al., 2012), and the MacConkey Agar medium (MAC) for coliform bacteria (Šyvokienė & Vosylienė, 2013).

2.5.2 | DGGE

Three fish from each replicate were euthanized using 2 ml/L of 2-phenoxyethanol. After dissection, intestines were taken out, pooled to exclude the variations (Liu et al., 2008; Spanggaard et al., 2000), and stored at −20°C until analysis.

The DNA extraction and DGGE analysis were performed according to the method described by Yu and Morrison (2004). In short, the DNA was eluted and the V3 region of the *rrs* gene was amplified by using the forward primer 357f (5′-CCTACGGGAGGCAGCAG-3′) and reverse 519r (5′-ATTACCGCGGCKGCTGG-3′). The PCR buffer, primers (each 500 nM), 200-μM dNTP, 1.75 mM MgCl2, 1.25 U Platinum® *Taq* DNA polymerase (Invitrogen, USA), and 670 ng/μl bovine serum albumin were added, and hot-start PCR was run. Following an initial denaturation, PCR cycles were performed, including negative controls. Before the DGGE, successful amplification of the V3 region of each PCR product was confirmed by 1.5% (w/v) agarose gel electrophoresis. Subsequently, aliquots (15 μl) were resolved in the polyacrylamide gel containing denaturants, and the DGGE gel was run at 60°C and 82 V for 15 hr using a DCode™ UMDS (Bio-Rad Laboratories, USA). The gel was then stained with GelStar® (Cambrex, USA), and the images were recorded by a FluorChem® Imager (Alpha Innotech, USA).

2.6 | Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) using SPSS version 20.0 following Tukey's multiple range tests to determine the statistical differences among treatments at a significance level of $p < .05$. Pearson's correlation analysis was also used to examine the relationships between PCR-DGGE band profiles.

3 | RESULTS

3.1 | Growth data

The growth and feed evaluation findings were given in Table 2. There were no significant differences between the groups in terms of weight gain, feed conversion rate (FCR), and specific growth rate (SGR) ($p > .05$).

TABLE 2 Growth performance after 60 days of feeding

	0% PHB (control)	2% PHB	5% PHB
Initial weight	2.76 ± 0.04	2.48 ± 0.06	2.47 ± 0.04
Final weight	9.14 ± 0.20	9.13 ± 0.39	8.79 ± 0.90
Wet weight gain (%)	231.55 ± 11.60	269.06 ± 15.39	257.64 ± 39.33
FCR	1.15 ± 0.01	1.08 ± 0.06	1.07 ± 0.13
SGR	1.99 ± 0.06	2.17 ± 0.07	2.09 ± 0.19

Note: Data were given as mean ± *SEM* (n = 4). No significant differences were observed between response variables.

TABLE 3 The body composition of fish fed the experimental diets at the end of the trial

	0% PHB (control)	2% PHB	5% PHB
Protein (% dry matter)	48.35 ± 0.45	49.03 ± 0.76	50.62 ± 1.32
Lipid (% dry matter)	21.13 ± 0.82	21.31 ± 0.72	21.14 ± 1.00
Ash (% dry matter)	10.90 ± 0.36	9.83 ± 0.26	11.17 ± 0.71
Moisture (%)	72.69 ± 0.31	72.85 ± 0.35	72.49 ± 0.50

Note: Data were given as mean ± *SEM* (n = 8). No significant differences were observed between response variables.

3.2 | Chemical analysis

The results of the chemical analysis were given in Table 3. There were no significant differences for crude protein, crude fat, crude ash, and moisture values between experimental groups ($p > .05$).

3.3 | Microbial findings

3.3.1 | Classical identification

According to the findings, there were no differences in total aerobic, psychotropic, and lactic acid bacteria counts ($p > .05$) (Table 4). However, the total Enterobacteriaceae and coliform bacteria counts were lower in the 5% PHB group than the control group ($p < .05$). Moreover, total coliform bacteria count was significantly lower in the 2% PHB group than in the control group ($p < .05$).

3.3.2 | DGGE results

The DGGE fingerprints revealed that the microbial profiles at 60 days in the intestine of fish that fed experimental diets did not differ within the respective communities; no significant differences were observed among the groups considering DGGE analysis ($p > .05$) (Figure 1).

