Promotors:

Prof. dr. ir. Peter Bossier (Flemish promoter)
Department of Animal Production, Faculty of BioScience Engineering
Ghent University, Gent, Belgium

Dr. ir. Peter De Schryver (Flemish co-promoter)
Department of Animal Production, Faculty of BioScience Engineering
Ghent University, Gent, Belgium

Present address:
INVE Technologies, Dendermonde, Belgium

Dr. Edgar C. Amar (Local promoter)
Fish Health Section, Research Division
Southeast Asian Fisheries Development Center-Aquaculture Department
Tigbauan, Iloilo, Philippines

Dean: **Prof. dr. ir. Marc Van Meirvenne**

Rector: **Prof. dr. ir. Rik Van de Walle**

GHENT UNIVERSITY

FACULTY OF BIOSCIENCE ENGINEERING

Artemia Reference Center

AMORPHOUS POLY-β-HYDROXYBUTYRATE (PHB) - ACCUMULATING *BACILLUS* SPP. AS BIOCONTROL AGENTS IN CRUSTACEAN CULTURE

JOSEPH LEOPOLDO Q. LARANJA, JR.

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Titel van het doctoraat in het Nederlands:

Amorfe Poly-beta-hydroxybuteraat (PHB)-geaccumuleerde *Bacillus* spp. als biologisch controlemiddel in crustaceeën

Illustration on the cover:

(1) Transmission electron micrograph of *Bacillus* sp. JL47 showing the intracellular amorphous PHB (front)
(2) Shrimp pond site in Bacolod, Philippines where the PHB-accumulating *Bacillus* sp. JL47 was obtained (back)

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Members of the examination committee (✍)

Prof. emer. dr. Patrick Sorgeloos (✍)
Department of Animal Production
Ghent University, Ghent, Belgium

Prof. dr. ir. Marc Verdegem (✍)
Aquaculture and Fisheries Group (AFI)
Wageningen University, The Netherlands

Prof. dr. ir. Frank Pasmans (✍)
Department of Pathology, Bacteriology and Poultry Diseases
Ghent University, Ghent, Belgium

Prof. dr. Daisy Vanrompay (✍)
Department of Animal Production
Ghent University, Ghent, Belgium

Prof. dr. ir. Peter Bossier (Flemish promoter)
Department of Animal Production, Faculty of BioScience Engineering
Ghent University, Ghent, Belgium

Dr. ir. Peter De Schryver (Flemish co-promoter)
Department of Animal Production, Faculty of BioScience Engineering
Ghent University, Ghent, Belgium

Present address:
INVE Technologies, Dendermonde, Belgium

Dr. Edgar C. Amar (Local promoter)
Fish Health Section, Research Division
Southeast Asian Fisheries Development Center - Aquaculture Department
Iloilo, Philippines

Chairman: **Prof. dr. ir. Pascal Boeckx**
Department of Applied Analytical and Physical Chemistry,
Faculty of Bioscience Engineering,
Ghent University, Ghent, Belgium

Secretary: **Prof. dr. ir. Peter Goethals**
Department of Applied Ecology and Environmental Biology,
Faculty of Bioscience Engineering,
Ghent University, Ghent, Belgium

This thesis is dedicated to my wife ***Rovelyn***

& to my children ***Xedric Llonel***, ***Xaraih Vlir*** & ***Xeraiah Elijah***

This thesis is dedicated to the loving memory of my mother, ***Angelita***
She left this world while I am writing the first few chapters of this book

FOREWORD

In the year 2050, the global population is expected to grow by another 2 billion from the current 7.3 billion people in the planet, thus placing a huge challenge of feeding our human population. According to FAO, the fisheries and aquaculture sector play a vital role in providing food for the people to eliminate world hunger, promoting health and reducing poverty. Although the fisheries sector provides more than half of aquatic food products worldwide, fish catches have been levelling off since the late 1980's and this is due to wild stock depletion. On the other hand, the aquaculture sector is considered as the fastest food-producing industry in the world with a huge potential to grow considerably in the future. However, its increased production, which is due to intensified culture methods, has been paralleled also by the occurrence of diseases, which hampered its progress. Thus, aquaculture scientists all over the world have been focusing their efforts in developing effective yet environmentally friendly measures to mitigate diseases to optimize (aqua)culture production without risking the environment and the human health. The application of biocontrol agents such as probiotics and the recently identified microbial agent –PHB, are some of the identified sustainable disease-controlling measures. In the context of these strategies mentioned, this research focuses on the potentials of using PHB-accumulating *Bacillus* spp. as new biocontrol agents for aquaculture. The approach of using amorphous PHB where the compound is still contained in (live) bacteria that are often associated with strong probiotic effects (e.g. *Bacillus* spp.) may further advanced the capabilities of both PHB and the probiotic strategy as important disease-controlling measures for aquaculture.

Indeed, it is worth mentioning that in reaching our goal for a food-secure and sustainable future, doing responsible fisheries and sustainable aquaculture practices are indispensable.

ACRONYMS AND ABBREVIATIONS

ABL. average body length
ABW. average body weight
AHL. acyl homoserine lactone
AHPNS/AHPND. acute hepatopancreatic necrosis syndrome/ acute hepatopancreatic necrosis syndrome/ acute hepatopancreatic necrosis disease
ANOVA. analysis of variance
AXOS. arabino-xylooligosaccharide
BFT. biofloc technology
BMN. baculoviral midgut gland necrosis
BOF. Bijzonder Onderzoeksfonds
cDNA. complementary DNA
CDW. cell dry weight
CFU. colony forming unit
CLO. cod liver oil
CMD. covert mortality disease
CoA. coenzyme A
CS. citrate synthase
CytK. cytotoxin K
DGGE. denatured gradient gel electrophoresis
DNA. deoxyribonucleic acid
EMS. early mortality syndrome
FA. fatty acid
FAO. food and agriculture organization
FASW. filtered autoclaved seawater
FOS. fructo-oligosaccharide
ftn. ferritin
GIT. gastro-intestinal tract
GOS. galactooligosaccharide
Hbl. hemolysin BL
HP. hepatopancreas
HPH. hepatopancreatic haplosporidiosis
HPM. hepatopancreatic microsporidiosis
HPV. hepatopancreatic parvovirus
Hsp70. heat shock protein 70
Hsps. heat shock proteins
IBs. inclusion bodies
IFS. International Foundation for Science
IHHNV. infectious hypodermal and hemtopoitic necrosis virus
IMNV. infectious mionecrosis virus

IMO. isomaltooligosaccharide
LAB. lactic acid bacteria
LB. luria bertani
LCFA. long chain fatty acid
lclPHA. long chain length polyhydroxyalkanoate
LGBP. lipopolysaccharide and beta-1,3-glucan binding protein
LPS. lipopolysaccharide
m/z. mass to charge
MALDI-TOF MS. matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MAMPs. microbe-associated molecular patterns
MBV. monodon baculovirus
mclPHA. medium chain length polyhydroxyalkanoate
MOS. mannanoligosaccharide
mRNA. messenger rna
NCBI. national center for biotechnology information
Nhe. nonhemolytic enterotoxin
PAMPs. pathogen-associated molecular patterns
PCR. polymerse chain reaction
PE. peroxinectin
PG. peptidoglycan
PGAPs. PHB granule-associated proteins
PHA. polyhydroxyalkanoate
PhaP. phasin
PHB. Poly-beta-hydroxybutyrate
PHB-HV. PHB-hydroxyvalerate
PL. postlarvae
PO. phenoloxidase
PP. polypropylene
proPO. prophenoloxidase
PRRs. pathogen/pattern recognition receptors
qPCR. quantitative PCR
QS. quorum sensing
REO. reovirus
RNA. ribonucleic acid
rpm. rounds per minute
S/N. signal to noise
SCFA. short-chain fatty acid
sclPHA. short chain length polyhydroxyalkanoate
SDS-PAGE. sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEAFDEC/AQD. Southeast Asian Fisheries Development Center/Aquaculture Department
SEM. standard error of the mean
SOD. superoxide dismutase
SP. serine protein
TCA. tricarboxylic acid

TGase. transglutaminase
UPGMA. unweighted pair group method with arithmetic mean
UV. ultraviolet
WSSV. white spot syndrome virus
XOS. xylooligosaccharide
YHV. yellow head virus

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GENERAL INTRODUCTION

CHAPTER 1

AQUACULTURE AND ITS DISEASES

1.1 AQUACULTURE AND ITS IMPORTANCE

Aquaculture comprises all forms of culture of aquatic animals (fish, crustaceans, molluscs, etc.) and aquatic plants (seaweeds, etc.) in fresh, brackish and marine waters. Aquaculture is considered as the fastest-growing food production sector globally, with an 8.6% increase in production per year since 1980 to 2012. Its global trend, being a lucrative industry has led to its ever continuing expansion and development and gained global importance being a major contributor of food fish (i.e. includes finfishes, crustaceans, molluscs, amphibians, freshwater turtles and other aquatic animals such as sea cucumbers, sea urchins, sea squirts and edible jelly fish produced for the intended use as food for human consumption). Of the total 158 million tonnes of total fish produced from capture and aquaculture in 2012, 42.2% was contributed by aquaculture as food fish (Figure 1.1). The aquaculture production has a reported value of 90.4 million tonnes (live weight equivalent) from which food fish comprised 66.6 million tonnes (US$ 137.7 billion) while 23.8 million tonnes (US$ 6.4billion) were aquatic algae (mostly seaweeds). In some countries, 22,400 tonnes (US$ 222.4 million) of non-food products such as pearls and sea shells (used for ornamental and decorative purposes) were also reported (FAO, 2014).

