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Growth enhancing, histomorphology and disease protective effects of poly-β-hydroxybutyrate dietry supplement on farmed seabass

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ABSTRACT

The aim of the present study is to evaluate the effects of polyβhydroxybutyrate (PHB) on the growth performance, organ histomorphology , transcription of immune-related genes, and resistance of cultured seabass (*Dicentrarchus labrax*) against *Vibrio anguillarum* infection. One hundred and twenty seabass were divided into four experimental groups in three replicates (n=3) fish were fed on basal diets supplemented with 0.0, 5.0, 10.0, and 15.0 g PHB per kg diet for eight weeks. The final body weight, weight gain, and weight gain percent were significantly increased in all PHB-groups compared with the controls ($P < 0.05$). No significant differences were noticed in the specific growth rate , the feed conversion ratio and survival rate . There was a clear dose-dependent increase in the mRNA expression values of interleukin-10 and complement C5 genes in the hepatic tissues of treated fish ($P < 0.05$). After challenge with *V. anguillarum*, the relative percent of survival was significantly increased depending on the increase of dose of PHB in each group compared with the control group. Histopathological examination of gills showed ballooning of primary gill lamellae ,curling of secondary lamellae and hyperplasia of lamellar epithelium. Liver showed congestion of hepatic blood sinusoids, vaccuolation of hepatic cytoplasm with presence of bacteria in hepatic tissues. Posterior kidney showed swollen and vaccuolation of epithelium lining renal tubules. Intestine showed desquamation of villus mucosa , necrosis with presence of bacteria in submucosa.

INTRODUCTION

Aquaculture provides the growing world population with a significant portion of the animal protein requirement. Mariculture is an essential alternative to Freshwater aquaculture due to growing shortage of freshwater and abundance of seawater. European sea bass (*Dicentrarchus labrax*) is now considered an important economic species in marine aquaculture. European seabass was produced in limited amounts in marine fish farms, which depend on seed collection from the wild. Recently,

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several marine fish hatcheries have been established with successful rearing of fish in an enclosed environment, eliminating the need to collect the seed from the wild. This is a necessary step to minimize the negative effect of wild marine fish seed on disease spread with cultured stocks. For example, in Egypt, European seabass constitutes 1.4% of the total fish catch from the egyptian Mediterranean coast **(GAFRD 2012).**

The uncontrolled antibiotics usage in treating bacterial diseases in aquaculture has been associated with the development of antibiotic resistant strains of *Vibrio anguillarum, Aeromonas hydrophila, Edwardsiella tarda, E. ictaluri,;* and *Yersenia ruckeri* ; increased antibiotic residues in fish tissues as well as increasing the environmental contamination **(Serrano, 2005)** In contrast, The non antibiotic growth promoter could clearly improve the health and growth of aquatic organisms **(Carbone and Faggio 2016).**

Many environmentally friendly biological products have been successfully used as immunostimulants such as Organic acids which are known to act as bacteriostatic and bactericidal against pathogenic bacteria **(Thompson and Hinton 1996 Ricke 2003 Vazquez et al. 2005).** The safety of Short chain fatty acids (SCFAs) and their salts are clearly known. Thus, they are usually used as antimicrobials in the feed industry of livestock. Butyrate, Format, propionate, acetate, and their salts are the most studied SCFAs. This is because of their effect on the physiological response and the host performance in three main ways: dietry effects, the local effects on the gastrointestinal tract of the animals, including fish or by direct metabolic effects . Generally, the mechanism of action of the SCFAs is the capability of reducing the pH of the feed with consequent inhibition of microbial growth reducing PH in the gastrointestinal tract including the stomach and the small intestines. Furthers; dissociation of the acids and production of anions in bacterial cells with consequent inhibition of the development of Gram negative bacteria **(Hoseinifar et al. 2017)**. SCFAs inhibit the growth of fish and shrimp pathogens such as *V. anguillarum, V. alginolyticus, V. pelagius,* and *V. campbellii* **(Vázquez et al. 2005; Defoirdt et al. 2006).**

Several problems have faced SCFA in aquaculture high solubility in water; loss of large particles by feeding from feed and dissolved in water. This may lead to higher feed doses which will eventually result in high stimulation of microbial growth in the water. poly-β-hydroxybutyrate (PHB) was proposed as a reliable solution to this problem . This compound serves as carbon reserve for bacteria and an intracellular energy source **(Madison and Huisman 1999, Tokiwa and Calabia 2004).** It is insoluble in water and biologically degradable into β-hydroxybutyric acid **(Defoirdt et al. 2007).**

The bacterial storage polymer poly-β-hydroxybutyrate (PHB) can act as an energy source for the fish ;increase their growth performance, and decrease the intestinal bacterial community structure. Thus it could be involved in the anti-infective strategy for aquaculture **(De Schryver et al. 2010).** PHB effectively act as growth promoter , enhances the resistance against pathogens and improves overall performance of farmed species and high survivability rate **(De Schryver 2010 Defoirdt et al. 2007 Nhan et al. 2010 Suguna et al. 2014 Halet et al. 2007 Laranja et al. 2014 Thai et al. 2014).** Therefore, the present study aimed to evaluate the effect of PBH on the growth performance, immune-related expression genes , histomorphology and its antibacterial effect against pathogenic *V. anguillarum* infection.

2. Material and Methods

2.1. Ethical approval

The authors followed all applicable international, national, and/or institutional guidelines for the care and use of fish.

2.2. Fish and acclimatization conditions

A total number of 120 sea bass (Dicentrarchus labrax) fingerlings (12.4±0.8 g) were purchased from a private fish farm (Alexandria governorate, Egypt) and transferred alive in polyethylene plastic bags supplemented with 2/3 air. Upon arrival to fish diseases unit in AHRI (Alexandria Provincial Lab.), they were acclimatized for 2 weeks in prepared glass aquaria (80 × 40 × 60 cm) contain (water sup-

plemented with 32 mg / L NaCl) with continuous aeration using electric air pumping compressors where (dissolved oxygen adjusted 6 mg / L), a water temp adjusted at 18°C. Fish were fed formulated feed (AQUA, Egypt) at 3% of their body weight per day. Table (1) shows the feed ingredients , formulation and proximate chemical composition of the control basal diet. The light system was programmed at a fixed 14 h light and 10 h dark. Each aquarium was supplied with an individual filter system.

Table (1) Ingredients and proximate chemical composition (g/kg on dry weight basis) of experimental diet.

Ingredients	**g/kg**
Soybean meal (42.7% CP)	300
Fish meal (65.0% CP)	400
Wheat gluten	50
Corn meal	100
Rice bran	60
Corn oil	20
Fish oil	10
Vitamin Premix[1]	30
Mineral Premix[2]	30
Total	1000
Proximate chemical composition	
Dry matter	93.2
Crude Protein	462
Crude lipids	135
Crude fibers	94
Ash	103

[1] Vitamin premix include (/kg in premix): vitamin A 67 IU, vitamin D 16.2 IU, vitamin E 7.4 g, vitamin K3 340 mg, vitamin B1 670 mg, vitamin B2 1000 mg, vitamin B6 800 mg, vitamin B12 1.4 mg, vitamin C 10 g, D-pantothenic acid 2.65 g, folic acid 330 mg, nicotinamide 5.35 g, choline chloride 35 g, biotin 34 mg, inositol 8 g.
[2] Mineral premix: Fe 14 g, Cu 350 mg, Zn 4 g, Mn 1.4 mg, Mg 10 g, Co 30 mg, I 40 mg, Se 35 mg.

