Immunostimulation in Early Life Stages of European Sea Bass (*Dicentrarchus labrax*)

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Life
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Summary

The reliable production of high quality juvenile fish is still hampered in marine aquaculture due to the enormous mortality in the larval stages, which is often caused by the outbreak of infectious diseases. Early life stages are extremely susceptible towards pathogens because they lack a mature immune system. Conventional disease prevention relies on the prophylactic use of antibiotics, selecting for antibiotic-resistant bacteria making treatments less and less efficient. Accordingly, the development of alternative health management strategies is one of the key factors for the progress towards a more sustainable aquaculture industry. A possibility to increase the disease resistance in early developmental stages is the stimulation of the immune system by steering the gastrointestinal microbiota. Even though it is recognized that a healthy gastrointestinal microbiota is a prerequisite for the host´s well-being, the mechanisms underlying the interdependency between gastrointestinal microbiota and immunity are not very well understood in fish. In my thesis, I explored different possibilities to stimulate the immune system in early life stages of European sea bass (*Dicentrarchus labrax*), a key species in European aquaculture. I investigated immunological processes on a cellular and molecular level as well as the larval microbiota, life history traits and disease resistance.

As a first step, I assessed the immunostimulatory potential of the probiotic bacterium *Bacillus subtilis* (strain NCIMB 3610) in juvenile sea bass. I enriched *Artemia* nauplii with the probiont and fed the juveniles over a period of 14 days. To evaluate the effect on the immune system, I combined immunological methods on the cellular and the molecular level. The cellular immune parameters, such as the relative number of monocytes and lymphocytes, and the proliferation of lymphocytes were examined in the spleen (central immune organ) and the blood (peripheral immune activity). Moreover, the expression of genes involved in innate, complement and adaptive immunity as well as growth was analyzed. Yet, no effect of *B. subtilis* on the immune response was detectable. However, the probiont influenced the expression of the main proteolytic enzyme trypsin.

As a next step, I investigated the capacity of poly-β-hydroxybutyrate (PHB) to stimulate the developing immune system in the most fragile life cycle stages of sea bass: the larval stages. To trigger the larval immune system at the earliest possible point in time, 2 different doses of PHB were applied from mouth opening (5 days post hatch) onwards until 22 days post hatch. I found evidence that PHB alters the larva-associated

microbiota composition as well as the expression of the antimicrobial peptides ferritin and dicentracin. Remarkably, PHB improved the survival of sea bass yolk-sac larvae making its application highly interesting for commercial hatcheries.

In a subsequent experiment, I fed PHB-enriched *Artemia* nauplii to sea bass post-larvae (28 days post hatch) over a period of 10 days and found evidence that PHB is able to stimulate the larval immune system. I observed an upregulation of genes involved in innate as well as adaptive immunity and growth. More precisely, the expression of the antimicrobial peptides dicentracin and hepcidin as well as the major histocompatibility complex class II and the insulin-like growth factor 1 was enhanced, which may improve robustness and increase resistance towards diseases in sea bass larvae.

The results of the first study indicate that the probiotic effects previously described for *B. subtilis* (NCIMB 3610) in sea bass might be attributed to direct antagonism towards pathogenic intestinal bacteria, a probiotic feature aside from immunostimulation. The findings demonstrate the complex nature of the efficacy of potential probionts and point out the necessity to disentangle the link between the gastrointestinal microbiota composition and the immune response in future research. Further, the second and third study provide first insights into the capability of PHB to improve survival and act as an immunostimulator in sea bass larvae, making the compound particularly valuable for sea bass hatcheries. The results indicate that PHB can be considered as a candidate immunoprophylactic measure in marine larviculture.

Zusammenfassung

Ein Engpass in der marinen Aquakultur ist die zuverlässige Produktion von Setzlingen, da larvale Stadien eine extrem hohe Sterblichkeit aufweisen, die oft durch den Ausbruch infektiöser Krankheiten bedingt ist. Frühe Lebensstadien sind extrem anfällig gegenüber Pathogenen, da ihr Immunsystem noch nicht voll funktionsfähig ist. Die (prophylaktische) Vergabe von Antibiotika hat zum Auftreten antibiotikaresistenter Bakterien geführt, die Antibiotikabehandlungen zunehmend unwirksam werden lassen. Dementsprechend ist die Entwicklung alternativer Methoden zum Vorbeugen von Krankheiten zwingend notwendig, um eine nachhaltige Weiterentwicklung des Aquakultursektors zu gewährleisten. Eine Möglichkeit um die Resistenz gegenüber Krankheiten in frühen Lebensstadien zu steigern, ist die Stimulierung des Immunsystems durch die Manipulation der gastrointestinalen Mikrobiota. Es ist bekannt, dass eine gesunde gastrointestinale Mikrobiota eine der Grundvoraussetzungen für das Wohlbefinden von Fischen ist. Allerdings ist die gegenseitige Wechselwirkung zwischen den Darmbakterien und dem Immunsystem in Fischen bisher kaum untersucht worden.

In meiner Doktorarbeit habe ich verschiedene Möglichkeiten erforscht, um das Immunsystem von frühen Lebensstadien des Europäischen Wolfsbarsches (*Dicentrarchus labrax*) zu stimulieren. Dabei habe ich nicht nur die Immunantwort auf der zellulären und molekularen Ebene, sondern auch Überlebensraten, Wachstum, Krankheitsresistenz und die larvale Mikrobiota untersucht.

In meinem ersten Experiment habe ich das immunstimulierende Potential des probiotischen Bakteriums *Bacillus subtilis* (Stamm NCIMB 3610) in juvenilen Wolfsbarschen erfasst. Dafür habe ich Lebendfutter mit *B. subtilis* angereichert und es über einen Zeitraum von 14 Tagen an die Fische verfüttert. Daraufhin habe ich zelluläre Immunparameter in der Milz und im Blut, sowie die Expression von Immun- und Wachstumsgenen gemessen. Dabei konnte kein Effekt auf die Immunantwort festgestellt werden, allerdings war die Expression des Verdauungsenzyms Trypsin von der Vergabe der probiotischen Bakterien beeinflusst.

In darauffolgenden Experimenten habe ich die Kapazität von Polyhydroxybuttersäure (PHB), das sich entwickelnde Immunsystem von Wolfsbarschlarven zu stimulieren, analysiert. Dazu habe ich PHB in 2 verschiedenen Dosen an Dottersacklarven verabreicht, von dem Moment an als sie ihr Maul öffneten (5 Tage nach dem Schlupf).

Ich fand Hinweise darauf, dass PHB einen Einfluss auf die Mikrobiota der Larven hat und das Expressionsmuster von Immungenen verändert. PHB hat weiterhin die Überlebensraten der Wolfsbarschlarven gesteigert, was ein enorm wertvolles Ergebnis für die kommerzielle Aufzucht von Wolfsbarschlarven darstellt.

Des Weiteren habe ich PHB an 28 Tage alte Wolfsbarschlarven über einen Zeitraum von 10 Tagen verfüttert, wobei ich eine Stimulierung des larvalen Immunsystems feststellen konnte. Sowohl Gene des angeborenen als auch erworbenen Immunsystems und zusätzlich ein Wachstumsgen wurden durch PHB hochreguliert. Dabei handelte es sich um die antimikrobiellen Peptide Dicentracin und Hepcidin, den Haupthistokompatibilitätskomplex Klasse II und den Insulinähnlichen Wachstumsfaktor 1. Damit konnte ich zeigen, dass PHB das Vermögen besitzt, das sich noch in der Entwicklung befindende Immunsystem von Wolfsbarschlarven zu stimulieren. Die beobachtete Anregung des larvalen Immunsystems könnte mit einer erhöhten Resistenz gegenüber Krankheiten einhergehen.

Die Resultate meiner ersten Studie indizieren, dass der probiotische Effekt, der für *B. subtilis* (NCIMB 3610) bei Wolfsbarschen beschrieben ist, eventuell auf seine direkte antagonistische Wirkung gegen pathogene Bakterien statt auf Immunstimulation zurückzuführen ist. Damit untermauern meine Ergebnisse wie hochgradig komplex die Wirkungsweise von potenziell probiotischen Bakterien ist. Die zweite und dritte Studie geben erste Einblicke in die Fähigkeit von PHB, das Überleben und die Immunantwort von Wolfsbarschlarven verbessern zu können. Diese Erkenntnisse zeichnen PHB als potentiellen Kandidaten für immunstimulierende Maßnahmen während der Aufzucht von marinen Fischlarven aus.

Introduction

1. Seafood & global food security

The ocean is home to a tremendously diverse spectrum of species. It has been harvested since prehistoric times and has, thus, been and remains an essential source of food for the world´s population. Seafood is still the major protein source for many people in developing countries that they can access and afford (Pauly & Zeller 2016). Seafood, in particular fish, has an excellent nutritional profile due to its high content of protein, unsaturated fatty acids, vitamins and minerals (FAO 2016a). By 2050, the world´s population is predicted to be 30% higher than today and the associated increasing demand for food will raise the already high pressure on natural resources (Jennings et al. 2016). However, the stocks of most commercially exploited fish species are already fully fished or even overfished (Worm & Branch 2012, Cardinale et al. 2013) (Fig. 1, top). This has led to the continuous stagnation of capture fisheries over the last decades, which is accompanied by a heavily growing aquaculture industry (Fig. 1, bottom).

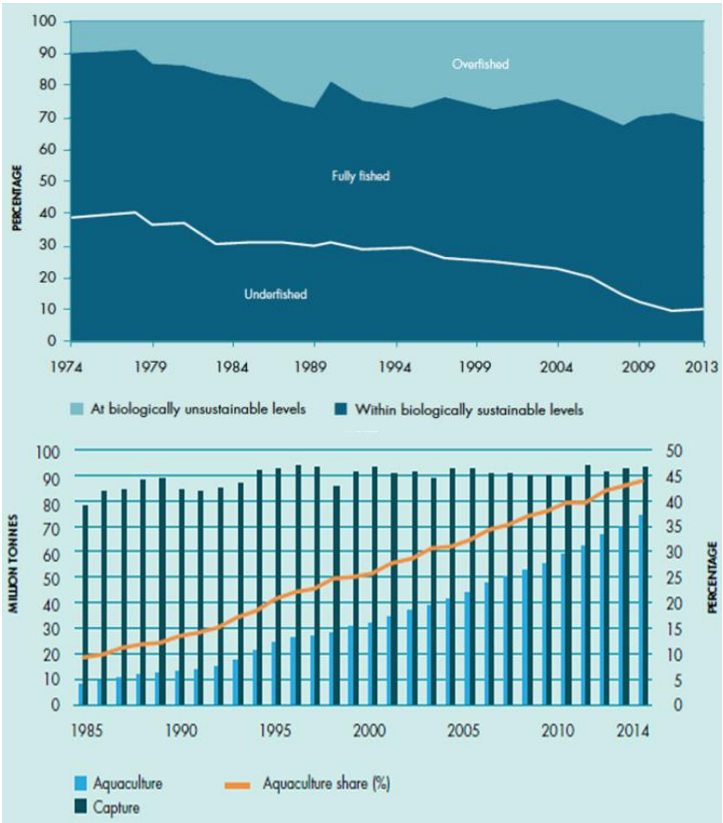


Fig. 1. Top: Global trends in the state of world marine fish stocks since 1974. **Bottom:** Global share of aquaculture in total production of aquatic animals. Both figures published by the FAO in 2016 (FAO 2016a).

The farming of aquatic organisms started as far as 6000 years ago (Builth et al. 2008). Nevertheless, aquaculture only recently became the fastest growing food producing sector worldwide. Whether or not fish farming can relieve the pressure on wild fisheries is an ongoing controversy (Naylor et al. 2000, Cao et al. 2015) far beyond the scope of this thesis and, hence, cannot be addressed here in detail. However, it has been stressed by natural scientists and economists that the prerequisite to avoid the collapse of the majority of stocks is to significantly improve the effectiveness of fisheries management (Quaas et al. 2016). Moreover, it has been criticized that intensive culture practices exert adverse environmental, socio-economic and health-related impacts (Cole et al. 2009, Martinez-Porchas & Martinez-Cordova 2012). As a consequence, researchers and policymakers all over the world are being challenged to develop new strategies for sustainable aquaculture practices (Diana et al. 2013, Jones et al. 2015). Furthermore, comprehensive policies and integrated management approaches are essential to ensure a responsible aquaculture development (Krause et al. 2015).

2. Infectious diseases in finfish aquaculture

Water is a much better medium for the growth of microorganisms than air (typically a million bacteria are present per milliliter seawater) (Gomez et al. 2013, Martin et al. 2016). Consequently, fish live in a pathogen-rich environment, where intensified rearing conditions additionally facilitate the outbreak and transmission of infectious diseases (Murray & Peeler 2005). A typical characteristic of intensive culture practices are high stocking densities, which induce crowding-stress, possibly leading to immune suppression and, ultimately, infections that can easily be transmitted from one fish to the other (Montero et al. 1999, Tort 2011). Bacterial and viral infections cause global economic losses in the range of several billion US$ per year and, consequently, are the most significant constraint to the development of the aquaculture industry (Defoirdt et al. 2011). Early life stages are most susceptible towards infectious diseases since they lack a mature immune system (Vadstein et al. 2012).

One of the most ubiquitous diseases is vibriosis (Toranzo et al. 2005). It is found in numerous cultured and wild fish species (Lafferty et al. 2015). The fatal septicemic disease is caused by several species of the bacterial genus *Vibrio* such as *V. anguillarum, V. harveyi* and *V. campbellii*. While not all strains of these species are pathogenic, some are highly virulent (Defoirdt, Boon, et al. 2007). Symptoms of vibriosis are hemorrhages,

diarrhea, skin lesions and tissue necrosis, resulting in mass mortalities reaching up to 100% (Frans et al. 2011).

The routine measure to combat infectious diseases in aquaculture is the application of antibiotics. Further, prophylactic antibiotics are used in a widespread and often unrestricted way to prevent diseases. This severe overuse of antimicrobial drugs has resulted in the emergence of antibiotic-resistant pathogenic bacteria, making treatments increasingly ineffective (Cabello 2006). Additionally, residual antibiotics remain in the environment surrounding aquaculture sites where they also select for antibiotic-resistant bacteria (Gormaz et al. 2014). Arising resistance genes can potentially be transmitted to terrestrial bacteria, including human and animal pathogens, by horizontal gene transfer. Additionally, drug-resistant pathogens might infect humans and animals directly (Heuer et al. 2009). Thus, the overuse of antibiotics does not only make disease control less and less efficient but is also a threat to the environment and the public health.

Vaccines play a crucial role in preventing diseases in aquaculture. However, their effect depends on immunological memory, a feature of adaptive immunity. They are, thus, not applicable during early developmental stages lacking a mature immune system (Sommerset et al. 2005). Moreover, there are a number of limitations concerning vaccine development and utilization. For instance, their mode of action is limited to one specific pathogen, often making them cost-ineffective (Sommerset et al. 2005, Ringø et al. 2014).

Hence, novel strategies for disease prevention and control are of paramount importance for the advancement of the aquaculture sector. Alternative biocontrol measures to successfully protect aquaculture organisms from diseases are mostly still in the research phase and include phage therapy, anti-virulence therapy (inhibition of virulence gene expression) and the administration of immunostimulating substances (Defoirdt et al. 2011). In my thesis, I focused on the latter.

3. The immune system of teleost fish

The immune response is the host´s defense against infections by pathogens, leading to disease protection (Murphy 2011). The prerequisite for this is the host´s ability to discriminate “self” from “nonself” by recognition of conserved molecular patterns (Medzhitov & Janeway 2002). The immune system of teleost fish consists of linked

innate and adaptive immunity, both encompassing cellular and humoral components (Foey & Picchietti 2014). The innate immune response is an evolutionarily ancient form of host defense (Janeway & Medzhitov 2002), while the adaptive immune system emerged only approx. 500 million years ago in jawed fish (Flajnik & Kasahara 2010). However, early life stages rely solely on innate immunity during their first weeks or month (depending on the species) until adaptive immune cells have finally developed (Rombout et al. 2005).

The epithelial mucosa of the skin, the gills and the digestive tract is the first barrier that pathogens encounter. Mucosal immunity compromises an array of innate and adaptive immune cells and molecules acting in concert to protect the host against pathogenic invasion (Gomez et al. 2013). An example for mucosa-associated lymphoid tissues (MALTs) is the gut-associated lymphoid tissue (GALT). Besides MALTs, central lymphoid organs such as thymus, head kidney and spleen play a crucial role in the teleost immune system (Tort et al. 2003). In European sea bass (*Dicentrachus labrax*), the GALT and the thymus were demonstrated to be the first tissue, respectively, organ to become lymphoid (Picchietti et al. 1997).

Innate immune mechanisms are immediately activated (within hours) and compose the first line of defense (Van Muiswinkel & Nakao 2014). Innate immune cells (phagocytes) recognize conserved microbe-associates molecular pattern (MAMPs) by means of germline-encoded, non-rearranging pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs) (Uribe et al. 2011). As a result, preformed soluble proteins, including antimicrobial enzymes (e.g. lysozyme), antimicrobial peptides (e.g. dicentracin) and complement molecules (e.g. complement component C3 and lectins), digest or lyse bacteria directly (Magnadottír 2006). Phagocytes are being divided into granulocytes (e.g. neutrophils) and agranulocytes (monocytes, the precursors of macrophages) (Whyte 2007). Once they are activated, downstream signaling pathways are induced resulting e.g. in the production of cytokines such as interleukins, which regulate a variety of cell functions (Zhu et al. 2012).

In contrast, the adaptive immune system reacts slowly (within days) but is highly specific and facilitates immunological memory, the prerequisite for vaccination (Van Muiswinkel & Nakao 2014). Lymphocytes (B and T cells) recognize a diverse variety of different antigens from pathogens, with the consequence that infected cells and extracellular pathogens are eliminated (Murphy 2011). Specificity is achieved by somatic

recombination (VDJ recombination), the rearrangement of immunoglobulin genes, which is initiated by enzymes expressed by recombination-activating genes (RAGs) (Zhu et al. 2012). B cells produce antigen-specific recognition proteins in the form of immunoglobulins, which are either membrane-bound or secreted as soluble antibodies (Tort et al. 2003, Salinas et al. 2011). T cells can detect the presence of intracellular pathogens (Fischer et al. 2006). Molecular fractions of antigens (epitopes) are presented on the surface of cells by 2 different classes of major histocompatibility complex (MHC) molecules. While MHC class I occurs on all cells, MHC class II is expressed on antigen-presenting cells (dendritic cells, macrophages, B cells). Epitopes represented by MHC class I molecules are recognized and killed by $CD8^+$ T cells. While the recognition of MHC class II-presented epitopes by $CD4^+$ T cells leads to the production of antibodies (Nakanishi et al. 2002, Tafalla et al. 2016).

4. Fish early life stages and the maturation of their immune system

Most economically important marine teleosts have extremely high fecundities, with females producing thousands to millions of eggs per spawning season. However, the variation in larval survival between the year-classes is massive and largely unpredictable and mortalities over 99% can occur between fertilization and recruitment of juveniles (Houde 1989, Jennings et al. 2001). Development (which can be divided into ontogeny and growth) and mortality are closely related. At hatch, fish larvae are not fully developed. Organogenesis is initiated during embryogenesis and continues during larval development (Rønnestad et al. 2013). The larva undergoes significant morphological and physiological changes until the individual finally becomes fully developed (Zambonino-Infante & Cahu 2001). The structural and functional development of the gastrointestinal (GI) tract, for example, is not completed before the transformation from larva to juvenile (Govoni et al. 1986). Accordingly, early life stages are most vulnerable (Pepin 1991, Hamre et al. 2013). Adverse environmental conditions including oxygen and prey deficiency as well as pathogen prevalence have the severest impact during early development (Rosenthal & Alderdice 1976). For example: The resistance towards starvation of larvae is all the lower, the younger (less developed and smaller) the larvae are. Therefore, it is not surprising that the rearing of marine fish larvae (larviculture) has only become viable on a commercial scale through major progress in the knowledge of their nutritional requirements (Coutteau et al. 1997, Sorgeloos et al. 2001). But even for fish species with established larval rearing protocols, high and unpredictable mortality

remains a challenging problem that needs to be addressed (Conceição et al. 2010). For the major Mediterranean farmed fish species, such as European sea bass and gilthead sea bream (*Sparus aurata*), the larval mortality in hatcheries is commonly around 90% (Valente et al. 2013). Besides inadequate nutrition, detrimental fish–microbe interactions have been recognized as one of the main problems in larviculture (Vadstein et al. 1993). Infections with opportunistic pathogens are a serious threat, especially for fish early life stages, since their immunological capacity is still gravely limited (Zapata et al. 2006, Vadstein et al. 2012). Young fish larvae are solely protected by their innate immune response while their adaptive immune system is still developing (Magnadottir et al. 2005, Magnadottír 2006). Innate and adaptive immune molecules including lysozymes, lectins and immunoglobulins (Ig) transferred from mother to offspring are mostly depleted during the first couple of days post hatch (dph) (Tanaka et al. 1999, Swain & Nayak 2009, Zhang et al. 2013). Fish innate immunity is activated right after fertilization and becomes functional at hatch (Vadstein et al. 2012). A complex network of innate defense mechanisms that include mucosal immune responses and inflammatory processes driven by cellular and humoral factors orchestrate the immunological protection during early development (Magnadottír 2006). The ontogenetic developmental pattern of the adaptive immune system in fish is species specific, whereby T cell development precedes generally B cell development (Vadstein et al. 2012). The primary organ for the differentiation and maturation of T cells is the thymus (first organ to become lymphoid) and for B cells the head kidney (Chistiakov et al. 2007).

