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Effect of poly-β-hydroxybutyrate on growth and disease resistance of Nile tilapia *Oreochromis niloticus* juveniles

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ABSTRACT

The growth promoting effect of the bacterial storage compound poly-β-hydroxybutyrate (PHB) has been studied for young fish of high trophic level (European sea bass) and intermediate trophic level (Siberian sturgeon). Here, the effect of PHB on growth, digestive enzyme activities, body composition and diseases resistance of juvenile Nile tilapia (*Oreochromis niloticus*) of low trophic level was investigated. Although dietary PHB supplementation (5, 25 and 50 g PHB kg^{-1} formulated semi-purified diet) during 28 days resulted in a trend of increased weight gain, there was no significant difference in the mean final body weight (258–284 mg) when compared to the fish from the control group (on average 218 mg). Lipase activity increased significantly with about 20–40% by the supplementation of PHB in the diet, which may have led to the significant increase in total lipid content with about 10% in the PHB treatment groups. However, the profile of total (n-6) fatty acids (FAs), total monounsaturated FAs and total saturated FAs relative to the total lipid was similar among various PHB treatments. An additional challenge test on gnotobiotic Nile tilapia larvae using the pathogen *Edwardsiella ictaluri* gly09R showed that feeding challenged larvae with PHB-enriched *Artemia* nauplii resulted in a 20% higher survival as compared to the challenged control larvae. Overall, it is suggested that the trend of increased body weight gain resulted from intestinal lipid digestion, absorption and deposition and that PHB is effective as an antimicrobial agent for application in Nile tilapia larviculture.

1. Introduction

Short-chain fatty acids (SCFAs) have been suggested as an alternative to antibiotics to control diseases in aquaculture (Defoirdt et al., 2009). Their main limitation, however, is their polar nature making them highly soluble and easy to diffuse in the culture water. This results in the risk for a low uptake efficiency of the SCFAs by the animals when formulated in a diet and thus demands for large doses in order to be effective at the animal level (Defoirdt et al., 2011). A solution has been provided in the form of the bacterial storage compound PHB, the polymer of the SCFA β-hydroxybutyrate (β-HB). This carbon reserve and intracellular energy source for different species of bacteria (De Schryver et al., 2010; Defoirdt et al., 2011; Tokiwa and Calabia, 2007) is water insoluble and biologically degrades into β-HB upon entrance in the gastrointestinal tract (Anderson and Dawes, 1990; Doyle et al., 1991).

Several studies have confirmed the protective effect of PHB against bacterial infections in crustaceans (Defoirdt et al., 2007; Sui et al., 2012; Thai et al., 2014), but interestingly enough an increased culture performance in crustaceans has also been observed. For example, Nhan et al. (2010) and Sui et al. (2012) found an increased growth performance and survival in larval giant freshwater prawn (*Macrobrachium rosenbergii*) and larval Chinese mitten crab (*Eriocheir sinensis*), respectively, upon the use of crystalline PHB (i.e. PHB extracted from the bacterial cell) in the diet. Most recently, Thai et al. (2014) described that the supplementation of PHB in amorphous form (i.e. PHB still contained within a bacterial cell) to the diet was effective to boost the growth and development of *M. rosenbergii* larvae. The beneficial effects of dietary PHB supplementation on the growth performance of fish has only been investigated in a limited number of cases. In carnivorous European sea bass (*Dicentrarchus labrax*) juveniles a growth promoting effect was observed at 20 and 50 g PHB kg^{-1} diet (De Schryver et al., 2010) while for omnivorous Siberian sturgeon (*Acipenser baerii*) fingerlings no significant increase in weight was observed resulting from dietary PHB supplementation (Najdegerami et al., 2012). The protective effect of PHB against pathogenic diseases has, as far as known, not yet been investigated for fish.

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In this study, the effect of crystalline PHB administered via a semi-purified diet to Nile tilapia (*Oreochromis niloticus*) at the early juvenile stage was investigated in terms of survival and growth performance, the digestive enzyme activities and body lipid composition. Additionally, the protective effect of amorphous PHB supplied through *Artemia* to larval Nile tilapia challenged with the bacterial pathogen *Edwardsiella ictaluri* gly09R was investigated.

2. Materials and methods

2.1. Effect of crystalline PHB on growth of Nile tilapia juveniles

2.1.1. Experimental setting

Nile tilapia juveniles were obtained from the Aquaculture and Fisheries Group (AFI) of Wageningen University and Research Center (The Netherlands). After transport, juveniles were collected and acclimated in a 50 L aquarium for 14 days prior to stocking in the experimental units. During this period, they were fed a formulated experimental basal diet (without PHB supplementation) three times daily to apparent satiation (Table 1). The juveniles were in healthy condition at the end of the acclimation period as indicated by the absence of deformities and abnormal movement, the absence of clinical signs of bacterial and/or fungal infection and a good appetite and feeding intake. At the end of the acclimation period, fish with an average weight of 26.4 ± 2.4 mg were randomly distributed in 12 rectangular aquaria of 38 L at a density of 50 fish per aquarium. During the feeding experiment, water temperature was maintained at 28 ± 1 °C with pH ranging from 8.2 to 8.8. NH_4^+, NO_2^-, and NO_3^- levels never exceeded 0.05 mg N L^{-1}, 0.5 mg N L^{-1} and 2.5 mg N L^{-1}, respectively. The experiment was carried out under a 16:8 h light: dark light regime. Each tank was supplied in flow-through mode of dechlorinated, heated tap water at a water renewal rate of 5 L h^{-1} (hydraulic residence time of 7.6 h). In order to prevent feed loss during the feeding period by water exchange, the flow through system was only activated during the dark period. Water flow rate was checked and adjusted daily to ensure proper water exchange.

