Article accepted for publication in Aquaculture Research

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

Running title: Immunostimulation through PHB in sea bass larvae

Andrea Franke[a,*], Catriona Clemmesen[a], Peter De Schryver[b,1], Linsey Garcia-Gonzalez[c], Joanna J. Miest[a,2], Olivia Roth[a]

[a] GEOMAR Helmholtz Centre for Ocean Research Kiel, Evolutionary Ecology of Marine Fishes, Kiel, Germany

[b] Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Ghent, Belgium

[c] Flemish Institute for Technological Research (VITO), Mol, Belgium

[1] Present address: INVE Technologies N.V., Hoogveld 93, Dendermonde, Belgium

[2] Present address: University of Greenwich, Department of Life & Sports Sciences, Chatham Maritime, UK

* Corresponding author: Andrea Franke

Address: GEOMAR Kiel, Department of Evolutionary Ecology of Marine Fishes, Duesternbrooker Weg 20, 24105 Kiel, Germany

E-mail address: andreafranke@gmail.com

Tel.: +49 1577 3856727

Keywords: Immunostimulation, PHB, fish larvae, gene expression, *Dicentrarchus labrax*, aquaculture

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 the present study, the effect of feeding PHB-enriched *Artemia* nauplii on survival, growth and immune response in European sea bass (*Dicentrarchus labrax*) post-larvae was examined. Amorphous PHB was administered to 28 days old sea bass larvae 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 larvae. 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.

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 when the yolk absorption is completed (Magnadottir *et al.* 2005; Swain and Nayak 2009). In European sea bass larvae, for example, maternal IgM was not detectable anymore by day 5 post hatch (Breuil *et al.* 1997). Consequently, vaccination, the most important method for disease prevention in aquaculture, cannot be applied during the larval stages, since their mode of action depends on adaptive immunological memory (Sommerset *et al.* 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 *et al.* 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 (Ringø *et al.* 2011; Akhter *et al.* 2015).

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 *et al.* 2007; Wang *et al.* 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 *et al.* 2009). While it was shown that the cell growth of pathogenic bacteria belonging to genera like *Vibrio* and *Salmonella* (Van Immerseel *et al.* 2003; Defoirdt *et al.* 2007) 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 and Hill 2003). This may explain why gnotobiotic Nile tilapia (*Oreochromis niloticus*) larvae (Situmorang *et al.* 2015) 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 the present study, we hypothesize that PHB stimulates the immune system and improves survival as well as growth performance in European sea bass larvae. We used *Artemia* as live carriers to feed freeze-dried PHB-accumulated bacteria (*Alcaligenes eutrophus*) to sea bass larvae 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.

Materials and methods

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^{-1}, which was gradually decreased to 26 g L^{-1} until 14 dph and increased again afterwards to improve the efficiency of the swim bladder inflation (Saillant *et al.* 2003). The water temperature was increased stepwise from 15 °C 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 *et al.* (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^{-1}. The experiment was started after a three-day acclimation period at 28 dph under the following conditions: temperature 18.5 °C, salinity 32 g L^{-1}, photoperiod 16L: 8D and flow rate 0.4 L min^{-1}. The tank bottoms were siphoned daily to remove dead larvae, feces 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).

Experimental diets and feeding

Over the course of the experiment, starting at 28 dph, the sea bass post-larvae were fed three times a day at 10:00 h, 15:00 h and 20:00 h with instar II *Artemia* nauplii (Micro Artemia Cysts, OCEAN NUTRITION, USA) at densities of 8 mL^{-1}, 4 mL^{-1} and 4 mL^{-1}, respectively. The water flow was turned off for feeding between 10:00 h and 22:00 h. 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^{-1} 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 *et al.* 2000). The PHB solution consisted of freeze-dried PHB accumulated bacteria (*Alcaligenes eutrophus*) dissolved in UV-treated salt water (salinity: 32 g L^{-1}) at a concentration of 10^8 bacteria ml^{-1}. The bacteria had a PHB content of 75% of the cell dry weight and were produced as described in Thai *et al.* (2014).

Measured parameters

Growth performance and survival rate

After 10 days of treatment (38 dph), 20 larvae 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 h 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.

Gene expression analysis

After 3 and 10 days of treatment (31 and 38 dph, respectively) six larvae were randomly sampled from each tank, anaesthetized with MS 222 (SIGMA-ALDRICH, Germany), transferred into RNAlater and kept at 4 °C for 24 h before being stored at -20 °C. These two sampling points were chosen to assess the short-term and the mid-term effects of PHB administration. The first sampling point is crucial to detect potential effects of PHB on the innate immune system since 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 were 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 were 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 96x96 PCR+Melt v2 thermal cycling protocol with a Tm of 60 °C according to the manufacturer´s instructions.

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 & 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 package in R; Oksanen *et al.* 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, since 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 analyze 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. For a graphical representation of gene expression data, the $2^{-\Delta\Delta Ct}$ method (Livak and 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).

Results

Survival

The larval survival rates (Fig. 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, $p > 0.05$). Survival remained above 85% in both treatment groups.

Growth performance

The estimated growth-related parameters, such as total length, dry weight and Fulton´s condition factor K, were not affected by the PHB treatment over the course of the experiment. All parameters are presented in Table 2.

Gene expression

The expression of genes involved in immune response, growth, metabolism, antioxidant activity and stress-response were analyzed 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 showed that the expression of genes related to metabolism and growth (*fad6, tryp, gh, igf1*) differed significantly between fish larvae fed on PHB-enriched *Artemia* or control diet ($F_{1,4} = 23.6$, $p < 0.01$) for 3 days, while all other functional gene groups were not significantly affected by the treatment (Table 3). 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, $p < 0.05$) 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 (Suppl. Table S1).

