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The role of poly-β-hydroxybutyrate as protection against *Vibrio* infections in blue mussel larvae

Thesis submitted in fulfillment of the requirements for the degree of

Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title: De rol van poly - β - hydroxybutyraat als bescherming tegen *Vibrio* infecties bij mossel larven

Cover page: Epifluorescence microscopy of microalgae and PHB- fed two day old blue mussel larvae stained with Nile Blue A (Laboratory of Microbial ecology and technology, Ghent University)

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LIST OF ABBREVIATIONS AND UNITS

A199	aeromonas media strain
AMPs	antimicrobial peptides
ANOVA	analysis of variance
AOD	american oyster defensin
ARC4B5	PHB degrading isolated from blue mussel larvae
BLAST	basic local alignment search tool
BRD	brown ring disease
CA2	alteromonas sp.
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
Cg-Def1	*Crassostrea gigas* defensin 1
Cg-Def2	*Crassostrea gigas* defensin 2
C_T	threshold cycle
DGGE	denaturing gradient gel electrophoresis
D-larvae	two day old mussel larvae
DMSO	dimethyl sulfoxide
DNA	deoxyribo nucleic acid
DNA	deoxyribo nucleic cid
ECP	extracellular products
EDTA	ethylenediaminetetraacetic acid
EEC	european economic community
EPS	exopolysaccharides
FAASW	filter autoclaved artificial sea water
FAO	food and agriculture organization

FASW	filter autoclaved sea water
FNSW	filtered natural seawater
FOS	fructooligosaccharides
FTS	flow through system
GI	gastrointestinal
HPS	heat shock protein
HSP70	heat shock protein gene 70
HUFA	high unsaturated fatty acid
ICI	Imperial Chemical Industries
LB_{35}	luria-Bertani medium (salinity 35g L^{-1})
L-DOPA	L-3, 4- Dihydroxyphenylalanine
MB	marine broth
MCF	medium chain length
MGD1	mytilus galloprovincialis defensin 1
MGD2	mytilus galloprovincialis defensin 2
MOPS	3–(N–morpholino) propanesulfonic acid
n	number of replicates
ND	not determined
N-FMLP	N-formyl-methionyl-leucyl-phenylal-anine
NMS	*non-metric multidimensional scaling*
NS	not specific
OD	optical density
OsHV-1	ostreid herpesvirus 1
PCR	polymerase chain reaction

PHAs	polyhydroxyalkanoates
PHB	poly-β-hydroxybutyrate
PHB-A	amorphous PHB
PHB-C	crystalline PHB
PHB–HV	poly-β hydroxybutyrate–hydroxyvalerate
PO	phenoloxidase
ppA	prophenoloxidase activating enzyme
ppm	parts per million
proPO	prophenoloxidase
PRRs	pathogen recognition receptors
q-PCR	quatitative polymerase chain reaction
RNA	ribonucleic acid
RNS	reaction nitrogen species
ROS	reaction oxygen species
rRNA	ribosomal RNA
SCF	short chain length
SCFAs	short-chain fatty acids
SEM	standard error of the mean
SGR	specific growth rate
SM	Summer Mortality
SPSS	statistical Package for the Social Sciences
TCD	tissue culture dish
TOS	transgalactooligosaccharides
UK	United Kingdom

USA	United States of America
UV	ultra-violet
v/v	volume per volume
VAR	*Vibrio anguillarrum* related strain
VLB	*Vibrio-like bacteria*
VIED	Vietnam International Education and Development
β-HB	β-hydroxy butyric acid
Δt	rate of change
$\Delta\Delta C_T$	relative gene expression
%	percentage
µg	microgram
µl	microliter
µm	micrometer
^0C	degree Celsius
cm	centimeter
g	gram
h	hour
kDa	kilo-Daltons
L	liter
mg	milligram
ml	milliliter
mM	millimolar
rpm	rotations per minute
V	voltage

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Chapter 1

General introduction

Aquaculture production is expected to play a crucial role in meeting the growing demand for aquatic products, especially since capture fisheries have markedly stagnated with little likelihood of increased production in the foreseeable future. Currently, aquaculture is the fastest growing sector in the food production industry with an average yearly growth rate of more than eight percent over the past two decades (FAO, 2014a). In 2011, total global aquaculture production of mollusks reached 14.4 million tonnes, accounting for 22.9% of total aquaculture production of 62.7 million tonnes (FAO, 2013).

Bivalve mollusks are an important global food commodity (Sauvé, 2013). Natural populations cannot meet the increasing demand for seed due to overexploitation, which has led to the development of aquaculture techniques (seed capture, hatchery practices) (Avendaño and Riquelme, 1999). Hatcheries have several advantages, such as the possibility to satisfy seed requirements out of the natural growing season, the supply of genetic strains with improved biological characteristics or the supply of new bivalve (exotic) species. Nevertheless, the culture of bivalves in hatcheries is frequently affected by severe disease outbreaks, mainly caused by bacterial infections of members of the genus *Vibrio*, resulting in the loss of complete batches and compromising the regular production and the economic viability of the industry. There are many descriptive studies detailing these outbreaks, but only a few focus on the control of microbiota. The unique characteristics of bivalve aquaculture must be considered in the design of improved hatchery systems. The classical treatment is directed towards the complete removal of bacteria from the culture seawater. However, this objective is on the one hand unfeasible, because the cultures are not axenic, and on the other hand undesirable since some bacteria enhance larval development (Prado et al., 2010). Larvae and bacteria, including both beneficial and potentially pathogenic ones, share a common environment. The filter feeding behavior of larvae increases the strong influence of these bacterial populations on the hosts.

The particularities of bivalve aquaculture in hatcheries must be considered to design methods of control. A number of methods have already been developed to control the proliferation of pathogens and to maintain a healthy microbial environment in aquaculture

systems. Classically, the water is subjected to treatments with the aim to reduce the associated bacterial population. Decantation and subsequent filtration of natural seawater are common practices, while additional procedures such as ultraviolet radiation or chemical treatment have also been employed to attain the complete elimination of harmful microbiota from the culture water. However, this objective is not practical as bacterial populations, or, at least, part of them, have a beneficial effect on the larval development (Hidu and Tubiash, 1963).

While vaccination has been a major success in fish culture (Romalde et al., 2002, Herrera and Toranzo, 2005), vaccination procedures cannot be applied successfully to mollusks because they have only an innate immune system and lack an adaptive immune system. However, a number of alternative strategies have been tested to enhance larval cultures performance of bivalves by modifying the microbiota or by interfering with the innate immune system. Among these are the use of probiotics (Douillet and Langdon, 1994), immunostimulants (Bachère, 2003), and antimicrobial peptides (Defer et al., 2009). However, the implementation of these alternative techniques should be based on the understanding of the mechanisms involved and their putative consequences (Marques et al., 2005). Therefore, new solutions need to be developed and validated for the control of bacterial diseases in larval cultures of bivalves in hatcheries.

Poly-β-hydroxybutyrate (PHB), the simplest and most common member of the polyhydroxyalkanoates (PHAs), a class of linear polyesters produced in nature by bacteria out of sugar or lipids, is considered to have a high potential in human medicine as a degradable implant material because of its non- toxic nature. (Freier et al., 2002). Several studies have demonstrated that PHB might constitute an ecologically and economically sustainable alternative strategy to fight infections in aquaculture (Defoirdt et al., 2006b), It has been suggested that, PHB can be degraded into β-hydroxy short-chain fatty acids (SCFAs). SCFAs specifically downregulate virulence factor expression and positively influence the gastrointestinal microflora of the host (Defoirdt et al., 2009). Currently, there is considerable interest in SCFAs as bio-control agents in animal production. Therefore, PHB could be a potential candidate for the biological control of bacterial infection in bivalve larvae culture as it could be a source of SCFAs after depolymerization of particulate PHB, provided the latter can be easily accumulated by the filter-feeding mollusk larvae.

In this study, the blue mussel *Mytilus edulis* was selected as a model species because it is an important aquaculture bivalve species in Europe with high production (Lobel et al., 1990) and because of the relative easiness of handing larvae of this species under lab condition. The overall objective of the present thesis is to investigate whether the application of PHB in larval cultures of the blue mussel, *M. edulis,* can increase survival. It has been suggested that PHB can boost survival in aquaculture species by acting as a bacteriostatic compound and/or by stimulating the immune system. Yet up to now it has not been verified where PHB is also instrumental in sustaining a high survival in mollusk larviculture **(chapter 3)**, neither had it been verified what the mode of action of this compound could be. Possible mode of action could be a direct inhibition of pathogens (chapter 4), an impact on the diversity of the microbial composition in the intestinal tract of the larvae reducing negative host microbial interactions **(chapter 3)** but also a potential stimulation of the innate immunity of the mussel larvae by **(chapter 5).**

As a general introduction to the results chapters the production of bivalve mollusks in aquaculture is reviewed. The advantages and constraints of larval production are described, jointly with an overview of the most common diseases in hatchery-reared bivalve larvae and common strategies to control pathogenic bacterial infection **(Chapter 2)**.

- In the results chapters, the beneficial effect of PHB is investigated together with a verification of some putative mode of action.

- In order to do so the effect of PHB on blue mussel larvae was identified **(Chapter 3).** This was achieved through the subsequent 3 experimental approaches:

    - By evaluating the blue mussel larvae's ability to ingest PHB using fluorescent microscopy of the gut. Different forms of PHB (crystalline PHB vs. amorphous PHB *Ralstonia eutropha* containing 75% DW of PHB) were supplemented in the culture water.

    - By determining the optimal PHB dosage for mussel larvae culture in a large-scale experiment where three dosages were tested. Larvae survival, and growth and settlement were the monitored parameters.

- By verification of the effect of PHB on the diversity of the microbial community composition in the digestive tract of mussel larvae using DGGE (denaturing gel gradation electrophoresis) on amplified 16S rDNA fragments.

- In addition the antimicrobial activity and the protection offered by PHB-containing bacteria against different pathogenic *Vibrio* strains was verified using *in vivo* challenge tests with mussel larvae in a nearly-gnotobiotic system. Furthermore, to document a potential mode of interaction between PHB and a particular *Vibrio* strain, the effect of hydroxybutyric acid on the growth and virulence factor of *Vibrio* pathogenic bacteria at two pH levels (pH7 and 8) was studied by *in vitro* experiments **(Chapter 4).**

- Finally, to monitor the immune reaction in mussel larvae in attempt to understand why PHB enhances their survival of mussel. The innate immune gene expression and phenoloxidase enzyme activity of mussel larvae challenged with *Vibrio coralliilyticus* was analyzed. In addition the production of selected antimicrobial peptides during specific bacterial infections was monitored **(Chapter 5).**

- Finally an overall discussion is presented, the main conclusions are drawn and perspectives for further research are elaborated in **Chapter 6.**

Chapter 2

Literature review

2.1. The importance of aquaculture

Aquaculture comprises all forms of culture of aquatic animals and plants in fresh, brackish and marine environments (Pillay and Kutty, 2005). The role of aquaculture in ensuring a consistent supply of aquatic species for human consumption cannot be overstated. Seafood production, especially fish, is important for future global food security. Fish, as well as other aquaculture products such as mollusks and crustaceans are nutritionally very important to humans, as it provides all essential amino acids and is an excellent source of proteins, trace elements and polyunsaturated fatty acids (Kris-Etherton et al., 2002). Currently, we face an increase in global population that is expected to grow by another 2 billion to reach 9.6 billion people by 2050 with a concentration in coastal urban areas (Glenn, 2014). The world fish consumption per capita apparently increased from an average of 9.9 kg in the 1960s to 17.0 kg in the 2000s and 18.9 kg in 2010, with preliminary estimates for 2012 pointing towards further growth to 19.2 kg (Belton and Thilsted, 2014). Fish consumption is not equally distributed geographically. For example, in 2014 fish consumption in Africa (Congo, Liberia) was 9.7 kg/person/year while fish consumption in Vietnam amounted to 38.3 kg/person/ year. The driving force behind the impressive surge has been a combination of population growth, rising incomes, and urbanization (resulting in better distribution channels) interlinked to the high expansion of fish production.

According to the available statistics collected globally by FAO, world aquaculture production attained another all-time high of 90.4 million tonnes (live weight equivalent) in 2012 (US$144.4 billion) (Figure 2.1), including 66.6 million tonnes of food fish (US$137.7 billion) and 23.8 million tonnes of aquatic algae (mostly seaweeds, US$6.4 billion) (FAO, 2014b). Not only is aquaculture important for future global food security but it is also the most sustainable form of animal production. With growing global population and declining natural resources aquaculture is increasingly being viewed as the most environmentally sustainable form of animal production. The food conversion rate (FCR) of fish production (between 1 and 2 in aquaculture setting) is less than all other production animals, including cattle (FCR >6), swine (FCR < 3.5), poultry (FCR < 2), while bivalves being primary consumers, are even more efficient (FAO, 2012). For bivalve spat a FCR of 0.4 is generally accepted in hatcheries (pers.comm).

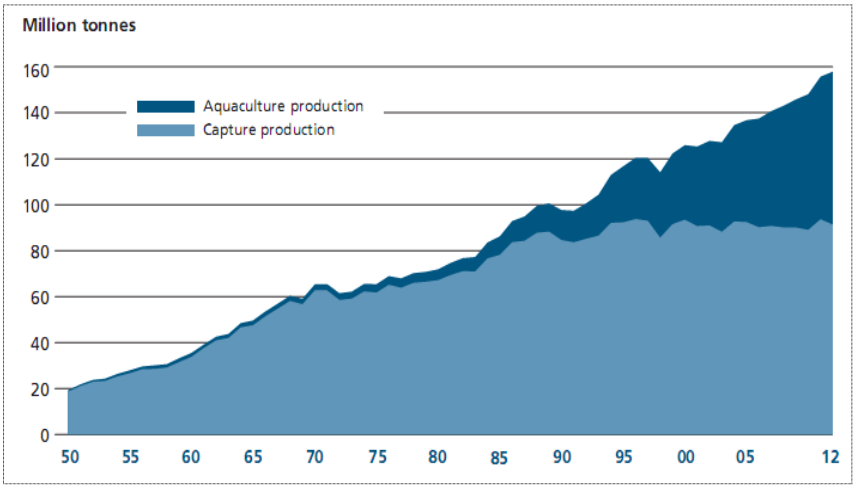

Figure 2. 1: World capture fisheries and aquaculture production (FAO, 2014b)

The global food sector is facing various challenges related to the recovering economy and demographic issues, including growing urbanization. In particular, urbanization will bring with it changes in lifestyles and consumption patterns. In combination with income growth, it may accelerate the ongoing diversification of diets in developing countries (Garrett and Ruel, 2000). Therefore, demand for fish products is expected to continue to rise in the coming decades. However, future increases in per capita fish consumption will depend on the availability of fishery products. With capture fisheries production stagnating, significant increases in food fish production are forecasted to come from aquaculture.

2.2. Bivalve mollusks in aquaculture

Bivalve mollusks (oysters, mussels, clams, and scallops) form a significant part of the world's fisheries production. Bivalve mollusks represented almost 10 % of the total world fishery production, but 26 % of volume and 14 % in value of the total world aquaculture production. World bivalve production has increased substantially in the last 50 years, going from nearly 1 million tons in 1950 to about 13.9 million tons in 2010 (FAO, 2014b).

China is by far the leading producer of bivalve mollusks, with 10.35 million tons in 2010, representing 70.8 % of the global molluscan shellfish production and 80 % of the global bivalve mollusk aquaculture production. All Chinese bivalve production is cultured. The other main bivalve producers in 2010 were Japan (819,131 tons), the USA (676,755 tons), the Republic of Korea (418,608 tons), Thailand (285,625 tons), France (216,811 tons), Spain

(206,003 tons) and Chile (300,000 tons). By species, the bivalve mollusk production from aquaculture in 2013 consisted of 37 % (clams, cockles, arkshells), 36 % (oysters), 13 % (mussels) and 14 % (scallops, pectens) with an impressive growth in the production of oysters, clams, cockles and ark shells since the early 1990s (Figure 2.2) (FAO, 2015)

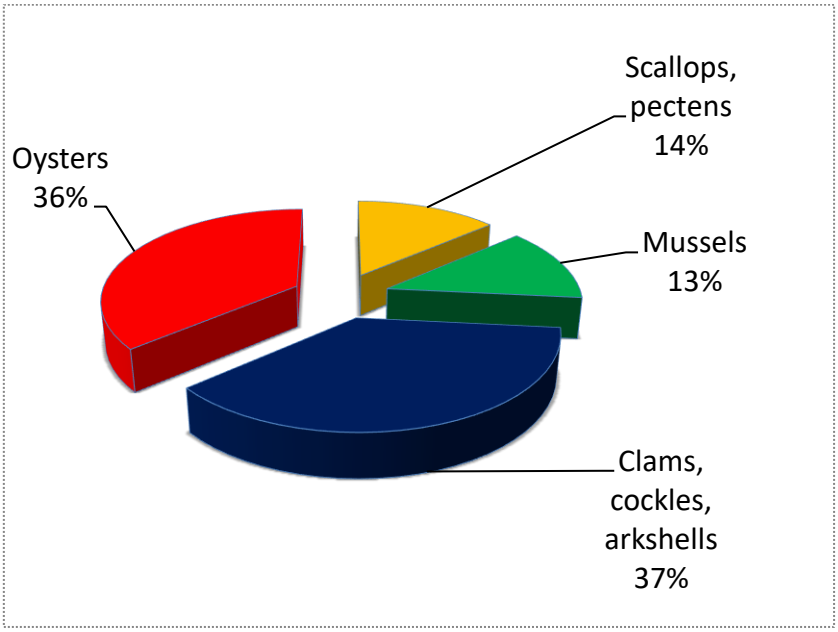

Figure 2. 2: Global bivalve mollusk production by species (FAO, 2015)

2.3. Introduction to the Blue mussel *Mytilus edulis*

2.3.1. Taxonomy and distribution of *Mytilus edulis*

Mytilus edulis is a taxon belonging to a group of three closely related taxa of blue mussels known as the *M. edulis* complex (de Meeiis and Renaud, 1997). This group comprises of *M. edulis*, *M. galloprovincialis* and *M. trossulus*. The taxonomy of these species is based on their morphological and genetic differences, and they can hybridize when present in the same locality (Gosling, 1984, McDonald et al., 1991). Linnaeus, 1758, described the scientific classification of *Mytilus edulis* (Figure 2.3) as:

Kingdom: Animalia

Phylum: Mollusca

Class: Bivalvia

Order: *Mytiloida*

Family: *Mytilidae*

Genus: *Mytilus*

Species: *Mytilus edulis* Linnaeus,

English name: Blue mussel

Figure 2. 3: Blue mussel *Mytilus edulis*

Mytilus edulis has a wide distribution pattern, extending from the Artic towards the mild subtropical regions. In European waters, blue mussels are distributed from the White Sea in the North (Russia), over the North Sea (The Netherlands and Belgium) and the Atlantic Ocean (the United Kingdom and Ireland) to the Atlantic coast in the South (Southern France) (see the red circle in Figure 2.4) (Gosling, 2003, Beaumont et al., 2007, FAO, 2016). They occur most abundantly in the tidal and sub-tidal zones, ranging from the poly-haline to mesohaline estuarine environments. They tend to proliferate on the rocky shores of the coastlines, bays, and river mouths which usually show high levels of nutrient loads (coming from the land), a necessary condition for phytoplankton blooms (Newell and Moran, 1989). Salinity has relatively little effect on growth and survival of both larvae and adult in contrast to other exogenous factors such as temperature or food availability (Widdows, 1991).

Adult mussels have been reported to survive to harsh winter periods even when the sea is covered by a thick ice layer (Hatcher et al., 1997). Low temperatures however, significantly influence the growth and the development of larvae, particularly temperatures lower than 16^0C. The strongest growth of larvae is obtained at temperatures between 16 -22^0C but this range varies among larvae originating from different geographic regions (Widdows, 1991). Brenko and Calabrese (1969) also recorded optimum larval growth at 20^0C within a salinity

range of 25 to 30 g L^{-1}. Decreases in growth rate were experienced at both 10^0C and 25^0C, especially at low (20 g L^{-1}) and high (40 g L^{-1}) salinities.

Figure 2. 4: Worldwide distribution of bivalves. *Mytilus edulis* lives in the Arctic regions and European waters (red circle) (Gosling, 2003).

2.3.2. Life cycle of blue mussel

The common blue mussel (*Mytilus edulis*) has several stages in its life cycle (Figure 2.6). The first stage occurs when two adult mussels produce and release a large amount of sperm and eggs into the water where the fertilization takes place. The sperm is released before the eggs, in fact sperm triggers the release the eggs (Gosling, 2003). Females can produce up to 8 million eggs per individual and for each egg there are about 10 000 spermatozoa released by males (Zaidi et al., 2003). The spawning season depends on different factors such as the environmental water temperature, food availability, salinity and occurs from early spring (April) till the end of summer (September) (Gabbott, 1976). Hatching usually occurs on average 24 hours after fertilization, forming a ciliated embryo (trochophore larva). The ciliated velum serves both to propel the larva and filter food particles. These embryos continue to undergo a series of different veliger stages as explained below. After 2 days of hatching, trochophore larvae have developed the first larval shell called prodissoconch I

secreted by the shell gland and the mantle epithelium. Based on the shape, larvae at this stage are also known as “D-larvae” or “straight hinged” larvae. As the veligers continue to develop, the second shell called prodissoconch II is secreted by the mantle. Larvae at this stage are called a veliconcha and distinguished themselves from the D-larva by the development of umbo on the top of larval shell (Figure 2.5).

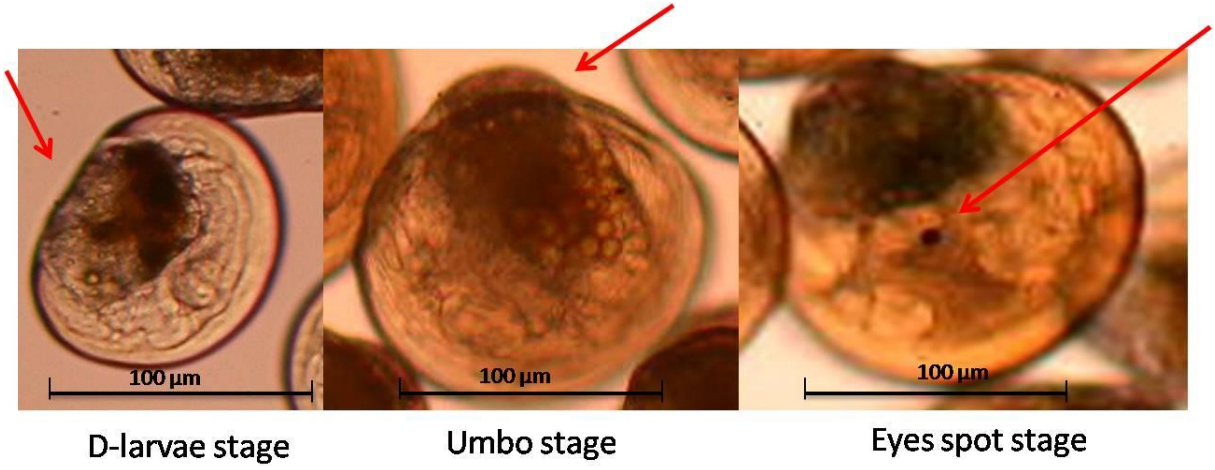

Figure 2. 5. Blue mussel larvae development

This larval stage can last for a week depending on environmental conditions. During that time it grows in size and develops a pair of photosensitive pigmented eye spots and an elongated foot with a byssal gland, and is termed the pediveliger. Once entering the pediveliver stage it starts searching sites and substrates for settlement. Once settled by secreting the byssal thread to attach to the suitable substrate, the larvae lose or reabsorb the velum and undergo metamorphosis stage to develop into spat which resemble the adults in shape (Newell and Moran, 1989). The life stages and characteristics are presented in Table 2.1.

Table 2. 1 Life stages and characteristics of the blue mussel (Newell and Moran, 1989)

Stage	Size	Age and characteristic
Fertilized egg	68-70 (µm)	0 – 5 h non motile
Trochophore	70 -110 (µm)	5 – 24 h ciliated and motile
Veliger larva		Up to 35 days; start feeding using velum
- Prodissoconch I	110 (µm)	Straight – hinged shell
- Prodissoconch II	260 (µm)	Occurring umbo
- Eyed larvae	220 – 260 (µm)	Development of eye spots
- Pediveliger	260 (µm)	Development of food
Spat	0.26 – 1.5 mm	Up to 6 months, temporarily attached to filamentous substrates
Juvenile	Up to 2 years	Up to 2 years, sexually immature

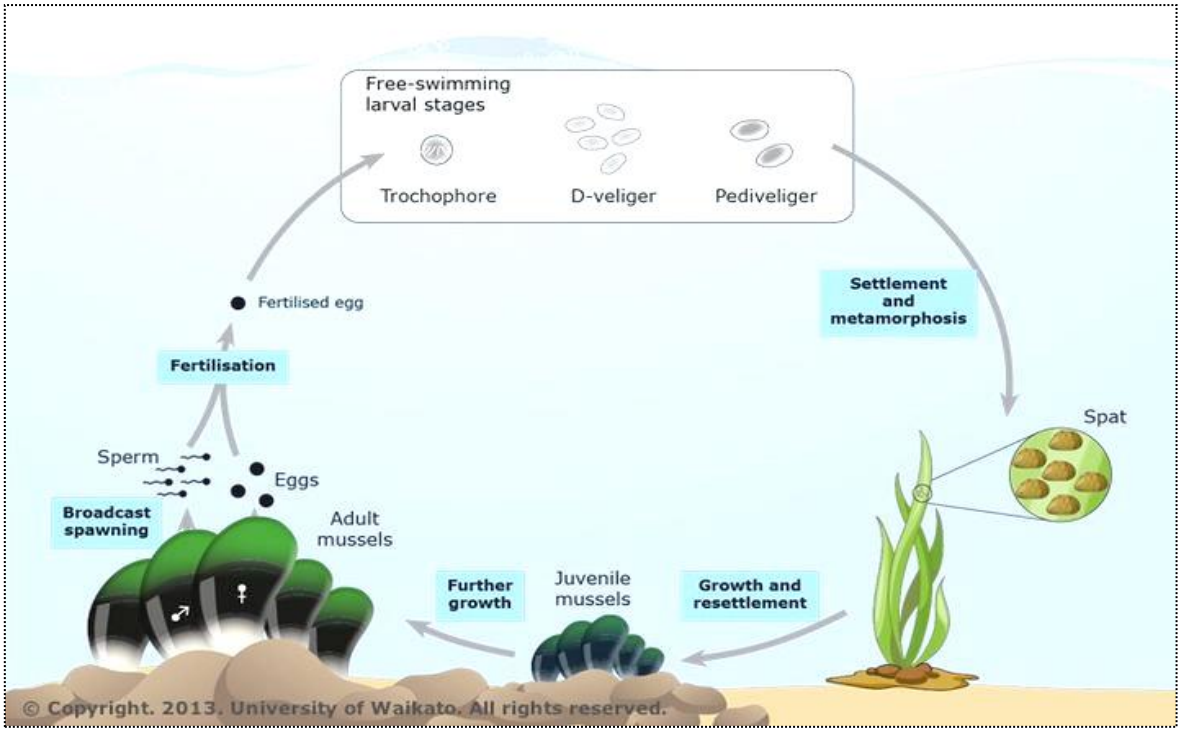

Figure 2. 6: Schematic illustration of reproduction cycle (Waikato, 2013)

2.3.3. The importance of European mussel aquaculture

There were reports in France in the 13th century of cultivation on wooden stakes (Garen et al., 2004). Blue mussels are also among the top 5 economically important species that contribute to the worldwide production of bivalves. In 2013 global production of blue mussel *M. edulis* was 197.831 metric tonnes (mt) of which 184.873 mt (93.45%) was produced in Europe (FAO, 2014b).

Over the last 50 years, the major producers of blue mussel *M. edulis* have been Denmark, France, Ireland, the Netherlands, United Kingdom, Sweden and Norway (Lee et al., 2012) (Figure 2.7) while *M. galloprovincialis* (Mediterranean mussel) has mainly been produced in Spain and Italy (Smaal, 2002)

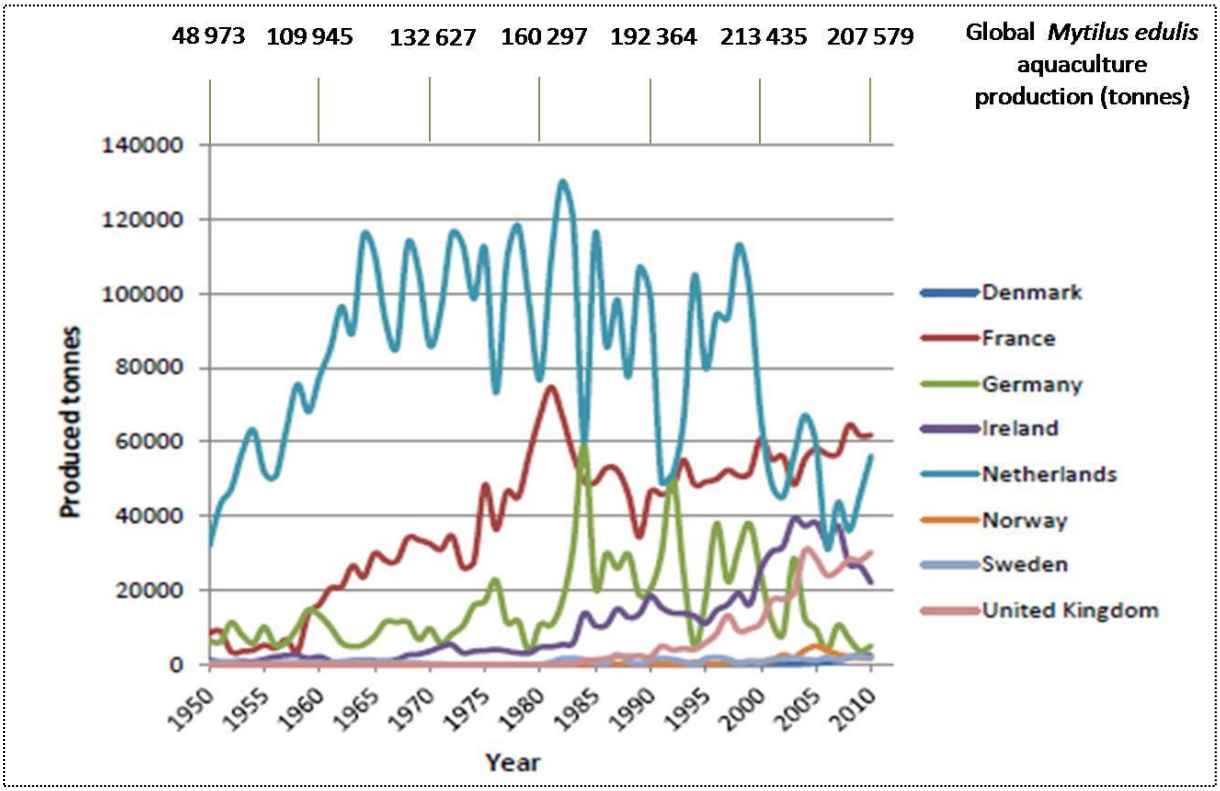

Figure 2. 7: Production of the blue mussel, *Mytilus edulis* in the world and in Europe (Lee *et al.*, 2012, FAO, 2014b)

The majority of seed production sources from natural populations although increasingly, stocks are approaching or have exceeded maximum sustainable yields. Stock enhancement through the capture of larvae while still relying on natural seed in both extensive and

intensive forms of culture is a common practice worldwide. However the reliability of natural recruitment can never be guaranteed, and conflicts over the use of the coastal zone are becoming ever more pressing. One solution for meeting the growing demand for seed by the mussel industry is seed production in hatcheries. However, production costs are still too high in Europe compared to the wild-caught seed to turn it into a profitable business. Hatchery production of spat could provide a back-up when natural spat fall fails, reduce the pressure on the natural populations and eventually would allow for selection.

Mussels are farmed using long lines, rafts or a mixture of long lines and poles installed in shallow intertidal areas. Aypa (1990) describes three main categories of culture methods for mussel cultivation, bottom culture growing mussels directly on the bottom, intertidal and shallow water culture in the intertidal zone, and deep water culture (Figure 2.8).

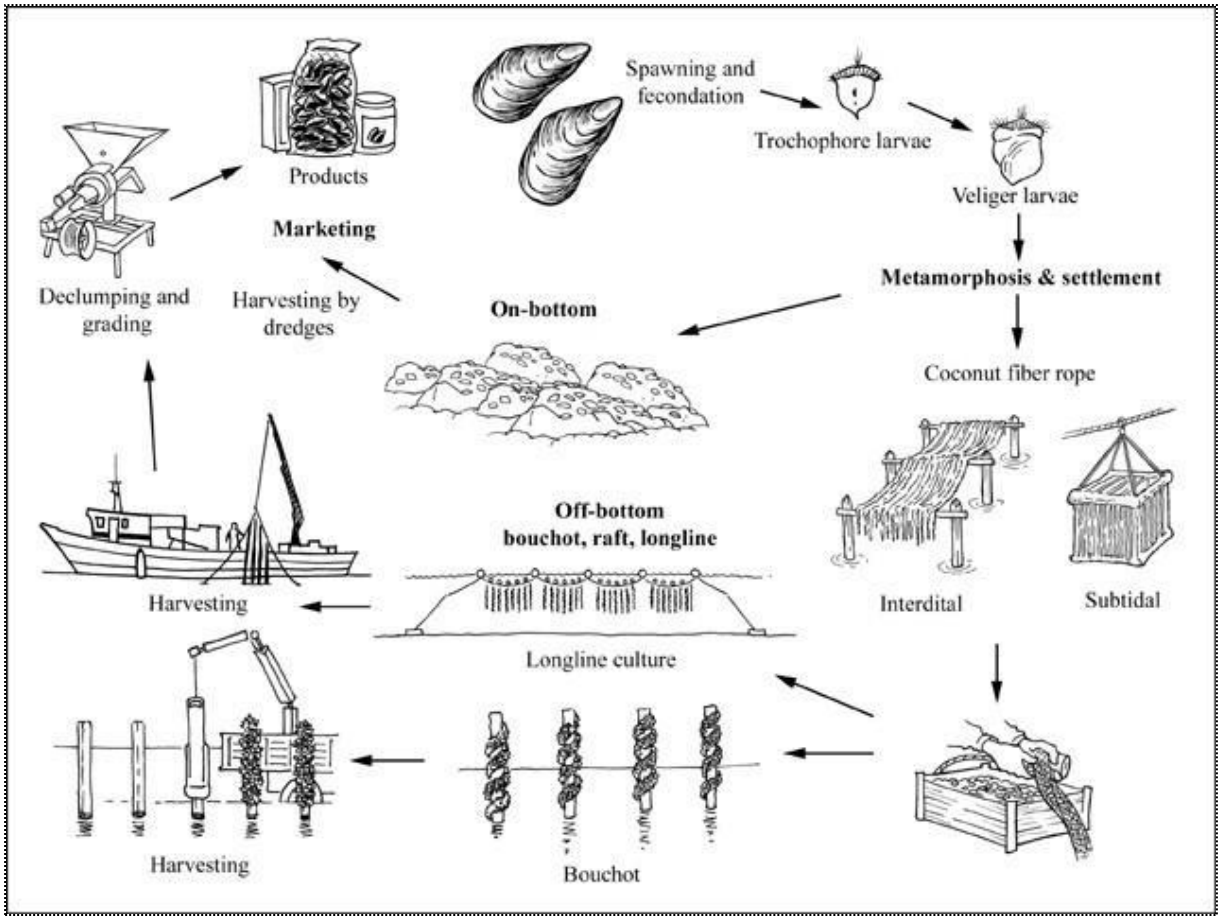

Figure 2. 8: Production cycle of *Mytilus edulis* (FAO, 2014b)

The largest bivalve markets in the European Union (EU) are France, Italy, and Spain. France is the largest EU importer of mussels (FAO, 2015). Meanwhile, smaller sized markets include the Netherlands, Italy, Belgium and Denmark. Ireland and France provide the market with oysters, while Denmark, Ireland, The Netherlands, and Spain provide mussels. The trade is mainly intraregional between the EU Member States with a smaller contribution from third countries. Only a few third countries, such as Chile and New Zealand, can penetrate the EU markets with their mussel species.

2.3.5. Feeding behavior and digestive system of blue mussel

The blue mussel is a filter feeder, a feeding process that is well conserved amongst most bivalve species. Food particles are taken up from the incoming water current, followed by selection, transport, ingestion and finally digestion as described below (Jorgensen, 1990, Gosling, 2003).

The water, with suspended organic particles such as unicellular phytoplankton, enters the mussel through the inhalant opening. With the help of cilia, located on the gills, particles of interest are trapped in mucus and transported along the labial palps towards the mouth. The food particles are then, again assisted by cilia, transported through the esophagus and directed towards the stomach. The stomach is surrounded by a dark digestive gland. Due to mechanical and enzymatic processes, the food particles are digested extracellularly. These digested end-products are then absorbed in the hemolymph (Figure 2.9).

Via the hemolymph this transport of nutrients could, for example, end up in the mantle tissue where it is stored as the metabolic reserve glycogen. The waste materials and rejected products are converted to faeces in the intestine and excreted through the anus. They find their way back into the seawater through the exhalant opening (Jorgensen, 1990, Gosling, 2003). The selection of particles of interest is a selective process. The unwanted particles are rejected and exit the mussel, as pseudofaeces, together with the waste water through the inhalant opening (Jorgensen, 1996).

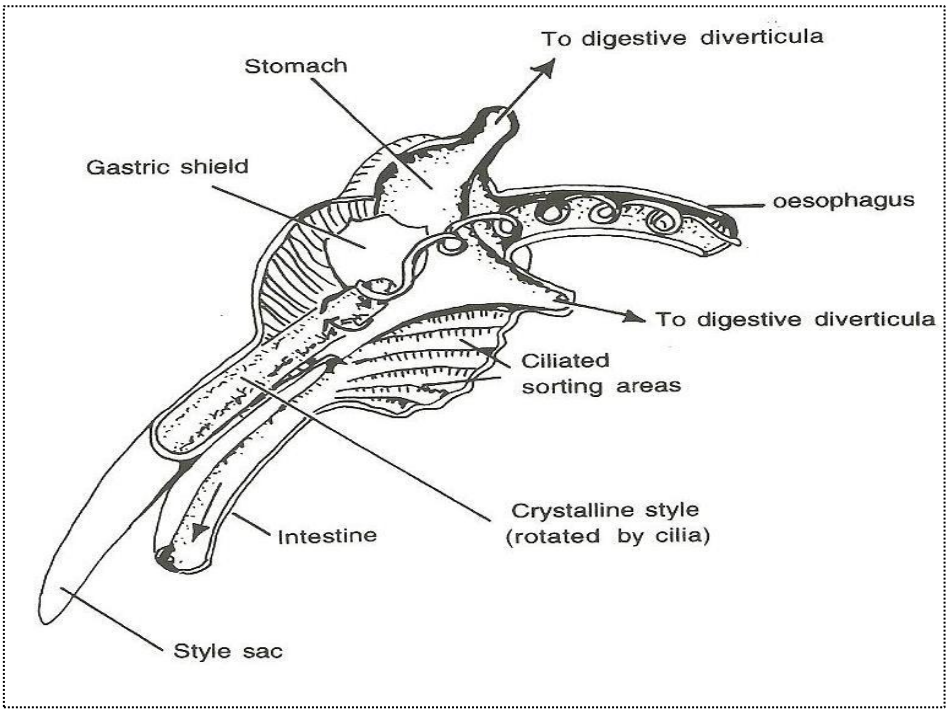

Figure 2. 9. Digestive system of *M. edulis*: Illustration of the stomach and digestive gland (Gosling, 2003)

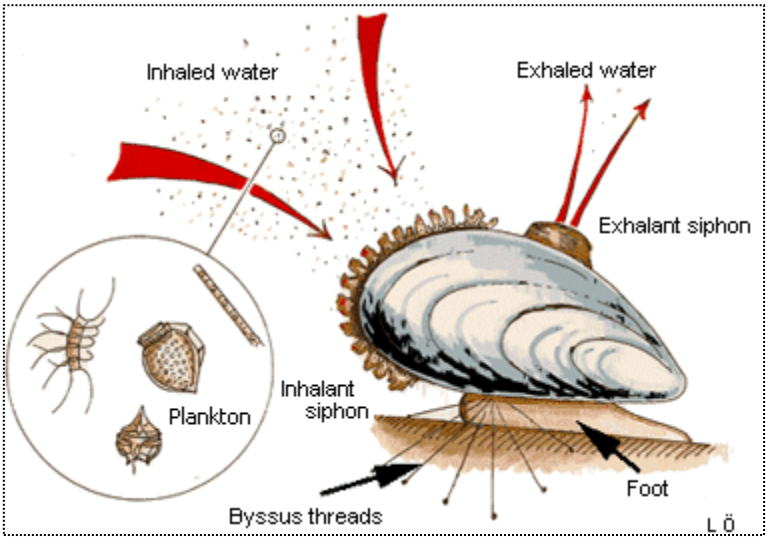

Figure 2. 10: Schematic illustration of filter feeding process (Aquascope, 2000).