2.1.2. Experimental diets and feeding regime

A basal semi-purified diet (control diet) was formulated to contain approximately 450 g crude protein kg^{-1} (Balarin and Halfer, 1982) and 150 g lipid kg^{-1} (Ng and Chong, 2004) based on the feedstuff values reported in NRC (1993) (Table 1). The PHB experimental diets were the basal diet supplemented with three levels of crystalline PHB (5, 25, and 50 g kg^{-1} diet; Goodfellow Cambridge Limited, Huntingdon, England), at the expense of α-cellulose. The moist mixture was extruded through a 3-mm diameter meat grinder (Hobart Corp., Troy, Idaho, USA). The resulting moist pellets were air-dried at room temperature to a moisture content of about 100 g kg^{-1}. Pellets were ground into small pieces, sieved to obtain appropriate sizes (300–500 μm) and stored at −20 °C until used. The fish were fed daily during 28 days at a fixed feeding level of 20% on fish wet body weight (BW) (Ng and Romano, 2013) in three equal meals given every 4 h between 09:00 and 17:00. The feed amount was adjusted daily based on mortality and weekly based on the average weight of the fish in the tank. Each experimental diet was tested in triplicate.

Table 1
Formulation of the basal semi-purified diet (control diet) for Nile tilapia juveniles used in this study.

Ingredients	g kg^{-1}
Carboxymethyl cellulose (Sigma)	20
Vitamin C (Stay-C 35% VDS)	0.6
L-methionine (Sigma)	5
Vitamins + minerals mix (VDS)	12.5
L-lysine (Sigma)	15
Choline chloride	1.8
α-cellulose	50
Corn meal (bio planet)	100
Fish herring meal (VDS)	200
Corn gluten meal (Sigma)	500
Fish oil (VDS)	20
Soybean oil	75
Vitamin E (95%)	0.1
Analyzed nutrient content	g kg^{-1} DM
DM (g kg^{-1})	922
Crude protein	524
Crude fat	146
Ash	54
Total carbohydrates[a]	276

[a] Calculated as follows: total carbohydrates = 1000 − (crude protein + crude fat + ash).

2.1.3. Fish survival and growth parameters

Six juveniles from each replicate tank were randomly collected at the end of the trial minimally 14 h after the last feeding to measure the final wet BW and length. The average wet BW gain of the fish in a tank over the 28 days was calculated by subtracting the weight of the fish sampled on the final day of the experiment with the average weight of the fish as measured at the beginning of the experiment, and then taking the average. The specific growth rate (SGR) was calculated as follows:

$$\text{SGR}(\%\ \text{weight increase per day}) = \frac{(\ln W - \ln W_0)}{t}$$

where W is the average BW after 28 days, W_0 is the average initial BW, and t is experimental period (28 days). The same approach was used to calculate the feed conversion ratio (FCR), expressed as the feed consumption over the weight increase of the fish per treatment. Fish survival for individual treatments was determined as the number of surviving fish at the end of experimental period relative to the number of fish at the beginning of the experimental period.

2.1.4. Fish sampling and analyses

2.1.4.1. Assessment of digestive enzyme activities. Whole-body homogenates were used instead of intestine homogenates to provide enough wet sample for enzymatic assays due to the small size of the fish. Fish samples were taken minimally 14 h after the last feeding. Three fish from each replicate tank were randomly sampled, euthanized with an overdose of tricaine mesylate (0.1% tricaine methanesulfonate, MS-222; Sigma Chemical Co., St Louis, USA) and stored at −80 °C until homogenization. Samples were homogenized on ice in 50 mM Tris–HCl buffer (pH 7.5) at a 5:1 ratio (w/w) in an electric homogenizer (Heidolph, Instruments Switzerland). The homogenate was centrifuged at 10,000 g for 20 min at 4 °C and the supernatant collected and stored in small aliquots (100–200 μL) at −80 °C until the spectrophotometric assays of digestive enzyme activities. Lipase activity, trypsin activity, pepsin activity, and amylase activity were quantified according to the procedures of Iijima et al. (1998),Holm et al. (1988), Zambonino-Infante and Cahu (1994) and Métais and Bieth (1968), respectively. All assays of digestive enzyme activities were carried out in triplicate using the Bio-Rad Benchmark Plus microplate spectrophotometer and Falcon flat-bottom 96-well microplates (Fisher Scientific). All pH values for buffers were measured at room temperature, and all reagents were purchased from Sigma–Aldrich Chemical. Each enzyme activity was measured in each individual fish. The total protein content was measured according to the method of Bradford (1976) using bovine serum albumin as standard. The specific activity of measured enzymes is expressed as unit enzyme activity per mg protein (U mg^{-1} protein).