2.3. Feed preparation and Experimental setup

The experiment consisted of 120 culured sea bass divided into four groups (10 fish /each) with three replicates. For eight weeks, fish in each aquarium were fed with a different concentration of experimental feed diet at 3% of their body weight per day.

PHB was purchased from Sigma-Aldrich, the USA, to be incorporated in the experimental feed. chloroform and distilled water (80:20) mixture was made for dissolving PHB. The feed particles were coated with the PHB solution by spraying it homogeneously. The coated feed particles were air dried for 2 days in atmospheric air. there were four different treatments: (1) a control (0.0 PHB/kg diet), (2) 5% PHB (5 g PHB/kg diet), (3) 10% PHB (10 g PHB/kg diet), and (4) 15% PHB (15 g / kg diet) according to **(De Schryver et al. 2010).**

Fish were fed one of the tested diets by hand up to visual satiation three times per day at 8:00, 11:00, and 14:00 h for eight weeks. Settled fish wastes were cleaned daily by siphoning off half of the aquarium's water, which was replaced by well-aerated water from a water

storage tank.

2.4. Water quality parameters

The water quality was assessed on regular basis the pH was measured using a pH meter (Digital Mini-pH Meter, USA) ; The unionized ammonia (NH3) was measured using special kits (HACH Co., Loveland, CO., USA); the temperature was measured with a thermometer ; Dissolved oxygen (DO) measured with a portable oxygen meter (Jenway, London, UK) When the levels of these water quality parameters exceeded 0.1, 1.0, and 20 mg L−1, respectively, 50% of the water was replaced according to **(Boyd and Tucker 2012).**

2.5. Growth performance

After 60 days of the feeding trial, fish from each aquarium were collected, counted, and bulk weighed. Growth performance was evaluated, and feed utilization was calculated as follows:

Weight gain (WG) = $W_2 – W_1$

Weight gain (WG) % = 100 $(W_2 – W_1) / W_1$

Specific growth rate (SGR; %g/day) = 100 [Ln W_2 (g) – Ln W_1 (g)] / T; where W_2 is final weight, W_1 is initial weight, and T is the experimental period (day)

Feed conversion ratio (FCR) = feed intake / weight gain

Fish survival (SR) (%) = 100 (fish number at final / fish number at initial)

2.6. Gene expression analysis

At day 60 of feeding trial, fish were randomly sampled for immune-related gene analysis, including interleukin-10 (*IL-10*) and complement (*C5*) genes . Primers used were supplied from Metabion (Germany). Primer sequences, target genes, amplicons sizes, and cycling conditions for SYBR green rt-PCR were listed in table (2).

Table (2) Primers sequences of the target genes and cycling conditions for SYBR green RT-PCR.

Target gene	Primers sequences	Reverse transcription	Primary denaturation	Amplification (40 cycles)			Dissociation curve (1 cycle)			Reference
				Secondarydenaturation	Annealing	Extension	Secondary denaturation	Annealing	Final denaturation	
EF-1α	CCTTCAACGCTCAGGTCATC TGTGGGCAGTGTGGCAATC	50˚C 30 min	94˚C 15 min	94˚C 15 sec	62˚C 30 sec	72˚C 30 sec	94˚C 1 min	62˚C 1 min	94˚C For 1 min	**Gröner et al. 2015**
IL-10	CTGCTAGATCAGTCCGTCGAA GCAGAACCGTGTCCAGGTAA				60˚C 30 sec			60˚C 1 min		**Staden et al. 2016**
C5	TGG-CAAGGACTTTTTCTGCT AG-CACAGGTATCCAGGGTTG				60˚C 30 sec			60˚C 1 min		**Syahputra et al. 2019**

EF-1α: Eukaryotic translation elongation factor 1 alpha, *IL-10*: Interleukin 10, *C5*: Complement C 5

2.6.1. RNA extraction

RNA extraction from tissue samples was applied using QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) when 30 mg of the tissue sample was added to 600 µl RLT buffer containing ten µl β-mercaptoethanol per 1 ml. For homogenization of samples, tubes were placed into the adaptor sets, fixed into the clamps of the Qiagen tissue Lyser. Disruption was performed in 2 minutes high-speed (30 Hz) shaking step. One volume of 70% ethanol was added to the cleared lysate. The steps werecompleted according to the Purification of Total RNA from Animal Tissues protocol of the QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH).

2.6.2. SYBR green rt-PCR

Primers were utilized in a 25- µl reaction containing 12.5 µl of the2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Germany, GmbH), 0.25 µl of Revert Aid Reverse Transcriptase (200 U/µL) (Thermo Fisher), 0.5 µl of each primer of 20 pmol concentration, 8.25 µl of water, and 3 µl of RNA template. The reaction was performed in a Strata gene MX3005P real-time PCR machine.

2.6.3. Analysis of the SYBR green rt-PCR results

The Stratagene MX3005P software determined amplification curves and ct values. To estimate the variation of gene expression on the RNA of the different samples, the CT of each sample was compared with that of the positive control group according to the "ΔΔCt" method **(Yuan et al. 2006)** using the following ratio: -

Whereas ΔΔCt = ΔCt reference – ΔCt target

ΔCt target = Ct control –Ct treatment and ΔCt reference = Ct control- Ct treatment

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ΔCt target = Ct control – Ct treatment and ΔCt reference = Ct control- Ct treatment.

2.7. Sampling for Histopathology

All fish were fasted for a period of 24 h, immediately before the sampling. to avoid the stress on fish during sampling The sampling containers were supplied with buffered tricaine methanesulphonate (MS-222, 25 mg/L), mixed with water **(Matsche 2011).**

All fish were necropsied for collecting livers and posterior kidneys of fish in the control group, PHB-fed group, *V. anguillarum* infected group, and PHB-infected group. For intestine samples, fish were incised, and the whole intestinal tracts were carefully separated. Liver, kidney, intestine, and gills were collected carefully and rapidly placed in 10% neutral buffered formalin then after 48 h, fixed tissues were processed in ascending grades of alcohol, cleared by xylene, and finally embedded in paraffin wax to prepare 5 µm thick sections stained with hematoxylin and eosin (H&E) stain for microscopic examination **(Suvarna et al. 2013).** Later, several representative photomicrographs were captured with a digital camera (Labomed LC-1 CMOS, Labomed, USA) connected to a microscope (Labomed LB-212).

2.8. Challenge test

The bacterial strains were isolated and fully identified using standard modern molecular techniques (RT- PCR) from seabass by the unit of Bacteriology, AHRI (Damnhour provincial lab) . The lethal dose (LD50) of *V. anguillarum* for seabass was determined as the following method, fish were intraperitoneally (IP) injected with different doses of 24-h live bacteria, and fish mortality was observed for 5 days post-infection. The LD50, which resulted in 50% fish mortality, was 1×10^7 CFU/ ml, and a sublethal dose was used for the bacterial challenge. After the feeding trial, fish per each treatment were collected, pooled, and randomly restocked at a density of 10 fish per 100-L tank in duplicates under the same rearing conditions of the experiment. Afterward, fish were injected with 200 µL of phosphate-buffered saline (PBS) containing 1×10^6 CFU of V. anguillarum, which intraperitoneally injected in seabass **(Azad et al. 2004).** Fish of each treatment were fed on the corresponding diets during the challenge test. All fish were observed daily to record any mortalities for 10 days. The cumulative fish mortality was recorded at the end of the experiment.

The relative percent of survival (RPS %) was calculated according to the following formula:

RPS % = [1- % of mortality in experimental group / % of mortality in control group] × 100

2.9. Statistical analysis

Data are shown as mean ± SE of three replicates. Before statistical analysis, all data were tested for normality of distribution and homogeneity of variances using Kolmogorov–Smirnov and Bartlett's tests, respectively. Differences between means were tested at a 5% probability level using the Duncan test as a post-hoc test ($P < 0.05$ was considered statistically significant). All the statistical analyses were done via SPSS program version 20 (SPSS, Richmond, VA, USA).