The application of PHB over a period of 10 days, however, enhanced the immune response of sea bass larvae significantly. The multivariate analysis showed a significant difference between the two treatments for expression of genes involved in innate immunity ($F_{1,4}$ = 9.2, $p < 0.01$) and adaptive immunity ($F_{1,4}$ = 6.9, $p < 0.01$), while all other functional gene groups were not significantly affected by the treatment (Table 3). The subsequent univariate analyses (Suppl. 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, $p < 0.05$; Fig. 3 and 4). While the expression of dicentracin in sea bass larvae fed with a PHB-enriched diet was slightly enhanced (1.7 ± 0.13-fold, ΔCt = 3.1 ± 0.13; F = 10.8) compared to larvae 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 3 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).

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 larvae 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 (Rosenthal and Alderdice 1976; Pepin 1991). 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.* 2015). 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 four weeks (8 to 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 (Sui *et al.* 2012; Hung *et al.* 2015). 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 (Weltzien *et al.* 2000, Azain 2004, Defoirdt *et al.* 2009, De Schryver *et al.* 2010). 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 (Nhan *et al.* 2010, De Schryver *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 post-larvae 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 homeostasis have been addressed (Kepka *et al.* 2014; Miest and 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 fish larvae 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 (Moriyama *et al.* 2000; Carnevali *et al.* 2006). A significantly elevated *igf1* expression alongside a significantly higher weight was e.g. 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 larvae. A dose-dependent growth promoting effect of PHB has been observed in earlier studies, e.g. 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 of the GI pH, which inhibits the growth of certain pathogenic bacteria (Defoirdt *et al.* 2007, De Schryver *et al.* 2010, De Schryver *et al.* 2011). Accordingly, it could be demonstrated that PHB effectively enhances the disease resistance in aquatic invertebrates (Sui *et al.* 2012; Ludevese-Pascual *et al.* 2016) and fish. Dietary PHB protected gnotobiotic Nile tilapia larvae (Situmorang *et al.*

2015) as well as conventional adult Mozambique tilapia (Suguna *et al.* 2014) from pathogens, resulting in higher survival rates after bacterial challenge 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 and Balcázar 2008; Sekirov and 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).

In order to estimate the potential immunomodulatory effect of PHB in fish larvae, the expression of genes involved in the immune response was analyzed 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 larvae 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 defense in teleost fish (Terova *et al.* 2009; Alvarez *et al.* 2014). Their antimicrobial activity has been demonstrated against a broad spectrum of pathogens such as bacteria, viruses and fungi (Salerno *et al.* 2007; Alvarez *et al.* 2014). 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 *et al.* 2014). The immunomodulatory activity of PHB is as well likely to be mediated through direct interactions with PRRs being expressed e.g. 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 and Blatter 2014; Kim *et al.* 2014; Shapiro *et al.* 2014), resulting from their binding to G protein-coupled receptors (GPRs) (Tazoe *et al.* 2008) being highly expressed in monocytes and granulocytes (Brestoff and 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* (Knight *et al.* 1998; Delamarre *et al.* 2003; Cuesta *et al.* 2006). Thus, *mhc class II* expression might be upregulated in sea bass larvae fed dietary PHB, since 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 analyzed 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 *et al.* 1993; Ladi *et al.* 2006; Luckheeram *et al.* 2012), 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 post-larvae, 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, e.g. 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.

Acknowledgements

This work was part of the FINEAQUA-project (grant number 12212001) funded by the Program for the Future - Economy (financed by the European Regional Development Fund, the Federal Republic of Germany and the state of Schleswig-Holstein). Olivia Roth was supported by Deutsche Forschungsgemeinschaft (DFG) and VolkswagenStiftung. Peter de Schryver was supported as a postdoctoral fellow by the Research Fund - Flanders (FWO), Belgium. The authors would like to thank Fabian Wendt for his excellent technical support and Anne Beemelmanns, Franziska Brunner, Isabel Keller and Daniel Bray for statistical advice. Additionally, we would like to thank Ramona Beckmann, Hanna Schade and Martina Stiasny for their help during the experiment and in the laboratory as well as Yasmin Appelhans for proofreading the manuscript. Special thanks go to Robert Tillner.

References

Akhter N., Wu B., Memon A.M. & Mohsin M. (2015) Probiotics and prebiotics associated with aquaculture: A review. *Fish and Shellfish Immunology* **45**, 733–741.

Alvarez C.A., Guzman F., Cardenas C., Marshall S.H. & Mercado L. (2014) Antimicrobial activity of trout hepcidin. *Fish and Shellfish Immunology* **41**, 93–101.

Anderson G., Jenkinson E.J., Moore N.C. & Owen J.J.T. (1993) MHC class II-positive

epithelium and mesenchyme cells are both required for T-cell development in the thymus. *Nature* **362**, 70–73.

Azain M.J. (2004) Role of fatty acids in adipocyte growth and development. *Journal of Animal Science* **82**, 916–924.

Bookout A.L. & Mangelsdorf D.J. (2003) Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. *Nuclear Receptor Signaling* **1**, 1–7.

Brestoff J.R. & Artis D. (2013) Commensal bacteria at the interface of host metabolism and the immune system. *Nature Immunology* **14**, 676–684.

Breuil G., Vassiloglou B., Pepin J.F. & Romestand B. (1997) Ontogeny of IgM-bearing cells and changes in the immunoglobulin M-like protein level (IgM) during larval stages in sea bass (*Dicentrarchus labrax*). *Fish and Shellfish Immunology* **7**, 29–43.

