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Immunostimulatory effects of dietary poly-β-hydroxybutyrate in European sea bass postlarvae

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Abstract

The stable production of high-quality fry in marine aquaculture is still hampered by unpredictable mortality caused by infectious diseases during larval rearing. Consequently, the development of new biocontrol agents is crucial for a viable aquaculture industry. The bacterial energy storage compound poly-β-hydroxybutyrate (PHB) has been shown to exhibit beneficial properties on aquatic organisms such as enhanced survival, growth, disease resistance and a controlling effect on the gastrointestinal microbiota. However, the effect of PHB on the developing immune system of fish larvae has so far not been investigated. In this study, the effect of feeding PHB-enriched *Artemia* nauplii on survival, growth and immune response in European sea bass (*Dicentrarchus labrax*) postlarvae was examined. Amorphous PHB was administered to 28-day-old sea bass postlarvae over a period of 10 days. The survival and growth performance were monitored, and the expression of 29 genes involved in immunity, growth, metabolism and stress-response was measured. While the expression of the insulin-like growth factor 1 (*igf1*), an indicator of relative growth, was upregulated in response to feeding PHB, the larval survival and growth performance remained unaffected. After 10 days of PHB treatment, the expression of the antimicrobial peptides dicentracin (*dic*) and hepcidin (*hep*) as well as *mhc class IIa* and *mhc class IIb* was elevated in the PHB fed postlarvae. This indicates that PHB is capable of stimulating the immune system of fish early life stages, which may be the cause of the increased resistance to diseases and robustness observed in previous studies.

KEYWORDS
aquaculture, *Dicentrarchus labrax*, fish larvae, gene expression, immunostimulation, PHB

1 | INTRODUCTION

The intensive production of marine fish larvae constitutes a major bottleneck in aquaculture, due to high and unpredictable mortality, mainly caused by the outbreak of infectious diseases (Vadstein et al., 2012). Especially the early life stages are highly susceptible towards pathogens, because they lack a mature immune system (Vadstein, 1997). During the first weeks after hatch, marine fish larvae mainly rely on their innate immune response, while the adaptive immune system is still developing (Magnadottír, 2006). Maternally derived immune factors are mostly exhausted as early as the yolk absorption is completed (Magnadottir, Lange, Gudmundsdottir, Bøgwald & Dalmo, 2005; Swain & Nayak, 2009). In European sea bass larvae, for example, maternal IgM was not detectable anymore by day 5 post hatch (Breuil, Vassiloglou, Pepin & Romestand, 1997). Consequently, vaccination, the most important method for disease

prevention in aquaculture, cannot be applied during the larval stages, as their mode of action depends on adaptive immunological memory (Sommerset, Krossøy, Biering & Frost, 2005). Furthermore, the standard practice for disease control, the prophylactic application of antibiotics, has selected for antibiotic-resistant bacteria, making treatments ineffective as well as being a threat to the public health and the environment (Defoirdt, Sorgeloos & Bossier, 2011). Therefore, the development of new biocontrol agents for disease prevention is crucial to improve animal welfare, ensure the consumers' health and reduce economic losses (Defoirdt et al., 2011). Several alternative strategies, such as the prophylactic application of prebiotics, probiotics and immunostimulants, have been proposed to reduce the infection risk and, thus, prevent diseases in aquaculture (Akhter, Wu, Memon & Mohsin, 2015; Ringø, Olsen, Gonzalez Vecino, Wadsworth & Song, 2011).

One possibility is the application of the bacterial energy storage compound poly-β-hydroxybutyrate (PHB), the polymer of the short-chain fatty acid (SCFA) β-hydroxybutyrate (β-HB). Under conditions of nutrient depletion and carbon excess, PHB is accumulated as a cellular carbon reserve by a wide range of bacterial genera such as *Alcaligenes*, *Bacillus* and *Pseudomonas* (Suriyamongkol, Weselake, Narine, Moloney & Shah, 2007; Wang, Sharma-Shivappa, Olson & Khan, 2012). The compound has been shown to increase growth and survival of some aquatic species, including penaeid shrimps (*Penaeus monodon*) (Laranja et al., 2014), blue mussels (*Mytilus edulis*) (Hung et al., 2015) and European sea bass (*Dicentrarchus labrax*) juveniles (De Schryver et al., 2010). Additionally, dietary PHB altered the microbial community of the gastrointestinal (GI) tract in European sea bass juveniles (De Schryver et al., 2011). After uptake of PHB-accumulated bacteria, PHB polymers can be gastrointestinally degraded into oligomers and monomers (SCFAs), lowering the pH in the host's gut (Defoirdt, Boon, Sorgeloos, Verstraete & Bossier, 2009). While it was shown that the cell growth of pathogenic bacteria belonging to genera like *Vibrio* and *Salmonella* (Defoirdt et al., 2007; Van Immerseel et al., 2003) is suppressed by SCFAs, beneficial bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. may profit from the lower gut pH, improving the GI health of the host organism (Cotter & Hill, 2003). This may explain why gnotobiotic Nile tilapia (*Oreochromis niloticus*) larvae (Situmorang, De Schryver, Dierckens & Bossier, 2016) and rainbow trout (*Oncorhynchus mykiss*) fry (Najdegerami et al., 2015a) fed with a PHB-enriched diet and subsequently challenged with pathogenic bacteria exhibited an increased resistance against the infection. Nevertheless, the specific mode of action of PHB remains unknown. It is, however, hypothesized that its monomer β-HB is able to stimulate the immune system in fish (Montalban-Arques et al., 2015). So far, it has only been shown that PHB enhances the immune response in adult Mozambique tilapia (*Oreochromis mossambicus*) when measuring serum parameters as well as antibody response (Suguna et al., 2014).