3. RESUITS

3.1. Growth performance

Table (3) shows the growth performance parameters and feed utilization indices of cultured seabass fed diets supplemented with PHB for eight weeks. There were no significant differences in the initial body weight of the fish. The FBW, WG, and WG (%) were statistically increased in a dose dependent manner compared with those reared in the control group ($P < 0.05$). No significant differences were found in the SGR and FCR values among all experimental groups. The SR % was similar in all groups and equal to 100% and this suggest that the feed additive did not harm the fish.

Table (3) Effect of dietary poly-beta-hydroxybutyrate (PHB) supplementation on growth performance and survival rates of European seabass (*Dicentrarchus labrax*).

Parameters	PHB levels				SEM	*P* value
	Control (0.0)	5 g / kg feed	10 g / kg feed	15 g / kg feed		
IBW (g)	12.20	12.00	12.10	12.20	0.143	0.454
FBW (g)	17.90 d	23.80 c	27.60 b	35.50 a	0.551	< 0.001
WG (g)	5.70 c	11.80 bc	15.50 b	23.30 a	0.454	< 0.001
WG (%)	46.72 d	98.33 c	128.09 b	190.98 a	4.211	< 0.001
SGR (% g / day)	2.983	3.001	2.999	2.986	0.014	0.454
FCR	1.42	1.41	1.44	1.42	0.021	0.869
SR (%)	100.0	100.0	100.0	100.0	0.00	1.00

IBW: Initial body weight, FBW: Final body weight, WG: Weight gain, WG%: Weight gain percentage, SGR: Specific growth rate, FCR: Feed conversion ratio, SR: Survival rates.

3.2. Gene transcription

There were significant upregulations of IL-10 and C5 genes in the liver in a dose-dependent manner compared with the controls ($P < 0.05$). Figure(1) shows the mRNA expression values of immune related genes in liver of cultured seabass fed diets supplemented with PHB for eight weeks.

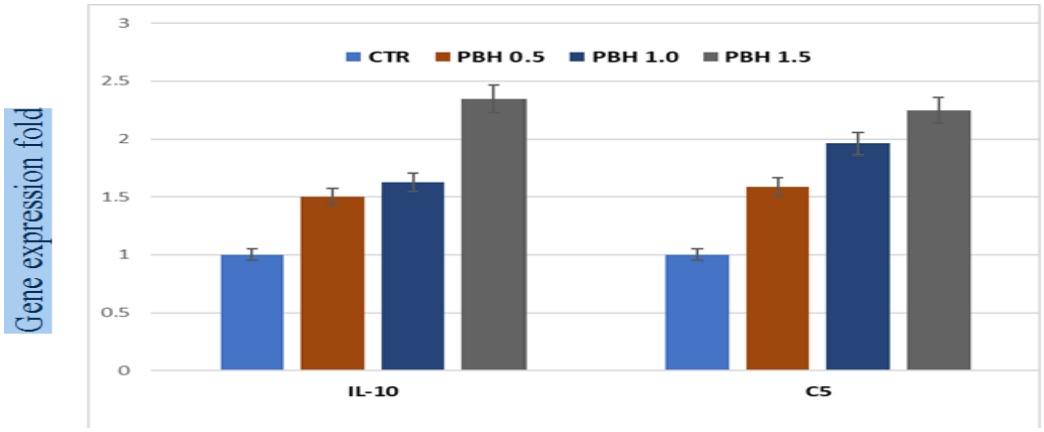

Fig. (1) Expression folds of interleukin-10 (*IL-10*) and complement (*C5*) genes in the liver of European sea bass (*D. labrax*) fed diets containing different levels of poly-β-hydroxybutyrate (PBH) for 4 weeks.

3.3. Histomorphological studies

In this study, the intestine, posterior kidney, and gills of the PHB-fed fish group had a normal histomorphological structure as well as the control group. Fat vacuoles with varying sizes are present in hepatocytic cytoplasm are observed in fish-fed BHP-based diet compared to fish of the control group. Fish groups fed a PHP-based diet and experimentally infected with *V. anguillarum* show fewer histopathological lesions than fish groups fed basal feed only and infected with *V. anguillarum* and all listed in table (4).

Table (4) Histopathological scoring of seabass infected with *Vibrio anguillarum* and fed basal diet compared to Groups of seabass fed on poly–β– hydroxybutyrate (5,10&15g PHB per kg diet) and infected with *Vibrio anguillarum.*

Histopathological findings	Group of seabass infected with *Vibrio anguillarum* and fed basal diet.	Groups of seabass fed on poly – β – hydroxybutyrate and infected with *Vibrio anguillarum*		
		5g PHB per kg diet	10g PHB per kg diet	15g PHB per kg diet
Gills:				
-Balooning of primary gill lamellae.	+	+	+	+ _
-Curling of secondary gill lamellae.	+	+	+	+
-Hyperplasia of lamellar epithelium.	_	_	+	
Liver:				
-Blood vessels.	+			
*congestion.	+	+	+	+
*presence of bacteria.		+	+	±
-Vaccuolation of hepatocytic cytoplasm.	+	+	+	++
Kidney:				
-Effusion of blood cells into interstitial tissue.	+	+	_	_
-Distortion of glomerular corpuscle.	+	_	_	_
-Swollen and vaccuolation of epithelium linning of proximal and distal renal tubules.	+	+	+	+
Intestine:	+	+	±	_
-Desquamation of villus mucosa.	+	+	±	_
-Presence of bacteria in submucosa.	+			
-Necrosis .		+	±	_

3.3.1 Histomorphology of liver

Liver of seabass showed normal hepatopancreatic tissue of control fish group, vacuolation of hepatocytic cytoplasm with the normal architecture of fish fed PHB –based diet with the presence of bacteria in the lumen of blood vessel of fish infected with *V. anguillarum* in fish fed PHB-based diet 0.5 g, 1.0 g and 1.5 g (Fig. 2).