2.1.4.2. Total protein and lipid content analyses. At the end of the experiment, remaining fish from each replicate tank were pooled (total wet weight of 2–3 g) and the total protein and lipid content in the whole body of fish from each treatment was analysed following standard methods. Nitrogen content was analyzed by the Kjeldahl method (AOAC, 2000) and crude protein content was estimated by multiplying the nitrogen percentage by 6.25. Lipid content in the whole body was analyzed by extraction following the modified Folch method (Folch et al., 1957) illustrated in Ways and Hanahan (1964). Fatty acid (FA) composition was determined by gas chromatography of the fatty acid methyl esters (FAME) following the modified procedure of Lepage and Roy (1984).

2.2. Effect of amorphous PHB on disease resistance of Nile tilapia larvae

2.2.1. Disinfection protocol to obtain axenic Nile tilapia larvae

Nile tilapia eggs of 3 days post fertilization (3 dpf) were collected from female breeders, pooled, disinfected and axenically hatched as described by Situmorang et al. (2014).

2.2.2. Bacterial strain and culture conditions

E. ictaluri gly09R with multiple antibiotics resistance as described in Situmorang et al. (2014) was used in gnotobiotic challenge tests. The strain was stored in Brain Heart Infusion (BHI) broth (FLUKA, Sigma–Aldrich, USA) supplemented with 20% (v/v) glycerol at −80 °C. It was then grown in BHI broth containing 10 mg L^{-1} ampicillin, rifampicin, kanamycin, trimethoprim and gentamycin, and incubated overnight on a horizontal shaker at 160 rpm at 27 °C. The bacterial suspensions were harvested by centrifuging at 1000 × *g* for 10 min and washed twice using 0.2 μm filtered autoclaved synthetic freshwater (Situmorang et al., 2014). The optical density was measured using a spectrophotometer (Genesys 20, Thermospectronic) at a wavelength of 550 nm and the bacterial density was estimated based on the McFarland standard (BioMérieux, Marcy L'Etoile, France).

2.2.3. Bacterial challenge test for gnotobiotic Nile tilapia larvae supplied with poly-β-hydroxybutyrate (PHB)

At 5 days after hatching (DAH 5), 160 axenic larvae were distributed over 500 mL sterile glass bottles containing 200 mL 0.2 μm filtered autoclaved synthetic freshwater at a density of 10 fish per bottle. The fish were subjected to 4 treatments with four replicates during 12 days:

(1) No pathogenic challenge; no PHB supplementation (control).
(2) No pathogenic challenge; PHB supplementation.
(3) Pathogenic challenge; no PHB supplementation.
(4) Pathogenic challenge; PHB supplementation.

The pathogenic challenge was done daily during the 11 days experimental period via the culture water and via the live food (*Artemia* nauplii). For the challenge via the culture water bacteria were added in the bottles at a density of 10^6 CFU mL^{-1}. For the challenge via the live food, axenic *Artemia* nauplii were obtained following the procedure of Marques et al. (2004). The axenic *Artemia* nauplii were harvested, washed using 0.2 μm filtered autoclaved synthetic freshwater, counted and diluted to a density of 100 nauplii mL^{-1}. The pathogen was added to the axenic *Artemia* nauplii at a density of 10^8 CFU mL^{-1}. Bacteria loaded *Artemia* nauplii were harvested after 1 h and washed twice using 0.2 μm filtered autoclaved synthetic freshwater, counted, and added into the bottles containing the fish.

For the fish supplied of PHB, axenic *Artemia* nauplii at a density of 100 *Artemia* mL^{-1} were enriched in a 100 mg L^{-1} amorphous PHB suspension for 1 h. The amorphous PHB consisted of the bacterium *Alcaligenes eutrophus* containing 70% PHB on dry weight and was produced according to Thai et al. (2014). The enriched *Artemia* were subsequently washed with 0.2 μm-filtered autoclaved synthetic freshwater and added into the bottles.

The total number of *Artemia* nauplii added in each bottle (independently of the treatment) was 20 per larva. As such, the control group fish were fed 20 axenic non-enriched *Artemia* nauplii per larva. PHB-supplemented fish were fed 10 PHB-enriched *Artemia* nauplii per larva with an extra addition of 10 axenic *Artemia* nauplii per larva (for unchallenged fish) or 10 pathogen-enriched *Artemia* nauplii per larva (for challenged fish). The challenged fish without PHB supplementation were fed 10 axenic *Artemia* nauplii and 10 pathogen-enriched *Artemia* nauplii per larva. Mortality was determined daily and dead fish were removed daily during the experiment. The experimental design for the bacterial challenge test was approved by the ethical committee of Ghent University under the file number EC2014/041.

2.3. Statistical analyses

For the growth test, normalization of the distribution of the survival and final BW data were done using arcsine and log transformation, respectively. Comparison of the fish survival, final BW and length, SGR, FCR, and digestive enzyme activities were done using one-way analysis of variance (ANOVA). Grouping of treatments based on significant differences in mean values was done using a Tukey post-hoc test (0.05 level of confidence). For the comparison of the cumulative mortality of fish larvae in the challenge test, data were arcsine transformed before a one-way analysis of variance (one-way ANOVA) was performed. A Duncan test was performed on the transformed data for multiple comparisons among means (0.05 level of confidence). STATISTICA statistical software (version 7.0) was used for all statistical analyses.