In this study, we hypothesize that PHB stimulates the immune system and improves survival as well as growth performance in European sea bass postlarvae. We used *Artemia* as live carriers to feed freeze-dried PHB-accumulated bacteria (*Alcaligenes eutrophus*) to sea bass postlarvae over a period of 10 days. Using gene expression analyses, we aimed to provide new insights into the capability of PHB to act as a stimulator for a developing immune system. Therefore, we carried out an extensive analysis on the expression of genes involved in immunity as well as growth, metabolism and stress. This is the first study to assess the potential immunomodulating effect of PHB in fish larvae.

2 | MATERIALS AND METHODS

2.1 | Larval rearing

European sea bass (*Dicentrarchus labrax*) larvae were purchased from a commercial hatchery (Ecloserie Marine de Gravelines, France) at 3 days post hatch (dph) and reared in a flow-through system at GEOMAR Kiel (Germany) in three green stocking tanks until 25 dph. Each tank was filled with 30 L Baltic Sea water (5 μm-filtered and UV-treated) with an artificially increased salinity (SEEQUASAL, Germany) of 32 g/L, which was gradually decreased to 26 g/L until 14 dph and increased again afterwards to improve the efficiency of the swim bladder inflation (Saillant, Fostier, Haffray, Menu & Chatain, 2003). The water temperature was increased stepwise from 15 to 18.5°C, and oxygen was maintained above 80% saturation throughout the experiment. The larvae were kept in the dark until first feeding at 7 dph and under a natural photoperiod regime (16L:8D) thereafter. For further details, see Tillner, Rønnestad, Dhert and Ueberschär (2014). The sea bass larvae were fed on rotifers (*Brachionus plicatilis*) from 7 dph on. The rotifers were reared in sterile filtered Baltic Sea water and fed on resuspended *Nannochloropsis* spp. concentrate (BLUEBIOTECH, Germany). From 23 to 25 dph, the sea bass larvae were fed on instar I *Artemia* nauplii and afterwards on instar II *Artemia* nauplii (Micro Artemia Cysts, OCEAN NUTRITION, USA). The *Artemia* eggs were incubated in 5 μm-filtered and UV-treated sea water according to the manufacturer's instructions. Prior to feeding, rotifers and instar II *Artemia* nauplii were enriched with essential fatty acids (S.presso, INVE, Belgium; applied according to instructions). At 25 dph, the larvae were randomly distributed into six experimental tanks (total volume: 65 L, used volume: 30 L) at a density of 40 larvae/L. The experiment was started after a 3-day acclimation period at 28 dph under the following conditions: temperature 18.5°C, salinity 32 g/L, photoperiod 16L:8D and flow rate 0.4 L/min. The tank bottoms were siphoned daily to remove dead larvae, faeces and debris.

The experiment was approved by the ethical committee of Kiel University (Germany) under the file number V 312-7224.121-19 (24-2/13).

2.2 | Experimental diets and feeding

Over the course of the experiment, starting at 28 dph, the sea bass postlarvae were fed three times a day at 10:00, 15:00 and 20:00 hours with instar II *Artemia* nauplii (Micro Artemia Cysts, OCEAN NUTRITION, USA) at densities of 8, 4 and 4 mL^{-1}

respectively. The water flow was turned off for feeding between 10:00 and 22:00 hours. Three tanks, respectively, were randomly assigned to the following treatments: (1) PHB treatment (*Artemia* enriched with PHB), (2) control treatment (*Artemia* without PHB enrichment). For both treatments, instar II *Artemia* nauplii were enriched with highly unsaturated fatty acids (S.presso, INVE, Belgium) according to the manufacturer's instructions. For the PHB treatment, instar II *Artemia* nauplii were enriched afterwards with a freshly prepared PHB solution at a density of 500 nauplii/ml for 60 min under gentle aeration directly before feeding. *Artemia* are non-selective filter feeder, and it was demonstrated that they are able to accumulate bacteria when incubated in bacterial suspensions (Makridis, Fjellheim, Skjermo & Vadstein, 2000). The PHB solution consisted of freeze-dried PHB-accumulated bacteria (*Alcaligenes eutrophus*) dissolved in UV-treated salt water (salinity: 32 g/L) at a concentration of 10^8 bacteria/ml. The bacteria had a PHB content of 75% of the cell dry weight and were produced as described in Thai et al. (2014).

2.3 | Measured parameters

2.3.1 | Growth performance and survival rate

After 10 days of treatment (38 dph), 20 postlarvae were randomly sampled from each tank, anaesthetized with MS 222 (SIGMA-ALDRICH, Germany), transferred into Eppendorf vials with sea water and immediately frozen on dry ice. The samples were stored at −80°C. For growth analysis, the total length (cm) of thawed larvae was measured. Subsequently, the larvae were briefly rinsed in distilled water to avoid salt residues, freeze-dried for 18 hr at −55°C (Alpha1-4 freeze dryer, CHRIST, Germany) and weighed (Microbalance SC2, SARTORIUS, Germany) in order to determine the larval dry weight (mg).

Furthermore, Fulton's condition factor (K) was calculated according to the equation:

$$K = \frac{W}{L^3}$$

where W equals the dry weight (mg) and L the total length (cm) of the larvae. For calculating survival rates, dead larvae were removed from the tanks and counted daily.

2.3.2 | Gene expression analysis

After 3 and 10 days of treatment (31 and 38 dph respectively), six postlarvae were randomly sampled from each tank, anaesthetized with MS 222 (SIGMA-ALDRICH, Germany), transferred into RNAlater and kept at 4°C for 24 hr before being stored at −20°C. These two sampling points were chosen to assess the short-term and the midterm effects of PHB administration. The first sampling point is crucial to detect potential effects of PHB on the innate immune system as it is known to react immediately (Magnadottír, 2006).