3.3.3 | Histological findings

No histopathological anomalies were found in the foregut cross-sections of experimental groups at the initial and 60th days of the experiment (Figure 2).

TABLE 4 The findings of gut microbial flora according to the classical identification method (log CFU g^{-1})

	0% PHB (control)	2% PHB	5% PHB
Total aerobic bacteria	7.75 ± 0.50	7.03 ± 0.33	6.12 ± 0.74
Total psychotropic bacteria	7.30 ± 0.68	6.19 ± 0.25	5.68 ± 0.99
Total Enterobacteriaceae	6.57 ± 0.43a	5.89 ± 0.36ab	4.95 ± 0.33b
Total coliform	5.19 ± 0.29a	4.39 ± 0.05b	3.78 ± 0.11b
Total lactic acid bacteria	1.61 ± 0.05	1.62 ± 0.06	1.61 ± 0.05

Note: Data were given as mean ± *SEM* (n = 4). Means within the same row sharing different letters are significantly different ($p < .05$).

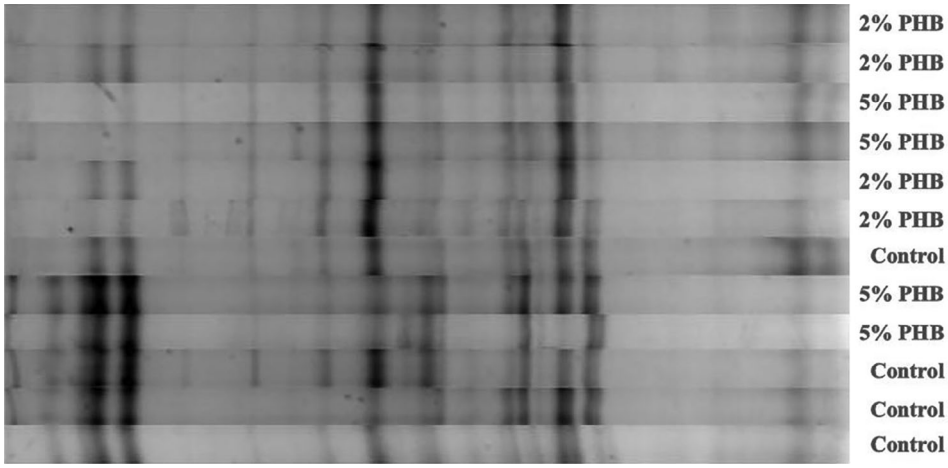

FIGURE 1 PCR-DGGE profiles of the gut samples of different treatment groups

In the cross-sections, the villi normally extended into the intestinal lumen and increased the surface area for absorption. The expansion of connective tissue components in the villi was uniform, and there was no infiltration. There were no abnormalities in the submucosa, muscularis mucosa, serosa, nor in the enterocytes of the simple cylindrical epithelium (microvilli) that laid the villus surface. The brush-border like microvilli folds, which increase the absorption surface in the gut, and located in the apical region of the enterocytes, and the goblet cells were in normal structure (Figure 2).

There were no histopathological anomalies in the foregut cross-sections of the 2% and 5% PHB supplemented groups on the 60th day of the PHB administration (Figure 2). The sections observed were histologically similar to the cross-sections of the control group.

However, considering histomorphological measurements, it was observed that the mean villus length (614.25 ± 11.8 and 608.47 ± 14.59 μm) and width (95.28 ± 8.55 and 104.41 ± 8.77 μm) were statistically different in 2 and 5% PHB groups than in the control group (Table 5) ($p < .05$).

There were no differences between the foregut samples of the groups in terms of enterocyte length & width, supranuclear enterocyte length, goblet cell length, and the mean goblet cell count (Table 6), and no histopathological findings in the liver cross-sections (Figure 3).