Bayne and Widdows (1978) determined the filtration rate of *M. edulis* to be 1.34-2.59 L h^{-1}. Winter (1973) found that filtration rate in mussels increases with body size. On average, mussels with shell lengths of 8.5 mm filtered algal cells out of water at a rate of approximately 20 ml h^{-1} whereas mussels with shell lengths of 56.5 mm filtered at a rate of

about 1300 ml h^{-1}. Winter (1973) also found that halving the algal concentration approximately doubled the filtration rates. This large filter capacity allows the mussel to ingest unselectively not only food particles and organic matter but also bacteria, toxic substances, parasite larvae and even chemical pollutants. Because of this, the blue mussel can concentrate several contaminants at high levels in its tissue and is thereby an ideal biological marker for monitoring aquatic environments (Widdows et al., 2002, Brenner et al., 2014). This characteristic also requires that mussels are produced in "clean" environments and/or that they are regularly monitored for contaminants, including amongst others faecal coliforms and marine toxin (depuration is often applied depending on the status of the production area).

The life of marine bivalve mollusks is complex and includes a planktonic larval stage. Both larvae and many adult bivalves feed by capturing microscopic particulates, inclusive detritus, bacteria, microalgae and small animals (seston). Sestonic particles captured by bivalve suspension feeders typically have diameters in the order of 1 to 100 µm: bacteria to microplankton (Figure 2.11) and occur in seasonally variable amounts in seawater. The size of particle captured depends on the different stage of life cycle of these species (Raby et al., 1997)

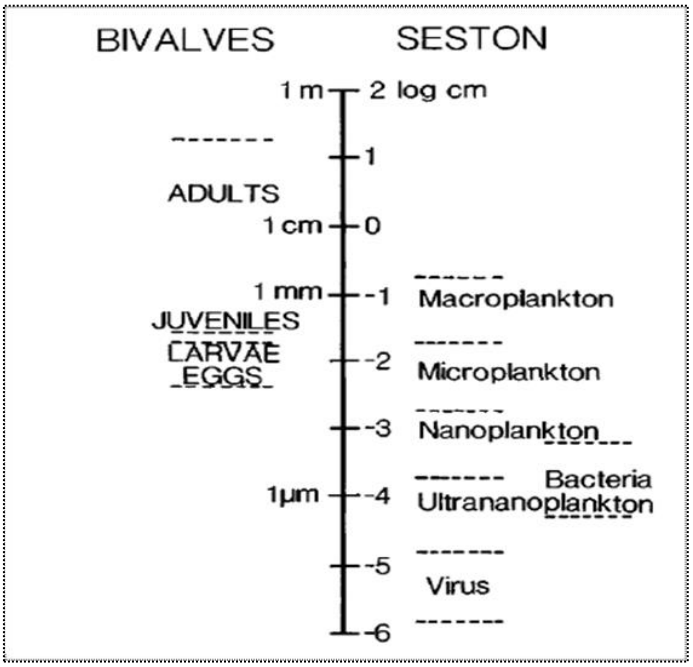

Figure 2. 11: Comparative sizes of suspension-feeding bivalves and their potential food (seston). Adapted from Wildish and Kristmanson (1993).

2.4. The immune system of bivalves

In common with most invertebrates, bivalve mollusks are characterized by an innate, non-specific immune system. This means that they react towards foreign components without any memory regarding previous contacts and they do not use antibodies, the latter being elements of the adaptive immune system (Roch, 1999, Gosling, 2003). The innate immune system in bivalves comprises both cellular and humoral components, which play a vital role in protecting the animals against foreign invaders namely bacterial, fungal, and viral pathogens (Canesi et al., 2002b, Iwanaga and Lee, 2005)

The cellular response of the innate immune system is based on the activity of the hemocytes, which resemble the monocytes and macrophages of vertebrates in both structure and function. The hemocytes, circulating in the hemolymph, are responsible for different cell-mediated reactions such as phagocytosis and subsequent cytotoxic reactions (Lee, 2005). Phagocytosis generally starts with the recognition of the foreign particle such as a bacterium. This particle, engulfed by the phagosome, is further processed. Hemocytes granules fuse with phagosome. This allows effector molecules to come into contact with the foreign particles helping to destroy them. This effectors include the production of reactive species of oxygen (ROS) through the NADPH oxidase complex and/or defense molecules (antimicrobials, hydrolytic enzymes) (Bachère et al., 2015). These cellular components of the immune system are also involved in other important physiological functions such as digestion and nutrient transport, wound healing and shell excretion (Lee, 2005, Bettencourt et al., 2009).

The humoral component immune system of bivalves consists of the blood cells, the hemocytes, and of soluble Hemolymph factors, that operate in a co-ordinated way to provide protection from invading micro-organisms (Canesi et al., 2006). Hemolymph serum contains humoral defense factors, such as soluble lectins, hydrolytic enzymes and antimicrobial peptides (Canesi et al., 2002a, Pruzzo et al., 2005, Bachère *et al.*, 2015) (Figure 2.12). Bivalve hemocytes are extremely heterogeneous; classifications proposed for different bivalve species are reported elsewhere (Fryer and Bayne, 1996, Ottaviani and Franceschi, 1998, Hine, 1999).

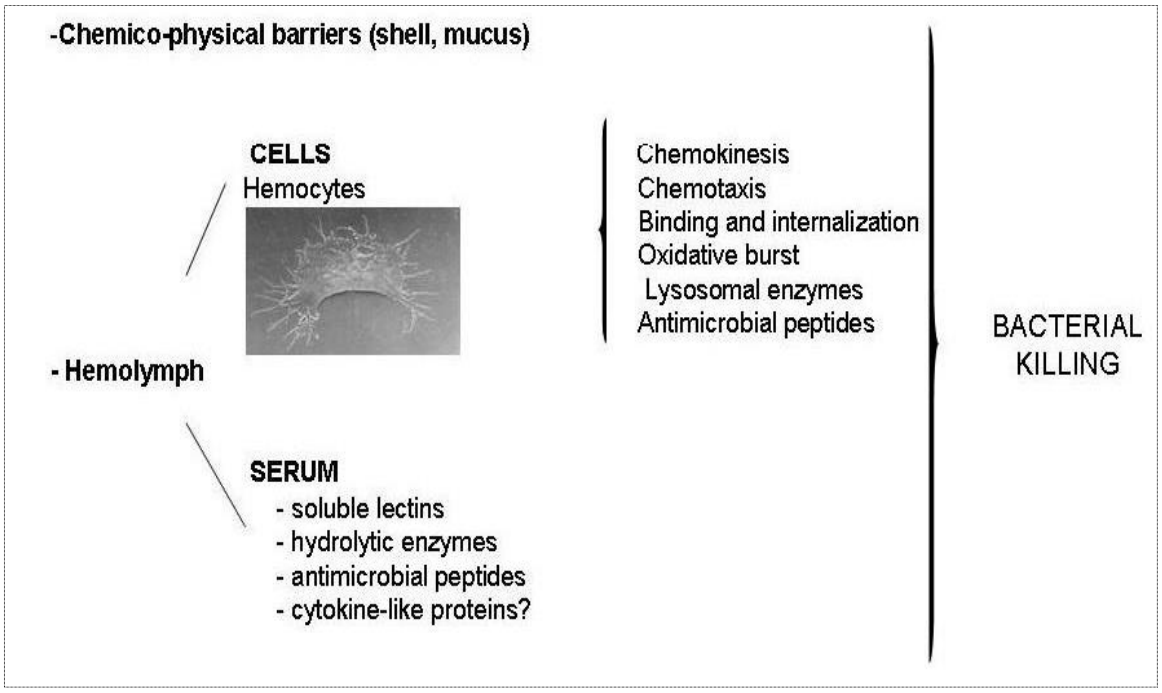

Figure 2. 12: Mechanisms involved in the bactericidal activity of bivalve hemolymph (Canesi *et al.*, 2006)

2.4.1. Cellular immune response

Cellular response is carried out by hemocytes and in *Mytilus edulis,* they can be subdivided into two categories, namely granulocytes and hyalinocytes or agranulocytes. Granulocytes are the most abundant hemocytes in bivalve and have a large variability in number, type of granules and degree of phagocytic activity. For example in *Mytilus campechiensis,* the percentage of granulocytes and agranulocytes is 58.4 and 41.6, respectively (Huffman and Tripp, 1982). The latter group, agranulocytes, has few or no granules and are less phagocytic then granulocytes (Gosling, 2003, Song et al., 2010). The classification of hemocytes for different bivalve mollusk species summarized in Table 2.2.

Table 2. 2 Classification of the hemocytes for different bivalve

Bivalves species	Hemocytes	Reference
Clam *Tapes philippinarum*	-Granulocytes (80.1%) -Hyalinocytes (32.2%) -Hemoblast (18.9%) -Serous cells (0.8%)	Cima et al. (2000)
Giant Clam *Tridacna crocea*	-Eosinophilic granular -Hemocytes -Agranular cells -Morula-like cells	Nakayama et al. (1997)
Meretrix meretrix	-Agranular hemocytes -Lymphoid hemocytes -Large granular hemocytes -Small granular hemocytes	Yanyan et al. (2006)
Anodont (Anodonta cygnea)	-Granulocytes (eosinophilic and basophilic granulocytes and an intermix) -Agranulocytes (hyalinocytes and blast-like cells, vesicular cells)	Salimi et al. (2009)

There are different types of cellular defense mechanisms in bivalves, namely hemocytosis, phagocytosis and encapsulation.

Hemocytosis, or hemocyte proliferation, is the first response to an infection or presence of a non-self-particle. This involves an increase of circulating hemocytes, which move towards the infected of injured tissue (Gosling, 2003). This movement of the cells is activated by chemo-attractant substances, such as opsonines (for example, the extrapallial protein in *Mytilus galloprovincialis* (MgEP)), to which the hemocytes are sensitive to. (basically opsonization of microorganisms has been shown to occur through plasma proteins like Cg-EcSOD, which promotes b-integrin-mediated phagocytosis (Duperthuy et al., 2011)). This movement is implemented by two motile responses, namely chemotaxis and chemokinesis.

Chemotaxis is a directional movement and in *M. edulis* for example, is triggered by the presence of lipopolysaccharides from *Escherichia coli* and *Serratia marcescens*. Chemokinesis is a random, indirectional migration of the hemocytes and for example stimulated by N-formyl-methionyl-leucyl-phenylal-anine (N-FMLP) in the blue mussel (Canesi *et al.*, 2002b, Song *et al.*, 2010).

Myticin class C of mussel *Mytilus galloprovincialis* (Myt C) is known as an antimicrobial peptide, and it is the most abundantly expressed gene in cDNA after immune stimulation of *Mytilus galloprovincialis* (Costa et al., 2009). Recently, Balseiro et al. (2011) showed that this AMP (Myt C) could be acting as immune system modulator molecule because its over-expression was able to alter the expression of mussel immune-related genes such as *Myticin B, Mytilin B*, and *lysozyme*. Moreover, the in *vitro* results indicate that Myt C-peptides have antimicrobial and chemotactic properties. The result of this study suggested that Myt C should be considered not only as an AMP but also as the first chemokine/cytokine-like molecule identified in bivalves.

After this movement, non-self-recognition and attachment of the hemocyte to the targeted particle occurs and, the particle is enclosed in a vesicle in the cell by endocytosis. This vesicle is also called a primary phagosome and fuses together with a lysosome to form a phagolysosome. In this structure, the engulfed particle is destroyed by lysosomal enzymes, reactive oxygen intermediates, nitric oxide and antimicrobial factors (Gosling, 2003, Song *et al.*, 2010). Antimicrobial factors including cytoskeleton modification, internalization, and destruction of the engulfed target within phagosomes (Song *et al.*, 2010). This mechanism is called phagocytosis and is illustrated in Figure 2.13.

Nodulation is one of cellular defense mechanisms against bacterial, fungal and viral infection in insect (Drosophila) and other invertebrates. Nodulation is mediated by hemocytes aggregate formation around bacteria and fungi (Lavine and Strand, 2002). Encapsulation is similar to nodulation and refers to hemocyte aggregation around larger pathogens like parasitoids and nematodes (Gandhe et al., 2007). The nodulation mechanism has been exited in bivalve Eastern oyster *Crassostrea virginica.* However, the study of nodule formation in bivalves is very rare.

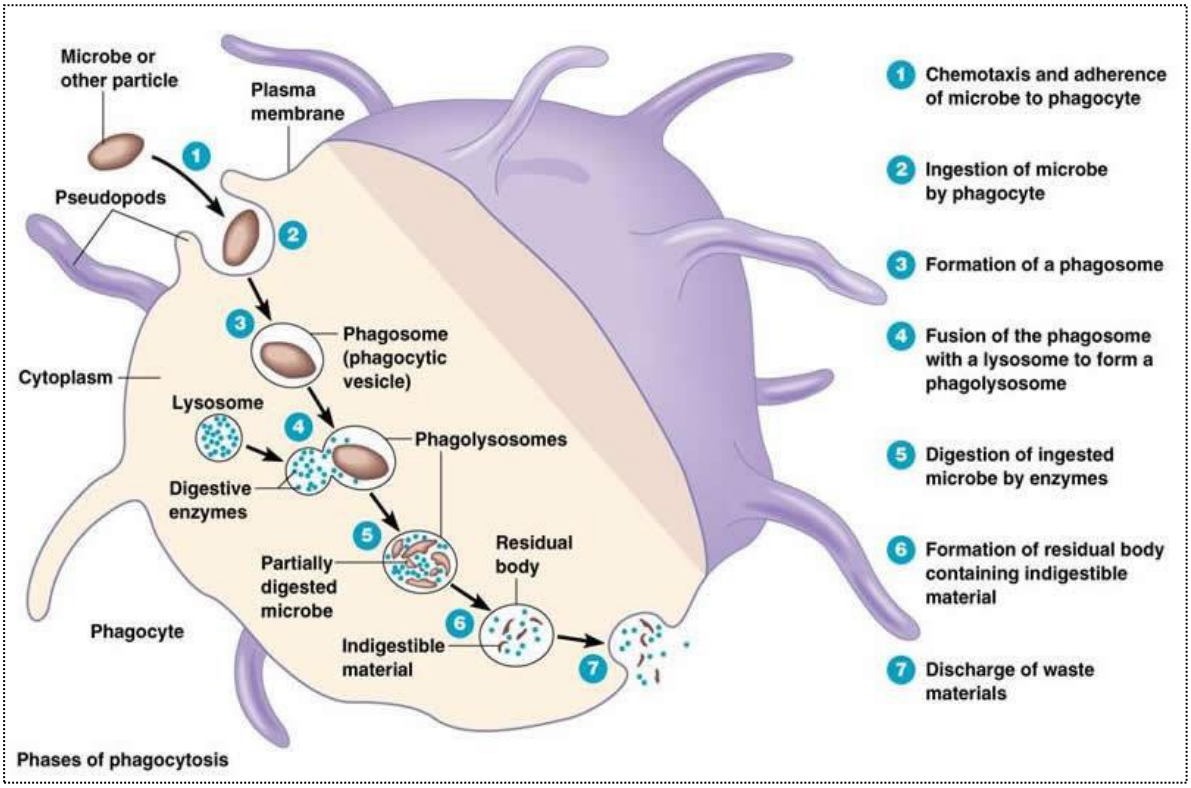

Figure 2. 13: Schematic illustration of phagocytosis (Anonymous, 2016).

Encapsulation is the cellular immune response to foreign particles that are too large to be phagocytosed. In this mechanism the foreign particle is encapsulated with hemocytes and cytotoxic products, such as degradative enzymes and free radicals, are released to destroy the particles (Song *et al.*, 2010).

2.4.2. Humoral immune defense

Humoral response is carried out by humoral defense factors that are present in the Hemolymph, as mentioned above. These factors can cause a direct cytotoxic effect on pathogens and a lot of soluble molecules in the hemolymph can be categorized as humoral defense factors. It should be noted that some of these factors are present in hemocytes and are thereby also involved in the cellular defense. The most abundant humoral defense factors in *Mytilus* species are (soluble) lectins, hydrolytic and lysosomal enzymes such as phosphatase and lysozyme and antimicrobial peptides (Canesi *et al.*, 2002b, Pruzzo *et al.*, 2005).

2.4.2.1. Lectins

Lectins are sugar-binding proteins that can interact specifically and reversibly with membrane glycoproteins or glycolipids of bacteria or other invaders (Renwrantz, 1990). Thereby, lectins are involved in several immune functions, such as self/non-self recognition and associated effector mechanisms (Song *et al.*, 2010).

Lectins can have a direct or indirect role in the phagocytosis process. Lectins can cause agglutination and thereby potentiate the phagocytosis process by immobilization of bacteria. On the other hand, lectins can also act as bridge molecules between bacteria and hemocytes and thereby act as opsonization for phagocytosis (Pruzzo *et al.*, 2005). There exist many lectins, and they can be distinguished from each other by differences in molecular size, subunit structure, agglutination properties, and sugar-binding specificity (Canesi *et al.*, 2002b). There are some important lectins such as MytiLec which is a α-Gal-binding lactic that was isolated from the Mediterranean mussel *Mytilus galloprovincialis* (family Mytilidae) (Hasan et al., 2015) and C-type GalNAc-specific lectins recovered from the sea cucumber *Cucumaria echinata* which interact with target membranes and exhibit strong hemolytic activity and cytotoxicity through pore formation (Hatakeyama et al., 1996).

2.4.2.2. Lysozymes

Lysozyme is a widely distributed anti-bacterial molecule (basically mostly a family of molecules) present in numerous animals including bivalves. It is a hydrolytic protein that can break the glycosidic union of the peptidoglycans of the bacteria cell wall. It is present in the Hemolymph and the cells, and its release is associated with physiological changes, age, stress, (Lopez et al., 1997, Cronin et al., 2001) and bacterial infections (Li et al., 2008). Several studies have shown that lysozymes are able to kill Gram-negative bacteria, but some have a wider activity. It was demonstrated for example that: an antibacterial lysozyme-like protein from Icelandic scallops could inhibit the growth of all Gram-positive and Gram-negative bacteria. Moreover, purified oyster plasma lysozyme inhibited the growth of Gram-positive bacteria (e.g., *Lactococcus garvieae, Enterococcus sp.*) and Gram-negative bacteria (e.g., *Escherichia coli, Vibrio vulnificus*)(Yue et al., 2011).

Lysozyme expression has been detected and localized in cells of digestive tubules, gill and mantle (Bachère *et al.*, 2015). Although lysozyme activity was first reported in mollusks over 30 years ago, complete sequences were published only recently, including those from *M. edulis*, *M. galloprovincialis*, and two different forms in *Crassostrea virginica*, *C. gigas*, *Ostrea edulis* and *Chlamys farreri* (Bachali et al., 2002, Itoh and Takahashi, 2007, Li *et al.*, 2008). Several types of lysozymes have been purified, from the best-known chicken-type (c-type) and goose-type (g-type) to the more recently identified invertebrate-type (i-type)(Nilsen and Myrnes, 2001). Such i-type lysozymes have been identified in several bivalve mollusk, including the Icelandic scallop, *Chlamys islandica* (Nilsen and Myrnes, 2001) and the blue mussel, *Mytilus edulis.* In the Mediterranean mussel, *M. galloprovincialis*, lysozyme has been found localized within granular hemocytes. Higher levels of activity have been detected in hemocytes compared with plasma in both *M. edulis* and the carpet shell clam, *Ruditapes decussatus* (Li *et al.*, 2008).

2.4.2.3. Antimicrobial peptides (AMPs):

AMPs are a major component of the innate immune defense system of bivalves. AMPs are molecules with a mass less than 10 kDa which shows antimicrobial and antifungal properties (Tincu and Taylor, 2004). Currently, ca. 10 different types of AMPs have been identified in the mussel. They can be categorized into four different groups according to their antimicrobial and antifungal action and to their primary structure (hydrophobic, cationic or amphipathic). These four groups with their associated classes are called defensins, mytilins, myticins and mytimycins (Canesi *et al.*, 2002b, Tincu and Taylor, 2004, Song *et al.*, 2010). Many antimicrobial peptides are located in epithelia (Schröder, 1999) where they prevent invasion by pathogens while others may be especially abundant in circulating cells.

In bivalves, AMPs were first purified from the blue mussel (*M. edulis*) hemocyte granules (Charlet et al., 1996) and classified into three families: *defensins,* related to arthropod defensins, *mytilins* and *mytimycin*. These three AMPs are present in different quantities (Gestal et al., 2008). Moreover, the genes are differentially regulated according to the challenging bacteria (Cellura et al., 2007). In *M. galloprovincialis*, all of the peptides possess eight cysteine's arranged in particular conserved arrays (Mitta et al., 2000a, Mitta et al., 2000d). The typical structure of these peptides is shared by the new *myticin* class, *myticin C*,

recently described in *M. galloprovincialis* (Pallavicini et al., 2008). The genes encoding these proteins showed a high polymorphic variability (Padhi and Verghese, 2008), which has been suggested to account for the high resistance of the Mediterranean mussel to disease (Costa et al., 2008). This variability was also observed in clam and mussel *mytilins* (Gestal et al., 2007, Parisi et al., 2009). In the oyster *C. gigas*, a molecular biological approach revealed the presence of two isoforms of a defensin-like protein (Gueguen et al., 2006, Gonzalez et al., 2007). In contrast to the one from the mussel, the oyster defensin was not altered after a bacterial challenge (Gueguen *et al.*, 2006).

Table 2. 3: Antimicrobial peptides of two closely related mussel species and their classes (Tincu and Taylor, 2004)

Species	Peptide	Class
Mytilus edulis	Defensins A and B	β-3
	Mytilin A and B	β-4
	Mytimycin	β-6
Mytilus galloprovinciallis	Myticin A and B	β-4
	Defensins 1 and 2	β-4
	MytilinB, C, D and G1	β-4

2.4.2.4. Phenoloxidase (PO)

Phenoloxidase is a critical component of the immune system of mollusks (Asokan et al., 1997, Lopez *et al.*, 1997). As the product of a complex cascade of reactions, PO is generated from prophenoloxidase (proPO) through a limited proteolysis by a proPO activating enzyme (ppA) (Coles and Pipe, 1994). *In vivo* ppA is activated in the presence of microbial polysaccharides, such as lipopolysaccharides, peptidoglycans and 1,3-β-glucans. Upon binding of these type of molecules to specific pattern-recognition receptors ppA is activated, and as a consequence, active ppA converts proPO to PO (Aspan et al., 1995, Ballarin et al., 1998). Finally, PO is involved in melanization, encapsulation, wound healing, phagocytosis, and pathogen extermination (Bai et al., 1997, Newton et al., 2004, Munoz et al., 2006). In

bivalve mollusks, PO is mainly found in the Hemolymph (Asokan *et al.*, 1997, Munoz *et al.*, 2006), and exits both in the soluble or cellular form: the soluble form is always involved in humoral immunity, while the cellular PO, which binds to the surface of hemocytes is more associated with cell-mediated immunity (Ling and Yu, 2005, Hellio et al., 2007), although there is other PO-like activity (Rizzi et al., 1994). Significant differences in phenoloxidase activity have been observed in bivalves exposed to environmental stressors or pollution (Kuchel et al., 2010, Luna-Acosta et al., 2010). Phenoloxidase enzymes can be detected in all life stages of *M. edulis* (Coles and Pipe, 1994, Dyrynda et al., 1995, Luna-Acosta et al., 2011) and Pacific oyster *C. gigas* (Thomas-Guyon et al., 2009). PO enzymes are most commonly found in adult hemocytes (Luna-González et al., 2003) but embryonic cells, which possess some phagocytic capacity, possess a restricted level of PO activity as well (Dyrynda *et al.*, 1995, Luna-González *et al.*, 2003) and it has been suggested that it plays an important function in non-self-recognition and host immune reactions in the early life stages of bivalve (Thomas-Guyon *et al.*, 2009).

2.4.2.5. Production of reactive oxygen species (ROS)

Reactive oxygen (ROS) and nitrogen species (RNS) are naturally produced in all cells and organisms. ROS generation may result in oxidative stress (Donaghy et al., 2015). In mammals, all the blood phagocytes contain granules rich in lysosomal enzyme, which degranule during phagocytosis to bring about the killing an degradation of endocytosis pathogens (Pipe, 1992). In bivalve mollusks, ROS is produced in hemocytes as part of their basal metabolism as well as in response to endogenous and exogenous stimuli (Donaghy *et al.*, 2015). The major reaction cascades from the initial pathogen contact through oxidative killing are summarized in Figure 2.14.

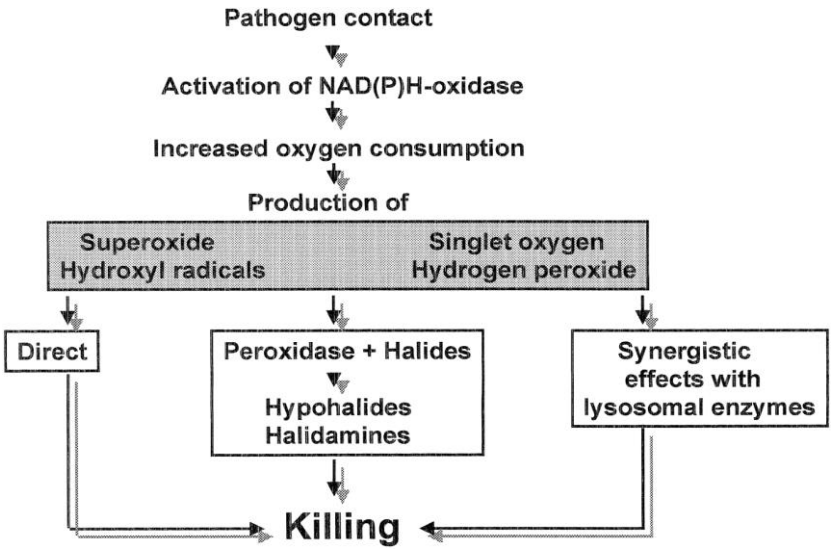

Figure 2. 14: Diagram of the major reaction cascades from the initial pathogen contact through oxidative killing. Adapted from Roch (1999).

2.4.2.6. Cytotoxic enzymes activity

In many invertebrate species, several kinds of immune-related humoral activities have been reported. Bivalves possess various types of non-specific humoral defense molecules including agglutinins, opsonizing lectins, bactericidins, lysozymes and serine proteases. In the blue mussel, *M. edulis*, a modification of the *in vitro* plaque assay has been employed to demonstrate the presence of cytolytic molecules (another name is cytotoxic hemocytes (Wittke and Renwrantz, 1984, Leippe and Renwrantz, 1988). Also, the plasma of the Mediterranean mussel, *M. galloprovincialis,* contains cytotoxic activity against both vertebrate (erythrocytes and mouse tumor) and protozoan cells. The prokaryotic bacteria, *Escherichia coli* and *Vibrio alginolyticus*, were not sensitive to this cytotoxicity (Hubert et al., 1996). Injection of erythrocytes stimulated the cytotoxic activity of the plasma with a maximum 2 days post-injection, suggesting that cytotoxic molecules are involved in immune defense (Hubert et al., 1997). The activity was still present in dialyzed samples but was destroyed by heating at 45°C. Purification by anion exchange chromatography followed by gel filtration revealed a 320-kDa cytotoxic polymeric protein (Roch et al., 1996). Composed of three different proteins (25 – 320 kDa), the complex acts through a hetero-polymerization process after binding to target cell membranes as revealed by ultrastructural observation (Hubert *et al.*, 1997).

2.4.3. Difference in the immune system between bivalve adult and larvae

Very little is known about the immune system of bivalve mollusk larvae although some studies reported that larvae of certain groups of mollusks may have a stronger innate internal defense than others, or perhaps there are differences in the maturation rate of the immune system during the early development (Luna-Gonzalez et al., 2002).

Dyrynda *et al.* (1995) found that larvae have non-specific defense barriers to bacterial invasion as well as specific immune functions. Non-specific immune defense includes external and mucosal barriers and some adaptive components that are transferred from the mother, such as agglutinins, precipitins, lysins and immunoglobins on the egg membrane surface (Mulero et al., 2007). External and mucosal barriers are the first line of defense since infection usually requires intimate association with larvae surfaces (Olafsen, 2001, Gomez-Leon et al., 2008).

In a study of the immune system of larvae of blue mussel *M. edulis* Dyrynda *et al.* (1995) demonstrated some immune defense functions including production of degradative enzymes phenoloxidase and arylsulphatase, phagocytosis of *E. coli* cells and generation of reactive oxygen metabolites. Immunity attributes were compared to those of adults in Table 2.4 (Dyrynda *et al.*, 1995), which indicates that some elements of the immune system in adults bivalves also appear in the trochophore and veliger larvae.

Recently, Song et al. (2016) reported that the newly hatched, trochophore stages of the Pacific oyster are not only critical for oyster development in general, but also critical for the ontogenesis of the immune system. Phagocytosis was firstly observed in the early D-veliger larvae, 17 hour after fertilization

The adult bivalve immune system does not appear to operate autonomously in regulation and action as previously thought, but rather may interact with the nervous and endocrine systems (Koller, 1990). These links may increase the complexity of the immune response and potentially make it more sensitive to environmental stressors (Parry and Pipe, 2004).

Table 2. 4: Summary of defense mechanism recorded in blue mussel (*Mytilus edulis*)

Activity	Adults	Larvae	Reference
Degradative enzyme	+	+	(Pipe, 1990)
Phagocytosis	+	+	(Noël et al., 1994)
Cytotoxic reaction	+	ND*	(Leippe and Renwrantz, 1988)
Reaction oxygen metabolites generation	+	ND	(Pipe, 1992)
Nitric oxide generation	+	ND	(Ottaviani et al., 1990)

(*)ND: not determined

2.5. Constraints of bivalve aquaculture

One of the main problems in the aquaculture of bivalve mollusks is occurring mortality, which severely reduces production. These outbreaks of disease affect larval, and postlarvae in the hatchery, as well as juvenile and adults, cultured in the natural environment. Research has been conducted to determine the aetiology, epidemiology and control measures for these epizootics (Renault, 1996). Bacteria, viruses, fungi, and protozoan parasites have caused major epizootics in bivalve mollusks. This is probably facilitated by considerable (commercial) movement of life animals, explaining the appearance and the spread of some infectious diseases in several countries around the world (Renault, 1996). In this chapter, we will focus on bacteria and viral diseases of bivalve mollusks that have been reported so far, and their economic impact on the bivalve mollusk aquaculture.

2.5.1. Bacterial diseases

For the main microbial diseases affecting cultured marine bivalves the aetiologic agents have been characterized, and their pathogenesis and pathogenicity have been studied. Several recent bivalve-interaction models have been studied for the brown ring disease, juvenile oyster disease, Pacific oyster nocardiosis and summer mortalities of oysters (Paillard et al., 2004). In addition, the taxonomy and phylogeny of new potential bivalve pathogens and

their virulence factors have been established. Facing the difficulty of identifying bacterial strains associated with molluscan diseases (mainly *vibriosis*), polyphasic approaches have been developed to correlate the phenotype and genotype of potential pathogens (Paillard *et al.*, 2004).

So far, in bivalve mollusk hatcheries, massive mortalities can result in the complete loss of the production stock, with serious economic consequences. In most cases, the studies have demonstrated that the problems are caused by bacterial pathogens, the primary etiological agents being members of genus *Vibrio*, *Pseudomonas*, *Aeromonas,* and *Vibrio*–like bacteria (VLB) (Tubiash et al., 1965, Tubiash et al., 1970, Sugumar, 1998, Beaz-Hidalgo et al., 2010). Although all life larval stages are vulnerable, the larvae are particularly exposed to a high concentration of potentially pathogenic bacteria associated with tank surface, moribund larvae, or organic detritus (moribund larvae or organic debris) during the temporary fixation of the larvae on the bottom of the tank (Sutton and Garrick, 1993).

Vibrio alginolyticus has been reported as one of the most pathogenic bacteria for molluscan larvae (Tubiash and Otto, 1986, Luna-Gonzalez *et al.*, 2002). For example, survival rates of *Mytilus galloprovincialis* larvae exposed to *Vibrio alginolyticus* were found to be between 64-95% 48 h post challenge for the different bacterial concentrations. Minimum survival (64%) was found in 3×10^4 CFU ml^{-1} and the maximum in the control treatment (95%) (Anguiano-Beltrán et al., 2004).

Together with the species *V. alginolyticus*, both *V. tubiashii*, and *V. anguillarum* were recognized as the primary causal agents of the "bacillary necrosis" (Tubiash *et al.*, 1970, Tubiash and Otto, 1986). The disease is characterized by bacterial colonization of the mantle, velum disruption, abnormal swimming, visceral atrophy, and lesions in the organs among other signs. Another characteristic sign of larval *vibriosis* in hatcheries is the appearance of the phenomenon called "spotting," defined as an accumulation of moribund larvae agglutinated at the bottom of the tanks (DiSalvo et al., 1978). Table 2.5 provides a summary of the most recent studies on bivalve larvae *vibriosis*.

Vibriosis is not only the main cause of massive mortalities in larval stages but also effects juveniles and adults (Paillard *et al.*, 2004). A total of 90 samples of adults blue mussels grown in Germany were analyzed. The analysis revealed the presence of *Escherichia coli* and

Salmonella spp. but also may *Vibrio spp*. *Vibrio spp*. was detected in 74.4% of the samples analyzed in this study. Among *Vibrio* isolates, *Vibrio alginolyticus* was the species most frequently detected (51.2%), followed by *Vibrio parahaemolyticus* (39.5%) and *Vibrio vulnificus* (3.5%). *V. parahaemolyticus* and *V. vulnificus* were not found in samples collected at low water temperatures (Lhafi and Kuhne, 2007).

In France, classical *Vibrio* infections are associated with the phenomenon known as Summer Mortality (SM) in juvenile Pacific oysters (*Crassostrea gigas*). SM affects juvenile oyster populations during the warmer months when the water temperature is ≥ 18 ^{0}C and reproduction takes place (Berthelin et al., 2000). This phenomenon has been associated with stress situations, low dissolved oxygen or presence of toxic substances in the sediment (Cheyney et al., 2000). Lipp et al. (1976) were the first to observe that the oyster Hemolymph had high levels of *Vibrios* that were causing death and later studies identified *V. splendidus* as the causal agent of SM (Lacoste et al., 2001, Gay et al., 2004). More recently, Allain et al. (2009) suggested the possible role of *V. harveyi* as the aetiological agent of SM, since it was detected in most samples of affected oysters during the 2008 warm season and was able to produce mortality in experimental challenges. The most accepted theory today is that the oyster SM cannot be attributed to one bacterial pathogen or to the Oyster *Herpesvirus alone*, but to a complex interaction between the physiological and/or genetic state of the host, environmental factors and the presence of various opportunistic infectious *Vibrio* species (Paillard *et al.*, 2004, Pruzzo *et al.*, 2005, Labreuche et al., 2006).

Another serious mollusk disease is the Brown Ring Disease (BRD), caused by *V. tapetis* (Borrego et al., 1996). It has been widely studied since it is the primary disease with bacterial etiology in adult clams (*Ruditapes semidecussatus* and *Ruditapes decussatus)* (Paillard *et al.*, 2004). Susceptibility to *V. tapetis* infections is species specific, causing greater physiological disturbances and mortality in *R. semidecussatus* than in other species of clams (*R. decussatus* and *Mercenaria mercenaria)* or in the oyster *C. Virginia* (Allam et al., 2001, Allam et al., 2006). Environmental factors (i.e. temperature and salinity) play a role in the development of BRD, which tends to be more frequent in the spring and winter as the optimum growth temperature for *V. tapetis* is 15 ˚C (Paillard et al., 1994, Paillard *et al.*, 2004).

Table 2. 5: Experimental infection of bivalve larvae, juvenile and adults with pathogenic *Vibrio spp*. Adapted from Romalde and Barja (2010).

Pathogenic species	Bivalve species*	Life stage	Reference
V. alginolyticus	*M.galloprovincialis*	Larvae	Anguiano-Beltrán *et al.* (2004)
	R. decussates	Larvae	Gómez-León et al. (2005)
V. splendidus biovar II	*R. decussates*	Larvae	Gómez-León *et al.* (2005)
	C. gigas	Juveniles	Waechter et al. (2002)
V. splendidus –like	*P. maxima*	Larvae	Sandlund et al. (2006)
	C. gigas	Adults	Gay *et al.* (2004)
V. aestuarianus	*C. gigas*	Adults	Garnier et al. (2008)
V. tubiashii	*C. gigas*	Larvae	Hasegawa et al. (2008)
V. parahaemolyticus *V. vulnificus* *V. alginolyticus*	*Mytilus edulis*	Adults	Lhafi and Kuhne (2007)
Vibrio sp.	*C.gigas; O. edulis*	Larvae	Estes et al. (2004)
	C. virginica	Larvae	Gomez-Leon *et al.* (2008)
	C. gigas; C. virginica	Juveniles	Gay *et al.* (2004); Gomez-Leon *et al.* (2008)

*Abbreviations: C = *Crassostrea*, M = *Mytilus*, O = *Ostrea*, P= *Pecten*, R = *Ruditapes*.

2.5.2. Viral diseases

In general, viruses can be significant infectious agents in bivalve species. Particularly in early life stages of bivalves, there appears to be mortality associated with Herpes-like and irido-like viruses (Elston, 1997). The herpes-like virus affects bivalve populations in many countries including Australia, Asian countries and especially in France where infected hatchery-reared larval Pacific oysters showed cumulative infection rates of 60-100% at 7-11 days post fertilization (Hine et al., 1992). Larval hatchery-reared Pacific oysters in France experienced

abnormal mortality and morbidity associated with a herpes-like viral infection (Nicolas et al., 1992) and mass mortality of 80-90% observed during the summer. Le Deuff et al. (1994) reported experimental transmission of the infection from a population of infected oyster larvae to larvae obtained from a pathogen-free broodstock but maintained by axenic methods. Within 48 h of inoculating a virus suspension into the cultures of axenic larvae, the animals exhibited a high rate of morbidity, and virus-like particles were observed in connective tissue cells around the velum. Le Deuff *et al.* (1994) later showed that the viral infection would produce complete viral particles and 80-90% mortality in oyster larvae reared at 25-26 °C but not in larvae reared at 22-23 °C. At the lower temperature, the authors reported nuclear alterations but no viral particles were found. This could be interpreted as latent infections. Elston (1997) also found viral infections developing in larvae from three out of four broodstock batches obtained from different areas in Atlantic France.

Larval hatchery-reared Pacific oysters in France experienced unusually high mortality and morbidity rates associated with a herpes-like viral infection (Nicolas *et al.*, 1992). The infection was associated with cellular changes in oyster seed, collected from survivors of populations that had undergone 80-90% mortality during the summer for at least 20 years (Renault et al., 1994, Goulletquer et al., 1998, Soletchnik et al., 2007). This syndrome has been reported in most Pacific oyster producing countries, such as Japan, USA and Australia. The first description of this phenomenon was made in the 1940s in Japan, where *C. gigas* is endemic. Herpes virus also causes mortality in different bivalve aquaculture species. For example, abnormal summer mortalities associated with the detection of ostreid herpesvirus 1 (OsHV-1) have been reported among larvae and spat of the Pacific oyster *C. gigas* in France (Schikorski et al., 2011), as well as in farmed seed and adult green-lipped mussels (*Perna canaliculus*) from New Zealand. Cumulative mortality of 50-100% associated with apparent virus infection was reported in green lipped mussel seed during the summer and autumn, while mortality in adults was only 2-5% (Jones et al. (1996). In addition, summer mortality occurred in different mollusks species and regions such as the blue mussel (*M. edulis*) in Denmark (Rasmussen, 1986), abalone (*Haliotic spp.*) farm in Australia (Hooper et al., 2007, Tan et al., 2008, Corbeil et al., 2012).