The global population is expected to grow by another 2 billion by 2050 from the current 7.3 billion, placing a huge challenge of feeding our human population. According to the Food and Agriculture Organization (FAO) (2014), the world's per capita apparent fish consumption has increased from an average of 9.9 kg in 1960s to 19.2 kg in 2012. This high demand for food fish has put pressure on our natural resources, making our fishery resources more vulnerable for exploitation. In fact, fish catches have been levelling off since the late 1980s (Figure 1.1) and this is due to wild stock depletion (Pauly, et al., 2002). In order to cope up with the increasing demand of fish supply, it is expected that the aquaculture sector, being the fastest food-producing sector, has a significant role in feeding this planet. It is expected that aquaculture will continue to grow at significant rates and will remain the fastest increasing food production system in the future. Estimations showed that aquaculture production will even have to increase to 80 million tonnes (excluding aquatic plants) by the year 2050 to meet the market demand (FAO, 2006).

Status on the world's capture fisheries and aquaculture production

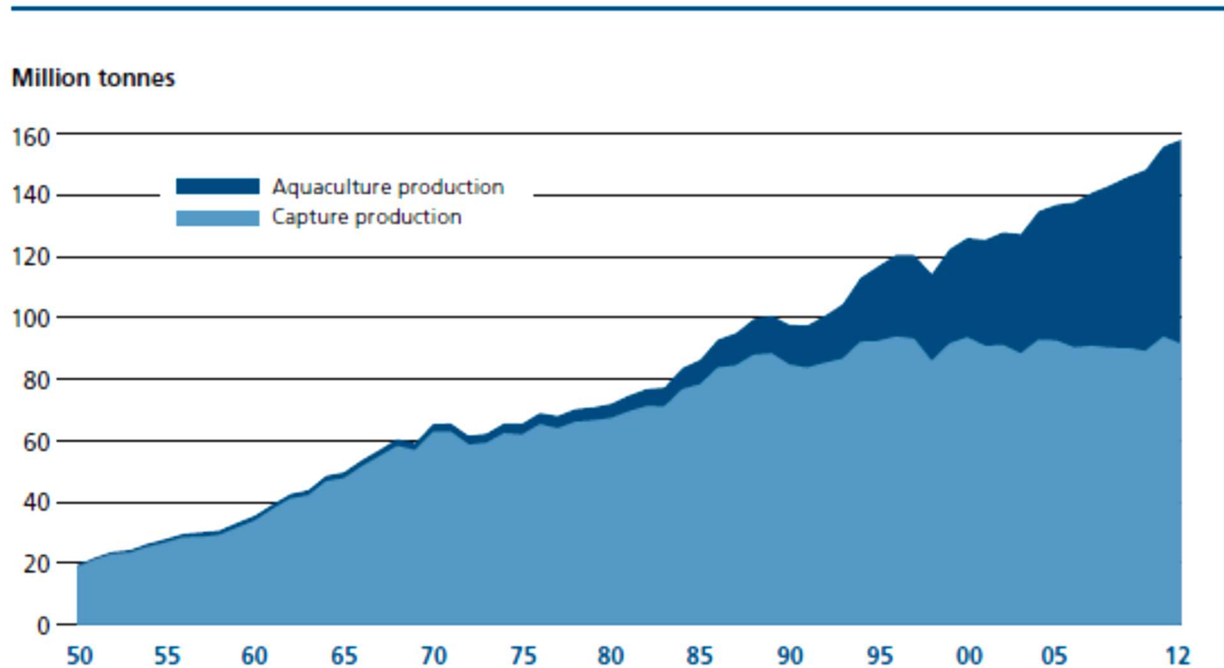

Figure 1.1 World capture fisheries and aquaculture production from 1950- 2012 (FAO, 2014)

In addition, since fish continues to be one of the most-traded food commodities worldwide, the sector has been providing jobs for millions of people. In 2012, a recorded 58.3 million people were engaged in the primary sector of capture fisheries and aquaculture and about 18.9 million were engaged in fish farming (FAO, 2014). Aquaculture provides jobs from basic labour to highly skilled technical jobs hence the sector has been offering jobs to all levels of workers in the market. Likewise, the sector also offers jobs in rural settings, wherein local people can benefit and have the chance to improve their quality of life. Indeed, FAO (2014) estimates that fisheries and aquaculture assure the livelihoods of 10-12% of the world's population.

Other positive impacts of aquaculture especially on biodiversity were also identified and among those are the following: (1) Production of fish from aquaculture can reduce the pressure on wildstocks, thus, minimizing the overexploitation of marine resources; (2) Stocking of cultured organisms from aquaculture systems may help to enhance the depleted stocks especially those organisms that have limited reproductive success; (3)

The effluents and waste from aquaculture can increase local production, abundance and diversity of species; (4) Destructive land-use practice such as slash-and-burn agriculture can be replaced by sustainable practice such as aquaculture ponds which will generate income and will aid the locals in improving their quality of life (Diana, 2009).

Conversely, the intensification of aquaculture activities to increase fish production has however created some negative impacts to the coastal ecosystem and the environment. For example, the conversion of mangrove areas and coastal wetlands to fishponds resulted in losses of essential ecosystem services generated by mangroves, including the provision of nursery habitat for diverse aquatic species, coastal protection, flood control, sediment trapping and water treatment (Naylor, et al., 2000). Furthermore, the global growth of aquaculture industries has brought an increase in the discharge of substantial amounts of polluting effluents containing uneaten feeds and feces. This organic enrichment causes environmental deterioration of both the receiving water bodies and sediments. Nitrogenous wastes (e.g. ammonium and nitrite) that exceeds the assimilative capacity of receiving waters lead to deterioration in water quality that is toxic to fish and shrimp (Hargreaves, 1998). Organic enrichment can also lead to an increase presence of pathogenic bacteria and are reservoirs of viruses associated with organic detritus (Chávez-Crooker and Obreque-Contreras, 2010). Furthermore, unrestricted and excessive use of prophylactic antibiotics to control disease infections in farmed (aquaculture) animals has brought potential risk to human health and the environment. Such unsustainable practices could lead to the emergence of antibiotic-resistant bacteria in aquaculture environments, the increase of antibiotic resistance in fish pathogens, the transfer of these resistance determinants to bacteria of land animals and then to human pathogens, and the alterations of the microbiota both in sediments and in the water column (Cabello, 2006). Furthermore, the use of antibiotics that have to be mixed with fish food also creates problems for industrial health and increases the opportunities for the presence of residual antibiotics in fish meat and fish products. Aquaculture can also lead to potential threats to aquatic biodiversity and among which are the possibilities: (1) the escapement of aquatic animals and their potential hazard as invasive species (2) genetic alteration of existing stocks from escaped hatchery products/ cultured species (3)

the overexploitation of wild fish stocks as source of fish meal and fish oil and (4) the disease or parasite transfer from captive to wild stocks (Diana, 2009).

1.1.1 CRUSTACEAN AQUACULTURE

Crustaceans are considered as high value aquaculture products with high worldwide demand. In terms of volume, it accounted for 9.7% (6.4 million tonnes) of the total food fish produced worldwide in 2012. Despite this modest volume, it represented 22.4% (US$ 30.9 billion) of the total value of the world aquaculture production (Table 1.1), thus ranked second in terms of percentage value shared in the overall aquaculture produced (FAO, 2014). In the current FAO data (2016 online database), crustacean aquaculture has a reported volume of 6.7 million tonnes with an estimated economic value of US$ 34.8 billion in 2013 (FAO, 2016). The major contributors of crustacean products come from the Asia region with a shared production of around 89.39% followed by the Americas with 10.36%. In all these regions, Penaeid shrimps have been consistently the most produced species (4.5 million tonnes; US$ 22.7 billion) followed by freshwater crustaceans (2 million tonnes; US$ 11 billion) (Table 1.2). The giant tiger prawn *Penaeus monodon* and western white-leg shrimp *Litopenaeus vannamei* are the two major Penaeid shrimp species cultured and produced worldwide, likewise, the freshwater prawn *Macrobrachium rosenbergii* is the major commodity produced in freshwater (FAO, 2016; New, 2005).