The blood flow was macroscopically normal throughout the liver. No abnormalities were observed in the portal vena, central vena, and portal artery endothelium. Epithelial cells laying the bile ducts were normal. Hepatopancreas

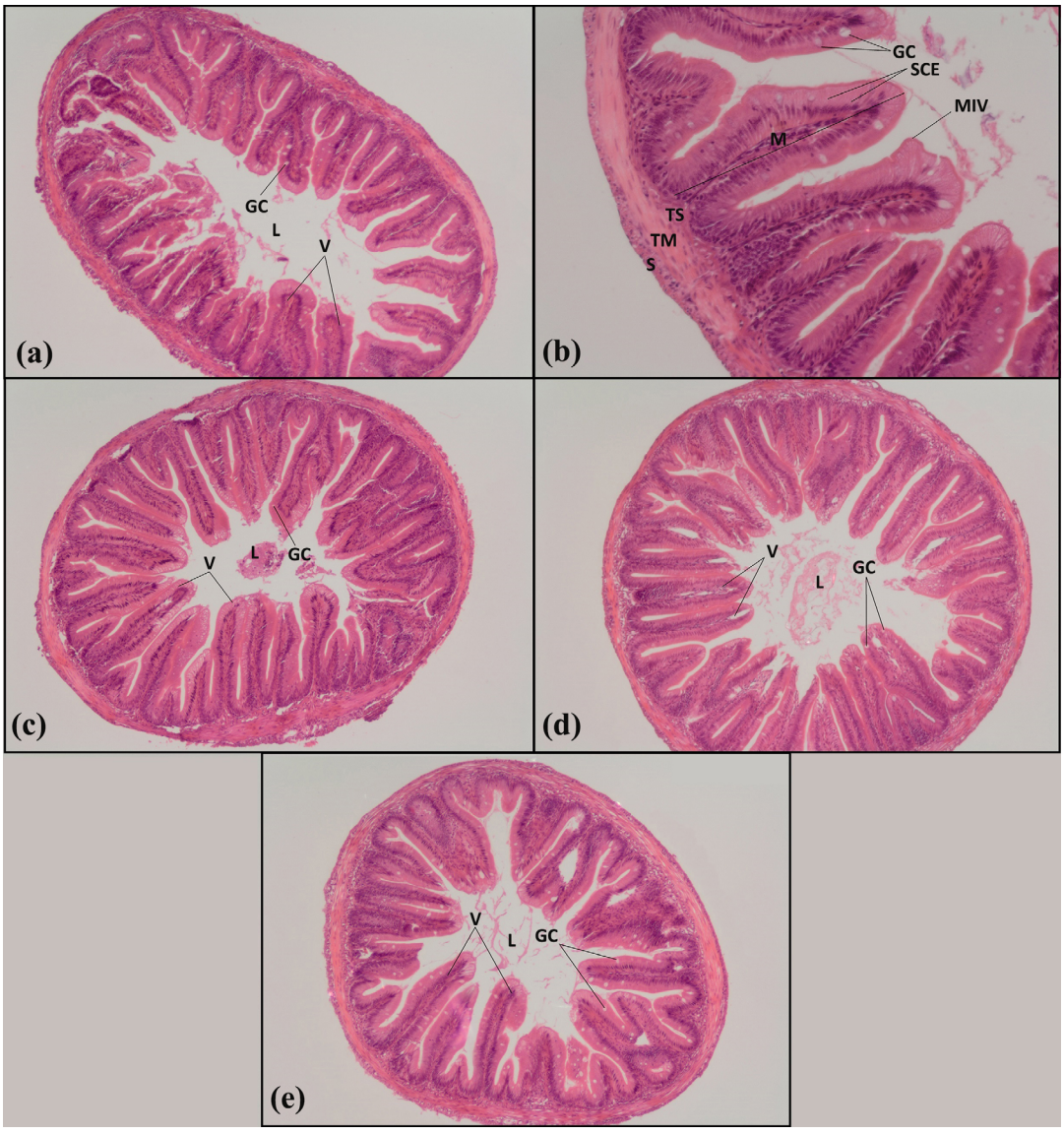

FIGURE 2 Foregut cross-sections (4× magnification) of *O. mykiss* at (a), (b) initial day, (c) 60th day for the control group, (d) 60th day for the 2% PHB group, (e) 60th day for the 5% PHB group. Abbreviations: GC, goblet cell; L, lumen; M, mucosa; MIV, microvillus; S, serosa; SCE, single-layered cylindrical epithelium (enterocyte); TM, tunica muscularis; TS, tunica submucosa; V, villus

did not show any anomalies. Hepatocytes were in the usual form and did not exhibit any hyperplasic or hypertrophic activity. Kupffer cells exhibited similar routine findings (Figure 3).