3. Results

3.1. Effect of crystalline PHB on growth of Nile tilapia juveniles

3.1.1. Fish survival and growth parameters

Following the 14 days acclimatization period during which the fish were fed the experimental basal diet without PHB supplementation, the fish readily started consuming the experimental

Table 2
Survival and growth parameters (mean ± standard deviation) of Nile tilapia juveniles fed four experimental diets. No significant differences were found in the fish survival, final body weight and length, and FCR (number of replicates per treatment = 3; number of fish sampled per replicate to determine growth parameters = 6).

Treatment	Survival (%)	Final body weight (mg)	Final body length (cm)	SGR (% BW day^{-1})	FCR
Control	87 ± 2^a	218 ± 101^a	2.3 ± 0.4^a	5.27 ± 0.05^a	1.0 ± 0.3^a
5 g kg^{-1} PHB	74 ± 8^a	284 ± 61^a	2.6 ± 0.3^a	5.39 ± 0.07^b	0.9 ± 0.0^a
25 g kg^{-1} PHB	86 ± 10^a	269 ± 68^a	2.5 ± 0.4^a	5.24 ± 0.05^a	0.9 ± 0.1^a
50 g kg^{-1} PHB	88 ± 3^a	258 ± 48^a	2.5 ± 0.4^a	5.21 ± 0.03^a	0.8 ± 0.1^a

Different superscript letters within a column denote significant differences ($P \leq 0.05$).

Table 3
Digestive enzyme activities (mean ± standard deviation) of Nile tilapia juveniles following 4 weeks of feeding with different experimental PHB-supplemented diets (number of replicates per treatment = 3; number of fish sampled per replicate = 3).

Treatment	Digestive enzyme activities (U mg protein^{-1})			
	Lipase	Trypsin	Amylase	Pepsin
Control	0.021 ± 0.003^{a}	0.035 ± 0.017^{a}	0.457 ± 0.039^{a}	0.253 ± 0.010^{a}
5 g kg^{-1} PHB	0.025 ± 0.004^{ab}	0.032 ± 0.017^{a}	0.533 ± 0.041^{a}	0.261 ± 0.021^{a}
25 g kg^{-1} PHB	0.029 ± 0.004^{b}	0.033 ± 0.014^{a}	0.489 ± 0.042^{a}	0.272 ± 0.014^{a}
50 g kg^{-1} PHB	0.029 ± 0.001^{b}	0.047 ± 0.016^{a}	0.547 ± 0.043^{a}	0.245 ± 0.022^{a}

Activities are expressed as follows: Lipase as mmole of substrate hydrolysed min^{-1} mg^{-1} protein; Trypsin activity as mmole of BAPNA hydrolysed min^{-1} mg^{-1} protein; Amylase mg starch hydrolysed min^{-1} mg^{-1} protein; Pepsin activity as mmole of tyrosine released min^{-1} mg^{-1} protein. Different superscript letters within a column denote significant differences ($P \leq 0.05$).

diets and no clear changes in feeding response were observed when the fish were provided with feed. At the end of the feeding trial, mortality had occurred in each treatment due to aggressive behavior of some fish in each tank. This mortality was slightly higher in the 5 g kg^{-1} treatment but there was no significant difference in survival between the different treatments (Table 2). After 28 days of feeding, a trend of increased final BW and length could be observed for the fish fed with PHB although there were no significant differences among all treatments. Only at a dose of 5 g PHB kg^{-1} diet the SGR was higher than for the control treatment (Table 2). The FCR- expressing the amount of feed dry matter needed per unit of fish weight gain - was not significantly different for the fish from the four experimental diet treatments.

3.1.2. Digestive enzyme activities

After 28 days of feeding with the experimental diets, no significant differences were observed for trypsin, amylase and pepsin activity among the various treatments (Table 3). Feeding the fish with the higher levels of PHB (25 g kg^{-1} and 50 g kg^{-1}) significantly increased the activity of lipase as compared to the control group which had the lowest lipase activity.

3.1.3. Fish body composition

A similar average total protein content was observed for the fish of all treatments with values of 58.2 ± 1.5%, 59.4 ± 1.0%, 58.6 ± 1.7% and 59.4 ± 2.1% on dry weight (DW) in the control, 5 g kg^{-1}, 25 g kg^{-1} and 50 g kg^{-1} PHB treatment groups, respectively ($P > 0.05$). Dietary PHB supplementation significantly increased the whole-body total lipid content with 2–3% on DW when compared to the control treatment (Table 4).

3.1.4. Fatty acid profile

The fish FA profile (content and composition) following 28 days of feeding on different diets is presented in Table 4. The contents of saturated and monounsaturated FAs were significantly higher in the 5 g kg^{-1} and 25 g kg^{-1} PHB treatment groups as compared to the control treatment, while a significantly higher content of total (*n*-6) FAs was observed in the 50 g kg^{-1} PHB treatment group. However, PHB did not significantly influence the lipid composition as similar values were observed among treatments when the FAs were expressed as percentage on the total lipid content. PHB had no significant influence on content and composition of C20:5*n*3 (eicosapentaenoic acid, EPA), C22:6*n*3 (docosahexaenoic acid, DHA), total (*n*-3) FAs, nor the (*n*-6)/(*n*-3) ratio.