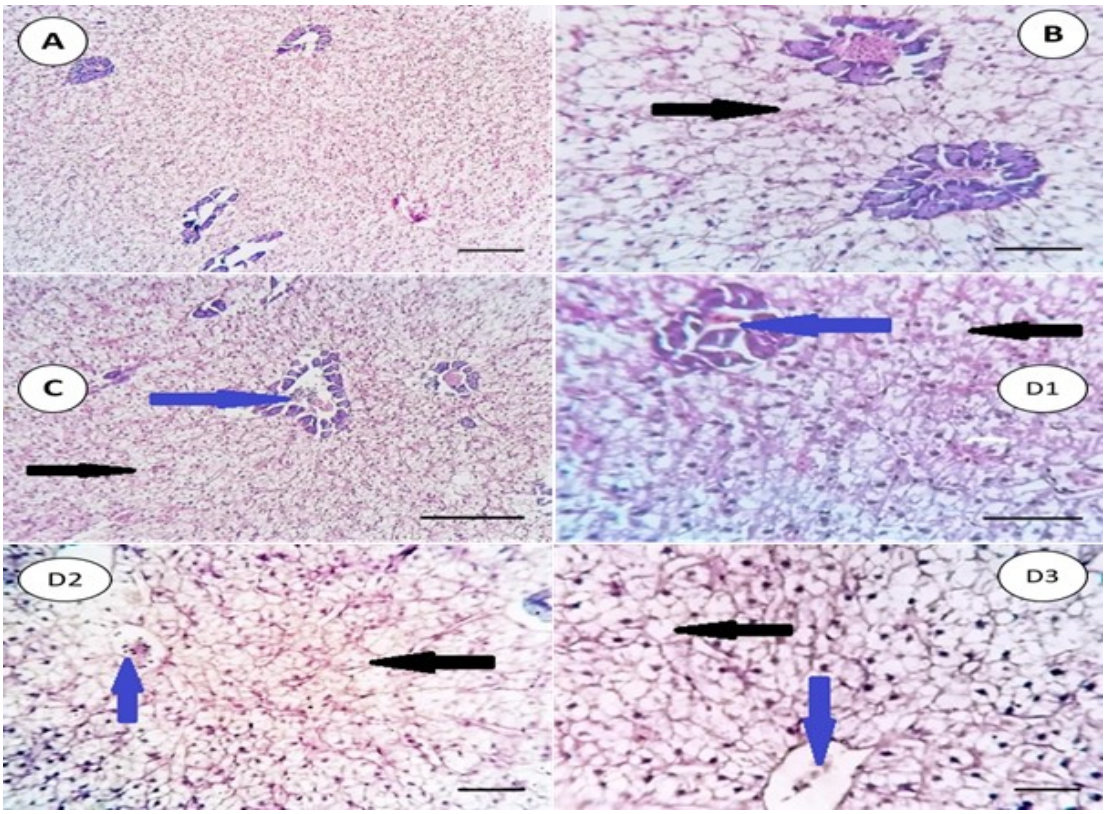

Fig. (2)
Liver of European seabass showed normal hepatopancreatic tissue of control fish (A; H & E, X100), vacuolation of hepatocytic cytoplasm (black arrow) with the normal architecture of fish fed PHB –based diet (B; H & E, X400), vacuolation of hepatocytic cytoplasm (black arrow) with the presence of bacteria in the lumen of the blood vessel (blue arrow) of fish infected with *V. anguillarum* (C; H & E, X250), vacuolation of hepatocytic cytoplasm (black arrow) in fish fed PHB-based diet 0.5 gm with the presence of bacteria in the lumen of the blood vessel (blue arrow) (D1; H & E, X400), vacuolation of hepatocytic cytoplasm (black arrow) in fish fed PHB-based diet 1.0 gm with the presence of bacteria in the lumen of the blood vessel (blue arrow) (D2; H & E, X 250), and sever vacuolation of hepatocytic cytoplasm (black arrow) in fish fed PHB-based diet 1.5 gm with the presence of fewer bacteria in the lumen of the blood vessel (blue arrow) (D3; H &E, X 250).

3.3.2. Histomorphology of intestine

The intestine of the seabass showed normal intestinal villi of control fish. Long intestinal villi with normal architecture and goblet cells of fish-fed PHB –based diet. Intestinal villi with bacteria in the submucosa of fish infected with *V. anguillarum* Necrosis and desquamation of villus mucosa of fish fed PHB-based diet 0.5 gm with the presence of bacteria in the submucosa. Necrosis and desquamation of villus mucosa of fish fed PHB-based diet 1.0 gm with bacteria in the submucosa. Normal villus mucosa with numerous goblet cells of fish-fed PHB-based diet 1.5 gm with the presence of bacteria in the submucosa (Fig. 3).

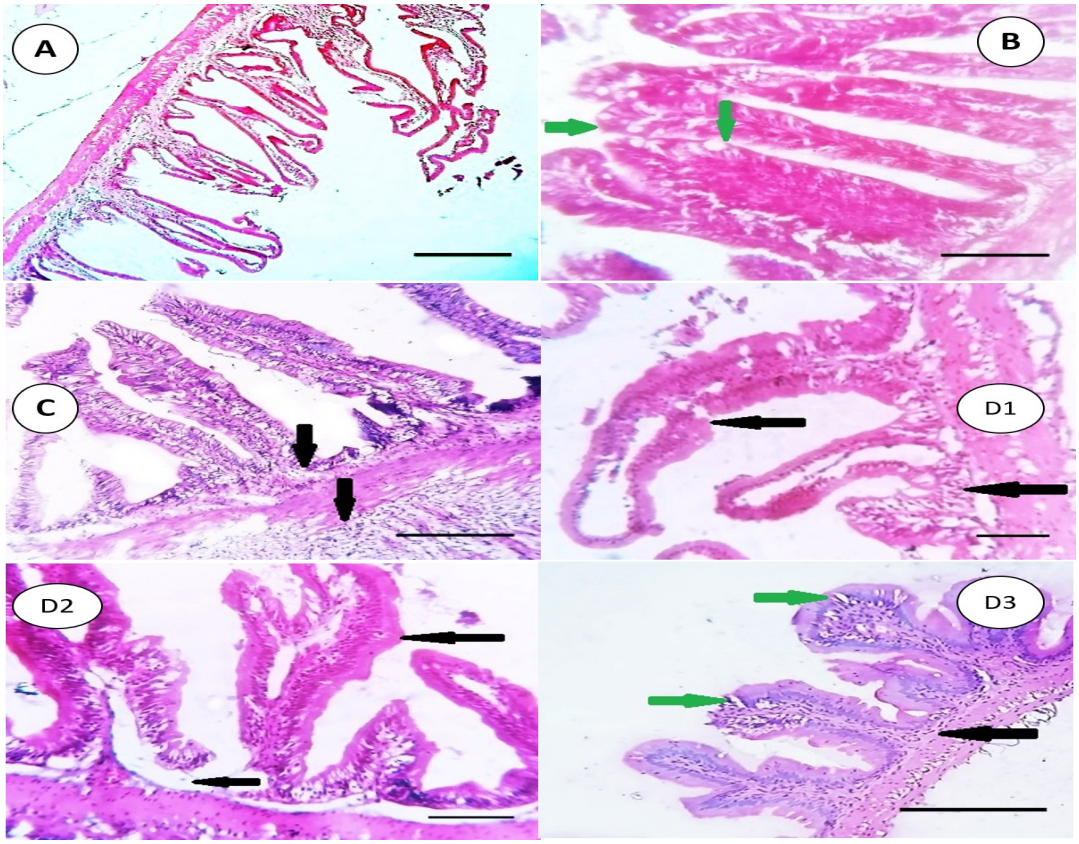

Fig. (3)
The intestine of European seabass showed normal intestinal villi of control fish (A; H&E, X100), long intestinal villi with normal architecture and goblet cells (green arrow) of fish fed PHB –based diet (B; H & E, X400), intestinal villi with the presence of bacteria in the submucosa (black arrows) of fish infected with *V. anguillarum* (C; H & E, X250), necrosis and desquamation of villus mucosa (black arrows) of fish fed PHB-based diet 0.5 gm with the presence of bacteria in the submucosa (D1; H & E, X400), hyperplasia and desquamation of villus mucosa (black arrows) of fish fed PHB-based diet 1.0 gm with the presence of bacteria in the submucosa (D2; H & E, X250), and normal villus mucosa with numerous goblet cells (green arrows) of fish fed PHB-based diet 1.5 gm with the presence of bacteria in the submucosa (D3; H & E, X250).