There were no significant differences between the liver cross-sections of fish treated with 2% PHB, 5% PHB, and the control group on the 60th day of the experiment (Figure 3). No histopathological findings were found in these sections. The mean hepatocyte diameters of the samples of 2 and 5% PHB supplemented groups were 8.56 ± 0.95 and 8.41 ± 0.96 μm, respectively (Table 6).

TABLE 5 The differences in the mean villi lengths (VL) and widths (VW)

	Initial	Final		
		0% PHB	2% PHB	5% PHB
VL (μm)	541.28 ± 18.25a	552.56 ± 15.27a	614.25 ± 11.8b	608.47 ± 14.59b
VW (μm)	70.35 ± 6.21b	73.41 ± 7.98b	95.28 ± 8.55a	104.41 ± 8.77a

Note: Data were given as mean ± *SEM* (n = 10). Means within the same row sharing different letters are significantly different ($p < .05$).

TABLE 6 The mean enterocyte length (EL), enterocyte width (EW), supranuclear enterocyte length (SNEL), goblet cell length (GCL), number of goblet cells per villus (GCPV), hepatocyte diameters (HD), and statistical differences between groups

	Initial	Final		
		0% PHB	2% PHB	5% PHB
EL (μm)	64.28 ± 4.46	66.86 ± 5.89	69.42 ± 4.89	68.47 ± 4.87
EW (μm)	9.56 ± 2.19	10.27 ± 2.54	10.88 ± 2.38	11.27 ± 2.97
SNEL (μm)	23.75 ± 2.47	19.87 ± 3.24	21.78 ± 2.5	19.56 ± 3.14
GCL (μm)	12.49 ± 2.46	11.59 ± 3.88	12.63 ± 2.39	11.68 ± 3.21
GCPV	4.2 ± 0.52	3.98 ± 0.44	4.49 ± 0.69	4.57 ± 0.27
HD (μm)	8.35 ± 1.37	9.21 ± 1.89	8.56 ± 0.95	8.41 ± 0.76

Note: Data were given as mean ± *SEM* (n = 10). No significant differences were observed between response variables.

4 | DISCUSSION

The environmental and other biological factors can adversely affect the health of fish and can cause both economic and ecological problems. In this respect, various protective and prophylactic applications are made to protect and improve the health of the fish. One of these applications is the use of probiotic and prebiotic additives, which are known to have positive effects on fish health. Although there have been some prebiotic additives used to control the microbial activity in the gastrointestinal tract of the fish, the studies conducted to observe the effects of the PHB on fish are very limited. Accordingly, in the present study, three different experimental diets containing 0, 2, and 5% PHB were prepared and fed to fish for 60 days to determine the effects of dietary PHB on rainbow trout fry.

Considering the data obtained, PHB has been found not to affect the growth and survival rates of rainbow trout fry in this trial ($p > .05$). In a similar study, Najdegerami et al. (2017) investigated the effects of dietary PHB (2%), mannan-oligosaccharide (MOS) (0.2%), and their mixture (2% PHB + 0.2% MOS) on rainbow trout fingerlings; and found neither positive nor negative changes in the growth and survival parameters of fish as well. Literature reports that the dietary PHB is known not to affect the growth performance of blue mussel larvae (Van Hung et al., 2015), while it adversely affects the growth performance of Siberian sturgeon larvae (Najdegerami et al., 2015). Nevertheless, a growth-promoting effect of the PHB was also reported for Siberian sturgeon larvae, giant freshwater prawn larvae, Chinese mitten crab larvae, Asian tiger shrimp larvae, Pacific white shrimp, and rainbow trout juveniles in different studies (Duan et al., 2017; Laranja et al., 2014; Najdegerami, 2020; Najdegerami et al., 2012; Nhan et al., 2010; Sui, Cai, Sun, Wille, & Bossier, 2012). Such effect of the PHB polymers is explained by researchers, as it degrades into short-chain fatty acid oligomers and monomers, it could then decrease the intestinal pH of the investigated organism and improve the activity of the digestive enzymes, and provide better nutrient absorption. Alternatively, the monomers and oligomers of the PHB might act as additional energy source itself (Azain, 2004; Baruah