3.2. Effect of PHB on disease resistance of Nile tilapia larvae

The protective effect of PHB for sterile Nile tilapia larvae challenged with pathogenic *E. ictaluri* gly09 was assessed. In all treatments, larval mortality was first observed on the 6th day after challenge and no significant differences were observed between the treatments until 9 days after challenge (Table 5). On this day, the mortality of the challenged larvae that were fed with PHB was significantly lower than the mortality of the challenged larvae not fed with PHB. At the same time, their mortality was not significantly different from the unchallenged larvae. At 10 and 11 days after challenge, the mortality of the challenged larvae fed with PHB was still significantly lower than the challenged larvae that were not fed with PHB. However, their mortality was significantly higher than that of the unchallenged larvae.

4. Discussion

Although positive effects of PHB on the growth performance of European sea bass juveniles have been reported by De Schryver et al. (2010) no significant differences in final body weight were found between the different treatment groups in the current study. Despite the trend of a higher mean final body weight observed in

Table 4
Whole-body total lipid content (% on DW; mean ± standard deviation), the content of the main groups of long chain fatty acids (C14–C24) as % on DW (mean ± standard deviation), and the composition of the main groups of long chain fatty acids (C14–C24) as % on total lipid content (in parenthesis; mean ± standard deviation) in Nile tilapia juveniles fed with different PHB experimental diets (number of replicates per treatment = 3; number of fish pooled per replicate for FAME analysis = 10).

Treatment	Total lipid	FA composition						(*n*-6)/(*n*-3) ratio
		Saturated	Mono-unsaturated	EPA	DHA	Total (*n*-6)	Total (*n*-3)	
Control	25.40 ± 0.33^{a}	7.22 ± 0.16^{a} (28.44 ± 0.62^{a})	5.99 ± 0.16^{a} (23.59 ± 0.61^{a})	0.06 ± 0.03^{a} (0.24 ± 0.01^{a})	1.45 ± 0.14^{a} (5.71 ± 0.54^{a})	7.18 ± 0.03^{a} (28.27 ± 0.12^{a})	1.91 ± 0.13^{a} (7.53 ± 0.51^{a})	3.76 ± 0.25^{a}
5 g kg^{-1} PHB	28.26 ± 0.13^{b}	8.23 ± 0.35^{bc} (29.13 ± 1.25^{a})	6.68 ± 0.23^{b} (23.64 ± 0.80^{a})	0.07 ± 0.06^{a} (0.26 ± 0.02^{a})	1.65 ± 0.02^{a} (5.83 ± 0.08^{a})	7.61 ± 0.47^{ab} (26.94 ± 1.67^{a})	2.13 ± 0.23^{a} (7.54 ± 0.80^{a})	3.58 ± 0.16^{a}
25 g kg^{-1} PHB	27.69 ± 1.06^{b}	8.47 ± 0.17^{c} (30.58 ± 0.60^{a})	6.79 ± 0.25^{b} (24.51 ± 0.89^{a})	0.07 ± 0.08^{a} (0.26 ± 0.03^{a})	1.51 ± 0.13^{a} (5.45 ± 0.48^{a})	7.32 ± 0.41^{ab} (26.44 ± 1.48^{a})	1.95 ± 0.16^{a} (7.03 ± 0.56^{a})	3.76 ± 0.10^{a}
50 g kg^{-1} PHB	27.92 ± 0.13^{b}	7.82 ± 0.24^{ab} (27.99 ± 0.86^{a})	6.36 ± 0.06^{ab} (22.78 ± 0.23^{a})	0.07 ± 0.02^{a} (0.27 ± 0.01^{a})	1.63 ± 0.02^{a} (5.82 ± 0.07^{a})	8.09 ± 0.23^{b} (28.95 ± 0.81^{a})	2.10 ± 0.03^{a} (7.52 ± 0.10^{a})	3.85 ± 0.14^{a}

Different superscript letters within a column denote significant differences ($P \leq 0.05$).

Table 5
Effect of PHB on the cumulative mortality (%, mean ± standard deviation) of gnotobiotic Nile tilapia larvae challenged with pathogenic *E. ictaluri*gly09R via culture water and *Artemia* nauplii (number of replicates per treatment = 4; initial number of fish larvae per replicate = 10). DAC = days after challenge. Different superscript letters within the same row denote significant differences ($P \leq 0.05$).

	Larvae not challenged, not fed with PHB	Larvae not challenged, fed with PHB	Larvae challenged, not fed with PHB	Larvae challenged, fed with PHB
6 DAC	3 ± 5^{a}	3 ± 5^{a}	3 ± 5^{a}	3 ± 5^{a}
7 DAC	3 ± 5^{a}	3 ± 5^{a}	10 ± 8^{a}	3 ± 5^{a}
8 DAC	8 ± 5^{a}	3 ± 5^{a}	13 ± 13^{a}	5 ± 6^{a}
9 DAC	10 ± 0^{a}	3 ± 5^{a}	30 ± 18^{b}	10 ± 8^{a}
10 DAC	20 ± 12^{a}	18 ± 10^{a}	60 ± 8^{c}	40 ± 12^{b}
11 DAC	28 ± 10^{a}	20 ± 8^{a}	70 ± 8^{c}	50 ± 8^{b}