However, one of the major constraints for the production of these crustaceans has always been the frequent occurrence of diseases wherein some are even considered “emerging diseases” (Thitamadee, et al., 2016). Hence, finding strategies to control these diseases has been the primary focus for research and development even to date.

Table 1.1 World production of farmed species groups from inland aquaculture and mariculture in 2012 (FAO, 2014).

	Inland aquaculture	Mariculture	Quantity subtotal		Value subtotal	
	(Million tonnes)	(Million tonnes)	(Million tonnes)	(Percentage by volume)	(US$ million)	(Percentage by value)
Finfish	38.599	5.552	44.151	66.3	87 499	63.5
Crustaceans	2.530	3.917	6.447	9.7	30 864	22.4
Molluscs	0.287	14.884	15.171	22.8	15 857	11.5
Other species	0.530	0.335	0.865	1.3	3 512	2.5
Total	**41.946**	**24.687**	**66.633**	**100**	**137 732**	**100**

Table 1.2 Production of farmed crustaceans from different regions in 2013 (FAO, 2016)

Continent	Farmed species	Quantity		Value	
		(tonnes)	(Percentage by volume)	(US$ thousand)	Percentage by value)
Africa	Freshwater crustaceans	8		167	
	Crabs, sea spiders	6		42	
	Penaeid shrimps	11 513		79 279	
Total Africa		11 527	0.17	79 487	0.23
Americas	Freshwater crustaceans	48 769		140 318	
	Penaeid shrimps	646 246		3 405 303	
Total Americas		695 015	10.36	3 545 622	10.20
Asia	Crabs, sea spiders	302 247		989 269	
	Freshwater crustaceans	1 904 733		10 945 234	
	Lobster, spiny-rock lobsters	1 045		16 268	
	Penaeid shrimps	3 791 261		19 096 767	
Total Asia		5 999 286	89.39	31 047 538	89.33
Europe	Crab, sea spiders	1		5	
	Freshwater crustaceans	101		626	
	Penaeid shrimps	135		1 701	
Total Europe		237	0.00	2 332	0.01
Oceania	Crabs, sea spiders	4		54	
	Freshwater crustaceans	161		3 564	
	Penaeid shrimps	5 448		79 168	
Total Oceania		5 613	0.08	82 785	0.24
Grand Total		**6 711 678**	**100**	**34 757 764**	**100**

1.2 DISEASE: A MAJOR CONSTRAINT IN AQUACULTURE

Disease is considered as a primary constraint to the growth of many aquaculture species and is responsible for tremendous economic losses of the aquaculture industry. Aside from these major economic losses, diseases have also caused considerable financial impact on investors' confidence as well as a direct threat to the world's food security/availability. Such losses have also posed a direct threat to the livelihoods of the communities that relies solely their income and employment on aquaculture. For example, in southern Vietnam, approximately 1200 families dependent on rice-shrimp culture have experienced annual losses of >US$ 300,000 due to shrimp diseases (Subasinghe, et al., 2001).

In many cases, the occurrence of these diseases are associated with poor husbandry practice (Kautsky, et al., 2000). Some farmers tend to increase stocking densities and intensify production to yield higher income and fast investment returns without considering proper biosecurity measures. For example, the observed boom-and-bust pattern of the shrimp industry in Thailand, Philippines, Indonesia and China in the 1990's were attributed to husbandry-related factors such as excessive stocking that goes beyond the carrying capacity of the environment, poor water quality and self-pollution (Kautsky, et al., 2000; Primavera, 1997; Spaargaren, 1998). It was observed that the intensification of aquaculture systems often leads to several detrimental culture conditions like overcrowding due to higher stocking density, accumulation of metabolic waste, insufficient oxygen supply, animal injury due to crowding and other stressors that can significantly affect the health of the cultured animal (Ashley, 2007; Kautsky, et al., 2000). Furthermore, the expansion and diversification of aquaculture based on movements of live aquatic animals and animal products (broodstock, seed and feed) has facilitated the introduction and spread of pathogens and diseases into many aquaculture systems which resulted in even more disease problems (Subasinghe, et al., 1996).

While these factors clearly initiate disease problems in the aquaculture systems, the disease-causing microorganisms are also ubiquitous in the marine environment. Unlike in terrestrial settings, the cultured animals in aquaculture settings are living in water and unavoidably are in constant contact with its potential pathogens. Noteworthy, these potential pathogens are able to maintain themselves in the external environment of the

animal (water) and can propagate independently of the host (Verschuere, et al., 2000). These pathogens can then be ingested by the animals through feeding or during drinking for osmoregulation. As a result, highly unpredictable survival rates (especially at the larval stages) are likely observed due to bacterial infection. In some conditions, the host and its pathogen are coexisting within the system showing little or no adverse effects to the animal (Kautsky, et al., 2000; Lavilla-Pitogo and de la Pena, 1998; Olafsen, 2001). It is when the animal is exposed to different physiological stresses that the disease starts to occur due to the lowering of its immune defense system (Rottmann, et al., 1992).

1.2.1 DISEASES IN SHRIMP

The same as with other aquaculture species, infectious diseases remain a significant problem to crustacean aquaculture. In the case of tropical shrimp production, approximately 40% (>3 billion US$) is estimated to be lost annually due to infectious diseases (Lundin, 1996; Stentiford, et al., 2012). Shrimp diseases are generally caused by pathogenic viruses, bacteria, fungi and parasites (Table 1.3) and among these disease-causing agents, major shrimp losses and mortalities were caused by pathogenic bacteria and viruses (Stentiford, et al., 2012). In the context of this research, pathogenic *Vibrio* species are emphasized, aside from the fact that they are also one of the economically most important pathogens in crustacean culture. Bacteria belonging to the *Vibrio* spp. (such as *V. campbellii, V. harveyii, V. parahaemolyticus* etc.) are considered as the most aggressive bacterial pathogens in crustaceans causing *Vibrio* disease also known as *Vibriosis*. Although this disease is also affecting other crustacean species, the most serious problems have been reported in penaeid culture, causing enormous losses and mortalities in shrimp (Table 1.4). *Vibrio* spp. are gram-negative bacteria, typically found in saltwater, are motile and have polar-flagella with sheaths. These bacteria may act as opportunistic agents (i.e. only causing disease when the host immune system is suppressed or if the animal is exposed to several stressors; or if signal molecules (called autoinducers) present in the environment reached a threshold level for a population-wide alteration in virulence genes, also referred as quorum sensing) or be true pathogens (i.e. can infect normal, healthy host with intact defense system) (Saulnier, et al., 2000). Clinical signs of *Vibriosis* in shrimp show localized cuticular lesions, oral and enteric infections to

septicaemia (Lightner, 1996). Melanisation and bioluminescence (Figure 1.2) are also signs of *Vibriosis*.

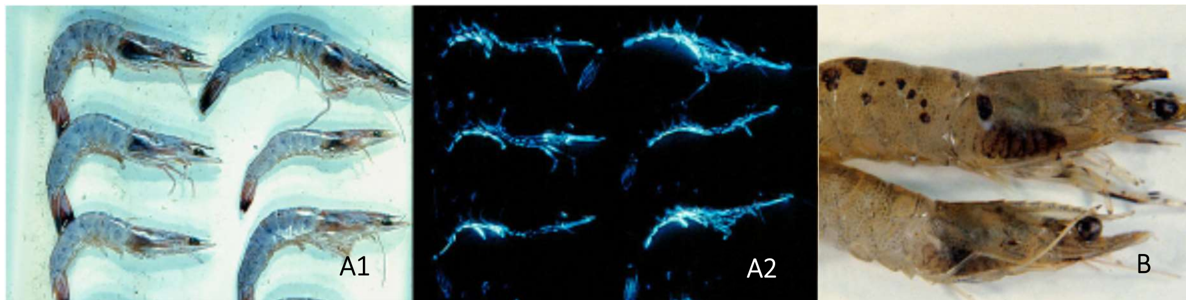

Figure 1.2 Luminescent *Vibriosis* (A1, A2) and melanisation in shrimp (B) [adapted from Hu (2014)]