Carnevali O., de Vivo L., Sulpizio R., Gioacchini G., Olivotto I., Silvi S. & Cresci A. (2006) Growth improvement by probiotic in European sea bass juveniles (*Dicentrarchus labrax*, L.), with particular attention to IGF-1, myostatin and cortisol gene expression. *Aquaculture* **258**, 430–438.

Cotter P.D. & Hill C. (2003) Surviving the acid test : Responses of gram-positive bacteria to low pH. *Microbiology and Molecular Biology Reviews* **67**, 429–453.

Cuesta A., Ángeles Esteban M. & Meseguer J. (2006) Cloning, distribution and up-regulation of the teleost fish MHC class II alpha suggests a role for granulocytes as antigen-presenting cells. *Molecular Immunology* **43**, 1275–1285.

De Schryver P., Dierckens K., Bahn Thi Q.Q., Amalia R., Marzorati M., Bossier P., Boon N. & Verstraete W. (2011) Convergent dynamics of the juvenile European sea bass

gut microbiota induced by poly-ß-hydroxybutyrate. *Environmental Microbiology* **13**, 1042–1051.

De Schryver P., Sinha A.K., Kunwar P.S., Baruah K., Verstraete W., Boon N., De Boeck G. & Bossier P. (2010) Poly-β-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, *Dicentrarchus labrax*. *Applied Microbiology and Biotechnology* **86**, 1535–1541.

Dedkova E.N. & Blatter L.A. (2014) Role of ß-hydroxybutyrate, its polymer poly-ß-hydroxybutyrate and inorganic polyphosphate in mammalian health and disease. *Frontiers in Physiology* **5**, 1–22.

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. *Biotechnology Advances* **27**, 680–685.

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

Defoirdt T., Sorgeloos P. & Bossier P. (2011) Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology* **14**, 251–258.

Delamarre L., Holcombe H. & Mellman I. (2003) Presentation of exogenous antigens on major histocompatibility complex (MHC) class I and MHC class II molecules is differentially regulated during dendritic cell maturation. *The Journal of Experimental Medicine* **198**, 111–122.

Dyer A.R., Barlow C.G., Bransden M.P., et al. (2004) Correlation of plasma IGF-I

concentrations and growth rate in aquacultured finfish: a tool for assessing the potential of new diets. *Aquaculture* **236**, 583–592.

Gómez G.D. & Balcázar J.L. (2008) A review on the interactions between gut microbiota and innate immunity of fish. *FEMS Immunology and Medical Microbiology* **52**, 145–154.

Hung N.V., De Schryver P., Thanh T., Garcia-Gonzalez L., Bossier P. & Nevejan N. (2015) Application of poly-β-hydroxybutyrate (PHB) in mussel larviculture. *Aquaculture* **446**, 318–324.

Kepka M., Verburg-van Kemenade B.M.L., Homa J. & Chadzinska M. (2014) Mechanisms involved in apoptosis of carp leukocytes upon invitro and invivo immunostimulation. *Fish and Shellfish Immunology* **39**, 386–395.

Kim C.H., Park J. & Kim M. (2014) Gut microbiota-derived short-chain fatty acids, T cells, and inflammation. *Immune network* **14**, 277–288.

Knight J., Stet R.J. & Secombes C.J. (1998) Modulation of MHC class II expression in rainbow trout *Oncorhynchus mykiss* macrophages by TNF and LPS. *Fish and Shellfish Immunology* **8**, 545–553.

Ladi E., Yin X., Chtanova T. & Robey E.A. (2006) Thymic microenvironments for T cell differentiation and selection. *Nature Immunology* **7**, 338–343.

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. *Veterinary Microbiology* **173**, 310–317.

Livak K.J. & Schmittgen T.D. (2001) Analysis of relative gene expression data using real-

time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **25**, 402–408.

Lucas A. (2011) Package amap: Another Multidimensional Analysis Package. *R package version 0.8-7*.

Luckheeram R.V., Zhou R., Verma A.D. & Xia B. (2012) $CD4^{+}$T Cells: Differentiation and Functions. *Clinical and Developmental Immunology* **2012**, 1–12.

Ludevese-Pascual G., Laranja J.L.Q., Amar E.C., Sorgeloos P., Bossier P. & De Schryver P. (2016) Poly-ß-hydroxybutyrate-enriched *Artemia* sp. for giant tiger prawn *Penaeus monodon* larviculture. *Aquaculture Nutrition*, 1–8.

Magnadottír B. (2006) Innate immunity of fish (overview). *Fish and Shellfish Immunology* **20**, 137–151.

Magnadottir B., Lange S., Gudmundsdottir S., Bøgwald J. & Dalmo R.A. (2005) Ontogeny of humoral immune parameters in fish. *Fish and Shellfish Immunology* **19**, 429–439.

Makridis P., Fjellheim A.J., Skjermo J. & Vadstein O. (2000) Control of the bacterial flora of *Brachionus plicatilis* and *Artemia franciscana* by incubation in bacterial suspensions. *Aquaculture* **185**, 207–218.

Miest J.J. & Hoole D. (2015) Time and concentration dependency of MacroGard® induced apoptosis. *Fish and Shellfish Immunology* **42**, 363–366.

Mitter K., Kotoulas G., Magoulas A., et al. (2009) Evaluation of candidate reference genes for QPCR during ontogenesis and of immune-relevant tissues of European seabass (*Dicentrarchus labrax*). *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* **153**, 340–347.

Montalban-Arques A., De Schryver P., Bossier P., Gorkiewicz G., Mulero V., Gatlin D.M. & Galindo-Villegas J. (2015) Selective manipulation of the gut microbiota improves immune status in vertebrates. *Frontiers in Immunology* **6**, 1–14.

Moriyama S., Ayson F.G. & Kawauchi H. (2000) Growth regulation by insulin-like growth factor-I in fish. *Bioscience, Biotechnology, and Biochemistry* **64**, 1553–1562.