the PHB treatment groups (258–284 mg) when compared to the control group (218 mg) it can thus only be concluded that PHB did not have negative effects on the growth of Nile tilapia juveniles. The significantly higher SGR in the 5 g kg^{-1} PHB treatment can likely be explained by the slightly higher mortality due to aggressive behavior resulting in a higher contribution of stronger/larger fish in the calculation of the final average body weight for this treatment. The observations on the effect of PHB on the growth of the fish correspond with the study by Najdegerami et al. (2012) where, despite the observation of higher final body weight in PHB treatments, no significant effect of PHB on the growth performance of Siberian sturgeon fingerlings could be concluded. The use of PHB to significantly promote fish growth thus seems to be species specific. Possibly, the fact that sea bass is carnivorous while sturgeon and tilapia are omnivorous plays a role. Fish of different trophic levels indeed differ in anatomy and functionality of the digestive tract which may influence the metabolization of feed components (Smith, 1980). In addition to species, the age or developmental stage of the fish will also be of importance in the PHB effect as it was most recently found that PHB did induce significant growth promoting effects in tilapia fry (unpublished data). For Siberian sturgeon larvae, then again, PHB seemed to negatively affect the growth performance (Najdegerami et al., 2015). Further research focussing specifically on the digestibility of PHB in fish of different trophic level and/or age could provide more information on this aspect.

Digestive enzyme activities can be used as indicators of digestive processes and fish nutritional conditions (Lazo et al., 2000; Ueberschar, 1988). It has been reported that manipulation of diets causes immediate changes in activities of digestive enzymes (Mohapatra et al., 2012). This specifically holds true for fishes with relatively broad diets (German et al., 2004). In this study, dietary PHB supplementation resulted in a significantly higher lipase activity in the 25 and 50 g kg^{-1} PHB-fed fish group, while a similar activity of trypsin, amylase, and pepsin was observed among all treatments. Lipases are the enzymes responsible for the breakdown of dietary lipid complexes within the intestinal lumen (Karasov and Hume, 1997). The 20–40% increase in lipase activity in the 25 and 50 g kg^{-1} PHB treatment groups suggests that the dietary PHB stimulated the digestion of dietary lipids, providing more fatty acids available for absorption and esterification into complex lipids. Such increase has earlier been shown to be physiologically relevant as a study by Essa et al. (2010) reported a higher growth and feed utilization resulting from a 35–80% increase in gut lipase activity in Nile tilapia fingerlings (~25 g). The higher lipase activity may thus have contributed to the increase in total lipid content as observed in the fish from the PHB treatment groups. Higher lipid deposition, however, does not equate to faster growth and may indicate an inefficient use of nutrients (Hixson, 2014). Indeed, Hanley (1991) indicated that tilapia that were able to store significant quantities of lipid in their carcass and viscera could not utilize this energy source to improve growth.

The increased lipid deposition can be undesirable from a commercial perspective because it would mean that the higher weight of the fish in the PHB treatments (although not significant) results from extra lipid and not extra muscle or "real growth". On the other hand, the increase in total lipid content can be favorable for human consumption to a certain degree, as lipids play an essential role as energy and FAs source for humans, both for immediate utilization by the body and in laying down a storage depot (adipose tissue) for later utilization when food intake is reduced. They can also act as a vehicle for the ingestion and absorption of fat-soluble vitamins (Medeiros and Wildman, 2013). Fats as a source of essential FAs (omega-3 and omega-6) are specifically of interest as they for example serve as specific precursors for eicosanoids that regulate numerous cell and organ functions in humans (FAO, 2010; Uauy et al., 2000). It was thus interesting to verify if the increment of total lipid resulting from PHB supplementation corresponded to an increase in essential FAs in the fish or that more neutral (or non-essential) fat which does not contribute to the quality of the tilapia as a consumption product was accumulated.

Earlier, it was reported by Najdegerami et al. (2012) that PHB significantly increased the linoleic acid (LA) and total (*n*-6) FAs content of the liver of Siberian sturgeon fingerling fed a 5 g kg^{-1} PHB diet. In another study, PHB was found to increase the total monounsaturated and total (*n*-3) FAs content of giant tiger prawn postlarvae (Ludevese-Pascual, personal communication). Similarly, an increasing trend was observed in the total saturated, total monounsaturated FAs and total (*n*-6) FAs content (% on DW) for the Nile tilapia juveniles fed with PHB in this study. However, PHB did not affect the composition of FAs groups when expressed as % on total lipid content. This indicates that the fish FA profile was not affected by PHB supplementation. The FAME analysis procedure performed in this study only measured the long chain fatty acids (C14–C24) and did not provide information on the composition of SCFAs (C2–C6) or medium chain fatty acids (C8–C12). As the degradation of dietary PHB is hypothesized to results in the release of SCFAs, likely β-HB (De Schryver et al., 2010; Defoirdt et al., 2009), it will be interesting to evaluate the effect of PHB on the fish SCFAs profile in future research.