For the quantification of mRNA as a measure of gene expression levels, the RNA of single whole larvae was extracted using a RNeasy 96 Universal Tissue Kit (QIAGEN, Germany) according to the manufacturer's instructions. RNA concentration was measured by spectrophotometry (NanoDrop ND-1000, VWR, Germany) and normalized to a common concentration with RNAse free water. 500 ng RNA was reverse transcribed into cDNA, including a gDNA wipeout step (QuantiTect Reverse Transcription Kit, QIAGEN, Germany). The cDNA was stored at −80°C until further use.

Primers (METABION, Germany) for all genes of interest as well as for reference genes were taken from the literature (Mitter et al., 2009; Sarropoulou et al., 2009) or designed with Primer3 (version 0.4.0), using *D. labrax* sequences from GenBank (Table 1). The primers were tested for functionality and efficiency against a serial dilution of *D. labrax* cDNA together with EvaGreen qPCR Mix Plus Rox (SOLIS BIODYNE, Estonia), using a StepOnePlus Real-Time PCR System (THERMO FISHER SCIENTIFIC, Germany). The cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by 95°C for 15 s, 60°C for 1 min and 95°C for 15 s.

A qPCR BioMark™ HD System (FLUIDIGM, Germany) running a 96.96 Dynamic Array™ IFC (Gene Expression chip) was used to measure the expression profiles of 29 genes in the larval samples. Briefly, 1.3 μl cDNA per sample was mixed with TaqMan-PreAmp Master Mix (THERMO FISHER SCIENTIFIC, Germany) and a 500 nM primer pool of all primers and pre-amplified (10 min at 95°C; 16 cycles: 15 s at 95°C and 4 min at 60°C). The obtained PCR products were diluted 1:10 with low EDTA-TE buffer and pipetted into the sample inlets on the chip together with SsoFast EvaGreen Supermix with Low Rox (BIO-RAD, Germany) and DNA Binding Dye Sample Loading Reagent (FLUIDIGM, Germany). Samples were distributed randomly across the chip, including no template controls (NTC) and controls for gDNA contamination. Primers (50 μM) mixed with Assay Loading Reagent (FLUIDIGM, Germany) and low EDTA-TE Buffer were loaded onto the chip in technical triplicates per sample. The chip was primed and the run subsequently performed using the GE Fast 96 × 96 PCR + Melt v2 thermal cycling protocol with a Tm of 60°C according to the manufacturer's instructions.

2.4 | Statistical analyses

Technical triplicates were used to calculate the mean cycle threshold value (Ct), the standard deviation (SD) and the coefficient of variation (CV) per sample for the gene expression analysis. Samples with a CV larger than 4% were excluded from the analysis, as in accordance with Bookout and Mangelsdorf (2003). The expression stability of genes was calculated using qbase$^+$ (BIOGAZELLE, Belgium), and the geometric mean Ct of the three most stable genes (*actb*, *l13a*, *hsp90*; $M < 0.5$) was used to normalize target genes (calculation of ΔCt-values). *Actb* and *l13a* were also identified as suitable reference genes for sea bass by Mitter et al. (2009).

All statistical analyses were carried out in RStudio (version 0.98.1103). Permutational multivariate analyses of variance (PERMANOVA) were performed (adonis function of the vegan

TABLE 1 Name, abbreviation and function of the 26 genes of interest and three reference genes. Genes were divided into the following five functional groups: (I) overall immune response, (II) innate immunity, (III) adaptive immunity, (IV) growth and metabolism and (V) stress. Forward (FW) and reverse (RV) primers were either designed using sequences from GenBank (see accession number) or taken from literature (see reference)

Group	Abbreviation	Gene name and function	Primer sequence	Accession No./Ref.
Overall immune response				
Innate immunity	*apoA1*	Apolipoprotein A1, antimicrobial protein	FW: ATACGTCCTGGCACTGATCC RV: AGCCTGACCTTGCTCACTGT	Sarropoulou et al. (2009)
	cc1	CC chemokine 1, chemotactic cytokine	FW: TGGGTTCGCCGCAAGGTTGTT RV: AGACAGTAGACGAGGGGACCACAGA	AM490065.1
	cox2	Cyclooxygenase-2, pro-inflammatory enzyme	FW: AGCACTTCACCCACCAGTTC RV: AAGCTTGCCATCCTTGAAGA	AJ630649.1
	Ifna1	Interferon, cytokine	FW: GTACAGACAGGCGTCCAAAGCATCA RV: CAAACAGGGCAGCCGTCTCATCAA	AM765846.2
	il1b	Interleukin 1 beta, pro-inflammatory cytokine	FW: GCGACATGGTGCGATTTCTCTTCTACA RV: GCTGTGCTGATGTACCAGTTGCTGA	AJ311925.1
	dic	Dicentracin, antimicrobial peptide	FW: AGTGCGCCACGCTCTTTCTTGT RV: TTGTGGATGGACTTGCCGACGTG	AY303949.1
	fer	Ferritin, antimicrobial peptide	FW: ATGCACAAGCTCTGCTCTGA RV: TTTGCCCAGGGTGTGTTTAT	Sarropoulou et al. (2009)
	hep	Hepcidin, antimicrobial peptide	FW: AAGAGCTGGAGGAGCCAATGAGCA RV: GACTGCTGTGACGCTTGTGTCTGT	DQ131605.1
	tlr1	Toll-like receptor 1, pattern recognition receptor	FW: GCCTCTGCCTCAATACCTGATCCCA RV: AACAACCTGTGCTTGGCCCTGTC	KX399287
	tlr9	Toll-like receptor 9, pattern recognition receptor	FW: TCTTGGTTTGCCGACTTCTTGCGT RV: TACTGTTGCCCTGTTGGGACTCTGG	KX399289
	tnfa	Tumour necrosis factor α, pro-inflammatory cytokine	FW: AGCCACAGGATCTGGAGCTA RV: GTCCGCTTCTGTAGCTGTCC	DQ070246.1
Adaptive immunity	*mhc class Ia*	Major histocompatibility complex I α, cell surface molecules	FW: TGTACGGCTGTGAGTGGGATGATGAG RV: AGCCTGTGGTCTTGGAGCGATGAA	JX171695.1
	mhc class IIa	Major histocompatibility complex II α, cell surface molecules	FW: AGTCCGATGATCTACCCCAGAGACAAC RV: ACAGGAGCAGGATAGAAACCAGTCACA	FN667955.1
	mhc class IIb	Major Histocompatibility Complex II ß, cell surface molecules	FW: GCTGGCAGACGCTGATTGGTTCT RV: TAACCAGAGGTTCTCTCAGGCTGGC	AM113471.1
	rag1	Recombination activating protein 1, involved in VDJ recombination	FW: CCAATTACCTGCACAAGACCCTGGC RV: GTTTGTTTGCCGACTCGTTCCCCT	FN687463.1
Complement system	*c3*	Complement component C3, classical and alternative pathway	FW: TGACGGAGAGCGGTGGTGAAATG RV: AGGCCATCCCTGGTTTGAAGTATTTGG	HM563078.1
	cla	C-Lectin-A, lectin pathway	FW: GATGGCAGCAAGCTCCGGTATTCA RV: TCTGACCTATGACCCCAGCCAACA	EU660935.1
	gal	Galectin, lectin pathway	FW: TGCAACTCTTACCAGGGAGGCAACT RV: GTCACGAGGAACTCTGTAGGGGTGA	EU660937.1
Apoptosis	*casp3*	Caspase 3, protease	FW: CTGATTTGGATCCAGGCATT RV: CGGTCGTAGTGTTCCTCCAT	DQ345773.1
	casp9	Caspase 9, protease	FW: GGCAGGACTCGACGAGATAG RV: CTCGCTCTGAGGAGCAAACT	DQ345776.1