Murphy K. (2011) *Janeway's Immunobiology*, 8th edn. Garland Science.

Najdegerami E.H., Bakhshi F., Tokmechi A., Shiri Harzevili A., Sorgeloos P. & Bossier P. (2015a) Dietary effects of poly-β-hydroxybutyrate on the growth performance, digestive enzyme activity, body composition, mineral uptake and bacterial challenge of rainbow trout fry (*Oncorhynchus mykiss*). *Aquaculture Nutrition*, 1–8.

Najdegerami E.H., Baruah K., Shiri A., Rekecki A., Van den Broeck W., Sorgeloos P., Boon N., Bossier P. & De Schryver P. (2015b) Siberian sturgeon (*Acipenser baerii*) larvae fed *Artemia* nauplii enriched with poly-ß-hydroxybutyrate (PHB): effect on growth performance, body composition, digestive enzymes, gut microbial community, gut histology and stress tests. *Aquaculture Research* **46**, 801–812.

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.

Oksanen J., Blanchet F.G. & Kindt R. (2012) Package Vegan: Community Ecology Package. Available at: https://cran.r-project.org/web/packages/vegan/index.html.

Pepin P. (1991) Effect of temperature and size on development, mortality, and survival rates of the pelagic early life history stages of marine fish. *Can. J. Fish. Aquat. Sci.*

48, 503–518.

Picchietti S., Guerra L., Buonocore F., Randelli E., Fausto A.M. & Abelli L. (2009) Lymphocyte differentiation in sea bass thymus: CD4 and CD8-α gene expression studies. *Fish and Shellfish Immunology* **27**, 50–56.

Picchietti S., Guerra L., Selleri L., et al. (2008) Compartmentalisation of T cells expressing CD8α and TCRβ in developing thymus of sea bass *Dicentrarchus labrax* (L.). *Developmental and Comparative Immunology* **32**, 92–99.

Ringø E., Olsen, R.E., Gonzalez Vecino J.L., Wadsworth S. & Song S.K. (2011) Use of immunostimulants and nucleotides in aquaculture: a review. *Journal of Marine Science: Research & Development* **2**, 1–22.

Rosenthal H. & Alderdice D.F. (1976) Sublethal effects of environmental stressors, natural and pollutional, on marine fish eggs and larvae. *Journal of the Fisheries Research Board of Canada* **33**, 2047–2065.

Saillant E., Fostier A., Haffray P., Menu B. & Chatain B. (2003) Saline preferendum for the European sea bass, *Dicentrarchus labrax*, larvae and juveniles: Effect of salinity on early development and sex determination. *Journal of Experimental Marine Biology and Ecology* **287**, 103–117.

Salerno G., Parrinello N., Roch P. & Cammarata M. (2007) cDNA sequence and tissue expression of an antimicrobial peptide, dicentracin; a new component of the moronecidin family isolated from head kidney leukocytes of sea bass, *Dicentrarchus labrax*. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* **146**, 521–529.

Sarropoulou E., Sepulcre P., Poisa-Beiro L., et al. (2009) Profiling of infection specific

mRNA transcripts of the European seabass *Dicentrarchus labrax*. *BMC Genomics* **10**, 1-18.

Sekirov I. & Finlay B.B. (2009) The role of the intestinal microbiota in enteric infection. *The Journal of Physiology* **587**, 4159–4167.

Shapiro H., Thaiss C.A., Levy M. & Elinav E. (2014) The cross talk between microbiota and the immune system: metabolites take center stage. *Current Opinion in Immunology* **30**, 54–62.

Situmorang M.L., De Schryver P., Dierckens K. & Bossier P. (2016) Effect of poly-β-hydroxybutyrate on growth and disease resistance of Nile tilapia *Oreochromis niloticus* juveniles. *Veterinary Microbiology* **182**, 44-49.

Sommerset I., Krossøy B., Biering E. & Frost P. (2005) Vaccines for fish in aquaculture. *Expert review of vaccines* **4**, 89–101.

Song S.K., Beck B.R., Kim D., Park J., Kim J., Kim H.D. & Ringø, E. (2014) Prebiotics as immunostimulants in aquaculture: A review. *Fish and Shellfish Immunology* **40**, 40–48.

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

Sui L., Cai J., Sun H., Wille M. & Bossier P. (2012) Effect of poly-β-hydroxybutyrate on Chinese mitten crab, *Eriocheir sinensis*, larvae challenged with pathogenic *Vibrio anguillarum*. *Journal of Fish Diseases* **35**, 359–364.

Suriyamongkol P., Weselake R., Narine S., Moloney M. & Shah S. (2007)

Biotechnological approaches for the production of polyhydroxyalkanoates in microorganisms and plants — A review. *Biotechnology Advances* **25**, 148–175.

Swain P. & Nayak S.K. (2009) Role of maternally derived immunity in fish. *Fish and Shellfish Immunology* **27**, 89–99.

Tazoe H., Otomo Y., Kaji I., Tanaka R., Karaki S.-I. & Kuwahara A. (2008) Roles of short-chain fatty acid receptors, GPR41 and GPR43 on colonic functions. *Journal of Physiology and Pharmacology* **59**, 251–262.

Terova G., Forchino A., Rimoldi S., Brambilla F., Antonini M. & Saroglia M. (2009) Bio-Mos: An effective inducer of dicentracin gene expression in European sea bass (*Dicentrarchus labrax*). *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* **153**, 372–377.

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

Therneau T. (2015) Package Survival: A Package for Survival Analysis in R. *R package version 2.38*.