5. Immunostimulation in marine larviculture

Fish early life stages lack a mature immune system and, consequently, are highly susceptible towards diseases (Vadstein 1997, Rombout et al. 2005). A possibility to increase the larval resistance towards diseases is the enhancement of its immune response, called immunostimulation (Kiron 2012). This includes the activation of humoral and cellular immunity through PRRs leading, for example, to an increased cytokine production, number of macrophages or lymphocytes (Magnadottir 2010).

A possibility to trigger an immune response is to steer the intestinal microbial community towards a, for the host, more beneficial direction by the application of prebiotics, probiotics or poly-β-hydroxybutyrate (Nayak 2010, Suguna et al. 2014,

Hoseinifar et al. 2015). This concept is based on the knowledge that the mucosal microbiota (including intestine, skin and gills) and the immune system are mutually interrelated (Maynard et al. 2012). Moreover, the intestinal microbiota has been demonstrated to be crucial for the maturation of the larval immune system (Hansen & Olafsen 1999, Maynard et al. 2012). Accordingly, the manipulation of the microbiota has the greatest effect on the developing immune system at the time of microbial colonization (Rombout et al. 2011). It has been shown that the microbiota regulates the expression of innate immune genes (such as the complement component C3) and the development of immune cells in gnotobiotic zebrafish (*Danio rerio*) larvae (Rawls et al. 2004, Kanther & Rawls 2010).

The successful stimulation of the larval immune system, however, is challenging due to various factors. Besides the circumstance that the effects of immunomodulatory compounds/probiotics have been demonstrated to be species-specific, the dose-response relationship, the duration of stimulation and administration procedures have to be evaluated (Vadstein 1997). Also, more knowledge is required on the developmental stage, at which immunostimulation is effective (Skjermo & Vadstein 1999). Moreover, the small size of fish larvae makes it difficult to find suitable methods to assess the stimulation of the immune system (Vadstein 1997).

5.1 Prebiotics

A prebiotic is defined as “a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of a limited number of bacteria in the colon and, thus, improves the host´s health” (Gibson & Roberfroid 1995, Gibson 2004). To be classified as a prebiotic, the food ingredients should be neither hydrolyzed nor absorbed in the upper part of the GI tract and be a selective substrate for beneficial commensal bacteria and, consequently, be able to alter the colonic microbiota towards a healthier composition (Fig. 2) (Lauzon et al. 2014, Song et al. 2014). Examples for well-studied prebiotics are β-glucans and mannan-oligosaccharides (Dalmo & Bøgwald 2008, Torrecillas et al. 2014).

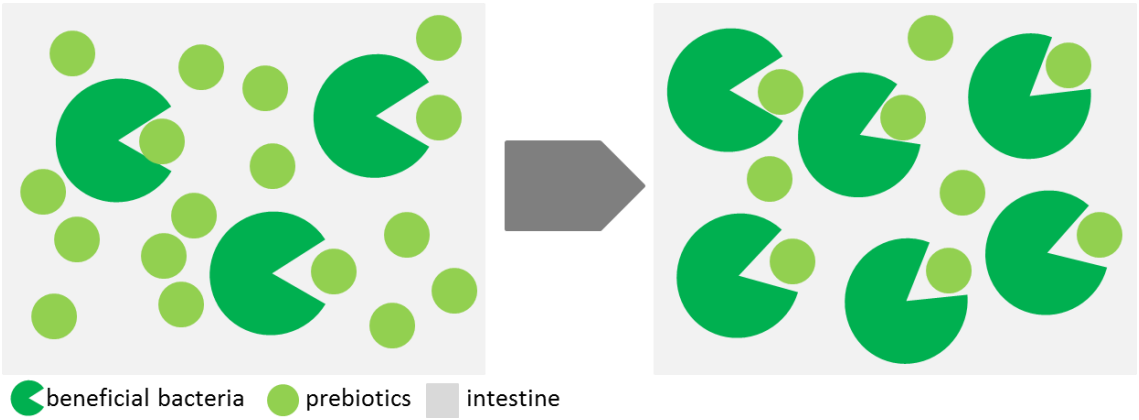


Fig.2. Non-digestible food ingredients stimulate the growth of beneficial bacteria in the GI tract of the host. Modified and redrawn after allergiesandyourgut.com.

5.2 Probiotics

The World Health Organization defines probiotics as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". Different microalgae and yeasts but especially a wide range of gram-positive (e.g. *Bacillus* spp., *Lactococcus* spp. and *Enterococcus* spp.) and gram-negative bacteria (e.g. *Vibrio* spp. and *Aeromonas* spp.) have been evaluated as probiotics (Fig. 3, left) (De et al. 2014).

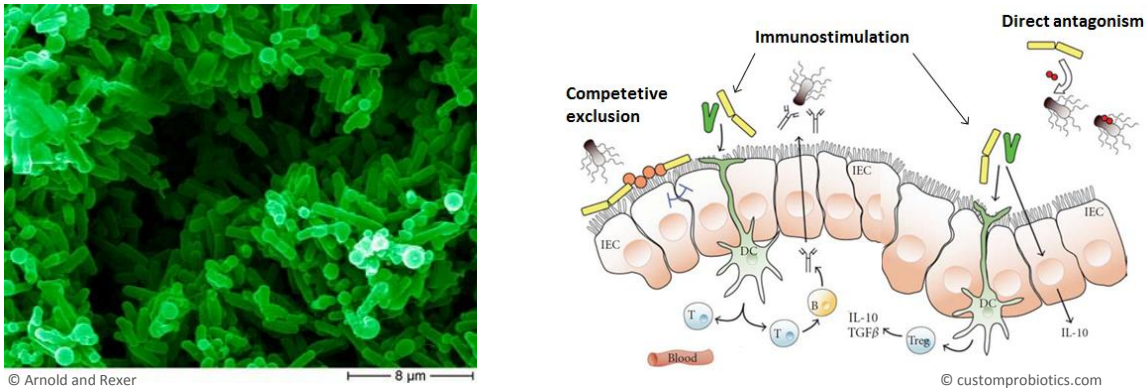


Fig. 3. Left: Electron microscopy image of *Bacillus subtilis.* **Right:** Mode of action of probiotics.

General consensus exists that the intestinal microbiota plays a key role in the health of the host (Gómez & Balcázar 2008, Pérez et al. 2010, Romero et al. 2014). Probiotics have the potential to beneficially modulate the composition of the intestinal microbiota and, thereby, to promote digestion, epithelial proliferation and disease resistance (Merrifield & Carnevali 2014, Ringø et al. 2016). The mode of action of probiotic bacteria differs

between fish species and depends not only on the bacterial species but on the strain, rendering the selection of potential probiotic candidates rather difficult (Pandiyan et al. 2013). Nonetheless, probiotic bacteria have the common ability to reduce the growth of pathogens by producing inhibitory substances or decreasing the intestinal pH (direct antagonism). They can also adhere to the intestinal mucus and, therefore, replace pathogenic bacteria (a process called competitive exclusion or barrier effect), which allows them to interact with the GALT through PRRs and to induce an immune response (Fig. 3, right) (Nayak 2010). In my thesis, I investigated the immunostimulatory capacity of a probiotic bacterial strain of the species *Bacillus subtilis*.

5.3 Poly-ß-hydroxybutyrate

Short-chain fatty acids (SCFAs) such as butyric and acetic acid are known to exhibit bactericidal activities (Bergeim 1940). Defoirdt et al. demonstrated that poly-β-hydroxybutyrate (PHB), the polymer of the SCFA β-hydroxybutyrate (β-HB), protected brine shrimp (*Artemia franciscana*) from pathogenic *Vibrio campbellii* (Defoirdt, Halet, et al. 2007). PHB particles and, respectively, PHB-containing bacteria (Fig. 4, left) were used and it was hypothesized that the PHB was degraded into the SCFA β-HB in the brine shrimp gut (Defoirdt, Halet, et al. 2007, Halet et al. 2007).

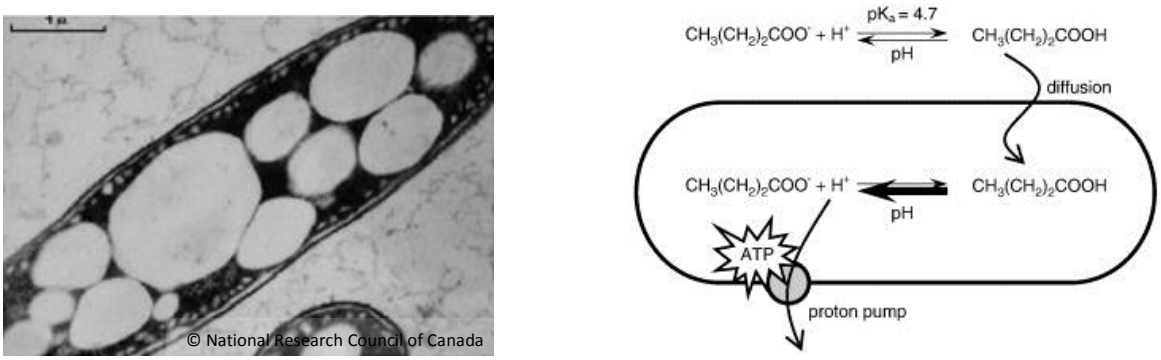


Fig. 4. Left: PHB granules in *Alcaligenes latus.* **Right:** Mechanism of the bacteriostatic activity of SCFAs using the example of butyric acid (© Defoirdt et al. 2009).

SCFAs exhibit their bacteriostatic effect by first passing bacterial cell membranes in their undissociated form and, subsequently, dissociating into anions and protons in the cytoplasm (Defoirdt et al. 2009). As a consequence, the cells have to export the excess of protons. This energy-consuming process leads to lower bacterial cell growth or even cell

death and lowers the pH in the gut (Fig. 4, right) (Defoirdt et al. 2009). While the growth of pathogenic bacteria is suppressed by SCFAs, beneficial bacteria may profit from the lower gut pH, improving the host´s gastrointestinal health (Cotter & Hill 2003) and potentially triggering an immune response. Moreover, it was hypothesized that β-HB itself is able to stimulate the immune system in fish (Montalban-Arques et al. 2015). In my thesis, I investigated the immunomodulatory effects of PHB in sea bass larvae.

6. European sea bass larvae

European sea bass (*Dicentrarchus labrax*) is the most important aquaculture fish species in the Mediterranean Sea and the Eastern Atlantic (FAO 2016b). However, the larval survival in hatcheries is commonly only around 10% (Valente et al. 2013). Females spawn approx. 200 000 eggs per kg body weight and have a mean weight of 5 to 7 kg (Sánchez Vázquez & Muñoz-Cueto 2014). Sea bass larvae hatch at a size of 3.5 mm (total length) with a yolk sac containing the vitellus and an oil globule (Fig. 5). At approx. 5 dph the still immobile larvae open their mouth. From 7-8 dph onwards larvae show periods of active swimming and start to feed (Sánchez Vázquez & Muñoz-Cueto 2014). In hatcheries, sea bass larvae are commonly fed on rotifers (*Brachionus* spp.) and brine shrimp (*Artemia* spp.), since the intensive cultivation of their natural diet (copepods) is not feasible. The nutritional value of rotifers and brine shrimp has to be improved to meet the larval requirements. Hence, the non-selective filter feeders are enriched with commercial emulsions containing highly-unsaturated fatty acids and vitamins (Lavens & Sorgeloos 1996).

Fig. 5. Newly-hatched European sea bass larvae.

In larvae of many cultured marine fish species, including sea bass, a delayed maturation of the lymphoid organs is observed compared to other fish species (Scapigliati et al. 1995). T cells appear around 28 dph in the thymus and the intestine (GALT), at 35 dph in the head kidney and 45 dph in the spleen, while B cells occur 45 dph in the head kidney and spleen and 90 dph in the thymus and intestine of sea bass (Scapigliati et al. 2002, Rombout et al. 2005). In contrast, the first B cells were detected already at 14 dph in common carp (*Cyprinus carpio*) (Rombout et al. 2005). The immune system of sea bass larvae is only competent for strong antibody production after 50 dph, and sea bass are not completely immunological mature before being 4-5 month old (Breuil et al. 1997, Dos Santos et al. 2000). In line with this, I focused on sea bass larvae and juveniles until the age of 3 month in my thesis. Considering the late maturation of the immune system in *D. labrax*, advances in larval immunostimulation research are crucial for the further development of sea bass larviculture. However, studies with immunostimulating compounds in *D. labrax* larvae are scarce so far. While the effects of probiotics (e.g. in regard to growth) are investigated, the effect on the immune system is not assessed (Touraki et al. 2012, 2013, Lamari et al. 2013, Hamza et al. 2016).

7. Methodical remarks

In my thesis, I aimed to investigate the stimulation of the immune system in early life stages of a key fish species in European aquaculture, the European sea bass. Accordingly, I performed my experiments in flow-through system, which mimics hatchery conditions on a smaller scale (Fig. 6).

Fig. 6. Experimental 60 L tanks for immunostimulation experiments in sea bass larvae and juveniles.

The number of available methodical approaches to analyze biological responses in fish larvae is limited due to their small and fragile nature. For example, young sea bass larvae are too tiny to dissect their intestinal tract intactly and have not enough tissue for the measurement of cellular immune parameters or standard immunoassays (e.g. lysozyme activity measurements). In my thesis, I therefore assessed the larval immune response on the gene expression level by means of the Fluidigm Biomark™ HD system, while the immune response could be additionally investigated on a cellular level in sea bass juveniles.

The probiotic bacteria and the PHB, respectively, were encapsulated in live feed (non-selective filter feeders) to be administered to different sea bass early life stages. Even though this is a commonly used method (Makridis et al. 2000, Najdegerami et al. 2015), the live feed was analyzed for probiotic bacteria (MiSeq sequencer, Illumina) and PHB (mass spectrometry of 13 C labelled PHB-containing bacteria), confirming their presence.

To assess the disease resistance of sea bass larvae after immunostimulation, I performed bacterial challenge tests using *Vibrio anguillarum* (Fig. 7). For this reason, it was necessary to find a *Vibrio* strain of intermediate pathogenicity. I assessed potentially suitable strains in bacterial challenge tests with sea bass larvae using different *V. anguillarum* strains (kindly provided by the Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Belgium). However, during subsequent experiments, the strain was considerably more virulent towards the sea bass larvae despite using the same experimental setup and conditions. This phenomenon, also observed in other research facilities, renders the reproducibility of bacterial challenge test extremely difficult (Frans et al. 2013).

Fig. 7. Experimental setup for bacterial challenge tests: flow-through system using 1.5 L aquaria.

Fish larvae inherit a high inter-individual variability making the detection of potential treatment effects challenging (Fuiman & Cowan 2003, Meyer et al. 2012). Therefore, the standard procedure in fish larval experiments is to sample more than one larva per tank to measure response variables (e.g. Miest et al. 2016). This inevitably results in pseudoreplication (non-independence among samples due to tank effects), which has to be considered during data analysis to avoid the inflation of statistical power. A suitable statistical test to analyze complex multivariate data (such as gene expression data) is a permutational multivariate analysis of variance (PERMANOVA), where the calculation of the p-value is based on the comparison of the actual data distribution with the permuted data distribution. By using permutations (data are randomly shuffled among the different treatments), the non-parametric test requires no specific assumption regarding the number of variables or the nature of their individual distribution (Anderson 2001). A random factor can be implemented in a PERMANOVA. However, this is only meaningful when it appears in every treatment (e.g. several families). In contrast, in the experimental setup I used, each tank appears in only one treatment, rendering a correct permutation void. Thus, the most conservative approach, averaging all larvae per tank, was applied and only the fixed factor “treatment” was included in the PERMANOVA. For univariate data analysis, I chose a mixed effect model, whereby the random factor “tank” was nested in the fixed factor “treatment”.

Thesis outline

I have structured the results of my thesis in 3 chapters. All chapters have the form of a manuscript including an Introduction, Material and Methods, Results and Discussion.

Chapter 1, entitled "The effect of probiotic *Bacillus subtilis* (NCIMB 3610) on survival, growth and immunity in European sea bass (*Dicentrarchus labrax*) juveniles", has been submitted to the journal of "Aquaculture Nutrition".

Chapter 2, entitled "Poly-β-hydroxybutyrate administration during early life: effect on survival, growth, microbial community, gene expression and disease resistance in European sea bass yolk-sac larvae", is currently being reviewed by my co-authors and will be submitted to the journal of "Fish and Shellfish Immunology".

Chapter 3, entitled "Immunostimulatory effects of dietary poly-β-hydroxybutyrate in European sea bass post-larvae", has been submitted to the journal of "Aquaculture".

The aim of my thesis was to contribute to the further development of larviculture towards more sustainability (reduction of antibiotic use) and efficiency (improved larval survival). Fish early life stages are especially susceptible towards pathogens since they lack a mature immune system. As a consequence, the production of offspring in marine fish species is still unstable due to high mortality rates mainly caused by infectious diseases. For this reason, I intended to investigate promising immunostimulatory candidates in early developmental stages of European sea bass since effective immunoprophylactic measures play a key role in the advancement of larviculture. The aim of my thesis was to gain unprecedented insights into the underlying mechanisms of immunostimulation at the cellular and gene expression level in fish early life stages.

Chapter 1

At the beginning of my thesis, I had the chance to learn how to culture sea bass larvae and, therefore, how to handle the difficulties associated with rearing animals at such a fragile and underdeveloped state. Thereupon, I performed my first experiment to evaluate the effect of the probiotic *Bacillus subtilis* strain NCIMB 3610 in 3-month-old sea bass. I investigated the immunomodulatory potential of the probiotic strain by measuring the immune response on the cellular as well as the gene expression level. I assessed the expression of over 20 genes associated with innate, complement and adaptive immunity but also stress and digestion. I detected that *B. subtilis* (NCIMB 3610)

affected the expression of trypsin, a major digestive enzyme, while the examined immune-related parameters were not influenced. My study emphasizes the complex nature of the mode of action of probiotics and demonstrates that their potential to exert protection against diseases must not necessarily be based on immunostimulation.

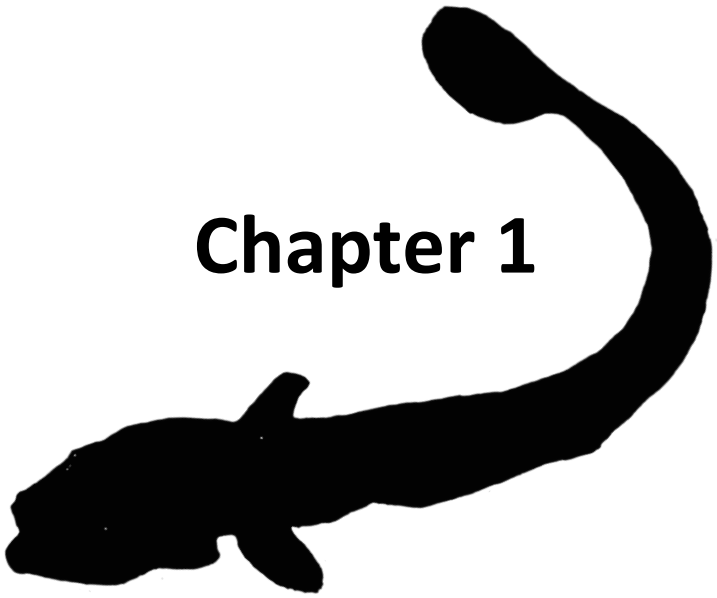
Chapter 2 and 3

Attending international workshops related to larviculture gave me the chance to establish a cooperation with the Laboratory of Aquaculture and Artemia Reference Center (Ghent University, Belgium). Hence, I got the opportunity to test the immunostimulatory capacity of poly-β-hydroxybutyrate (PHB), a proposed biocontrol agent for a sustainable production of aquatic organisms, in different sea bass larval stages.

The younger a fish larva, the lower its immunocompetence and, consequently, the higher the risk of infection resulting in mortality. For this reason, one of my main aims was to stimulate the developing immune system of fish larvae at the earliest point in time possible. During the experiment presented in chapter 2, I administered PHB to sea bass yolk-sac larvae from mouth opening onwards. At the end of this experiment, I challenged the sea bass larvae with pathogenic *Vibrio anguillarum* to assess their disease resistance. Besides, I collected data with respect to larval survival, growth performance, gene expression and microbiota. My data show that larval survival was increased, indicating that the application of PHB has the potential to reduce economic losses in sea bass hatcheries.

In a subsequent experiment, I fed PHB-enriched *Artemia* to sea bass post-larvae and measured the expression of 26 genes related to innate, complement and adaptive immunity as well as digestion, growth and stress. Furthermore, I performed a survival analysis and examined life-history parameters. The results are presented and discussed in chapter 3. They demonstrate that PHB has the capability to enhance the larval expression of genes involved in innate and adaptive immunity. Therefore, it is likely to improve the immune response in sea bass post-larvae.