The gnotobiotic challenge test was used to investigate if PHB has a similar disease protecting effect for fish as it does for crustaceans. In this study, *E. ictaluri* gly09R, a pathogen known to cause mortality in tilapia culture, was used (Soto et al., 2012). The experiment showed that PHB supplementation to gnotobiotic Nile tilapia larvae provided significant protection against the challenge, although the protection was not complete in comparison to a negative control. These observations are similar to the ones of Thai et al. (2014) in freshwater prawn and Laranja et al. (2014) in giant tiger prawn. While it has been shown that the use of PHB limits the pathogenicity and the presence of presumptive vibrios in shrimp (Defoirdt et al., 2007; Thai et al., 2014), it remains to be determined if PHB also worked through affecting the growth or activity of *E.*

*ictaluri*gly09R in the tilapia or that it may have acted as an immunostimulant as described by Baruah et al. (2015) for brine shrimp. The availability of the gnotobiotic Nile tilapia challenge test creates the opportunity for a detailed investigation of the effects of PHB exposure on pathogen growth and virulence under microbiologically controlled conditions.

5. Conclusion and perspectives

In general, the findings of this study suggest that PHB does not have a negative effect on the growth of Nile tilapia juveniles. A trend of increasing body weight was observed in all PHB treatment groups, however, the increase was not statistically significant compared to the control group. Furthermore, lipid digestion and deposition seemed to be increased by the dietary PHB supplementation. We hypothesize that the altered lipid utilization may have resulted from diet-induced alterations in the lipase activity. A challenge test making use of a gnotobiotic Nile tilapia larvae system confirmed that PHB is also effective as an antipathogenic strategy for fish. Further research needs to focus on the effect of PHB on the modulation of the digestive functionality in fish of different trophic levels and on determining the way in which PHB provides protection against pathogens in fish.