(Continues)

TABLE 1 (Continued)

Group	Abbreviation	Gene name and function	Primer sequence	Accession No./Ref.
Growth and metabolism	*gh*	Growth hormone	FW: GGCCAATCAGGACGGAGCAGAGAT	GQ918491.1
			RV: AGGTTCGTCTCAGCGACTCATCGG	
	igf1	Insulin-like growth factor 1	FW: TTCAAGGGCGCGATGTGCTGTATC	AY800248.1
			RV: GCCTCTCTCTCCACACACAAACTGC	
	fad6	Fatty acid desaturase-6, fatty acid synthesis	FW: GCTCAGCCTTTGTTCTTCTGCCTCC	FP671139.1
			RV: TGAGCAGTTGCCAGCATGATCGAG	
	tryp	Trypsin, protease	FW: CCTGGTCAACGAGAACTGGGTTGTG	AJ006882.1
			RV: GGATGACACGGGAGGAGCTGATGAA	
Stress	*cat*	Catalase, antioxidant	FW: TGATGGCTACCGCCACATGAACG	FJ860003.1
			RV: TTGCAGTAGAAACGCTCACCATCGG	
	hsp70	Heat shock protein 70, stress protection	FW: ACAAAGCAGACCCAGACCTTCACCA	AY423555.2
			RV: TGGTCATAGCACGTTCGCCCTCA	
Reference genes	*actb*	Beta-actin	FW: TGAACCCCAAAGCCAACAGGGAGA	AJ537421.1
			RV: GTACGACCAGAGGCATACAGGGACA	
	l13a	Ribosomal protein L13 a	FW: TCTGGAGGACTGTCAGGGGCATGC	Mitter et al. (2009)
			RV: AGACGCACAATCTTGAGAGCAG	
	hsp90	Heat shock protein 90	FW: GCTGACAAGAACGACAAGGCTGTGA	AY395632.1
			RV: AGATGCGGTTGGAGTGGGTCTGT	

package in R; Oksanen, Blanchet & Kindt, 2012) for each functional gene group to test for overall differences between the two treatments. PERMANOVAs using ΔCt-values are based on Pearson correlation distance matrices (amap package, Dist function; Lucas, 2011) and were run with 699 permutations. The multivariate model included treatment as a fixed factor, whereas ΔCt-values of all larvae per tank were averaged, as tank could not be implemented as a random factor in the PERMANOVA. Subsequently, a mixed-effect model, which included treatment as a fixed factor and tank as a random factor, was used to analyse each individual target gene and growth data respectively. All data were tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test). If the test assumptions were violated, data were Box-Cox transformed. All values are presented as mean ± SEM. For a graphical representation of gene expression data, the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001) was applied by calculating the ΔΔCt for each larva in relation to the mean ΔCt of the control treatment group. The survival data are presented by means of Kaplan–Meier curves and compared between treatment groups using a log-rank test (survival package in R; Therneau, 2015).

3 | RESULTS

3.1 | Survival

The larval survival rates (Figure 1) in the PHB treatment and the control treatment did not differ significantly from each other over the course of the experiment ($\chi^2 = 0.9$, $df = 1$, $p > .05$). Survival remained above 85% in both treatment groups.

3.2 | Growth performance

After a treatment of 10 days, the 38-day-old sea bass postlarvae had an average dry weight of 2.6 ± 0.12 mg in the control treatment and 2.0 ± 0.11 mg in the PHB treatment, a total length of 1.35 ± 0.02 cm (control) and 1.39 ± 0.02 cm (PHB) and a Fulton's condition factor K of 0.9 ± 0.02 mg/cm^3 (control) and 0.8 ± 0.02 mg/cm^3 (PHB) respectively. The mixed-effect model revealed that neither the dry weight ($F_{1,4} = 4.0$), nor the total length ($F_{1,4} = 0.9$), nor the condition factor ($F_{1,4} = 7.3$) were affected by the PHB treatment ($p > .05$).