Among 1200 specimens of *M. edulis* examined from three localities in Denmark, a total of 34 mussels with virus-associated granulocytomas were discovered. Confirmation that the virus was related to *Picornaviridae* was based on the following five observations. The virion was non-enveloped, the capsid was apparently icosahedral and measured approximately 27 nm, the virus was found only in the cytoplasm, and no viral DNA was seen (for the latter the Feulgen reaction was used for viral DNA detection. However, result of the Feulgen reaction was negative) (Rasmussen et al., 1985).

2.6. Solutions to control diseases in bivalve larvae culture

2.6.1. Water treatment

The influence of the environment on hatchery cultures is paramount. The bivalve larvae live in the water, sharing the environment with both beneficial bacteria and potential pathogens. The bacterial loads in bivalve larvae tanks and reported to vary between 10^5- 10^6 bacteria ml^{-1} (Nevejan et al., 2016). As bivalves release their young at an early ontogenic stage, these early life-history stages are especially sensitive to possible infections. In part, this is due to their filter-feeding behavior and the subsequent flow of sea water through the organism (Prado *et al.*, 2010). Some methodologies and technologies are currently used to remove the excess of organic matters and nutrients in aquatic systems. Different water treatments, such as filtration, pasteurization, Ozone, Ultraviolet (UV) radiation have been employed in mollusk hatcheries (Vasconcelos and Lee, 1972, Murchelano et al., 1975, Lodeiros et al., 1987). A recent alternative approach is the integration of a biological filter in recirculating systems (Marinho-Soriano et al., 2011) The core mechanism of such strategies is the utilization of living bacteria and algae to convert the toxic pollutants into the less toxic form or microbial biomass that can be fed to the culture animals.

Once most of the solid particles are eliminated from the seawater through decantation, the water is filtered. Filtration is an expensive treatment, with incremental costs with increased volume and the filtration range established. An advisable practice is to reduce the content of bacterial load and results show that this is better than using alternative systems like pasteurization, as demonstrated by Lewis et al. (1988) in a Pacific oyster hatchery. The main disadvantage of pasteurization is the risk of subsequent contaminations due to the necessity

of transporting the treated water through long pipes and the prolonged period of high temperature to cool down (Prado *et al.*, 2010).

To overcome those problems, disinfection with chlorine has been employed too, but it comes with many problems, like the interference with the larval mechanism of pumping, as described by Vasconcelos and Lee (1972) for cultures of *C. gigas,* and by Asokan et al. (2013) for a mussel hatchery. Some studies suggested that the reactions between chlorine and organic nitrogen in the water could produce residues toxic to marine organisms (Jorquera et al., 2002). Another alternative is ozonization, but its application to disinfect aquaculture systems can be complex and costly (Summerfelt, 2003). One of the most common treatments is the radiation of seawater with ultraviolet (UV) light, with unquestionable lethal effects on bacteria and viruses. However, there are disagreements about the actual effects when the water treatment is used in hatcheries. Vasconcelos and Lee (1972) found advantages in its use, as different bacterial populations, including bivalve pathogens, decreased. In larval cultures of *Ostrea edulis*, the population of *Vibrios* decreased with UV treatment (Lodeiros *et al.*, 1987, Sainz-Hernandez and Maeda-Martinez, 2005). Nevertheless, Brown (1981) found that the same treatment with UV tested against two pathogenic *Vibrio spp.* was effective against one of them, while the other strain was able to grow after an initial inhibition. Therefore, the effects of UV-radiation are not homogenous against all the bacterial populations, and more variability is added with factors like the dose, the water flow and the individual efficiency of the radiation unit (Prado *et al.*, 2010). In general, those treatments can reduce number of bacteria in water. For example the addition of ozone reduced the numbers of heterotrophic bacteria by $3\log_{10}$ units (Suantika et al., 2001). A reduction in bacterial numbers can only have advantages in terms of the hygienic quality of aquatic animal; however, attention should also be paid to the changes in bacterial communities imposed by the disinfection.

Moreover, it is known that the efficiency of the treatment is affected by the organic content of water and the presence of small particles to which bacteria are attached (Liltved and Cripps, 1999). In summary, these significant differences in the effectivity can lead to the selection of undesirable populations, including those resistant to the treatment, which would find an ecological niche favorable to their growth.

Currently many bivalve mollusk hatcheries are using a flow through system (FTS) for growing larvae. There are some important advantages larval handling is reduce and the water parameters are less fluctuating because the feeding with algae happens also continuously. These stress-reducing conditions make the larvae less susceptible for disease. Besides a better disease control, larval tanks can hold a much higher larval density in comparison to batch culture and labor cost are reduced (Magnesen and Jacobsen, 2012). However, one should take into account that it is impossible to create a stable bacteria community in the tanks under these conditions and the development of bivalve- specific recirculating systems offers a window of opportunities.

2.6.2. Antibiotics

Antibiotics have been extensively used as therapeutic treatments to disease in bivalve hatcheries since Davis and Chanley (1956) showed their ability to reduce larval mortalities in hard clam *Mercenaria mercenaria*. However, in parallel with studies confirming the beneficial effects, other works described toxic effects, as well as a lack of effectiveness or consistency in the results obtained. Jeffries (1982) observed that the treatment with chloramphenicol achieved a slight recovery in a larval culture of *C. gigas*, but at the same time the larvae ceased swimming and therefore feeding. Uriarte et al. (2001) reported that chloramphenicol added to the food two times per day resulted in better survival and growth rates in Chilean scallops (*Argopecten purpuratus*) between the early larvae and pediveliger stages cultured in a closed system. Similar benefits were reported for larval stages of the great scallop (*Pecten maximus)* (Torkildsen et al., 2005). In experiments with the blue mussel, *M. edulis*, the treatment with chloramphenicol resulted in good survival but slow development, showing an opposite effect to the combination of ampicillin and streptomycin, with excellent development but low survival (Prado *et al.*, 2010).

However, the massive use of antibiotics has led to the development of multiple drug resistant bacteria (Karunasagar et al., 1994, Tendencia and de la Peña, 2001) and even the risk of transmission of resistant plasmids among aquatic-associated pathogens to human pathogens, with the subsequent risk for the hatchery workers (Jeffries, 1982, McPhearson et

al., 1991, Spanggaard et al., 1993). Also, the water exchange between the hatchery and the environment may spread resistant bacteria (McPhearson *et al.*, 1991, Kemper, 2008).

As a result of the growing awareness that antibiotics should be used with more care, more strict regulations on antibiotic usage in aquaculture and on the acceptable residue levels of various agents in the aquatic product have been set (Bachère, 2003, Defoirdt et al., 2011). For instant, nowadays, chloramphenicol is prohibited in any veterinary applications to avoid the health hazards to humans by food consumption at least within the EU (Annex IV of Directive 2377/90/EEC), due to its potential toxic effects in humans as well as to the difficulty in determining security levels of its residues (Prado *et al.*, 2010).

The final objective of all the methods cited above is to obtain a substantial reduction of number bacteria within the hatcheries, but this goal does not seem reasonable, since the bacteria population, or, at least, part of it, may have a beneficial effect on larval development. In addition, a lack of microbiota can favor the colonization of the system by non-desirable microorganisms, due to a lack of natural competitors, in an environment with a regular input of organic matter and optimal conditions (Romalde and Barja, 2010).

2.6.3. Immunostimulants

A set of different substances such as beta-glucans, bacterial products, and plant constituents may directly activate the innate defense mechanisms by acting on receptors. This may trigger the production of anti-microbial molecules. These immunostimulants are often obtained from bacterial sources, brown or red algae. Terrestrial fungi are also exploited as a source of novel potential substances. The use of immunostimulants, as dietary supplements, can improve the innate defense of animals providing resistance to pathogens during periods of high stress (Bricknell and Dalmo, 2005).

Heat Shock Protein (HSP) was considered as an immunostimulant in different bivalve mollusk species, including the mud clam *Mya arenaria* (Abele et al., 2002) and Pacific oyster *C.gigas* (Lang et al., 2009). These studies found that transcription after heat shock increased for genes putatively encoding heat shock proteins and genes for proteins that synthesize lipids, whereas transcription decreased for genes of proteins that mobilize lipids and detoxify reactive oxygen species. Hong et al. (2006) reported that the genomic DNA of *Escherichia*

coli, which contains the un-methylated CpG motif, was used to evaluate the immunostimulating effect of bacterial DNA on innate immune responses in the bivalve mussel *Hyriopsis cumingii* Lea. These results showed that the bactericidal activity of the hemocytes was significantly increased when the cells were incubated with 50 or 100 μg ml^{-1} bacterial DNA for 12 and 24 h. Antibacterial activity, lysozyme activity, and prophenoloxidase (proPO) production of hemolymph were also increased, when the bivalve mollusk was injected with 50 or 100 μg ml^{-1} of bacterial DNA for 12 and 24 h. These activities returned to the control level after 48 h. In summary, bacterial DNA with unmethylated CpG motif (Unmethylated CpG motifs are prevalent in bacterial but not vertebrate genomic DNAs) could activate some parameters of the immune system of bivalve mollusk both *in vivo* and *in vitro*.

In conclusion, immunostimulants can reduce the losses caused by disease in aquaculture; however, they may not be effective against all diseases. For the efficient use of immunostimulants, the timing, dosages, method of administration and the physiological condition of aquatic animal, including bivalve mollusks, need to be taken into consideration.

2.6.4. Prebiotics and probiotics

Since the concept of prebiotics was introduced, it has attracted scientific and commercial interest. The main characteristic of prebiotics is the selective stimulation of intestinal bacteria growth, contributing to the hosts' health and welfare. This requires prebiotics to be non-digestible and resistant to gastric acidity, bile salts, and hydrolysis by the host's enzymes (Glenn and Roberfroid, 1995, Roberfroid, 2007). Symbiotics, products that contain both probiotics and specific prebiotics that promote the growth of the probiotic strains, are considered a good solution in many cases (Apajalahti et al., 2004, Cheng et al., 2005, Bomba et al., 2006). Recently, the use of prebiotics has become popular for a number of aquaculture species, both fish and shrimp (Yousefian and Amiri, 2009). Some of the more commonly used prebiotics in animal aquaculture feeds include inulin, fructooligosaccharides (FOS) and transgalactooligosaccharides (TOS) (Vulevic et al., 2004), which function to increase feed intake, growth rate and food digestibility of aquatic animals (RingØ et al.,

2010). In bivalve mollusk species, the use of prebiotics for larval stages has not been as widely used compared to probiotics.

Verschuere et al. (2000) gave a definition of probiotics, appropriated to be used within the field of aquaculture, taking into account the close interaction of the animal with the environment. According to these authors a probiotic would be a live microbial additive with a beneficial effect on the host, modifying the microbiota associated with the host or the environment, ensuring an optimal use of the feed or improving its nutritional value, improving the host response to the disease, or getting a better quality of its environment.

To date, the use of probiotics in aquaculture has focused mainly on fish and crustacean production, with scarce literature considering their application in bivalve mollusk aquaculture. Lodeiros et al. (1989) published the first work on the effect of antibiotic-producing marine bacteria on the larval survival of scallop *Pecten ziczac* and the best results were achieved using a live bacterial culture.

Douillet and Langdon (1993) conducted a set of experiments with axenic oyster larvae *(C. gigas),* fed with a mono-algal axenic diet and inoculated with different marine bacteria. Most of the isolates assayed had negative or neutral variable effects, and only the strain CA2 (*Alteromonas sp.)* showed a beneficial impact on the growth and survival of oyster larvae. In non-axenic larval cultures, Douillet and Langdon (1994) found that the effect of strain CA2 depended on its concentration, being detrimental to larval growth and survival when administered at 10^7 CFU ml^{-1} whereas at 10 to 10^6 CFU ml^{-1} larval survival was not affected and growth was enhanced. The optimal concentration, 10^5 CFU ml^{-1}, was within the constant values for bacterial populations in bivalve cultures obtained by plate counting, between 10^4 and 10^6 CFU ml^{-1} (Jeanthon et al., 1988). They pointed out the beneficial effects in terms of the percentage of larvae that metamorphosed to spat and spat size after 30 days; a possible indirect beneficial effect was improved growth and development during the larval phase.

The group of Riquelme (Riquelme et al., 1996, Riquelme et al., 1997, Riquelme et al., 2001), performed the most comprehensive assessment on the benefits of probiotic bacteria in bivalve cultures. Their results demonstrated that a strain of *Pseudoalteromonas haloplanktis* was effective against different bacterial species, including members of genus *Vibrio.* Further, they found that strain 11 (*Pseudomonas sp)* (one strain among 506 isolates) was effective in

the larval cultures of *Argopecten purpuratus* against pathogenic *Vibrio anguillarum*-related (*VAR)* strain.

The term 'probiotic' was also used by Gibson et al. (1998) in a study on the protection of C. gigas larvae by the strain A199 (*Aeromonas media*), against the infection by *Vibrio tubiashii*. The strain *A199* showed a broad spectrum of *in vitro* inhibition, including members of genus *Vibrio* and *Aeromonas*. The conditioning of larvae with the probiotic strain (10^4 CFU ml^{-1}) allowed the survival of larval cultures inoculated with the pathogen (10^2,10^3 and 10^5 CFU ml^{-1}).

Nakamura et al. (1999) reported the beneficial effects of strain S21, isolated from seawater, against pathogenic *Vibrios*, as demonstrated by the protection offered to Pacific oyster *C. gigas* larvae against *V. alginolyticus.* These results demonstrated that the inoculation of probiotic at 10^4 or 10^5 CFU ml^{-1} immediately after the pathogenic strain (10^5 CFU ml^{-1}) reduced the larval mortality from 91.6% to 53.1 and 78.0%, respectively. In any case, the potential probiotic maintained their numbers in the medium, avoiding the risk of a dangerous proliferation.

Kesarcodi-Watson et al. (2009) first screened probiotics for effective use with green shell mussel larvae (*Perna canaliculus*), and has demonstrated the benefit of including test animals in the initial screening stages. It should also be considered that when screening for effective probiotics, it is best to use bacteria associated with or existing in the natural environment of the host. Effective application in flow-through hatchery production conditions is achieved by switching off the water flow for just 2 h with daily probiotic addition. A later study of Kesarcodi-Watson et al. (2010) demonstrated that both strains of *Alteromonas macleodii* 0444 and *Neptunomonas* sp. 0536, were two novel probiotics for effective use on hatchery-reared Green shell mussel larvae, *Perna canaliculus.*

In general, the positive effect of probiotic bacteria in bivalve mollusk species was demonstrated in those studies. However, there are some limitations to the use of probiotics in aquaculture. Because, the most important limitation to the use of probiotics is that in many cases they are not able to maintain themselves in sufficient numbers in the standing microbial communities, and so they need to be added regularly and at high concentrations making this technique less cost effective (Defoirdt et al., 2007b). Probiotics that were mostly

selected *in vitro* based on the production of inhibitory compounds might fail to produce these compounds *in vivo*. Also, food safety issues and difficulties regarding incorporation of viable cells in feed, maintaining them stable during storage have put a constraint on the full-scale application of probiotics (Merrifield et al., 2010).

2.6.5. Antivirulence therapy

Antivirulence therapy is a promising alternative approach to treating bacterial diseases. Because bacteria use virulence factors to cause infection, preventing pathogens from producing and using such factors is a promising strategy for disease control. This therapy is thus based on a thorough understanding of the mechanisms that pathogenic bacteria use to cause diseases (Defoirdt, 2014). The major advantage of antivirulence therapy is that there will be less or no interference with non-target organisms, such as the commensal microbiota. This is because antivirulence therapy specifically targets virulence gene expression and regulation (Defoirdt, 2013, Defoirdt, 2014). Antivirulence therapy consists on one hand of specifically inhibiting one or more virulence factors. On the another hand, antivirulence therapy can interfere with the mechanisms that control the expression of virulence factors. These mechanisms are for instance cell-to-cell communication (or quorum sensing) and host-pathogen signaling. So for example, quorum sensing disrupting agents can prevent bacteria from attaching to the host and are in this way effective in disease control (Defoirdt, 2014).

2.7. The use of poly–β–Hydroxybutyrate (PHB) as a tool to control disease in aquaculture seed production

2.7.1. Introduction

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyalkanoates that accumulate intracellularly as carbon and energy storage materials in numerous microorganisms, usually when grown under limitations of oxygen, nitrogen, phosphate, sulphur, or magnesium and in the presence of excess carbon (Anderson and Dawes, 1990, Valappil et al., 2007). PHA is typically produced as a polymer of 10^3 to 10^4 monomers (Suriyamongkol et al., 2007) which exists as discrete granules, with about 5 to 13 granules per cell and with diameters of 0.2 to

0.5 µm (Figure 2.15) (Lee, 1996a, Sudesh et al., 2000). The properties of PHAs are similar to those of conventional petrochemical-based synthetic thermoplastics and can hence potentially replace them. Furthermore, they become completely degraded to carbon dioxide and water under aerobic conditions, and to methane and carbon dioxide under anaerobic conditions by microorganisms in the environment (Anderson and Dawes, 1990, Lee, 1996b).

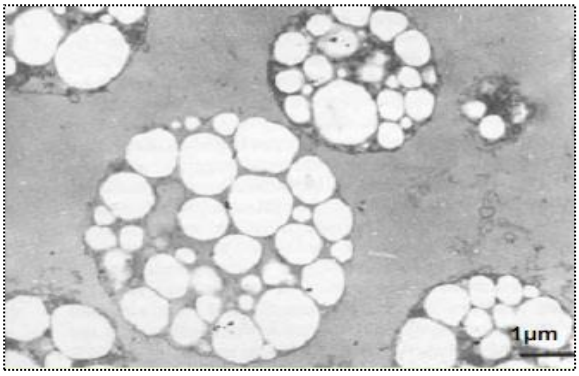

Figure 2. 15: *Pseudomonas putida* cells accumulating PHB inclusion under electron microscopy (Luengo et al., 2003).

PHAs can be classified as either short-chain-length (SCF) PHAs with C_3-C_5 hydroxyacids as monomers or medium-chain-length (MCF) PHAs with C_6-C_{16} hydroxyacids as monomers. The SCF-PHAs have properties close to the conventional plastics (stiff and brittle) while MCF PHAs are flexible, present low crystallinities, tensile strengths and melting points. Certain bacteria can synthesize PHAs containing both SCF and MSF monomer units (Philip et al., 2007). The molecular weight of PHAs varies between 2×10^5 and 3×10^6 Daltons (Figure 2.16). The composition and molecular weight of the synthesized polymer are governed by two factors: the microbial strains and the provided substrate (mainly the carbon source) (Valappil *et al.*, 2007). The best-known member of the PHAs is poly-β-hydroxybutyrate (PHB), containing repeat units of (R)-3 hydroxybutyrate (Lee, 1996b).

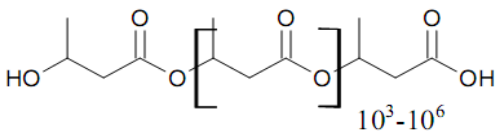

Figure 2. 16: General structural formula of poly–β–hydroxybutyrate

2.7.2. The production of polyhydroxyalkanoates

Previous studies have shown that several bacteria can accumulate high levels of PHA per dry cell weight (Kim et al., 1994, Wang and Lee, 1997). They can be divided into two groups, of which the first includes *Ralstonia eutropha, Protomonas extorquens*, and *Pseudomonas oleovorans*. These bacteria require limitations of essential nutrients (such as nitrogen, magnesium, phosphorous or sulphur) while those of the second group, i.e., *Alcaligenes latus* and *Azotobacter vinelandii*, require no such nutrient limitations (Lee, 1996b). Nonetheless, the PHA content can be increased if a nutrient limitation is applied (Wang and Lee, 1997).

The production of PHAs can be performed from a wide variety of renewable resources, including pure sugars (glucose, xylose, sucrose) (Quillaguamán et al., 2006, Quillaguamán et al., 2007), biomacro-molecules (starch, cellulose) (Keenan et al., 2006, Halami, 2008), and by-products (molasses, whey, wheat bran, corn steep liquor) (Ahn et al., 2000, Vijayendra et al., 2007), as well as fossil resources (methane, mineral oil, lignite, hard coal) (Reddy et al., 2003, Mothes et al., 2008), chemicals (acetate, propionic acid, butyric acid) (Shi et al., 1997) and carbon dioxide (Ishizaki et al., 2001). The metabolic pathway of PHA synthesis in bacteria from different carbon sources is summarized in Figure 2.17. The carbon source is first transferred from the extracellular environment into the cells by a specific transport system or diffusion. The carbon source is then converted into an (R)-hydroxyacyl-CoA thioester (a substrate of the PHA synthase) by anabolic or catabolic reactions or both. Finally, PHA synthases, which are the key enzyme of PHA synthesis, use (R)-hydroxyacyl-CoA thioesters as the substrate and catalyzes the formation of PHA inclusions with a concomitant release of coenzyme A (Steinbüchel and Valentin, 1995).

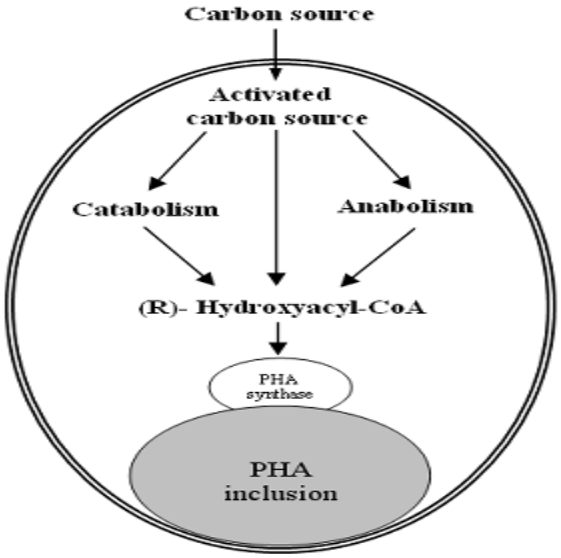

Figure 2. 17: A schematic representation of the biosynthesis of PHA in bacteria (adapted from Steinbüchel and Valentin (1995))

Metabolic pathways and possible options for production of (R)-3-HB are described in Figure 2.18. For the production of (R)-3-HB, pathways leading to PHB production and degradation have to be exploited. Recently, a metabolic pathway for the production of PHB and *in vivo* hydrolysis to release (R)-3-HB in the culture supernatant was investigated (Shiraki et al., 2006). Culture conditions would affect the metabolic shift of the PHB pathway; thereby enhancing the extracellular production of (R)-3-HB in the culture supernatant. Furthermore, it may be possible to introduce PHB knock-out in the PHB metabolic pathway of *R. eutropha*. This process would involve the conversion of acetoacetyl-CoA to acetoacetate (in the presence of acetoacetyl-CoA synthetase), which finally could be converted to (R)-3-HB.

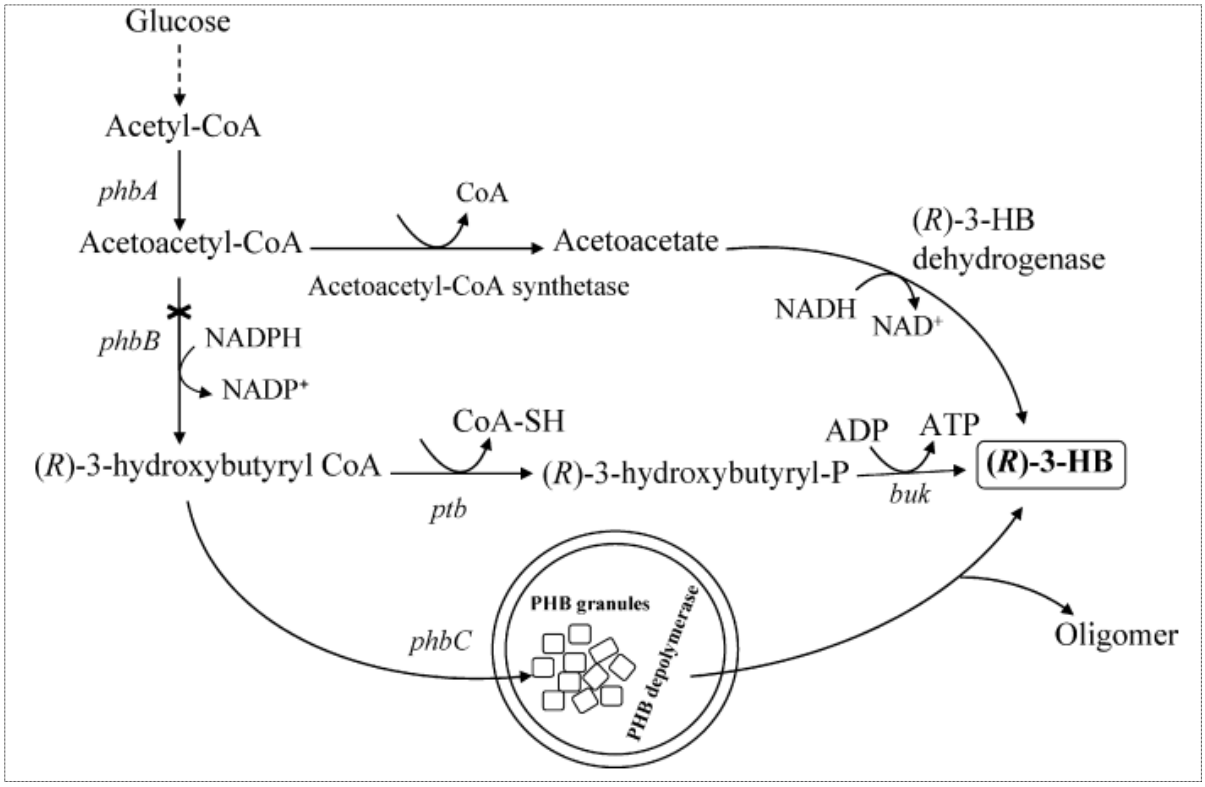

Figure 2. 18: Metabolic pathway for microbial production of (R)-3-hydroxybutyric acid (Tokiwa and Ugwu, 2007).

2.7.3. PHB production in *Ralstonia eutropha*

Ralstonia eutropha (formerly named *Alcaligen eutrophus*) has been studied most extensively due to its ability to accumulate a large amount of PHB from pure carbon sources, for example, glucose, fructose and acetic acid. Imperial Chemical Industries (ICI, UK) has been producing PHB on a large scale from glucose, and poly3-hydroxybutyrate-co-3-hydroxyvalerate (P(3HB-co-3HV)) from a mixture of glucose and propionic acid by the fed-batch culture of *R. eutropha*. Initially, the cells are grown in a glucose-salts medium containing calculated amounts of phosphate to support the desired amount of cell growth. Cells encounter phosphate limitation after about 60 h and accumulate PHB during the next 40–60 h from supplied glucose. By controlling the glucose concentration at 10–20 g l^{-1} during fed-batch culture the final cell mass, PHB concentration and PHB content of 164, 121 g l^{-1}, and 76%, respectively, is obtained for 50 h, resulting in a high productivity of 2.42 g (l^{-1} h^{-1}) (Kim and Chang, 1995). Several carbon sources other than glucose have also been used as the substrate for PHB production by *Ralstonia eutropha* (Table 2.6).

Table 2. 6: The evaluation of various PHB production systems reported for wild-type PHB producing strains (Tokiwa and Ugwu, 2007).

Strain	Carbon substrate	PHB analysis	Culture time (h)	Culture mode	PHB con. (gl^{-1})	PHB prod. ($gl^{-1}h^{-1}$)	PHB content (%)	Reference
A. latus (DSM 1123)	Sucrose	GC	20	Feb-batch	98.7	4.94	88	Wang and Lee (1997)
A. eutrophus (NCIMB 11599)	Glucose	GC	50	Feb-batch	121	2.42	76	Kim *et al.* (1994)
R.eutropha H16	Soybean oil	GC	96	Feb-batch	95.8	1.00	76	Kahar et al. (2004)
Azotobacter chrococcum	Starch	GC	-	Continuous	30.1	0.87	75	Du et al. (2001)
A. latus (DSM 1123)	Sucrose	GV	93	Feb-batch	4.9	0.05	63	Grothe et al. (1999)
R. eutropha WSH3	Tapioca	GC	59	Feb-batch	61.5	1.04	58	Kim and Chang (1995)
A. latus (DSM 1123)	Sucrose	GC	18	Feb-batch	71.4	3.97	50	Yamane et al. (1996)

CDW=cell dry weight; conc.= concentration; GC= gas chromatography; GV= gravimetric; prod.= productivity.

2.7.4. Mechanism of antibacterial activity of poly-β-hydroxybutyrate

Poly-β hydroxybutyrate (PHB) is a short chain fatty acid polymer of biological origin, which is insoluble in water. PHB is degraded into water-soluble monomers or oligomers by the action of PHB depolymerase enzymes secreted in the gastrointestinal passage of many aquatic animals (Liu et al., 2010). PHB is produced by a wide variety of microorganisms as an intracellular energy and carbon storage compound (Anderson and Dawes, 1990, Madison and Huisman, 1999). PHB is deposited intracellularly in the form of inclusion bodies in the fluid, amorphous state (Amor et al., 1991). After death and cell lysis, the polymer is released

in a partially crystalline state (Doi et al., 1995). The ability to degrade extracellular PHB depends on the secretion of extracellular PHB depolymerase enzymes and is widely distributed in bacteria and fungi (Jendrossek, 1998, Jendrossek and Handrick, 2002). The extracellular PHB depolymerase of *Comamonas testosterone* is well characterized; the enzymes were found to hydrolyse PHB into β-hydroxybutyrate monomers (Mukai et al., 1993, Kasuya et al., 1994).

PHB has been shown to effectively exhibit some antimicrobial, insecticidal and antiviral activities (Tokiwa and Ugwu, 2007). Philip *et al.* (2007) mentions for example the commercial product Nodax which is a copolymer containing mainly 3(HB) and small quantities of MCL monomers. It can degrade anaerobically and hence can be applied as a coating for urea fertilizers. It is used in rice fields as herbicide and insecticide. As such, it may also be applied as a means to control infectious disease in aquaculture systems (Defoirdt *et al.*, 2009). The antimicrobial activity of β-hydroxybutyrate acid is thought to be comparable to that of other short-chain fatty acids (SCFAs) (Defoirdt *et al.*, 2007b). The main bacteriostatic and/or bactericidal mechanism of SCFAs (and organic in general) is believed to be caused by the undissociated form of the acid (Ricke, 2003). In the undissociated form, the short-chain fatty acids can pass the cell membrane of bacteria and dissociate in the more alkaline cytoplasm, thereby increasing the intracellular concentration of protons (Kashket, 1987, Cherrington et al., 1991). Consequently, the cells have to spend energy in order to maintain the intracellular pH at the optimal level. This energy cannot be used for other metabolic processes and therefore, the growth of the cells is inhibited (Defoirdt *et al.*, 2007b).

In fact, it has been suggested that short-chain fatty acids (SCFAs) could be useful as biocontrol agents to control bacterial diseases in animal production and more specifically aquaculture (Defoirdt *et al.*, 2009). Several studies have demonstrated that SCFAs inhibit the growth of enterobacteria like *Salmonella* Typhimurium, and *Shigella flexneri* (Cherrington *et al.*, 1991). In another report, the well-known bacterial storage compound PHB, a polymer of the SCFA ß-hydroxybutyrate, was also shown to protect *Artemia* larvae from the virulent *V. campbellii* strain. The degraded product can exert its beneficial effects like other SCFA do (Defoirdt *et al.*, 2007b). In several experiments with *A. fransiscana*, this approach increased

the survival of the animals by up to 73% following a challenge with the pathogen *V. campbellii* (Halet et al., 2007, Defoirdt *et al.*, 2007b).

2.7.5. The immunostimulants properties of the Poly-β-hydroxybutyrate monomer

In addition to its antimicrobial function, SCFA butyrate also induces heat shock protein (Hsp) 25 in rat intestinal epithelial cells and protects the latter against oxidant injury (Ren et al., 2001). HSPs are a group of highly conserved proteins of which expression is constitutive or inducible under different conditions. Hsps, particularly Hsp70, have strong cytoprotective effects and behave as molecular chaperones for maintaining proper protein folding, disaggregating, and refolding misfolded protein as well as targeting damaged proteins for degradation (Hishiya and Takayama, 2008, Tutar and Tutar, 2010). Besides these, Hsp70 also generates protective immunity against many diseases as demonstrated in a wide variety of animal models (Johnson and Fleshner, 2006). Baruah et al. (2015) suggested that PHB conferred protection to *Artemia* host against *V. campbellii* by a mechanism of inducing heat shock protein (Hsp) 70. Additionally, further findings also showed that this salutary effect of PHB was associated with the generation of protective innate immune responses, especially the pro-phenoloxidase and transglutaminase immune systems – phenomena possibly mediated by PHB-induced Hsp70. From overall results, it can be concluded that PHB induces Hsp70 production which might contribute in part to the protection of *Artemia* against pathogenic *V. campbellii* (Baruah *et al.*, 2015).

Yik Sung et al. (2007) and Baruah et al. (2013) demonstrated a correction between the amount of induced Hsp70 and the degree of protective immune responses against diseases in animals. Baruah *et al.* (2015) determined that PHB not only regulates the expression of innate immune related genes in *Artemia* but also increases the activity of phenoloxidase in *Artemia* treated with PHB and simultaneously challenged with *V. campbellii.* Further, Suguna et al. (2014) recently reported the immunostimulatory efficacy of poly-β hydroxybutyrate–hydroxyvalerate (PHB–HV) extracted from *Bacillus thuringiensis B.t.*A102 in another aquaculture species, the tilapia *Oreochromis mossambicu*. These results revealed that all the doses of PHB–HV supplementation in feed were effective in stimulating nonspecific immune mechanisms.

As a conclusion from the research mentioned above, it can be stated that SCFAs and β-hydroxybutyrate acid showed good potential as antimicrobial and immunostimulant compounds to fight infections in aquaculture. However, the mode of action of PHB in aquaculture species needs to be further studied e.g. in relation to its capacity to regulate the immune response.

2.7.6. The use of poly-β-hydroxybutyrate in aquaculture

Bacterial disease outbreaks are considered a significant constraint to the development of the aquaculture sector, as well terrestrial animal production. The ban on the use of antibiotics to control diseases in these production sectors has challenged researchers throughout the world to look for alternative biocontrol strategies (Nicolas et al., 2007, Sapkota et al., 2008). As PHB has been shown to effectively exhibit some antimicrobial, insecticidal and antiviral activities (Tokiwa and Ugwu, 2007) it is increasingly being tested as an antimicrobial compound to control bacterial infection in aquaculture research, especially in larval rearing. Recently, a high number of trials with PHB aquatic animals have been done, demonstrating positive effects on survival, growth rate and disease resistance. It also seems to act as an antimicrobial agent and specially enhances "positive" shifts in the microbial community composition in the digestive tract of aquaculture animals.

Defoirdt *et al.* (2007b) started to investigate whether poly-β-hydroxybutyrate (PHB) could be used as an elegant method to deliver SCFAs to the brine shrimp gut. The addition of 1000 mg L^{-1} commercial PHB particles (average diameter 30 µm) to the culture water offered a complete protection (no significant mortality when compared with uninfected nauplii) from the pathogenic *V campbellii* (Defoirdt *et al.*, 2007b). In a follow-up study, it was shown that the addition of 10^7 CFU ml^{-1} of PHB-containing *Brachymonas* bacteria (corresponding to ~10 mg L^{-1} PHB) also completely protected the shrimp from the Vibrios (Halet *et al.*, 2007). Based on these results it was hypothesized that the PHB polymer is (at least partially) degraded to β-hydroxybutyrate in the *Artemia* gut and that the release of this SCFA protects the shrimp from the pathogen (Defoirdt et al., 2007a). Recently, a number of studies have found a positive effect of PHB treatment on the survival of either the larval or juvenile stage of a number of aquaculture species. For example, Nhan et al. (2010) reported the increased

survival and growth in the larvae of the giant fresh water prawn, following PHB enrichment at 5 g L^{-1} in combination with/or highly unsaturated fatty acids (HUFA). Similar beneficial effects have been reported in another study (Ludevese-Pascual personal communication, Ugent) in which survival rates of tiger shrimp *Penaeus monodon* postlarvae increased via PHB incorporated in *Artemia* nauplii. Laranja et al. (2014) reported that PHB is accumulated in *Bacillus spp*, improving the survival, growth and robustness of *Penaeus monodon* (Fabricius, 1798) postlarvae. In other crustacean aquaculture species, enhanced growth, survival and protection from *Vibrio anguillarum* was found in the zoea larvae of the Chinese mitten crab (*Eriocheir sinensis)* (Sui et al., 2012). Beneficial effects of PHB have also been reported in fish culture systems. For example, (Situmorang et al., 2016) reported that PHB also acted as a protecting agent for Nile tilapia larvae, resulting in a 20% increase in larval survival following a gnotobiotic challenge test with the pathogen *Edwardsiella ictaluri* gly09R.

Further investigations are required to verify the effect of PHB in the different animal species, with focus on using PHB supplementations in fish diets. De Schryver et al. (2010) replaced the sea bass *(Dicentrachus labrax*) diet partially with 2%, 5%, and 10% of the dry feed weight with PHB. They documented that PHB could serve as an energy source in sea bass juveniles (as in a diet with 100% PHB, survival was considerably higher than with starved animals) and significantly increased growth rate and decreased FCR when present at 2% and 5% in the diets. In addition, a reduction of the gut pH from 7.7 to 7.2 was observed, suggesting that PHB can be degraded in the intestine, leading to an increased production of short-chain fatty acids. Based on molecular analysis, higher dietary PHB levels induced larger changes in the bacteria community composition and the highest bacteria range-weighted richness in the fish intestine was observed in PHB treatments.

In the later experiment, Najdegerami et al. (2012) confirmed the positive effect of PHB on growth performance of Siberian sturgeon (*Acipenser baerii*) fingerlings and microbial community in its gastrointestinal tract. The results indicated that a 2% replacement in the diet with PHB improved weight gain, specific growth rate (SGR) and survival in the sturgeon fingerlings during the 10-week experimental period. Juveniles fed with 2% PHB enriched diet

exhibited the highest intestinal microbial species richness. The development of bacteria belonging to the genera *Bacillus* and *Ruminococcaceae* was promoted.

Based on current results, the use of PHB looks promising for aquaculture, especially in improving growth and survival in larval culture systems. However, in terms of effectiveness, the use of bacteria cells that contain PHB-A (amorphous PHB particles) can be considered as an effective alternative for PHB-C (crystalline) particles (Halet *et al.*, 2007). It is reported that the extraction of PHB from the cells amount to about 30% of the total production cost of the pure/ crystalline PHB-C (Mudliar et al., 2008). Consequently, the absence for the need of PHB extraction in the application of amorphous PHB will considerably decrease the application costs of PHB. It was estimated that the price of PHB contained within bacterial cells will be about 40% lower than that of pure extracted crystalline PHB (De Schryver *et al.*, 2010). Overall, more research is needed on PHB degradation, metabolism and immune response, in different aquaculture species in order to effectively and efficiently apply PHB as immunostimulants/additives in aquaculture production.

Chapter 3

Application of poly-β-hydroxybutyrate (PHB) in mussel larviculture

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Abstract

In this study, the effect of poly-β-hydroxybutyrate (PHB) delivery on the performance (survival, growth and metamorphosis) of blue mussel (*Mytilus edulis*) larvae was studied for the first time. Upon addition of PHB in either crystalline (i.e. extracted from the bacterial cell) or amorphous (i.e. still contained in the bacterial cell) form to a standard algal diet at concentrations of 0.1 mg L^{-1}, 1.0 mg L^{-1} and 10.0 mg L^{-1}, no significant improvement in growth performance or metamorphosis was observed. However, a concentration of 1.0 mg L^{-1} amorphous PHB almost doubled the survival of the larvae in three independent experiments. A total of 22 PHB-degrading bacterial strains could be isolated from the PHB treated mussel larvae of which 16 could be characterized to belong to the genus *Pseudoalteromonas*. The application of these in combination with PHB however, did not further increase the culture performance of the mussel larvae. Where previous studies suggested that the PHB effect could be related to a change in the intestinal microbial community of the treated animals, molecular fingerprinting analysis of the mussel larvae associated microbiota did not show a relationship between changes in the microbiota composition and the improved survival following PHB treatment. Overall, this study showed for the first time that PHB can be applied in blue mussel larviculture to increase the survival of the cultured animals.