Aside from existing shrimp diseases, the industry has also been devastated with new/emerging diseases. By definition, “emerging diseases” are considered as a new disease, new presentation of a known disease, or either existing diseases in a new geographical area (Brown and Bolin, 2000). Indeed in recent years, a new emerging shrimp disease called acute hepatopancreatic necrosis syndrome/disease (AHPNS/AHPND) also known as early mortality syndrome (EMS) was reported to be seriously affecting the *P. monodon*, *P. vannamei* and *P. chinensis* culture (FAO, 2014). Since its outbreak in China in 2009, it has then spread through Southeast Asia such as Vietnam, Malaysia, Thailand (FAO, 2014), transmitted as far as Mexico (Nunan, et al., 2014) and recently in the Philippines (de la Peña, et al., 2015). The disease has decreased the annual shrimp output of about 30-70% and the global shrimp industry has an estimated losses of US$1 billion per year (FAO, 2014). AHPND-infected shrimp showed symptoms like pale and atrophied hepatopancreas (HP) together with an empty stomach and midgut (Figure 1.3). Histological examinations showed that the disease causes sloughing of the HP tubule epithelial cells into the HP tubule lumens (Lightner, et al., 2012; Tran, et al., 2013). Mortality typically occurs in the first 30-45 days of culture and can begin as early as 10 days after stocking (Browdy, et al., 2014). The causative agent of AHPND is a strain of *Vibrio parahaemolyticus* (VP_{AHPND}) (Lightner, et al., 2012) carrying a unique extra chromosomal virulence plasmid pVA1 (Yang, et al., 2014). The

pVA1 plasmid encodes the binary toxin Pir-like ToxA (12.7 kDa) and ToxB (50.1 kDa) (Han, et al., 2015; Lee, et al., 2015) and were reported to be the causative agent of AHPND in shrimp (Lee, et al., 2015; Sirikharin, et al., 2015). These toxins are homology to the binary insecticidal Pir-toxin previously described from the nematode –associated genera *Photorhabdus* and *Xenorhabdus* (Forst, et al., 1997; Waterfield, et al., 2005). Furthermore, it showed that the plasmid is stably inherited via a post segregational killing system and is disseminated by a conjugative transfer (Lee, et al., 2015). In the meantime, there are already three AHPND-causing plasmids identified, namely pVH_{vo} (accession no. KX268305; 69,142 bp) which was detected in *V.owensii* strain SH-14 (Liu, et al., 2015), pVPA3-1 (accession no. KM067908; 69,168bp) and pVA1 (accession no. KP324996; 70,452 bp) which were detected in *V. parahaemolyticus* (Han, et al., 2015; Yang, et al., 2014). Detection of AHPND can be done by polymerase chain reaction (PCR) method using designed primers targeting the AHPND toxin genes.

Other emerging shrimp diseases such as hepatopancreatic microsporidiosis (HPM) (caused by a microsporidian parasite, *Enterocytozoon hepatopenaei*) (Tourtip, et al., 2009), hepatopancreatic haplosporidiosis (HPH) (caused by still unnamed haplosporidian parasite) (Utari, et al., 2012), aggregated transformed microvilli (caused unknown) (Sriurairatana, et al., 2014) and covert mortality disease (CMD) (caused by covert mortality nodavirus) (Zhang, et al., 2014) have also been reported recently.

Table 1.3 Some of the shrimp diseases caused by virus, bacteria, fungi and parasites

Viral diseases	Reference	Bacterial and fungal diseases	Reference	Other parasitic diseases	Reference
White spot disease (*white spot syndrome virus; WSSV)	(Flegel and Lio-Po, 2009; Liu, et al., 2009)	*Vibriosis* (*pathogenic *Vibrio* species)	Refer to table 1.4	Epicomensals: Protozoon gill disease & surface fouling disease (*Leucothrix mucor,* **Zoothamnium* sp., *Epistylis* sp., and *Vorticella* sp.)	(Lightner, 1985; Overstreet, 1973)
Infectious mionecrosis (*infectious mionecrosis virus; IMNV)	(Senapin, et al., 2007)	Rickettsia (**Rickettsia*-like bacterium)	(Krol, et al., 1991)		
Yellow head disease (*yellow head virus; YHV)	(Boonyaratpalin, et al., 1993; Flegel, 1997)	Larval mycosis (**Lagenidium callinectes, *Sirolpidium* sp.and **Haliphthoros* sp.)	(Lightner and Sindermann, 1988)	Gregarine disease (*Gregarines; *Paraophloidina scolecoides*)	
Baculoviral midgut gland necrosis (*BMN virus)	(Momoyama and Sano, 1989)	Fusariosis (**Fusarium solani*)	(Colorni, 1989)		
Penaeus monodon-type baculovirus (MBV) group	(Lightner, et al., 1985; Lightner, et al., 1983b)			Microspordiosis (*Microsporidians)	(Pasharawipas and Flegel, 1994)
Infectious hypodermal and hematopoietic necrosis (*IHHN virus)	(Lightner, et al., 1983a; Lightner, et al., 1983b)				
Hepatopancreatic parvovirus disease (*Hepatopancreatic parvovirus)	(Lightner and Redman, 1985)			Haplosporidian disease (*Haplosporidians)	(Dykova, et al., 1988)
Reoviruses disease (*REO) group	(Adams and Bonami, 1991; Tsing and Bonami, 1987)				
Taura syndrome (*Taura syndrome virus)	(Bonami, et al., 1997)				
Rhabdovirus disease (*Rhabdovirus)	(Lu and Loh, 1992)				

Etiology of the disease

Table 1.4 List of selected *Vibrio* spp. causing *Vibriosis* disease in selected crustacean species [redrafted from Baruah (2012)]

Causative organism	Host organisms	Disease characteristics	References
V. campbellii/V. harveyi	Brine shrimp (*Artemia franciscana*)	Between 45-80% mortality	(Soto-Rodriguez, et al., 2003)
	Kuruma prawn (*Penaeus japonicus*)	High mortality	(Liu, et al., 1996a)
	Ridgeback prawn (*Sicyonia ingentis*)	Detachment of mid gut epithelium resulting in up to 55% mortality	(Martin, et al., 2004)
	Rock lobster (*Jasus verreauxi*)	Up to 75% mortality	(Diggles, et al., 2000)
	Tiger prawn (*Penaeus monodon*)	Mass mortality	(Karunasagar, et al., 1994)
	White shrimp (*Litopenaeus vannamie*)	Up to 85% mortality in nauplii	(Aguirre-Guzmán, et al., 2004)
	Marine shrimp (*Penaeus merguiensis*)	70-100% mortality	(Sae-Oui, et al., 1987)
V. parahaemolyticus	White shrimp (*Litopenaeus vannamie*)	Gill necrosis; lethargy and significant mortality;	(Aguirre-Guzmán, et al., 2010)
	Freshwater prawn (*Macrobrachium rosenbergii*)	Black coloration on the carapace; red discoloration of the exoskeleton; loss of appendages within 6 days and 80% mortality	(Sudheesh and Xu, 2001)
	Tiger prawn (*Penaeus monodon*)	Mass mortality	(Sung, et al., 2001)
V. alginolyticus	Freshwater prawn (*Macrobrachium rosenbergii*)	Mortalities of 80-100%	(Jayaprakash, et al., 2006)
	Tiger prawn (*Penaeus monodon*); Kuruma prawn (*Penaeus japonicus*)	Mass mortality	(Lee, et al., 1996a; Lee, et al., 1996b)
V. anguillarum	Brine shrimp (*Artemia franciscana*)	Significant mortality	(Defoirdt, et al., 2005)

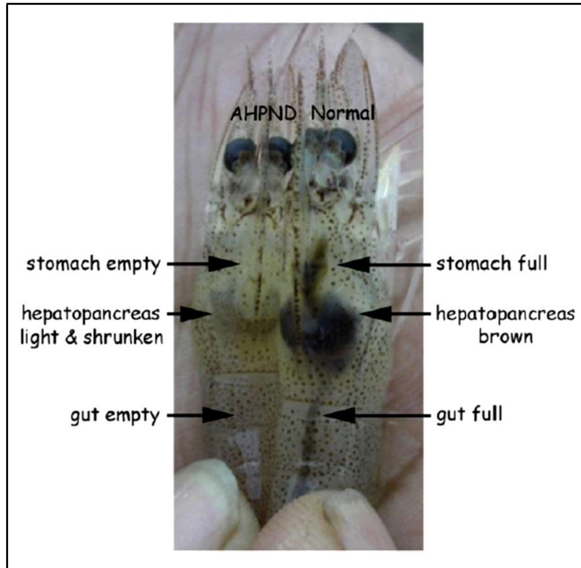

Figure 1.3 Gross signs of AHPND and non-AHPND shrimp

1.3 RATIONALE, OBJECTIVES AND OUTLINE OF WORK

The latest FAO report (2014) showed that almost half of aquatic food products worldwide are produced from the aquaculture industry, thus making the sector a significant contributor of food fish products for the current 7.3 billion people on the planet. This industry is considered as the fastest food-producing sector in the world and has immensely developed due to its intensified culture methods. However, its increased production has been paralleled by the occurrence of diseases, which hampered its progress **(Chapter 1).** Due to the adverse effects of using antibiotics to fight disease infections in aquaculture, aquaculture scientists all over the world have been focusing their efforts on finding equally effective yet sustainable measures for controlling diseases. To date, several alternative strategies have been suggested **(Chapter 2)**, and most of these methods are still in the research phase while only few have been tested in real aquaculture settings. The application of probiotics (beneficial microorganisms) and poly-β-hydroxybutyrate (PHB) (an antimicrobial compound produced by bacteria) are some of the strategies identified as highly promising to replace the use of antibiotics. Probiotics application is considered as the most widely studied alternative strategy to control diseases, in fact some of these products are already commercially available and are tested in the field (e.g. in shrimp ponds) (Wang, et al., 2005), however for PHB, to our knowledge, no report has yet been made about is application in real aquaculture settings. Actually, only few studies have been done so far to explore its beneficial effects and most of these studies were conducted using crystalline PHB (i.e. the biopolymer is already extracted from the bacterial cell). Hence, in this research, a novel approach of using *Bacillus* species (known probiotic bacteria in aquaculture) containing higher amount of amorphous PHB (i.e. the biopolymer is still inside a bacterial cell) as an alternative strategy to fight disease infection in crustacean larviculture (or aquaculture in general) is explored.