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References

AOAC, 2000. in: Horwitz, W. (Ed.), Official Methods of Analysis of AOAC International. AOAC, Arlington, VA, USA.
Anderson, A.J., Dawes, E.A., 1990. Occurrence metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54, 450–472.
Balarin, J.D., Halfer, R.D., 1982. The intensive culture of tilapia in tanks, raceways and cages. In: Muir, J.F., Roberts, R.J. (Eds.), Recent Advances in Aquaculture. Crom Helm, London, pp. 265–356.
Baruah, K., Huy, T.T., Norouzitallab, P., Niu, Y., Gupta, S.K., De Schryver, P., Bossier, P., 2015. Probing the protective mechanism of poly-ß-hydroxybutyrate against vibriosis by using gnotobiotic *Artemia franciscana* and *Vibrio campbellii* as host-pathogen model. Sci. Rep. 5, 9427.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
De Schryver, P., Sinha, A., Kunwar, P., Baruah, K., Verstraete, W., Boon, N., De Boeck, G., Bossier, P., 2010. Poly-ß-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, *Dicentrarchus labrax*. Appl. Microbiol. Biotechnol. 86, 1535–1541.
Defoirdt, T., Halet, D., Vervaeren, H., Boon, N., De Wiele, T., Sorgeloos, P., Bossier, P., Verstraete, W., 2007. The bacterial storage compound poly-ß-hydroxybutyrate protects *Artemia franciscana* from pathogenic *Vibrio campbellii*. Environ. Microbiol. 9, 445–452.
Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P., 2009. Short-chain fatty acids and poly-β-hydroxyalkanoates: (new) biocontrol agents for a sustainable animal production. Biotechnol. Adv. 27, 680–685.
Defoirdt, T., Sorgeloos, P., Bossier, P., 2011. Alternatives to antibiotics for the control of bacterial disease in aquaculture. Curr. Opin. Microbiol. 14, 251–258.
Doyle, C., Tanner, E., Bonfield, W., 1991. In vitro and in vivo evaluation of poly hydroxybutyrate and of poly hydroxybutyrate reinforced with hydroxyl apatite. Biomaterials. 12, 841–847.
Essa, M., El Serafy, S., El Ezabi, M., Daboor, S., Esmael, N., Lall, S., 2010. Effect of different dietary probiotics on growth, feed utilization and digestive enzymes activities of Nile tilapia, *Oreochromis niloticus*. J. Arab. Aquat. Soc. 5, 143–162.
FAO, 2010. Fats and fatty acids in human nutrition: Report of an expert consultation. Food and Agriculture Organization of the United Nations, Rome.
Folch, J., Lees, M., Sloane-Stanley, G., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.
German, D.P., Horn, M.H., Gawlika, A., 2004. Digestive enzyme activities in herbivorous and carnivorous Prickelback fishes (*Teleostei: Stichaeidae*): ontogenetic, dietary and phylogenetic effects. Physiol. Biochemi. Zool. 77 (5), 789–804.
Hanley, F., 1991. Effects of feeding supplementary diets containing varying levels of lipid on growth, food conversion and body composition of Nile tilapia *Oreochromis niloticus* (L.). Aquaculture 93, 323–334.
Hixson, S., 2014. Fish nutrition and current issues in aquaculture: the balance in providing safe and nutritious seafood, in an environmentally sustainable manner. J. Aquac. Res. Development. 5, 234–243.
Holm, H., Krogdahl, A., Hanssen, L.E., 1988. High and low inhibitor soybean meals affect human duodenal proteinase activity differently: in vitro comparison of proteinase inhibition. J. Nutr. 118, 521–525.
Iijima, N., Tanaka, S., Ota, Y., 1998. Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream *Pagrus major*. Fish Physiol. Biochem. 18, 59–69.
Karasov, W., Hume, I., 1997. The vertebrate gastrointestinal system. In: Dantzler, W. (Ed.), Handbook of Physiology. Oxford University Press, New York, pp. 407–480 Section 13: Comparative Physiology.
Laranja, J.L.Q., Ludevese-Pascual, G.L., 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 (Fabricius, 1798) postlarvae. Vet. Microbiol. 173, 310–317.
Lazo, J.P., Holt, G.J., Arnold, C.R., 2000. Ontogeny of pancreatic enzymes in larval red drum *Sciaenops ocellatus*. Aquacult. Nutr. 6, 183–192.
Lepage, G., Roy, C.C., 1984. Improved recovery of fatty acid through direct transesterification without prior extraction or purification. J. Lipid Res. 25, 1391–1396.
Marques, A., Dhont, J., Sorgeloos, P., Bossier, P., 2004. Evaluation of different yeast cell wall mutants and microalgae strains as feed for gnotobiotically grown brine shrimp Artemia franciscana. J. Exp. Mar. Bio. Ecol. 312, 115–136.
Medeiros, D.M., Wildman, R.E.C., 2013. Advance Human Nutrition, 3rd ed. Jones & Barlett Learning, Burlington, USA, pp. 391.
Métais, P., Bieth, J., 1968. Determination de l'amylase par une microtechnique. Annales de Biologie Clinique (Paris) 26, 133–142.
Mohapatra, S., Chakraborty, T., Prusty, A.K., Das, P., Paniprasad, K., Mohanta, K.N., 2012. Use of different microbials probiotics in the diet of rohu, *Labeo rohita* fingerlings: effects on growth, nutrient digestibility and retention, digestive enzyme activities and intestinal microflora. Aquacult. Nutr. 18, 1–11.
Najdegerami, E.H., Tran, T.N., Defoirdt, T., Marzorati, M., Sorgeloos, P., Boon, N., Bossier, P., 2012. Effects of poly-beta-hydroxybutyrate (PHB) on Siberian sturgeon (*Acipenser baerii*) fingerlings performance and its gastrointestinal tract microbial community. FEMS Microbiol. Ecol. 79, 25–33.
Najdegerami, E.H., Baruah, K., Shiri, A., Rekecki, A., Van den Broeck, W., Sorgeloos, P., Boon, N., Bossier, P., De Schryver, P., 2015. 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. 46, 801–812.
Ng, W.K., Romano, N., 2013. A review of the nutrition and feeding management of farmed tilapia throughout the culture cycle. Rev. Aquacult. 5, 220–254.
Ng, W.K., Chong, C.Y., 2004. An overview of lipid nutrition with emphasis on alternative lipid sources in tilapia feeds. In: Bolivar, R.B., Mair, G.C., Fitzsimmons, K. (Eds.), New Dimensions in Farmed Tilapia. Sixth International Symposium on Tilapia in Aquaculture. American Tilapia Association, USA, pp. 241–248.
Nhan, D.T., Wille, M., De Schryver, P., Defoirdt, T., Bossier, P., Sorgeloos, P., 2010. The effect of poly β-hydroxybutyrate on larviculture of the giant freshwater prawn *Macrobrachium rosenbergii*. Aquaculture 302, 76–81.
Situmorang, M.L., Dierckens, K., Mlingi, F.T., Van Delsen, B., Bossier, P., 2014. Development of a bacterial challenge test for gnotobiotic Nile tilapia *Oreochromis niloticus* larvae. Dis. Aquat. Organ. 109, 23–33.
Smith, L.S., 1980. Digestion in teleost fishes. Fish Feed Technology—ADCP/REP/80/11. Food and Agriculture Organisation of the United Nations, Rome, pp. 395.
Soto, E., Maziel, A., Riofrio, A., Martinez, A., Cabrejos, M.E., 2012. *Edwardsiella ictaluri* as the causative agent of mortality in cultured Nile tilapia. J. Aquat. Anim. Health 24, 81–90.
Sui, L., Cai, J., Sun, H., Wille, M., Bossier, P., 2012. Effect of poly-beta-hydroxybutyrate on Chinese mitten crab, *Eriocheir sinensis*, larvae challenged with pathogenic *Vibrio anguillarum*. J. Fish Dis. 35, 359–364.
Thai, T.Q., Wille, M., Garcia-Gonzalez, L., Sorgeloos, P., Bossier, P., 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. Appl. Microbiol. Biotechnol. 98, 5205–5215.
Tokiwa, Y., Calabia, B.P., 2007. Biodegradability and biodegradation of polyesters. J. Polym. Environ. 15, 259–267.
Uauy, R., Mena, P., Rojas, C., 2000. Essential fatty acids in early life: structural and functional role. Proc. Nutr. Soc. 59, 3–15.
Ueberschar, B., 1988. Determination of the nutritional condition of individual marine fish larvae by analyzing their proteolytic enzyme activities with a highly sensitive technique. Meeresforschung 32, 144–154.
Ways, P., Hanahan, D.J., 1964. Characterization and quantification of red cell lipids in normal man. J. Lipid Res. 5, 318–328.
Zambonino-Infante, J.L., Cahu, C.L., 1994. Early weaning of sea bass (*Dicentrarchus labrax*) larvae with a compound diet: effect on digestive enzymes. Comp. Biochem. Physiol. 109A, 212–222.