3.3 | Gene expression

The expression of genes involved in immune response, growth, metabolism, antioxidant activity and stress-response was analysed and classified into the following functional gene groups: (I) *overall immune response* (innate and adaptive immunity, complement system and apoptosis), (II) *innate immunity*, (III) *adaptive immunity*, (IV) *growth and metabolism*, (V) *stress*. All genes included in the study (Table 1) were expressed at day 31 and 38 ph (corresponding to 3 and 10 days of treatment respectively).

The multivariate analysis (PERMANOVA) showed that the functional gene groups *overall immune response* ($F_{1,4} = 1.9$), *innate immunity* ($F_{1,4} = 3.1$), *adaptive immunity* ($F_{1,4} = 1.8$) and *stress* ($F_{1,4} = 3.6$) were not significantly affected by the treatment ($p > .05$) at 31 dph. In contrast, the expression of genes related to *metabolism and growth* (*fad6*, *tryp*, *gh*, *igf1*) differed significantly between sea bass postlarvae fed on PHB-enriched *Artemia* or control diet ($F_{1,4} = 23.6$, $p < .01$) for 3 days. The univariate

analyses of the four genes involved in *metabolism and growth* revealed that 3 days of PHB treatment only increased the insulin-like growth factor 1 (*igf1*) expression (2.0 ± 0.19-fold, ΔCt = 9.0 ± 0.24; *df* = 1, *F* = 9.8, $p < .05$) compared to the control treatment (1.2 ± 0.17-fold, ΔCt = 9.9 ± 0.16; Fig. 2) while the expression of *fad6*, *tryp* and *gh* was not significantly affected (Table S1).

The application of PHB over a period of 10 days, however, enhanced the innate and the adaptive immune response of sea bass postlarvae significantly. The multivariate analysis (PERMANOVA) showed a significant difference between the two treatments for expression of genes involved in *innate immunity* ($F_{1,4} = 9.2$, $p < .01$) and *adaptive immunity* ($F_{1,4} = 6.9$, $p < .01$), while the functional gene groups *overall immune response* ($F_{1,4} = 6.5$), *growth and metabolism* ($F_{1,4} = 1.1$) and *stress* ($F_{1,4} = 3.4$) were not significantly affected by the treatment. The subsequent univariate analyses (Table S1) revealed that the expression of the antimicrobial peptides dicentracin (*dic*) and hepcidin (*hep*) as well as the major histocompatibility complex class II (*mhc class IIa* and *mhc class IIb*) was significantly upregulated in the PHB treatment (for all 4 genes: df = 1, $p < .05$; Figures 3 and 4). While the expression of dicentracin in sea bass postlarvae fed with a PHB-enriched diet was slightly enhanced (1.7 ± 0.13-fold, ΔCt = 3.1 ± 0.13; *F* = 10.8) compared to postlarvae fed on the control diet (1.1 ± 0.11-fold, ΔCt = 3.8 ± 0.14), the expression of hepcidin was highly upregulated in the PHB treatment group (21.3 ± 5.00-fold, ΔCt = 4.9 ± 0.53; *F* = 15.4) compared to the control (1.7 ± 0.35-fold, ΔCt = 8.4 ± 0.38). The expression of *mhc class II* genes was approximately three times higher due to dietary PHB administration (*mhc class IIa*: 3.6 ± 0.70-fold, ΔCt = 6.7 ± 0.28; *F* = 14.3; *mhc class IIb*: 2.8 ± 0.47-fold, ΔCt = 6.4 ± 0.24; *F* = 8.3) than in the control group (*mhc class IIa*: 1.1 ± 0.12-fold, ΔCt = 8.2 ± 0.17; *mhc class IIb*: 1.1 ± 0.08-fold, ΔCt = 7.6 ± 0.11).

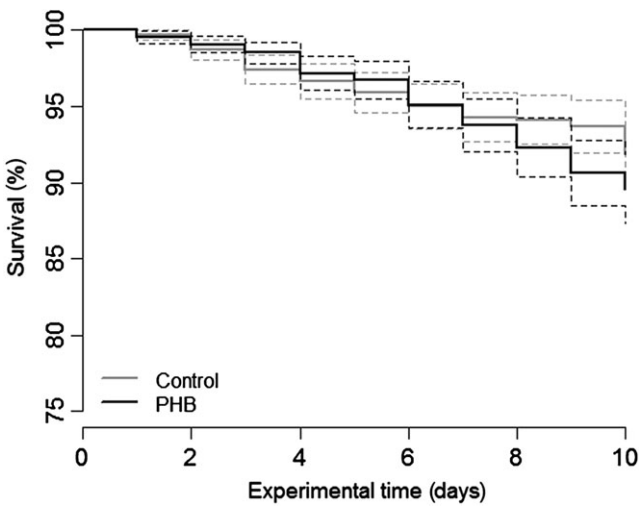

FIGURE 1 Kaplan–Meier survival curves of sea bass postlarvae fed with *Artemia* enriched with PHB (black) or without PHB (grey) over a period of 10 days (from 28 to 38 dph). The dashed lines represent the 95% confidence intervals