The general objective of this research is to evaluate the application and mode of action of amorphous poly-β-hydroxybutyrate-accumulating *Bacillus* spp. as effective biocontrol agents for crustacean (larvi)culture. In order to accomplish this objective, the following specific goals are set as follows:

First, is to isolate, screen and characterize for poly-β-hydroxybutyrate-accumulating *Bacillus* spp. from shrimp pond sediments with superior PHB accumulation capability as criterion **(Chapter 3)**. To facilitate the rapid screening and characterization of the PHB-accumulating *Bacillus* spp., two steps were employed in this work: (1) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used for dereplication (i.e. bacterial grouping) and (2) PHB quantification using a spectrophotometric method.

Second, is to determine if these superior PHB-accumulating *Bacillus* strains selected and identified from chapter 3 could provide beneficial effects to selected crustacean hosts. In this research, two in *vivo* experiments were performed. The first *in vivo* work was conducted in a model culture system using gnotobiotically-cultured *Artemia franciscana* **(Chapter 4)**. This study aimed to determine if the *Bacillus* sp. JL47 (the superior PHB accumulating isolate identified in chapter 3) could provide protection to gnotobiotic *Artemia franciscana* against pathogenic *Vibrio campbellii* LMG 21363 infection. Furthermore, this study also wanted to examine if the protective effects of the *Bacillus* sp. JL47 in gnotobiotic *Artemia* differ when the *Bacillus* strain contains low or high amounts of intracellular amorphous PHB. The second *in vivo* work was conducted in *P. monodon* postlarvae in actual culture systems in nursery tanks **(Chapter 5)**. This experiment aimed to investigate the effects of two superior PHB-accumulating *Bacillus* isolates (JL47 and JL1) and a mixed bacterial culture (mBC) containing PHB-accumulating *Bacillus* spp. on the growth, survival and robustness of *P. monodon* postlarvae. The protective effect of the tested PHB-accumulating *Bacillus* spp. added to the water or through the feed in *P. monodon* exposed to pathogenic *V. campbellii* LMG 21363 was also assessed.

Third, this research also aimed to investigate the mode of action of the PHB-accumulating *Bacillus* sp. JL47, being the superior PHB-accumulating *Bacillus* strain identified in this work. This part was divided into two experiments as follows: the first experiment aimed to investigate the molecular mechanisms involved in the protective capability of PHB-accumulating *Bacillus* sp. JL47 in shrimp (as demonstrated in chapter 5) by looking at its effects on the expression of the immune-related genes prophenoloxidase, transglutaminase and heatshock protein 70 in *P. monodon* before and

after the *Vibrio campbellii* LMG 21363 challenge **(Chapter 6)**. In the second experiment, the effect of phasin protein- a PHB granule associated protein present at the surface of the intracellular amorphous PHB- on the survival of challenged gnotobiotic *Artemia* was determined. In this work, the phasin gene in *Bacillus* sp. JL47 was cloned, overproduced in *E. coli* and was subsequently fed in gnotobiotic *Artemia*. The effects of the phasin protein on the survival of *Artemia* challenged with pathogenic *Vibrio campbellii* LMG 21363 was determined **(Chapter 7)**.

Finally, the general discussion of the results obtained in these studies, conclusions as well as possibilities for further research are presented (**Chapter 8)**.

PART 1

REVIEW

CHAPTER 2
STRATEGIES TO CONTROL DISEASE INFECTIONS IN AQUACULTURE

Joseph Leopoldo Q. Laranja[1,2], Edgar C. Amar[2], Peter De Schryver[1], Peter Bossier[1]

[1]Laboratory of Aquaculture and *Artemia* Reference Center, Ghent University, 9000 Gent, Belgium
[2]Aquaculture Department, Southeast Asian Fisheries Development Center, 5021 Tigbauan, Iloilo, Philippines

ABSTRACT

Disease outbreaks are being considered as a significant constraint on aquaculture production and trade. As described in the previous chapter (Chapter 1), disease has brought major socio-economic losses in the aquaculture sector, as well as threats to food availability/security and the livelihood of vulnerable sectors in the society. The traditional approach of using chemicals and antibiotics has brought limited success in the past because it instead brought more harm than good. The current disease control measures developed to replace the use of antibiotics has now focused on the pathogen-host-environment interaction, wherein the strategy/agent(s) could be acting at the host, at the host's environment or at the pathogen level. Among those, the application of probiotics and Poly-β-hydroxybutyrate are among the identified promising disease control agents. In the context of this work, probiotics (i.e. *Bacillus* species) and PHB are reviewed extensively.

Keywords: disease control, probiotics, *Bacillus* species, Poly-β-hydroxybutyrate

2.1 Disease prevention

In the quest for disease management and control, the FAO recognized disease prevention (i.e. preventing the introduction and establishment of a disease agent) as the preferred health management since it can be considered as the most practical and straightforward. Disease prevention can be attained by observing good husbandry practice in all levels of the culture activity such as by following strict quarantining and inspecting of (newly acquired) stocks to prevent transmission of pathogens between farms, or if necessary avoid movement of animals between sites/farms, observing good sanitary measures in the culture system (i.e. disinfection of tanks, eggs, rearing water) and proper preparation prior to re-stocking (i.e. fallowing) (Subasinghe, 2001). Eliminating factors that causes physiological stress to the cultured animals will also reduce infection such as improving the water quality of the rearing water (i.e. water treatment), maintaining desired physicochemical parameters (i.e. salinity, temperature, dissolve oxygen etc.), avoid high stocking density and proper handling. The use of quality feeds to meet the nutritional requirement and physical health of the animal and good feeding practice to eliminate wastage of feeds that may contribute to the detrimental condition of the water should also be observed.

However, due to the fact that the risk of contamination cannot always be eliminated in any aquaculture systems because of the constant interaction of the cultured animal to its potential pathogens that are omnipresent in the aquatic environment, it is suggested that the application of other sustainable disease control measures (e.g. use of probiotics, PHB, etc.) together with good husbandry practice is advisable to attain optimal culture conditions of the animals.

2.2 Antibiotics and chemicals: does more harm than good

In the traditional concept of disease control, it was suggested that removing all the possible pathogenic bacteria, for example by using antibiotics and other chemicals, in the culture system could provide safer culture conditions for the animals. However, this approach has brought limited success in the past because of the observed major

drawbacks and limitation. Due to the wide and massive (mis)use of antibiotics in aquaculture, it has resulted in resistance development in various aquaculture pathogens (Table 2.1). Hence, in several cases, antibiotic treatment can no longer be effective in treating bacterial diseases. Furthermore, the indiscriminate use of antibiotics has also posed a direct threat to the human health and the environment. These antibiotic resistant genes that have emerged or evolved in the aquaculture environments have been shown to be horizontally transferred to terrestrial and human pathogens and thus the pathogens have acquired a wider resistance to antibiotics such as those that are normally used in aquaculture (Angulo, 1999; Cabello, 2006). Likewise, the presence of antibiotic residues in commercialized aquaculture products from excessive use of antibiotics in culturing these animals may generate human health problems such as allergies and toxicity (Cabello, 2006).

The application of antibiotics and other chemicals (i.e. disinfectants) in the culture system will not only eliminate pathogenic bacteria but also remove the normal microbes and beneficial bacteria present. This approach was suggested to be not sustainable and effective because it will disturb or destroy the existing microbial ecosystem that is supposed to “balance” the system by controlling the proliferation of opportunistic pathogens (De Schryver, et al., 2014). In the ecology theory of *r/K* selection (MacArthur and Wilson, 2015), the presence of slow-growing bacteria (referred as *K*-strategist) eliminates the niches of the fast-growing ones (referred as *r* strategist), which include many disease-causing *Vibrio* spp. In this context, the presence of a stable/mature microbial ecosystem in the culture system is suggested to be important and needs to be managed (e.g. by using prebiotics, PHB) rather than to eliminate it.