Keywords: Mussel larvae, crystalline PHB, amorphous PHB, PHB-degrading bacteria*, Mytilus edulis*

3. 1. Introduction

Mass mortalities of bivalve larvae caused by bacterial infections are reported regularly (Nicolas et al., 1996, Romalde et al., 2013). For example, mass mortality of early stage oyster larvae has been attributed to *Vibrio tubiashii* infections in 2006 and 2007, resulting in 59% loss in hatchery production (Elston et al., 2008). To eliminate pathogenic bacteria, many bivalve hatcheries use different water treatments such as filtration, pasteurization, ozonation and UV radiation (Verschuere *et al.*, 2000). The use of chemotherapeutic agents is widespread in mollusk hatcheries, although showing inconsistent results (Prado *et al.*, 2010). In addition, many hatcheries use antibiotics to culture the larval phases (Nicolas *et al.*, 1996, Sugumar, 1998, Uriarte *et al.*, 2001, Cabello, 2006). However, due to rising concerns about public health and environmental safety issues, the use of antibiotics are being discouraged worldwide (Schneider, 2003). Attempts have been made to enhance the culture of larval bivalves by controlling the microbiota in the culture water to mitigate the effects of pathogens in bivalve hatcheries. Of these methods, prebiotics and probiotics have gained a lot of interest recently (Gibson *et al.*, 1998, Ruiz-Ponte et al., 1999, Riquelme et al., 2000, Kesarcodi-Watson et al., 2008, Kesarcodi-Watson *et al.*, 2010, Prado *et al.*, 2010). For example, the addition of the probiotic *Alteromonas* sp. (CA2 strain) resulted in an enhanced survival and growth rate of Pacific oyster larvae (Douillet and Langdon, 1993) while Riquelme and colleagues (Riquelme *et al.*, 1997, Riquelme *et al.*, 2000, Riquelme *et al.*, 2001) used three strains of probiotic bacteria - *Vibrio* sp. C33, *Pseudomonas* sp. 11 and *Bacillus* sp. B2 – to enhance the survival of scallop (*Argopecten purpuratus*) larvae. As illustrated by these examples, the search for novel sustainable alternatives for maintaining healthy bivalve larvae has become increasingly important.

The bacterial storage compound poly-β-hydroxybutyrate (PHB) has been applied as a prebiotic and proved to act beneficially for a variety of aquatic animals (De Schryver *et al.*, 2010). PHB is a natural polymer that is accumulated by a wide variety of microorganisms that produce this polymer of the fatty acid β-hydroxybutyrate as an intracellular energy and carbon storage compound (Anderson and Dawes, 1990). Within a bacterial cell, PHB is present in a non-crystalline amorphous state whereas upon lysis of the cell the PHB becomes (partially) crystalline (Jendrossek and Handrick, 2002). The use of PHB for aquaculture

purposes was introduced in 2007 when Defoirdt *et al.* (2007b) and Halet *et al.* (2007) described the effectiveness of the compound to protect *Artemia franciscana* larvae against *Vibrio* infection. It was suggested that this was due to the gastrointestinal production of the monomer β-hydroxybutyrate (β-HB) that had an anti-pathogenic activity. Since then, the application of PHB has been successfully tested on a variety of aquaculture species: PHB significantly increased the growth rate of larvae of giant freshwater prawn (*Macrobrachium rosenbergii*) when supplied through live food (Nhan *et al.*, 2010) and of juvenile European seabass (*Dicentrarchus labrax*) when included in the diet at 2 and 5% (w/w) (De Schryver *et al.*, 2010). Survival and growth rate of Siberian sturgeon (*Acipenser baerii*) fingerlings was improved when included in the diet at 2% (w/w) (Najdegerami *et al.*, 2012). It also protected zoea larvae of the Chinese mitten crab (*Eriocheir sinensis*) against *V. anguillarum* (Sui *et al.*, 2012) and larvae of giant freshwater prawn against *V. harveyi* BB120 infection (Thai et al., 2014).

The ability to produce extracellular PHB depolymerase enzymes is widely distributed among bacteria and fungi (Jendrossek and Handrick, 2002). Liu *et al.* (2010) isolated PHB-degrading bacteria from the gastrointestinal tract of juvenile European sea bass, juvenile Siberian sturgeon and giant freshwater prawn larvae. They evaluated their efficiency to increase the beneficial effect of PHB in a synergetic approach and the results provided perspectives to improve the gastrointestinal health of aquatic animals.

In this study, the impact of PHB on the culture performance of blue mussel larvae was investigated for the first time. PHB was supplied in crystalline and amorphous form at different concentrations to the culture water of blue mussel larvae (*Mytilus edulis*) and the survival, growth and metamorphosis success of the larvae was assessed. PHB-degrading bacteria associated with the larvae were isolated and supplemented, and changes in the intestinal microbial community composition of the larvae were investigated. The table below gives an overview of the experiments. Experiment 1 lasted for 18 days (22 March – 9 April, 2013); experiment 2 lasted for 14 days (8 May – 22 May, 2013) and experiment 3 for 18 day (22 June – 10 July, 2013).

Table 3. 1 Overview of the experiments

Experiment (Exp.)	Treatment	Periods of experiment		Evaluation
		Larvae	spat	
Exp. 1 (n=3) Effect of different PHB form	Control PHB-C 0.1 mg L^{-1} PHB-C 1.0 mg L^{-1} PHB-A 0.1 mg L^{-1} PHB-A 1.0 mg L^{-1}	18 day Sample for MC analysis	15 day	- Larvae performance - Spat performance - Microbial community (MC)
Exp. 2 (n=5) Optimization of PHB-A concentration in larvae culture	Control PHB-A 1.0 mg L^{-1} PHB-A 10.0 mg L^{-1}	14 day Sample for MC analysis	15 day	- Larvae performance - Spat performance - MC
Exp.3 (n=4) Impact of PHB degrader bacteria in larvae culture	Control PHB-A 1.0 mg L^{-1} PHB-A 1.0 mg L^{-1} + PHB degrader PHB degrader	18 day	15 day	- Larvae performance - Spat performance

3. 2. Materials and methods

3. 2.1. Crystalline PHB and amorphous PHB

Two forms of PHB were used in this study. Crystalline PHB particles (PHB-C) were obtained from Goodfellow (Huntingdon, England) and amorphous PHB (PHB-A) consisting of a freeze-dried *Ralstonia eutropha* culture containing 75% cell dry weight of PHB was obtained from VITO (Mol, Belgium). Both of them were ground and sieved through a 30 µm mesh before addition into the culture water to make sure that the particles could be filtered out of the water by the larvae.

3. 2. 2. Detection of ingested PHB by fluorescence microscopy

Before performing the experiments, fluorescence microscopy was used to verify the ability of mussel larvae to ingest PHB. For that purpose crystalline PHB was stained with Nile blue. 500 µl Nile blue A (NBA) was added to 500 mg sonicated PHB in 30 ml of deionized water for

20 minutes. It was consequently centrifuged at 5000 rpm for 10 minutes. The solution was resuspended to wash 3 times with deionized water. Four day post-hatching D-larvae were starved for 24 h to eliminate all algae present in the intestines. Then, the larvae were supplied with 30 µm sieved and Nile Blue A stained PHB (Ostle and Holt, 1982) at a concentration of 1 g L^{-1} in 0.2 µm filtered natural seawater (FNSW). After two hours, the larvae were collected on a 60 µm sieve, rinsed with seawater to remove all PHB particles from the outer surface of the larvae and examined under a fluorescence microscope (Axioskop II, Zeiss, Jena, Germany) operating at 488 nm wavelength and using a total magnification of 630 X. The microscope was equipped with a Peltier-cooled single-chip digital camera (Orca Illm; Hamamatsu, Massay, France) connected to a PC. Larvae that were not supplied PHB served as control.

3. 2. 3. Experimental design

3. 2.3.1. Mussel larvae source and experimental conditions

Broodstock animals were supplied by the commercial mussel producer Roem van Yerseke in The Netherlands. Upon arrival in the laboratory, they were rinsed with FNSW. Spawning was induced by thermal shocks, by emerging the animals alternately in water baths of 15°C and 25 °C every 15 minutes. Individual matured males and females were identified at the time of gamete release and isolated into individual beakers. Eggs and sperm of individual females and males, respectively, were pooled before fertilization was carried out. Fertilized eggs were regularly stirred with a plunger during 30 min before being rinsed with FNSW and put in a 20 L incubator rectangular tank. Two days after fertilization, hatched D-larvae were concentrated on a sieve (60 µm), rinsed with FNSW and counted before stocking in 8L Züger bottles (Urbányi et al., 2008). The larvae from all treatments were fed every 2 days with an algae diet consisting of *Isochrysis galbana* and *Chaetoceros calcitrans* (1:1, based on cell numbers) for the first 4 days. From day 4 onwards *Tetraselmis suecica* was added as well (1:1:1, based on cell numbers). The total concentrations gradually increased from 3 x 10^4 CFU mL^{-1} at the start of the experiment to 1 x 10^5 CFU mL^{-1} at the end of the experiment in steps of 1 x 10^4 CFU mL^{-1} per 2 days.

Water was changed completely every two days. At that occasion, feed was administered and samples were taken for larval survival and height measurements. During the experiments, gentle bottom aeration was provided to supply oxygen and enhance larvae suspension. The water temperature of the larvae culture was on average 17 ± 0.5 °C during experiment 1 and 3 and 19 ± 0.5 °C during experiment 2.

Three different experiments were performed:

- In experiment 1, the effects of PHB-C and PHB-A were compared. The larvae were stocked in the Züger bottles at a density of 5 larvae mL^{-1}. The following 5 treatments were conducted in triplicate for 18 days: (1) Control, consisting of the algae diet only, (2) PHB-C 0.1, consisting of adding crystalline PHB at 0.1 mg L^{-1} to the algae diet, (3) PHB-C 1.0, consisting of adding crystalline PHB at 1.0 mg L^{-1} to the algae diet, (4) PHB-A 0.1, consisting of adding amorphous PHB at 0.1 mg L^{-1} to the algae diet, and (5) PHB-A 1.0, consisting of adding amorphous PHB at 1.0 mg L^{-1} to the algae diet. The parameters as described under 3.2.3.2 were assessed on Day 14 and on Day 18 post-hatching. At the end of experiment 1, PHB-degrading bacteria were isolated from the mussel larvae (see 3.2.4).

- In experiment 2, the optimal concentration of PHB-A for the mussel larvae was determined as PHB-A showed better effects than PHB-C in experiment 1. The larvae were stocked at a density of 10 larvae mL^{-1} in the Züger bottles and the following 3 treatments were conducted in quintuplicate for 14 days: (1) Control, consisting of the algae diet only, (2) PHB-A 1.0, consisting of adding amorphous PHB at 1.0 mg L^{-1} to the algae diet and (3) PHB-A 10 mg L^{-1}, consisting of adding amorphous PHB at 10 mg L^{-1} to the algae diet. The parameters as described under 3. 2.3.2 were assessed on Day 10 and on Day 14 post-hatching.

- In experiment 3, the optimal dose of PHB-A from experiment 2 was combined with the strongest bacterial PHB degrader (isolate ARC4B5 obtained from experiment 1, see 3.2.4) to examine whether the effects of amorphous PHB could be further enhanced. Larvae were stocked at a density of 10 larvae mL^{-1} in the Züger bottles and the following 4 treatments were conducted in quadruplicate for 18 days: (1) Control, consisting of the algae diet only, (2) PHB-A 1.0, consisting of adding amorphous PHB at 1.0 mg L^{-1} to the algae diet, (3) PHB-A 1.0 + PHB degrader, consisting of adding amorphous PHB at 1.0 mg L^{-1} and 10^6 colony forming units (CFU) mL^{-1} of ARC4B5 to the algae diet and (4) PHB-degrader, consisting of

adding 10^6 CFU mL^{-1} of ARC4B5 to the algae diet. The parameters as described under 3.2.3.2 were assessed on Day 14 and on Day 18 post-hatching.

3. 2.3.2. Larval survival, height measurement, and settlement

The survival and growth of the larvae in experiments were determined as follows. For sampling, all larvae were collected on a 60 to 90 µm sieve depending on the average larval size, and concentrated in 1.5 L of FNSW. A plunger was used to distribute larvae homogenously in the beaker before taking a subsample of 2 mL in four replicates. Dead and living larvae were distinguished under a light microscope (Euromex, Holland) using Lugol's solution (Sullivan and Gifford, 2009). Deterioration of organ structure and empty shells denoted moribund or dead individuals. The growth was determined by measuring the shell height of fifty larvae that were randomly sampled from a 2 mL subsample. The shell height, being the largest distance from the ventral side to the straight-hinge, was measured using the automated image analysis software Clemex Vision PE version 3.6.002 based on images obtained with a light microscope at a magnification of 400x, equipped with a MicroPublisher 3.3 camera (QImaging, China) directly connected to the computer with the software.

At the end of each experiment, all larvae from each Züger bottle were harvested on a 150 µm sieve. After re-suspension, 1000 larvae from each replicate were stocked in a downweller, consisting of a PVC cylinder (Ø 12cm, H 14cm) provided with a bottom sieve of 150 µm. The downwellers were placed in a rectangular tank (water bath) containing 60 L of FNSW of 17 ± 0.5 °C, making sure that each tank contained 1 replicate of each treatment. The larvae were fed a combination of the three microalgae species that were administered during the larval stage at a concentration of 1 x 10^5 CFU mL^{-1} without any addition of PHB particle or amorphous PHB. The water was changed completely every 2 days and after 15 days, settlers (young spat) were counted and the shell height of fifty settlers from each replicate was measured.

3. 2.4. Isolation, culture and identification of PHB-degrading strains

At the end of experiment 1, larvae from each PHB treatment were collected to isolate PHB-degrading bacteria from the intestinal tract. About ten thousand larvae from each replicate were collected on a sterile 100 µm sieve and rinsed twice with 0.2 µm filtered autoclaved

artificial sea water (FAASW) (35 g L^{-1} Instant Ocean®, Aquarium Systems Inc., Sarrebourg, France). The larvae were transferred to a sterile plastic bag containing 2 ml of FAASW and homogenized with a stomacher blender (400SN, Seward Medical, London, UK) for 5 min. A 70 µL aliquot of the homogenate was added either to 10 mL marine broth (MB - Difco™ Marine Broth 2216) to boost bacterial growth prior to PHB degrader selection or to 10 mL PHB minimal medium (FAASW containing 0.2 g L^{-1} NH_4Cl and 1.0 g L^{-1} crystalline PHB particles, average diameter 30 µm) to directly select for PHB degraders. The cultures were incubated at 28°C on an orbital shaker (130 rpm) for 1 and 3 days, respectively. Both cultures were sub-cultured twice by transferring 1 ml of culture medium into 100 mL fresh PHB minimal medium (which was incubated at 28°C on an orbital shaker (130 rpm) for 3 days) before being plated on marine agar where single colonies could be picked. Morphologically different colonies were incubated in PHB minimal medium for 72h and subsequently purified by streak plating on marine agar resulting in 22 isolates. Extracellular PHB depolymerase production was assessed qualitatively by spotting the isolates on PHB agar (being PHB minimal medium supplemented with 15 g L^{-1} agar) or PHB agar supplemented with 10% LB medium as the carbon sources (Defoirdt *et al.*, 2007b, Liu *et al.*, 2010). The plates were incubated at 28°C and examined daily for the presence of clearing zones around the colonies. PHB-degrading bacteria were stored in 20% glycerol at -80 °C until further use.

A 1465 base pair fragment of the 16S rRNA gene of the 22 isolated PHB-degrading strains was amplified using the universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') (Jiang et al., 2006). PCR was performed using a GeneAmps PCR system 2700 thermal cycler using the following program: 95°C for 5 minutes, 30 cycles of 95°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes and finally an extension period of 72 °C for 10 minutes. The resulting PCR product was purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, The Netherlands) and sequenced at the Center for Medical Genetics (Ghent University Hospital, Belgium) using the PCR primers. The nucleotide sequences were analyzed using the VECTOR NTI ADVANCE program, version 11.5 and homology searches were completed with the BLAST server of the National Centre for Biotechnology Information using the BLAST algorithm (Altschul et al., 1997) for comparison of the nucleotide query sequence against a nucleotide sequence database (BLASTN).

3. 2.5. Molecular fingerprinting of larval associated bacterial community

3. 2.5.1. DNA extraction and PCR amplification

From each replicate, about five thousand mussel larvae were collected on a 100 µm sieve at the end of the experiments 1 and 2 and rinsed three times with FAASW. The larvae were transferred to a sterile 2 ml tube and stored at -20°C until further processing. The extraction of DNA from the samples was carried out with a DNeasy Blood and Tissue Kit (Qiagen, The Netherlands) according to the manufacturer's instruction. PCR amplification targeting the V3 region of the 16S rRNA gene was conducted as described by Boon et al. (2002) using bacterial primer 338f (5'-ACTCCTACGGGAGGCAGCAG-3') with a 40-base GC clamp attached to its 5' end and universal primer 518r (5'-ATTACCGCGGCTGCTGG-3').

3. 2.5.2. Denaturing gradient gel electrophoresis (DGGE)

A Bio-Rad D Gene system (Bio-Rad, Hercules, CA, USA) was used to perform DGGE analysis (Boon *et al.*, 2002). The denaturing gradient of the gel ranged between 45% and 60%. Electrophoresis was performed with a constant voltage of 38 V at 60°C for 16 h. Bands were visualized using a UV transilluminator after staining the gel for 20 minutes in a 1% gel-red solution.

3. 2.5.3. Processing DGGE images and statistical analysis

The DGGE patterns were processed with BIONUMERICS software version 5.0 (Applied Maths, Sint-Martens-Latem, Belgium) and analyzed as described by Bakke et al. (2013). A 1% band position tolerance limit was maintained for considering bands as being identical. As DGGE analysis of the DNA of the algae used as feed also resulted in bands, the peak areas in each sample pattern located at the position of the algal bands were deleted prior to analysis of the gels.

3. 2.6. Statistics

The survival data of larvae and spat were arcsine transformed to satisfy the homogeneity of variance requirement. The survival, height and the settlement success of the mussel larvae in the different treatments were compared by one-way ANOVA analysis using SPSS (version

16.0). A Duncan post-hoc test was used for identification of significant differences between the means ($p \leq 0.05$). For analysis of the DGGE patterns from experiment 1 and 2, a matrix of Bray – Curtis similarities (Bray and Curtis, 1957) between the samples from each experiment (all loaded on a single DGGE gel) was constructed using the software Primer6 (Bakke *et al.*, 2013). A non-metric multidimensional scaling (NMS) plot was constructed using the software SPSS (version 20.0). To identify significant differences between the microbial communities in the different treatment, an ANOSIM analysis of the Bray-Curtis similarity values was performed (Clarke, 1993) using Primer6. The ranked similarity R was determined by performing 999 permutations.

3. 3. Results

3. 3.1. PHB ingestion by mussel larvae

Epifluorescence images of larvae showed that larvae fed with Nile Blue A stained PHB-particles were brightly fluorescent in the stomach area (Figure 1). Fluorescence was already visible 1 hour after supplementation of PHB but maximum intensity was reached after 2 hours. Fluorescence of the stained PHB lasted for 5 hours.

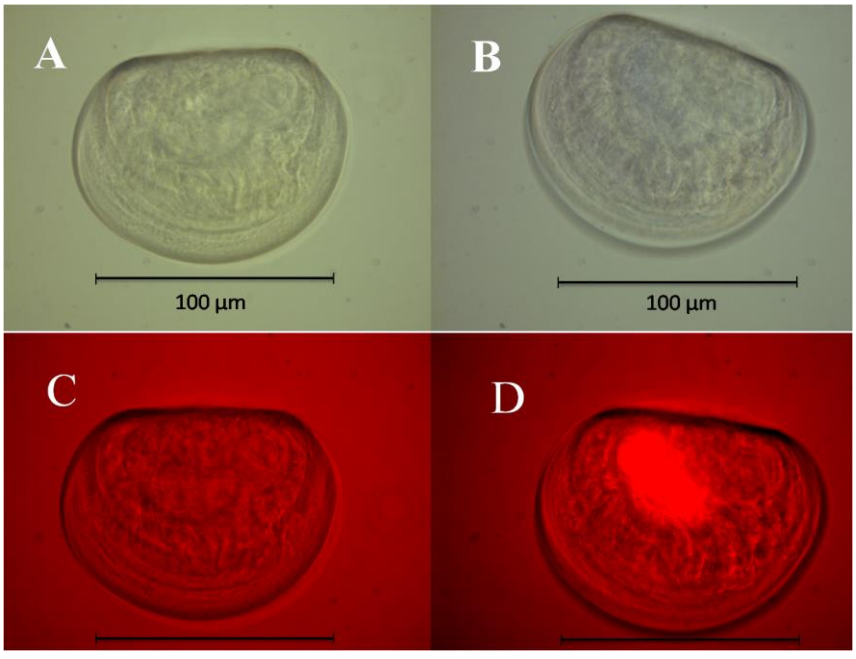

Figure 3. 1: Light (A and B) and epifluorescence microscopy (C and D) images of starved larvae (A and C) and larvae fed with Nile Blue A stained PHB for 2 hours (B and D)

3. 3.2. Larval survival and growth

In experiment 1, the larvae fed the PHB-A 1.0 mg L^{-1} diet survived significantly better than the larvae from the control treatment at day 14 and day 18 (Table 3. 2). The larvae from the PHB-C 0.1, PHB-C 1.0 and the PHB-A 0.1 treatment tended to survive better than the larvae from the control treatment, but the differences were not significant anymore on day 18. At the onset of the experiment, the larvae had an average shell height of 70 µm. At 14 days and 18 days post hatching, the larvae supplied with PHB in either crystalline or amorphous form were not bigger than the control larvae.

There were no significant positive effects of PHB on the settlement success of the mussel spat (Table 3. 3). There was also no significant difference in spat size between the treatments, although the spat of the control treatment had a tendency to be larger than the spat from the PHB treatments.

Table 3. 2: Survival (%) and shell height (µm) of mussel larvae subjected to different PHB treatments in experiment 1 and 3 (Day 14 post-hatching and Day 18 post-hatching) and in experiment 2 (Day 10 post-hatching and Day 14 post-hatching)

Treatment	Survival	Shell height	Survival	Shell height
Experiment 1 (n = 3)	Day 14 post-hatching		Day 18 post-hatching	
Control	33.9 ± 6.1^{a}	157.5 ± 3.1^{a}	25.9 ± 8.9 ^{a}	185.5 ± 1.9^{a}
PHB-C 0.1 mg L^{-1}	56.2 ± 5.6^{b}	146.2 ± 0.8^{a}	35.4 ± 6.4^{ab}	187.3 ± 0.2^{a}
PHB-C 1.0 mg L^{-1}	50.4 ± 4.1^{ab}	148.2 ± 7.0^{a}	32.3 ± 2.2^{ab}	183.5 ± 8.4^{a}
PHB-A 0.1 mg L^{-1}	56.0 ± 4.0 ^{b}	145.1 ± 2.8^{a}	39.7 ± 7.1^{ab}	197.4 ± 1.3^{a}
PHB-A 1.0 mg L^{-1}	58.3 ± 6.9^{b}	142.6 ± 2.4^{a}	50.2 ± 6.2^{b}	180.8 ± 4.5^{a}
Experiment 2 (n = 5)	Day 10 post-hatching		Day 14 post-hatching	
Control	42.9 ± 3.0^{a}	160.5± 2.3^{b}	26.5 ± 1.9^{a}	177.1 ± 1.0^{b}
PHB-A 1.0 mg L^{-1}	53.8 ±1.2^{b}	159.4 ± 2.0^{b}	40.3 ± 2.4^{b}	177.9 ± 3.4^{b}
PHB-A 10.0 mg L^{-1}	39.4 ± 2.5^{a}	99.3 ± 0.9^{a}	23.0 ± 0.8^{a}	113.9 ± 5.7^{a}
Experiment 3 (n = 4)	Day 14 post-hatching		Day 18 post-hatching	
Control	49.0 ± 2.5^{a}	138.7 ± 1.0^{a}	33.5 ± 3.0^{a}	189.8 ± 4.4^{a}
PHB-A 1.0 mg L^{-1}	56.5 ± 5.2^{ab}	159.8 ± 4.9^{b}	47.7 ± 4.0^{b}	186.8 ± 3.5^{a}
PHB-A 1.0 mg L^{-1} + PHB degrader	59.3 ± 4.5^{ab}	122.2 ± 3.0^{c}	31.6 ± 6.0^{a}	149.1 ± 0.5^{b}
PHB degrader	68.8 ± 4.8^{b}	122.8 ± 3.0^{c}	32.9 ±3 5.5^{a}	141.5 ± 3.0^{b}

Values represent means ± standard error. Data in the same column and within an experiment that are labelled with a different superscript letter are significantly different ($p \leq 0.05$)

Table 3. 3: Settlement success (%) and shell height (µm) of mussel spat subjected to different PHB treatments in experiment 1, 2 and 3

Treatment	Settlement success	Shell height (µm)
Experiment 1 (n = 5)		
Control	41.4 ± 10.8^{a}	388.2 ± 5.3^{a}
PHB-C 0.1 mg L^{-1}	43.3 ± 2.7^{a}	347.2 ± 18.1^{a}
PHB-C 1.0 mg L^{-1}	27.7 ± 4.2^{a}	352.9 ± 15.7^{a}
PHB-A 0.1 mg L^{-1}	43.0 ± 9.6^{a}	365.5 ± 3.4^{a}
PHB-A 1.0 mg L^{-1}	51.2 ± 5.1^{a}	352.1 ± 13.8^{a}
Experiment 2 (n=5)		
Control	35.5 ± 2.1^{a}	433.8 ± 11.3^{a}
PHB-A 1.0 mg L^{-1}	30.5 ± 4.9^{a}	428.9 ± 9.0^{a}
PHB-A 10.0 mg L^{-1}	24.1 ± 3.4^{a}	418.9 ± 14.6^{a}
Experiment 3 (n = 4)		
Control	20.4 ± 7.3^{ab}	375.6 ± 13.2^{a}
PHB-A 1.0 mg L^{-1}	34.3 ± 4.0^{b}	333.6 ± 5.2^{ab}
PHB-A 1.0 mg L^{-1} + PHB degrader	15.9 ± 3.9^{a}	314.0 ± 4.5^{bc}
PHB degrader	13.4 ± 1.4^{a}	279.3 ± 16.9^{c}

Values represent means ± standard error. Data in the same column and within an experiment that are labelled with a different superscript letter are significantly different ($p \leq 0.05$)

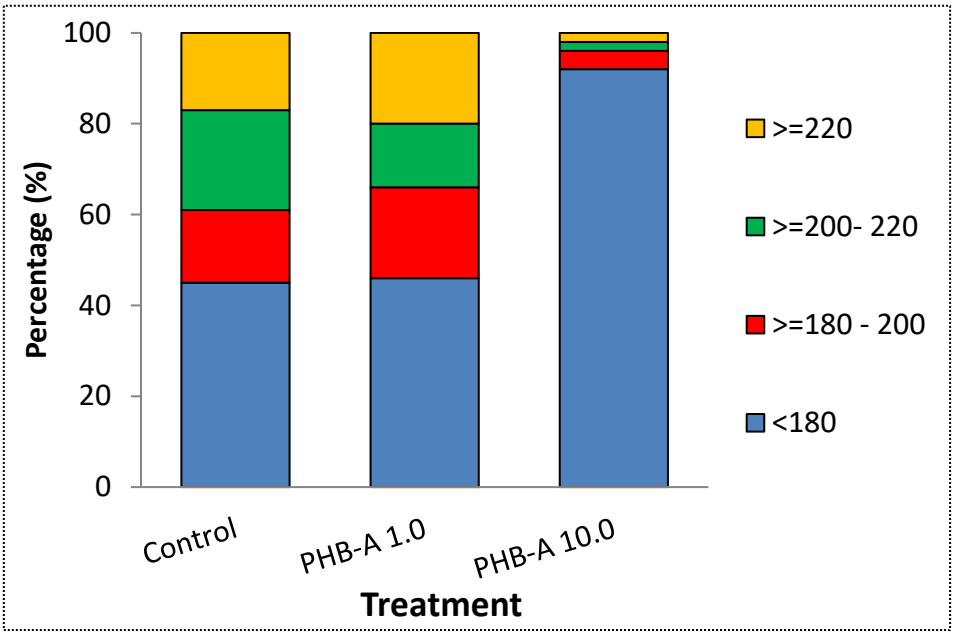

Figure 3. 2: Size class distribution of mussel larvae on day 14, subjected to different feeding regimes in experiment 2 (n =5)

Similar to experiment 1, the PHB-A 1.0 mg L^{-1} treatment in experiment 2 showed a significantly higher survival as compared to the control treatment while the growth was not significantly different (Table 3.2). The addition of 10 mg L^{-1} PHB-A to the rearing water induced no difference in survival of the mussel larvae as compared to the control, but significantly suppressed the growth. A total of 92% of the larvae in this treatment were smaller than 180 µm while this was only 45% and 46% in the control treatment and the PHB-A 1.0 mg L^{-1} treatment, respectively (Figure 3. 2). There were no significant effects of PHB-A on the settlement success and the growth of the mussel spat (Table 3. 3).

The addition of the PHB-degrading isolate ARC4B5 in experiment 3 did not enhance the positive effect of PHA-A 1.0 mg L^{-1} on the larval survival (Table 3. 2). On the contrary, the presence of the PHB-degrader significantly decreased the growth of the larvae and the settlement success. The resulting spat were also smaller (Table 3. 3).

Based on the results of Table 3.2 and Table 3.3, the total yield of mussel spat under difference experimental conditions was calculated Table 3.4

Table 3. 4. The final yield of mussel spat under different experimental conditions

Treatment	Yield (from egg to spat, %)
Experiment 1 (n = 5)	
Control	10.7
PHB-C 0.1 mg L^{-1}	15.3
PHB-C 1.0 mg L^{-1}	8.9
PHB-A 0.1 mg L^{-1}	17.0
PHB-A 1.0 mg L^{-1}	25.7
Experiment 2 (n=5)	
Control	9.4
PHB-A 1.0 mg L^{-1}	12.3
PHB-A 10.0 mg L^{-1}	5.5
Experiment 3 (n = 4)	
Control	6.8
PHB-A 1.0 mg L^{-1}	16.3
PHB-A 1.0 mg L^{-1} + PHB degrader	5.0
PHB degrader	4.4

3. 3.3. Isolation and identification of PHB-degrading bacteria

A total of 16 different isolates were obtained from the mussel larvae fed with either crystalline PHB or amorphous PHB. A homology search revealed that all isolates belonged to the genus *Pseudoalteromonas*. Isolate ARC4B5 was most closely related to the marine bacterium *Pseudoalteromonas* species Strain X153 (GenBank accession number AJ581533) and showed the highest PHB-degrading capacity according to a PHB depolymerase agar assay (data not shown). Therefore it was selected for use in experiment 3.

The nucleotide sequences of the isolates were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank) with the accession numbers KJ775741, KJ775742, KJ775743, KJ775744, KJ775745, KJ775746, KJ775747, KJ775748, KJ775749, KJ775750, KJ775751, KJ775752, KJ775753, KJ775754, KJ775755, KJ775756.

3. 3.4. Microbial community analysis

In experiment 1, NMS ordination based on Bray-Curtis similarities revealed that the microbiota associated with the mussel larvae seemed to group together according to treatment (amorphous or crystalline) (Figure 3. 3). The analysis of similarities indeed showed a significant global R value of 0.751 (p-value of 0.001) suggesting that similarity between samples within a treatment is higher than the similarity between samples of different treatments. Upon pairwise comparisons of treatments, however, no significant differences could be determined due to the low number of replicates per treatment.

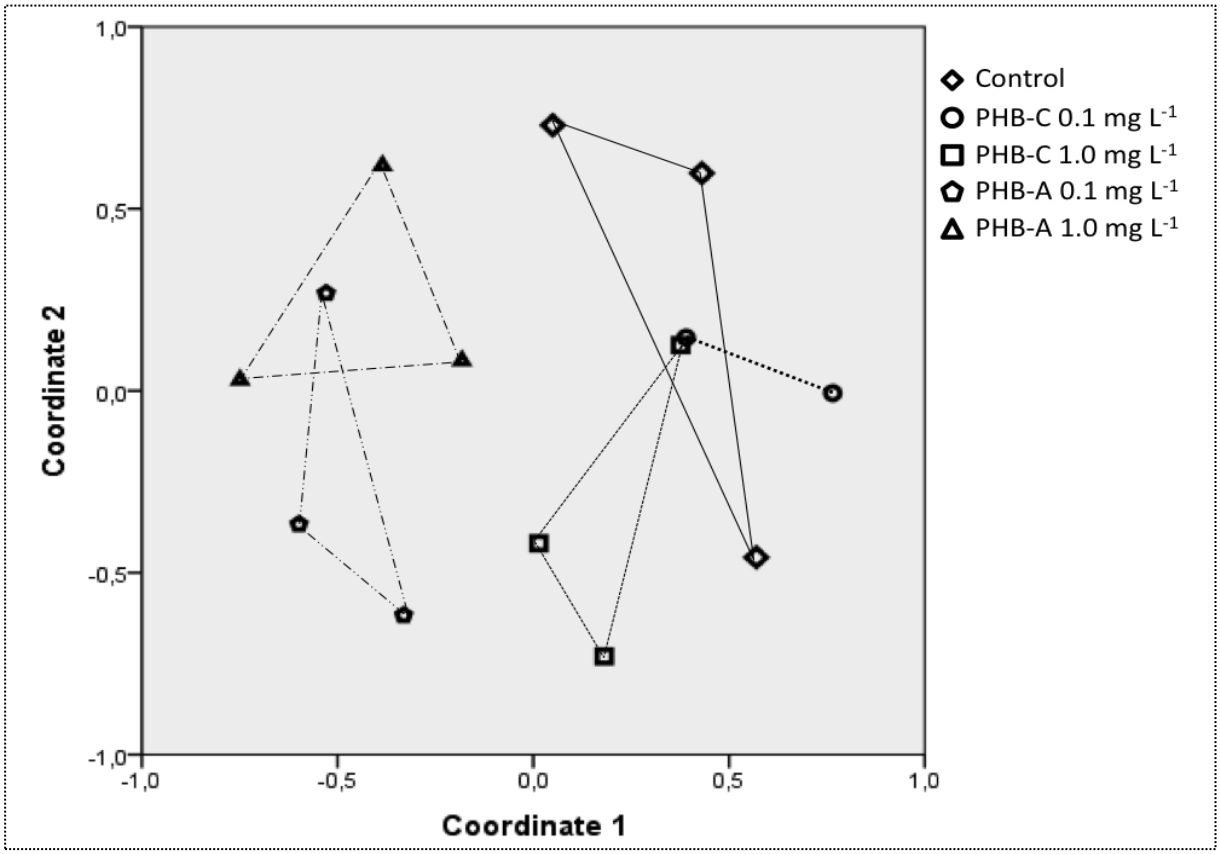

Figure 3. 3: NMS ordination based on Bray Curtis similarities for the bacterial communities associated with the larvae subjected to different feeding regimes in experiment 1. One replicate sample from treatment PHB-C 0.1 mg L-1 was left out of the analysis because it showed an abnormal low number of bands on the DGGE pattern

The NMS plot from experiment 2 suggests a considerable overlap in the composition of the microbial communities associated with the larvae from the different treatments (Figure 3. 4). The non-significant differences between the microbial communities were confirmed by the global R-value of only 0.027 and the p-value of 0.346.

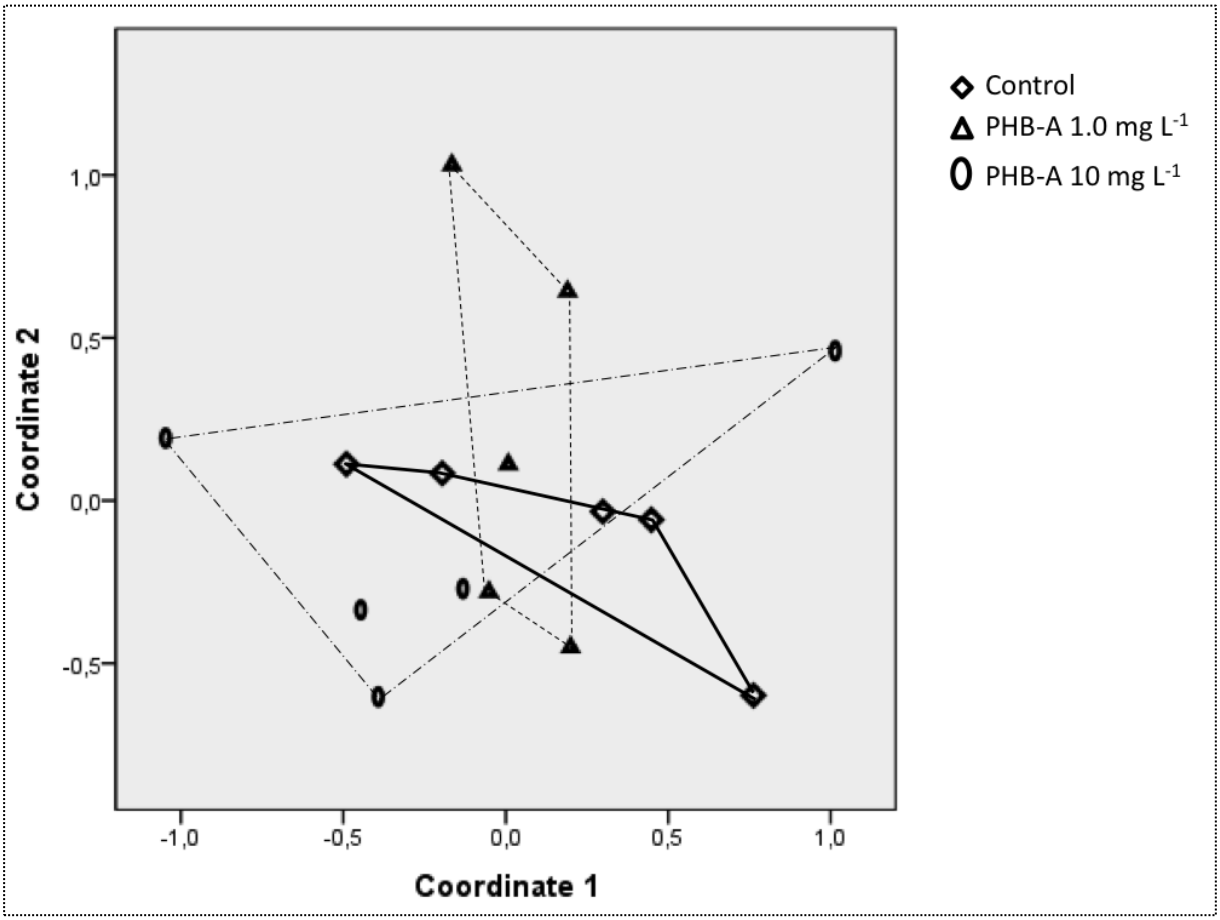

Figure 3. 4: NMS ordination based on Bray Curtis similarities for the bacterial communities associated with the larvae subjected to different PHB concentrations in experiment 2.

3. 4. Discussion

Many studies focus on the development of sustainable strategies to improve the culture performance and disease resistance of aquatically cultured animals. In this study, it was shown for the first time that the supplementation of PHB can improve the survival of bivalve larvae.

Even though mussel larvae are known filter feeders, a prerequisite for the experiments was to determine whether PHB particles are taken up from the rearing water when offered to the larvae. Defoirdt *et al.* (2007b) described the use of fluorescent labeling to visualize the

ingestion of PHB by *Artemia franciscana* larvae and it proved to be effective to demonstrate the ingestion of PHB by mussel larvae within a time frame of two hours. The supplementation of crystalline PHB to the culture water of the mussel larvae did not significantly improve the survival, except for a temporary effect at the lowest concentration until 14 days post hatching. This observation does not correspond with the results of previous studies. A beneficial effect of PHB on survival was reported for *Artemia franciscana* larvae (Defoirdt *et al.*, 2007b), giant fresh water prawn larvae (Nhan *et al.*, 2010, Thai *et al.*, 2014), and Chinese mitten crab larvae (Sui *et al.*, 2012). The application method of PHB in these studies (with the exception of the study on *Artemia franciscana*), however, was different as PHB was administered either by addition to formulated feeds or by the enrichment of live feed. This may partly explain the differences in observed effects. It is also possible that the absence of improved survival is due to the mussel larvae not being able to metabolize the crystalline PHB, despite their proven ability to ingest it, as D-stage mussel larvae have an immature digestive system (Seafood-Industry-training-Organisation, 2006) that may prevent them from efficiently metabolizing the crystalline PHB. In contrast, the supplementation of amorphous PHB at a concentration of 1.0 mg L^{-1} did have a positive effect on the larval survival. This may be due to the amorphous PHB being more biodegradable than the crystalline PHB. Halet *et al.* (2007) made similar observations in case of *Artemia franciscana* and ascribed this to the smaller size of the amorphous PHB and the lower degradability of PHB in a crystalline structure. The crystalline particles in this study have an average diameter of 30 µm, whereas the PHB containing bacteria are on average 1 µm in size. It cannot be overlooked, however, that the PHB is delivered to the mussel larvae encapsulated within microbial biomass. This biomass may also have unintentionally influenced the survival (for example by uncharacterized immunological stimulation). This aspect needs to be further investigated.