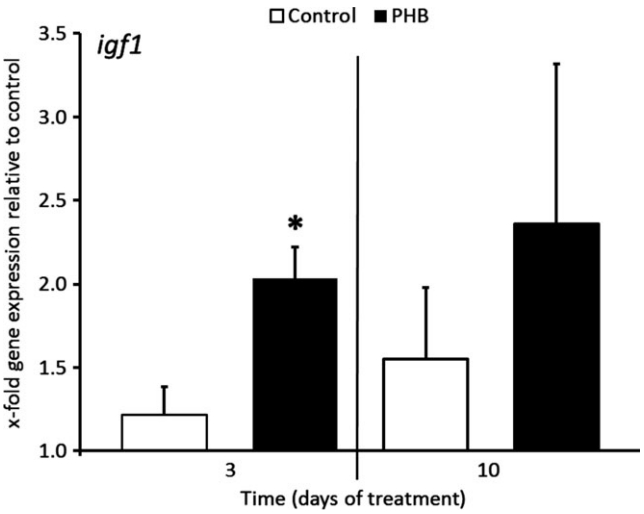

FIGURE 2 Gene expression of insulin-like growth factor 1 (*igf1*) in sea bass postlarvae fed with *Artemia* nauplii with PHB (black bars) or without PHB enrichment (white bars). Larval samples were taken at 31 and 38 dph (3 and 10 days of treatment respectively). The figure displays the x-fold gene expression to the control. Data are presented as mean ± SEM. The asterisk represents the level of significance (*$p < .05$)

4 | DISCUSSION

The revelation of the manifold disadvantages concerning the widespread overuse of antibiotics in animal production has encouraged researchers all over the world to investigate alternative biocontrol compounds (Defoirdt et al., 2011). In the present study, the effects of the bacterial energy storage compound PHB on sea bass postlarvae were investigated with respect to survival, growth and gene expression.

Early developmental stages, such as larvae, are known to be the most vulnerable life cycle stages, exhibiting high and unpredictable mortality (Pepin, 1991; Rosenthal & Alderdice, 1976). Fish larvae only rely on their innate immune system while their adaptive immune system is still developing, making them highly susceptible to infectious diseases (Magnadottír, 2006). Thus, the effect of potential immunostimulatory compounds such as PHB might vary significantly between different life stages. To the best of our knowledge, there are only one study on the effect of PHB on conventional and one on gnotobiotic fish larvae (Najdegerami et al., 2015b; Situmorang et al. 2016). However, the influence on the larval immune response has so far not been addressed.

In the current study, larval survival rates were not affected by PHB administration. The same result was identified in an experiment with Siberian sturgeon (*Acipenser baerii*) larvae fed with PHB-enriched *Artemia* from first feeding onwards over a period of 4 weeks (8–35 dph) (Najdegerami et al., 2015b). In contrast, blue mussel larvae fed with a PHB-supplemented diet directly after hatch over a period of 10 days showed a significantly higher survival compared to the control (Hung et al., 2015; Sui, Cai, Sun, Wille & Bossier, 2012). Interestingly, in a study with Chinese mitten crabs

(*Eriocheir sinensis*), the beneficial effect of PHB on larval survival was not yet present after 8 days of treatment, but could only be observed after 10 days of PHB administration (Sui et al., 2012). Regarding the effect of PHB on the growth performance, it is hypothesized that PHB is gastrointestinally degraded either by digestive enzymes, PHB degrading bacteria or a combination of both into β-HB oligomers and monomers, which could then be used as an additional energy source by the organism (Azain, 2004; De Schryver et al., 2010; Defoirdt et al., 2009; Weltzien, Hemre, Evjemo, Olsen & Fyhn, 2000). However, in the present study, none of the estimated growth-related parameters such as total length, dry weight and Fulton's condition factor K were affected by the PHB treatment. In Siberian sturgeon larvae, dietary PHB decreased growth (Najdegerami et al., 2015b), whereas it increased growth in giant freshwater prawn larvae, Chinese mitten crab larvae and sea bass juveniles (De Schryver et al., 2010; Nhan et al., 2010; Sui et al., 2012; Thai et al., 2014), while no effect was observed on larval size in blue mussels (Hung et al., 2015). Generally, the effect of PHB on larval survival and growth performance seems to be species-specific as well as depend on the onset, dose and duration of the PHB supplementation and the developmental stage of the investigated organism. In the current study, PHB was fed to sea bass postlarvae over a duration of 10 days, hence, it cannot be ruled out that PHB applied at an earlier larval stage in a different dose and/or over a longer period of time would have resulted in a positive effect on survival and growth performance.

For various immunostimulating substances, potential negative effects on cellular homoeostasis have been addressed (Kepka, Verburg-van Kemenade, Homa & Chadzinska, 2014; Miest & Hoole, 2015). However, PHB did not affect the expression of the studied stress- and apoptosis-related genes (*cat*, *hsp70*, *hsp90*; *casp3*, *casp9*). Thus, there is no indication that PHB induced cellular stress or cytotoxicity.

The expression of genes related to growth and metabolism (*fad6*, *tryp*, *gh*, *igf1*) differed significantly between sea bass postlarvae fed for 3 days on PHB-enriched *Artemia* or the control diet. In contrast to our results on growth-related parameters, the insulin-like growth factor 1 (*igf1*) was significantly enhanced after 3 days of PHB supplementation, while *fad6*, *tryp* and *gh* were not significantly affected by the PHB treatment. *Igf1* can be used as an indicator of relative growth (Dyer et al., 2004). It is involved in the differentiation and proliferation of cells (in particular myoblasts) as well as in the protein, lipid and carbohydrate metabolism promoting muscle and cartilage growth (Carnevali et al., 2006; Moriyama, Ayson & Kawauchi, 2000). A significantly elevated *igf1* expression alongside a significantly higher weight was, for example, found in sea bass fry treated with probiotic *Lactobacillus* species (Carnevali et al., 2006). After 10 days of PHB supplementation, only a trend towards a higher *igf1* expression remained. This might indicate that the PHB was not administered in an adequate dose to induce a clearly persisting growth promoting effect in sea bass postlarvae. A dose-dependent growth promoting effect of PHB has been observed in earlier studies, for example when dietary PHB at a low, medium and high dose was administered to juvenile sea bass. While the low and medium dose enhanced growth and caused a controlling effect on the GI microbiota, the high dose showed no effect (De Schryver et al., 2010). The observed change in the intestinal microbial community is hypothesized to develop due to the degradation of PHB into SCFAs, causing a decrease in the GI pH, which inhibits the growth of certain pathogenic bacteria (De Schryver et al., 2010, 2011; Defoirdt et al., 2007). Accordingly, it could be demonstrated that PHB effectively enhances the disease resistance in aquatic invertebrates (Ludevese-Pascual et al., 2017; Sui et al., 2012) and fish. Dietary PHB protected gnotobiotic Nile tilapia larvae (Situmorang et al. 2016) as well as conventional adult Mozambique tilapia (Suguna et al., 2014) from pathogens, resulting in higher survival rates after bacterial challenge