Chapter 1



The effect of probiotic *Bacillus subtilis* (NCIMB 3610) on survival, growth and immunity in European sea bass (*Dicentrarchus labrax*) juveniles

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Abstract

The outbreak of infectious diseases in fish farms is starting to be recognized as one of the most severe constraints to aquaculture production. Conventionally, disease prevention has relied on the prophylactic use of antibiotics selecting for antibiotic-resistant bacteria. Accordingly, the development of alternative health management strategies is one of the key factors for the progress towards a more sustainable aquaculture industry. Potential biocontrol applications are, for instance, immunostimulating dietary supplements such as probiotics. A broad variety of bacteria has been evaluated, identifying *Bacillus subtilis* as a promising probiotic candidate for sea bass. The present study aimed to improve the performance and immunocompetence in European sea bass (*Dicentrarchus labrax*) juveniles by administering the probiotic strain *Bacillus subtilis* NCIMB 3610. The bacteria were encapsulated in instar II *Artemia* and fed to the juveniles over a period of 14 days. Survival and growth performance of juveniles were not influenced by the *B. subtilis* application. The expression of 17 genes involved in innate, complement and adaptive immunity as well as apoptosis was not altered after 7 and 14 days of treatment, respectively. However, after 7 days of *B. subtilis* administration, the proteolytic enzyme trypsin was downregulated compared to the control, while there was no difference in trypsin expression between the treatments after 14 days. Furthermore, cellular immune parameters such as the relative number of monocytes and lymphocytes and the proliferation of lymphocytes were neither affected

in the spleen nor in the blood of juvenile sea bass. However, it cannot be excluded that *B. subtilis* (NCIMB 3610) exerts its probiotic effects through direct antagonism or competitive exclusion. The current study highlights the complex nature of probiotic efficacy and points out the necessity to disentangle the link between intestinal microbiota composition and immune response.

1. Introduction

Intensive aquaculture practices render the transmission of infectious diseases leading to high mortality especially during the rearing of early life stages (Woo & Gregory 2014, Lafferty et al. 2015). Accordingly, the stable production of high quality juveniles is still a major bottleneck in the aquaculture industry (Valente et al. 2013). The overuse of antimicrobial drugs has selected for resistant bacteria, making antibiotics increasingly ineffective and raising concerns regarding the environment as well as the public health (Romero et al. 2012). Hence, the establishment of alternative methods such as the application of probiotics, prebiotics and immunostimulants to prevent diseases is essential to improve the productivity and sustainability in aquaculture.

According to the World Health Organization, probiotics are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host”. It is generally recognized that the gastrointestinal (GI) microbiota is a key component to maintain the host´s well-being (Gómez & Balcázar 2008, Romero et al. 2014). Probiotic bacteria have the potential to beneficially modulate the composition of the GI microbiota and, thereby, to enhance digestive functions, epithelial proliferation and disease resistance (Merrifield & Carnevali 2014). Among the numerous advantageous effects of probiotics, the stimulation of the immune system is outstanding (Akhter et al. 2015). The mode of action of probiotics is strictly strain-specific, entailing that the evaluation of potential probiotic candidates is challenging (Pandiyan et al. 2013, De et al. 2014). However, probiotic bacteria have the mutual ability to repress the growth of pathogens by decreasing the intestinal pH or by producing inhibitory substances (direct antagonism). Furthermore, they adhere to the intestinal mucus potentially not only extruding pathogenic bacteria through competitive exclusion but interacting with the gut-associated lymphoid tissue (GALT) inducing an immune response (Nayak 2010).

A diverse range of bacteria has been considered for the application in the aquatic environment including gram-positive bacteria of the genera *Bacillus, Lactobacillus* and *Vagococcus* (Newaj-Fyzul et al. 2014). Probiotic studies performed with aquaculture fish species focus mostly on the evaluation of the host´s disease resistance (in terms of survival) after probiotic administration (Gildberg & Mikkelsen 1998, Sorroza et al. 2012, Touraki et al. 2013). In contrast, the intrinsic modulation of the immune response in early life stages upon administration of probiotics has been examined less commonly.

In European sea bass (*Dicentrarchus labrax*), the most important commercially farmed fish in the Mediterranean Sea and the Eastern Atlantic (FAO 2016), the probiotic strain *Bacillus subtilis* NCIMB 3610 was shown to increase the larval survival after a challenge test with pathogenic bacteria (Touraki et al. 2012). Hence, the present study aimed at investigating the immunostimulatory capacity of this strain. *B. subtilis* (NCIMB 3610) was administered to sea bass juveniles over a period of 14 days and the effect on survival, growth performance and cellular immune parameters was assessed. Furthermore, an analysis on the expression of genes involved in innate, complement and adaptive immunity, digestion and stress was carried out. The ultimate aim of the current study was to determine the suitability of *B. subtilis* (NCIMB 3610) as an immunostimulating supplement for an early life stage of a key fish species in marine aquaculture.

2. Materials and methods

2.1. Bacterial strain

Bacteria were kindly provided by the Max Rubner-Institut (Kiel, Germany). The *Bacillus subtilis* strain NCIMB 3610 (originating from the National Collection of Industrial, Food and Marine Bacteria, UK) was sub-cultured on nutrient agar (3 g L^{-1} meat extract, 5 g L^{-1} peptone, 15 g L^{-1} agar; VWR) at 37 °C for 2 days. Bacteria from 4 agar plates were collected using 20 mL quarter-strength Ringer´s solution, which was used for inoculation of nutrient broth in a 10 L fermenter at a constant pH of 7.0 under aerobic conditions (pO_2 was set to 10 % saturation obtained by aeration and stirring). Bacteria were harvested aseptically by centrifugation (6,000 xg), re-suspended in a sterile storage solution (5 g L^{-1} casein peptone, 5 g L^{-1} casein hydrolysate, 5 g L^{-1} glucose, 20 g L^{-1} NaCl in distilled water) and stored at 4 °C during use. Cell counts were obtained by plating serial dilutions of bacteria on nutrient agar.

2.2. Origin of fish

European sea bass (*Dicentrarchus labrax*) larvae were purchased from a commercial hatchery (Ecloserie Marine de Gravelines, France) and reared in a flow-through system at GEOMAR Kiel (Germany) in 3 stocking tanks with a volume of 65 L (water was 5 µm-filtered and UV-treated). The initial salinity of 32 PSU was gradually decreased to 26 PSU 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 14 to 17 °C and oxygen was maintained above 80% saturation. The larvae were kept in the dark until first feeding at 7 dph and under a natural photoperiod regime (16L: 8D) thereafter. Until 26 dph the larvae were fed on instar I *Artemia* nauplii (AF 430 Artemia, INVE) and subsequently on instar II *Artemia* nauplii, enriched with essential fatty acids (S.presso, INVE, applied according to instructions), 3 times a day at increasing densities. For further details see Tillner et al. (2014). From 50 dph onwards the temperature and the salinity were gradually decreased to 13 ± 0.5 °C and 20 ± 1 PSU.

2.3. Experimental design and preparation of feed

3-month-old sea bass juveniles were randomly distributed into 6 experimental 65 L tanks. The experimental conditions were as follows: temperature 13 ± 0.5 °C, salinity 20 ± 1 PSU, photoperiod 16L: 8D and flow rate 0.5 L min^{-1}. The experiment was started after a 3-day acclimation period. The juveniles had an initial average weight of 255 ± 13 mg. Three tanks, respectively, were assigned to the following treatments: (1) probiotic treatment (*B. subtilis* strain NCIMB 3610 encapsulated in *Artemia*), (2) control treatment (*Artemia* without *B. subtilis*). Sea bass juveniles were fed 2 times a day at 10:00 h and 20:00 h with instar II *Artemia* nauplii at a density of 14 mL^{-1}. For both treatments, *Artemia* (Sanders) were cultured and enriched with highly unsaturated fatty acids (S.presso, INVE) according to the manufacturers´ instructions. Instar II *Artemia* nauplii were harvested and thoroughly rinsed with fresh water. For the probiotic treatment, instar II *Artemia* nauplii were subsequently allowed to graze on live *B. subtilis* for 30 min at a density of 50 nauplii mL^{-1} under gentle aeration. Therefore, a bacterial suspension in sea water (UV-treated) was prepared with a final concentration of 10^7 CFU mL^{-1}. *Artemia* density, bacterial concentration and grazing time were chosen as in accordance with Makridis et al. (2000), who demonstrated that non-selective filter feeders such as

Artemia accumulate bacteria when incubated in bacterial suspensions. The tank bottoms were siphoned daily to remove dead fish, feces and debris.

2.3. Measured parameters

2.3.1. Growth performance and survival rate

At the start of the experiment, after 7 and 14 days of treatment, 5 juveniles were randomly sampled from each tank, anaesthetized with MS 222 (Sigma-Aldrich), transferred into Eppendorf vials with seawater and immediately frozen on dry ice. Samples were stored at -80 °C. For growth analysis, the total length (mm) and weight (mg) of thawed fish was measured. The specific growth rate (SGR, % d^{-1}) of juveniles was calculated as follows:

$$SGR = 100 * \frac{lnW_{t2} - lnW_{t1}}{\Delta t}$$

where W_{t2} equals the mean weight at second sampling, W_{t1} equals the mean weight at the start of the experiment and Δt is the time (d) between start and second sampling. Furthermore, Fulton's condition factor (K) was calculated according to the equation:

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

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

2.3.2. Cellular immune parameter analysis

After 14 days of treatment, 5 sea bass juveniles were randomly sampled from each tank, anaesthetized with MS 222 (Sigma-Aldrich) and dissected for immunocompetence measurements. The spleen (central immune organ) and blood (peripheral immune activity) were used to examine cellular immune parameters by means of flow cytometry (BD Accuri™ C6 flow cytometer and software, BD Biosciences). After dissection, the spleen was pressed through a 40 µm Falcon™ cell strainer (Fisher Scientific) to obtain a uniform single-cell suspension from the tissue. Spleen cells and blood, respectively, were suspended in RMPI-1640 cell medium (Sigma-Aldrich). All steps for the isolation of leucocytes were performed on ice and with refrigerated reagents.

The proportion of monocytes (innate immune cells) and lymphocytes (adaptive immune cells) relative to the live cells was measured immediately after adding propidium iodide to the cell suspensions. Cells were distinguished according to their size and complexity (whereas monocytes are larger and of higher complexity than lymphocytes). For cell cycle analysis, the living cells in suspension were killed with ethanol and, subsequently, stained with propidium iodide. As a parameter for the activity of the adaptive immune system, the relative number of lymphocytes in the S, G_2 and M phase of the cell division cycle in contrast to the resting phase $G_{0/1}$ was assessed. For further details see Roth et al. (2011).

2.3.3. Gene expression analysis

After 7 and 14 days, 5 sea bass juveniles were randomly sampled from each tank, anaesthetized with MS 222 (Sigma-Aldrich) and decapitated. The spleen was removed immediately, transferred into RNAlater and kept at 4 °C for 24 h before being stored at -20 °C.

For the quantification of mRNA as a measure of gene expression levels, RNA of the spleen (central immune organ) was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer´s instructions. RNA concentration was measured by spectrophotometry (NanoDrop ND-1000, Peqlab) and normalized to a common concentration with RNase free water. 500 ng RNA was reverse transcribed into cDNA, including a gDNA wipeout step (Qiagen QuantiTect Reverse Transcription Kit). The cDNA was stored at -80 °C until further use.

Primers (Metabion) for all 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), using a StepOnePlus Real-Time PCR System (Applied Biosystems). 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) running a 96.96 Dynamic Array™ IFC (Gene Expression chip) was used to measure the expression profiles of 21 target genes as well as 3 reference genes in the samples (Table 1). Briefly, 1.3 µl cDNA per sample were mixed with TaqMan-PreAmp Master Mix (Applied Biosystems) 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) and DNA Binding Dye Sample Loading Reagent (Fluidigm). 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) and low EDTA-TE Buffer were loaded onto the chip in triplicates, resulting in the measurement of technical triplicates per sample. The chip was primed and the run performed subsequently using the GE Fast 96x96 PCR+Melt v2 thermal cycling protocol with a Tm of 60 °C according to the manufacturer´s instructions. For further details, see Beemelmanns & Roth (2016) and Miest et al. (2016).

Table 1

Name, abbreviation and function of the 21 genes of interest and 3 reference genes. Genes are organized by functional groups: (I) immunity (all immune genes), (II) innate immunity, (III) adaptive immunity, (IV) stress and (V) digestion. Forward (FW) and reverse (RV) primers were either designed using sequences from GenBank (see accession number) or taken from literature (see reference).

Functional Group		Abbreviation	Gene name and function		Primer sequence	Accession No. / Ref.
Immunity	Innate immunity	*cc1*	CC chemokine 1, chemotactic cytokine	FW	TGGGTTCGCCGCAAGGTTGTT	AM490065.1
				RV	AGACAGTAGACGAGGGGACCACAGA	
		ifn	Interferon, cytokine	FW	GTACAGACAGGCGTCCAAAGCATCA	FQ310507.3
				RV	CAAACAGGGCAGCCGTCTCATCAA	
		il1b	Interleukin 1 beta, pro-inflammatory cytokine	FW	GCGACATGGTGCGATTTCTCTTCTACA	AJ311925.1
				RV	GCTGTGCTGATGTACCAGTTGCTGA	
		il8	Interleukin 8, pro-inflammatory cytokine	FW	CGCTGCATCCAAACAGAGAGCAAAC	AM490063.1
				RV	TCGGGGTCCAGGCAAACCTCTT	
		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	*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	
Stress		*cat*	Catalase, antioxidant	FW	TGATGGCTACCGCCACATGAACG	FJ860003.1
				RV	TTGCAGTAGAAACGCTCACCATCGG	
		hsp70	Heat shock protein 70, stress protection	FW	ACAAAGCAGACCCAGACCTTCACCA	AY423555.2
				RV	TGGTCATAGCACGTTCGCCCTCA	
Digestion		*fad6*	Fatty acid desaturase-6, fatty acid synthesis	FW	GCTCAGCCTTTGTTCTTCTGCCTCC	FP671139.1
				RV	TGAGCAGTTGCCAGCATGATCGAG	
		tryp	Trypsin, protease	FW	CCTGGTCAACGAGAACTGGGTTGTG	AJ006882.1
				RV	GGATGACACGGGAGGAGCTGATGAA	
Reference		*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	

2.4. Statistical analysis

For gene expression analysis, technical triplicates were used to calculate the mean cycle threshold value (Ct), the standard deviation (SD) and the coefficient of variation (CV) per sample. 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) and the geometric mean Ct of the 3 most stable genes (*actb*, *l13a*, *hsp90*; M < 0.5) was used to normalize the target genes (calculation of ΔCt-values). For graphical representation of gene expression data in response to *B. subtilis*, the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001) was applied by calculating the ΔΔCt for the target gene in relation to the mean ΔCt of the respective gene in the untreated control.

All statistical analyses were carried out in RStudio (version 0.98.1103). Permutational multivariate analyses of variance (PERMANOVA) were performed (vegan package, adonis function; Oksanen et al. 2012) for each functional gene group to test for overall differences between the 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 fish 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 as well as growth data and cellular immune parameters. Proportional and percentage data were arcsine transformed. All data were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test). Survival data are presented by means of Kaplan-Meier curves and compared between treatment groups using a log-rank test (survival package; Therneau 2015).

3. Results

3.1. Growth performance and survival

Neither the estimated growth-related parameters, such as total length, weight, specific growth rate and Fulton´s condition factor K (Table 2 and Suppl. Table S1), nor the survival

(χ^2 = 2.1, df = 1, p > 0.05, Fig. 1) of juvenile sea bass were affected by *B. subtilis* (NCIMB 3610) application.

Table 2

Growth-related parameters of sea bass juveniles fed *B. subtilis* encapsulated in *Artemia* or a control diet (*Artemia* without *B. subtilis*) over a period of 7 and 14 days, respectively. Values represent mean ± SEM.

Growth-related parameters	7 days of treatment		14 days of treatment	
	B. subtilis	Control	*B. subtilis*	Control
Weight (mg)	324 ± 19	380 ± 30	345 ± 22	403 ± 19
Total length (mm)	30 ± 0.5	32 ± 0.8	31 ± 0.5	33 ± 0.6
SGR[1] (% d^{-1})	-	-	2.2 ± 0.4	3.3 ± 0.3
Condition[2] (mg mm^{-3})	1.14 ± 0.03	1.11 ± 0.03	1.14 ± 0.03	1.16 ± 0.03

[1] Specific growth rate = $100 * (\ln W_{t2} - \ln W_{t1}) / \Delta t$

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

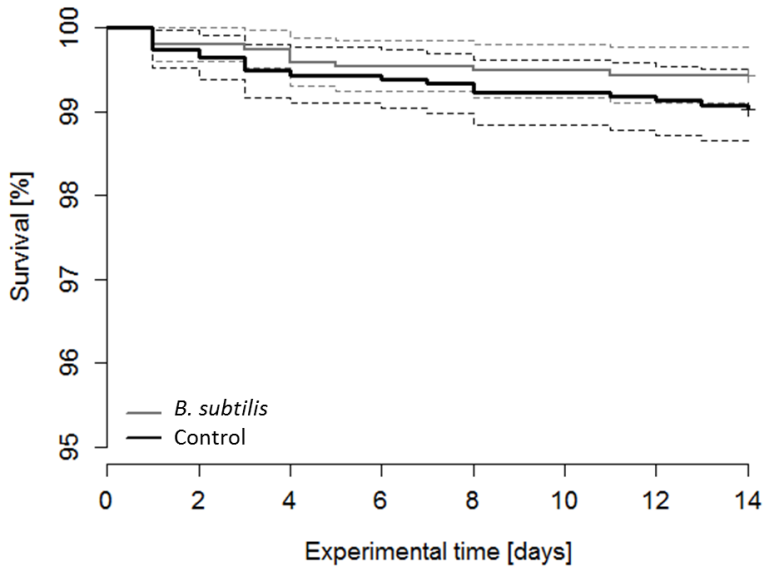

Fig. 1. Kaplan-Meier survival curves of sea bass juveniles fed *B. subtilis* encapsulated in *Artemia* (grey) or a control diet (*Artemia* without *B. subtilis* in black) over a period of 7 and 14 days, respectively. The dashed lines represent the 95% confidence intervals.

3.2. Cellular immune parameters

Administering *B. subtilis* (NCIMB 3610) over a period 14 days had no effect on the relative number of monocytes, the relative number of lymphocytes and the proliferation of lymphocytes in spleen, respectively, blood of juvenile sea bass (Fig. 2, Suppl. Table S2).

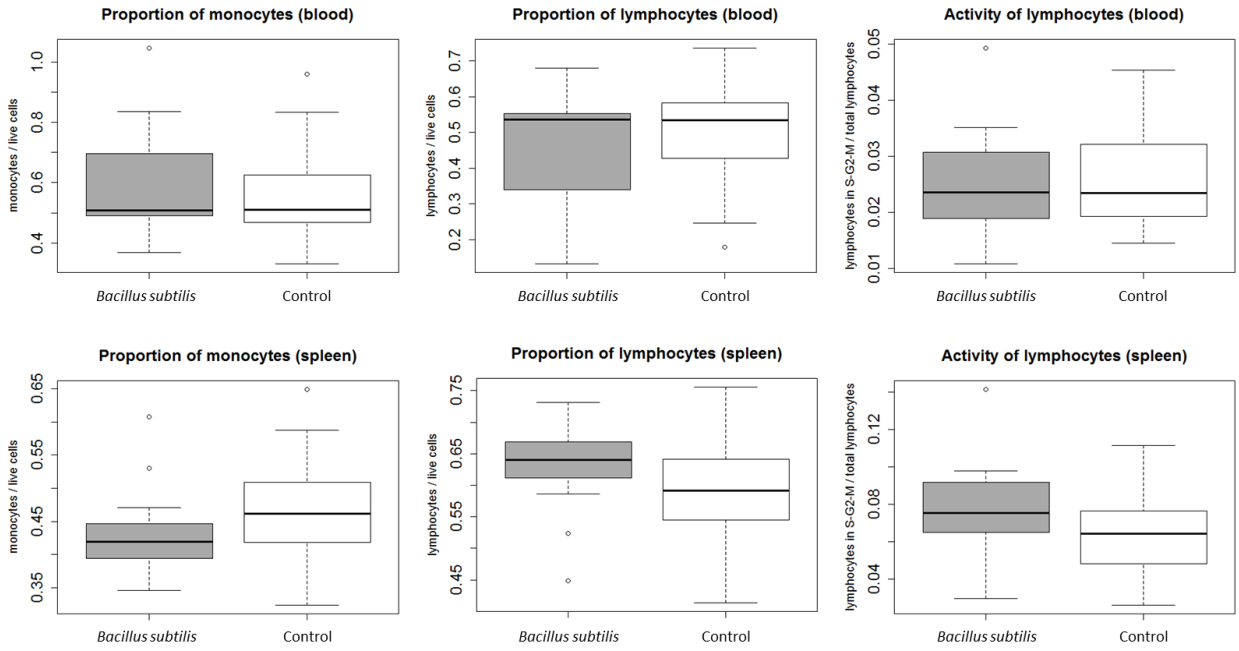

Fig. 2. Effect of *B. subtilis* application on cellular immune parameters of juvenile sea bass after 14 days of treatment. Box-whisker plots show median, upper and lower quartile, 1.5 × IQR and outliers as individual points.

3.3. Gene expression

The expression of 21 genes involved in the immune response, apoptosis, digestion, antioxidant activity and stress-response were analyzed and classified into the following functional gene groups: (I) all immune genes, (II) innate immunity, (III) adaptive immunity, (IV) stress and (V) digestion (Table 1).