3.3.3. Histomorphology of kidney

The kidney of the seabass showed normal renal tissue of control fish. Normal proximal, distal renal tubules and renal corpuscle with effusion of blood cells into the renal interstitial tissue of fish fed PHB –based diet, normal proximal, distal renal tubules, and renal corpuscle with effusion of blood cells into renal interstitial tissue and presence of bacteria in the renal vessel of fish infected with *V. anguillarum*. The lining epithelium of Proximal renal tubules are swollen, a wide lumen of distal renal tubules with large renal corpuscles of fish infected with *V. anguillarum* and fed PHB-based diet 0.5 gm .lining epithelium of Proximal renal tubules are swollen, vacuolation of the cytoplasm of lining epithelium of distal renal tubules, large renal corpuscles, presence of bacteria in renal interstitial tissue of fish infected with *V. Anguillarum* and fed PHB-based diet 1.0 g, lining epithelium of Proximal renal tubules are swollen, vacuolation of the cytoplasm of lining epithelium of distal renal tubules, large renal corpuscles, presence of bacteria in renal interstitial tissue of fish infected with *V. anguillarum* and fed PHB-based diet 1.5 gm (Fig. 4).

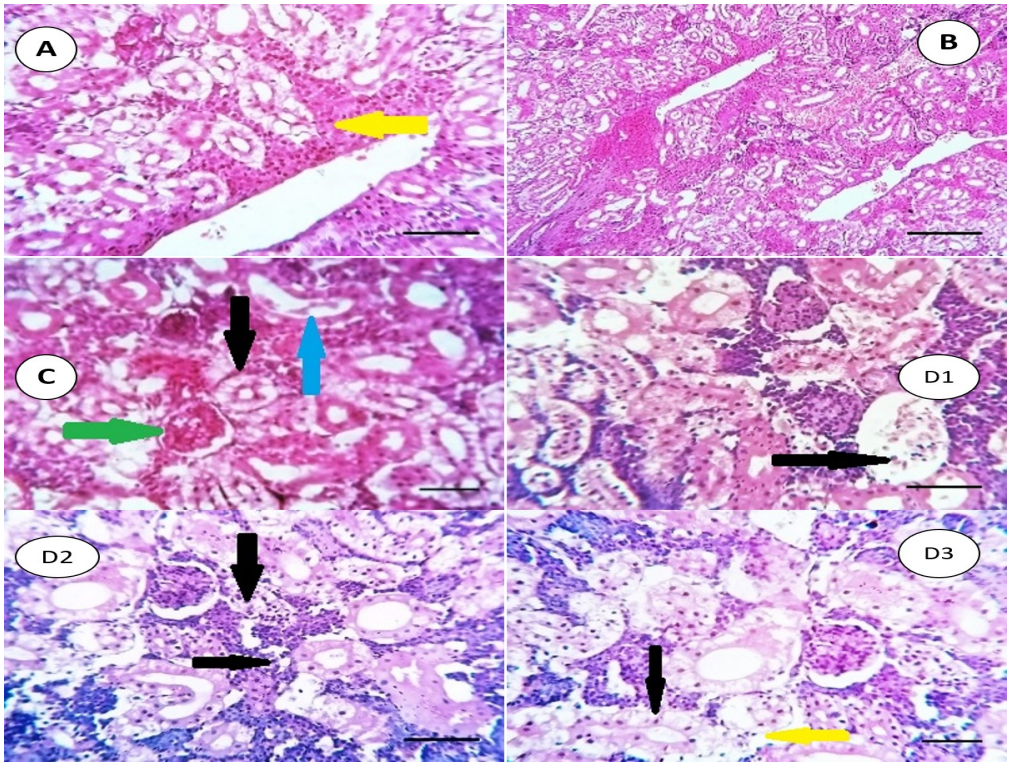

Fig. (4)
Kidney of European seabass showed normal renal tissue of control fish (A; H & E, X100), normal proximal, distal renal tubules and renal corpuscle with effusion of blood cells into renal interstitial tissue (yellow arrow) of fish fed PHB –based diet (B; H & E, X250), normal proximal, distal renal tubules and destorted renal corpuscle with presence of bacteria in renal vessel (black arrow) of fish infected with *V. anguillarum* (C; H & E, X400), lining epithelium of proximal renal tubules are swollen (black arrow), wide lumen of distal renal tubules (blue arrow) with large renal corpuscles (green arrow) of fish infected with *V. anguillarum* and fed PHB-based diet 0.5 gm (D1; H & E, X250), lining epithelium of proximal and distal renal tubules are swollen and vacuolated (black arrow), large renal corpuscles, presence of bacteria in renal interstitial tissue (yellow arrow) of fish infected with *V. anguillarum* and fed PHB-based diet 1.0 gm (D2; H & E, X400), lining epithelium of proximal and distal renal tubules are swollen and vacuolated, large renal corpuscles, presence of bacteria in renal interstitial tissue (black arrows) of fish infected with *V. anguillarum* and fed PHB-based diet 1.5 gm (D3; H & E, X400).

3.3.4. Histomorphology of gills

Gills of seabass showing normal primary gill and secondary gill lamellae of the control fish group. Normal primary gill lamellae, increased number of secondary gill lamellae of fish fed PHB –based diet. Primary gill lamellae were showing ballooning dilatation and curling of secondary lamellae of fish infected with *V. anguillarum*. Primary gill lamellae showing ballooning dilatation and curling of fish infected with *V. anguillarum* and fed PHB-based diet 0.5 g and 1.0 g. Congestion of Primary gill lamellae, ballooning of secondary lamellae, and hyperplasia of gill epithelial cells of fish infected with *V. anguillarum* and fed PHB-based diet 1.5 g (Fig. 5).