Table 2.1 The different classes of antibiotics used in aquaculture, their importance for human medicine and examples of (multi)resistant pathogenic bacteria isolated from aquaculture settings [adapted from Defoirdt et al., *(2011a)*].

Drug classes	Importance for human medicine[a]	Example	Resistant bacteria	Multiple[b] resistance?	Isolated from	Reference
Aminoglycosides	Critically important	Streptomycin	*Edwardsiella ictulari*	yes	Disease striped catfish (*Pangasianodon hypophthalmus*), Vietnam	(Dung, et al., 2008)
Amphenicols	Important	Florfenicol	*Enterobacter spp. and Pseudomonas spp.*	yes	Freshwater salmon farms, Chile	(Fernández-Alarcón, et al., 2010)
Beta-lactams	Critically important	Amoxicillin	*Vibrio spp., Aeromonas spp. and Edwardsiella tarda*	yes	Different aquaculture settings, Australia	(Akinbowale, et al., 2006)
Beta-lactams	Critically important	Ampicillin	*Vibrio harveyi*	Yes	Shrimp farms and coastal waters, Indonesia	(Teo, et al., 2000)
Fluoroquinolones	Critically important	Enrofloxacin	*Tenacibaculum maritimum*	Yes	Diseased turbot (*Scophthalmus maximus*) and sole (*Solea senegalensis)*, Spain and Portuga	(Avendaño-Herrera, et al., 2008)
Macrolides	Critically important	Erythromycin	*Salmonella spp*	Yes	Marketed fish, China	(Broughton and Walker, 2009)

Nitrofurans	Critically important	Furazolidone	*Vibrio anguillarum*	Yes	Diseased sea bass and sea bream, Greece	(Smith and Christofilogiannis, 2007)
Nitrofurans	Important	Nitrofurantoin	*Vibrio harveyi*	Yes	Diseased penaeid shrimp, Taiwan	(Liu, et al., 1996b)
Quinolones	Critically important	Oxolinic acid	*Aeromonas spp., Pseudomonas spp. and Vibrio spp.*	Yes	Pond water, pond sediment and tiger shrimp (*Penaeus monodon*), Philippines	(Tendencia and de la Peña, 2001)
Sulphonamides	Important	Sulphadiazine	*Aeromonas spp.*	Yes	Diseased katla (*Catla catla*), mrigel (*Cirrhinus mrigala*) and punti (*Puntius* spp.), India	(Das, et al., 2009)
Tetracyclines	Highly important	Tetracycline	*Aeromonas hydrophila*	Yes	Water from mullet and tilapia farms, Egypt	(Ishida, et al., 2010)
Tetracyclines	Highly important	Oxytetracycline	*Aeromonas salmonicida*	Yes	Atlantic salmon (*Salmo salar*) culture facilities, Canada	(McIntosh, et al., 2008)

[a]On the basis of World Health Organization Expert Consultations on "Critically Important Antimicrobials for Human Medicine"(Heuer, et al., 2009). [b]Resistance to antibiotics belonging to different classes in at least one of the isolates.

2.3 ALTERNATIVE DISEASE CONTROL STRATEGIES

In the concept of disease occurrence, there is a complex interaction between the cultured animal (host), the environment (where the animal is cultured) and the microbiota present (both pathogenic and beneficial; host and/or environmentally-associated) (Snieszko, 1973). It was suggested that in attaining a best management strategy, prevention and control should take into account the different aspects of the pathogen-host-environment interaction for a more holistic and effective approach (Figure 2.1) (Defoirdt, et al., 2007). The current disease control measures developed to replace the use of antibiotics focused on these interactions, wherein the strategy/agent(s) could be acting at the host, at the host's environment or at the pathogen. It is more advantageous if the control agent(s) has several modes of action, or if necessary, a combination of these strategies to maximize the chances of success in controlling diseases.

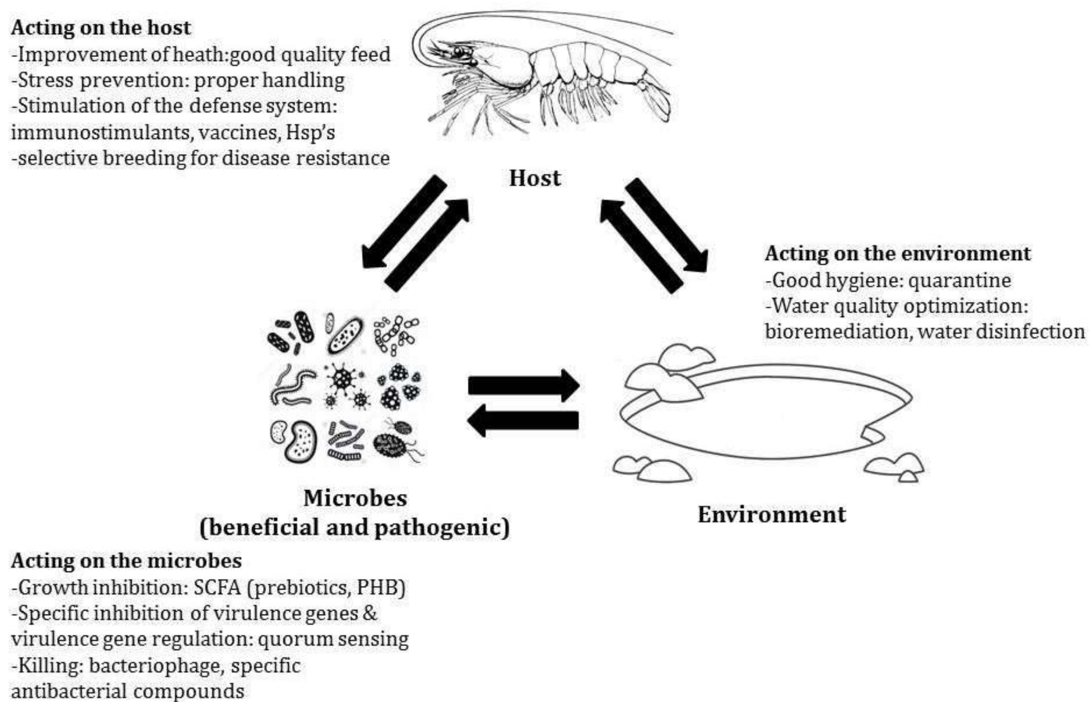

Figure 2.1 Schematic overview of different strategies to prevent and control diseases without using antibiotics [redrawn from Defoirdt et al., *(2011a)*]

2.3.1 STRATEGIES ACTING AT THE HOST

As opportunistic infectious agents abound in most aquatic systems, in addition to the several stressors that will be possibly encountered by the aquatic animal during culture, enhancing its immune response is an effective strategy to avoid disease infection. Treatments that focus on enhancing the host health by stimulating its innate immune system and/or triggering its specific immune response cover this technique. Among these strategies are as follows:

2.3.1.1 Immunostimulants

Immunostimulants, are defined as “naturally occurring compounds that modulate the immune system by increasing the host’s resistance against diseases that in most circumstances are caused by pathogens” (Bricknell and Dalmo, 2005). In aquaculture, immunostimulants have been used as feed additives and have been proven effective in reducing disease losses. The most commonly used immunostimulants are the β-glucans which can be derived from the cell wall of some plants, seaweeds, fungi, bacteria, mushrooms, and yeast (Volman, et al., 2008). Of these different β-glucans, the most potent immune-system enhancers are the β-1,3/1,6-glucans and is commonly obtained from baker’s yeast *Saccharomyces cerevisiae* (Meena, et al., 2013). Other compounds that were also investigated as immunostimulants are alginates which can be extracted from brown algae (e.g. *Laminaria hyperborean*, *Ascophyllum nodosum* and *Macrocystis pyrifera*) or bacteria (e.g. *Azotobacter vinelandii*) (Remminghorst and Rehm, 2006), plant extracts (Galina, et al., 2009), chitin (Sakai, et al., 1992), and nucleotides (Burrells, et al., 2001). Non-digestible carbohydrates (i.e. prebiotics, see further) and bacterial supplementation (i.e. probiotics; see further) have also been suggested to induce immunostimulation in aquatic animals (Nayak, 2010; Panigrahi, et al., 2009; Song, et al., 2014). However, long term administration of immunostimulants may decrease its efficacy and immune-suppression may occur at overdoses hence the timing and dose of immunostimulant application should be considered to make these compounds more effective (Sakai, 1999).