The multivariate analysis showed that after 7 days of *B. subtilis* (NCIMB 3610) application only the expression of the genes related to digestion differed significantly between the treatments ($F_{1,4}$ = 142.5, p < 0.01, Suppl. Table S3). The subsequent univariate analyses revealed that the expression of trypsin ($F_{1,4}$ = 9.1, p = 0.05, Fig. 3) was significantly down-

regulated in the *B. subtilis* treatment (0.47 ± 0.09-fold, ΔCt = -0.97 ± 0.31) compared to the control (1.3 ± 0.28-fold, ΔCt = -2.47 ± 0.36), while there was no effect on the expression of the fatty acid desaturase-6 ($F_{1,4}$ = 1.9, p < 0.23).

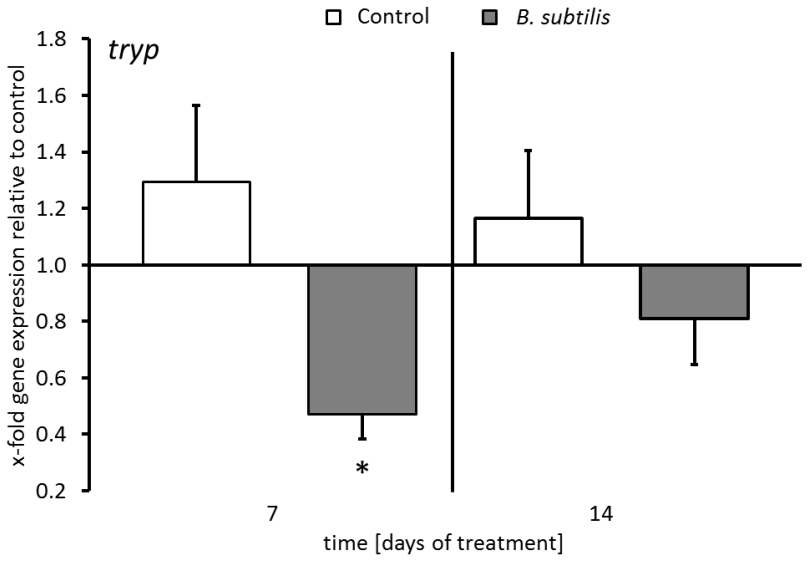

Fig. 3. Gene expression of trypsin (*tryp*) in sea bass juveniles fed *B. subtilis* encapsulated in *Artemia* or a control diet (*Artemia* without *B. subtilis*). Samples were taken after 7 and 14 days of treatment, respectively. The figures display the x-fold gene expression to the control. Data are presented as mean ± SEM. The asterisk represents the level of significance (*: p < 0.05).

4. Discussion

The aquatic environment is more supportive towards pathogenic bacteria than the terrestrial environment. Pathogens reach high densities in the water, especially under intensive culture conditions, and are ingested by the animals with the feed or by drinking, causing the outbreak of diseases (Defoirdt et al. 2011). An enhanced disease resistance of the host to bacterial pathogens, however, can be achieved by the prophylactic administration of probiotics which beneficially alters the gastrointestinal (GI) microbiota, thereby improving the immunocompetence (Sahu et al. 2008). The capability of probiotics to modulate the GI microbiota does not appear to be restricted to the stage of its maturation (during larval development) as various studies have demonstrated probiotic effects across all life-history stages (for review see Ringo et al. 2016).

In the present study, the effect of orally administered *B. subtilis* on sea bass juveniles has been investigated with special emphasis on the immune response. The *B. subtilis* strain NCIMB 3610 was chosen based on a study by Touraki et al. (2012) suggesting the strain as a probiotic candidate for sea bass. They administered the probiotic strain to sea bass larvae over a period of only 5 days and observed an enhanced disease resistance (by performing *Vibrio anguillarum* bath challenges), however, the impact on the immune system was not determined. Since a vast number of potential probiotics has been studied (Newaj-Fyzul et al. 2014), the obtained results are discussed within the context of other *B. subtilis* studies (whenever possible).

Survival and growth performance of juvenile sea bass were not affected by the application of *B. subtilis* in the current study. Nevertheless, it has to be mentioned that very few fish died over the course of the experiment. Hence, it is plausible that a potential treatment effect could not be detected with respect to survival. Regarding the growth performance, the *B. subtilis* strain ATCC 6633, for example, was shown to improve the weight gain in adult Nile tilapia (*Oreochromis niloticus*) when fed over a period of 4 weeks (10^7 CFU g^{-1} dry feed) (Aly et al. 2008). The specific growth rate was enhanced in rainbow trout (*Onchorhynchus mykiss*) fry fed with a mixture of *B. subtilis* and *B. licheniformis* over a period of 4 weeks. Nevertheless, no effect was observed at a concentration below 10^9 CFU g^{-1} dry feed (Bagheri et al. 2008). In both cases, the period of probiotic administration was longer than in the present investigation. Thus, the experimental time of 2 weeks might have been too short to promote the growth-performance in sea bass juveniles. Albeit, the general question arises if a long-term application of probiotics would be economically profitable for fish farmers which, however, can only be answered by cost-benefit analyses for each individual aquaculture farm.

After 7 days of *B. subtilis* administration, trypsin (*tryp*) was downregulated compared to the control, while there was no difference in *tryp* expression between the treatments after 14 days. Trypsin is a major intestinal digestive enzyme hydrolyzing proteins. Probiotic bacteria including *Bacillus* spp. are known to secrete a wide range of exoenzymes and to digest proteins in the host´s intestine, therefore, contributing to the feed digestion (Ghosh et al. 2002, Vine et al. 2006). Accordingly, it has been demonstrated in several fish species that the enzymatic activity in probiotic treatments was higher than in the control group (Yanbo

& Zirong 2006, Suzer et al. 2008). As a consequence, the host itself would need to produce less digestive enzymes as observed in the present study. Further investigations analyzing the GI microbiota and the expression of other digestive enzymes would be necessary to answer why *tryp* was not downregulated anymore after 14 days of *B. subtilis* administration. A possible explanation would be that the *B. subtilis* application shifted the GI microbial community over the first days affecting the enzymatic activity until a new state was reached in which the *tryp* expression was unaffected.

The stimulation of the immune system is of special interest for the aquaculture sector as in high-density fish cultures the occurrence and transmission of infectious diseases are increased compared to natural conditions (Bondad-Reantaso et al. 2005). In this study, we focused on the influence of *B. subtilis* administration on cellular immune parameters and the expression of immune-related genes in sea bass juveniles. The relative number of monocytes, respectively, lymphocytes and the proliferation of lymphocytes neither have been affected in the spleen nor in the blood of juvenile sea bass. Furthermore, the expression of the 17 analyzed genes involved in innate, complement and adaptive immunity as well as apoptosis has not been altered in sea bass spleen. The review of research literature regarding the immunostimulatory potential of probiotics revealed a contradictory picture, indicating the complex nature of probiotic efficacy. Experimental outcomes have been demonstrated to depend on multiple factors such as probiotic concentration (Bagheri et al. 2008), period of administration and stocking density of fish (Telli et al. 2014). Furthermore, results are highly variable even when using the same probiotic strain in the same fish species (Merrifield, Bradley, et al. 2010). Two separate studies, for example, administering a mixture of *B. subtilis* and *B. licheniformis* to rainbow trout, resulted in an altered (Merrifield, Bradley, et al. 2010), respectively, unaltered total number of leucocytes (Merrifield, Dimitroglou, et al. 2010). In another rainbow trout study, the expression of innate immune genes (*il1b* and *tnfa*) was not found to be affected when feeding *B. subtilis*-supplemented dry feed (Panigrahi et al. 2007). While gene expression studies after *B. subtilis* application are scarce, other probiotics such as lactic acid bacteria have been shown to alter the expression of pro-inflammatory cytokines in fish including sea bass (Picchietti et al. 2009, Perez-Sanchez et al. 2011, Kim et al. 2013).

In conclusion, *B. subtilis* (NCIMB 3610) application neither influenced survival nor growth performance in juvenile sea bass. However, the expression of *tryp* was altered confirming the close relationship between GI microbiota and digestion. The examined cellular immune parameters and immune gene expression were not affected by *B. subtilis* (NCIMB 3610) administration. The obtained results are in contrast to our expectations since the strain was shown to prevent sea bass larvae from vibriosis (Touraki et al. 2012). However, the stimulation of the immune system is just one of the features of probiotics. *B. subtilis* (NCIMB 3610) might have protected the sea bass larvae from vibriosis through direct antagonism or competitive exclusion while having no significant impact on the immune system. Probiotic bacteria were found to decrease the activity and growth of pathogenic bacteria by producing inhibitory substances (direct antagonism) and/or to adhere to the intestinal mucosa, thus, preventing the pathogen from colonizing the intestine, however, these effects to not necessarily lead to immunostimulation (Pandiyan et al. 2013).

Besides, it is worth noticing that even though the number of the measured immune cells was not affected by *B. subtilis* (NCIMB 3610), their bactericidal activity might have been enhanced. Furthermore, the set of genes investigated in the current study covered a broad spectrum of immune-related genes involved in innate, complement and adaptive immunity. Nonetheless, it can of course not be excluded that other immune-related genes were affected. The current study highlights the complex nature of probiotic efficacy and reveals the necessity of microbiota and transcriptome analyses in future probiotic studies to elucidate the link between intestinal microbiota composition and immune response.

Acknowledgement

This work was performed within the frame of the project FINE-Aqua (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. The authors would like to thank Robert Tillner for providing the juvenile fish, Wilhelm Bockelmann from the Max Rubner-Institute for providing the bacteria, Fabian Wendt for his excellent technical support, Isabel Keller and Anne Beemelmanns for statistical advice, Joanna Miest for her help during the experiment and in the laboratory and

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Supplementary material

Table S1

Statistical mixed-effect model results for the growth-related parameters of sea bass juveniles after 7 and 14 days of *B. subtilis* treatment, respectively. *F*-statistics (*F*) and p-values (p) are shown. Freedom/residual degrees of freedom for all groups: 1/4.

Growth-related parameters	7 days of treatment		14 days of treatment	
	F	p	*F*	p
Weight (mg)	0.56	0.50	3.13	0.14
Total length (mm)	0.83	0.41	2.79	0.17
SGR[1] (% d^{-1})	-	-	4.08	0.11
Condition[2] (mg/mm^3)	0.02	0.88	0.11	0.76

[1] Specific growth rate = $100 * (\ln W_{t2} - \ln W_{t1}) / \Delta t$

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

Table S2

Statistical mixed-effect model results for the cellular immune parameters measured in the spleen and blood of juvenile sea bass after 14 days of *B. subtilis* treatment. Degrees of freedom/residual degrees of freedom: 1/4.

Cellular immune parameters	spleen		blood	
	F	p	*F*	p
Relative number of monocytes	1.09	0.35	0.07	0.80
Relative number of lymphocytes	1.05	0.36	0.09	0.78
Proliferation of lymphocytes	1.22	0.33	0.03	0.87

Table S3

PERMANOVA results for gene expression profiles measured in the spleen of juvenile sea bass. The permutational multivariate analysis of variance is based on Pearson correlation distance matrix. The effect of *B. subtilis* application on gene expression levels was tested for different functional gene groups. *F*-statistics (*F*), R^2 and p-values (p) are shown. Degrees of freedom/residual degrees of freedom for all groups: 1/4.

	7 days of treatment			14 days of treatment		
Functional group	*F*	R^2	p	*F*	R^2	p
All immune genes	2.5	0.38	> 0.05	1.0	0.20	> 0.05
Innate immunity	4.3	0.52	> 0.05	3.6	0.48	> 0.05
Adaptive immunity	2.2	0.36	> 0.05	0.5	0.11	> 0.05
Digestion	142.5	0.97	**< 0.01**	2.3	0.37	> 0.05
Stress	1.0	0.35	> 0.05	9.1	0.69	> 0.05

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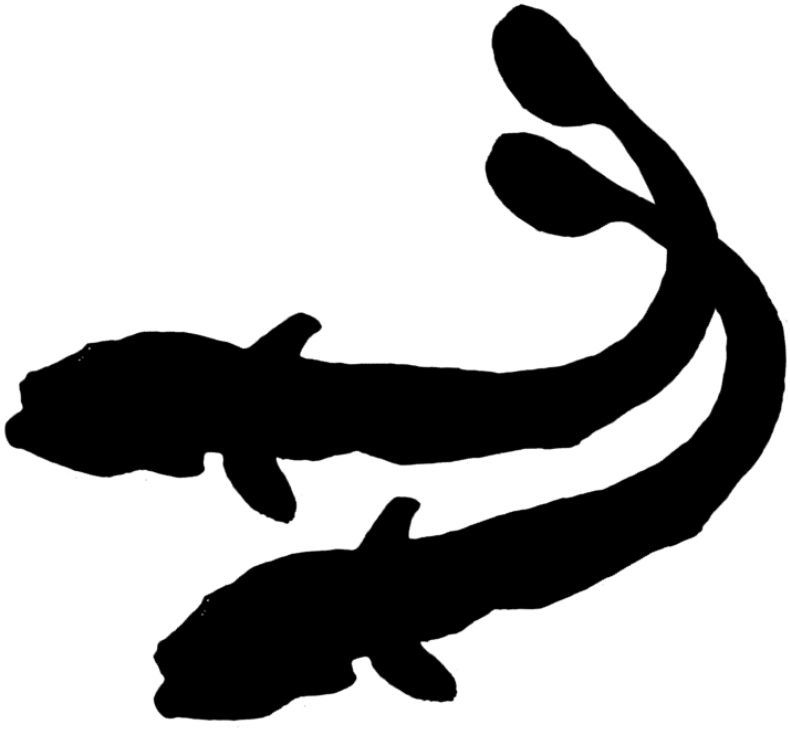
Chapter 2



Poly-β-hydroxybutyrate administration during early life: effect on survival, growth, microbial community, gene expression and disease resistance in European sea bass yolk-sac larvae

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Highlights

- Poly-β-hydroxybutyrate (PHB) improved survival in sea bass yolk-sac larvae.
- PHB altered the expression of the antimicrobial peptides ferritin and dicentracin.
- PHB altered the larva-associated microbiota composition at the phylum level.

Keywords: Immunostimulation, PHB, fish larvae, early development, bacterial challenge test, *Dicentrarchus labrax*, aquaculture

Abstract

The reliable production of marine fish larvae is still one of the major bottlenecks in aquaculture due to high mortalities mainly caused by the outbreak of infectious diseases. Therefore, the present study aimed to explore the capacity of poly-β-hydroxybutyrate (PHB)

to improve the larval performance and immunity in European sea bass (*Dicentrarchus labrax*) yolk-sac larvae in order to evaluate if PHB is a suitable candidate for immunostimulation in sea bass larviculture. To stimulate the developing larval immune system at the earliest possible point in time, PHB was applied from mouth opening onwards until 22 days post hatch (dph) and larval survival, growth performance, microbiota, gene expression profiles as well as disease resistance were assessed. PHB administration improved larval survival and, furthermore, altered the larva-associated microbiota composition at the phylum level. A bacterial challenge test (from 23 dph onwards) using a pathogenic *Vibrio anguillarum* strain, revealed that the disease resistance in PHB treated larvae was not different from the control (no PHB). The expression of 26 genes involved in immunity, growth, metabolism and stress-response was measured at 11 and 22 dph. PHB affected the expression of the antimicrobial peptides ferritin (*fer*) and dicentracin (*dic*), however, the response to PHB was inconsistent and weaker than detected in sea bass post-larvae (see chapter 3). Thus, the present study highlights the need for more research focusing on the immunostimulation at different points in time during early development.

1. Introduction

In marine aquaculture, the stable and cost-effective production of high quality fish larvae is still hampered due to low survival during larval rearing, mainly caused by infectious diseases (Conceição et al. 2010, Valente et al. 2013). Fish early life stages are highly susceptible towards pathogens, because their immunocompetence is still severely limited (Vadstein et al. 2012). For many aquaculture-relevant fish species, it has been reported that maternally-transferred immune molecules are mostly depleted during the first couple of days after hatch (Lillehaug et al. 1996, Tanaka et al. 1999, Hanif et al. 2004, Swain & Nayak 2009). In European sea bass larvae, for example, maternal IgM was not detectable anymore by day 5 post hatch (Breuil et al. 1997). Accordingly, fish larvae mainly rely on their innate immune response, while adaptive immunity still needs to be acquired (Magnadottir et al. 2005, Magnadottír 2006). As a consequence, vaccines are not applicable during larval stages, as their effect depends on immunological memory, a feature of adaptive immunity (Sommerset et al. 2005). Moreover, the standard method to combat diseases with antimicrobial drugs has selected for antibiotic-resistant bacteria, making treatments less

effective and additionally being a risk to the environment and the human health (Romero et al. 2012). Hence, investigating new biocontrol agents for disease prevention is crucial to evolve a sustainable aquaculture industry with enhanced larval survival rates and, therefore, reduced economic losses (Defoirdt et al. 2011). An alternative method is the stimulation of the immune response in fish larvae to improve their health and, thus, prevent the outbreak of diseases (Bricknell & Dalmo 2005, Ringø et al. 2011, Song et al. 2014, Akhter et al. 2015).

A potential biocontrol strategy is the application of poly-β-hydroxybutyrate (PHB), the polymer of the short-chain fatty acid (SCFA) β-hydroxybutyrate (β-HB) (Defoirdt et al. 2009). PHB is an energy storage compound accumulated by a wide range of bacterial genera such as *Alcaligenes* and *Bacillus* (Suriyamongkol et al. 2007, Wang 2012). In various aquatic organisms, including shrimps (*Penaeus monodon*) (Laranja et al. 2014), Rainbow trout (*Oncorhynchus mykiss*) and European sea bass (*Dicentrarchus labrax*) juveniles (De Schryver et al. 2010, Najdegerami, Bakhshi, et al. 2015), PHB has been demonstrated to increase survival and growth. Furthermore, dietary PHB altered the gastrointestinal (GI) microbiota in European sea bass juveniles and Siberian sturgeon (*Acipenser baerii*) juveniles (De Schryver et al. 2011, Najdegerami et al. 2012). The compound can be intestinally degraded into SCFAs, thereby, lowering the pH in the host's gut (Defoirdt et al. 2009). While beneficial bacteria such as lactic acid bacteria profit from a lower intestinal pH, it has been shown that the cell growth and multiplying of pathogenic bacteria like *Vibrio* spp. is suppressed by SCFAs (Cotter & Hill 2003, Van Immerseel et al. 2003, Defoirdt et al. 2007). A disease protecting effect of PHB has been demonstrated, for example, in *Artemia franciscana* (Defoirdt et al. 2007), Chinese mitten crab (*Eriocheir sinensis*) larvae (Sui et al. 2012) and gnotobiotic Nile tilapia (*Oreochromis niloticus*) larvae (Situmorang et al. 2015). Since SCFAs are known to play a central role in mammalian immunity, it is hypothesized that β-HB is capable of modulating the immune response in fish (Montalban-Arques et al. 2015). However, in teleosts PHB has so far only been shown to enhance serum immune parameters as well as antibody response in adult Mozambique tilapia (*Oreochromis mossambicus*) (Suguna et al. 2014) but not in early life stages.

In the current study, it has been investigated if the application of PHB modulates the immune response and the microbial community in European sea bass yolk-sac larvae. The

aim was to stimulate the developing larval immune system at the earliest possible point in time. Therefore, freeze-dried PHB-accumulated bacteria (*Alcaligenes eutrophus*) were administered to sea bass larvae from mouth opening onwards and the effect on larval survival, growth performance, microbiota and disease resistance has been assessed. Furthermore, an extensive analysis on the expression of genes involved in immunity and additionally metabolism, growth and stress was carried out.

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 dph and randomly distributed over 15 green tanks (100 larvae L^{-1}) at GEOMAR Kiel, Germany. Each tank was filled with 30 L Baltic Sea water (5 µm-filtered and UV-treated) with an artificially increased salinity (Seequasal) of 32 PSU, which was gradually decreased to 26 PSU until 14 dph and increased to 32 PSU again afterwards to improve the efficiency of swim bladder inflation (Saillant et al. 2003). The water flow was gradually increased from 0.05 to 0.1 L min^{-1} until the end of the experimental period. The water temperature was increased stepwise from 15 to 18 °C and oxygen was maintained above 89% saturation throughout the experiment. Larvae were kept in the dark until first feeding at 8 dph and thereafter under a natural photoperiod regime (16L: 8D). Tank bottoms were siphoned daily to remove dead larvae, feces and debris.

2.2. Experimental treatments and feeding

Two different PHB doses (low and high) were administered from 2 different points in time onwards (mouth opening and first feeding), respectively, resulting in the following treatments: (1) Low PHB dose administered from mouth opening on (LMO), (2) Low PHB dose from first feeding on (LFF), (3) High PHB dose from mouth opening on (HMO), (4) High PHB dose from first feeding on (HFF) and (5) No PHB administration (Control). Each treatment was triplicated.