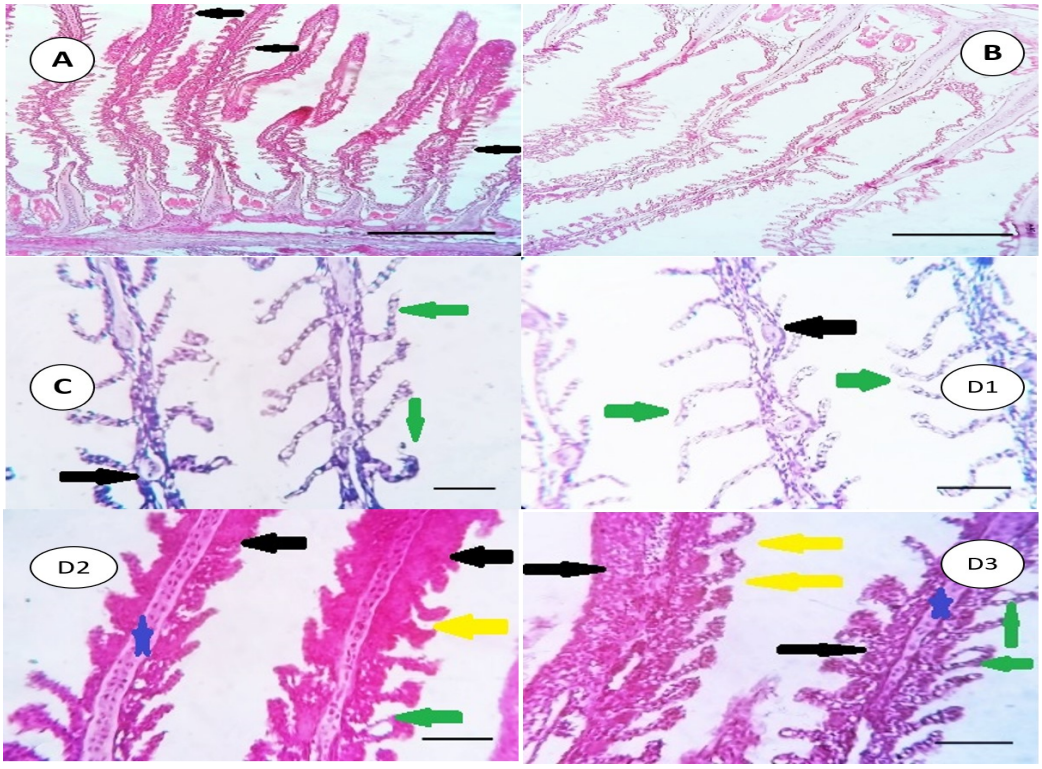

Fig. (5)
Gills of European seabass showing normal primary gill and secondary gill lamellae of control fish (A; H & E, X100), normal primary gill lamellae and increased number of secondary gill lamellae (black arrows) of fish fed PHB –based diet (B; H & E, X100), primary gill lamellae showing ballooning dilatation (black arrow) and curling of secondary lamellae (green arrows) of fish infected with *V. anguillarum* (C; H & E, X250), primary gill lamellae showing ballooning dilatation (black arrow) and curling (green arrows) of fish infected with *V. anguillarum* and fed PHB-based diet 0.5 gm (D1; H & E, X400), ballooning dilatation (green arrows) and curling of secondary lamellae (yellow arrows) and hyperplasia of gill epithelial cells (black arrows), congestion of gill lamellae (blue star) of fish infected with *V. anguillarum* and fed PHB-based diet 1.0 gm (D2; H & E, X250), congestion of primary gill lamellae (blue star), ballooning of secondary lamellae (green arrow), curling of secondary lamellae (yellow arrow) and hyperplasia of gill epithelial cells (black arrows) of fish infected with *V. anguillarum* and fed PHB-based diet 1.5 gm (D3; H & E, X250).

3.4. Relative percent of survival

Relative percent of survival (%) of cultured sea bass, *D. labrax*, fed diets containing different poly-β-hydroxybutyrate (PBH) levels for 8 weeks and post-challenged by *V. anguillarum* infection for 10 days. There was significant increase in RPS% in a dose-dependent manner compared with the controls ($P < 0.05$) whereas the lowest mortalities were found in PHB15% group and highest mortalities were found in the control group Figure(6).

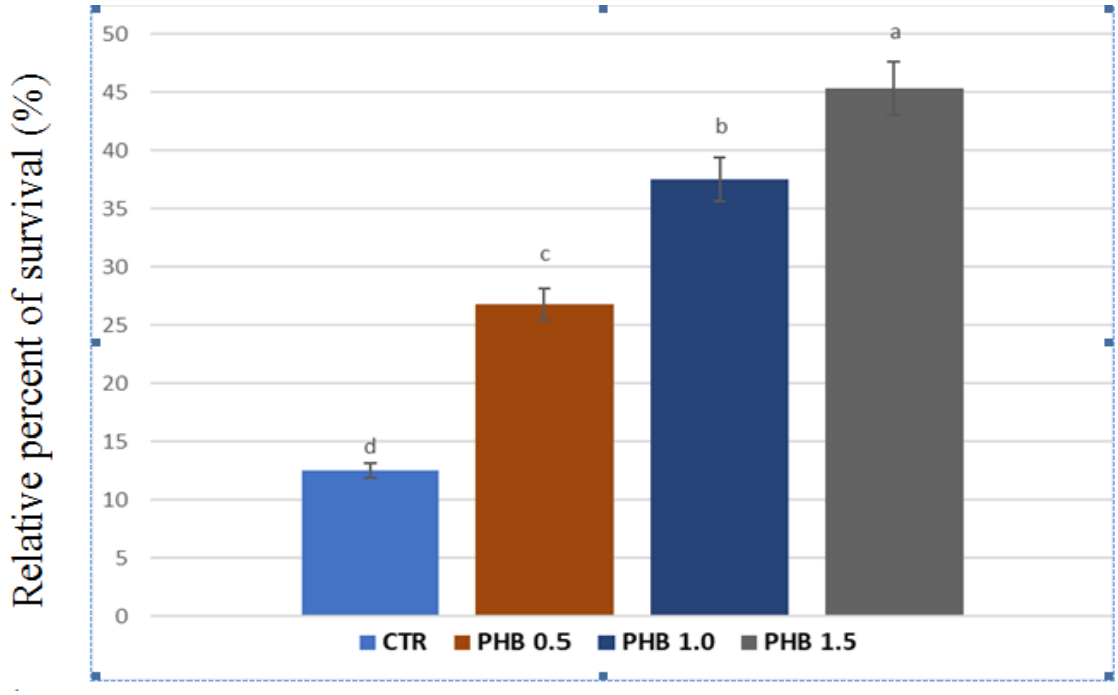

Fig. (6)
Relative percent of survival (%) of European sea bass, *D. labrax*, fed diets containing different poly-β-hydroxybutyrate (PBH) levels for 8 weeks and post-challenged by *V. anguillarum* infection for 10 days. Bars assigned by different letters have significantly differed at $P < 0.05$.

DISCUSSION

Antibiotics have been widely used to prevent diseases for a long time as therapeutic agents and also growth promoters in aquaculture and animal production , European Union and other countries have banned the use of antibiotics routinely to promote growth and prevent disease in animals, which poses a challenge to farmers **(Acar et al. 2000; Hong et al. 2015)**, the possible application of PHB as a biocontrol agent for farmed aquatic animal facilitates its use in aquaculture **(De Schryver et al. 2010 and Acar et al. 2000)** . this biopolymer can be degraded into short-chain fatty acids (SCFAs) which have highly antimicrobial properties **.**They are capable of acting as bacteriostatic and bactericidal effects towards pathogenic bacteria **(Ricke 2003).**

In aquaculture, SCFAs have high ability to inhibit the growth of fish and shrimp pathogens such as *Vibrio anguillarum, Vibrio alginolyticus* **(Vázquez et al. 2005 Defoirdt et al. 2006).** Anti-pathogenic effects of PHB can be either by degrading the gut bacteria of cultured aquatic animals **(Liu et al. 2010)** or could be also (partially) degraded by the animal digestive enzymes **(Defoirdt et al. 2007).** SCFAs are capable of acting as antibacterial properties depending on the physicochemical characteristics of the external environment and on the physiological status of the organisms **(Ricke 2003)**. Also, PHB enhancing the immune response of the immune system either by affecting both specific and non specific immune system the obtained results here in this study indicate that PHB can boost the immune status of fish via rising the expression of C5 and IL10 genes. In tilapia *Oreochromis mossambicus* where in the fish showed a significant immunostimulatory effect on both nonspecific (i.e., increased lysozyme, total peroxidases and antiprotease activity) and the specific (i.e., increased antibody response) immunity of the fish after feeding with diet containing PHB-hydroxyvalerate (PHB-HV) extracted from *Bacillus thuringiensi* **(Suguna et al. 2014)**. The PHB is considered a source of energy, as the PHB is degraded and absorbed in the intestines resulting in the transport of free fatty acids