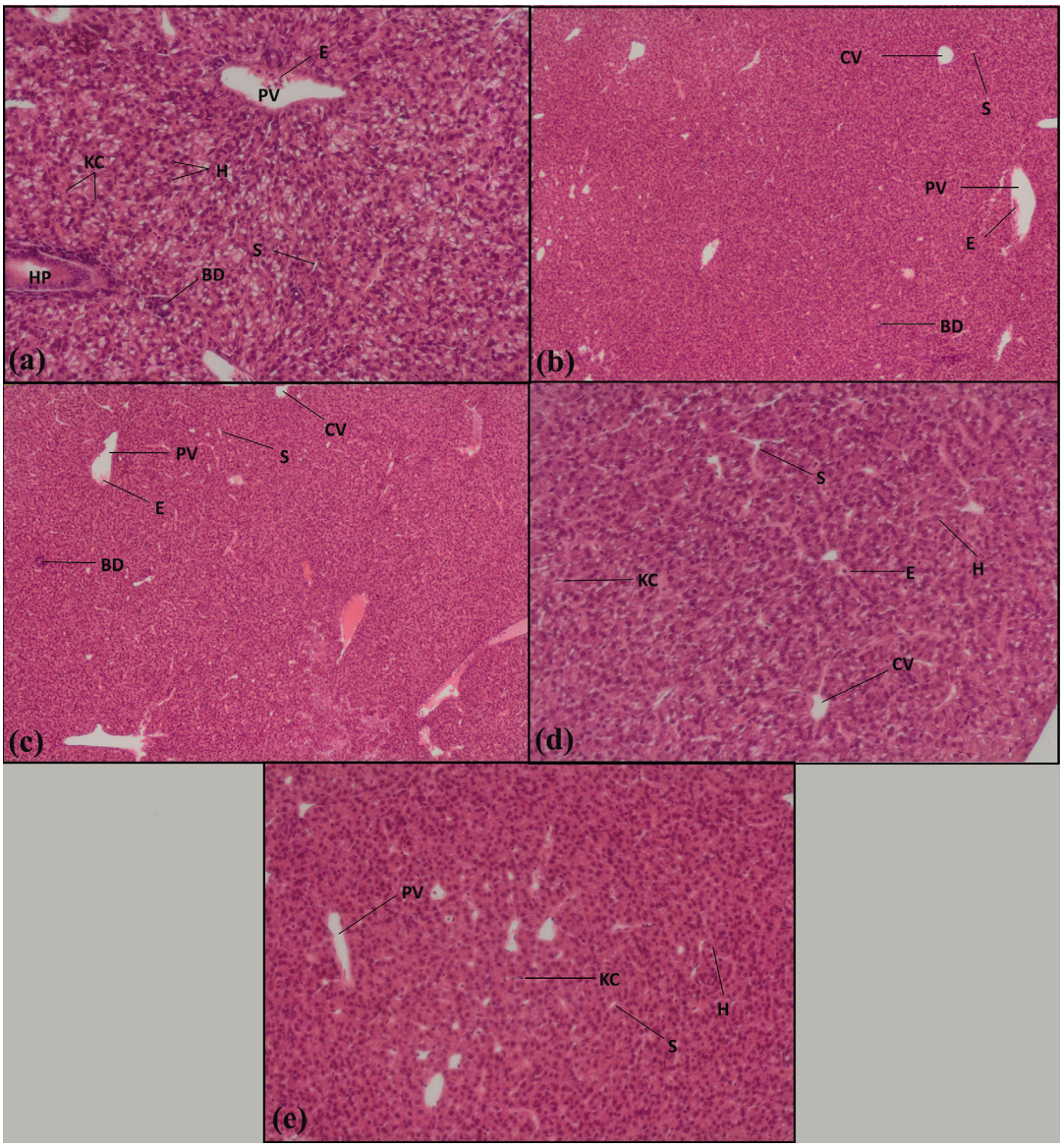

FIGURE 3 Liver cross-sections (4× magnification) of *O. mykiss* at (a), (b) initial day, (c) 60th day for the control group, (d) 60th day for the 2% PHB group, (e) 60th day for the 5% PHB group. Abbreviations: BD, bile duct; CV, coronary vein; E, erythrocyte; H, hepatocyte; HP, hepatopancreas; KC, kupffer cell; PV, portal vein; S, sinusoid