Amorphous PHB was administered in the form of freeze-dried PHB accumulated bacteria (*Alcaligenes eutrophus*) produced as described by Thai et al. (2014). For details on differences between amorphous and crystalline PHB, respectively, see Hung et al. (2015). Bacteria with 2.5% and 75% PHB on cell dry weight (low and a high PHB content) were used. From mouth opening (MO at 5 dph) onwards, PHB at a low and high dose, respectively, was added directly to the tank water of the treatments LMO and HMO. Therefore, PHB accumulated bacteria were dissolved in UV-treated salt water (32 PSU). The PHB solutions were added daily at 10:00 h at a concentration of 10^6 bacteria mL^{-1} tank water until the end of the experiment (22 dph). The water flow was turned off between 10:00 h and 18:00 h. Furthermore, all PHB treatments (LMO, HMO, LFF and HFF) were fed with PHB-enriched rotifers (*Brachionus plicatilis*) from first feeding (FF at 8 dph) onwards over a period of 14 days. The control treatment was fed on rotifers that were not enriched with PHB. Rotifers were reared in sterile filtered Baltic Sea water, fed on resuspended *Nannochloropsis* spp. concentrate (BlueBioTech, Germany) and enriched with essential fatty acids (S.presso, INVE, applied according to instructions). Sea bass larvae were fed 3 times a day at 10:00 h, 15:00 h and 20:00 h (feeding ratio = 2:1:1). The rotifer density was increased over the course of the experiment from 4 to 12 mL^{-1} (first meal). For the PHB treatments, rotifers were enriched in freshly prepared PHB solutions at a density of 1000 ind. mL^{-1} for 30 min under gentle aeration directly before feeding. The PHB solutions for the rotifer enrichment consisted of freeze-dried PHB accumulated bacteria with a low and high PHB content, respectively, dissolved in salt water (UV-treated, 32 PSU) at a concentration of 10^8 bacteria mL^{-1}.

2.3. Measured parameters

2.3.1. Growth performance and survival

At the end of the experiment (22 dph), 10 larvae were randomly sampled from each tank, killed with an overdose of MS 222 (Sigma-Aldrich), transferred into Eppendorf vials with sea water and immediately frozen on dry ice. Samples were stored at -80 °C. For growth analysis the total length (mm) of thawed larvae was measured. Afterwards larvae were briefly rinsed in distilled water to avoid salt residues, freeze-dried for 18 h at -55 °C (Alpha1-4 freeze dryer, Christ) and weighed (Microbalance SC2, Sartorius) in order to determine the larval dry weight (µg).

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

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

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

2.3.2. Gene expression analysis

At 11 dph, 18 larvae and at 22 dph 6 larvae were randomly sampled from each tank, killed with an overdose of MS 222 (Sigma-Aldrich), transferred into RNAlater and kept at 4 °C for 24 h before being stored at -20 °C. These 2 sampling points were chosen to assess short-term and mid-term effects of PHB administration on young sea bass larvae.

For the quantification of mRNA as a measure of gene expression levels, RNA of whole larvae was extracted using the RNeasy 96 Universal Tissue Kit (Qiagen) according to the manufacturer´s instructions.

At 11 dph, single larvae were still too small to obtain enough RNA for gene expression measurements. Therefore, 6 times 3 larvae were pooled per tank for RNA extraction. At 22 dph 6 single larvae per tank were used. RNA concentration was measured by spectrophotometry (NanoDrop ND-1000, Peqlab) and normalized to a common concentration with RNase free water. 500 ng RNA were reverse transcribed into cDNA, including a gDNA wipeout step (Qiagen QuantiTect Reverse Transcription Kit). The cDNA was stored at -80 °C until further use.

Primers (Metabion) for all 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), using a StepOnePlus Real-Time PCR System (Applied Biosystems). 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.

Table 1

Name, abbreviation and function of the 26 genes of interest and 3 reference genes. Genes were divided into the following functional groups: (I) immunity, (II) growth and metabolism, (III) 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.
Immunity	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	
		ifn	Interferon, cytokine	FW	GTACAGACAGGCGTCCAAAGCATCA	FQ310507.3
				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 Iα*	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		*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	

A qPCR BioMark™ HD System (Fluidigm) running a 96.96 Dynamic Array™ IFC (Gene Expression chip) was used to measure the expression profiles of 26 target genes as well as 3 reference genes in the larval samples. Briefly, 1.3 µl cDNA per sample were mixed with TaqMan-PreAmp Master Mix (Applied Biosystems) 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) and DNA Binding Dye Sample Loading Reagent (Fluidigm). 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) and low EDTA-TE Buffer were loaded onto the chip in triplicate resulting in the measurement of technical triplicates per sample. The chip was primed and subsequently the run was performed using the GE Fast 96x96 PCR+Melt v2 thermal cycling protocol with a Tm of 60 °C according to the manufacturer´s instructions.

2.3.3. Larva-associated microbiota analysis

At the end of the experiment (22 dph), 5 larvae were randomly sampled from each tank, killed with an overdose of MS 222 (Sigma-Aldrich), transferred into ethanol and stored at 4 °C. Since the larvae were too small to remove the intestinal tract, whole single larvae were used for DNA extraction (DNeasy 96 blood & tissue kit, Qiagen). Extracted DNA was stored at -20 °C until further use. For the 16S rRNA gene-based characterization of the larva-associated microbiota, DNA including a negative and a positive (*Vibrio* sp.) control was amplified (Phusion High-Fidelity DNA Polymerase, Thermo Fisher Scientific) using the primers F515 and R806 for the variable region 4 of the 16S rRNA gene (Caporaso et al. 2011). Both primers contained adapters, barcodes, pad and linker sequences as described by Kozich et al. (2013). PCR cycling conditions for DNA amplification were as follows: 98 °C for 30 sec, followed by 30 cycles of 98 °C for 9 s, 55 °C for 15 sec and 72 °C for 20 sec, followed by 10 min at 72 °C. To eliminate primer dimers, the PCR products were purified using a MinElute 96 UF PCR purification kit (Qiagen). Subsequently, the DNA concentration of every sample was measured by spectrophotometry (NanoDrop ND-1000, Peqlab). Approx. 30 ng DNA per sample were pooled and a gel extraction was conducted (NucleoSpin

gel and PCR clean-up kit, Macherey-Nagel). The extraction products were fluorometrically quantified (Qubit fluorometer, Invitrogen) and then pooled in equimolar amounts. Thereafter, the purified 16S rRNA amplicons were sequenced on a MiSeq sequencer (Illumina) as described in Kozich et al. (2013). MiSeq sequence data were assembled and filtered using mothur (version 1.16.1; see Kozich et al. 2013). Sequence reads were merged and aligned against the SILVA alignment database (release 119), all sequences that did not cover the variable region 4 were removed (SILVA alignment position 1968 to 11550) (Pruesse et al. 2007). To reduce sequencing noise, a preclustering step (2 bp difference) was performed (Huse et al. 2010) and chimeric sequences were removed using UCHIME as implemented in mothur (Edgar et al. 2011). The taxonomy of all sequences was estimated using the classify.seqs function in mother against the RDP database (Cole et al. 2005) using a bootstrap cutoff of 80%. The sequences were clustered at the 0.03 difference level to obtain operational taxonomic units (OTUs). Furthermore, species richness, Simpson´s evenness and inverse Simpson´s diversity were calculated in mothur based on a dataset subsampled to a number of 10,000 reads per sample.

2.3.4. Bacterial challenge test

To investigate the protective effect of PHB against vibriosis, sea bass larvae were exposed to pathogenic *Vibrio anguillarum* in a bath challenge. Therefore, 100 larvae were taken out of each tank at 22 dph and randomly distributed over 2 aquaria with a volume of 1.5 L (resulting in 50 larvae per aquaria). One aquaria, respectively, was used for the bacterial challenge test (group BC) and the other one as an unchallenged control group (group UC). The experimental conditions were as follows: temperature 18 °C, salinity 32 PSU, sea water was 5µm-filtered and UV-treated, photoperiod 16L: 8D. The virulent *V. anguillarum* strain 87-9-117 was obtained from the Laboratory of Aquaculture and Artemia Reference Center (Ghent, Belgium) and preserved in 25% glycerol at -80 °C. Bacteria were cultured on 101 nutrient agar (5 g peptone, 3 g meat extract, 30 g NaCl, 15 g agar, 1 L distilled water) overnight at 25 °C. Subsequently, single colonies were picked and grown in 101 nutrient broth (5 g peptone, 3 g meat extract, 30 g NaCl, 1 L distilled water) overnight at 25 °C and 200 rpm in an incubator shaker. Bacteria were harvested by centrifugation at 2000 rpm for 10 min, washed twice in filtered and autoclaved seawater (32 PSU) and added to the rearing

water of group BC at a final density of 10^7 CFU mL^{-1}. After 24 h of challenge, the tank water was completely replaced in groups BC and UC. Afterwards the water flow was turned on overnight at a flow rate of 0.01 L min^{-1}. The survival of the sea bass larvae was monitored over a period of 4 days. Larvae were not fed over the course of the experiment and dead larvae were removed daily.

2.4. Statistical analyses

All statistical analyses were carried out in RStudio (version 0.98.1103). For parametric statistics, 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.

Survival data are presented by means of Kaplan-Meier curves and compared between treatment groups using a log-rank test (survival package, Therneau 2015). Post-hoc pairwise comparisons were performed in order to determine statistical differences between the respective treatments. To analyze growth data, a mixed effect model which included treatment as a fixed factor and tank as a random factor was used (lme4 package, Bates et al. 2015).

For gene expression analysis, the technical triplicates were used to calculate the mean cycle threshold value (Ct), the standard deviation (SD), and the coefficient of variation (CV) per sample. 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) and the geometric mean Ct of the 3 most stable genes (reference genes *actb*, *l13a*, *hsp90*; M < 0.5) was used to normalize the target genes (calculation of ΔCt-values). Permutational multivariate analyses of variance (PERMANOVA) were performed for each functional gene group to test for overall differences between the treatments. PERMANOVAs using ΔCt-values are based on Pearson distance matrices and were run with 999 permutations (adonis and Dist function, vegan and amap package, Lucas 2011, Oksanen et al. 2012). Multivariate effects were assessed on data averaged within experimental tank. 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. Post-hoc pairwise comparisons were performed using the lsmeans function (lsmeans package,

Lenth 2016). For graphical representation of gene expression data in response to PHB, the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001) was applied by calculating the ΔΔCt for each target gene in relation to the mean ΔCt of the respective target gene in the untreated control. For graphical representation of the expression of all 26 target genes over the larval development, the $2^{-\Delta Ct}$ method (Wang et al. 2004) was applied by using expression data of the untreated control only.

The microbiota data could not be analyzed for the whole data set due to an insufficient number of reads for many larval samples. Only samples with >10,000 reads were included in the statistical analysis, lowering the sample size to 1 to 4 larvae per tank and making it difficult to account for a potential tank effect. Hence, tank was not implemented in the statistical analyses, instead only treatment was included as a fixed factor using each larva instead of each tank as a replicate. To test for differences in the microbial community compositions between the treatments, bacterial phyla and OTUs were analyzed performing PERMANOVAs based on Euclidean distance matrices (adonis and vegdist function, vegan package, 999 permutations, Oksanen et al. 2012). In addition, a principle component analysis (PCA) for graphical visualization was implemented based on differences in the bacterial phyla composition according to the PHB treatments (ade4 package, Dray & Dufour 2007). To analyze the species richness, Simpson´s evenness and inverse Simpson´s diversity, analyses of variance (Anova) were performed (stats package, R Core Team 2016).

3. Results

3.1. Survival and growth performance

Around first feeding (8 dph), mortality was highly variable between the tanks across all treatments. Therefore, differences in survival were only examined after the onset of exogenous feeding. The treatments were significantly different from each other ($\chi^2 = 175$, df = 4, $p < 0.001$, Fig. 1). Larval survival in treatments LMO, HMO and HFF was significantly higher than in the control treatment ($\chi^2_{LMO} = 37$, $\chi^2_{HMO} = 134$, $\chi^2_{HFF} = 56$, df = 1, $p < 0.001$) while LFF and control ($\chi^2_{LFF} = 1.1$, df = 1, $p = 0.30$) were not significantly different from each other. Treatment HMO exhibited the highest survival rate.

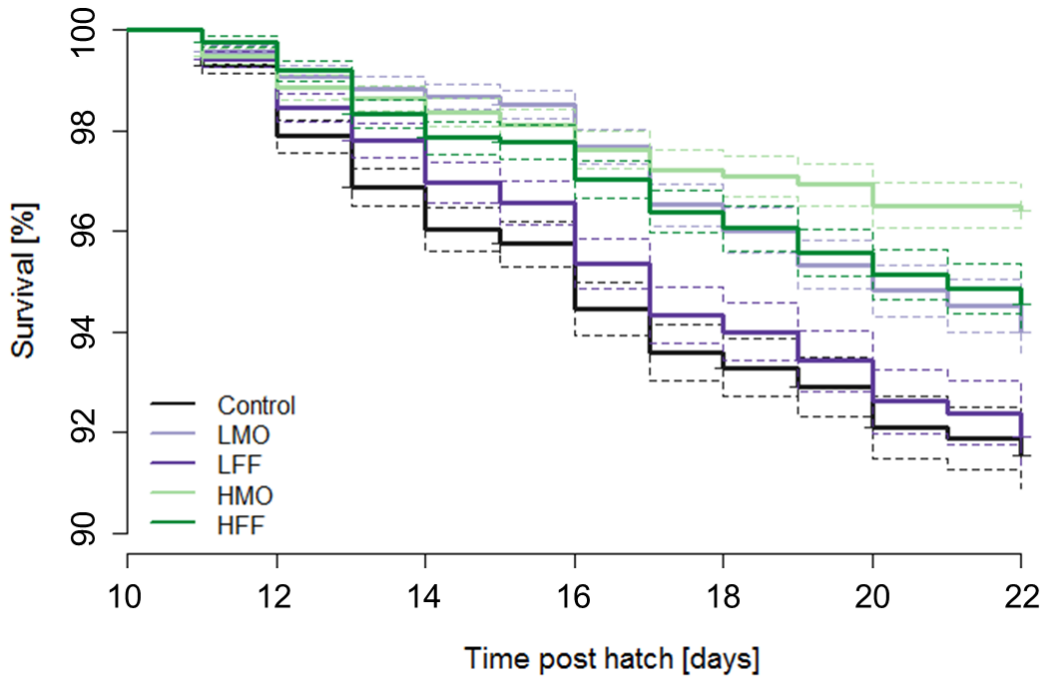

Fig. 1. Kaplan-Meier survival curves of sea bass larvae (after 10 dph) administered a low PHB dose from mouth opening on (LMO), a low PHB dose from first feeding on (LFF), a high PHB dose from mouth opening on (HMO), a high PHB dose from first feeding on (HFF) or no PHB (control), respectively. The dashed lines represent the 95% confidence intervals.

The estimated growth-related parameters such as total length (mm), dry weight (µm) and Fulton´s condition factor K (µg mm^{-3}) were not affected by the PHB treatment over the course of the experiment. However, there was a trend towards a higher larval weight in all PHB treatments compared to the control. All results are presented in Table 2.

Table 2

Growth-related parameters of sea bass larvae administered a low PHB dose from mouth opening on (LMO), a low PHB dose from first feeding on (LFF), a high PHB dose from mouth opening on (HMO), a high PHB dose from first feeding on (HFF) or no PHB (control), respectively. 10 larvae per tank were sampled at 22 dph. Values represent mean ± SEM. Further, the mixed-effect model results are shown (*F*-value (*F*), p-value (p), degrees of freedom/residual degrees of freedom for all parameters: 4/10).

Growth-related	22 dph (end of experiment)						
parameters	Control	LMO	LFF	HMO	HFF	*F*	p
Dry weight (µg)	314 ± 18	386 ± 32	332 ± 25	378 ± 25	364 ± 23	0.7	0.61
Total length (mm)	8.0 ± 0.1	8.3 ± 0.2	8.2 ± 0.1	8.4 ± 0.2	8.4 ± 0.1	0.5	0.72
Condition[1] (µg mm^{-3})	0.61 ± 0.03	0.63 ± 0.03	0.58 ± 0.03	0.62 ± 0.02	0.61 ± 0.03	0.6	0.66

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

3.2. Gene expression

The expression of 26 genes involved in immune response, apoptosis, growth, metabolism, antioxidant activity and stress-response were analyzed and classified into the following functional gene groups: (I) immunity, (II) growth and metabolism, (III) stress. All genes included in the study (Table 1) were expressed at day 11 and 22 ph (Fig. 2).

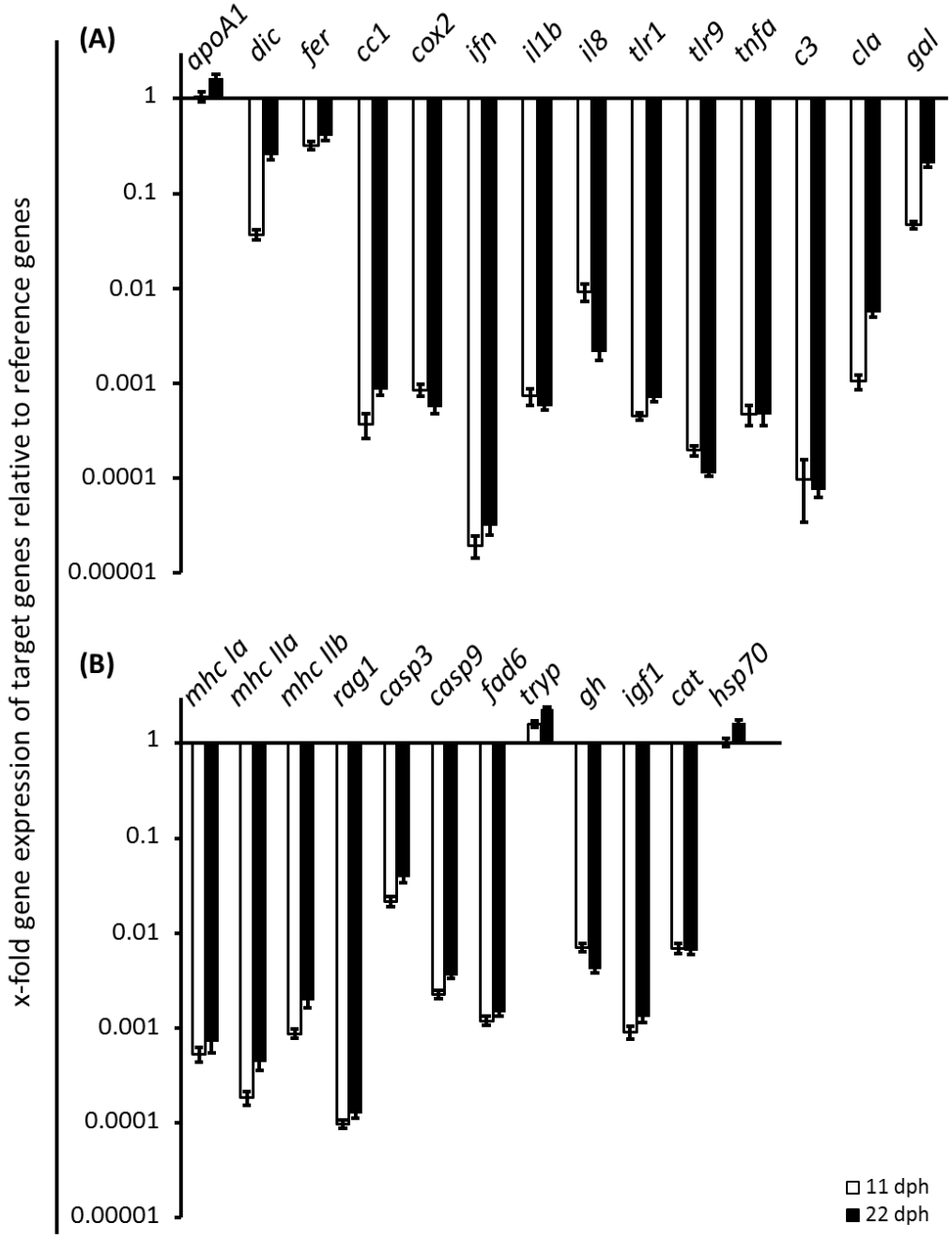

Fig. 2. Expression of all target genes in sea bass larvae at 11 dph (white) and 22 dph (black) in the untreated control. **(A)** innate immunity and complement system, **(B)** adaptive immunity, apoptosis, metabolism, growth and stress. The figures display the x-fold gene expression of the target genes relative to the geometric mean Ct of the reference genes. Data are presented as mean ± SEM. For information on the gene functions see Table 1.

The multivariate analysis showed that the expression of the immune-related genes differed significantly between the treatments at 11 dph ($F_{4,10}$ = 5.0, p < 0.05) and 22 dph ($F_{4,10}$ = 2.8, p < 0.05) , while the other functional gene groups were not affected by the administration of PHB (Suppl. Table S1).

The subsequent univariate analyses (Suppl. Table S2) revealed that the impact of PHB on immune-related gene was driven by ferritin expression at 11 dph ($F_{4,10}$ = 4.0, p < 0.05) and the dicentracin expression at 22 dph ($F_{4,10}$ = 3.6, p < 0.05).

The expression of ferritin was significantly down-regulated in treatment LFF (0.7 ± 0.07-fold, ΔCt = 2.6 ± 0.22) compared to the control (1.0 ± 0.04-fold, ΔCt = 1.8 ± 0.09; Fig. 3). At this point in time (11 dph), the treatment LFF corresponded to 3 days of a low PHB dose encapsulated in the live feed. The treatments LMO (ΔCt = 1.9 ± 0.24), HMO (ΔCt = 2.2 ± 0.16) and HFF (ΔCt = 1.9 ± 0.22) were not statistically different from the control.