2.3.1.2 Heat shock proteins

Heat shock proteins (Hsps), also called molecular chaperones, are conserved proteins (16-100 kDa) produced by all organisms when they are exposed to cellular stress (Welch, 1993). Hsps, in general, are involved in protein biogenesis and protein homeostasis in the cell, including folding of nascent polypeptides, translocation of proteins across intracellular membranes, assembly/disassembly of multi-sub-unit oligomers, process of endocytosis, regulation of apoptosis and cytoskeletal organization (Schmitt, et al., 2007; Young, et al., 2004). However, accumulating evidence suggests that Hsps play a significant role in the regulation of the immune response in both vertebrates and invertebrates (Feder and Hofmann, 1999; Sung, et al., 2011). For example, feeding the brine shrimp larvae with prokaryotic or eukaryotic Hsp70 showed stimulation of the innate immune response of the brine shrimp (e.g. prophenoloxidase) resulting in the protection of the larvae against pathogenic *Vibrio* infection (Baruah, et al., 2011). Furthermore, Hsps were also suggested to be involved in the organism's ability to acquire tolerance to environmental extremes, referred as cross tolerance (Clegg, et al., 2000). Due to the potential application of Hsps as immunoprophylactic strategy in aquatic animals, Hsp inducing compounds (induce Hsp production inside the host) were also suggested (Baruah, et al., 2012). Although the application of Hsps and Hsp inducing compounds were found promising, the molecular mechanisms of triggering potent immune responses by Hsps are yet to be clearly defined.

2.3.2 STRATEGIES ACTING AT THE MICROBES

The aquaculture system contains high quantity and diversity of microbes (pathogenic and beneficial) and these microbes are in constant contact with the cultured animals. Microbial intervention through an environmentally friendly approach has been suggested as an alternative method of heath management in aquaculture. These interventions may target the pathogens or the beneficial micro-organisms present in the host environment (water) or inside the intestinal tract of the host with the aim of having a beneficial effects to the cultured animal. The following alternative strategies are as follows:

2.3.2.1 Bacteriophages

Bacteriophages were discovered in the 1920s as viral infections of bacteria (Duckworth, 1976). These bacterial viruses have genetic material in the form of either deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), encapsulated in a protein (Clark and March, 2006). Phages infect bacteria in two ways as follows: when phages multiply vegetatively, they kill their (bacterial) host at the end of the growth cycle through lysis, referred as lytic life cycle or phages will undergo a lysogenic state wherein prophages grow vegetatively and integrate their genome and replicate themselves with the host chromosome for many generations (Haq, et al., 2012). Prophages switch to lytic cycle when host DNA is damage or when the host is in imminent cell death (Inal, 2003). A major advantage of phage therapy is that non-target microbiota are not affected because of its narrow host range (Nakai and Park, 2002). At a single dose, phages are more effective than antibiotics because they can replicate at the site of infection with less or no side effects (Häusler, 2006). However, some limitations of using bacteriophages are that they are sometimes strain specific rather than species specific, resistance can also be developed by alteration of phage attachment sites and the possible transfer of virulence factors (Austin, et al., 2003; Defoirdt, et al., 2007). Hence, it is suggested that before using bacteriophages for therapy, phages should first be tested if they carry any virulence genes and if they are safe to use. Furthermore, applying cocktails of phages or by using phage components instead of intact phage were also suggested to avoid resistance development (Defoirdt, et al., 2007; Inal, 2003).

2.3.2.2 Quorum sensing disruption

Quorum sensing (QS) or bacterial cell-cell communication, is a mechanism in which bacteria coordinate the expression of certain genes in response to their population density by producing, releasing, and detecting small signal molecules called autoinducers (Waters and Bassler, 2005). When autoinducers are accumulated and reached a threshold concentration, it initiates a signal transduction cascade that culminates in a population-wide alteration in gene expression (Camilli and Bassler, 2006). In many aquaculture pathogens containing a QS system, QS has been found to regulate their virulence factor expression (such as lytic enzymes, toxins, siderophores and adhesion

molecules) (Bassler, et al., 1993; Defoirdt, et al., 2004). It has been shown that inactivating the QS system of QS pathogens can result in decrease virulence or its virulence factors (Swift, et al., 1999; Wu, et al., 2001). Hence, disrupting the QS system in these QS pathogens has been suggested as a new anti-infective strategy in aquaculture. Among the several techniques identified for QS disruption were as follows: a) inhibition of signal molecule biosynthesis b) application of QS antagonist (e.g. natural or synthetic furanones) c) chemical inactivation of QS signals by oxidised halogen antimicrobials and d) signal molecule biodegradation/inactivation by bacteria (e.g. *N*-acyl homoserine lactone (AHL) degrading bacteria) (for review see Defoirdt et al., (2004)). Although this novel technique showed promising results in aquaculture, most of these studies were still conducted in laboratory scale and under controlled conditions (Defoirdt, et al., 2006b; Tinh, et al., 2007). Hence, to understand the impacts and applicability of QS disruption technique in real aquaculture settings, more studies will be required in this field.

2.3.2.3 Prebiotics

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species, already resident in the gut and thus attempt to improve host health (Gibson and Roberfroid, 1995). To be identified as prebiotics, the following criteria were suggested: it must be neither hydrolysed, nor absorbed in the upper part of the gastro-intestinal tract (GIT), there is a selective fermentation by potentially beneficial bacteria in the gut (e.g. lactic acid bacteria), there is alteration in the composition of the gut microbiota towards a healthier composition and preferably induce effects which are beneficial to the host health (Glenn and Roberfroid, 1995). The suggested beneficial effects of prebiotics include: supporting the beneficial indigenous bacteria in the gut while decreasing the growth of (potential) pathogens, enhancing the host immune system, inhibit carcinogenesis and tumor growth, improve mineral absorption, cholesterol removal, reduction of cardiovascular disease, prevention of obesity, colitis and constipation prevention (Abrams, et al., 2005; Gibson, et al., 1995; Patel and Goyal, 2012; Pierre, et al., 1997). The common prebiotics established in fish to date include inulin, fructo-oligosaccharides (FOS), short chain FOS, mannanoligosaccharides (MOS), galacto-oligosaccharides

(GOS), xylo-oligosaccharides (XOS), arabino-xylooligosaccharides (AXOS), isomaltooligosaccharides (IMO) and GroBiotic® (Ringø, et al., 2010). Prebiotic application like FOS has been shown to enhance growth rate of some aquatic animals such as Atlantic salmon, hybrid tilapia, turbot larvae, and soft shell turtle (see further review of Ringø et al., (2010). Furthermore, feeding Raftilose® P95 (FOS) to turbot larvae resulted to a possible increase of *Bacillus* spp. in the gut because the *Bacillus* spp. were the only bacteria isolated in the gut of the larvae. Several reports have shown that dietary supplementation of prebiotics in terrestrial animals has some favourable growth effects on selected health-promoting bacteria such as *LactoBacillus* and *Bifidobacterium* in the intestine of these animals while limiting the growth of potentially pathogenic bacteria such as *Salmonella, Listeria* and *Escherichia coli* (Schroeter and Klaenhammer, 2009). However, such information is lacking for aquatic animals. Hence, more studies on the effects of prebiotics on the microbial communities of cultured aquatic animals are needed to assess the effectiveness of prebiotics in aquaculture.

2.3.2.4 Green water technique

The green water technique refers to the culture of aquatic animals (like shrimp) in ponds where microalgae such as *Chlorella* grow abundantly. Tendencia and dela Peña (2003) reported that *V. harveyi* disappeared from seawater containing *Chlorella* after two days of incubation. Further observations of Tendencia et. al., (2005) showed that the presence of more than one species of microalgae (such as *Chaetoceros, Thalassiosira, Naviucla, Nitszchia, Melosira* and *Fragilaria* spp.) in the rearing water of shrimp has attributed to a higher shrimp survival with a decrease density of luminous bacteria. While these studies show the possible antimicrobial effect of microalgae against luminescent *Vibrio* spp., more research is needed to clarify the mechanisms behind the decreasing effect of green water on luminescent *Vibrio* spp, or on potential pathogens in general, in the water.

2.3.3 Strategies acting at the environment

In aquaculture setting, the cultured animal is living in water therefore the water quality is a very important factor to look at in attaining optimal culture conditions for the

animal. Several strategies are identified to improve the quality of water in the aquaculture system and this could be done by either removing the potentially pathogenic microorganisms in the rearing water and/or by removing unwanted toxicants such as excess organic material, nitrogen or phosphorous that may cause physiological stress to the cultured animal.

2.3.3.1 Water disinfection

Preventing the introduction or establishment of disease agent in the water through disinfection has been explored to avoid disease occurrence in the aquaculture system. One disinfection strategy is by ozone application. Ozone is a powerful oxidising agent that destroys the cell membrane of microorganisms, thus, it can kill pathogens that are potentially present in the water. Ozone can also improve the quality of the water by oxidising larger/complex molecules into smaller biodegradable form, oxidises nitrite or nitrate, breaks apart refractory organic molecules and enhances fine solid molecule removal by changing the particle size and surface properties (Aguilar, et al., 2005; Summerfelt, 2003). However, applying ozone to disinfect aquaculture system influents or effluents can be quite complex and costly. Ozone application requires high level of control because ozone gas is also toxic to aquatic life and even to humans (Wedemeyer, et al., 1979). Another approach of disinfecting the water is through ultraviolet (UV) irradiation. UV treatment in water has been used to kill or neutralize pathogens by denaturing the DNA of the microorganism (Summerfelt, 2003). Its effectiveness is dependent on the UV transmittance hence the UV light source, the quantity of its energy transmitted as well as its UV ray penetration in the water should be considered. However, the common downside with these disinfection techniques mentioned is that they will not only target the pathogens present but also the beneficial bacteria, hence both applications can potentially disturb the natural microbial balance in the water.