The supplementation of PHB to the culture water of the mussel larvae did not result in a faster growth nor in an improvement of the settlement success, not even for the PHB-A 1.0 mg.L^{-1} treatment that induced an increase in survival. De Schryver *et al.* (2010) in contrast found that supplementing the diets of European sea bass with 2% or 5% crystalline PHB led to a significant increase in fish weight gain as compared to the normal feed treatment (0% PHB). Nhan *et al.* (2010) and Thai *et al.* (2014) demonstrated that the development of giant

freshwater prawn larvae fed with *Artemia* nauplii enriched with PHB in crystalline and amorphous form, respectively, was significantly faster. Similarly, Sui *et al.* (2012) showed a growth promoting effect of crystalline PHB on Chinese mitten crab larvae. As it was previously suggested that PHB and/or its degradation products may be used as a fuel for development and growth (Thai *et al.*, 2014), this does not seem to be the case for mussel larvae. Maybe the monomer of PHB, 3-hydroxybutyrate, is not used as a ketone body by bivalves whereas this is the case for vertebrates and shrimp (Weltzien et al., 1999).

At this stage the exact mechanism by which the PHB polymer is broken down inside the intestinal tract of animals is not known, i.e. whether it is mainly driven by physicochemical processes or by biological activity of the host and/or microorganisms present in the gut (Nhan *et al.*, 2010). Defoirdt *et al.* (2007b) and (Liu *et al.*, 2010) suggest that the metabolism of PHB in brine shrimp larvae occurs at least partially by enzymatic activity of the host while microbial activity may also play a role. This was based on the observation that the disease resistance against the pathogen *V. campbellii* LMG21363 increased by the addition of PHB-degrading bacteria, compared to the PHB treatment alone. To maximize the chance that PHB-degrading isolates can persist and function in the specific ecological niche of the host gastrointestinal tract, it is advisable to isolate them from this specific environment (Reid et al., 2003). In the current study, all 16 (out of 22) PHB-degrading isolates seemed to belong to the genus *Pseudoalteromonas*; isolate ARC4B5 showed the strongest PHB-degrading activity and was thus selected for experiment 3. Its application, however, did not result in an increase in survival and even seemed to lower the growth performance of the larvae. These observations partly are in correspondence with the findings of Arlette et al. (2004), who found a *Pseudoalteromonas* isolate that improved the survival of infected scallop (*Pecten maximus*) larvae but also decreased their growth. Sandaa et al. (2008) describe a strain of *Pseudoalteromonas* spp. as an opportunistic pathogen of *P. maximus* larvae. In our study, no significant difference in survival between the control treatment and the *Pseudoalteromonas* sp. treatment was observed.

It was earlier suggested that the beneficial effects associated with PHB may result from a change in the gastrointestinal microbial community composition (De Schryver et al., 2011). The differences in survival rates of mussel larvae in the different treatments could, however, not be explained by differences in their respective microbial communities based on the

molecular analyses of the larvae associated microbial communities that were performed in this study.

In conclusion, our results revealed that amorphous PHB, encapsulated in *Ralstonia* microbial biomass, at a concentration of 1 mg L^{-1} had a positive effect on the survival of blue mussel larvae. The total yield from egg to settled spat of 2 weeks old was 1.3 (experiment 2) to 2.4 (experiment 1 and 3) times higher than in the control treatment. Further research needs to be performed to establish why amorphous PHB beneficially influences larval survival and not larval growth. In addition, it is advised to determine if PHB may be used as an antimicrobial compound to control pathogenic disease in blue mussel larvae like it does for other aquatic species.

Acknowledgements

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Chapter 4

Does *Ralstonia eutropha*, rich in poly-β hydroxybutyrate (PHB), protect blue mussel larvae against pathogenic *vibrios*?

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Abstract

Disease outbreaks during larval rearing of commercial bivalve species are an important impediment to the development of the mollusk aquaculture sector. Current research focuses on finding alternatives to antibiotics and in this study the use of the natural polymer poly-β-hydroxybutyrate (PHB) as a biological agent to control bacterial pathogens of blue mussel *(Mytilus edulis)* larvae was investigated.

Blue mussel larvae were supplied with amorphous PHB (PHB-A: lyophilized *Ralstonia eutropha* containing 75% PHB) at a concentration of 1 mg L^{-1} or 10 mg L^{-1} for 6 or 24 hours, after which they were exposed to either the rifampicin resistant pathogen *Vibrio splendidus* or *Vibrio coralliilyticus* at a concentration of 10^5 CFU mL^{-1} in well-plates containing 2 ml of filtered-sterilized seawater enriched with 0.1% (v/v) of Luria-Bertani medium (salinity 35 g L^{-1}) and 10 mg L^{-1} rifampicin. Unchallenged larvae (with and without PHB-A) were used as negative controls. LB_{35} (0.1%) medium was added to the water culture to stimulate the growth of (only) the rifampicin-resistant pathogens. The survival of the larvae that were PHB-A pretreated (1 mg L^{-1}) for 6 h before the challenge was higher than when the pre-treatment started 24 h before challenge. After 96h of pathogen exposure, the survival of PHB-A treated mussel larvae was 1.41 and 1.76 fold higher than the survival of larvae not treated with PHB-A when challenged with *V. splendidus* and *V. coralliilyticus* respectively. An increase in PHB-A concentration from 1 to 10 mg L^{-1} significantly increased larval survival

after 72 h and 48 h of a challenge with *V. splendidus* in the groups pretreated with PHB-A for 6 h and 24 h respectively. The increased PHB-A concentration, however, did not lead to a significant difference in the protection of mussel larvae against *V. coralliilyticus* A possible mode of action includes the depolymerization of PHB by enzymatic or microbial activity into the water-soluble short-chain fatty acid monomer, β-hydroxybutyrate (β-HB) that acts as a microbial control agent. Therefore, growth inhibition of four concentrations of β-HB (1, 5, 25 and 125 mM) on the two pathogens was tested *in vitro* in LB_{35} medium, buffered at two different pH values (pH7 and pH8). The highest concentration of 125 mM significantly inhibited the pathogen growth in comparison to the lower levels. The effect of β-HB on the virulence of the tested pathogenic *Vibrios* revealed a variable pattern of responses. The highest β-HB concentration inhibited some virulence factors such as the production of the lytic enzymes hemolysis, phospholipase but also stimulated the production of caseinase in *V. splendidus* at pH7 and in *V. coralliilyticus* at pH8. β-HB had no effect on the production of gelatinase and lipase or biofilm formation.

4.1. Introduction

The production of many bivalve mollusk species depends on hatchery production of seed, yet the intensive production of bivalve larvae is characterized by mass mortalities caused by bacterial pathogens (Hada et al., 1984, Prado *et al.*, 2010) In many cases, bacteria of the genus *Vibrio* have been found to be the principle causative agents, with mortality often exceeding 90% (Takahashi et al., 2000). Therefore, *Vibriosis* is considered a significant disease threat for hatchery-reared larvae. To prevent infection by pathogenic *Vibrio* strains many hatcheries adopt the use of antibiotics during larval culture (Nicolas *et al.*, 1996, Uriarte *et al.*, 2001) although this is known to lead to the development of drug resistance in pathogenic strains (Nicolas *et al.*, 1996, Cabello, 2006, Fernandes Cardoso de Oliveira et al., 2010). For this reason, the focus has shifted towards applying alternative biocontrol techniques in bivalve rearing systems to prevent disease outbreaks. Probiotics, for example, are considered a possible solution for controlling bacterial infections in bivalve larvae (Douillet and Langdon, 1993, Kesarcodi-Watson et al., 2012). Alternatively, Defoirdt *et al.* (2009) suggested that the polymer of the short-chain fatty acid (SCFA) β-hydroxybutyrate (ß-HB), the well-known bacterial storage compound poly-β-hydroxybutyrate (PHB), could be used to protect aquatic animals from pathogenic bacteria. Polyhydroxybutyric acid is an important member of the family of polyhydroxyalkanoates (PHAs), and it can be degraded by bacteria into the monomer β-HB (Kato et al., 1992, Patnaik, 2005). β-hydroxybutyrate acid is known to have some antimicrobial, insecticidal and antiviral activities (Tokiwa and Ugwu, 2007). In addition, β-hydroxybutyrate is a known ketone body (Jendrossek and Handrick, 2002) that serves an important role in the energy metabolism of a large number of animals (Nehlig, 2004). PHB can be supplied in either the crystalline (i.e. extracted from the bacterial cell) or amorphous form (i.e. still contained within the bacterial cell) which after ingestion, is thought to be depolymerized.Positive effects of PHB on growth and survival were shown for a number of aquaculture species such as juvenile European sea bass (De Schryver *et al.*, 2010), juvenile Siberian sturgeon (Najdegerami *et al.*, 2012), larval giant freshwater prawn (Nhan *et al.*, 2010, Thai *et al.*, 2014), larval giant tiger prawn (Laranja *et al.*, 2014), larval Nile tilapia (Situmorang *et al.*, 2016) and larval Chinese mitten crab (Sui *et al.*, 2012). .

Most recently, Hung et al. (2015) observed a 50 % increase in blue mussel larvae survival when amorphous PHB (PHB-A) was supplied to the culture water at a concentration of 1 mg L^{-1}. However, no effect on larval growth or survival after metamorphosis was observed. It was suggested that the increase in larval survival may have resulted from the antimicrobial activity of the compound against opportunistic bacteria that were naturally present in the water although that assumption was not verified.

This study investigated the effect of amorphous PHB in blue mussel larvae cultures *in vivo* when challenged with the pathogenic bacteria *Vibrio splendidus* and *Vibrio coralliilyticus*. Furthermore, the impact of βHB on the growth and some virulence factors of *V. splendidus* or *V. coralliilyticus* was examined *in vitro*. The *in vitro* trials were carried out at pH7 and pH8. Normal acidity of seawater is pH 8, but previous research demonstrated that β-HB was more efficient as antimicrobial agent at a lower pH of 7 (Defoirdt *et al.*, 2007b). In addition, Defoirdt *et al.* (2009) hypothesized that a possible effect of PHB supplementation, is the lowering of the pH value in the intestines of the host.

4. 2. Materials and Methods

4. 2.1. Bacterial strains and growth conditions

The pathogenic bacteria used in this study were *V. coralliilyticus* DO1 (Genbank accession number EU358784) and *V. splendidus* 0529 (Genbank accession number EU358783) since there are no pathogens described specifically for *Mytilus edulis* larvae to our knowledge. The chosen pathogens were earlier reported to be pathogenic towards green shell mussel (*Perna canaliculus*) larvae (Kesarcodi-Watson et al., 2009) and were kindly provided by Dr. Kesarcodi-Watson (Cawthron Institue, New Zealand) for the current study.

Before use, the strains were made rifampicin resistant. Firstly, each bacterial strain stocked in 40 % glycerol at - 80 °C was grown overnight at 18 °C in Luria-Bertani broth supplemented with 35 g L^{-1} Instant Ocean sea salt (Aquarium Systems, Sarrebourg, France) (LB_{35}). Further, a volume of 500 µL was transferred into 5 mL of fresh LB_{35} containing rifampicin (which was dissolved in DMSO) at a final concentration of 50 mg L^{-1}. It took a few days before the medium showed signs of growth. Afterward, 50 µl of this medium was plated on LB_{35} plates

containing 50 mg L^{-1} rifampicin. A single colony was picked up from the plate, and the whole procedure was repeated. The resistant strains were finally stored in 40 % glycerol at – 80 °C.

For use in the experiments, the rifampicin resistant bacteria were grown overnight in LB_{35} under constant agitation (130 rpm) at 18 °C, the temperature at which the challenges were carried out. Cell densities were measured spectrophotometrically at 600 nm with an OD of 1 corresponding to about 1.2 x 10^9 colony forming units (CFU) mL^{-1}. The final concentration of pathogenic bacteria used in all challenge experiments was 10^5 CFU mL^{-1}.

4. 2.2. Amorphous PHB (PHB-A)

The amorphous PHB consisted of lyophilized *Ralstonia eutropha* containing 75% PHB on cell dry weight and was produced as described by Thai *et al.* (2014). The PHB-A was suspended at 50 mg L^{-1} in FASW and sonicated for homogenization using an ultrasonic machine (Branson 1200, USA). A final concentration of 1 mg L^{-1} and 10 mg L^{-1} PHB was utilized in the challenge experiments.

4. 2.3. Experimental mussel larvae

Mature adult mussels were obtained from Roem van Yerseke (Yerseke, The Netherlands) the day before spawning. The spawning and fertilization protocol were followed as described by Hung *et al.* (2015). After fertilization, the development of the fertilized eggs was regularly monitored. When the morula stage was reached in the majority of the embryos, they were rinsed with filter autoclaved sea water (FASW) on a 30 µm sieve to wash away remaining sperm. Next, the embryos were transferred to a glass bottle containing 2L of FASW at 18 °C and a mixture of the antibiotics rifampicin, kanamycin, and ampicillin (10 mg L^{-1} each). After 48 hr, D-veliger larvae were obtained and washed at least five times with FASW to remove the antibiotics. The larvae were re-suspended in fresh FASW, and the density was determined. All manipulations were performed under laminar flow.

4. 2.4. Blue mussel larvae challenge tests

The protective effect of PHB-A against bacterial infection in blue mussel larvae was evaluated in a number of challenge tests. The basis of each challenge test consisted of

adding 100 mussel larvae into 24 inner wells (to avoid plate edge effects) of a sterile 48 well-plate (Tissue Culture Dish, Thermo Scientific). Each well contained 1 ml of FASW supplemented with LB_{35} (0.1% v/v) and rifampicin (10 mg L^{-1}). These 24 wells were divided into 6 treatments with 4 replicate wells per treatment. This set-up was also applied in 24 well-plates for challenge tests. The plates were incubated at 18°C under a light regime 12D: 12L for all experiments. During the challenge test, the suspension in all wells was pipetted very gently twice a day to re-suspend the PHB-A in the water column. Each challenge test was repeated at least once. Only one sample experiment is shown here, since the results were very consistent. Other results can be found in Appendix-C (see page on 189.)

Each challenge test consisted of following six treatments:

1) Non-treated larvae ("control")
2) Larvae supplied with PHB-A immediately after stocking in the plates ("PHB-A")
3) Larvae challenged with *V. coralliilyticus* at 6 or 24 h after stocking in the plates ("*V. coralliilyticus")*
4) Larvae challenged with *V. splendidus* at 6 or 24 h after stocking in the plates ("*V. splendidus")*
5) Larvae supplied with PHB-A immediately after stocking in the plates and challenged 6 or 24 h later with *V. coralliilyticus* ("PHB-A + *V. coralliilyticus*")
6) Larvae supplied with PHB-A immediately after stocking in the plates and challenged 6 or 24 h later with *V. splendidus* ("PHB-A + *V. splendidus*")

In each challenge test, the survival of the larvae was verified at 24h, 48h, 72h, and 96h after introduction of the pathogens (except for control). At each of these time points, the larvae of 3 well-plates were killed and stained with Lugol's solution. Under the microscope, survival was determined based on the presence of lugol coloured internal structures that included the vellum, cilia, and stomach. Deterioration of organ structures and empty shells were denoted as moribund or dead individuals (Figure 4.1). The conditions of the different challenge tests are as explained in Table 4.1.

Table 4. 1: Overview of the different blue mussel larvae challenge tests

	Pathogens	PHB concentration	Period of PHB treatment before challenge
Challenge test 1	*V. coralliilyticus* *V. splendidus*	1 mg L^{-1}	6 h
Challenge test 2	*V. coralliilyticus* *V. splendidus*	1 mg L^{-1}	24 h
Challenge test 3	*V. coralliilyticus* *V. splendidus*	10 mg L^{-1}	6 h
Challenge test 4	*V. coralliilyticus* *V. splendidus*	10 mg L^{-1}	24 h

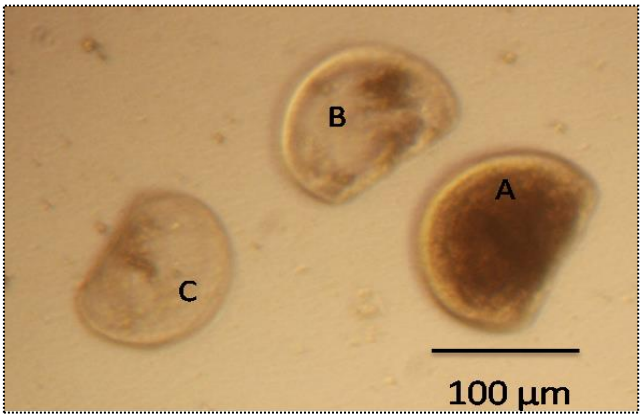

Figure 4. 1: Blue mussel larvae after Lugol staining (x 400). Living larvae (A) are completely dark while partly (B) or entirely (C) empty shells are counted as dead larvae.

4. 2.5. Effect of β-HB on growth of *V. coralliilyticus* and *V. splendidus*

V. coralliilyticus and *V. splendidus* were grown overnight as described above and set at an OD_{600} value of 1.0 in fresh LB_{35}. The bacterial suspensions were inoculated (2% v/v) in 200 µl LB_{35} containing different concentrations (0 mM, 1.0 mM, 5.0 mM, 25 mM and 125 mM) of β-

HB (sodium β-hydroxybutyrate, Sigma-Aldrich, Germany) in a 96-well plate (n = 4 for each pathogen-concentration combination). The assay was performed at pH 7 and pH 8 in the presence of a 1M MOPS buffer. The plates were incubated in a spectrophotometer (Tecan I-Control, Belgium) and the optical density of each well at 600 nm was measured every hour for 24 h.

4. 2.6. Effect of β-HB on virulence factors of *V. coralliilyticus* and *V. splendidus*

For all virulence factors considered, filtered sterilized β-HB was added to the medium at concentrations of 0 mM, 1 mM, 5 mM, 25 mM, or 125 mM. The pH of the medium was consequently adjusted at 7 or 8 by the addition of 0.1 N HCl or 0.1 N NaOH.

All assays were performed in triplicate in at least two independent experiments with consistent results.

4. 2.6.1. Swimming motility

A swimming motility assay was performed using LB_{35} plates with 2% agar (Yang and Defoirdt (2015)). The pathogens were spotted at the center of the plates, and the diameter of the motility zones was measured after 24 h of incubation at 18 °C.

4. 2.6.2. Lytic enzyme production: caseinase, gelatinase, hemolysin, lipase and phospholipase

All assays were performed according to Natrah et al. (2011). The caseinase assay plates were prepared by mixing equal volumes of autoclaved double strength LB_{35} agar and a 4% skimmed milk powder suspension (Oxoid, UK), sterilized separately at 121 °C for 5 min. Colony diameters and clearing zones were measured after 3 days of incubation at 28 °C.

Gelatinase assay plates were prepared by mixing 0.5% gelatin (Sigma-Aldrich) into LB_{35}. After incubation for 7 days, saturated ammonium sulfate (80%) in distilled water was poured over the plates and after 2 min, the diameters of the clearing zones around the colonies were measured.

Hemolytic assay plates were prepared by supplementing autoclaved LB_{35} agar with 5% defibrinated sheep blood (Oxoid, Basingstoke, and Hampshire, UK). Colony diameters and clearing zones were measured after 2 days of incubation at 18 °C.

For the lipase and phospholipase assays, LB_{35} agar plates were supplemented with 1% Tween 80 (Sigma-Aldrich) and 1% egg yolk emulsion (Sigma-Aldrich), respectively.The diameter of the opalescent zones was measured after 3 days of incubation at 18 °C.

4. 2.6.3. Biofilm formation and exopolysaccharide production

A biofilm formation assay was conducted in 96 well plates as described by Li et al. (2014) using LB_{35} broth. The exopolysaccharide (EPS) production assay was performed in a similar way. For the quantification of exopolysaccharides, calcofluor white staining (Sigma-Aldrich) was used as described by Brackman et al. (2008)

4. 2.7. Data analysis and statistics

Larval survival data in challenge tests were subjected to a logistic regression analysis using GenStat (VSN International) version 16. The effects of varying concentrations of β-HB on the virulence factors of the pathogenic bacteria were determined by first checking the assumptions associated with ANOVA and in case any of the assumptions was violated, non-parametric equivalents were considered. In effect, since the mean response was expected to vary with a concentration level of β-HB, we were interested in testing trends in response to increasing levels. Therefore, the Jonckheere Terpstra test for ordered alternatives was applied. In situations where this test failed to indicate the presence of trends, the Kruskal-Wallis test was used. All tests between the different concentrations of β-HB were tested at the 5% significance level with the ANOVA Dunn-Bonferroni posthoc analysis to account for multiplicity. All analyses were carried out using Statistical Package for the Social Sciences version 23 (SPSS. Inc., Chicago. IL, USA) and R version 3.2.2 software packages.

4. 3. Results

4. 3.1. Survival of mussel larvae in challenge tests

The supplementation 6h before the introduction of the pathogens *V. splendidus* and *V.coralliilyticus* of PHB-A at a concentration of 1 mg L^{-1}, significantly increased larval survival measured 96 h after the challenge (Figure 4.2, challenge 1). For all other larval pretreatments in combination with *V. coralliilyticus*, no significant difference in survival with the challenged control was observed.

There was a significantly increased protection of mussel larvae against *V. splendidus* for 48 h in the 24h pretreated PHB-A group in comparison to the non-pretreated challenged group. (Figure 4.2, challenge 2). Compared to the challenged larvae that did not receive PHB-A, the increase in PHB-A concentration from 1 to 10 mg L^{-1} significantly increased larval survival after 72 h and 48 h of a challenge with *V. splendidus* in the groups pretreated with PHB-A for 6h and 24 h respectively. The increased PHB-A concentration, however, did not lead to a significant difference in the protection of mussel larvae against *V. coralliilyticus* (Figure 4.2, challenge 3 &4).

4. 3.2. Effect of β-HB on growth of *V. coralliilyticus* and *V. splendidus*

The impact of β-HB on the growth of the pathogens was species and pH dependent. The growth of the two bacterial strains in LB_{35} was significantly inhibited at the highest β-HB concentration of 125 mM at pH7 and pH8 except for the combination *V.coralliilyticus* at pH7 and pH8 (Figure 4.3).

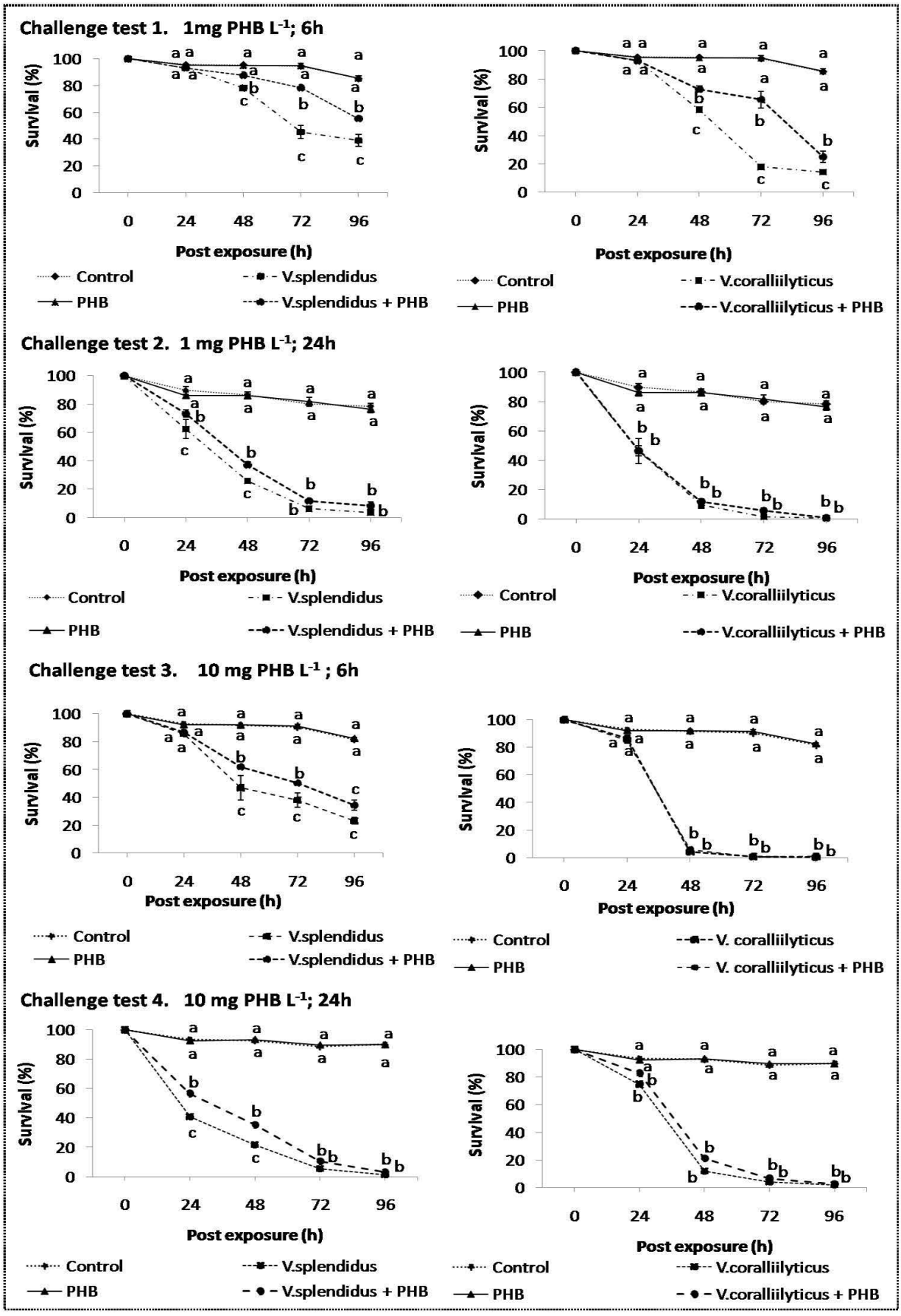
Challenge test 1. 1mg PHB L-1; 6h
Survival (%)
Post exposure (h)
Control
PHB
V.splendidus
V.splendidus + PHB
V.coralliilyticus
V.coralliilyticus + PHB
Challenge test 2. 1 mg PHB L-1; 24h
Survival (%)
Post exposure (h)
Control
PHB
V.splendidus
V.splendidus + PHB
V.coralliilyticus
V.coralliilyticus + PHB
Challenge test 3. 10 mg PHB L-1 ; 6h
Survival (%)
Post exposure (h)
Control
PHB
V.splendidus
V.splendidus + PHB
V. coralliilyticus
V. coralliilyticus + PHB
Challenge test 4. 10 mg PHB L-1; 24h
Survival (%)
Post exposure (h)
Control
PHB
V.splendidus
V.splendidus + PHB
V.coralliilyticus
V.coralliilyticus + PHB

Figure 4. 2: Survival of blue mussel larvae challenged with *V. coralliilyticus* or *V. splendidus* at 10^5 CFU mL^{-1} under different experimental conditions. In challenge test 1 and 2, PHB was supplemented at a concentration of 1 mg L^{-1} 6 h and 24 h respectively before the challenge, in challenge test 3 and 4, PHB was supplemented at a concentration of 10 mg L^{-1} 6 h and 24h respectively before the challenge. Values are presented as means ± standard error (SE) of 3 replicate plates. The survival in both the control treatment (larvae only) and the PHB-A treatment without challenge was similar and therefore cannot be distinguished in the graphs. Values at the one-time point (just 24 h) that are marked with a different letter are significantly different ($P < 0.05$).

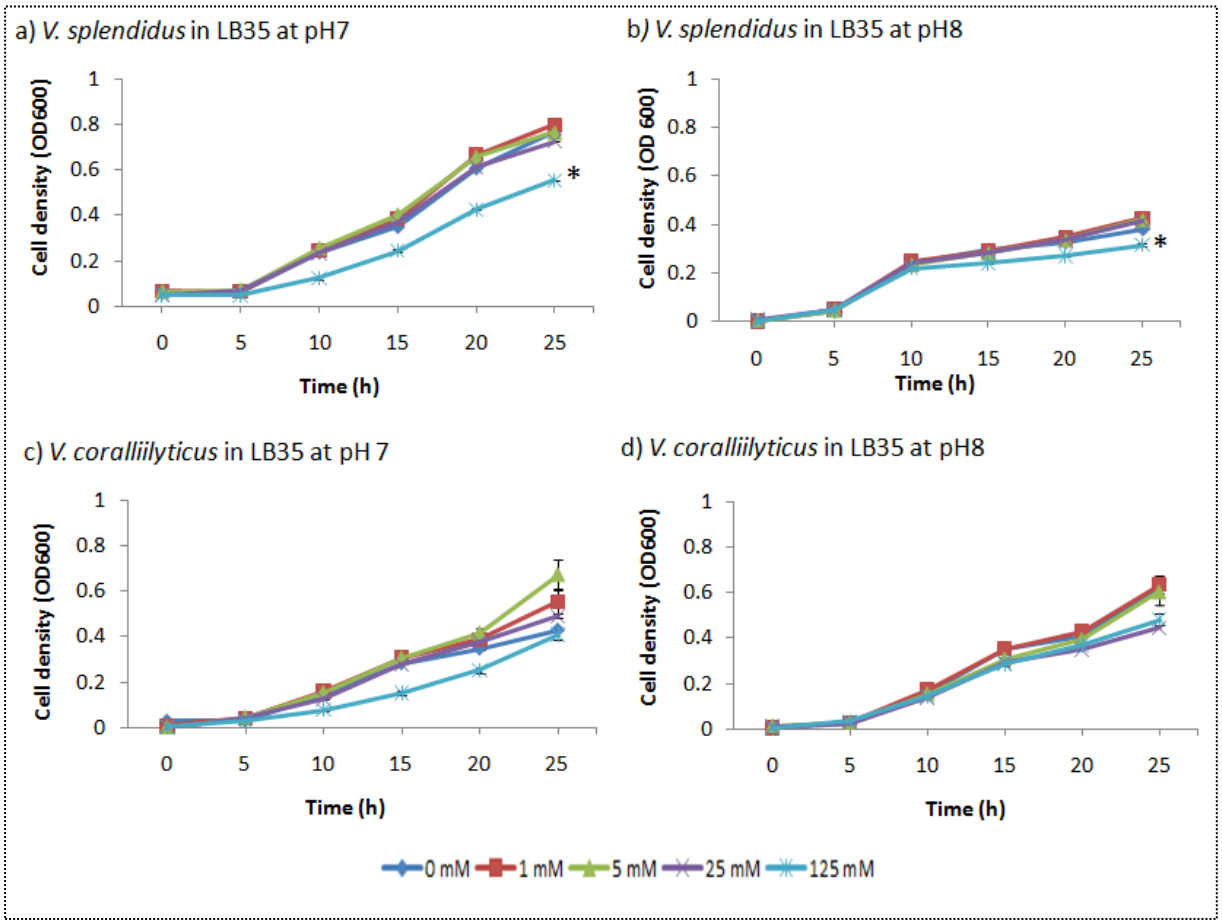

Figure 4. 3: Growth curves of *V. splendidus* and *V. coralliilyticus* (OD_{600}) in LB_{35} at pH7 and pH8 with different concentrations of 0, 1, 5, 25, and 125 mM β-HB. Data are expressed as mean ± Standard Error (SE) of four replicates. An asterisk denotes a significant difference between the highest concentration of β-HB and the other concentrations. Error bars presented in the graph are often too small to be visible.

4. 3.3. Effect of β-HB on virulence factors

4. 3.3.1. Swimming motility

Both *Vibrio* spp. used in this study exhibited swimming motility although patterns appeared to differ between the two species (Table 4.2). The swimming motility of *V. splendidus* was significantly higher with 25 mM β-HB at neutral pH, whereas no significant differences were found between the different β-HB concentrations at pH8. The swimming motility of *V. coralliilyticus* was only significantly affected at the highest concentration of 125 mM β-HB at pH 7. At pH 8, no significant effects as compared to the control could be observed.

Table 4. 2: Swimming motility zone of *V. splendidus* and *V.coralliilyticus* after 24h incubation on soft agar at pH7 and pH8 with different β-HB concentrations.

	V. splendidus		*V. coralliilyticus*	
β-HB (mM)	pH7	pH8	pH7	pH8
	Motility zone (mm)	Motility zone (mm)	Motility zone (mm)	Motility zone (mm)
0	40.0 ± 0.7^{a}	33.5 ± 0.5^{a}	45.3 ± 0.3^{b}	47.5 ± 1.0^{ab}
1	38.0 ± 2.0^{a}	30.0 ± 0.7^{a}	44.8 ± 0.3^{ab}	45.0 ± 0.0^{a}
5	46.8 ± 1.0^{ab}	32.5 ± 1.0^{a}	46.0 ± 0.7^{b}	45.8 ± 0.8^{ab}
25	57.3 ± 3.5^{b}	31.8 ± 2.3^{a}	41.3 ± 0.5^{ab}	49.8 ± 0.9^{b}
125	43.8 ± 0.9^{ab}	30.8 ± 1.7^{a}	35.3 ± 1.0^{a}	46.0 ± 0.7^{ab}

Data are expressed as mean ± Standard Error (SE) of four replicates. Values in the same column with different letters indicate significant differences in motility zones between β-hydroxybutyrate concentrations ($p<0.05$). All pairwise comparisons were done using the Dunn-Bonferroni method.

4. 3.3.2. Lytic enzymes activity

The activity of different lytic enzymes (caseinase, gelatinase, lipase, hemolysin, and phospholipase) of *V. splendidus* and *V. coralliilyticus* upon supplementation of varying

concentrations of β-hydroxybutyrate at different pH levels is shown both *Vibrio* strains tested positive for all virulence factors in the absence of β-HB. Overall, the colonies and clearing zones on the caseinase assay plates were noticeably smaller at the highest concentration of β-HB. The ratio between the clearing zone and colony diameter was significantly higher at 125 mM β-HB as compared to 0 mM (except for *V. splendidus* at pH8).

A significantly stronger gelatinase activity was observed at 5 mM β-HB for *V. coralliilyticus* at pH8. For the other concentrations of β-HB, there were no significant differences in the control treatment of 0 mM β-HB for both pathogens at both pH values (Table 4. 2).

There was no significant effect of β-HB on lipase at any of the β-HB concentrations for both pathogens. The ratio in *V. splendidus* was bigger than in *V. coralliilyticus*, whereas the colonies in *V. splendidus* were smaller than in *V. coralliilyticus*.

On the contrary, an effect of β-HB on the hemolytic activity was observed in both pathogens and both pH levels. The highest level of β-HB always showed a significant difference as compared to the control treatment (0 mM βHB), being higher for *V. splendidus* at pH 7 and lower in all other cases.

While phospholipase activity was significantly decreased at 125 mM for *V. splendidus* at both pH7 and pH8, there was no significant effect for *V. coralliilyticus.*

Table 4. 3: The activity of different lytic enzymes (caseinase, gelatinase, lipase, hemolysin, and phospholipase) of *V. splendidus* and *V. coralliilyticus* upon supplementation of varying concentrations of β-hydroxybutyrate at various pH levels

β-HB (mM)	pH7			pH8		
	Colony diameter (mm)	**Clearing zone** (mm)	**Ratio**	**Colony diameter** (mm)	**Clearing zone** (mm)	**Ratio**
Caseinase/ *V.splendidus*						
0	18.0 ± 0.0	22.0 ± 0.0	1.2 ± 0.0^{a}	25.5 ± 1.0	29.5 ± 1.7	1.2 ± 0.0^{ab}
1	20.0 ± 0.7	24.5 ± 1.2	1.2 ± 0.0^{a}	26.0 ± 1.1	31.3 ± 1.8	1.2 ± 0.0^{ab}
5	20.0 ± 0.4	23.5 ± 0.6	1.2 ± 0.0^{a}	25.5 ± 0.5	29.3 ± 0.8	1.1 ± 0.0^{a}
25	17.3 ± 0.9	21.3 ± 1.3	1.2 ± 0.0^{a}	19.0 ± 0.0	24.0 ± 0.0	1.3 ± 0.0^{b}
125	5.0 ± 0.0	10.0 ± 0.0	2.0 ± 0.0^{b}	18.5 ± 0.3	21.8 ± 0.3	1.2 ± 0.0^{ab}
Caseinase / *V.coralliilyticus*						
0	19.0 ± 0.6	24.3 ± 0.3	1.3 ± 0.0^{a}	24.5 ± 0.3	29.3 ± 0.5	1.2 ± 0.0^{a}
1	21.8 ± 0.3	26.0 ± 0.4	1.2 ± 0.0^{a}	26.0 ± 0.0	30.0 ± 0.0	1.2 ± 0.0^{a}
5	18.5 ± 0.9	21.0 ± 1.0	1.1 ± 0.0^{a}	25.3 ± 0.3	28.8 ± 0.8	1.1 ± 0.0^{a}
25	17.0 ± 0.4	20.8 ± 0.3	1.2 ± 0.0^{a}	22.8 ± 0.9	24.8 ± 0.3	1.1 ± 0.0^{a}
125	5.0 ± 0.0	10.0 ± 0.0	2.0 ± 0.0^{b}	17.0 ± 0.0	23.0 ± 0.0	1.4 ± 0.0^{b}
Gelatinase/*V.splendidus*						
0	18.5 ± 1.6	34.3 ± 0.5	1.9 ± 0.1^{ab}	19.0 ± 0.6	35.3 ± 0.3	1.9 ± 0.1^{ab}
1	15.3 ± 0.5	30.5 ± 1.0	2.0 ± 0.0^{b}	15.0 ± 1.3	32.5 ± 1.0	2.2 ± 0.1^{b}

5	25.3 ± 1.9	35.0 ± 3.1	1.4 ± 0.1^{a}	20.0 ± 0.0	35.0 ± 0.0	1.8 ± 0.0^{ab}
25	22.8 ± 1.0	34.0 ± 0.7	1.5 ± 0.0^{ab}	22.3 ± 1.0	35.5 ± 0.5	1.6 ± 0.1^{a}
125	14.5 ± 1.2	26.0 ± 0.8	1.8 ± 0.1^{ab}	16.0 ± 0.0	30.0 ± 0.0	1.9 ± 0.0^{ab}
Gelatinase/ *V.coralliilyticus*						
0	21.5 ± 1.2	33.5 ± 0.5	1.6 ± 0.1^{ab}	19.5 ± 0.3	33.0 ± 0.6	1.7 ± 0.0^{a}
1	13.8 ± 1.3	29.0 ± 0.6	2.1 ± 0.2^{b}	16.0 ± 0.7	33.0 ± 0.6	2.1 ± 0.1^{ab}
5	19.8 ± 1.3	32.8 ± 1.1	1.7 ± 0.1^{ab}	14.0 ± 0.0	33.3 ± 0.5	2.4 ± 0.0^{b}
25	27.8 ± 3.0	38.0 ± 0.0	1.4 ± 0.2^{a}	16.0 ± 2.0	33.3 ± 0.8	2.1 ± 0.3^{ab}
125	15.3 ± 0.9	27.3 ± 0.5	1.8 ± 0.1^{ab}	15.8 ± 0.5	32.0 ± 0.0	2.0 ± 0.1^{ab}
Lipase/ *V.splendidus*						
0	7.6 ± 0.2	16.5 ± 0.3	2.2 ± 0.1^{a}	7.5 ± 0.3	14.5 ± 0.3	1.9 ± 0.0^{ab}
1	6.8 ± 0.6	13.5 ± 0.6	2.0 ± 0.1^{a}	7.5 ± 0.5	12.5 ± 0.6	1.7 ± 0.1^{a}
5	7.8 ± 0.3	15.8 ± 0.5	2.0 ± 0.1^{a}	8.1 ± 0.1	16.3 ± 0.3	2.0 ± 0.0^{b}
25	8.3 ± 0.3	16.3 ± 0.5	2.0 ± 0.1^{a}	8.0 ± 0.4	16.0 ± 0.8	2.0 ± 0.0^{b}
125	8.3 ± 0.1	15.5 ± 0.3	1.9 ± 0.0^{a}	7.8 ± 0.3	14.5 ± 0.3	1.9 ± 0.1^{ab}
Lipase/ *V.coralliilyticus*						
0	15.5 ± 0.9	19.5 ± 0.5	1.4 ± 0.1^{a}	14.8 ± 0.3	19.8 ± 0.3	1.3 ± 0.0^{a}
1	14.0 ± 0.6	18.5 ± 0.6	1.3 ± 0.0^{a}	15.0 ± 0.4	19.0 ± 0.4	1.3 ± 0.0^{a}
5	13.8 ± 1.3	18.8 ± 0.9	1.4 ± 0.1^{a}	12.8 ± 0.5	20.3 ± 0.9	1.6 ± 0.1^{a}
25	17.3 ± 0.8	21.3 ± 0.6	1.2 ± 0.0^{a}	14.5 ± 0.5	19.3 ± 0.8	1.3 ± 0.0^{a}
125	13.8 ± 0.5	18.5 ± 0.3	1.3 ± 0.0^{a}	13.8 ± 0.5	18.3 ± 0.6	1.3 ± 0.0^{a}

Hemolytic/ *V.splendidus*						
0	18.8 ± 0.6	15.5 ± 0.6	0.8 ± 0.1^{a}	11.3 ± 1.1	19.5 ± 1.0	1.8 ± 0.1^{a}
1	9.3 ± 0.1	12.3 ± 0.1	1.3 ± 0.0^{ab}	10.5 ± 0.3	12.5 ± 0.3	1.2 ± 0.0^{bc}
5	18.3 ± 1.1	16.0 ± 0.3	0.9 ± 0.2^{ab}	14.8 ± 2.0	13.5 ± 0.9	0.9 ± 0.1^{c}
25	11.5 ± 0.7	13.8 ± 0.4	1.2 ± 0.1^{ab}	9.0 ± 0.4	12.5 ± 0.3	1.4 ± 0.1^{b}
125	7.0 ± 0.0	10.5 ± 0.1	1.5 ± 0.0^{b}	9.0 ± 0.0	12.3 ± 0.3	1.4 ± 0.0^{b}
Hemolytic/ *V.coralliilyticus*						
0	9.0 ± 0.0	15.5 ± 0.3	1.7 ± 0.0^{a}	9.0 ± 0.0	18.0 ± 0.0	2.0 ± 0.0^{a}
1	9.5 ± 0.3	11.8 ± 0.3	1.2 ± 0.0^{ab}	9.0 ± 0.0	12.0 ± 0.0	1.3 ± 0.0^{ab}
5	10.0 ± 0.0	12.0 ± 0.0	1.2 ± 0.0^{ab}	9.0 ± 0.0	12.0 ± 0.0	1.3 ± 0.0^{ab}
25	10.0 ± 0.4	11.8 ± 0.3	1.2 ± 0.0^{ab}	9.5 ± 0.3	12.0 ± 0.0	1.3 ± 0.0^{ab}
125	10.8 ± 0.3	13.0 ± 0.0	1.2 ± 0.0^{b}	11.5 ± 0.5	13.3 ± 0.5	1.2 ± 0.0^{b}
Phospholipase/ *V.splendidus*						
0	10.0 ± 0.0	14.5 ± 0.3	1.5 ± 0.0^{a}	11.0 ± 0.6	14.3 ± 0.3	1.3 ± 0.1^{a}
1	12.0 ± 0.0	15.8 ± 0.3	1.3 ± 0.0^{ab}	13.5 ± 0.5	15.5 ± 0.5	1.1 ± 0.0^{ab}
5	10.0 ± 0.3	13.0 ± 0.0	1.3 ± 0.0^{ab}	12.8 ± 0.5	15.8 ± 0.5	1.2 ± 0.0^{a}
25	11.8 ± 0.3	15.8 ± 0.3	1.3 ± 0.0^{ab}	13.0 ± 0.0	15.3 ± 0.3	1.2 ± 0.0^{a}
125	11.0 ± 0.3	13.5 ± 0.3	1.2 ± 0.0^{b}	10.5 ± 0.3	11.5 ± 0.3	1.1 ± 0.0^{b}
Phospholipase/ *V.coralliilyticus*						
0	13.8 ± 0.3	18.8 ± 0.8	1.4 ± 0.1^{ab}	15.3 ± 0.5	18.5 ± 0.5	1.2 ± 0.0^{ab}
1	14.5 ± 0.3	21.0 ± 0.0	1.4 ± 0.0^{ab}	13.0 ± 0.0	16.3 ± 0.3	1.3 ± 0.0^{a}

5	12.5 ± 0.5	17.0 ± 0.0	1.4 ± 0.1^{ab}	12.0 ± 0.0	16.0 ± 0.0	1.3 ± 0.0^{a}
25	13.0 ± 0.0	15.0 ± 0.0	1.2 ± 0.0^{b}	12.0 ± 0.0	14.0 ± 0.0	1.2 ± 0.0^{ab}
125	13.3 ± 0.3	15.5 ± 0.3	1.2 ± 0.0^{b}	12.0 ± 0.0	13.3 ± 0.5	1.1 ± 0.0^{b}

Data are expressed as mean ± standard error (SE) of four replicates. Ratio values for each virulence factor-species combination in the same column indicated with a different letter are significantly different ($P < 0.05$). All pair wise comparisons were done using Dunn-Bonferroni method. The ratio of colony diameter and clearing zone data were transformed (normality and homogeneity) to satisfy assumptions of ANOVA.