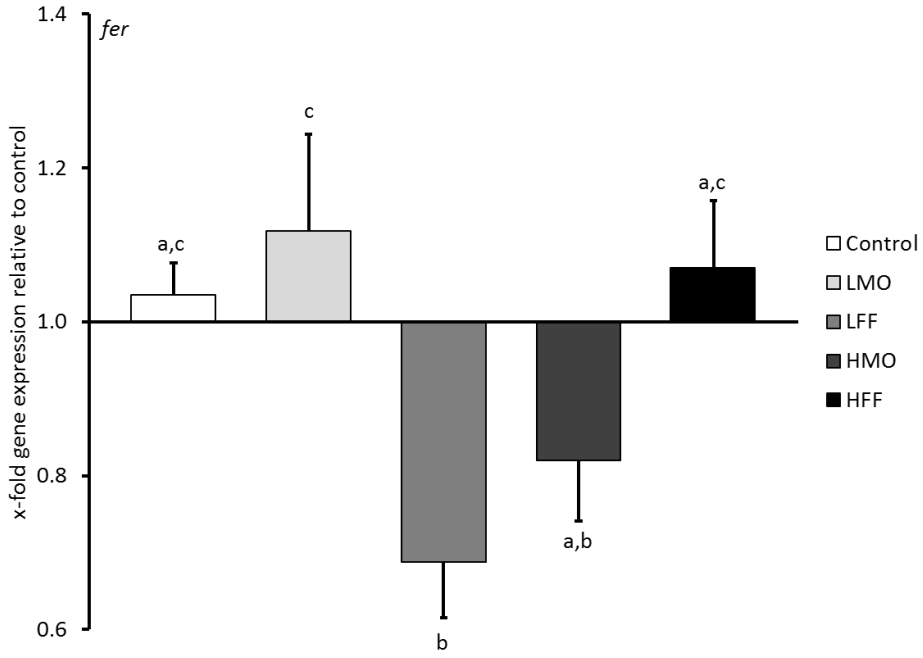

Fig. 3. Gene expression of ferritin (*fer*) in sea bass larvae administered a low PHB dose from mouth opening on (LMO), a low PHB dose from first feeding on (LFF), a high PHB dose from mouth opening on (HMO), a high PHB dose from first feeding on (HFF) or no PHB (control), respectively. Larval samples were taken at 11 dph. The figure displays the x-fold gene expression to the control. Data are presented as mean ± SEM. Treatments with different letters are significantly different at p < 0.05.

The dicentracin expression was significantly decreased in treatment LMO (0.8 ± 0.08-fold, ΔCt = 3.0 ± 0.18) compared to the control (1.1 ± 0.11-fold, ΔCt = 2.5 ± 0.13; Fig. 4). At this point in time (22 dph), the treatment LMO corresponded to 17 days of a low PHB dose administered via the tank water and 14 days of PHB encapsulated in the live feed. The treatments LFF (ΔCt = 2.6 ± 0.14), HMO (ΔCt = 2.2 ± 0.11) and HFF (ΔCt = 2.5 ± 0.12) did not differ from the control.

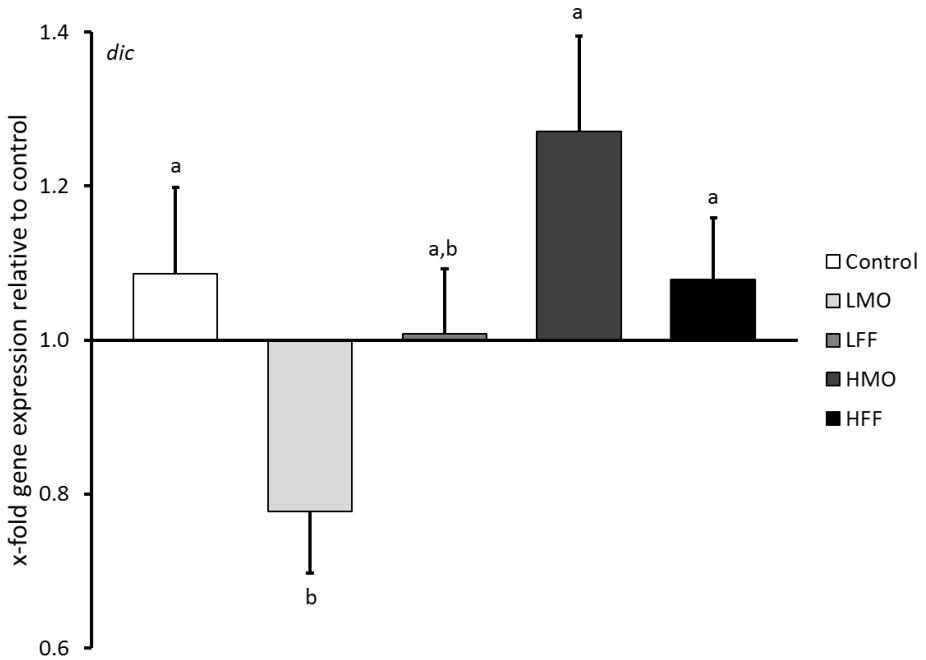

Fig. 4. Gene expression of dicentracin (*dic*) in sea bass larvae administered a low PHB dose from mouth opening on (LMO), a low PHB dose from first feeding on (LFF), a high PHB dose from mouth opening on (HMO), a high PHB dose from first feeding on (HFF) or no PHB (control), respectively. Larval samples were taken at 22 dph. The figure displays the x-fold gene expression to the control. Data are presented as mean ± SEM. Treatments with different letters are significantly different at $p < 0.05$.

3.3. Larva-associated microbiota

While the bacterial phyla composition differed significantly between the treatments ($F_{4,27}$ = 1.9, $p < 0.05$, Fig. 5), there was no difference at the OTU level ($F_{4,27}$ = 1.0, $p = 0.53$).

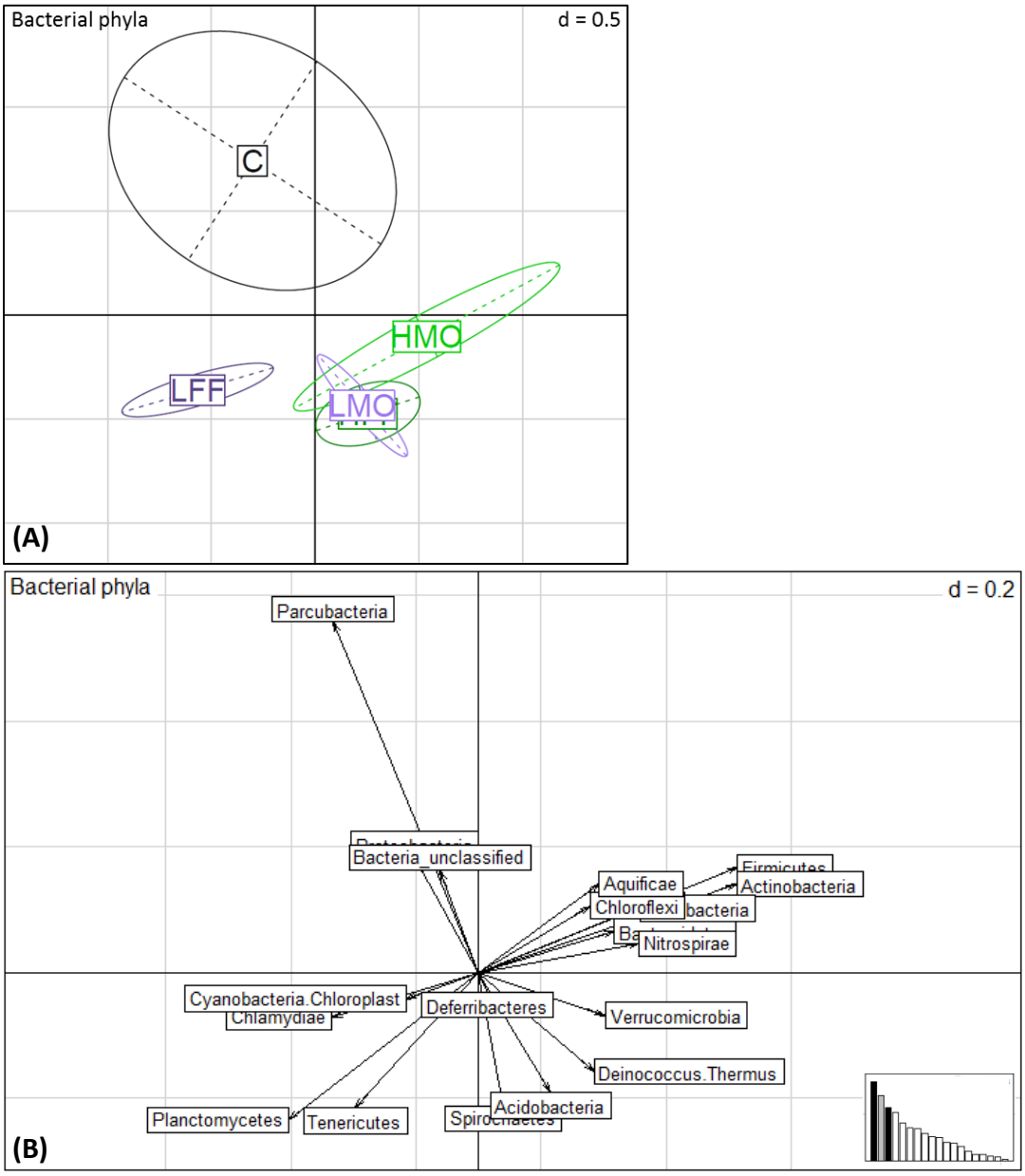

Fig. 5. (A) PCA plot visualizing the bacterial phyla composition in European sea bass larvae (22 dph) administered a low PHB dose from mouth opening on (LMO), a low PHB dose from first feeding on (LFF), a high PHB dose from mouth opening on (HMO), a high PHB dose from first feeding on (HFF) or no PHB (control), respectively. **(B)** The corresponding scatterplot represents the contribution of each variable (bacterial phyla) to the total variability. The Eigenvalues are represented by a bar chart in the lower right corner. Principle component 1 retains 15.5% and principle component 2 retains 12.9% of variance.

Bacteria belonging to the phyla Proteobacteria and Bacteroidetes accounted for more than 90% of the microbial community in all treatments except for LMO where they represented 74.5%. While unclassified bacteria accounted for 18.6% in the group LMO, they represented less than 3.7% in all other experimental groups (Fig. 6). The phylum Firmicutes accounted for 2.5% and 1.3% of the bacterial community in the treatments HMO and LMO, respectively, while it was less than 0.8% in the all other groups.

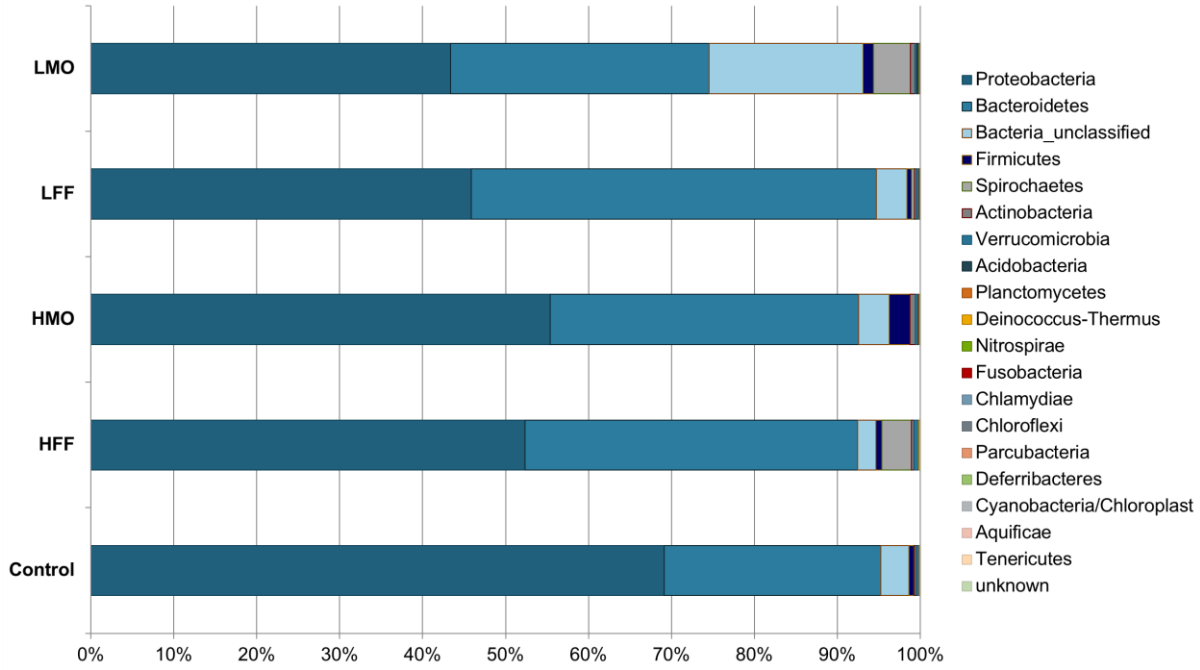

Fig. 6. Bacterial phyla composition in European sea bass larvae (22 dph) administered a low PHB dose from mouth opening on (LMO), a low PHB dose from first feeding on (LFF), a high PHB dose from mouth opening on (HMO), a high PHB dose from first feeding on (HFF) or no PHB (control), respectively.

Furthermore, the species richness, Simpson´s evenness and inverse Simpson´s diversity were not affected by the PHB treatment (Table 3).

Table 3

Microbial community parameters of sea bass larvae administered a low PHB dose from mouth opening on (LMO), a low PHB dose from first feeding on (LFF), a high PHB dose from mouth opening on (HMO), a high PHB dose from first feeding on (HFF) or no PHB (control), respectively. Values represent mean ± SEM. Further, Anova results are shown (*F*-value (*F*), p-value (p), degrees of freedom/residual degrees of freedom for all parameters: 4/27).

Microbial community	Second sampling point (22 dph)						
parameters	Control	LMO	LFF	HMO	HFF	*F*	p
Species richness	235 ± 25	268 ± 48	226 ± 13	272 ± 28	306 ± 8	0.3	0.86
Simpson´s evenness	0.04 ± 0.007	0.05 ± 0.009	0.04 ± 0.011	0.05 ± 0.014	0.03 ± 0.004	0.7	0.57
Inv. Simps. diversity	9 ± 1.8	12 ± 2.8	10 ± 2.4	16 ± 6.8	9 ± 1.4	0.7	0.59

3.4. Bacterial challenge test

Unchallenged larvae survived significantly better than larvae challenged with the *Vibrio anguillarum* strain 87-9-117 (χ^2 = 900, df = 1, $p < 0.001$). The survival of the *V. anguillarum* challenged larvae differed significantly between the PHB treatments (χ^2 = 10, df = 4, $p < 0.05$, Fig. 7). While the larval survival in the control group was not different from any PHB treated group, the survival in group LMO was significantly higher than in group HMO (χ^2 = 6.5, df = 1, $p < 0.05$) and group HFF (χ^2 = 5.4, df = 1, $p < 0.05$).

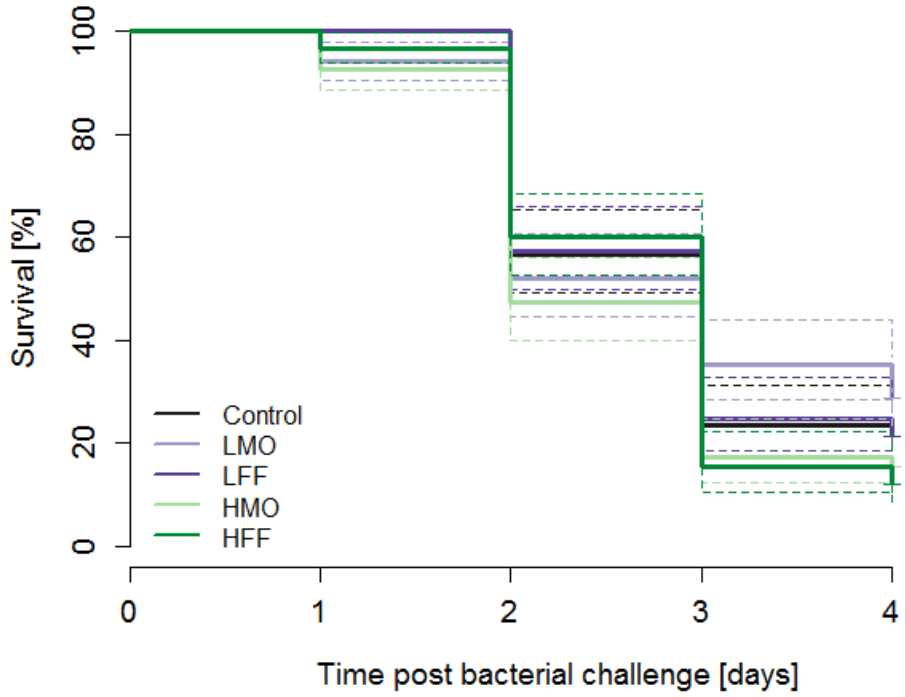

Fig. 7. Kaplan-Meier survival curves of sea bass larvae challenged with *V. anguillarum* at 22 dph after being administered a low PHB dose from mouth opening on (LMO), a low PHB dose from first feeding on (LFF), a high PHB dose from mouth opening on (HMO), a high PHB dose from first feeding on (HFF) or no PHB (control), respectively. The dashed lines represent the 95% confidence intervals.

4. Discussion

In the present study, the effects of the bacterial energy storage compound PHB on sea bass yolk-sac larvae were examined with respect to survival, growth performance, disease resistance and immunity. Early fish larvae depend mainly on their innate immune response since their adaptive immune system is still developing, making them extremely susceptible to infections (Castro et al. 2015). The maturation of the immune system is influenced by the intestinal microbiota, which starts to build up from mouth opening on and was shown to be altered by PHB supplementation (Gómez & Balcázar 2008, De Schryver et al. 2011, Maynard et al. 2012, Najdegerami et al. 2012). Accordingly, the focus of this study was to investigate the impact of PHB on the developing larval immune system and microbiota, when administered at the earliest possible point in time (mouth opening).

The larval stage is the most vulnerable of the life cycle stages, suffering from high mortality rates (Rosenthal & Alderdice 1976, Pepin 1991) which are additionally found to be extremely variable during critical developmental periods such as first feeding (Voss et al. 2001). This was as well shown for sea bass larvae where the highest mortality rates occurred between 6 and 10 dph around the onset of exogenous feeding (Hatziathanasiou et al. 2002). Similar results were obtained in the present study, where mortality during this period was highly variable between the tanks independent of the respective treatment. Therefore, the survival analysis was started at the end of the critical first feeding period. Survival was significantly higher in the treatments LMO, HMO and HFF compared to the control whereby the administration of PHB in a high dose from mouth opening on (HMO) resulted in the best larval survival.

The larval growth performance was not significantly affected by PHB administration. However, there was a trend towards a higher larval weight in all PHB treatments compared to the control. Larvae that received a low, respectively, high PHB dose from mouth opening on (LMO and HMO) tended to have higher weights at the end of the experiment. Treatments LMO and HMO were the only experimental groups being provided with PHB via the tank water before larvae were able to feed. An improvement in survival and growth after PHB administration was also found in blue mussel (*Mytilus edulis*) (Hung et al. 2015) and Chinese mitten crab (*Eriocheir sinensis*) larvae (Sui et al. 2014), giant tiger prawn (*Penaeus monodon*) post-larvae (Laranja et al. 2014) and sea bass juveniles (De Schryver et al. 2010). It is hypothesized that PHB is gastrointestinally degraded into the SCFA β-HB (by digestive enzymes, PHB degrading bacteria or both), which promotes growth and survival by being an additional energy source for the host (Weltzien et al. 2000, Azain 2004, Defoirdt et al. 2009). SCFAs are known to be the main energy sources of intestinal cells and to mediate their proliferation, differentiation and mucin production (Roy et al. 2015). Morover, SCFAs lower the intestinal pH which may enhance the activity of digestive enzymes leading to a better nutrient absorption (Lückstädt 2008). This additional energy might be the reason for the observed enhanced survival in sea bass larvae provided with PHB.

The expression of 26 genes involved in the innate and adaptive immune response, the complement system, apoptosis, digestion, growth and oxidative damage was analyzed in order to estimate the effect of PHB on larval immunity, metabolism and stress. 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, the larval immune gene expression profiles differed significantly between the PHB treatments at 11 and 22 dph, respectively. The analyses of the single genes revealed that this effect was driven by a differential expression of the antimicrobial peptides *fer* and *dic*. This indicates that the observed changes in immune gene expressions are moderate and, therefore, cannot be detected for most individual genes. The differences in the expression of *fer*, respectively, *dic* between the treatments did not show a clear pattern as observed in sea bass post-larvae, where several immune-related genes were up-regulated in response to PHB administration (see chapter 3). Nevertheless, it has to be taken into account that the methodological approach only allowed to analyze the expression of a limited number of genes. Since the immune response in fish early life stages is driven by a complex network of innate defense mechanisms (Vadstein et al. 2012), it cannot be excluded that PHB may have induced changes in the expression of immune genes not assessed in this study. It was hypothesized that the metabolic product of PHB, the SCFA β-HB, might stimulate the immune system in fish in a similar way as in mammals by binding to G protein-coupled receptors (GPRs) (Montalban-Arques et al. 2015). Mammalian GPR43 for example recognizes butyrate and is highly expressed in macrophages and neutrophils (Brestoff & Artis 2013). Both cell types are known to be present already in fish early life stages (Rombout et al. 2005). The immune response might be modulated in addition through changes in the intestinal microbial community caused by the degradation of PHB lowering the luminal pH (Gómez & Balcázar 2008, Defoirdt et al. 2009). In Siberian sturgeon larvae, for example, administered PHB from first-feeding on, the intestinal microbiota was altered (Najdegerami, Baruah, et al. 2015). In the current study, it was only possible to perform a preliminary microbiota analysis due to an insufficient number of reads for many larval samples. However, the larva-associated bacterial phyla composition differed between the treatments in the analyzed data set. Interestingly, the treatment HMO, exhibiting the best survival and a trend towards an increased growth performance, had the highest proportion of bacteria belonging to the phylum Firmicutes. The phylum includes a variety of probiotic bacteria such as *Bacillus* spp.

that have been demonstrated to confer a health benefit to the host (Gómez & Balcázar 2008, Akhter et al. 2015). The larval bacterial communities on the OTU level as well as the bacterial species richness, evenness and diversity were not affected by the administration of PHB. In contrast, the application of PHB increased the bacterial evenness and diversity in sea bass juveniles (De Schryver et al. 2010, De Schryver et al. 2011) and Siberian sturgeon juveniles (Najdegerami et al. 2012). These results indicate that the mode of action of PHB might vary between different life cycle stages.