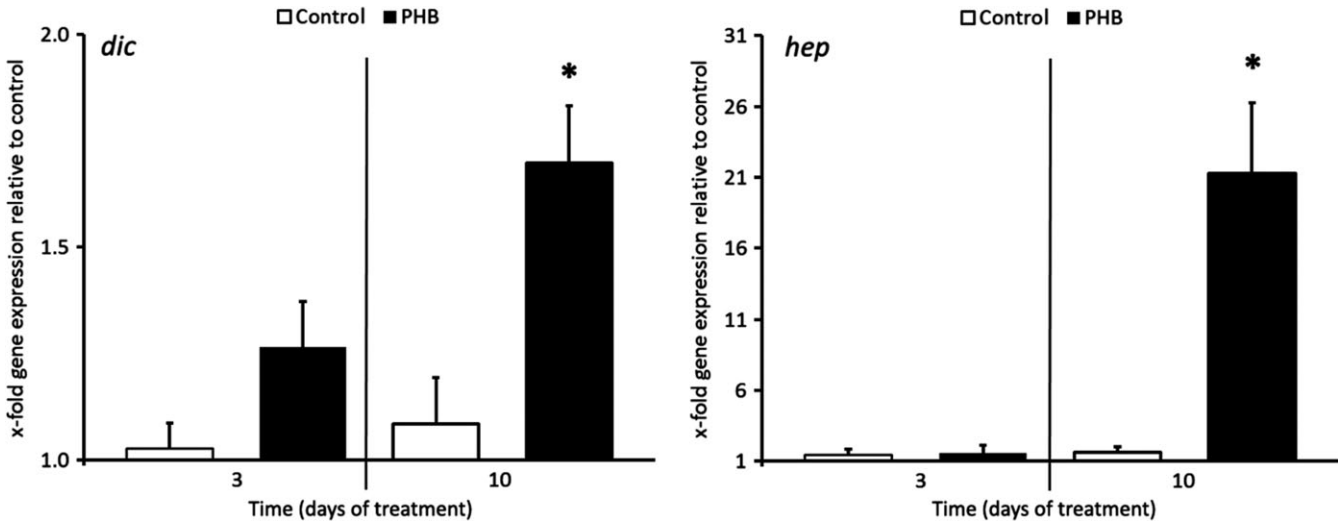

FIGURE 3 Gene expression of dicentracin (*dic*) and hepcidin (*hep*) in sea bass postlarvae fed with *Artemia* nauplii with PHB (black bars) or without PHB enrichment (white bars). Larval samples were taken at 31 and 38 dph (3 and 10 days of treatment respectively). The figures display the x-fold gene expression to the control. Data are presented as mean ± SEM. The asterisk represents the level of significance (*$p < .05$)

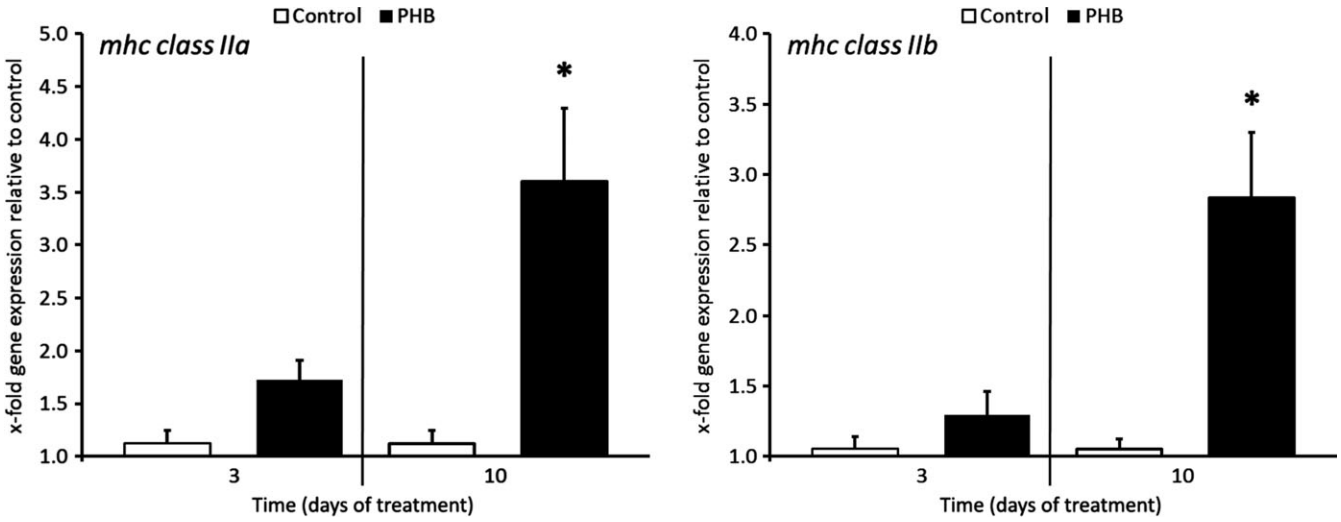

FIGURE 4 Gene expression of *mhc class IIa* and *mhc class IIb* in sea bass postlarvae fed with *Artemia* nauplii with PHB (black bars) or without PHB enrichment (white bars). Larval samples were taken at 31 and 38 dph (3 and 10 days of treatment respectively). The figure displays the x-fold gene expression to the control. Data are presented as mean ± SEM. The asterisk represents the level of significance (*$p < .05$)

tests. Moreover, a lowered GI pH promotes the growth of specific beneficial bacteria, which can trigger an immune response via microbe-associated molecular patterns (MAMPs) as described for prebiotics (Gómez & Balcázar, 2008; Sekirov & Finlay, 2009; Song et al., 2014). Indeed, it has been demonstrated that dietary PHB enhanced serum lysozyme, peroxidase and antiprotease activity as well as antibody response in adult tilapia (Suguna et al., 2014).