Tillner R., Rønnestad I., Dhert P. & Ueberschär B. (2014) The regulatory loop between gut cholecystokinin and tryptic enzyme activity in sea bass (*Dicentrarchus labrax*) larvae is influenced by different feeding regimes and trigger substances. *Aquaculture* **420–421**, 139–146.

Torrecillas S., Montero D. & Izquierdo M. (2014) Improved health and growth of fish fed mannan oligosaccharides: Potential mode of action. *Fish and Shellfish*

Immunology **36**, 525–544.

Vadstein O. (1997) The use of immunostimulation in marine larviculture: Possibilities and challenges. *Aquaculture* **155**, 401–417.

Vadstein O., Bergh Ø., Gatesoupe F.-J., Galindo-Villegas J., Mulero V., Picchietti S., Scapigliati G., Makridis P., Olsen Y., Dierckens K., Defoirdt T., Boon N., De Schryver P. and Bossier P. (2012) Microbiology and immunology of fish larvae. *Reviews in Aquaculture* **4**, 1–25.

Van Immerseel F., De Buck J., Pasmans F., et al. (2003) Invasion of *Salmonella enteritidis* in avian intestinal epithelial cells in vitro is influenced by short-chain fatty acids. *International Journal of Food Microbiology* **85**, 237–248.

Wang B., Sharma-Shivappa R.R., Olson J.W. & Khan S.A. (2012) Upstream process optimization of polyhydroxybutyrate (PHB) by *Alcaligenes latus* using two-stage batch and fed-batch fermentation strategies. *Bioprocess and Biosystems Engineering* **35**, 1591–1602.

Weltzien F.A., Hemre G.I., Evjemo J.O., Olsen Y. & Fyhn H.J. (2000) β-Hydroxybutyrate in developing nauplii of brine shrimp (*Artemia franciscana* K.) under feeding and non-feeding conditions. *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology* **125**, 63–69.

Fig. 1. Kaplan-Meier survival curves of sea bass larvae 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.

Fig. 2. Gene expression of insulin-like growth factor 1 (*igf1*) in sea bass larvae 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 (*: $p < 0.05$).

Fig. 3. Gene expression of dicentracin (*dic*) and hepcidin (*hep*) in sea bass larvae 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 (*: $p < 0.05$).

Fig. 4. Gene expression of *mhc class IIa* and *mhc class IIb* in sea bass larvae 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 (*: $p < 0.05$).

Table 1

Name, abbreviation and function of the 26 genes of interest and 3 reference genes. Genes were divided into the following 5 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	Sarropoulou et al. 2009
				RV	AGCCTGACCTTGCTCACTGT	
		cc1	CC chemokine 1, chemotactic cytokine	FW	TGGGTTCGCCGCAAGGTTGTT	AM490065.1
				RV	AGACAGTAGACGAGGGGACCACAGA	
		cox2	Cyclo-Oxygenase-2, pro-inflammatory enzyme	FW	AGCACTTCACCCACCAGTTC	AJ630649.1
				RV	AAGCTTGCCATCCTTGAAGA	
		Ifna1	Interferon, cytokine	FW	GTACAGACAGGCGTCCAAAGCATCA	AM765846.2
				RV	CAAACAGGGCAGCCGTCTCATCAA	
		il1b	Interleukin 1 beta, pro-inflammatory cytokine	FW	GCGACATGGTGCGATTTCTCTTCTACA	AJ311925.1
				RV	GCTGTGCTGATGTACCAGTTGCTGA	
		dic	Dicentracin, antimicrobial peptide	FW	AGTGCGCCACGCTCTTTCTTGT	AY303949.1
				RV	TTGTGGATGGACTTGCCGACGTG	
		fer	Ferritin, antimicrobial peptide	FW	ATGCACAAGCTCTGCTCTGA	Sarropoulou et al. 2009
				RV	TTTGCCCAGGGTGTGTTTAT	
		hep	Hepcidin, antimicrobial peptide	FW	AAGAGCTGGAGGAGCCAATGAGCA	DQ131605.1
				RV	GACTGCTGTGACGCTTGTGTCTGT	
		tlr1	Toll-like receptor 1, pattern recognition receptor	FW	GCCTCTGCCTCAATACCTGATCCCA	KX399287
				RV	AACAACCTGTGCTTGGCCCTGTC	
		tlr9	Toll-like receptor 9, pattern recognition receptor	FW	TCTTGGTTTGCCGACTTCTTGCGT	KX399289
				RV	TACTGTTGCCCTGTTGGGACTCTGG	
		tnfa	Tumor necrosis factor α, pro-inflammatory cytokine	FW	AGCCACAGGATCTGGAGCTA	DQ070246.1
				RV	GTCCGCTTCTGTAGCTGTCC	
	Adaptive immunity	*mhc class Ia*	Major Histocompatibility Complex I α, cell surface molecules	FW	TGTACGGCTGTGAGTGGGATGATGAG	JX171695.1
				RV	AGCCTGTGGTCTTGGAGCGATGAA	
		mhc class IIa	Major Histocompatibility Complex II α, cell surface molecules	FW	AGTCCGATGATCTACCCCAGAGACAAC	FN667955.1
				RV	ACAGGAGCAGGATAGAAACCAGTCACA	