et al., 2005, 2007; De Schryver et al., 2010; Defoirdt, Boon, Sorgeloos, Verstraete, & Bossier, 2009; Lückstädt, 2008; Najdegerami et al., 2012, 2017; Sui et al., 2012; Weltzien, Hemre, Evjemo, Olsen, & Fyhn, 2000). According to Najdegerami et al. (2017), despite the necessary pH level and enzyme activities for degradation of PHB was achieved, the growth and survival parameters were not affected as expected. Therefore, the authors have mentioned that the inert property of PHB might have been originated from the doses of the application. Thus, in the present study, we investigated an additional dose as 5% PHB; however, it is also found to be ineffective for rainbow trout fry. Therefore, the relation between PHB and growth appears to be independent of the dose of the application. The PHB supplementation did not cause any significant improvement in the growth performances of European sea bass larvae (De Schryver et al., 2011; Franke et al., 2017; Franke, Clemmesen, et al., 2017), whereas in another study, it

increased the growth rate of the same species' juveniles (De Schryver et al., 2010). Accordingly, a correlation between the size or developmental stage of fish and PHB might be probable in terms of growth rate.

Regarding survival rates, no significant differences were determined among the groups fed with different ratios of PHB. Consequently, PHB is found to be ineffective for survival rates of rainbow trout fry in this experiment. Najdegerami et al. (2017) reported a confirmatory effect of the PHB on rainbow trout fingerlings in their research as well. Another similar result was also expressed in an experiment with European sea bass post-larvae, in which Franke, Clemmesen, et al. (2017) fed the fish with PHB-enriched *Artemia* nauplii and reported that the PHB did not affect the survival rates of the animals. However, in a different study, PHB-enriched *Artemia* increased the survival rates of the yolk-sac larvae of the same species (Franke, Roth, et al., 2017). Other contradicting results on the effects of dietary PHB are also present in the literature. For instance, Najdegerami et al. (2012) investigated the effects of PHB supplemented diets on Siberian sturgeon and reported that the survival rates of the 2% PHB group showed statistically better results than the groups 5% PHB and control. Nevertheless, in another study, PHB-enriched *Artemia* reduced survival rates of the same species (Najdegerami et al., 2015). On the contrary, De Schryver et al. (2010) fed European sea bass juveniles with PHB supplemented diets, which improved survival rates of experimental fish for the first 3 weeks; however, ended up with a relatively decreased survival for the groups 100% PHB and control at the end of the experiment. Najdegerami (2020) reported positive effects of PHB on the survival of rainbow trout juveniles challenged with *Yersinia ruckeri*. Relevant to the previous studies, the PHB seemed to have divergent effects on the growth and survival performances of the animals investigated. In this respect, it is hard to state that the PHB can be a certain promoter for such parameters. However, even though it is difficult to assume the effectiveness of PHB on the growth and survival rates of the experimental animals, similar to that of Najdegerami et al. (2017) our findings somewhat confirmed the inert feature of PHB on those parameters for rainbow trout.