To estimate the potential immunomodulatory effect of PHB in fish postlarvae, the expression of genes involved in the immune response was analysed in the present study. It has to be noticed that PHB was administered in form of freeze-dried PHB-accumulated bacteria and that a direct effect of these bacteria on the larval immune system cannot be excluded. However, previous studies using bacteria accumulated with different PHB doses showed that the level of PHB was the main driver for the observed effects (e.g. disease resistance) (Laranja et al., 2014).

In the current study, PHB administration over a period of 10 days enhanced the innate and adaptive immune gene expression in sea bass postlarvae significantly. The expression of the antimicrobial peptides (AMPs) *dic* and *hep* was significantly upregulated in the PHB treatment. Being quickly mobilized due to rapid diffusion rate, AMPs play a crucial role in the first line of innate immune defence in teleost fish (Alvarez, Guzman, Cardenas, Marshall & Mercado, 2014; Terova et al., 2009). Their antimicrobial activity has been demonstrated against a broad-spectrum of pathogens such as bacteria, viruses and fungi (Alvarez et al., 2014; Salerno, Parrinello, Roch & Cammarata, 2007). Thus, the upregulation of AMPs is considered to be advantageous especially for fish early life stages lacking a fully functional adaptive immune system. An enhanced expression of *dic* was also shown after incorporation of yeast cell wall extracts (Bio-Mos®) in the diet of sea bass juveniles (Terova et al., 2009). The immunostimulating effect of Bio-Mos® is probably based on the activation of pattern recognition receptors (PRR) triggering an immune response to the non-self substance (Torrecillas, Montero & Izquierdo, 2014). The immunomodulatory activity of PHB is as well likely to be mediated through direct interactions with PRRs being expressed, for example, on macrophages and neutrophils (Montalban-Arques et al., 2015). This ligand–receptor interaction activates signal transduction molecules, such as NF-κB, that stimulate immune cells (Song et al., 2014). It has previously been shown that SCFAs like β-HB have immunomodulatory effects in mammals (Dedkova & Blatter, 2014; Kim, Park & Kim, 2014; Shapiro, Thaiss, Levy & Elinav, 2014), resulting from their binding to G protein-coupled receptors (GPRs) (Tazoe et al., 2008) being highly expressed in monocytes and granulocytes (Brestoff & Artis, 2013). Even though specific receptors for SCFAs in fish cells have not yet been described in the literature, gene orthologs of mammalian GPR41 and GPR43 can be found in zebrafish (*Danio rerio*) (Montalban-Arques et al., 2015). Therefore, it can be hypothesized that β-HB can stimulate the immune system in fish as a ligand for GPRs in similar ways as they do in mammals (Montalban-Arques et al., 2015).

The expression of *mhc class IIa* and *mhc class IIb* was significantly upregulated after 10 days of PHB treatment. MHC class II molecules are expressed predominantly by antigen-presenting cells (APCs) such as macrophages, granulocytes and dendritic cells. The presence of antigens triggers the maturation of APCs accompanied by an increased expression of *mhc class II* (Cuesta, Ángeles Esteban & Meseguer, 2006; Delamarre, Holcombe & Mellman, 2003; Knight, Stet & Secombes, 1998). Thus, *mhc class II* expression might be upregulated in sea bass postlarvae fed dietary PHB, as the compound modulates the GI microbiota altering the antigen pattern. After antigens are taken up and degraded within APCs, their peptide fragments are displayed by MHC class II molecules at the cell surface and recognized by $CD4^+$ T cells (Murphy, 2011). In sea

bass larvae reared at 15 ± 1°C, the expression of *cd4* could not be detected until 39 dph but from 51 dph onwards (no measurements were performed between 40 and 50 pdh) (Picchietti et al., 2009). Sea bass larvae analysed here were 38 days old but reared at a higher temperature. Consequently, they most likely were in a developmental stage where *cd4* expression is about to appear. In mammals, the development of T cell precursors into TCR^+ cells expressing CD4 is induced by MHC class II molecules (Anderson, Jenkinson, Moore & Owen, 1993; Ladi, Yin, Chtanova & Robey, 2006; Luckheeram, Zhou, Verma & Xia, 2012), and a similar process is suggested to occur in teleosts as well (Picchietti et al., 2008). Hence, the upregulated *mhc class II* expression observed in the present study might enhance the performance of the still developing adaptive immune system by inducing differentiation of immature T cells into $CD4^+$ T cells.

In conclusion, this study demonstrates that PHB stimulated immune gene expression in sea bass postlarvae, possibly leading to heightened protection against pathogens. Hence, PHB can be considered as a potential biocontrol agent in fish larviculture, being additionally safe for the consumers' health and the environment. The question to what extent PHB could modulate the immune response in fish larvae should be addressed in future studies testing various PHB concentrations and administration times. Furthermore, it would be valuable to investigate the effect of PHB on the entire immune response, for example through transcriptome analyses, which could then be linked to immune and physiological parameters. Additionally, microbiota analyses and challenge tests with pathogenic bacteria should be taken into considerations in follow-up studies to elucidate the link between immune response, intestinal microbiota and disease resistance.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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