		mhc class IIb	Major Histocompatibility Complex II ß, cell surface molecules	FW	GCTGGCAGACGCTGATTGGTTCT	AM113471.1
				RV	TAACCAGAGGTTCTCTCAGGCTGGC	
		rag1	Recombination activating protein 1, involved in VDJ recombination	FW	CCAATTACCTGCACAAGACCCTGGC	FN687463.1
				RV	GTTTGTTTGCCGACTCGTTCCCCT	
	Complement system	*c3*	Complement Component C3, classical & alternative pathway	FW	TGACGGAGAGCGGTGGTGAAATG	HM563078.1
				RV	AGGCCATCCCTGGTTTGAAGTATTTGG	
		cla	C-Lectin-A, lectin pathway	FW	GATGGCAGCAAGCTCCGGTATTCA	EU660935.1
				RV	TCTGACCTATGACCCCAGCCAACA	
		gal	Galectin, lectin pathway	FW	TGCAACTCTTACCAGGGAGGCAACT	EU660937.1
				RV	GTCACGAGGAACTCTGTAGGGGTGA	
	Apoptosis	*casp3*	Caspase 3, protease	FW	CTGATTTGGATCCAGGCATT	DQ345773.1
				RV	CGGTCGTAGTGTTCCTCCAT	
		casp9	Caspase 9, protease	FW	GGCAGGACTCGACGAGATAG	DQ345776.1
				RV	CTCGCTCTGAGGAGCAAACT	
Growth & 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	

Table 2

Growth-related parameters of sea bass larvae fed with *Artemia* enriched with or without PHB over a period of 10 days (from 28 to 38 dph). Values represent mean ± SEM. In addition, *F*-statistics (*F*) and p-values (p) calculated with a mixed-effect model are shown. Degrees of freedom/residual degrees of freedom: 1/4.

Growth-related parameters	Control	PHB treatment	*F*	p
Dry weight (mg)	2.6 ± 0.12	2.0 ± 0.11	3.98	0.12
Total length (cm)	1.4 ± 0.02	1.4 ± 0.02	0.90	0.40
Condition[1] (mg cm^{-3})	0.9 ± 0.02	0.8 ± 0.02	7.31	0.06

[1] Fulton´s condition factor K = (W/L^3)

Table 3

PERMANOVA results for larval gene expression profiles. The permutational multivariate analysis of variance is based on Pearson correlation distance matrix. The effect of the experimental treatment (PHB administration) on overall gene expression levels was tested for different functional gene groups. *F*-statistics (*F*) and p-values (p) are shown. Degrees of freedom/residual degrees of freedom for all groups: 1/4.

	3 days of treatment (31 dph)		10 days of treatment (38 dph)	
Functional group	*F*	p	*F*	p
Overall immune response	1.9	> 0.05	6.5	> 0.05
Innate immunity	3.1	> 0.05	9.2	**< 0.01**
Adaptive immunity	1.8	> 0.05	6.9	**< 0.01**
Growth & metabolism	23.6	**< 0.01**	1.1	> 0.05
Stress	3.6	> 0.05	3.4	> 0.05

Table S1

Univariate statistical results (mixed-effect model) for larval gene expression at 31 dph and 38 dph. *F*-statistics (*F*) and p-values (p) are shown. Degrees of freedom/residual degrees of freedom: 1/4.

		3 days of treatment (31 dph)	
Functional group	**Gene**	*F*	p
Growth and metabolism	*gh*	0.86	0.41
	igf1	9.80	0.04
	fad6	1.18	0.34
	tryp	3.61	0.13
		10 days of treatment (38 dph)	
Functional group	**Gene**	*F*	p
Innate immunity	*apoA1*	2.10	0.22
	cc1	4.60	0.10
	cox2	2.43	0.19
	ifn	3.61	0.13
	il1b	4.46	0.10
	dic	10.85	**0.03**
	fer	3.46	0.14
	hep	15.36	**0.02**
	tlr1	1.95	0.24
	tlr9	1.00	0.38
	tnfa	1.12	0.35
Adaptive immunity	*mhc class Ia*	0.75	0.44
	mhc class IIa	14.35	**0.02**
	mhc class IIb	8.27	**0.04**
	rag1	1.58	0.28