There was no significant effect of β-HB on biofilm formation for both pathogens at both pH values compared to the control treatment (Figure 4. 4 a & b). The exopolysaccharide production more or less reflected the tendencies observed for the biofilm formation (Figure 4. 4 c & d). A significant increase was found though at 5 mM and pH8 in the case of *V. splendidus* (Figure 4. 4 c).

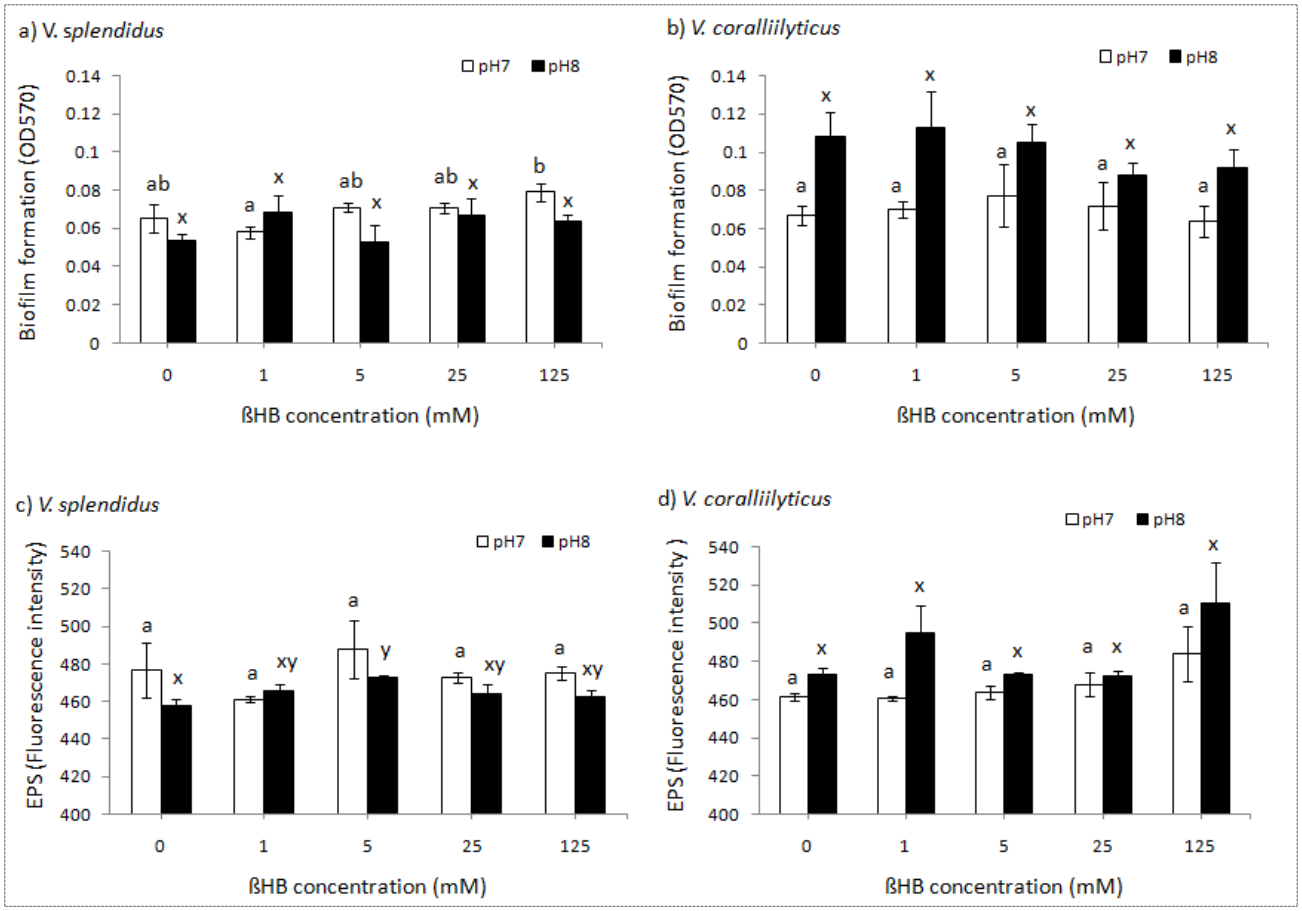

Figure 4. 4: Biofilm formation (a & b) and exopolysaccharide production (c & d) of *V. splendidus* and *V.coralliilyticus* after 4 days incubation at 18°C in different ß-HB concentrations at pH7 and pH8 (average ± standard error of four replicates). Different letters indicate significant differences between β-HB levels (a,b for comparison at pH7; x,y for comparison at pH8). All pairwise comparisons were done using Dunn-Bonferroni method.

4. 4. Discussion

The results obtained in the present work demonstrated that the administration of PHB-A had a positive effect *in vivo* on the survival of blue mussel larvae when it was administered 6 h before the challenge with V. *splendidus* or V. *coralliilyticus.* To our knowledge, this is the first time that PHB-A has been tested as a protective compound for blue mussel larvae in controlled challenge tests. It was also confirmed that the two tested vibrios are also virulent for blue mussel larvae.

Currently, the exact mode of action by which PHB induces its protective effects is still not clear. In general, it is assumed that the compound is bio-converted to its monomer β-HB upon ingestion and that this compound then acts as an antimicrobial agent (Defoirdt *et al.*,

2009), provides direct energy to the host (Fernández, 2001) or acts as an immunostimulant (Baruah *et al.*, 2015). In previous studies, PHB-A has been shown to significantly increase the survival of Artemia when challenged with *V. campbellii* under both gnotobiotic and conventional conditions (Halet *et al.*, 2007, Defoirdt *et al.*, 2007b). Consistent with a previous report on *Artemia* (Defoirdt *et al.*, 2007b), our results provided evidence that the addition of amorphous PHB to the mussel larvae culture water significantly improves the protection of larvae against both pathogens *V. splendidus* and *V. coralliilyticus*, although there were major differences in the effective PHB-A dose between the two studies. In the Defoirdt *et al.* (2007b) study, maximum and complete protection against *V. campbellii* was obtained at a PHB-A concentration of 1000 mg L^{-1} while the lower PHB-A concentration of 100 mg L^{-1} still provided significant protection. In contrast, in the present study, an increase in PHB-A concentration from 1 mg to 10 mg PHB-A mL^{-1} did not improve the larval survival. This result supports our previous *in vivo* study (Hung *et al.*, 2015) where the beneficial effects of PHB-A in the culture of blue mussel larvae were optimal at 1 mg L^{-1}, with inferior results obtained at the higher concentration of 10 mg L^{-1}.

Another interesting observation that was noted in this study was that the time window between treatment and challenge influenced the outcome of the challenge. When the pretreatment with PHB-A was administered 24h before the challenge, no positive effect on survival was observed. In this case, it is possible that the PHB-A had been rapidly degraded by the natural enzymes or resident PHB-degraders in the digestive system of the larvae. Therefore, PHB-A treated mussel larvae failed to accumulate enough PHB concentration when pretreated 24h before the challenge (in contrast to the 6 h pre-treatment).

In order to determine the effect of the monomer β-HB on the pathogens in this study, an effort was made to measure the concentration of β-HB in the mussel larvae themselves. However, due to the small dimensions of the mussel larvae, values remained below the detection limit. In another study, however, it was found that the β-HB concentration in *Artemia* nauplii supplied with PHB ranged between 1 and 8 mM (De Schryver et al., unpublished data). Based on these values, it was decided to determine the effect of β-HB on the growth and virulence factor activity of the pathogens at concentrations of 1 and 5 mM, this being a realistic estimation of the PHB concentration in the gut of blue mussel larvae treated with PHB. In addition, higher PHB concentrations of 25 and 125 mM were also

included to provide a better insight on the effect of β-HB. The effect of these concentrations were verified at two pH values. Indeed, pH of the environment is a very important factor since the growth-inhibitory effect of β-HB was earlier shown to decrease with increasing pH. At pH 5, the growth of *V. campbellii* was completely inhibited; at pH 6, growth was delayed and at pH 7, no inhibition could be observed (Defoirdt *et al.*, 2006b). The pH-dependency can be explained by the fact that fatty acids can pass the cell membrane only in their undissociated form, which will be more pronounced at lower pH (see the Henderson-Hasselbach equation (Sun et al., 1998)).

From the in vitro assays, it became clear that the pathogens' growth was not inhibited at the lower concentrations of β-HB. Only a concentration of 125 mM β-HB significantly inhibited the growth of both pathogens *V. splendidus* and *V. coralliilyticus* at neutral pH (except for *V. coralliilyticus* at alkaline pH). This indicates that the beneficial effect of PHB on larval survival was unlikely the consequence of β-HB reducing the growth of the pathogens as it is exactly these lower concentrations that can be expected in the gastrointestinal environment of the blue mussel larvae. For this reason, it was determined if there could have been an effect of β-HB on the virulence factors of the pathogens.

A proper evaluation of the *in vitro* test requires a better understanding of the general and specific stress response capabilities of pathogens. The degree of pathogenicity, also termed virulence depends on the expression of virulence factors, i.e. gene products that enable the pathogen to infect and damage the host, including proteins involved in motility and adhesion of the pathogen to the host, protection from host defence mechanism and host tissue degradation, iron acquisition and toxins (Chen et al., 2005, Defoirdt, 2014).

Bacterial motility is now considered as an important virulence factor for many pathogens. It is essential for pathogenic bacteria during the initial phases of infection as it helps them to overcome repulsive forces between the bacterial cell and the host tissues and hence, facilitates attachment to the host (McCarter, 2001). Our results indicated that the effect of β-HB on the swimming motility of pathogens depended both on the species and concentration. *V. splendidus*' swimming activity significantly increased at 25 mM of β-HB while *V. coralliilyticus*' motility was significantly decreased at 125 mM (pH7).

Biofilm formation is an ancient and integral component of the prokaryotic life cycle and is a critical factor for survival in diverse environments (Hall-Stoodley et al., 2004). This process in *Vibrios* depends on specific genes (flagella, pili, and exopolysaccharide biosynthesis) and regulatory processes (Yildiz and Visick, 2009). In the present study, β-HB did not exert any influence on the biofilm formation of the two *Vibrio spp*. investigated. Irrespective of the presence or absence of β-HB, biofilm formation seemed to be very low in both *Vibrio spp*. For *Vibrio fischeri,* Chavez-Dozal and Nishiguchi (2011) found biofilm formation at an OD_{562} to be 0.83 and 1.10 at 20 and 25°C, respectively, whereas our results showed minimum-maximum values of 0.04-0.10 and 0.04-0.15 in *V. splendidus* and *V. coralliilyticus*, respectively. The small biofilm formation observed in the present study could be related to the sub-optimal incubation temperature used (18 °C). This context applies in particular for *V. coralliilyticus*. Several studies have identified *V. coralliilyticus* as a mesophilic bacterium with its virulence factors upregulated at elevated temperatures (Kimes et al., 2012, Frydenborg et al., 2014). This is not applicable however for *V. splendidus* that grows at low temperatures, being psychrotroph (Pujalte et al., 1999).

Extracellular proteases have been identified to play a significant role in virulence and pathogenicity of many bacteria (Cai et al., 2007). Defoirdt (2014) found that lytic enzymes produced by aquaculture pathogens include hemolysins and proteases. Haemolysin is one of the virulence factors of pathogens described for shrimp, fish (Liu et al., 1996, Sun et al., 2007) and bivalve mollusks (Nottage and Birkbeck, 1990). Secreted phospholipases are thought to function in phosphate acquisition, carbon source acquisition, and in some cases as virulence factors for pathogenic species (Schmiel and Miller, 1999). The results of the assays indicated that β-HB significantly affected the virulence factors only in a limited number of cases (Table 4. 4).

Table 4. 4: Summary of the effects of β-HB on selected virulence factors of mussel larvae *Vibrio* pathogens

	V. splendidus		*V. coralliilyticus*	
	pH 7	pH 8	pH 7	pH 8
Caseinase	125 mM ↑	No effect	No effect	125 mM ↑
Gelatinase	No effect	No effect	No effect	No effect
Lipase	No effect	No effect	No effect	No effect
Hemolysin	125 mM ↑	1, 5, 25 ,125 mM ↓	125 mM ↓	125 mM ↓
Phospholipase	125 mM ↓	125 mM ↓	No effect	No effect
Swimming motility	25 mM ↑	No effect	125 mM ↓	No effect
Biofilm formation	No effect	No effect	No effect	No effect
Exopolysaccharide	No effect	5 mM↑	No effect	No effect

Focusing on the concentrations that can be considered to be relevant to the gastrointestinal environment of blue mussel larvae (range 1- 25mM), only one beneficial effect of β-HB for the host can be reported: at a β-HB concentration of 1, 5 and 25mM, the hemolytic enzyme activity in *V. splendidus* is inhibited at pH8. The hypothesis is that PHB may affect the virulence factor activity of the pathogens under investigation and as such protect the blue mussel larvae from infection, is here with rejected. It is likely that the protective effect of PHB on blue mussel larvae involves other mechanisms such as immunostimulation (Baruah *et al.*, 2015) or the direct provision of energy (De Schryver *et al.*, 2010).

In conclusion, the present study indicated that PHB-A can partly protect mussel larvae against specific pathogenic bacteria and that the effect of PHB-A on larval survival in the challenge experiments depended on both PHB-A concentration and exposure time. In general, pretreatment of mussel larvae with PHB-A at a concentration of 1 mg L^{-1} resulted in better larval survival when administered 6h before the challenge, compared to

administration of PHB-A 24h before the challenge. Larvae with the 6h pretreatment showed a 55% and 25% increase in survival after exposure to the pathogens *V. splendidus* and *V. coralliilyticus,* respectively, as compared to challenged larvae that were not treated with PHB-A. Increasing the pretreatment from 1 to 10 mg PHB-A L^{-1} did not improve the protection of mussel larvae against the challenge unless treatment was suspended PHB-A at 6h challenged with *V. splendidus*. This result is in accordance with the previous mussel larvae experiments confirming that PHB-A of 1 mg L^{-1} is an optimal concentration in mussel larvae culture (Hung *et al.*, 2015).

The in *vitro* experiments suggested that β-HB affected the virulence factor activity of the pathogens rather than growth, although the responses didn't follow a clear pattern. The highest β-HB concentration of 125 mM inhibited some virulence factors of the pathogenic bacteria such as hemolysis, phospholipase, but also stimulated caseinase production. The lowest β-HB concentrations inhibited a hemolytic enzyme (1 mM) in contrast, EPS was stimulated at (5mM) β-HB in *V. splendidus* at pH8. There was no effect on the production of the lytic enzymes gelatinase and lipase and the formation of biofilm were not affected by the presence of β-HB at any concentration. The *in vitro* and *in vivo* tests of this study confirmed that both *Vibrio coralliilyticus* and *V. splendidus* are also virulent for blue mussel larvae.

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Chapter 5

Ralstonia eutropha, containing high poly-β-hydroxybutyrate levels (PHB-A), regulates the immune response in mussel larvae challenged with *Vibrio coralliilyticus*

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Abstract

Marine invertebrates rely solely on innate immune mechanisms that include both humoral and cellular responses. Antimicrobial peptides (AMPs), lysozyme and phenoloxidase activity, are important components of the innate immune defense system in marine invertebrates. They provide an immediate and rapid response to invading microorganisms. The impact of amorphous poly-β-hydroxybutyrate (PHB-A) (1mg PHB-A L^{-1}) on gene expression of the AMPs mytimycin, mytilinB, defensin and the hydrolytic enzyme lysozyme in infected blue mussel larvae was investigated during "*in vivo*" challenge tests with *Vibrio coralliilyticus* (10^5 CFU mL^{-1}). RNAs were isolated from mussel larvae tissue, and AMPs were quantified by q-PCR using the 18SrRNA gene as a housekeeping gene. Our data demonstrated that AMPs genes had a tendency to be upregulated in challenged mussel larvae, and the strongest expression was observed from 24h post-exposure onwards. The presence of both PHB-A and the pathogen stimulated the APMs gene expression however; no significant differences were noticed between treatments and exposure time. Looking at the phenoloxidase activity in the infected mussels, it was observed that the addition of PHB-A induced increased activity.

5.1. Introduction

In common with other invertebrates, bivalves rely solely on innate immunity, which by definition lacks adaptive characteristics, to combat against invading pathogens. Antimicrobial peptides appear to be one of the actors in innate immunity that have been conserved during evolution, although their involvement in anti-infectious processes is different according to species, cell type, and tissue (Mitta et al., 2000b). Many antimicrobial peptides are located in epithelia (Schröder, 1999) where they prevent invasion by pathogens while others may be especially abundant in circulating cells. They are recognized to be a major component of the innate immune defense system in bivalve mollusks as well (Cellura *et al.*, 2007).

The innate immunity in mollusks is not well understood. Nevertheless, pathogen recognition receptors (PRRs) have been identified in some species (Araya et al., 2010). Once the presence of microbes is detected, these intruders are phagocytosed and destroyed by toxic radicals, lysozyme and antimicrobial peptides, which are involved in bacterial killing by destabilizing their membrane permeability (Hancock and Rozek, 2002). Jenssen et al. (2006) described in detail the interaction process between the peptide and target cell. It is thought to occur through electrostatic bonding between the cationic peptide and the negatively charged components present on the bacterial outer envelope, such as phosphate groups within the lipopolysaccharides of Gram-negative bacteria or lipoteichoic acids present on the surface of Gram-positive bacteria.

In mussels, four AMPs (defensin, mytilin, myticin and mytimycin) which play a key role in the immune defense were identified and characterized (Mitta *et al.*, 2000b). Different mussel species have various AMPs genes, e.g., myticin appears in *Mytilus galloprovincialis* only, whereas mytimycin occurs in *M. edulis* only (Tincu and Taylor, 2004). Some of them have a wide spectrum of action while others are target-specific. For instance, mytimycin is described as being strictly anti-fungal, mytilin acts extracellularly, whereas myticin can act either intracellularly (during phagocytosis) or extracellularly (Mitta *et al.*, 2000b). Mitta *et al.* (2000b) also reported that defensin producing granulocytes are concentrated in the intestinal epithelia, whereas mytilin and myticin expressing granulocytes are well represented in gills suggesting that the type of AMP may dictate granulocyte allocation in

different organs in mussels. *M. galloprovincialis* defensin 1 (MGD1) is an original member of the arthropod defensin family due to the presence of 2 extra-cysteines and one modified amino acid (Mitta et al., 1999b). Defensin, mytilin and myticin were determined *in vitro* to have antimicrobial activity. Differences in expression of these AMPs genes were recorded when mussel *M. galloprovincialis* adults were challenged with various factors such as bacterial infections or by heat shock (Cellura *et al.*, 2007). To our knowledge, nothing is known about the genes that code for these antimicrobial peptides in blue mussel *M. edulis* larvae or about the regulation of their expression. Besides the AMP gene expression, another critical component of the immune system of bivalves namely phenoloxidase activity (PO) was examined. As the product of a complex cascade of reactions, PO is generated from proPO through a limited proteolysis by a proPO activating enzyme (Asokan *et al.*, 1997), and is involved in melanization, encapsulation, wound healing, phagocytosis, and pathogen extermination (Bai *et al.*, 1997, Munoz *et al.*, 2006). The soluble form of PO is always involved in humoral immunity while the cellular PO that binds to the surface of hemocytes, is more associated with cell-mediated immunity (Hellio *et al.*, 2007). Bacterial infection can cause a significant increase in PO in the hemolymph of the bivalves *Crassostrea madrasensis* and *Chlamys farrization* (Cong et al., 2008).

The compound poly-ß-hydroxybutyrate (PHB), a polymer of the short-chain-fatty acid ß-hydroxybutyrate, was proven to protect experimental animals against a variety of bacterial diseases, including vibriosis in farmed aquatic animals, albeit through undefined mechanisms (Baruah *et al.*, 2015). Recent research demonstrated that amorphous PHB-A, namely *Ralstonia* cells containing more than 75% PHB on dry weight basis, in particular increases the survival of mussel larvae (*M. edulis*) (Hung *et al.*, 2015) and protects them during *in vivo* challenge tests with *V. coralliilyticus* and *V. splendidus* in the previous study (chapter 4).

This study focuses on the detection and regulation of the three AMPs mytimycin, mytilinB, defensin and the hydrolytic lysozyme in *M. edulis* larvae. On a second level the impact of PHB-A on the expression of these genes and on PO activity regulation was investigated during challenge tests with the pathogen *V.coralliilyticus*.

5. 2. Materials and methods

5.2.1. Bacterial strains and growth conditions

Vibrio coralliilyticus is a Gram-negative marine bacterium isolated at the Glen Haven Aquaculture Centre (New Zealand) during 2004 and 2005. This selected strain was reported as a pathogenic *Vibrio* for bivalve larvae culture (Kesarcodi-Watson *et al.,* 2009b). *V. coralliilyticus* used in the experiments was mutated to a rifampicin resistant strain (in previous study - chapter 4). Before use, 10 µl of the stored cultures (in 40% glycerol at -80 °C) were plated on Luria- Bertani plates to which 35g L^{-1} of Instant Ocean® (LB_{35} agar) was added and incubated for 24 h at 17 °C. Single colonies were picked from the plates and cultured overnight in fresh LB_{35} at 17 °C under constant agitation (150 min^{-1}) before each experiment.

5.2.2. Spawning procedure and larvae handling

Mature blue mussels *(Mytilus edulis)* were transported from the mussel producer Roem van Yerseke, in The Netherlands to the Laboratory of Aquaculture and Artemia Reference Center, Ghent University, Belgium, under cooled conditions. The protocol for mussel spawning and fertilization was followed as described in Hung *et al.* (2015). The development of the fertilized eggs was regularly monitored. When the morula stage was reached in the majority of the embryos, the embryo solution was sieved through a 30 µm sieve. The remaining sperm was washed away with 0.22 µm filtered and autoclaved seawater (FASW). After this washing step, the embryos were transferred to a 2L glass bottle containing fresh FASW at 18 °C and a mixture of antibiotics (10 mg l^{-1}) to minimize bacterial interference (in previous study-chapter 4). After 48h, the straight-hinged D-larvae were washed at least five times with FASW to remove every trace of antibiotics and then re-suspended in fresh FASW. All manipulations were performed under a laminar flow hood to avoid contamination before the start of the challenge tests.

5.2.3. Challenge tests

The first experiment was carried out in 48-well tissue culture dishes (TCD), and the second, third and fourth experiment in 1L bottles. Survival was only measured in the small-scale test

while the larvae in the bottle experiment were also used for tissue collection. Samples from the second and third experiment were used to measure AMPs gene expression and PO activity, respectively. The fourth experiment was carried out to evaluate a wider time window of gene expression for the different AMPs (till 48 h, based on the results of second experiment, in which the signal of expression was low).

Each treatment was performed in triplicate, each experiment in TCD test was repeated at least once, and only one result is presented in this study. Plate sterility of the control treatments was checked at the end of the challenge by plating 100 µl of the culture water on LB_{35} plates and incubating at 17 °C. If the control was contaminated, the results were not considered, and the experiment was repeated.

5.2.3.1. Experiment 1

A completely randomized experimental design was followed to evaluate the effect of PHB-A on *V. coralliilyticus* exposure in mussel larvae. The challenge test was performed in 48-well TCDs. Each well contained approximately 100 two-day-old D-larvae. The rearing water contained LB_{35} (0.1% v/v) and rifampicin (10 mg L^{-1}) to suppress growth of ambient bacteria. In treatment 1 (control), larvae were neither PHB-A treated nor challenged, in treatment 2 the mussel larvae were challenged after 6 h with the rifampicin resistant strain *V.coralliilyticus* at a concentration of 10^5 CFU mL^{-1} while in treatment 3 the larvae were supplemented with PHB-A at a concentration of 1 mg L^{-1}. PHB-A consists of a freeze-dried *Ralstonia eutropha* culture containing 75% PHB on cell dry weight (VITO, Mol, Belgium). In treatment 4, the larvae were supplemented with PHB-A 6 h before being challenged with *V.coralliilyticus*. During the challenge test, larvae were pipetted twice a day to ensure that PHB-A particles were suspended in the water column and available for uptake by the larvae. The survival of the larvae was determined 24h and 48h after the addition of the pathogen.

5.2.3.2. Experiment 2, 3 and 4

The larvae were submitted to the same treatments as in Experiment 1, control PHB-A unfed larvae were compared with PHB-A fed larvae challenged with 10^5 CFU mL^{-1} of *V. coralliilyticus*. Three replicate glass bottles of 1L, containing 50,000 D-larvae each, were sacrificed at each sampling point. Room temperature was 17°C. All the larvae of the three replicate bottles were collected separately on a 60 µm sieve. For experiment 2, mussel

larvae were pretreated with PHB-A for 6h before exposure to the pathogen and samples were collected at the sampling points 3h, 6h, and 12h (counted just after adding the pathogen), and no sampling was done at 24h and 48h. For experiment 3, larvae were collected at 48h after the addition of the pathogen and PO was measured in challenged larvae at 12h, 24h, 36h and 48h while for experiment 4, the larvae were collected at 0h, 3h, 6h, 9h, 18h, 24h and 48h. The larvae were washed with AFSW and transferred to 2 ml centrifuge tubes, weighed, flash-frozen in liquid nitrogen and stored at -80°C for PO measurement and RNA extraction respectively. Procedure of four experiment was summarized in the Table below:

Table 5. 1: Summary of the challenge tests with *V. coralliilyticus* (10^5CFU mL^{-1}) in the presence of PHB-A (1 mg L^{-1}) added 6 h before the pathogen

Challenge experiment (Exp.)	Vessel	Sampling point	Evaluation
Exp.1	Tissue culture Dishes (TCD)	48h	Survival of larvae
Exp.2	Glass bottle of 1 L	0, 3, 6, 9, 12, 24, 48, 72 and 96 h	Time window
Exp.3	Glass bottle of 1 L	0, 3, 6 and 12 h	AMPs gene expression
Exp.4	Glass bottle of 1 L	12,24,26 and 48h	Phenoloxidase activity

5.2.4. Standardization of quantitative real-time qRT-PCR for mussel larvae

5.2.4.1. Design of gene-specific primers

The sequences of mytimycin, mytilinB, defensin, and lysozyme from two blue mussel species *M. galloprovincialis* and *M. edulis* were selected from the GenBank (http://www.ncbi.nlm.nih.gov). Specific primers for 18srRNA, mytimycin, mytilinB, lysozyme and defensin genes were designed using the Primer3.0 software

(http://biotools.umassmed.edu/bioapps/primer3). In this study, the 18srRNA gene was retained as the housekeeping gene.

5.2.4.2. Gradient PCR

The stock and working solutions of the primers were made at concentrations of 100 µl and 20 µM respectively. The gradient PCR was performed with Bio-Rad My Cycler^{TM} thermocycler at temperatures varying between 52 and 66°C. Pure species of PCR products were obtained by electrophoretic separation. The PCR products were run on a 2% agarose gel in 0.5X TBE (Tris-borate-EDTA) buffer for 75 minutes at 400 volts.

5.2.4.3. Bulk PCR and Band elution of PCR product

To have sufficient amounts of PCR product for cloning and sequencing a total of 50 µl of the reaction at each particular temperature was loaded and ran on a 1.5% agarose gel in 1X TBE (Tris-borate-EDTA) buffer stained with Gel Red. The specific bands of interest were cut and purified from gel slices, following the instructions of the manufacturer (Wizard SV Gel and PCR Clean –up System, Promega, USA).

5.2.4.4. Cloning

Ten ligation mixes were prepared in advance, containing 5 µl ligation buffer, 1 µl vector, 3 µl insert, and 1 µl T4 enzyme. 50 µl of thawed TOP10 competent *E.coli* was supplemented with 2 µl of a ligation mixture and incubated on ice for 20 minutes. Further, a heat shock of 42°C was applied for 70 seconds after which the *E.coli* were immediately placed back in the ice. 950 µl of SOC medium (Tryptone 2%, Yeast Extract 0.5%, NaCl 0.05%, KCl 0.02%) was added and the pH adjusted to at 7. Next, 1.8 ml of filtered glucose 20% and 0.5 ml filtered $MgCl_2$ (2M) was added, and the mixture incubated at 37°C at 210 rpm for 1hour. Afterward, a sample of 30, 150, 300 µl was spread on LB supplemented ampicillin (100 mg ml^{-1}) plates and grown in an incubator at 37°C for 16 hours. A single colony was picked up, and colony PCR was performed to see only for positive clones. These clones were picked and grown overnight in LB broth containing ampicillin.

5.2.4.5. Plasmid extraction and sequencing

The protocol for plasmid extraction is described by the manufacturer of the kit (Wizard Plus Minipreps DNA purification system, Omega). NanoDrop 2000 (Thermo Scientific) measured

the concentration of that plasmid. The number of plasmid copies was calculated according to the formula of Whelan et al. (2003) and used to build the standard curve for each gene.

The concentration of the primers was between approximately 10 and 20 ηg μl^{-1} and the final volume was 10 µl. Primers T7 and SP6 were used as reverse and forward sequencing primers, respectively. The genomic sequencing company LGC (Germany) conducted the sequencing. The software Vector NTI version 15.0 was used to edit the sequence database. The selected primer sequences were blasted on NCBI to verify their specificity for the respective AMPs genes.

5.2.5. Real-time qPCR standardization of primer concentrations

The concentration of genes was optimized based on the manufacturer's instructions (Thermo Scientific Maxima SYBR Green/ROX qPCR Master Mix (2X)). Each primer was prepared at different concentrations varying from 0.1 to 0.35 µM to select the optimal level for defining the standard curve. A standard curve was drawn by plotting the natural log of the threshold cycle (C_T) against the natural of the number of molecules. The plasmids were serially diluted from 10^2 to 10^6 plasmid copy μl^{-1}. The standard curve was run in triplicate per sample in the StepOneTM Real-Time PCR System thermal cycler (Applied Biosystems, Belgium). The concentration of the primer that gave an optimal efficiency of 90 – 110 % with a slope between -3.1 and -3.6 was selected to determine the standard curve for C_T value calculations.

5.2.6. Total RNA extraction and Reverse Transcription

Before the RNA extraction, the larvae were homogenized using the stomacher homogenizer machine (Minibeabbeater, Biospec products, Branson 1200 Model B1200E-1, USA). Tissue debris of the larvae was removed using the Qiashredder apparatus (Qiagen, Hilden, Germany) to avoid clogging of the RNA extraction columns. Total RNA was extracted using the SV Total RNA Isolation System kit (Promega, USA) following the manufacturer's instructions. Extracts were subsequently treated with DNase I (Fermentas, Germany) to remove the remaining DNA. The RNA concentration was checked with the NanoDrop 2000 (Thermo Scientific) and adjusted to 500 ng μl^{-1} in all samples. The complete DNA degradation within the RNA samples was confirmed by running the DNase-treated RNA sample in the

PCR. After confirmation of the RNA quality by electrophoresis, it was stored at – 80°C for subsequent use.

Reverse transcription was performed with a RevertAidTM H minus First strand cDNA synthesis kit (Fermentas GmbH, Baden-Württemberg, Germany) following the manufacturer's instructions with some modifications (Ruwandeepika et al., 2011). Briefly, a mixture of 1 µg RNA and 1 µl random hexamer primer solution was prepared. Then, 8 µl of the reaction mixture containing 4 µl of 5x reaction buffer (0.25 mol^{-1} Tris-HCl pH 8.3, 0.25 mol^{-1} KCl, 0.02 mol^{-1} $MgCl_2$, 0.05 mol^{-1} DTT), 2 µl of 0.01 mol^{-1} dNTP mix, 20 units of ribonuclease inhibitor, 200 units of RevertAidTM H minus M-MuLV Reverse Transcriptase was added. The reaction mixture was incubated for 5 min at 25°C followed by 60 min at 42°C. The reaction was terminated by heating at 70°C for 5 min and then cooled to 4°C. cDNA samples were checked by PCR and stored at −20°C for further use.

5.2.7. Real-time PCR

Real-time PCR was used to quantify the level of expression of the selected antimicrobial peptide genes. This procedure was performed using Maxima® SYBR Green/ROX qPCR Master Mix (Fermentas, Fisher Scientific, Erembodegem, Belgium) as described previously by Yang et al. (2014) with some modifications. Briefly, the reaction was performed in a StepOneTM Real-Time PCR System thermal cycler (Applied Biosystems, Belgium) in a total volume of 25 µl, containing 12.5 µl of 2 × SYBR Green master mix, 300 nM of forward and reverse primers and 2 µl of template cDNA. The thermal cycle parameters used for the real-time amplification were an initial activation at 50°C for 2 minutes, initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and primer annealing at 60°C (gene 18srRNA), 63°C (genes mytimycin, and mytilinB) , and 58°C (genes lysozyme and defensin) and elongation at 60°C for 1 min. Dissociation curve analysis in the real-time PCR was performed to check for the amplification of untargeted fragments. Data acquisition was carried out with the StepOneTM Software.

5.2.8. Real-time PCR analysis ($2^{-\Delta\Delta C}{}_T$ Method)

The real-time PCR was validated by amplifying serial dilutions of cDNA synthesized from 0.5 µg of RNA isolated from mussel larvae samples. Serial dilutions of cDNA were amplified by

real-time PCR using gene specific primers. ΔC_T (average C_T value of target – average C_T value of 18srRNA) was calculated for the different dilutions and plotted against the cDNA concentration. The slope of the graph was almost equal to 0 for all four target genes. Therefore, the amplification efficiency of reference and the target genes was considered to be equal. Based on this precondition, real-time PCR data were analyzed using the $2^{-\Delta\Delta C_T}$ method (Schmittgen and Livak, 2008).

The relative expression was calculated as Relative expression = $2^{-\Delta\Delta C_T}$.

$$\text{With } \Delta\Delta C_T = \Delta C_{T,\text{target}} - \Delta C_{T,\text{ control}}$$

$$\Delta C_{T,\text{target}} = C_{T,\text{ target, time x}} - C_{T,\text{ 18srRNA, time x}}$$

$$\Delta C_{T,\text{ control}} = C_{T,\text{ control, time 3}} - C_{T,\text{ 18srRNA, time 3}}$$

RNA extracts from unchallenged mussels taken at 3h were used as a reference: expression in this sample was set at 1 and all other data were normalized accordingly. The 18SrRNA gene was used as a reference gene.

5.2.9.Phenoloxidase activity

The protocol as reported for *Artemia* Baruah et al. (2011) was slightly modified for use in mussel larvae (De Rijcke et al., 2015). Briefly, based on sufficient cellular material (> 0.1 g), the enzymatic transformation of L-3, 4-dihydroxyphenylalanine (L-DOPA) to dopachrome can be used to determine the phenoloxidase (PO) activity of tissues. Before the actual PO analysis, the homogenizing buffer (pH 7.5) was made (0.43% NaCl, 1.25 mM EDTA, 0.5% Triton-X, 5 mM $CaCl_2$). With this buffer, a 0.5 mM stock solution of L-DOPA was prepared (all chemicals purchased from Sigma-Aldrich, Germany). Then, larvae (10% w:w) were homogenized in the buffer using a pestle and stored overnight at 4^0C. Tube filters (0.22 µm) removed cellular debris the following day through centrifuging (10,000 g, 20 min). Triplicate 20 µl aliquots of the resulting larval protein extracts were placed in a 96-well tissue culture plate, and 200 µl of the L-DOPA solution was added to each well. Blank homogenizing buffer samples were included for determining the non-enzymatic dopachrome production. The tissue culture plates were dark-incubated at 30^0C, and absorbance (λ = 490 nm) was measured twice a day for the next 48 hours using the Tecan spectrophotometer (Tecan I-

Control). For all treatments, differences in average optical densities at time t and 0 were calculated.