In previous studies, it was demonstrated that PHB increased disease resistance in aquatic organisms. Survival was enhanced when PHB was provided during bacterial challenge tests with axenic Nile tilapia larvae (Situmorang et al. 2015), Chinese mitten crab larvae (Sui et al. 2012) and *Artemia* (Defoirdt et al. 2007, Halet et al. 2007). It was concluded that PHB inhibited the multiplying of the pathogenic bacteria directly. SCFAs such as β-hydroxybutyric acid can pass bacterial cell membranes and release protons in the cytoplasm which have to be exported in order to maintain a stable pH. This energy consuming process suppresses the growth of the pathogenic bacteria (Defoirdt et al. 2009). In the current study we wanted to investigate if the immunostimulating effects of PHB may increase the robustness of sea bass larvae during a *V. anguillarum* infection. To disentangle the immediate antimicrobial effect of the compound and its immunostimulatory capacities, sea bass larvae were not supplied with PHB during the bacterial challenge test. Compared to the control, none of the PHB treatments had a significant effect on the survival of sea bass larvae challenged with the pathogenic *V. anguillarum* strain 87-9-117. However, the survival was the highest in larvae administered a low PHB dose from mouth opening onwards (LMO). The observed weak effect of PHB administration on disease resistance coincides with the weak effect on the larval immune gene expression. However, when shrimp (*Penaeus monodon*) post-larvae were infected with *V. campbellii* after being fed over 30 days with a PHB-supplemented diet, survival was increased compared to the control (Laranja et al. 2014). The different results obtained in sea bass larvae and shrimp post-larvae might have manifold reasons. Variation in dose and period of time of PHB administration as well as the virulence of the bacteria used for the challenge test might be an explanation. While less than 25% of the sea bass larvae survived in the control treatment (no PHB) challenged with *V. anguillarum*, over 60% of the shrimp post-larvae survived. This indicates that the *Vibrio* strain used in the

present study was most likely too virulent to enable the detection of a potential protective effect of PHB. Furthermore, possible differences in the mode of action of PHB between teleost fish and crustaceans have to be taken into consideration and addressed in future studies.

In conclusion, our study indicates that administering amorphous PHB to sea bass larvae already before the onset of first feeding via the tank water had a positive influence on their survival. A prolongation of the time span of the PHB application is advised for further research to investigate if a significant growth-promoting effect can be obtained. The impact of PHB on the immune gene expression of sea bass yolk-sac larvae was inconsistent and weaker than detected in sea bass post-larvae (see chapter 3). During their first weeks of life, fish larvae undergo significant morphological and physiological changes including the maturation of the immune system. Thus, the mode of action of PHB in early, respectively, late larval stages might vary substantially. The current study highlights the need for more research focusing on the immunostimulation at different points in time during early development. In order to elucidate life-stage dependent differences, future studies on fish larvae analyzing an extended set of immune-related genes are necessary.

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Supplementary material

Table S1

PERMANOVA results for larval gene expression profiles. The permutational multivariate analyses of variance are based on a Pearson 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: 4/10.

	11 dph		22 dph	
Functional group	*F*	p	*F*	p
Immunity	5.0	**< 0.05**	2.8	**< 0.05**
Growth & metabolism	1.4	> 0.05	1.1	> 0.05
Stress	0.6	> 0.05	0.8	> 0.05

Table S2

Univariate statistical results (mixed-effect model) for all genes of functional group (I) immunity. Shown are *F*-values (*F*) and p-values (p). Degrees of freedom/residual degrees of freedom: 4/10.

	11 dph		22 dph	
Gene	*F*	p	*F*	p
apoA1	3.04	0.07	1.31	0.33
dic	1.52	0.27	3.60	**0.04**
fer	4.02	**0.03**	1.66	0.23
cc1	2.78	0.09	1.63	0.24
cox2	1.03	0.44	0.78	0.56
ifn	1.98	0.17	1.09	0.41
il1b	2.28	0.13	0.43	0.78
il8	3.04	0.07	2.07	0.16
tlr1	0.65	0.64	1.38	0.31
tlr9	1.55	0.26	0.83	0.54
tnfa	1.25	0.35	2.06	0.16
c3	0.24	0.91	0.24	0.91
cla	1.54	0.26	2.29	0.13
gal	0.18	0.95	2.41	0.12
mhc class Ia	0.37	0.83	1.48	0.28
mhc class IIa	0.35	0.84	1.12	0.40
mhc class IIb	0.87	0.51	2.00	0.17
rag1	0.91	0.49	0.08	0.99
casp3	0.62	0.66	1.70	0.23
casp9	0.33	0.85	1.29	0.34

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Chapter 3

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

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Highlights

- Poly-β-hydroxybutyrate (PHB) stimulates the innate and adaptive immune response in sea bass post-larvae.
- PHB enhances the expression of antimicrobial peptides and *mhc class II*.
- PHB increases the expression of a growth-related gene.

Keywords: Immunostimulation, PHB, fish larvae, immune system, early development, 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 as well as 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. Survival was monitored daily, but found not to be affected by the application of PHB. We monitored growth performance and measured the expression of 29 genes involved in immunity, growth, metabolism and stress-response using larval samples. While the expression of the insulin-like growth factor 1 (*igf1*), an indicator of relative growth, was upregulated in response to feeding PHB, the overall larval 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.

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 when the yolk absorption is completed (Magnadottir et al. 2005, Swain & 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 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 & Hill 2003). This may explain why gnotobiotic Nile tilapia (*Oreochromis niloticus*) larvae (Situmorang et al. 2015) and rainbow trout (*Oncorhynchus mykiss*) fry (Najdegerami, Bakhshi, et al. 2015) 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.

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) of 32 PSU, which was gradually decreased to 26 PSU 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). 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, 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 PSU, photoperiod 16L: 8D and flow rate 0.4 L min^{-1}. The tank bottoms were siphoned daily to remove dead larvae, feces and debris.

2.2. 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) 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) 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 salt water (UV-treated, 32 PSU) 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).

2.3. Measured parameters

2.3.1. 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), 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) and weighed (Microbalance SC2, Sartorius) 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 larvae were randomly sampled from each tank, anaesthetized with MS 222 (Sigma-Aldrich), 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.

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) according to the manufacturer´s instructions. RNA concentration was measured by spectrophotometry (NanoDrop ND-1000, Peqlab) and normalized to a common concentration with RNase free water. 500 ng RNA were reverse transcribed into cDNA, including a gDNA wipeout step (Qiagen QuantiTect Reverse Transcription Kit). The cDNA was stored at -80 °C until further use.

Primers (Metabion) 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), using a StepOnePlus Real-Time PCR System (Applied Biosystems). 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.

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 ref.).

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	
		ifn	Interferon, cytokine	FW	GTACAGACAGGCGTCCAAAGCATCA	FQ310507.3
				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		*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	

A qPCR BioMark™ HD System (Fluidigm) 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 (Applied Biosystems) 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) and DNA Binding Dye Sample Loading Reagent (Fluidigm). 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) 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.

2.3.3. 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) 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 & 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 (Fig. 1) in the PHB treatment and the control treatment did not differ significantly from each other over the course of the experiment (χ^2 = 0.9, df = 1, p > 0.05). Survival remained above 85% in both treatment groups.

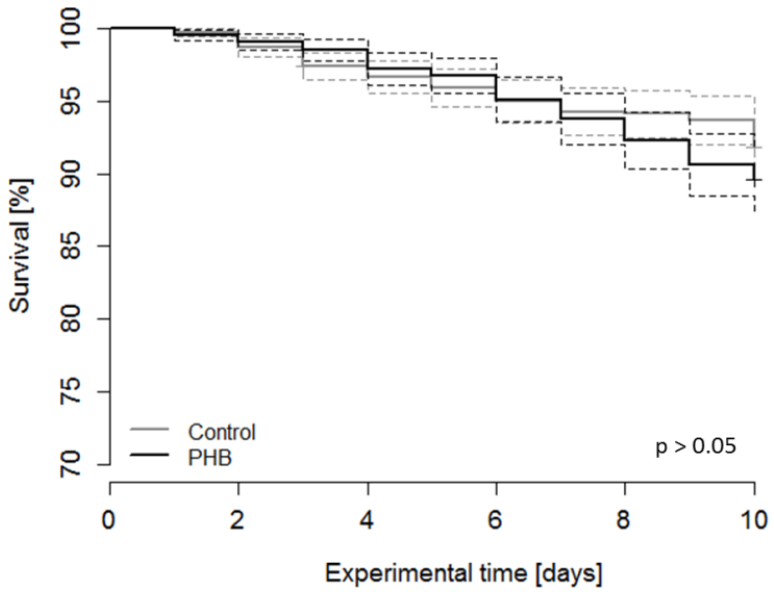

Fig. 1. Kaplan-Meier survival curves of sea bass larvae fed with *Artemia* enriched with PHB (black) or without PHB (grey) over a period of 10 days (from 28 to 38 dph). The dashed lines represent the 95% confidence intervals.

3.2 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.

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)

3.3 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 (Suppl. Table S1). 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 S2).

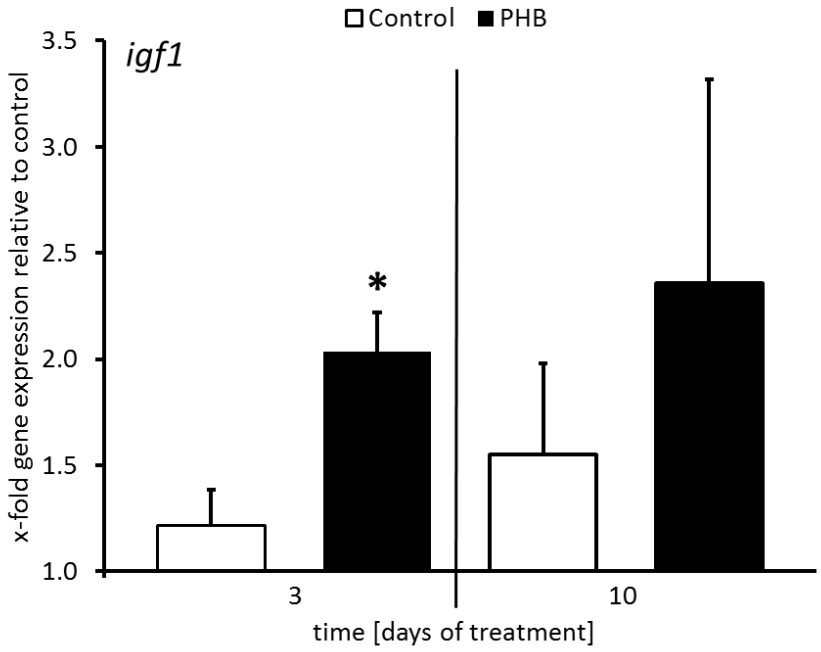

Fig. 2. Gene expression of insulin-like growth factor 1 (*igf1*) in sea bass larvae fed with *Artemia* nauplii with PHB (black bars) or without PHB enrichment (white bars). Larval samples were taken at 31 and 38 dph (3 and 10 days of treatment, respectively). The figure displays the x-fold gene expression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

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 (Suppl. Table S1). The subsequent univariate analyses (Suppl. Table S2) 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).

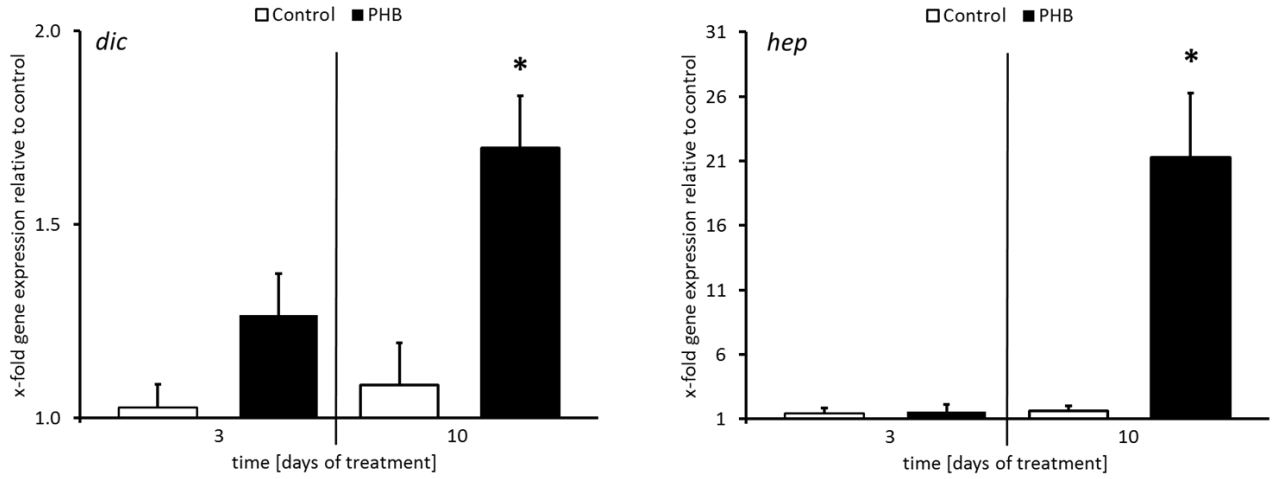

Fig. 3. Gene expression of dicentracin (*dic*) and hepcidin (*hep*) in sea bass larvae fed with *Artemia* nauplii with PHB (black bars) or without PHB enrichment (white bars). Larval samples were taken at 31 and 38 dph (3 and 10 days of treatment, respectively). The figures display the x-fold gene expression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

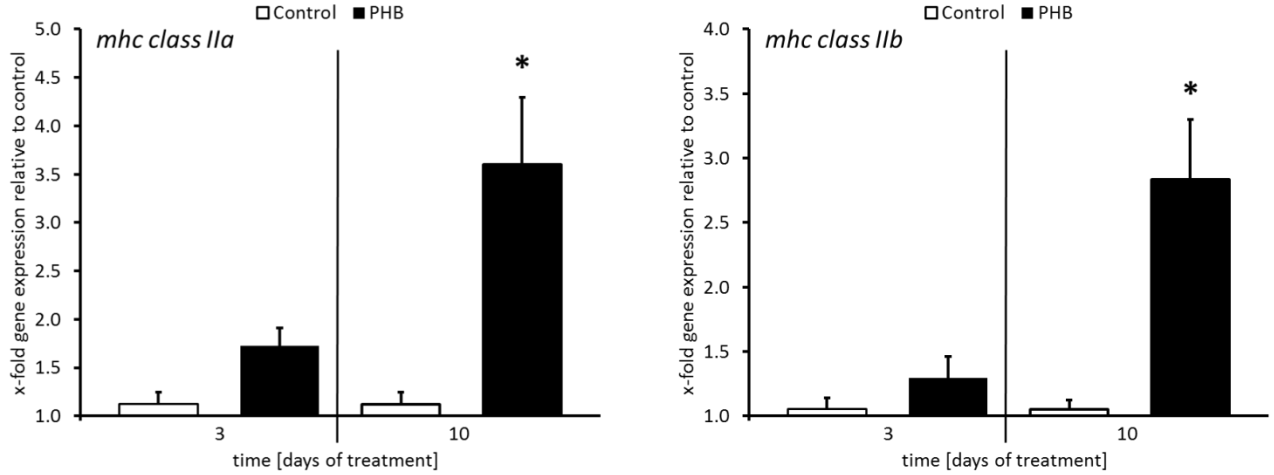

Fig. 4. Gene expression of *mhc class IIa* and *mhc class IIb* in sea bass larvae fed with *Artemia* nauplii with PHB (black bars) or without PHB enrichment (white bars). Larval samples were taken at 31 and 38 dph (3 and 10 days of treatment, respectively). The figure displays the x-fold gene

expression to the control. Data are presented as mean ± SEM. The asterisk represents the level of significance (*: $p < 0.05$).

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 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 & 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, Baruah, et al. 2015, 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, Baruah, et al. 2015). 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, Baruah, et al. 2015), 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 & 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 & 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).

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 & 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 & 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.

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Supplementary material

Table S1

PERMANOVA results for larval gene expression based on Pearson distance matrices. The effect of the experimental PHB treatment 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 S2

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	Genes	*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	Genes	*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

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Synthesis

In around 50 years from now almost 10 billion people will inhabit our planet, making it an unprecedented challenge for mankind to ensure food security. Aquaculture, being one of the most controversial food-producing industries, is expected to play a crucial role in meeting the increasing demand of animal protein (Young & Matthews 2010, FAO 2016a, Quaas et al. 2016). Nevertheless, the aquaculture sector is facing serious difficulties (Murray & Peeler 2005, Defoirdt et al. 2011). At present, one of the severest problems in marine larval aquaculture is the lack of possibilities to prevent the outbreak of infectious diseases, leading to high and unpredictable larval mortalities. Fish larvae are most susceptible towards infectious diseases since they lack a mature immune system (Vadstein et al. 2012), hence, researchers around the world are dedicated to develop new immunoprophylactic measures for fish early life stages.

In my thesis, I aimed to contribute novel knowledge regarding the stimulation of the still maturing immune system in early life stages of European sea bass (*Dicentrarchus labrax*), one of the economically most important fish species in European aquaculture (FAO 2016b). I will summarize and discuss the main findings of my PhD thesis with special emphasis on the key role of the gastrointestinal (GI) microbiota for fish immunity as well as give an outlook regarding potential future research directions.

1. Protecting the defenseless

The probability of a disease outbreak in fish larval aquaculture depends on manifold interacting factors. First and foremost, adequate physiochemical conditions such as a sufficient oxygen supply, appropriate temperature, salinity and light are a prerequisite for successful fish larvae culturing. Moreover, it is crucial to guarantee a good water quality (e.g. using (bio)filter, protein skimmer, UV-light) and maintain tank hygiene (e.g. remove dead larvae and feces) (Attramadal et al. 2012, 2016). Yet, high loads of bacteria including opportunistic pathogens and organic matter are introduced into the tanks with the live feed (Vadstein 1997). Since the immunological capacity of fish larvae is severely limited, they are highly susceptible towards pathogenic infections, which cause high mortalities during early life stages. However, the disease resistance of early developmental stages can be improved by immunostimulation resulting in enhanced survival (Fig.1).

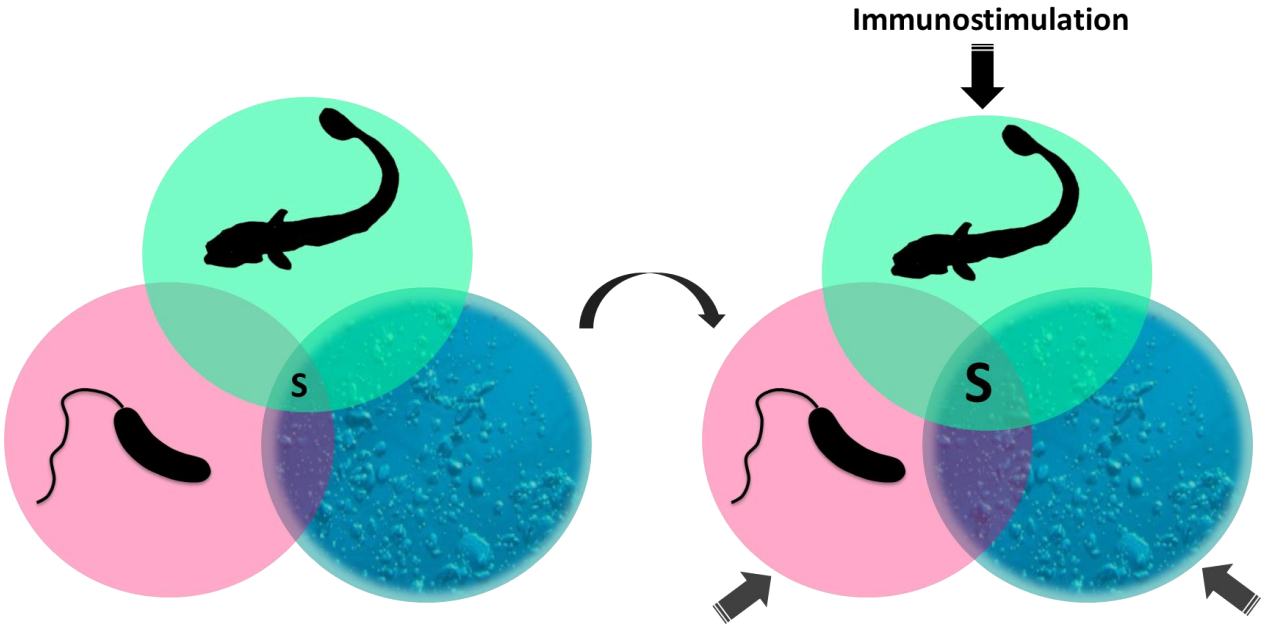

Fig. 1. The 3 factors "condition of the larvae", "microbial composition of the tank water" and "physiochemical conditions of the tank water" are interrelated and influence the probability of larval survival (S). All 3 factors can be manipulated in a way that larval survival is enhanced. One of these methods is immunostimulation. Redrawn after Vadstein 1997.