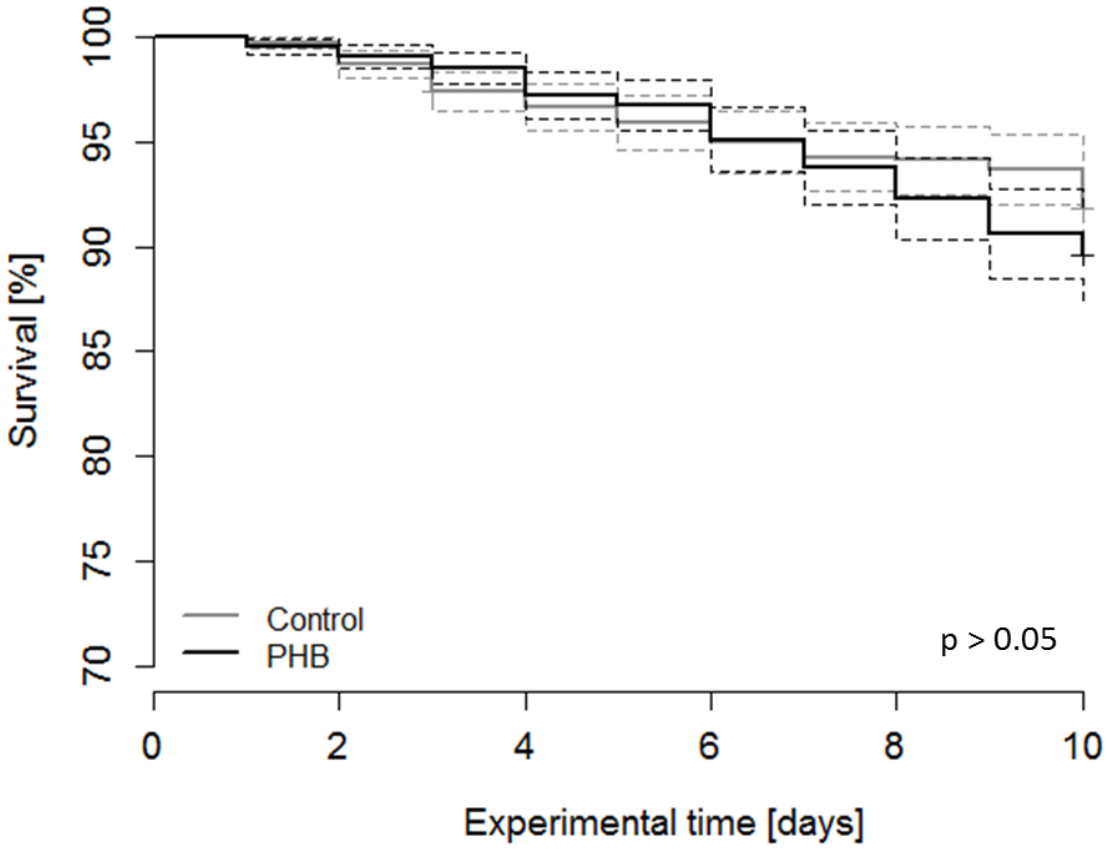

Fig. 1. Kaplan-Meier survival curves of sea bass larvae fed with *Artemia* enriched with PHB (black) or without PHB (grey) over a period of 10 days (from 28 to 38 dph). The dashed lines represent the 95% confidence intervals.

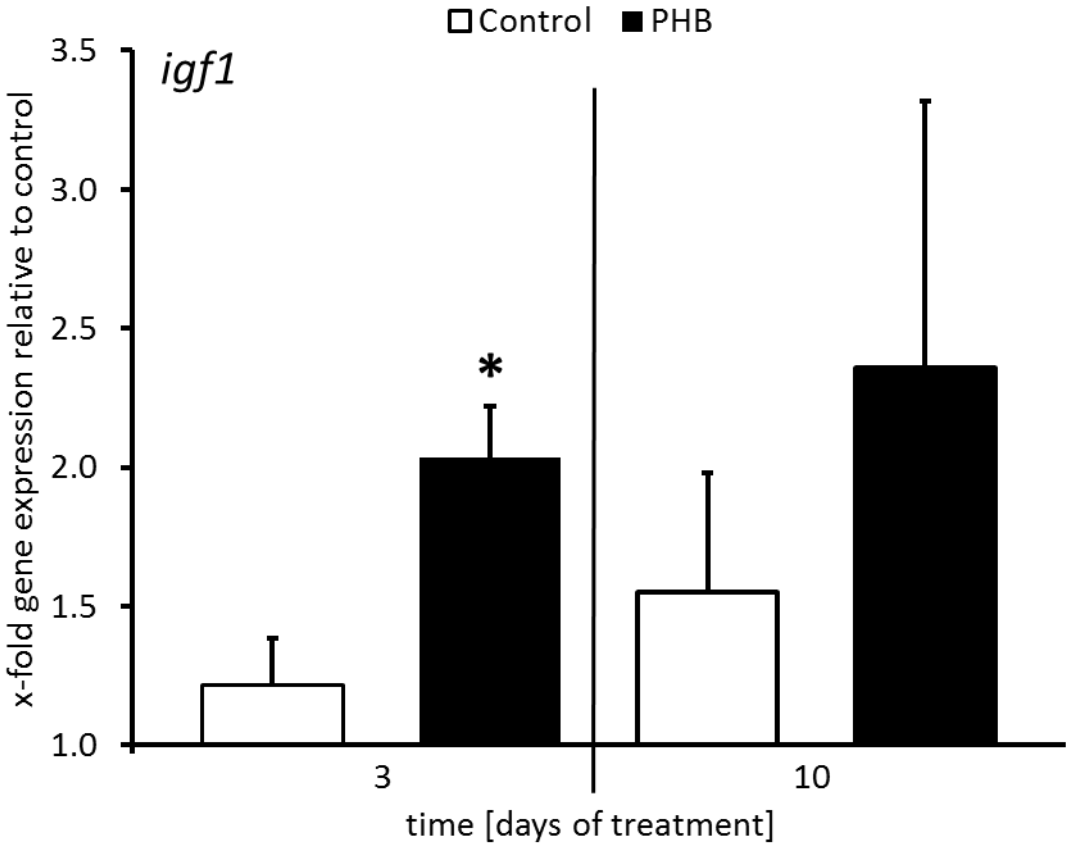

Fig. 2. Gene expression of insulin-like growth factor 1 (*igf1*) in sea bass larvae fed with *Artemia* nauplii with PHB (black bars) or without PHB enrichment (white bars). Larval samples were taken at 31 and 38 dph (3 and 10 days of treatment, respectively). The figure displays the x-fold gene expression to the control. Data are presented as mean ± SEM. The asterisk represents the level of significance (*: $p < 0.05$).