(FAs), consisting of long and short-chain fatty acids (LCFAs and SCFAs) in the blood of Nile tilapia **(Situmorang 2015).** Their results showed faster and higher presence of PHB in the intestine, liver, spleen, and kidney compared to the heart, blood, brain, and muscle of the fish. Necrosis and desquamation of intestinal villus mucosa and presence of bacteria were absent in group fed 15 g/ kg diet PHB. In which PHB has growth-promoting effect this related to its ability to improve the intestinal health status of the animal **(Silva et al. 2016)**. The present research on PHB was mainly focused on Weight gain and special growth rate, immune-related gene expression and histopathological changes of cultured sea bass. Weight gain and special growth rate were significantly higher in the group that fed 15 gm / kg basal feed conc of PHB than group fed on 10 and 5 conc of PHB than that in the control group after 60 days, according to a previous report **(Najdegerami et al. 2012).** However, the lowest values of WG and SG were obtained in 0.5 g group after 60-day feeding of PHB. It suggested that long-term feeding of a low concentration of PHB would have a negative effect on the growth of cultured seabass . PHB can enhance the immunity of cultured seabass Expression of immune-related genes, IL10 and C5 in sea bass was significantly upregulated in groups D/ C, and expression was downregulated in A and B groups at 60 days of feeding. The stimulatory effects of IL-10 include the increase in the cytotoxic activity of NK cells and the induction of proliferation of certain subsets of CD8+ T cells. Altogether, IL-10 is a cytokine with a key role in the termination of inflammatory responses , the stimulatory roles for IL-10 were described as IL-10 prevents apoptosis and successful restoration of homeostasis characterized by the development of long -lived memory cells to face future threats. Also IL-10 appeared to be fundamental in the maintenance of gill homeostasis. Its loss enhanced the inflammatory response to Resiquimod in the gills, supporting a potent anti-inflammatory function for this cytokine Also increases proliferation and MHC class II expression in B cells, and plays a stimulatory role in Ig class switching.. An anti-inflammatory role for IL-10 has been previously shown in carp **(Piazzon 2015)** and zebrafish gut **(Coronado 2019).** Moreover, suppression of T1 cell response and cytokine production was reported in a zebrafish *M. marinum* model.

For the first time, we knew the function of zebrafish IL-10 in the gills and its clear importance in preventing inflammation in this mucosal tissue. Expression analysis by RT-PCR in control fish showed a high basal expression in the head kidney, followed by gut and brain **(Bounocore et al. 2007).** The role of IL-10 in sea bass immune responses was illustrated by the expression of IL-1β and IL-10 in the head-kidney and spleen following intraperitoneal injection of UV-killed *Photobacterium damselae* sp. *piscicida*. So , the role of IL-10 in the resolution of inflammation is for the first time suggested in fish due to the delayed maximal mRNA levels of sbIL-10 compared to those of the pro-inflammatory IL-1β **.** In concerning to The complement system it is composed of more than 35 soluble plasma proteins that play very important role in innate and adaptive immunity **(Sunyer et al. 1999) (Gasque 2004).** It plays the role in defense against bacteria, viruses, fungi, and parasites. The role of fish complements in phagocytosis, respiratory burst, chemotaxis, and cell lysis **(Boshra et al. 2006).** The complement is one of the most important killing factors for clearing bacteria in teleosts because of its bactericidal activity which has been well recognized **(Ellis et al. 2001).** Histomorphology can be considered a potential indicator for the general health status of fish **(Rašković et al. 2013).** The histomorphology of the gills and intestine in this study showed that PHB keeps gills and intestine status healthy in which necrosis and desquamation of intestinal villus mucosa and presence of bacteria were absent in group fed 15 g/ kg diet PHB, curling and hyperplasia of gill lamellae were absent in group fed 10&15 g/ kg diet PHB **(Silva et al. 2016)**. PHB has growth-promoting effect this related to its ability to improve the intestinal health status, keep the length of both gill lamellae and intestinal villi. The adapting bacterial community would have caused the PHB degradation. Of course, the small intestine is the primary site of nutrient absorption so as a combination of both bacterial and fish enzymatic degradation of the PHB is also possible **(Cheng et al. 2012;**

Dawood et al. 2019; Dawood et al. 2020a).

Healthy intestinal tract and Long intestinal villi indicate efficient nutrient absorption leading to good performance **(Sklan et al. 2004 Huerta-Aguirre et al. 2019).** If PHB is not contained within abacterial cell it can be degraded by microbial extracellular hydrolytic enzymes, to obtain carbon and energy **(Gebauer and Jendrossek 2006).** The survivability of the sea bass affected by the partial substitution of the feed with PHB. Even in the control treatment, the overall mortality was not rather high . In other experiments using similar rearing tank set-ups, such mortalities did not occur **(De Schryver et al. 2010).**

5. CONCLUSIONS

The findings of this study showed that dietary supplementation with PHB for eight weeks significantly enhanced the growth rates, increased the expression of immune-related genes, improved the tissue histomorphology, and increased the protection of the treated fish against challenge with *V. anguillarum*. Further studies should be done to evaluate better the innate and non-specific immune responses of cultured seabass to understand better the mechanisms of action of dietary PHB on the treated fish. This study directs us to include PHB regularly in the diets of cultured seabass to improve the aquaculture industry.

Conflict of interests: None

REFERENCES

Acar J, Casewell M, Freeman J, Friis C, Goossens H. 2000. Avoparcin and virginiamycin as animal growth promoters: a plea for science in decision-making. Clinical Microbiology and Infection, 6(9): 477-482.

Azad IS, Thirunavukkarasu AR, Kailasam M, Rajan JJS. 2004. Virulence and histopathology of *Vibrio anguillarum* like (VAL) bacterium isolated from hatchery produced juveniles of *Lates calcarifer* (Bloch). Asian Fisheries Science, 17(1/2): 101-110.

Boshra H, Li J, Sunyer JO. 2006. Recent advances on the complement system of teleost fish. Fish & Shellfish Immunology, 20(2): 239-262.

Boyd CE, Tucker CS. 2012. Pond Aquaculture Water Quality Management. Kluwer Academic Publishers, Boston, MA, USA.

Buonocore F, Randelli E, Bird S, Secombes C J, Facchiano A, Costantini S Scapigliati G. 2007. Interleukin-10 expression by real-time PCR and homology modelling analysis in the European seabass (*Dicentrarchus labrax* L.). Aquaculture, 270(1-4): 512-522.

Carbone D Faggio C. 2016. Importance of prebiotics in aquaculture as immunostimulants. Effects on immune system of *Sparus aurata* and *Dicentrarchus labrax*. Fish & Shellfish Immunology, (54): 172-178.

Cheng Z, Gatlin DM, Buentello A. 2012. Dietary supplementation of arginine and/or glutamine influences growth performance, immune responses and intestinal morphology of hybrid striped bass (*Morone chrysops*× *Morone saxatilis*). Aquaculture, (362): 39-43.

Coronado M, Solis CJ, Hernandez PP, Feijóo CG. 2019. Soybean meal-induced intestinal inflammation in zebrafish is T cell-dependent and has a Th17 cytokine profile. Frontiers in Immunology, (10): 610.

Dawood MA, Magouz FI, Salem MF, Elbialy ZI,Abdel-Daim HA. 2019. Synergetic effects of *Lactobacillus plantarum* and β-glucan on digestive enzyme activity, intestinal morphology, growth, fatty acid, and glucose-related gene expression of genetically improved farmed tilapia. Probiotics and Antimicrobial proteins, 1-11.

Dawood MA, Metwally AES, El-Sharawy ME, Atta AM, Elbialy ZI, Abdel-Latif HM Paray BA. 2020. The role of β-glucan in the growth, intestinal morphometry, and immune -related gene and heat shock protein expressions of Nile tilapia (*Oreochromis niloticus*) under different stocking densities. Aquaculture, 523, 735205.

De Schryver P, Sinha AK, Kunwar PS, Baruah K, Verstraete W, Boon N, Bossier P. 2010. Poly-β-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European seabass, Dicentrarchus labrax. Applied Microbiology and Biotechnology, 86(5): 1535-1541.

Defoirdt T, Halet D, Sorgeloos P, Bossier P, Verstraete W. 2006. Short-chain fatty acids protect gnotobiotic *Artemia franciscana* from

pathogenic *Vibrio campbellii*. Aquaculture, 261(2): 804-808.

Defoirdt T, Halet D, Vervaeren H, Boon N, Van de Wiele T, Sorgeloos P, Verstraete W. 2007. The bacterial storage compound poly-β -hydroxybutyrate protects *Artemia franciscana* from pathogenic *Vibrio campbellii*. Environmental Microbiology, 9(2): 445-452.