Advances in the development of immunomodulating measures are of crucial importance in early life stages as vaccines cannot be applied until adaptive immunity has developed, which can take several months in marine fish species (Sommerset et al. 2005, Miest et al. 2016). In freshwater species such as the common carp (*Cyprinus carpio*), the first B cells are present at 14 days post hatch (dph) (Rombout et al. 2005). In contrast, a delayed maturation of the adaptive immune system is observed in marine fish including sea bass. In *D. labrax* first B cells do not occur before 45 dph and immunological maturity is only attained after 4-5 month (Scapigliati et al. 1995, 2002, Dos Santos et al. 2000). For the development of the host´s immune system, a healthy GI microbial community is a prerequisite since the microbiota and the immune system are mutually interrelated (Gómez & Balcázar 2008, Maynard et al. 2012). Accordingly, manipulating the host´s GI microbiota towards a beneficial composition potentially results in immunostimulation (Merrifield & Ringø 2014). In this respect, I aimed to steer the GI microbial community and, hence, trigger the immune system in sea bass early life stages by administering probiotic bacteria and poly-β-hydroxybutyrate (PHB), respectively.

In chapter 1, I applied the probiotic *Bacillus subtilis* strain NCIMB 3610 to sea bass juveniles. I administered the probiont in a concentration and over a period of time that has been shown to induce an immune response in fish (Kumar et al. 2008). However, the measured cellular immune parameters, such as the proportion of monocytes and lymphocytes, and the proliferation of lymphocytes as well as the immune gene expression were not altered by the probiotic treatment in my study. To elucidate the relation between the manipulation of the GI microbial composition and a potential immunostimulatory effect, microbiota analyses are crucial. Therefore, I aimed to analyze the GI microbiota of the sea bass juveniles. Yet, the extraction of microbial DNA from the GI tract failed, even though, the same DNA extraction kit was used successfully for the extraction of microbial DNA in sea bass larvae as well as for various other microbiota samples in our laboratory.

In a previous study, an enhanced disease resistance during a *Vibrio anguillarum* challenge test was observed in sea bass larvae after *B. subtilis* NCIMB 3610 application (Touraki et al. 2012). This result might rather be based on the direct antagonistic effect of the probiont towards pathogenic bacteria (which is the ability to reduce the growth of pathogens by producing inhibitory substances (Nayak 2010)) than on immunostimulation. Nevertheless, my results do not exclude that *B. subtilis* (NCIMB 3610) might potentially be capable to modulate the immune response in sea bass, since the ability of a probiotic bacteria to act immunostimulatory depends on manifold experimental factors. As such, the experimental conditions were potentially suboptimal for the bacterial strain and/or the appropriate immunological parameters were not assessed.

Results from probiotic studies differ even if the same probiotic bacteria strain is applied in the same fish species, demonstrating that the outcome of probiotic supplementation is highly variable, often not reproducible and, thus, unpredictable (Merrifield et al. (2010). But where does this lack of reproducibility possibly derive from? The particular mode of action of probiotics depends on the condition they encounter in the GI tract of the host. The probiont and the indigenous microbial population interact and the extent of the direct antagonistic activity of the indigenous microbiota towards the probiont determines the probiont´s success to exert its immunostimulatory features (Merrifield & Carnevali 2014). The indigenous microbial community, in turn, depends on the diet and the environment (it has to be kept in mind that marine fish drink water). Since the environmental bacterial communities and the feed differ between research

facilities/hatcheries, the GI microbiota and, therefore, the ability and efficacy of a probiont to act immunostimulating can vary even between fish of the same species, leading to non-repeatable results (Ringø et al. 2016).

Furthermore, the aspect that a probionts function and fitness can vary even within the same bacterial strain depending on the culture conditions (Gale & Epps 1942, Vine et al. 2006) seems often to be overlooked but complicates the comparability between studies performed in different laboratories additionally. My literature research revealed that the culture methods for the respective probiont differ between studies. For example, nutrient broth (Touraki et al. 2012) and tryptone soy broth (Aly et al. 2008) are used to culture *B. subtilis*, or commercial *B. subtilis* are used without providing any further information on the culture conditions (Telli et al. 2014). Moreover, live cells in commercial probiotic products will inevitably lose viability over time, implying that they contain varying proportions of viable-to-non-viable cells (Lauzon et al. 2014). In summary, probiotics have a mode of action, which varies not only between fish species but also depends on the probiont´s fitness and on the host´s diet and environment, constraining their utility as a preventive health care measure.

To overcome the above-discussed issues associated with probiotic bacteria, compounds that selectively steer the indigenous GI microbial community, such as poly-β-hydroxybutyrate (PHB) or prebiotics, are currently being evaluated in various aquaculture laboratories (Hoseinifar et al. 2015, Najdegerami et al. 2015, Situmorang et al. 2015). Accordingly, I investigated the effect of PHB on sea bass larvae in cooperation with the Laboratory of Aquaculture and Artemia Reference Center (Ghent University, Belgium) as a subsequent step (chapter 2 and chapter 3). PHB is the polymer of the short-chain fatty acid (SCFA) β-hydroxybutyrate (β-HB), which is hypothesized to be able to stimulate the immune system in fish, although the underlying mechanism is not known (Montalban-Arques et al. 2015).

PHB is often classified as a prebiotic which is a “non-digestible food ingredient that increases beneficial GI bacteria in the host” (Roberfroid 2005). However, while PHB acts like a prebiotic, it is hypothesized to be gastrointestinally degraded by host digestive enzymes into its oligomers and monomers (the latter being the SCFA β-HB), thereby lowering the pH in the host’s gut (Defoirdt et al. 2009). While beneficial bacteria such as lactic acid bacteria profit from a lower intestinal pH, it has been shown that the multiplying of pathogenic bacteria like *Vibrio* spp. is suppressed by SCFAs (Defoirdt et al.

2007). Accordingly, a disease protecting effect of the compound has been demonstrated in various aquatic organisms including fish (Situmorang et al. 2015, Ludevese-Pascual et al. 2016). Moreover, dietary PHB was demonstrated to alter the GI microbiota (De Schryver et al. 2010). In a study with sea bass juveniles, it was shown that the GI microbial community compositions converged between replicate tanks (overruling the tank effect) and were characterized by increased evenness (De Schryver et al. 2011). In other words: PHB exhibits a controlling effect on the GI microbiota resulting in a higher level of reproducibility between replicates which might translate into higher survival rates when rearing fish early life stages.

In the framework of my thesis, I administered PHB to 2 different sea bass larval stages and assessed among other things the expression of immune-related genes, the survival and the microbiota (the latter only for yolk-sac larvae). In sea bass yolk-sac larvae, PHB was administered in a low and a high dose, respectively, at 2 different points in time (chapter 2). The observed effect on the expression of the antimicrobial peptides (AMPs) dicentracin and ferritin was weak and no clear pattern could be detected. However, the survival was improved by the low and the high PHB dose, respectively, and the larva-associated microbiota composition was altered at the phylum level. In contrast, in sea bass post-larvae administered a high dose of PHB, I found an upregulation in dicentracin, hepcidin (both AMPs) and MHC class II but no effect on survival (chapter 3). The observed alterations in the immune gene expression might either be due to the manipulation of the GI microbial composition caused by the degradation of PHB (as described above) or due to the binding of β-HB to G protein-coupled receptors (GPRs) (Montalban-Arques et al. 2015). More precisely, changes in the GI microbiota composition can trigger an immune response via microbe-associated molecular patterns (MAMPs) as described for prebiotics (Sekirov & Finlay 2009, Song et al. 2014). Furthermore, SCFAs can bind to GPRs and exhibit immunomodulatory effects in mammals (Dedkova & Blatter 2014, Shapiro et al. 2014). Hence, in order to elucidate the differences in the immune gene expression patterns in yolk-sac larvae and post-larvae, information about the larval GI microbiota composition, respectively, GPRs would be required. Specific receptors for SCFAs such as GPRs are believed to be present in fish, however, have not been described yet (Montalban-Arques et al. 2015) and should, accordingly, be a subject of future research. In both of the PHB experiments that I conducted, larvae were sampled for subsequent microbiota analyses but, due to ongoing difficulties during the sequencing of the microbial DNA, only a few yolk-sac larvae and no

post-larvae could be analyzed so far. The majority of my samples is currently being sequenced, yet again because of previous technical issues. This is very unfortunate, since the larval microbial compositions could have shed light on the differences between yolk-sac and post larvae regarding their AMP expression. Furthermore, the number of genes, investigated in my thesis, was constrained by the applied method (Fluidigm qPCR BioMark™ HD System) and, therefore, conclusions about the ultimate extent to which PHB stimulates the immune system in larval sea bass cannot be drawn. The increased survival that I observed in sea bass yolk-sac larvae upon PHB administration might possibly be related to an enhanced immunity that remained undetected due to the limited number of analyzed genes. To achieve a more comprehensive overview of PHB as an immunomodulator, measurements of cellular and humoral immune parameters (e.g. macrophage activity and lysozyme activity) should be combined with full transcriptome sequencing (RNAseq) to get insights into the role of innate defense mechanisms at the cellular, humoral and molecular level.

2. Future research directions – The big picture

The view on innate immunity has been revolutionized during the last years as memory properties for innate immune cells have been described (Van der Meer et al. 2015). To define specific innate immunological memory the term “trained immunity” was introduced (Netea et al. 2011). However, the mechanisms underlying trained immunity are not yet unraveled, even though, there is growing evidence that trained immunity relies on epigenetic changes as well as cross-talk between different types of innate immune cells and immune components (Saeed et al. 2014, Beemelmanns & Roth 2016). Trained immunity can be induced after a primary infection or immunization and, subsequently, provide protection against a secondary infection aside from adaptive immunity (Petit & Wiegertjes 2016).

Upon broodstock vaccination, trans-generational immune priming can be observed in fish larvae (Hanif et al. 2004). In the offspring of vaccinated and non-vaccinated sea bream (*Sparus aurata*), transferred innate and adaptive immune components were shown to be depleted already at 8 dph. However, the intrinsic larval lysozyme and anti-protease activity increased afterwards and was higher in the offspring of vaccinated parents than in the offspring of non-vaccinated parents (Hanif et al. 2004). This effect

persisted until the end of the experiment at 100 dph and can be seen as an example for transgenerational trained immunity.

Moreover, indications for trained immunity induced by the prebiotic β-glucan have been recently reported in fish (Petit & Wiegertjes 2016) implying that immunostimulation can be a much more powerful tool than thus far anticipated. As a result, early immunostimulation in fish larvae could not only boost the innate immune system during the period of immunostimulation but have long term disease protecting effects beyond this period. In future research broodstock vaccination should be combined with early immunostimulation to elucidate potential interactions and additive effects regarding trained immunity.

Like all prebiotics β-glucan is known to modulate the GI microbiota (Carda-Diéguez et al. 2014), hence, trained immunity could be induced directly by the prebiotic or through changes of the GI microbial composition. To exploit the full potential of PHB to manipulate the host´s intestinal microbiota and to stimulate its immune system, we need to advance our knowledge of intestinal immunity in fish. The latter should thus play a key role in future immunology research. So far, it is ascertained that immune competent cells and structures in the GI mucosa of fish are more diffusely organized than in mammals (Rombout et al. 2011). Consequently, there is still an ongoing debate on whether these structures fulfill the definition of a true gut-associated lymphoid tissue (Tafalla et al. 2016). Nevertheless, the intestinal immune system comprises a unique array of innate and adaptive immune cells and molecules such as macrophages, granulocytes, T cells and B cells as well as AMPs, lysozyme and complement components (Gomez et al. 2013). In sea bass, for example, the first T cells appear approx. at 28 dph, not only in the spleen but also in the intestine (Rombout et al. 2005), demonstrating the importance of intestinal immunity for fish early life stages. However, since the GI tract is one of the main portals of pathogen entry, innate intestinal immunity is certainly crucial for the larval protection before adaptive immunity is fully functional. Even though the ontogenetic development of the adaptive immune system has been investigated considerably more in sea bass than in other marine fish species (Scapigliati et al. 2002, Rombout et al. 2005, Chistiakov et al. 2007), we lack insight into the immune response in newly-hatched larvae (neither in sea bass nor in other marine fish species; Vadstein et al. 2012). Future research in larval immunology should emphasize the influence of the microbial colonization of the GI tract and the other mucosal surfaces (skin and gills). The mucosal immune system has evolved to permit the colonization by diverse commensal

bacteria while fighting pathogenic bacteria (Salinas et al. 2011). The commensal microbiota is seen as an “extended self” that lives in symbiosis with the host forming a holobiont and is crucial for the development of the immune system (Gomez et al. 2013). To better understand the interplay between commensal as well as pathogenic bacteria and the intestinal immune system, underlying biological pathways need to be unraveled on the cellular, humoral and molecular level.

Axenic (germ-free) and gnotobiotic fish larvae are proposed as a suitable model system to elucidate complex host–microbe interactions (Dierckens et al. 2009, Forberg et al. 2011). In gnotobiotic studies, only a known composition of bacteria is added to axenic larvae. By comparing axenic vs. gnotobiotic larvae, the microbial specificity of cellular and molecular host responses such as intestinal cell proliferation or gene expression can be assessed. For instance, immune-related genes were shown to be differentially expressed in gnotobiotic cod (*Gadus morhua*) larvae and axenic control larvae, demonstrating that gnotobiotic model systems help to improve the understanding of the role microbes play in regulating the functional development of the host (Forberg et al. 2012).

Simultaneously, conventional fish larvae experiments should be designed using a holistic concepts by combining different methodical approaches and, hence, enable scientist to acquire novel basic knowledge. To elucidate the link between the GI microbial composition and the immune response in fish early life stages, it is essential to combine microbiota analyses with transcriptomics and/or proteomics. However, so far, these methods are not implied in most research steering host-microbial interactions to investigate the associated immune response. Consequently, often no attention is paid to the immunological pathways behind immunostimulation, even though over the last years a lot of research dealing with the administration of probiotics or immunostimulating substances has been performed (Rombout et al. 2011).

Considering the diversity of powerful molecular tools that have been developed over the last years, it is time to think big and collaboratively. Programs like the European Union´s program for Research and Innovation (Horizon 2020) enable gigantic international scientific cooperations, the perfect condition to use the complete variety of modern techniques available at different institutes. Research institutes with excellent larval aquaculture facilities and laboratories that have established different examination methods might collaborate to combine information obtained at all levels of biological

organization. This strategy may be the key to control the devastating disease problem in aquaculture and diminish the progressive increase of antibiotic resistance being a threat to one of the most important ecosystems on our planet and in the same moment to our health.

Immune genes that play a key role during early development in immunostimulated and control larvae should be identified, which can be achieved with differential gene expression analyses upon transcriptome sequencing (comparative transcriptomics) (Martin et al. 2016) and should be combined with microbiota analyses. However, the transcriptome does not account for post-transcriptional regulation of protein expression and often a poor correlation between changes in the transcript level and protein expression is revealed (Pandey & Mann 2000). In contrast, the proteome can provide valuable information of an organism's physiological state that is missed by the transcriptome (Rodrigues et al. 2012). To further correlate this information with the host´s actual health status, intestinal morphology (e.g. increased microvilli integrity), cellular immunity (monocyte, granulocyte and lymphocyte levels) and humoral components (e.g. lysozyme activity) need to be assessed.

This knowledge will place researchers in a better position to design new immunoprophylactic measures that specifically target stimulation in the fish mucosal immune system (Gomez et al. 2013). To understand how the GI microbial composition shapes and trains intestinal immunity will allow us to develop effective immunostimulating dietary supplements that steer the GI microbiota towards a beneficial composition.

An aspect that should be kept in mind for larval experiments is that the individual variability in larval life history traits is naturally enormous even between fish larvae of the same species in the same developmental stage (Meyer et al. 2012, Tillner et al. 2013). Accordingly, potential treatment effects are easily masked by the high individual variability and, therefore, hard to be statistically detected. Hence, experiments with fish larvae should be designed in a manner that maximizes statistical power by increasing the number of replicates (tanks) and pseudoreplicates (larvae per tank).

3. Final summary

In essence, the commercial rearing of marine fish larvae is still hampered by high and unpredictable mortality due to the outbreak of infectious diseases (Vadstein et al. 2012).

Vaccines are not applicable in larvae of many important marine aquaculture fish species due to their delayed maturation of adaptive immunity (Rombout et al. 2005). Moreover, the serious drawbacks of the overuse of antibiotics cannot be emphasized enough (Heuer et al. 2009). A preventive health care strategy of great potential is the improvement of the severely limited immunocompetence of fish early life stages (Kiron 2012). Immunostimulation, possibly resulting in trained immunity, can be achieved by a beneficial manipulation of the host´s GI microbial community. However, the underlying mechanisms regarding the interdependency of the GI microbiota and the immune response in fish are, thus far, not very well explored (Merrifield & Carnevali 2014, Hoseinifar et al. 2015, Ringø et al. 2016). As such, not only immunostimulation in fish early life stages but the entire research field of fish larval immunity is still in its infancy. The further advancement of knowledge in this area occupies a key role in the development towards a more successful and sustainable marine larviculture.

With my thesis, I contribute to this process by giving first insights into the potential of PHB to act as an immunostimulator and to improve survival in sea bass larvae, making the compound particularly valuable for marine larviculture. PHB is safe for the environment as well as the human health and, accordingly, should be further investigated as a candidate immunoprophylactic measure, which constitutes an alternative to conventional disease control methods.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation mit dem Titel:

Immunostimulation in Early Life Stages of European Sea Bass (*Dicentrarchus labrax*)

selbstständig, mit der Beratung meiner Betreuer, verfasst habe. Die Dissertation ist in Form und Inhalt meine eigene Arbeit und es wurden keine anderen als die angegebenen Hilfsmittel und Quellen verwendet. Die Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden.

Diese Arbeit wurde an keiner anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt und ist mein erstes und einziges Promotionsverfahren.

Teile dieser Arbeit wurden zur Begutachtung in Fachzeitschriften eingereicht. Die Koautoren aller Kapitel befinden sich zu Beginn des jeweiligen Kapitels in der Autorenliste. Der Anteil der Autoren an den Manuskripten wird im Abschnitt „Author contributions“ erläutert.

Kiel, den 08.11.16 _____

Author contributions

Chapter 1

Andrea Franke, Catriona Clemmesen & Olivia Roth, The effect of probiotic *Bacillus subtilis* (NCIMB 3610) on survival, growth and immunity in European sea bass (*Dicentrarchus labrax*) juveniles, Submitted to Aquaculture Nutrition

The experiment was designed by Andrea Franke, Catriona Clemmesen and Olivia Roth and was conducted by Andrea Franke. The samples and data were analyzed by Andrea Franke and Olivia Roth. Andrea Franke wrote the manuscript, including all figures, tables and statistical analyses assisted by Olivia Roth and Catriona Clemmesen.

Chapter 2

Andrea Franke, Olivia Roth, Peter De Schryver, Till Bayer, Linsey Garcia-Gonzalez, Sven Künzel, Joanna J. Miest & Catriona Clemmesen: Poly-β-hydroxybutyrate administration during early life: effect on survival, growth, microbial community, gene expression and disease resistance in European sea bass yolk-sac larvae

The experiment was designed by Andrea Franke, Catriona Clemmesen and Olivia Roth and was conducted by Andrea Franke and Joanna Miest. The PHB-containing bacteria were provided by Linsey Garcia-Gonzalez and Peter De Schryver. The samples were analyzed by Andrea Franke, Joanna Miest and Sven Künzel. The data were analyzed by Andrea Franke, Joanna Miest, Till Bayer and Olivia Roth. Andrea Franke wrote the manuscript, including all figures, tables and statistical analyses assisted by Olivia Roth, Till Bayer and Catriona Clemmesen.

Chapter 3

Andrea Franke, Catriona Clemmesen, Peter De Schryver, Linsey Garcia-Gonzalez, Joanna J. Miest & Olivia Roth, Immunostimulatory effects of dietary poly-β-hydroxybutyrate in European sea bass post-larvae, Submitted to Aquaculture

The experiment was designed by Andrea Franke, Catriona Clemmesen and Olivia Roth and was conducted by Andrea Franke. The PHB-containing bacteria were provided by Linsey Garcia-Gonzalez and Peter De Schryver. The samples and data were analyzed by Andrea Franke and Joanna Miest. Andrea Franke wrote the manuscript, including all

figures, tables and statistical analyses assisted by Olivia Roth, Joanna Miest, Peter De Schryver and Catriona Clemmesen.

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Talking about coffee. **Svend**, ich weiß ich habe es schon hundertmal gesagt, aber jetzt kann ich es endlich mal schriftlich festhalten: ich liebe deine (meine) Espressomaschine. Danke für all deine Hilfe egal was nicht (manchmal im wahrsten Sinne des Wortes) läuft, du bekommst es wieder hin. Computer, Drucker, Espressomaschine, Experimentierkram, bei dir ist man immer an der richtigen Adresse. Vielleicht sollte ich mal wieder mit auf See fahren oder ist das Fisch-Alphabet schon fertig?

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