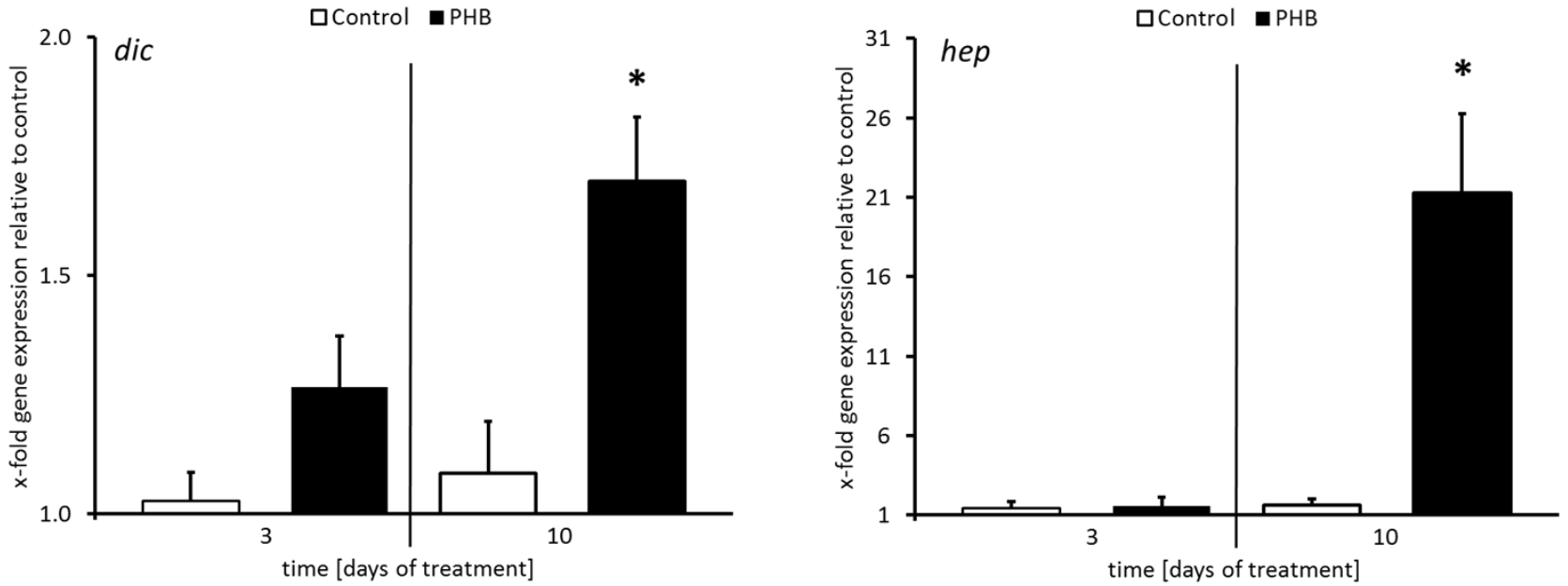

Fig. 3. Gene expression of dicentracin (*dic*) and hepcidin (*hep*) in sea bass larvae fed with *Artemia* nauplii with PHB (black bars) or without PHB enrichment (white bars). Larval samples were taken at 31 and 38 dph (3 and 10 days of treatment, respectively). The figures display the x-fold gene expression to the control. Data are presented as mean ± SEM. The asterisk represents the level of significance (*: $p < 0.05$).

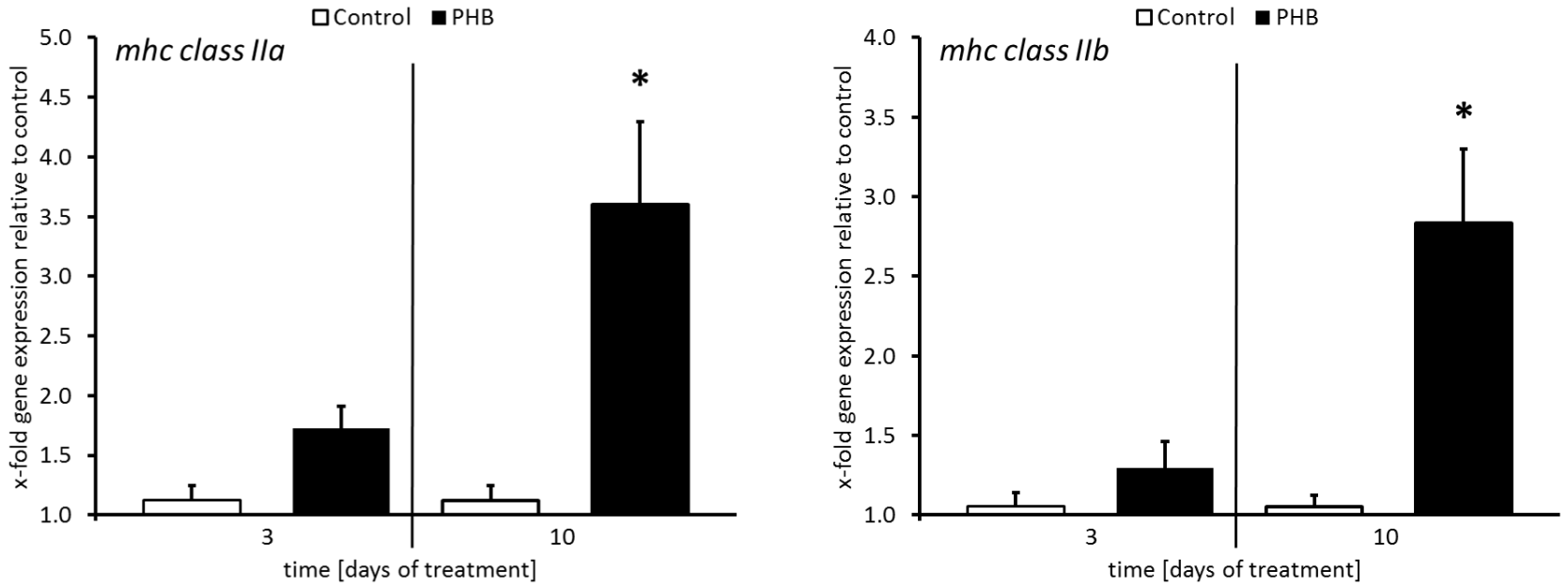

Fig. 4. Gene expression of *mhc class IIa* and *mhc class IIb* in sea bass larvae fed with *Artemia* nauplii with PHB (black bars) or without PHB enrichment (white bars). Larval samples were taken at 31 and 38 dph (3 and 10 days of treatment, respectively). The figure displays the x-fold gene expression to the control. Data are presented as mean ± SEM. The asterisk represents the level of significance (*: $p < 0.05$).