5.2.10. Statistics and analysis

Data analysis was carried out using the Statistical Package for the Social Sciences (SPSS version 23). Statistical significance of differences in survival between treatment groups was assessed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test.

To study the effect of the treatments on expression levels (ΔC_T) of the different genes and on PO a repeated measures ANOVA taking account of the replicate measurements was performed, with treatment and time as factors. The significance of the interaction between treatment and time was tested. In case the interaction effect was significant, a one-way ANOVA or t-test was performed for each time point followed by post-hoc tests with Bonferroni correction. For all statistical analysis a 5% significant level was used. The changes in expression between a 0.5 and 2- fold threshold are often not considered as biologically relevant (Figure 5.2, Figure 5.4, and Table 5.3).

5.3. Results

5.3.1. Survival

Survival of mussel larvae in Experiment 1 at 6h, 12h, and 24h was 100% for all treatments (data not shown). However, significant differences were observed between the treatments 48h post-exposure, whereby the addition of PHB-A lowered the mortality for the larvae challenged with *V. coralliitycus* (L+VC+PHB-A) compared to challenged larvae without PHB-A treatment (L+VC). The survival was highest for non-challenged larvae L (CT) and L+PHB-A (Figure 5.1).

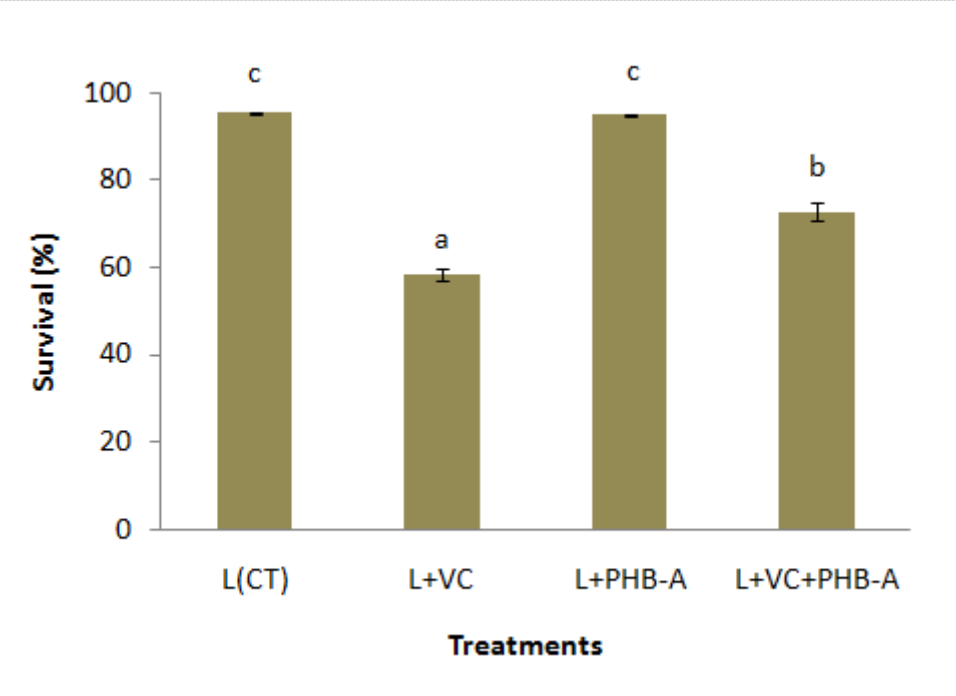

Figure 5. 1: Experiment 1. Survival (%) of mussel larvae after 48h exposure to *V. coralliilyticus* (10^5 CFU mL^{-1}) in the presence or not of PHB-A (1 mg L^{-1}). Data are expressed as mean ± Standard Error (SE) of three replicates. Different letters indicate significant differences ($p < 0.05$).

5.3.2. Design of immune gene primers for blue mussel larvae

Table 5.2 gives an overview of the primers that were designed to evaluate the gene expression of the 4 selected antimicrobial peptides in mussel larvae by qPCR, using 18srRNA as a housekeeping gene. A standard curve based on the amplification efficiency for each gene was established (Table 5.3).

Table 5. 2: Characteristics of the primers used for expression analysis with RT-qPCR

No	Gene	GenBank accession no.	Primer	Amplicon length (bp)	Annealing temperature
1	18srRNA	L33448.1	F: TTAAGAGGGACTGACGGGGG R: TTGGCAAATGCTTTCGCAGT	93	60 °C
2	Mytimicin	JN825739.1	F: CCATTGTTGGGACTGCACTG R: CGGTCCCCACGTTCATAACA	123	63 °C
3	MytilinB	AF177540.1	F: CAGAGGCAAGTTGTGCTTCC R: GGAATGCTCACTGGAACAACG	125	63 °C
4	Lysozyme	AF334662.1	F: CCAACGACTATTCATGTGCCT R: TCCCCTTGGACCTCCATTGT	122	58 °C
5	Defensin	JN935271.1	F: CCCAGCACCGATTCTAGGAC R: AAGCGCCATATGCTGCTACT	140	58 °C

Table 5. 3: RT-qPCR amplification efficiencies for all immune genes

No.	Gene	Funtion	Slope	R^2	Amplification efficiency (%)
1	18SrRNA	Housekeeping gene	-3.5	0.99	93
2	Mytimycin	Target gene	-3.2	0.99	103
3	MytilinB	Target gene	-3.1	0.98	109
4	Lysozyme	Target gene	-3.3	0.98	100
5	Defensin	Target gene	-3.3	0.98	102

5.3.3. Impact PHB-A and/or pathogen on the expression of the antimicrobial peptides (Experiment 2).

The addition of the pathogen (L+VC) led to a significant down regulation of mytimycin after 12 h in comparison to the control treatment. Remarkably none of the genes was upregulated upon *Vibrio* challenge neither at 6 nor 12 h. The addition of PHB-A on the other hand led to a significant up-regulation of mytimycin and defensin after 6 h relative to the control. This effect disappeared after 12h (Figure 5.2A & D). After 6 h, in the presence of *Vibrio*, PHB-A down regulates mytimycin and lysozyme. After 12 h this is only the case of lysozyme. The levels of defensin gene expression were equal for all treatments and time points except for treatment L+PHB-A+VC at 6h post-exposure (Figure 5.2D). The overall expression pattern, across treatments and time points, is rather similar for mytimycin and defensin. Both *Vibrio* challenge and PHB-A seem to down regulate lysozyme expression at both time points.

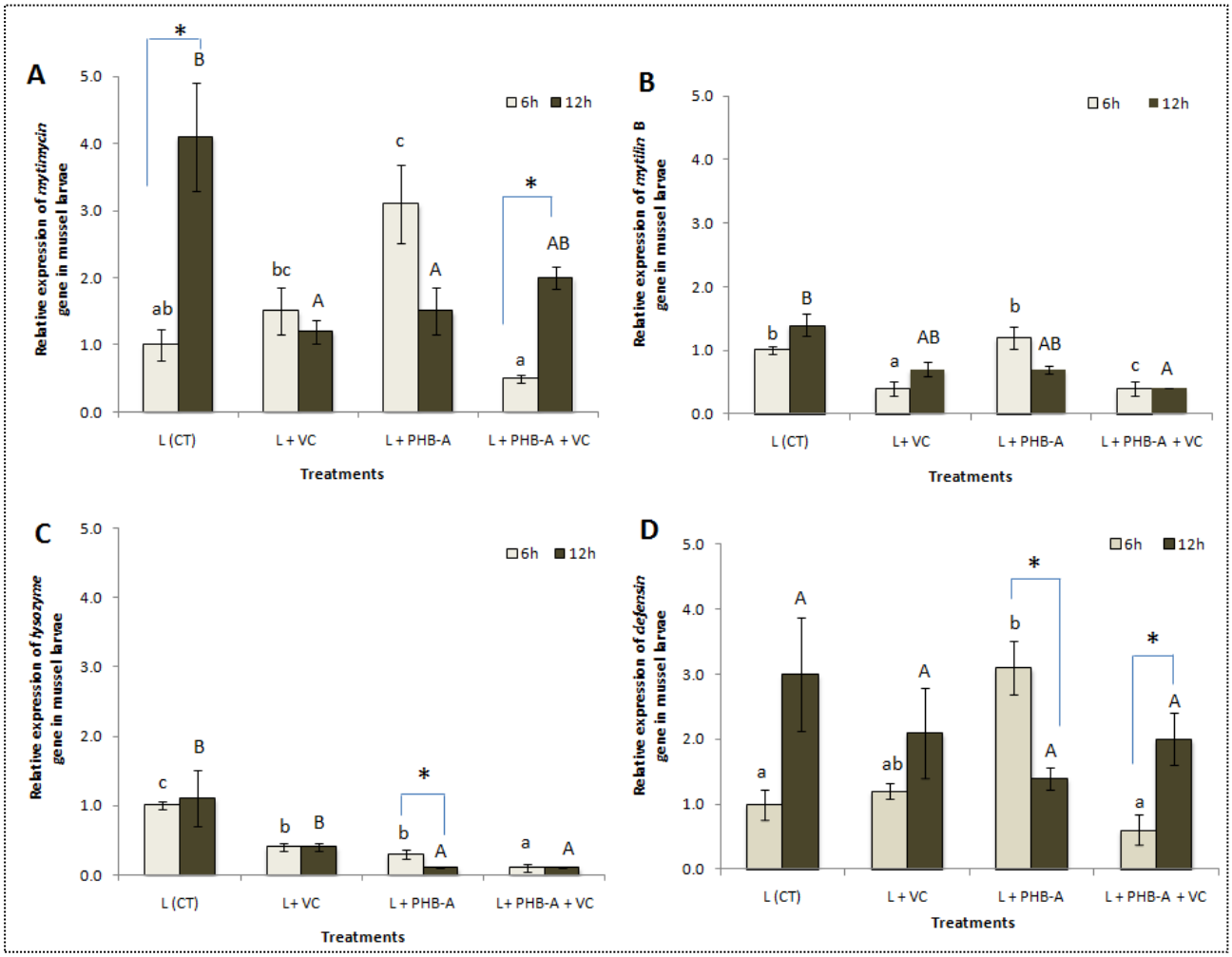

Figure 5. 2: Experiment 2. Expression of immune genes (A) mytimycin, (B) mytilinB (C) lysozyme, and (D) defensin in unchallenged (L) and challenged (L+VC) mussel larvae with *V.coralliilyticus* at 10^5 CFU mL^{-1} (n=3, mean ± standard error). PHB-A (1 mg L^{-1}) was added to the mussel larvae culture water 6 h before the pathogen. The expression of AMP genes in the control treatment (CT) was regarded as 1.0. Different small and capital letters indicate significant differences between the treatments for the sampling points 6 h and 12 h post challenge respectively. Asterisks denote a significant difference between sampling point 6 h and 12 h post challenge for a particular treatment (T-test, $P < 0.05$).

5.3.4. Phenoloxidase activity (Experiment 3)

PHB-A significantly increased the phenoloxidase activity during the 48h of the assay (Figure 5.3). However, not only PHB-A but also *V.coralliilyticus* triggered a significant increase in larval phenoloxidase activity ($p < 0.05$) as compared to the negative control (unchallenged). Especially, PO activity was significantly increased in the double treatment where the presence both of PHB-A and pathogen resulted in a higher PO activity relative to the control but also to the single treatments.

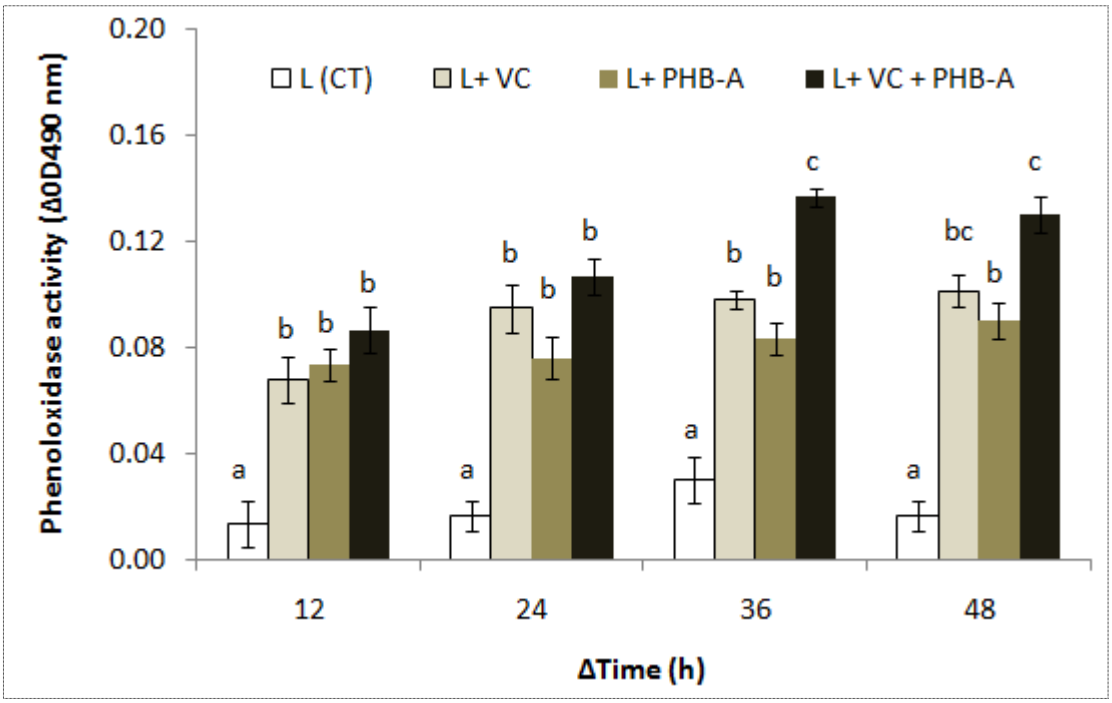

Figure 5. 3: Phenoloxidase activity (PO) in the mussel larvae collected after 48 h exposure to the different treatments (mean ΔOD±SE, n=3). Data represent the increase in dopachrome over a 48 hours time frame in which PO activity in the 4 samples was measured. Data are expressed as mean ± Standard Error (SE) of three replicates. Different letters indicate significant differences for a given time point ($P < 0.05$).

5.3.5. Time window of gene expression of AMPs (Experiment 4)

Overall, the expression of the AMPs genes in exposed mussel larvae appeared to be up-regulated during the challenge test with *V.coralliilyticus* and to rapidly increase from 24h onwards following exposure (Figure 5.4 A, B, and D). At 48 hrs, mytimycin, defensin, and mytilinB are expressed 53, 35.7 and 8.3-fold higher respectively, in comparison to the expression at time point 3h. During the 48h challenge test, the expression levels of the lysozyme are down-regulated (Figure 5.4C) except at sampling point 6h, when an up-regulation was noticed (Table 5.4). Differences between treatments because of a challenge at subsequent sampling point are shown Table 5.3.

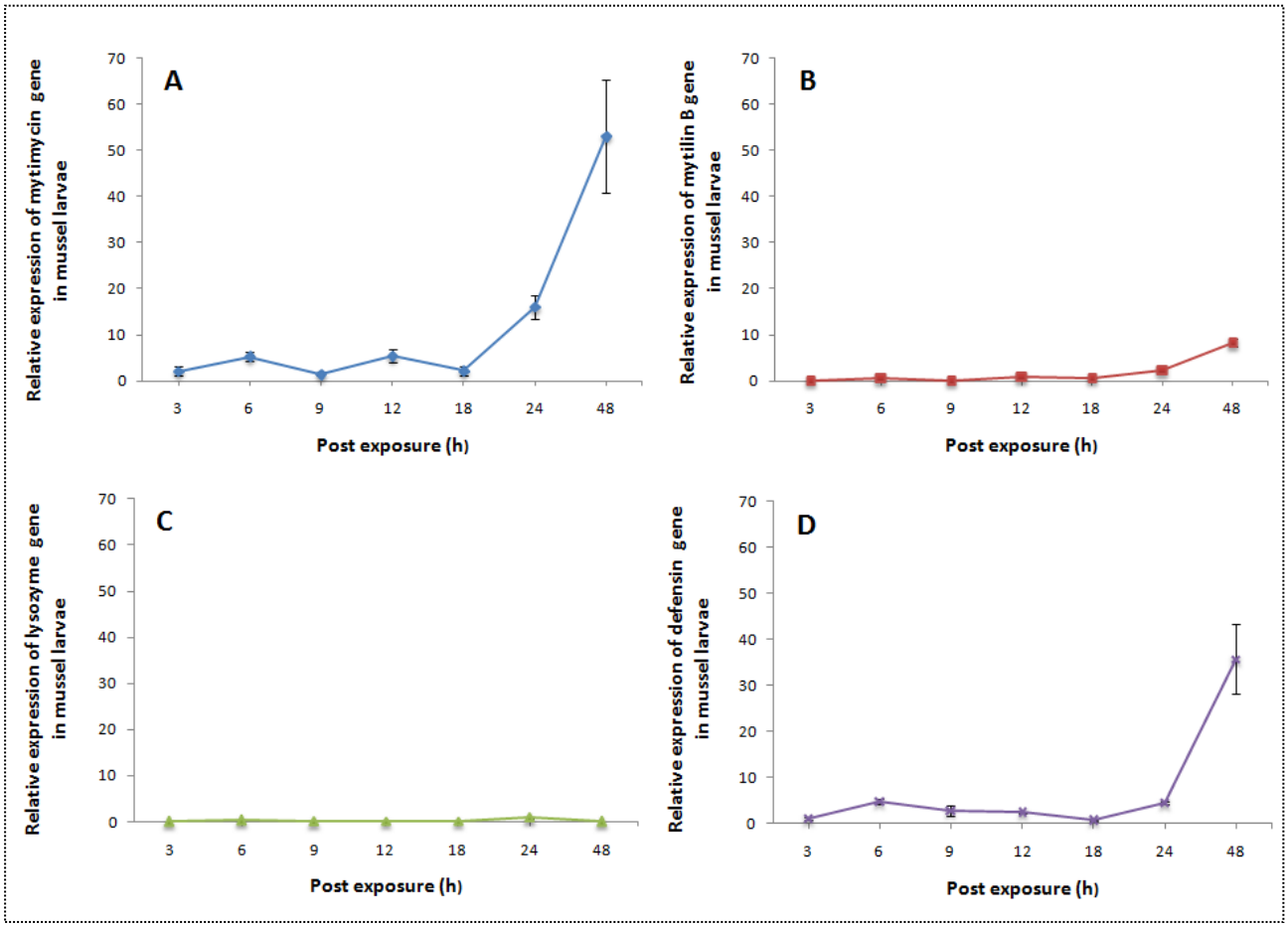

Figure 5. 4: Expression of immune genes (A) mytimicin, (B) mytilinB, (C) lysozyme and (D) defensin in challenged mussel larvae with *V. coralliilyticus* at 10^5 CFU mL^{-1} (n=3, mean ± standard error). The expression of AMPs gene in the negative control was regarded as 1.0 at 3h.

Table 5. 4:The outcome of independent t-test between challenged and un-challenged mussel larvae

Gene	Post exposure (h)				
	6	9	12	18	24
Mytimycin	*↑				*↑
MytilinB	*↓	*↓			*↑
Lysozyme	*↑	*↓	*↓		
Defensin	*↑		*↓		*↑

Asterisks denote a significant difference between challenged and un-challenged larvae at a particular sampling point relative to time point 3 h after exposure (t-test, $p < 0.05$). Symbols represent an up-regulation (↑), or down-regulation (↓). The sample of unchallenged treatment at 48h was not analyzed. The changes in expression between a 0.5 and 2- fold threshold are often not considered as biologically relevant, and are hence not included in the table. Difference in AMP expression in samples of challenged and unchallenged mussel larvae are verified by t-test at $P < 0.05$.

5.4. Discussion

The pathogenicity of this *V. corallilyticus* strain was again established in this study, confirming the validity of the strain as a pathogen using the described challenge protocol. Knowledge on the immune system in bivalve larvae is scare. In order to develop a tool box for studying the immune response in bivalve mollusk, several studies concentrated on designing specific primers for AMPs genes. Adults and larvae of *M. galloprovincialis* have been studied quite extensively: primers for mytilin, myticin, defensin, heat shock protein gene 70 (HSP70), mytilinB, lysozyme and for the house-keeping genes 18srRNA and 28srRNA have been reported (Mitta et al., 1999a, Mitta *et al.*, 1999b, Mitta *et al.*, 2000a, Mitta *et al.*, 2000b, Cellura *et al.*, 2007, Li *et al.*, 2008).

The primers developed for *M. galloprovincialis* were tested at several occasions during this research and did not work well for the blue mussel *M. edulis* larvae, challenged with *V. coralliilyticus*. In addition, the time window of gene expression of AMPs in the larval phase of

M. edulis is not determined yet. In fact, there is no information on the gene expression of mytimycin in this species although it is abundantly present in the hemolymph of *M. edulis*. Therefore it was necessary to first develop the primers for the 3 antimicrobial peptides that were reported to be present in *M. edulis* adults (Charlet *et al.*, 1996) as well as for the hydrolytic enzyme lysozyme.

The selection of the housekeeping gene 18srRNA for mussel larvae appeared to be the right choice, based on its expression in both untreated and treated mussel larvae and its stability in expression, independently from the treatment (data not shown). This allowed us to establish the kinetics of expression of the AMPs genes and lysozyme gene in blue mussel larvae in response to a *V.coralliilyticus* challenge.

Phenoloxidase activity is commonly found in invertebrates (Smith and Söderhäll, 1991) and is present in hemocytes of the adult mussel and larvae as well (Coles and Pipe, 1994, Dyrynda *et al.*, 1995). PO activity have been detected in all the disaggregated larval cells from *M. edulis*, showing a stronger reaction in the veligers (Dyrynda *et al.*, 1995) and also previously observed in cells from the inner mantle fold of the pediveliger of *O. edulis*, (Cranfield, 1974). Larval *M. edulis* cells are described to be capable of phagocytosis (Dyrynda *et al.*, 1995). Bacterial infection can cause a significant increase in PO activity in the whole hemolymph of adult bivalves *Crassostrea madrasensis* and *Chlamys farreri* (Cong *et al.*, 2008, Gijo Ittoop et al., 2010). Recently De Rijcke *et al.* (2015) determined a significant increase in PO activity in blue mussel larvae challenged with the toxin of harmful microalgae. The implications of an elevated larval PO activity are, however, largely unknown as the immunological role of PO is still poorly understood.

In this study, PO activity was detected in homogenates of *M. edulis* larvae and results show that PO enzyme activity was elicited in non-infected mussel larvae in response to larvae fed PHB-A. These findings are consistent with previous studies in other aquaculture species such as *Artemia* (Baruah *et al.*, 2011) and may at least partly explain why mussel larval survival increased when PHB-A was added to the rearing water in this study (Figure 1), also observed in previous experiments (Hung *et al.*, 2015).

For a long time, mollusk hemocytes have been reported to be responsible for bactericidal activities mediated against numerous toxic compounds (Li *et al.*, 2008), such as

antimicrobial peptides (Charlet *et al.*, 1996) and lysozyme (McHenery et al., 1979), and by phenoloxidase activity (Coles and Pipe, 1994). During the last decade, knowledge of immune processes in adult bivalves have been significantly improved by the development of genomic tools (Fleury et al., 2009, De Lorgeril et al., 2011, Fleury and Huvet, 2012). However, the immune characteristics of larvae remain under-investigated, notably due to the difficulty to isolate hemocytes from larvae. Elston (1980) observed phagocytes (described as coelomocytes) containing bacterial fragments in the visceral cavity of *C. virginica* veliger larvae. Dyrynda *et al.* (1995) have confirmed that some elements of the immune system in adult *M. edulis* are also present in the trochophore and veliger larvae of this species. Recently, several studies reported that hemocytes appear during the gastrular – trochophore developmental stages. At these stages, haemocyte generation/proliferation and induction of immune related genes are concomitant (Tirapé et al., 2007). In the mussel *Mytilus galloprovincialis*, the antimicrobial peptides mytilin and defensin have been found during and after larval metamorphosis (Mitta *et al.*, 2000a). Furthermore, lysozyme-like and hydrolysis enzyme activities are present in *C. gigas* larvae. Bivalve hemocytes seem to respond to bacteria stimulation with a burst of respiratory activity similarly to the respiratory burst of mammalian phagocytes, resulting in the generation of various free radicals or reactive oxygen species (ROS) that eliminate the phagocytized material (Labreuche et al., 2006, Lambert et al., 2007). ROS production are controlled by antioxidant defense systems to limit tissue peroxidation (Genard et al., 2013). The generation of ROS metabolites has been recorded in both trochophore and D-larvae cells of *M. edulis* (Dyrynda *et al.*, 1995).

Genard *et al.* (2013) reported that bacterial infections can have serious consequences for the survival of bivalve larvae. When a pathogen infects a host, multiple reactions occur, initiated both by the pathogen in an attempt to survive and multiply, and by the host in an attempt to eliminate the pathogen. For the host, changes induced by infection can be seen at several levels such as molecular, physiological and biochemical processes. First, infection induces the activation of both cellular and humoral immune responses that act together to kill and eliminate the infecting bacteria. In bivalves, immunity is constituted of innate processes including various serologically active molecules including antimicrobial factors and of the

phagocytosis accompanied by production of oxygen metabolites and the release of lysosomal enzymes.

After engulfment of microorganism, the phagosome undergoes maturation with acidification and sequential fusion with endosomal and lysosomal compartments including granules, which contain diverse families of antimicrobial peptides/proteins (Gueguen et al., 2009, Schmitt et al., 2012). The release into the phagosome of microcidal compounds leads to the rapid neutralization/degradation of the engulfed microorganisms. Among the hydrolytic enzymes that are released into the maturing phagosome, lysozymes are known play an important role in microbial destruction due to their lytic properties on the peptidoglycan of the bacteria cell wall (Hancock and Scott, 2000). It is likely that AMPs and lysozymes stored in hemocyte cytoplasmic granules are delivered to the phagosome to kill phagocytosed bacteria (Bachère *et al.*, 2015).

The existence and diversity of AMP has been revealed both in *M. edulis* (Charlet *et al.*, 1996) and *M. galloprovincialis* (Hubert et al., 1996). Cellura *et al.* (2007) described how AMPs respond specifically to the challenges, confirming that at least some of the innate immune mechanisms are specifically orientated. Mytimycin is linked to fungal infection (Charlet *et al.*, 1996, Sonthi et al., 2011) and also shows very low expression in the velum of *M. galloprovincialis* larvae, when challenged with *Vibrio anguillarum* (Balseiro et al., 2013). This is in contradiction to the earlier findings of Mitta *et al.* (2000a) who found that both mytilinB and defensin genes in *M. galloprovincialis* are constitutively expressed and not inducible following *Vibrio alginolyticus* (DSMZ 2171) bacterial challenge (possibly the host response is strain dependent). In addition, the expression of both genes mytilinB and defensin are recorded to be developmentally regulated, and neither gene is expressed in mussels until after larval settlement and metamorphosis according to Mitta *et al.* (2000a). This study, therefore, focused on immune gene expression in D-larvae both by PHB-A and Vibrio *coralliilyticus* at two specific time points namely 6 and 12 h after challenge. Overall the results seem to indicate that in general in this short time frame, neither PHB-A nor challenge seem to upregulate the measured immune genes (apart from some upregulation after 6h by PHB-A) This might indicate that the infection process had not started yet (in which case the fluctuations are stochastic) or that this particular species is able to control the host response

at the transcriptional level (assuming that infection had started). The PHB-A treatment did not have a major positive effect either and hence does not seem to contribute to the capacity of the host to maintain expression of the tested genes (in this time frame). In view of these observations immune genes expression was monitored over a longer time frame.

An experiment running over 48h demonstrated that mussel larvae (2-day old D-larvae) regulate the tested genes from 24h post-exposure onwards. Mytimycin is the AMPs gene with the highest expression in *M. edulis* larvae in response to the exposure to the pathogenic bacterium *V. coralliilyticus,* followed by defensin and mytilinB. This result indicates that D-larvae is either responding very late to the invading pathogen or that the pathogen is only invading the host after 24 h exposure when an increased expression of the tested genes becomes apparent. This is also in agreement with the results of the first experiment of this study where mortality only occurs after 48 h. The former explanation would be in accordance to the report of Balseiro *et al.* (2013) who stated that bivalve larvae are not entirely immune-competent to combat infections by pathogens.

However the PO activity measurement, in larvae 48 hours after exposure, seems to point in the other direction. Here PHB-A stimulates PO, possibly contributing to the capacity of the larvae to handle a Vibrio challenge, in which case PHB-A acts as an immunostimulant. PO activity is also increased upon Vibrio exposure, but that level of PO activity is insufficient for protection against Vibrio. A double exposure, PHB-A, and *Vibrio*, increased PO activity even further. These data seem to suggest that some immunological capacity is available in this developmental stages and that it can be steered, such as by the addition of PHB-A.

Taken together it is apparent that the immunological response in larvae as monitored by gene expression can only be interpreted at its best if simultaneously the infection process is visualized (by e.g. immunohistochemistry). Such data would allow a better understanding of the dynamics of the immune response.

In conclusion, this study reports the successful development of 5 primers (3 AMPs, 1 hydrolytic enzyme, 1 house-keeping gene) to monitor the immune response of blue mussel (*M. edulis)* larvae. PHB-A regulated immune gene expression was found in challenged mussel larvae, as revealed by PO activity in unchallenged and challenged (48h after exposure)

larvae. This may at least partly explain why larval survival increases in the presence of PHB-A.

It was demonstrated that 2 day-old blue mussel larvae activate the expression of the three AMPs, mytimycin, mytilinB and defensin upon invasion by the pathogen *V. coralliilyticus*. The level of expression is up- and down-regulated in the first 12hours after exposure in an unclear pattern, but rapidly increases from 24 to 48 h post-exposure. The expression of lysozyme, however, remains very low and stable during the first 48 h after challenge. Mytimycin gene is one of the genes with the strongest expression in blue mussel larvae.

Acknowledgments

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Chapter 6

GENERAL DISCUSSION

6.1. Introduction

In bivalve aquaculture species, susceptibility to viral and bacterial infections is higher in larvae than adults (Lane and Birkbeck, 2000, Arzul et al., 2001). Major knowledge gaps still exist in our understanding of the internal defense mechanisms in the early life-history of bivalves. To date, most immunological studies in bivalves have been performed on adults, probably because it is not possible to obtain blood samples from larvae and young juveniles needed to study the hemocytes responsible for the internal defense (Luna-González *et al.*, 2003). Several studies reported though that hemocyte appears during the gastrular – trochophore developmental stages and that, hemocyte generation/proliferation and induction of immune related genes are concomitant at these stages (Tirapé *et al.*, 2007). Therefore, this study accepted the challenge and concentrated on the gene expression of immune defense effectors in blue mussel (*Mytilus edulis*) larvae. Advances in characterization of these effectors in larvae may lead to a better understanding of the immune defense mechanisms and give new insights into health management and diseases control in the larval culture of bivalves.

Polyhydroxybutyrate (PHB) is a typical storage compound of carbon and energy that occurs widely in prokaryotes. This polymer can be accumulated up to 90% of the cellular dry weight of some bacteria (Uchino et al., 2008). PHB can be degraded extracellularly by many types of bacteria that secrete specific extracellular PHB depolymerases into the environment or by intracellular mobilization of PHB in the accumulating strain itself. Originally, the interest for PHB was based on its medical and industrial application possibilities (Anderson and Dawes, 1990, Chen and Wu, 2005), and it was not until recently that PHB was identified as a potential bio-control agent for aquaculture (Defoirdt *et al.*, 2007b). The use of crystalline PHB (i.e. extracted from the bacterial cells, PHB-C) as a bio-control agent has been tested and was found promising to control vibriosis in different aquaculture species (Nhan *et al.*, 2010, Sui *et al.*, 2012). However, the efficiency of PHB was shown to be considerably higher when supplied in the amorphous state (i.e. still contained in bacteria cell, PHB-A) (Halet *et al.*, 2007). Indeed, the impact of both forms of PHB on aquatic species is different due to a number of confounding factors such as the particular shape, size, digestibility, and others (cf

infra). In this study, PHB was applied as a pre-biotic compound in the larval culture of a model bivalve species, the blue mussel *Mytilus edulis* (Figure 6.1). The effects of crystalline and amorphous PHB were evaluated (1) based on the growth performance and survival of mussel larvae and early-set spat under normal rearing conditions, (2) based on the interference with virulence factors of mussel pathogens *in vitro* and the results of larval challenge tests *in vivo*, and (3) based on the larval innate immune response. In this chapter, our results are confronted with the existing literature and discussed in the light of research needs and application opportunities in hatcheries.

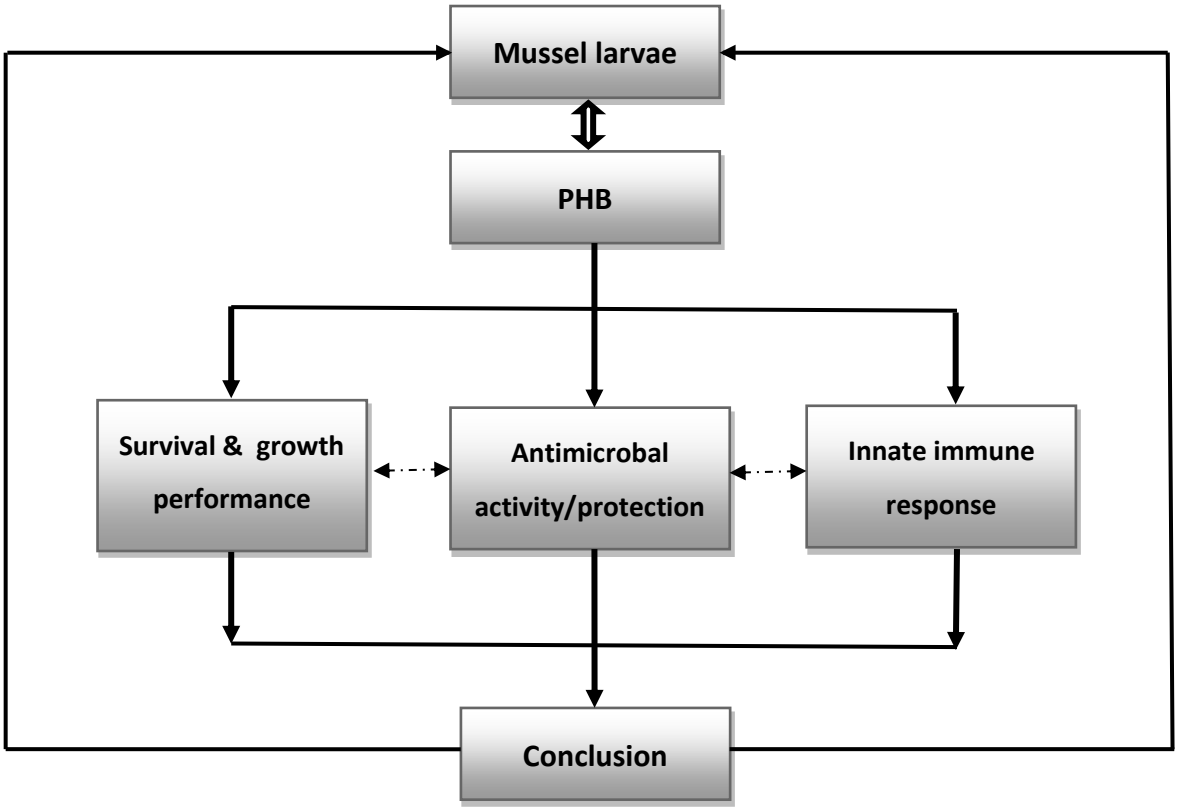

Figure 6. 1: Schematic overview of the study on the mode of action of PHB in mussel larvae culture.

6.2. The effect of PHB on bivalve larvae and spat performance

The successful application of PHB in aquaculture systems has been explored in different species and at different life stages. Based on these results, it can be stated that the beneficial effects of PHB, including acting as an antimicrobial agent and changing the microbial composition in the digestive tract of the cultured animal, depend on several

factors such as application method, a form of PHB, concentration and aquaculture species. Depending on the feeding behaviour of the target species, PHB-C can be administered either directly to the culture water (giant freshwater prawn larvae (Nhan *et al.*, 2010)), as a compound of formulated feeds (European seabass (De Schryver *et al.*, 2010), Siberian sturgeon (Najdegerami *et al.*, 2012)) or as enrichment of live feed (giant freshwater prawn larvae (Thai *et al.*, 2014), Chinese mitten crab (Sui et al., 2014). Blue mussel larvae are active filter feeders, so the direct application of both forms of PHB in the culture water proved to be successful (**Chapter 3**).

The supplementation of PHB-A into the rearing water of blue mussel larvae led to better performances than the supplementation of PHB-C. Indeed, this result may be due to their inability to efficiently metabolize PHB-C **(Chapter 3)**, despite their proven ability to ingest it. In contrast, the addition of PHB-A at a concentration of 1.0 mg L^{-1} did have a positive effect on the larval survival. This may be attributed to the fact that PHB-A is better biodegradable and has a smaller size than PHB-C (Halet *et al.*, 2007). At this stage the exact mechanism by which the PHB polymer is broken down inside the intestinal tract of animals is not known, i.e. whether it is mainly driven by physicochemical processes or by biological activity of the host and/or microorganisms present in the gut (Nhan *et al.*, 2010). Defoirdt *et al.* (2007b) and Liu *et al.* (2010) suggested that the metabolism of PHB in brine shrimp nauplii occurs at least partially by the enzymatic activity of the host. Based on the results of our study, it can be hypothesized that PHB-degrading enzymes that would enable mussel larvae to digest the compound are lacking since D-stage mussel larvae have an immature digestive system. Supplementing the strongest PHB-degrading bacterium ARC4B5, isolated from PHB-treated mussel larvae, to the larval cultures that received PHB-A, did not result in an increase in survival, on the contrary, seemed to lower the growth performance of the larvae. This is in contrast to the findings of Liu *et al.* (2010) who reported that the application of PHB-degraders as pre/probiotic in combination with PHB was better than PHB alone. As a consequence, it would seem that enzymes play a more important role in PHB-digestion than microbial activity. However, it is not clear whether the immature digestive system of D-stage larvae possess depolymerases.

It should also be kept in mind that PHB-A does not contain solely PHB, as is the case of PHB-C, but also dead bacterial cell walls. The latter may also trigger an increased immune response (Nathalie, 2001) and ultimately lead to less mortality. More and more evidence is being obtained in other species for this alternative explanation of the observed beneficial effect, basically indicating that PHB effects are multifactorial

The supplementation of PHB to the culture water of the mussel larvae did not result in faster growth nor in an improvement of spat performance in terms of growth or survival regardless form and concentration. In contrast, De Schryver *et al.* (2010) found that supplementing the diets of European sea bass with 2% or 5% crystalline PHB led to a significant increase in fish weight compared to fish fed the control feed (0% PHB). Nhan *et al.* (2010) and Thai *et al.* (2014) demonstrated the faster development of giant freshwater prawn larvae fed with *Artemia nauplii* enriched with crystalline or amorphous PHB respectively. Similarly, Sui *et al.* (2012) showed a growth- promoting effect of crystalline PHB on Chinese mitten crab larvae.

The positive effect of PHB on fish growth can be explained by the fact that the monomer β-hydroxybutyrate (β-HB) resulting from degradation (enzymatic or microbial) is known to be used as carbon and energy source for fish (Kato *et al.*, 1992, Patnaik, 2005, De Schryver *et al.*, 2010). Mussel larvae, however, do not seem to use PHB and/or its degradation products as fuel for development and growth. I hypothesize therefore that either the mussel larvae are not capable of degrading PHB or that the monomer β-HB is not used as a ketone body by this marine bivalve in contrast to vertebrates and crustaceans (Weltzien *et al.*, 1999). The latter is supported by the findings of Stuart and Ballantyne (1996) who did not detect any activity of the enzyme β-hydroxybutyrate dehydrogenase in the gills or digestive gland of adult *Mytilus edulis*, which indicates that the mussels are not capable of producing nor using β-HB. Besides, the lack of this enzyme seems to be general in marine mollusk. In other words, whilst PHB seems to stimulate the immune system of blue mussel larvae resulting in a positive effect on the survival this phenomenon has an energetic cost which affects growth, and the production of ketones does not seem to compensate for that since marine bivalves mollusks are not able to use β-HB ketones. All this requires further verification.