The endogenous gut microbiota has been implicated as a significant factor in the mucosal development and maturation of the fish intestine and immune system (Rawls, Samuel, & Gordon, 2004). Furthermore, it has been suggested that gastric populations aid digestive function and provide competition against pathogenic visitors to the intestine (He et al., 2009). Thus, in the present study, we investigated the bacterial flora in case any changes have been occurred in the gastric populations, regarding the information that the PHB promotes the health of the intestinal microflora of the experimental animals (Duan et al., 2017; Laranja et al., 2014; Najdegerami et al., 2017). As a result of the classical and DGGE identification analysis, the total count of both Enterobacteriaceae and coliform bacteria decreased significantly in the group 5% PHB, while only coliform count decreased in the group 2% PHB. It seems that the increased ratio of the PHB had a positive effect on reducing several harmful bacteria. However, the bacterial community determined by DGGE fingerprints as the total, psychrophilic, and lactic acid bacterial counts in the intestines did not differ among the treatments. It was stated in the literature that the application of PHB increased the bacterial evenness and diversity in European sea bass "juveniles" (De Schryver et al., 2010, 2011), whereas did not affect the species-richness, evenness, and diversity of the bacteria for yolk-sac larvae of the same species (Franke, Roth, et al., 2017). It was also demonstrated in previous studies that the PHB had inert properties on the intestinal microflora of experimental animals. For instance, Da Silva et al. (2016) investigated the effects of dietary butyrate and PHB on the performance of Pacific white shrimp and found that the shrimps fed with a 2% PHB diet did not alter significantly in intestinal bacterial counts. In another research, the intestinal microbiota of Siberian sturgeon larvae fed with PHB enriched *Artemia* nauplii did not differ concerning the control group (Najdegerami et al., 2015). Even though there are conflicting findings among experiments, the PHB found to be beneficial for the gastrointestinal tract of several species. The variations on the effect of PHB might be depending on the experimental species and, therefore, different microflora. In the present study, the reducing effect of PHB on Enterobacteriaceae and coliform counts corresponded with the previous findings. The decline in the number of harmful bacteria is important, as it reduces the number of bacterial groups and promotes an increment in the number of beneficial bacteria. However, because the total number of bacteria did not change, it can be recommended for further studies to use other methods such as the NGS (next-generation sequencing) analysis to identify the beneficial or harmful bacteria in the flora.

In terms of nutritional composition, no significant differences were determined in fish fed or non-fed with PHB diets. Najdegerami et al. (2015) reported that there was no effect of PHB-enriched *Artemia* on crude protein levels of Siberian sturgeon larvae, but was on the lipid content. According to unpublished data of Najdegerami et al. (2015), on the contrary, the PHB-supplemented feed reduced the lipid level of Siberian sturgeon juveniles (Najdegerami et al., 2015). In another study, Najdegerami (2020) reported that the protein and lipid levels were not affected by the inclusion of PHB in the diet, whereas ash was. Duan et al. (2017) also reported that the 5% PHB supplementation in the diet significantly increased both crude protein and lipid levels of Pacific white shrimp. According to these findings, it can be thought that the contradicting effects of PHB may depend on the delivery method, the developmental stage of the animal, or the species itself.

In our experiment, the steady values of body composition among the treatments led us to investigate the histopathological condition of fish. Considering the findings, the PHB administration did not cause any histopathological anomalies in the foregut and liver cross-sections of experimental fish. Besides, 2 and 5% PHB-supplemented diets increased the mean villus length and width, concerning the control group. It has previously been reported by researchers that the increased villus length provides notably better nutrient uptake (Spring & Privulesku, 1998). However, the results we obtained did not correspond with this information, because the growth and body composition of fish did not change among the treatments. Consequently, it has been thought that there might be an unidentified mechanism of action for PHB between villi size and body composition.

5 | CONCLUSION

In the light of the present study, even though the PHB did not significantly affect the growth and survival of rainbow trout fry, the resulting healthier intestinal flora makes it useful as long as the cost/benefit ratio was calculated well. Besides, the increased length and width of the villus, as well as the count of beneficial bacteria induced by PHB, make the determination of the increased bacteria species significant to clarify the mechanism of actions of PHB. With this regard, further experiments may also provide insight into shelf-life studies. However, considering previous studies, the effects of PHB seem very species-specific, even further, more specific to the life stage of the experimental animal. For this reason, it would be a better approach to take the factors as the developmental stage of the investigated organism or the species itself into consideration as well as the onset, form, duration of the supplementation, or delivery method for a better understanding of PHB.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Tolga Şahin: Experimental set-up, diet formulation and preparation, feeding, chemical analysis, analytical determinations, statistical analysis, interpretation of the data. **Sevdan Yılmaz:** Microbial work, statistical analysis, interpretation of the data. **İrfan Selçuk Yazıcı:** PCR-DGGE analysis, histological work, interpretation of the data. **Selçuk Berber:** Study design, interpretation of the data.

DATA AVAILABILITY STATEMENT

Research data are not shared.

ORCID

Tolga Şahin https://orcid.org/0000-0001-8232-3126
Sevdan Yılmaz https://orcid.org/0000-0002-4809-5809

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