Ellis AE. 2001. Innate host defense mechanisms of fish against viruses and bacteria. Developmental & Comparative Immunology, 25(8-9): 827-839.

GAFRD. 2012. General Authority for Fish Resources Development. Statistics book.

Gasque P. 2004. Complement: a unique innate immune sensor for danger signals. Molecular immunology, 41(11): 1089-1098.

Gebauer B, Jendrossek D. 2006. Assay of poly (3-hydroxybutyrate) depolymerase activity and product determination. Applied and Environmental Microbiology, 72(9):6094-6100.

Gröner F, Ziková A, Kloas W. 2015. Effects of the pharmaceuticals diclofenac and metoprolol on gene expression levels of enzymes of biotransformation, excretion pathways and estrogenicity in primary hepatocytes of Nile tilapia (*Oreochromis niloticus*). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, (167): 51-57.

Halet D, Defoirdt T, Van Damme P, Vervaeren H, Forrez I, Van de Wiele T, Verstraete W. 2007. Poly-β-hydroxybutyrate-accumulating bacteria protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*. FEMS Microbiology Ecology, 60(3): 363-369.

Hong C, Si Y, Xing Y, Li, Y. 2015. Illumina MiSeq sequencing investigation on the contrasting soil bacterial community structures in different iron mining areas. Environmental Science and Pollution Research, 22(14): 10788-10799.

Hoseinifar SH, Sun YZ, Caipang CM. 2017. Shortchain fatty acids as feed supplements for sustainable aquaculture: An updated view. Aquaculture Research, 48(4): 1380-1391.

Huerta-Aguirre G, Paredes-Ramo KM, Becerra -Amezcua MP, Hernández-Calderas I, Matadamas-Guzman M, Guzmán-García X. 2019. Histopathological analysis of the intestine from *Mugil cephalus* on environment reference sites. In: Pollution of Water Bodies in Latin America. Springer, pp. 319–328.

Laranja JLQ , Ludevese-Pascual GL, Amar E C, Sorgeloos P, Bossier P, De Schryver P. 2014. Poly-β-hydroxybutyrate (PHB) accumulating *Bacillus* spp. improve the survival, growth and robustness of Penaeus monodon postlarvae. Veterinary Microbiology, 173(3-4): 310-317.

Liu Y, De Schryver P, Van Delsen B, Maignien L, Boon N, Sorgeloos P, Defoirdt T. 2010 . PHB-degrading bacteria isolated from the gastrointestinal tract of aquatic animals as protective actors against luminescent vibriosis. FEMS Microbiology Ecology, 74 (1): 196-204.

Madison LL, Huisman GW. 1999. Metabolic engineering of poly (3-hydroxyalkanoates): from DNA to plastic. Microbiology and Molecular Biology Reviews, 63(1): 21-53.

Matsche MA. 2011. Evaluation of tricaine methanesulfonate (MS-222) as a surgical anesthetic for Atlantic Sturgeon Acipenser *oxyrinchus oxyrinchus*. J. Appl. Ichthyol. 27(2): 600–610.

Najdegerami E H, Tran T N, Defoirdt T, Marzorati M, Sorgeloos P, Boon N and Bossier P. 2012. Effects of poly-β-hydroxybutyrate (PHB) on Siberian sturgeon (*Acipenser baerii*) fingerlings performance and its gastrointestinal tract microbial community. FEMS Microbiology Ecology, 79(1): 25-33.

Nhan D T, Wille M, De Schryver P, Defoirdt T, Bossier P and Sorgeloos P. 2010 . The effect of poly β-hydroxybutyrate on larviculture of the giant freshwater prawn *Macrobrachium rosenbergii*. Aquaculture, 302(1-2): 76 -81.

Piazzon M C, Savelkoul H F, Pietretti D, Wiegertjes G F and Forlenza M. 2015 . Carp Il10 has anti-inflammatory activities on phagocytes, promotes proliferation of memory T cells, and regulates B cell differentiation and antibody secretion. The Journal of Immunology, 194(1): 187-199.

Rašković B, Jarić I , Koko V, Spasić M , Dulić Z , Marković Z and Poleksić V. 2013. Histopathological indicators: a useful fish health monitoring tool in common carp (*Cyprinus carpio* Linnaeus, 1758) culture. Open Life Sciences, 8(10): 975-985.

Ricke S C. 2003. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poultry Science, 82(4): 632-639.

Serrano P H. 2005 . Responsible use of antibiotics in aquaculture (Vol. 469). Food and Agriculture Organization.

Silva B C, Jesus G F A, Seiffert W Q , Vieira F N, Mouriño J L P, Jatobá A and Nolasco-Soria H. 2016. The effects of dietary supplementation with butyrate and polyhydroxybutyrate on the digestive capacity and intestinal morphology of Pacific White Shrimp (*Litopenaeus vannamei*). Marine and Freshwater Behaviour and Physiology, 49(6): 447-458.

Situmorang M L .2015 . Application of poly-β-hydroxybutyrate in growth and health promotion of Nile tilapia *Oreochromis niloticus* culture. PhD thesis, Ghent University, Belgium.

Sklan, D., Prag, T., and Lupatsch, I. (2004) Structure and function of the small intestine of the tilapia *Oreochromis niloticus* × *Oreochromis aureus* (Teleostei, Cichlidae). Aquac. Res. 35: 350–357.

Standen B T, Peggs D L, Rawling M D, Foey A , Davies S J, Santos G A and Merrifield D L. 2016. Dietary administration of a commercial mixed-species probiotic improves growth performance and modulates the intestinal immunity of tilapia, *Oreochromis niloticus*. Fish & Shellfish Immunology, 49, 427-435.

Suguna P, Binuramesh C, Abirami P, Saranya V, Poornima K, Rajeswari V and Shenbagarathai R . 2014. Immunostimulation by poly-β hydroxybutyrate–hydroxyvalerate (PHB–HV) from *Bacillus thuringiensis* in *Oreochromis mossambicus*. Fish & Shellfish Immunology, 36(1): 90-97.

Sunyer J O and Lambris J D. 1999 . Complement. Encyclopedia of life sciences.

Suvarna S, Layton C and Bancroft D. 2013. Bancroft's Theory and Practice of Histological Techniques. 7th Ed. Churchill Livingstone: 173-185.

Syahputra K, Kania P W, Al-Jubury A, Marnis H , Setyawan A C and Buchmann K. 2019. Differential immune gene response in gills, skin, and spleen of rainbow trout *Oncorhynchus mykiss* infected by *Ichthyophthirius multifiliis*. PloS one, 14(6): e0218630.

Thai TQ , Wille M, Garcia-Gonzalez L, Sorgeloos P, Bossier P and De Schryver P. 2014 . Poly-ß-hydroxybutyrate content and dose of the bacterial carrier for Artemia enrichment determine the performance of giant freshwater prawn larvae. Applied Microbiology and Biotechnology, 98(11): 5205-5215.

Thompson JL, Hinton M. 1996. Effect of short - chain fatty acids on the size of enteric bacteria. Letters in Applied Microbiology, 22(6): 408-412.

Tokiwa Y, Calabia BP. 2004. Review degradation of microbial polyesters. Biotechnology letters, 26(15): 1181-1189.

Vázquez JA, González M, Murado MA. 2005. Effects of lactic acid bacteria cultures on pathogenic microbiota from fish. Aquaculture, 245(1-4): 149-161.

Yuan JS, Reed A, Chen F, Stewart CN. 2006 . Statistical analysis of real-time PCR data. BMC Bioinformatics, 7(1): 1-12.