6.3. The effect of poly-β-hydroxybutyrate on the microbial composition in the digestive tract of blue mussel larvae

Establishing and maintaining beneficial interactions between the host and its associated microbiota are critical requirements for host health. Although the gut microbiota has previously been studied in the context of inflammatory diseases, it has recently become clear that this microbial community has a beneficial role during normal homeostasis, modulating the host's immune system (Sommer and Backhed, 2013). Concerted research efforts have concentrated on optimizing livestock production (both terrestrial and aquatic) with eco-friendly alternatives to the therapeutic use of antimicrobials. In several previous studies, PHB was used as a prebiotic in aquaculture species, offering some benefits to the host primarily via the direct or indirect modulation of the gut microbiota. PHB interacts with the microbiota inhabiting the gut and induces modifications at the microbial community level and consequently affects the host level. In fact, the presence of PHB in the gut of the animal has been demonstrated to induce a steering effect on the gut microbial community in juvenile European sea bass (De Schryver *et al.*, 2010), and in Siberian sturgeon fingerlings (Najdegerami *et al.*, 2012) where the gut pH decreased from 7.7 to 7.2 suggesting that the presence of PHB in the gut led to the increased production of short-chain fatty acids (De Schryver *et al.*, 2010). Nhan *et al.* (2010) reported that supplementation of PHB in the culture water resulted in a decreased number of *Vibrio spp*. in the intestinal tract of freshwater prawn larvae.

In our research (**Chapter 3**), it was shown for the first time that PHB tends to alter the gut microbiota in mussel larvae regarding relative abundance, dynamics, and genetic diversity. Bacteria that are enhanced by PHB may represent niche populations able to metabolize PHB during the gastrointestinal passage. The capacity to produce extracellular PHB depolymerase is a widespread phenomenon amongst bacteria and was demonstrated under aerobic, anaerobic and thermophilic conditions (Tokiwa and Ugwu, 2007). A total of 22 strains of PHB degraders were isolated from the digestive system of mussel larvae and their degrading properties demonstrated. Of these 22 strains, 16 PHB-degrading isolates seemed to belong to the genus *Pseudoalteromonas*, of which isolate ARC4B5 showed the strongest PHB-degrading activity.

Our study showed that the microbial community of mussel larvae fed PHB tended to be more diverse than the control treatment, although not significantly. As a consequence, no close link could be defined between survival and growth performance on the one hand and the intestinal microbiota community structure, richness and diversity in the mussel larvae on the other hand. This is in contrast to the findings of De Schryver *et al.* (2010) and Najdegerami *et al.* (2012) who observed significant differences. A possible explanation suggests that PHB degradation does not occur in the immature digestive tract of mussel larvae because the transit time of the food particles is too short (Bayne et al., 1987). Mussel larvae clear their gut in less than 2 hours which may prevent degradation of PHB both enzymatically and microbially.

6.4. Effect of poly-β-hydroxybutyrate on disease resistance of bivalve larvae

Poly-β-hydroxyalkanoate polymers can be degraded into β-hydroxy short-chain fatty acids of which β-hydroxybutyrate (β-HB) exhibits some antimicrobial, insecticidal, and antiviral activities. The antimicrobial activity of β-HB is thought to be comparable to that of other short chain fatty acids although the antibacterial mechanism(s) of these compounds are not completely understood (Ricke, 2003, Tokiwa and Ugwu, 2007). The antimicrobial effect of β-HB towards aquaculture pathogens *Vibrio campbellii* and *Vibrio harveyi* has previously been investigated by Defoirdt *et al.* (2007b), who demonstrated that the growth-inhibitory effect of β-HB was sensitive to the environmental pH. At pH 5, growth was completely inhibited, at pH 6, growth was delayed and at pH 7, no inhibition could be observed (Defoirdt *et al.*, 2006b). It is probable that this pH-dependency can be explained by the fact that fatty acids can pass the cell membrane only in their undissociated form, which is more prominent at lower pH (Sun *et al.*, 1998). In this study (**Chapter 4**), the effect of β-HB on the growth and virulence of the bivalve pathogens was assessed in *in vitro* experiments at pH 7 and 8. It was observed that β-HB affected the virulence factors' activity of the bivalve pathogens rather than the growth. The effect of β-HB on the virulence factors depended on the pathogenic strain, the environmental pH and did not allow for a clear pattern to be distinguished. The highest β-HB concentration (125 µM) inhibited some virulence factors of the pathogenic bacteria such as hemolysis, phospholipase, but also stimulated caseinase in *V. splendidus* at

pH7 and *V. coralliilyticus* at pH8. In addition, β-HB had no effect on gelatinase, lipase, and biofilm formation. The concentration of 125 µM is biologically speaking irrelevant for mussel larvae and hence the results should be put in the right perspective.

The effect of PHB-A on larval survival during challenge experiments depended on various factors: the pathogen, PHB-A concentration and exposure time. In general, survival of mussel larvae that received PHB-A at a concentration of 1 mg L^{-1} PHB-A 6h before the challenge test was higher compared to the survival of the larvae that received the PHB-A pre-treatment 24h before the challenge test. Larvae that received PHB-A 6h before the challenge showed a 55% and 25% increase in survival measured 96h after exposure to the pathogens *V. splendidus* and *V. coralliilyticus* respectively, as compared to the controls. Increasing the pretreatment from 1 to 10 mg PHB-A L^{-1} did not further improve the results.

These results validate *V. coralliilyticus* and *V. splendidus* as being pathogenic for *M. edulis* larvae. Furthermore, it was demonstrated that PHB-A as a pre-treatment (6h) offers protection to the mussel larvae and increases survival significantly during challenge tests.

6.5. Antimicrobial peptides (AMPs) immune response in bivalve mollusks

Bivalves lack an adaptive immune system and, therefore, do not possess immune memory. Alternatively, they have developed an innate immune system involving cell-mediated and humoral components used to recognize and eliminate pathogens (Gestal *et al.*, 2007). Antimicrobial peptides (AMPs) are essential elements of the innate host response to microbial invasion (Gestal *et al.*, 2007). The study of antimicrobial peptides is important to increase our basic knowledge on shellfish immunity, but also to evaluate the potential of these peptides as disease management tools in aquaculture.

They are usually characterized by their small size, heat-stability and a broad range of antimicrobial activity (Licheng et al., 2009). Antibacterial activity was first reported in mollusks in the ‘80s (Kubota et al., 1985) whereas the isolation and characterization of true AMPs from the mussels *Mytilus galloprovincialis* (Hubert *et al.*, 1996) and *M. edulis* (Charlet *et al.*, 1996) date back to 1996. Taking into account the features of their primary structure and their consensus cysteine array, these peptides were classified into four groups: i) mytilins, with five isoforms (A, B, C, D and G1) (Mitta *et al.*, 2000a, Mitta et al., 2000c), ii)

myticins, with three isoforms A, B (Mitta *et al.*, 1999a, Mitta *et al.*, 1999b), and C (Pallavicini *et al.*, 2008) iii) defensins, found in both mussel and oysters, with two isoforms (MGD1 and MGD2) in *M. galloprovincialis*; defensin A and B in *M. edulis*, two isoforms in the Pacific oyster, *Crassostrea gigas* (Cg-Def1 and Cg-Def2), and AOD (American oyster defensin) in *C. virginica* (Charlet *et al.*, 1996, Mitta *et al.*, 2000a, Seo et al., 2005, Gonzalez *et al.*, 2007) and iv) mytimicin, partially characterized from *M. edulis* plasma (Charlet *et al.*, 1996).

In a first research line (**Chapter 5**), three genes coding for the AMPs mytimycin, mytilin B, defensin and one gene coding for the hydrolytic enzyme (lysozyme) were selected to study the immune gene expression in blue mussel larvae when exposed to the pathogen *V. coralliilyticus*. The mytimicin gene is one of the AMPs only present in *M. edulis*, but not in *M. galloprovincialis* whereas mytilinB, and defensin exists in both *Mytilus* species (Tincu and Taylor, 2004). A first challenge was to develop the primers for AMPs that work specifically for blue mussel (*M. edulis*) larvae.

Next, these home-designed primers were used to optimize the Real-time PCR protocol for blue mussel larvae and to investigate the immune gene response of the mussel larvae when challenged with *V. coralliilyticus*, as opposed to the traditional approach where functional assays and histological studies determine the lesions and the interactions between host immune system and pathogens (Figueras and Novoa, 2004). To date, most of the information on mussel larval immunity is based on phagocytosis assays (García-García et al., 2008) chemo-illuminescence production and nitric oxide release (Costa et al., 2009). Information on gene expression of the immune response in mussel larvae tissue homogenates is scarce. Mitta *et al.* (2000a) analyzed the expression of the genes encoding for the AMPs mytimicin, mytilin B, defensin, and lysozyme in the hemocytes of adult animals subjected to various stress factors, as well as in tissue homogenates during larval development.

Overall the results seem to indicate that in a short time frame (up to 12 h), neither PHB-A nor challenge seem to up-regulate the measured immune genes (apart from some up-regulation after 6h by PHB-A). This might indicate that the infection process had not started yet (in which case the fluctuations are stochastic) or that this particular pathogenic strain is

able to control the host response at the transcriptional level (assuming that infection had started). The PHB-A treatment did not have a major positive affect either and hence does not seem to contribute to the capacity of the host to maintain expression of the tested genes (in this time frame). In view of these observations immune genes expression was monitored over a longer time frame.

When considering a time frame of 48 h, the response of exposed mussel larvae appeared soon after 3 hours, but a drastic increase in expression of mytimycin 48 h after exposure to the pathogen as well as induced up-regulation of defensin and mytilinB from 24h post-exposure onwards was observed. The latter is interesting since the expression of both genes mytilinB and defensin are reported to be developmentally regulated, and not being expressed in mussels until after larval settlement and metamorphosis (Mitta *et al.*, 2000a).

These results are in agreement with the results of Gestal *et al.* (2007) who found that the expression levels in carpet shells (*Ruditapes decussates)* increased from 24 to 48h after exposure to a mixture of both dead and alive *Vibrio anguillarum*. When comparing the gene expression of the 3 AMPs in infected mussel larvae, mytimicin was found to be highly expressed, suggesting that the production of this AMP is quite important as a protection mechanism in mussel larvae against invasive pathogenic bacteria.

This result indicates that D-larvae are either responding very late to the invading pathogen or that the pathogen is only invading the host after 24 h exposure when an increased expression of the tested genes becomes apparent. This is also in agreement with the results of the first experiment where mortality only occurs after 48 h. The main clinical signs observed in challenged scallop larvae for 24 h were bacterial swarms on the margins of the larvae, extension and disruption of the velum, detachment of velum cilia cells and digestive tissue necrosis (Beaz-Hidalgo *et al.*, 2010) and very high mortality (98 -100%) in challenged oyster larvae less than 48 h (Rojas et al., 2015).

In addition, AMPs are found to be abundant in hemocytes which are an important barrier defense in the mussel. Conversely, during pathogen exposure, the expression of the lysozyme gene was stable and seemed not to be induced when the mussel larvae were exposed to the pathogen *V. coralliilyticus.* This seems logical since lysozyme can be in both

the gut and the digestive system of the mussel. However, lysozyme were able to differentiate amongst bacteria species, even between two vibrio species. The expression of lysozyme was downregulate to *V. coralliilyticus* whereas may be strongly modulate to another stressors (Li et al., 2010). Further studies must address the behavior of the other known immune molecules in order to obtain a complete overview of the innate response.

The main goal of the present research was to study the gene immune reactions produced after stimulation with pathogenic *Vibrio* bacteria and PHB-A. This new information will contribute to increased knowledge about the defense mechanisms in this species, which is of major interest to the aquaculture sector.

6.6. Induction of phenoloxidase enzyme activity in the early life stage of bivalve larvae by PHB.

Phenoloxidase (PO) activity is considered a constituent of the immune system and is probably responsible, at least in part, for the defense mechanism upon non-self recognition in crustaceans, insects (Hernández-López et al., 1996) and bivalve mollusks (Coles and Pipe, 1994, Dyrynda *et al.*, 1995). PO is present in hemocytes of the adult mussel and also detected in disaggregated larval cells from *M. edulis*, showing a strong reaction in the veligers (Dyrynda *et al.*, 1995). PO activities always had a strong relationship with pathogen levels: f.ex. bacterial infection caused a significant increase in PO activity in the whole hemolymph of the bivalves *Crassostrea madrasensis* and *Chlamys farreri* (Cong *et al.*, 2008, Gijo Ittoop *et al.*, 2010). Recently De Rijcke *et al.* (2015) recorded a significant increase in PO activity in challenged blue mussel larvae with toxic harmful microalgae.

The presence of PO in the hemolymph of bivalve mollusks, and its stimulation by bacterial and fungal cell wall components in the adult mussel *Perna viridis* confirm that PO plays a role in the internal defense mechanism (Deaton et al., 1999). The implications of an elevated larval PO activity are, however, largely unknown as the immunological role of PO is still poorly understood. The current study (**Chapter 5**) indicated that the addition of PHB-A increased PO enzyme activity in the early life stages of blue mussel larvae. The combination of V. *coralliilyticus* and PHB-A stimulated the PO activity in mussel larvae, indicating that PHB-A treatment might facilitate a PO response to a pathogen.

6.7. The proposed model for future application of amorphous PHB in bivalve larvae culture.

Recently, under lab conditions, PHB has been successfully applied to some aquaculture species, resulting in an increase in survival, grow performance and disease resistance. In this thesis work, we demonstrated that the use of amorphous PHB-A was more advantageous than crystalline PHB, leading to higher larval survival both under challenged and unchallenged conditions. Possibly, the addition of the bacterial cell walls plays a role as well as an immune-stimulating agent. This research indicated that the application of PHB as a biological control against bacterial infection in bivalve mollusk aquaculture would be a valid option, and certainly has potential benefits for the bivalve hatchery industry. It was demonstrated that PHB-A acts mainly as an immune-stimulating compound, enabling the blue mussel larvae to protect themselves at least partly against the invasion of pathogens. The concentrations of PHB used in this study were significantly lower than those used for other aquaculture organisms which make it even more attractive as a disease control tool. The fact that PHB-A seems to have a positive role also opened the possibility to apply microbial biomass rich in PHB rather than PHB-C. PHB-C needs to be chemically extracted from PHB-rich microbial biomass, increasing the price of its production. A thorough validation of the benefits of PHB-A at a commercial hatchery level might provide the necessary data to verify the economic feasibility of PHB-A application.

6.8. General discussions

The survival of the mussel larvae almost doubled following the application of 1 mg L^{-1} PHB-A to the culture water of blue mussel larvae. This research demonstrated that amorphous PHB was more effective than crystalline PHB in mussel larvae culture. Both forms of PHB did not improve larval growth or metamorphosis and appeared not to be used as an energy source in mussel larvae. PHB-A can partly protect mussel larvae against the specific pathogenic bacteria *V. splendidus* and *V. coralliilyticus,* but the effect depended on both PHB-A concentration and exposure time. The beneficial effect of PHB-A could also be attributed to the presence of the dead bacterial cell walls, but this needs further investigation.

In general, pretreatment of mussel larvae with PHB-A at a concentration of 1 mg L^{-1} resulted in better larval survival when pretreated 6h before the challenge test compared to being treated 24h before the challenge test. B-HB had some impact on selected virulence factors of the pathogenic bacteria but there was no clear pattern, and the effects were only observed at concentrations that were not biologically relevant. Interestingly, the supplementation of PHB-A regulates gene expression in challenged mussel larvae but also triggers increased PO activity. The AMP gene expression in mussel larvae challenged with *V. coralliilyticus* was strongest from 24 h after challenge onwards. The expression of defensin is for the first time described in blue mussel larvae. Based on these results we can make a strong assumption that PHB-A has an effect on the immune system of the blue mussel larvae rather than having an anti-bacterial activity against the pathogens.

6.9. Further perspectives

1. By linking the changes in AMP gen-expression with the actual production of the AMP through Western blot (for which antibodies would be needed), one would gain a better insight in the biological meaning of the registered up- and downregulation of the responsible genes.

2. The expression of the AMP-genes has been described to depend on the nature of the stressor. It would be very interesting to monitor gene expression and the effect of PHB when comparing different stressors such as temperature shock and physical stress caused by handlings common in hatcheries (sieving f.ex.). It would give a better insight in the diversity of responses of the innate immune system of the mussel.

3. The regulation of AMP expression is species dependent. It would be interesting to run the experiments of Chapter 5 with Japanese oyster larvae *Crassostrea gigas*, the most important bivalve in terms of aquaculture production or with bivalve species that are renowned for their stress-sensitivity such as scallops. The genome of *C. gigas* is almost completely known and extensive literature is available on the genetic regulation of its innate immune system. As a result, changes in AMP production caused by PHB could be linked to the responsible genes.

4. The importance of the incubation time with PHB before exposure to the pathogen was obvious in our study. Best results were obtained with an incubation period of 6 h. Therefore application of PHB-A as a pre-treatment at different time slots (e.g. 18 h) could be interesting and should be related to a better understanding of the infection model of the pathogen under investigation. Mode of infection can be followed visually and histologically by fixating mussel larvae in parafilm at different time intervals. By labelling the pathogen with a green fluorescent protein (f.ex. (GFP-HI-610) (Rekecki et al., 2012), one can localize the pathogen in function of time (eventually immunohistochemistry techniques could be used as well, provided antibodies could be raised against the pathogens. Tissue damage could be visualized with standard staining methods (hematoxylin and eosin).

5. PHB-A contains not only PHB but also the bacterial cell wall. In addition, *Ralstonia eutropha* is a gram-negative bacterium possibly containing endotoxins in the cell wall which may also have an effect on mussel larvae when PHB-A is supplemented. Lee et al. (1999) produced poly (3-hydroxybutyrate) by cultivating several gram-negative bacteria, including *Ralstonia eutropha*, *Alcaligenes latus*, and recombinant *Escherichia coli*. When PHB was recovered by the chloroform extraction method, the endotoxin level was less than 10 endotoxin units (EU) per g of PHB irrespective of the bacterial strains employed and the PHB content in the cell. Therefore the endotoxins in the PHB-A used in our study is most probably negligible. Nevertheless, it would be interesting to separate the effect of the bacterial cell wall from PHB as such. It might not be easy to obtain PHB-A in a pure form. A density gradient centrifugation protocol has been described to isolate PHB, obtaining amorphous PHB with high purity (Nickerson, 1982). So it would be worthwhile to follow this possibility. It amorphous PHB could be obtained in this way with no contamination of other cellular polymers, such amorphous PHB could be of great help in unraveling the mode of action of PHB. Alternatively, we can evaluate the (interfering) effect of the cell wall by using *Ralstonia eutropha* with different PHB concentrations. This is only an approximation since the cell wall composition is most probably also affected by the different culture conditions that lead to different PHB cell concentrations.

6. The mode of action of poly-β-hydroxybutyrate in bivalve mollusk should be further investigated. This research should focus on its regulating effect on the microbial community

composition in the gastrointestinal tract of the animal, using different PHB-A concentrations. Microbial community analysis could be done on 16S rDNA by DGGE or by high throughput sequencing technology.. The latter technology has the advantage of generating a much more detailed picture of the microbial community composition, depending on the "sequencing depth". However, the downside of this method is the price of the analysis (although falling) and the overload of data that needs to processed and interpreted by specialists.

7. A combination of various immune-stimulating compounds (such as beta glucan) could further enhance the pre-biotic effect of PHB on bivalve larvae.

8. The application of PHB can be considered in bivalve hatcheries industry. It is anticipated that the necessary microbial biomass does not need to be added alive and rather small amounts need to be dosed. This makes the application of PHB in hatcheries economically feasible provided positive effects can be obtained.Research would still need to be done on e.g. dose, frequency of application and strains.

9. In an attempt to link virulence factor production with the observed *in vivo* mortality, challenged tests could be designed that compare the effect of the "wild type" pathogen with mutant pathogen that lack certain putative virulence factors and eventually such mutant that contain this virulence gene on a single copy plasmid (provided such strains can be engineered for the pathogenic strains that have been used in this study. This experimental approach could yet a much better insight into the importance of certain virulence factors in the pathogenesis.

APPENDIXES

REFERENCES/A

SUMMARY/SAMENVATTING/B

EXTRA-EXPERIMENT/C

ACKNOWLEDGMENTS/D

CURRICULUM VITAE/E

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SUMMARY

The blue mussel (*Mytilus edulis*) is an important species in Europe, not only as a model for environmental studies but also as aquaculture species. For this reason, the blue mussel was considered an ideal bivalve model species for this study. One of the main problems in the culture of bivalve mollusks are the repetitive episodes of mortality which seriously reduce the commercial production as illustrated by the phenomenon of summer mortality (SM) in juvenile oysters *Crassostrea gigas* in France, and brown ring disease (BRD) in adult carpet shells (*Ruditapes semidecussatus)* in Spain. Not only the grow out production but also bivalve hatcheries are hit by production losses. Disease outbreaks during larval stages are often caused by *Vibrionaceae* as primary agents. Traditional methods to combat bacterial diseases such as enhanced water treatment with UV and ozone or the use of antibiotics proved to be insufficient, costly and in the case of antibiotics even compromising for the environment and human health. In the last decade, a lot of research has been dedicated to finding alternative methods to control disease outbreaks. Several studies have demonstrated that the use of poly-β-hydroxybutyrate (PHB) might constitute an ecological and economical, sustainable alternative to fight infections in aquaculture. PHB is the most common polyhydroxyalkanoate that is accumulated as carbon and energy reserve by a wide variety of prokaryotes and may account for 90% of the cellular dry weight in some specific bacteria, e.g., *Ralstonia*. This PhD research is the first study to evaluate the use of PHB as an innovative production strategy to improve the performance of bivalve larvae. The following three approaches were used:

(1) First, the application of PHB in mussel larvae cultures in crystalline form (PHB-C: i.e. extracted from the bacterial cells) and amorphous form (PHB-A: i.e. still contained in the bacterial cells) was compared. PHB was supplied at concentrations of 0.1 mg L^{-1}, 1.0 mg $L^{-1,}$ and 10.0 mg L^{-1} to the culture water of the larvae, starting 2 days after fertilization. The results indicated that PHB-C was ineffective while the supplementation of 1 mg L^{-1} PHB-A combined with microalgae significantly increased the survival. It did not, however, stimulate the growth of the larvae and had no beneficial effect on the metamorphosis process. The gut

microbiota of the mussel larvae was not significantly modulated by PHB supplementation in terms of diversity and abundance and, therefore, could not be related to the larval performance. Upon further investigation, a total of 22 PHB-degrading isolates were identified from the mussel larvae fed with either crystalline PHB or amorphous PHB. 16 of which belonged to the genus *Pseudoalteromonas.* Hypothesizing better survival and growth by adding a strong PHB-degrading bacterium to the mussel culture in combination with amorphous PHB, the isolate ARC4B5 was administered at a concentration of 10^6 CFU $mL^{-1,}$ but the strain surprisingly suppressed larval growth.

(2) Secondly, the possible antimicrobial effect of PHB and its monomer in mussel larvae was investigated. Their protective effect against two pathogenic bacteria *Vibrio splendidus* and *Vibrio coralliilyticus* was studied both *in vitro* and *in vivo* at pH7 and pH8. pH-dependency can be explained by the fact that fatty acids can pass the cell membrane only in their undissociated form, which is more prominent at lower pH. For the *in vitro* tests, the monomer β-hydroxybutyrate (β-HB) was used since bacteria can degrade PHB into β-HB which is known to have antimicrobial activities. Only the highest concentration (125 mM) significantly decreased the growth of the pathogens compared to the other concentrations at both pH levels, except for *V. coralliilyticus* at pH8. The influence of β-HB on the virulence factors of the pathogens was dependent on the dosage, *Vibrio* species, and pH. A clear pattern could not be distinguished. The highest β-HB concentration (125 µM) inhibited some virulence factors of the pathogenic bacteria such as hemolysis, phospholipase, but also stimulated caseinase in *V. splendidus* at pH7 and *V. coralliilyticus* at pH8. In addition, β-HB had no effect on gelatinase, lipase, and biofilm formation. The concentration of 125 µM is biologically speaking irrelevant for mussel larvae and hence the results should be put in the right perspective

Concerning the *in vivo* tests, survival of mussel larvae that received PHB-A at a concentration of 1 mg L^{-1} PHB-A 6h before the challenge, was higher compared to the survival of the larvae that received the PHB-A pre-treatment 24h before the challenge. Larvae that received PHB-A 6h before the challenge showed a 55% and 25% increase in survival compared to the controls when measured 96h after exposure to the pathogens *V. splendidus* and *V. coralliilyticus* respectively.

(3) Finally, the impact of amorphous poly-β-hydroxybutyrate (PHB-A) (1mg PHB-A L^{-1}) on gene expression of the antimicrobial peptides (AMPs) mytimycin, mytilinB, defensin and the hydrolytic enzyme lysozyme in exposed mussel larvae was looked into during *in vivo* challenge tests with *V. coralliilyticus* (10^5 CFU mL^{-1}). Phenoloxidase (PO) activity in the challenged mussel larvae was also evaluated. RNAs were isolated from mussel larvae tissue, and AMPs were quantified by q-PCR using 18SrRNA gene as a housekeeping gene. The results of this study demonstrated PHB-A regulated immune gene expression in mussel larvae, as revealed by PO activity in unchallenged and challenged (48h after exposure) larvae. This may at least partly explain why larval survival increases in the presence of PHB-A.

It was demonstrated that 2 day-old blue mussel larvae activate the expression of the three AMPs, mytimycin, mytilinB and defensin upon invasion by the pathogen *V. coralliilyticus*. The level of expression is up- and down-regulated in the first 12hours after exposure in an unclear pattern, but rapidly increases from 24 to 48 h post-exposure. The expression of lysozyme, however, remains very low and stable during the first 48 h after challenge. The mytimycin gene is one of the genes with the strongest expression in blue mussel larvae.

In conclusion, it can be stated that the biological compound PHB supplied as freeze-dried *Ralstonia* can bring added value to bivalve larvae culture by its ability to fight pathogenic infections and stimulate immune gene expression of these species. The fact that PHB-A seems to have a positive role opens also the possibility to apply microbial biomass rich in PHB rather than PHB-C, which needs to be chemically extracted, increasing the price of its production. A thorough validation of the benefits of PHB-A at a commercial hatchery level might provide the necessary data to verify the economic feasibility of PHB-A application as a disease controlling tool.

SAMENVATTING

De blauwe mossel (*Mytilus edulis*) is een belangrijke soort in Europa, niet alleen vanuit ecologisch standpunt maar ook als aquacultuur soort. Dit is de reden waarom de blauwe mossel ook voor deze studie als model heeft gediend voor de klasse van de bivalven. Een van de belangrijkste problemen waarmee de sector te kampen heeft, zijn de recurrente periodes van mortalteit die de commerciële productie zware slagen kan toedienen zoals geïllustreerd door het fenomeen van zomermortaliteit bij juveniele holle oester *Crassostrea gigas* in Frankrijk and de bruine ringziekte bij adulte tapijtschelpen (*Ruditapes semidecussatus*) in Spanje. Niet alleen de schelpdierproducenten maar ook de broedhuizen van schelpdieren worden geconfronteerd met zware verliezen. Ziektes gedurende het larvale stadium worden vaak veroorzaakt door *Vibrionaceae* als primaire vectoren. Traditionele methoden om deze bacteriële ziekten te bestrijden zoals het doorgedreven zuiveren van het water met UV en ozon of het gebruik van antibiotica blijken niet zo efficiënt te zijn, kostelijk en bovendien, in het geval van antibiotica, zelfs compromitterend voor het milieu en de menselijke gezondheid. In het laatste decennium, is veel onderzoek gewijd aan het ontwikkelen van alternatieve methodes om het uitbreken van ziektes in de hand te houden. Verschillende studies hebben aangetoond dat het gebruik van poly-β-hydroxybutyrate (PHB) een ecologisch en economisch verantwoord alternatief kan betekenen om infecties in aquaculture te bestrijden. PHB is de meest voorkomende polyhydroxyalkanoate dat als koolstof en energie reserve wordt opgeslagen door een wijd gamma aan prokaryoten, tot zelfs 90% van het droog celgewicht in specieke bacteriële stammen zoals *Ralstonia*. Dit doctoraatsonderzoek heeft voor de eerste keer de applicatie van PHB in mollusken en dus ook in blauwe mossellarven geëvalueerd. Drie strategieën werden gevolgd:

(1) Als eerste werd de toediening van PHB in het kweekwater van mossel larven onder crystallijne vorm (d.i. geëxtraheerd uit de bacteriële cellen, PHB-C)) en amorfe vorm (d.i. nog ingecapseld in de gevriesdroogde bacteria, PHB-A) met elkaar vergeleken. PHB werd toegediend in concentraties van 0,1 mg L^{-1}, 1,0 mg L^{-1} and 10,0 mg L^{-1}, startend 2 dagen na de bevruchting van de eitjes. De resultaten toonden aan dat PHB-C geen impact had terwijl

de supplementatie van 1,0 mg L^{-1} PHB-A in combinatie met microalgen leidde tot significante verhoging van de larvale overleving. Er was echter geen effect op de larvale groei noch op het proces van de metamorphose. De samenstelling en rijkdom van de intestinale microbiële flora van de mossellarven was niet significant gewijzigd door de toediening van PHB en kon dus niet gerelateerd worden aan de larvale performantie. In een volgende stap werden in het totaal 22 PHB-afbrekende isolaten gekarakterizeerd afkomstig van mossel larven die PHB-C of PHB-A waren toegediend. Alle isolaten behoorden toe tot het genus *Pseudoalteromonas*. De hypothese dat de toevoeging van een sterk PHB-afbrekende bacterium een positieve invloed zou hebben op overleving en groei van de mossellarven wanneer die toegevoegd werd aan het kweekwater samen met PHB-A, werd ontkracht gezien de toevoeging van ARC4B5 aan 10^6 CFU mL^{-1} de larvale groei zelfs onderdrukte.

(2) Vervolgens werd de mogelijke anti-bacteriële werking van PHB en het afgeleid monomeer product onderzocht. Hun beschermende werking tegen twee pathogenen voor bivalven, *Vibrio splendidus* en *Vibrio coralliilyticus* werd zowel *in vitro* als *in vivo* uitgetest bij pH7 en pH8. De pH afhankelijkheid kan verklaard worden door het feit dat de vetzuren alleen in ongedissocieerde vorm doorheen de celwand kunnen passeren en deze vorm is meer vertegenwoordigd bij lage pH. Voor de *in vitro* tests werd de monomeer β-hydroxybutyrate (β-HB) gebruikt omdat bacteriën PHB degraderen naar β-HB dat bewezen anti-microbiële activiteiten heeft. Alleen de hoogste concentratie (125 mM) inhibeerde significant de groei van beide pathogenen onafhankelijk van de pH, met uitzondering van *V. coralliilyticus* by pH8. De impact van β-HB op de virulentie factoren van de pathogenen was afhankelijk van de concentratie, *Vibrio* soort en pH. Er kon geen duidelijk patroon waargenomen worden. De hoogste β-HB concentratie (125 µm) inhibeerde sommige virulentie factoren van de pathogenen zoals haemolyisis and phospholipase maar stimuleerde ook de productie van caseinase in *V. splendidus* bij pH7 en *V. coralliilyticus* bij pH8. Het gebruik van β-HB had geen effect op gelatinase, lipase en biofilm vorming. De concentratie van 125 µM is biologisch gesproken echter irrelevant voor mossellarven en de resultaten moeten dus voorzichtig geïnterpreteerd worden.

In de *in vivo* testen werd duidelijk dat de overleving van de mossel larven die PHB-A (1,0 mg L^{-1}) 6 hr voor de challenge toegediend kregen, hoger was dan de overleving bij larven die

PHB-A 24hr voor de challenge kregen. In vergelijking met de controle dieren, vertoonden deze eerstgenoemde larven een toename in overleving van 55% en 25% na 96h blootstelling aan de pathogenen *V. splendidus* en *V. coralliilyticus* respectivelijk.

(3) Tenslotte werd de impact van PHB-A (1,0 mg L^{-1}) op de genexpressie van de antimicrobiële peptides (AMPs) mytimycin, mytilinB, defensin en van het hydrolytisch enzyme lysozyme bestudeerd tijdens *in vivo* challenge tests met *V. coralliilyticus* (10^5 CFU mL^{-1}). Phenoloxidase activiteit (PO) in de larven werd ook gemeten. RNA werd geïsoleerd van gehomogeniseerde mossel larven en de expressie van de AMPs werd gekwantificeerd met q-PCR en met 18SrRNA gen als huishoudgen. Uit deze studie komt naar voor dat PHB-A de immuun genexpressie in larven kan regelen gezien de PO activiteit zowel in de controle als blootgestelde larven toeneemt na 48h. Dit zou deels kunnen verklaren waarom de larvale overleving toeneemt wanneer PHB-A wordt toegediend aan het kweekwater.

Er is aangetoond dat larven van 2 dagen-oud de expressie van drie AMPs, mytimycin, mytilinB en defensin activeren bij een invasie van de pathogeen *V. coralliilyticus*. Het expressie niveau gaat op en neer in de eerste 12 uren na blootstelling zonder patroon, maar stijgt snel na 24 tot 48 uur blootstelling. De expressie van lysozyme echter blijft heel laag en stabiel gedurende de eerste 48 uur na blootstelling. Het mytimycin-gen is een van de genen die het sterkst tot expressie komt in blauwe mossellarven.

Als besluit van deze studie kan gesteld worden dat PHB, aangeleverd in de vorm van gevriesdroogde *Ralstonia* een toegevoegde waarde kan bieden voor larvale cultuur van bivalven dankzij zijn directe werking op de pathogeen en stimulerend effect op de expressie van immuun-gerelateerde genen. Dit opent de mogelijkheid om microbiële biomassa rijk aan PHB te gebruiken eerder dan PHB-C dat eerst chemisch geëxtraheerd moet worden wat de productiekost opdrijft. Een grondige validatie van de voordelen van PHB-A op het niveau van een commercieel broedhuis kan de nodige data aanleveren om de economische haalbaarheid van het gebruik van PHB-A als ziekte controlerend middel te evalueren.

EXTRA- EXPERIMENT

Below are the results of an experiment supplementary to the results already shown in fig 4.2

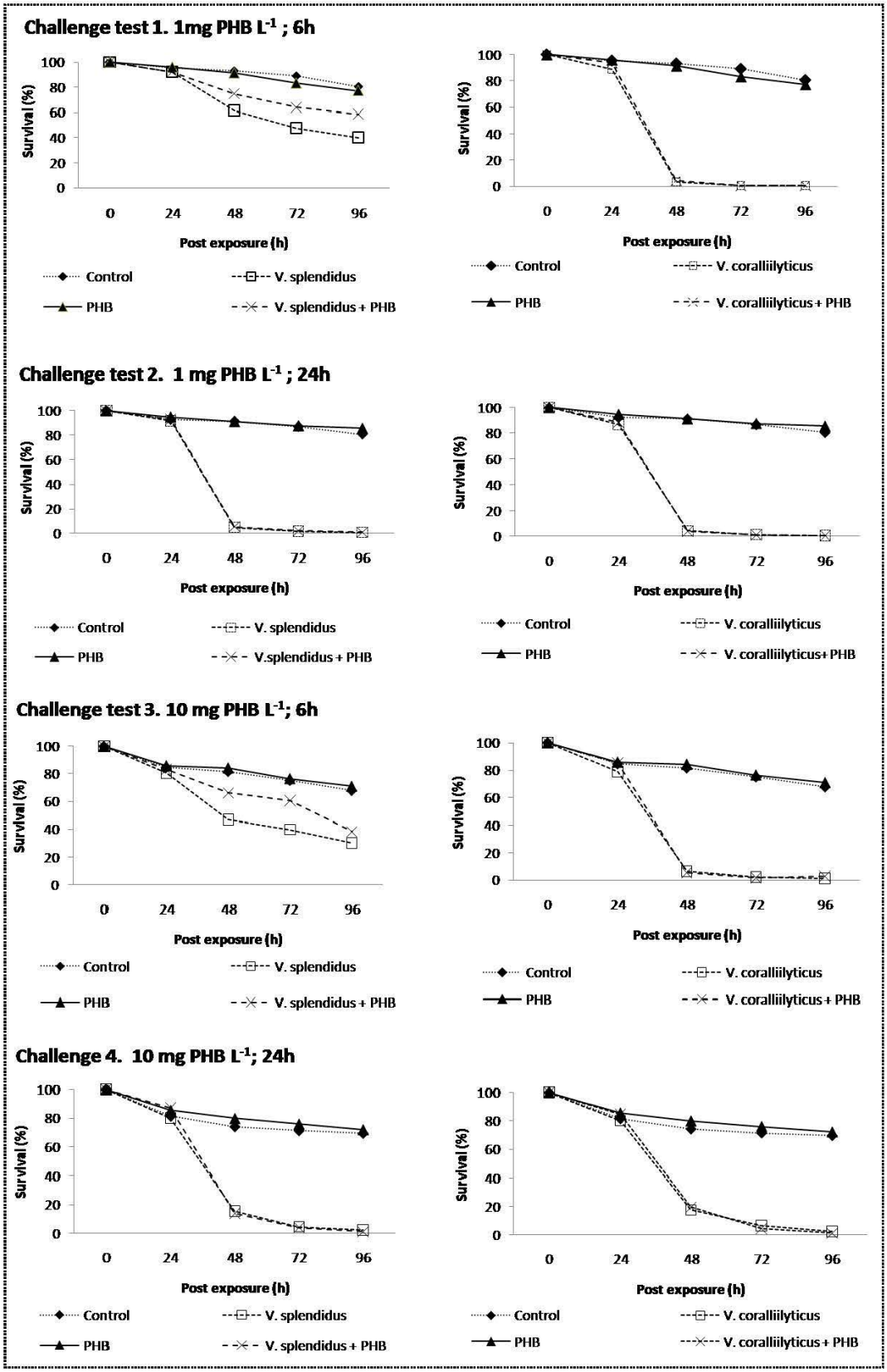

Figure 4.2. (Repeat experiment). Survival of blue mussel challenge with *V. coralliilyticus* and *V. splendidus* at 10^5CFU mL^{-1} under different experimental condition.

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Thuy, N.T.T., **Hung, N.V**., Thuy, T.N., 2009. Detecting parasites and bacteria in damselfish (*Dascyllus trimaculatus)* cultured at Nhatrang with the exophthalmia and ulcerative infection. Agriculture publishing house HCM, Vietnam, p: 694 – 702 (Abstract in English).

Hung, N.V., Bon, L.Q., Duy, V.D., Ngoc, H.T., Minh, D.M., 2008. Some classification and population genetic features of Brown- marbled grouper (*Epinephelus fuscoguttatus*, Forssakal 1775) in Khanh Hoa coastal area. Fisheries Technological Science No. 9/2008 ISSN 1859 -1299 p: 32 – 34 (Abstract in English).

E. DOCTORAL TRAINING SCHOOL / PROFESSIONAL TRAINING COURSES

- Specialist courses

- Applying Chemical Tools to study Biology, held by VIB, University of Antwerp, Belgium.

- Basis of biology for engineers, held by IMEC, University of Leuven, Belgium.

- Microbial Community Management in Aquaculture, held by ARC, Ugent, Belgium.

• Transferable skills

-Personal Effectiveness held by Doctoral Traning School, Ugent, Belgium.

-Project management held by Doctoral Traning School, Ugent, Belgium.

-Scientific writing skill, held by Doctoral Training School -VIB, Ugent, Belgium.

-Effective scientific communication held by Doctoral Traning School, Ugent, Belgium.

-Advanced Conference Skills in English held by Doctoral Traning School, Ugent, Belgium.

• Professional training courses

2009: Practice on molecular biotechnology held by Biotechnology Institute, Ha Noi, Vietnam.

2007: Grouper Hatchery Training course funded by Network of Aquaculture Centers in Asia – Pacific (NACA). Held at Brackishwater Aquaculture Development Center- Situbondo, Research Institute for Mariculture, Gondol, Indonesia.

2006: Intensive Aquaculture production, Environmental, considerations & Food safety. Held at Ministry of Agriculture and Rural Development, Center for International Agriculture Development Cooperation Cinadco, Israel.

2003: Babylon Seed production technology funded and held by Ocean Wave Seafood, Melbourne, Australia.

1999: Taxonomy and diversity of Tropical marine mollusk held by DANIDA, Denmark.

G. THESIS SUPERVISION

1. Tran Thanh Tam, 2013. The use of poly-β-hydroxybutyrate to increase robustness in blue mussel larviculture. Master of Sciences in Aquaculture at the Faculty of Bioscience engineering, Ghent University, Belgium.

2. Christine Llorente Ludevese, 2015. Antimicrobial effect of poly-β-hydroxybutyrate (PHB) on blue mussel larviculture. Master of Sciences in Aquaculture at the Faculty of Bioscience engineering, Ghent University, Belgium.