2.3.3.2 Bioremediation

Bioremediation refers to the treatment of water that will result to improving the quality of the water (Gatesoupe, 1999). For example, the application of microorganisms (e.g. nitrifying bacteria) to convert/break down and/or remove undesirable and toxic

substances to treat water pollutants or waste, referred as bioaugmentation, or the supply of compounds (e.g. crop by-products) to stimulate autochthonous bacteria with such capacities, referred as biostimulation, has been applied in aquaculture activities (Head and Oleszkiewicz, 2004; Krishnani, et al., 2006; Shan and Obbard, 2001).

2.4 PROBIOTIC APPLICATION IN AQUACULTURE

Probiotic application has been considered as the most widely studied alternative strategy to control diseases and is considered as one of the most significant technologies that has evolved in response to disease control (Browdy, 1998). The most widely quoted definition of probiotic was made by Fuller (1989) - that is "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Verschuere et al., (2000) however proposed a broader definition of probiotic that caters the application of probiotics in aquaculture and that is "a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment". The latter definition arose because of the different environmental conditions of the terrestrial animals and humans which the probiotic concept was first developed, as compared to aquatic animals like shrimp. The aquatic animals, unlike in terrestrial animals, are living in water and unavoidably are in constant contact with the surrounding bacteria that are present in the water. Hence, the interaction between the probiotics (together with other microorganisms present) and the host is not only limited in the intestinal tract but also in the gills, skin or in the host's environment (water). Indeed, studies show that the bacteria that are present in the aquatic environment have significant influence on the composition of the host's gut microbiota and vice versa (Cahill, 1990).

The most common microorganisms that were examined for use as probiotics in aquaculture comprised of Gram-positive (e.g. *Bacillus, LactoBacillus, Carnobacterium*, etc.), Gram-negative bacteria (*Aeromonas, Vibrio, Pseudomonas,* etc.) and some none bacterial candidates such as yeasts (*Saccharomyces, Debaryomyces,* etc.) and microalgae (Table 2.2). These probiotic microorganisms were reported to be either

improving the growth, survival and/or robustness of the animal, inhibiting the growth of pathogens or improving the quality of the culture water. See further review of Akhter, et al. (2015), Irianto and Austin (2002), Verschuere, et al. (2000) and Vine, et al. (2006).

Table 2.2 Different probiotics used for biological control in aquaculture

Putative probiotic strain	Source	Applied on	Method of application	Observations	Reference
Gram-positive bacteria					
Bacillus strain S11	*P. monodon* or mud and water from shrimp ponds	*P. monodon* larvae	Added to feed	Increase weight and survival of *P. monodon* larvae and postlarvae; decrease *P. monodon* mortality after *V. harveyi* challenge	(Rengpipat, et al., 2000)
B. subtilis strains L10 and G1	-	*Litopenaeus vannamei*	Added to feed	Upregulation of the genes related to secondary defense mechanism immune-related genes	(Zokaeifar, et al., 2012)
B. subtilis and LactoBacillus acidophilus	-	Nile tilapia (*O. niloticus*)	Added to feed	Increase bactericidal activity, haematocrit values and lysozyme activity	(Salah Mesalhy, et al., 2008)
B. megaterium, B. subtilis, B. polymyxa, B. licheniformis	*Commercial product (Biostart)	Channel catfish	Added to pond water	Improved growth rate and survival of catfish	(Queiroz and Boyd, 1998)
L. rhamnosus GG	Human origin	Nile tilapia (*O. niloticus*)	Bacterial pellet	Higher feed absorption and lymphocyte count	(Pirarat, et al., 2008)
Carnobacterium divergens	Atlantic salmon intestine	Atlantic cod	Added to feed	Decrease of mortality of fry challenged with *V. anguillarum*	(Gildberg, et al., 1997)
Carnobacterium sp.	Atlantic salmon intestine	Atlantic salmon	Added to feed	Antagonism against *A. hydrophila, A. salmonicida, Flavovacterium psychrophilum*	(Robertson, et al., 2000)

Pediococcus pentosaceus and *Staphylococcus hemolyticus*	Gut of *FarfantePenaeus californiensis* (brown shrimp)	*L. vannamei*	Sprayed in commercial feed	Decrease prevalence of White spot syndrome virus	(Leyva-Madrigal, et al., 2011)
Enterococcus faecium	*Mugil cephalus*	*Cyprinus carpio*	Injection and oral	Higher protection against *A. hydrophila*	(Gopalakannan and Arul, 2011)
Gram-negative bacteria					
Vibrio alginolyticus	Pacific ocean seawater	*L. vannamei* postlarvae	Added to culture water	Increase of survival and weight of *L. vannamei* postlarvae; decrease observation of *V. parahaemolyticus* in shrimp	(Garriques and Arevalo, 1995)
V. pelagius	Copepod-fed turbot larvae	Turbot larvae	Added to culture water	Decrease of mortality of turbot larvae challenged with *A. caviae*	(Ringo and Vadstein, 1998)
Pseudomonas fluorescens AH2	Iced freshwater fish (*Lates niloticus)*	Rainbow trout (*O. mykiss*)	Added to culture water	Growth rate improved and 46% reduction in mortality	(Gram, et al., 1999)
Aeromonas media A 199	-	Pacific oyster larvae	Added to culture water	Decrease of mortality and suppression of the pathogen *V. tubiashii*	(Gibson, et al., 1998)
Pseudoalteromonas aliena	Traditional fermented Korean food	Swimming crab zoea	Added in culture water	Mortality rate reduced	(Morya, et al., 2014)
Yeast					
Saccharomyces cerevisiae, S. exiguous, Phaffia rhodozoma	Commercial product	*L. vannamei*	Added in feed	Better survival	(Scholz, et al., 1999)

Debaryomyces hansenii CBS 8339	Dicentrarchus labrax gut	D. labrax larvae	Added in feed	Improved the survival and reduced malformation of larvae; high intestinal enzyme activities	(Tovar-Ramírez, et al., 2004)
Microalgae					
Tetraselmis suecica	Commercial product	*Salmo salar*	Added in feed	Inhibited the growth of *Aeromonas hydrophila, A. salmonicida, LactoBacillus* sp., *Serratia liquefaciens, Staphylococcus epidermidis, Vibrio anguillarum, V. salmonicida* and *Yersinia ruckeri* type I *in vitro;* inhibited infection in *S. salar*; reduced the mortalities caused by *A, salmonicida, Ser. liquefaciens, V. anguillarum, V. salmonicida* and *Y. ruckeri* type I.	(Austin, et al., 1992)
Isochrysis galbana	-	*Dicentrarchus labrax* larvae	Added in culture water	Significant improvement in survival; triggered digestive enzyme production	(Cahu, et al., 1998)

2.4.1 Mode of actions of probiotics

Several studies show the effectiveness of using probiotics in aquatic animals and their probiotic effects were demonstrated either *in vitro* or *in vivo* studies. The mechanisms by which these probiotics act to the host, to the pathogen or to the host's ambient environment are suggested as follows:

2.4.1.1 Production of inhibitory compounds

Due to competition between microbial communities, either for chemicals or energy, the secretion of chemical substances that have bacteriostatic (stops growth of bacteria without killing) or bactericidal (kills the bacteria) effects on other microbial populations are observed between microorganisms (Lemos, et al., 1991). These substances are maybe produced either primary or secondary metabolites and may have different mode of actions. For example, *B. subtilis* 3 strain or the *B. pumilus* has been shown to produce amicoumacin A antibiotic, a heat-stable and protease resistant antibiotic that is active against *Staphylococcus aureus, Shigella sonnei, Citrobacter freundii, Salmonella enteritidis, Proteus vulgaris, Pseudomonas aeruginosa, Escherichia coli* and *Heliobacter pylori* (Itoh, et al., 1981; Pinchuk, et al., 2001). Lactic acid bacteria are also known to produce bacteriocins, which have bactericidal activities (it affects membranes, DNA and protein synthesis) against its taxonomically close microorganism (Piard and Desmazeaud, 1992). Furthermore, the alteration in the environmental pH in the gut due to the secretion of short chain fatty acids (SCFA) such as lactic, acetic, butyric and propionic acids can cause microbial inhibition, which some pathogens cannot withstand the change in pH (Fooks and Gibson, 2002).

2.4.1.2 Decrease virulence

Virulence gene expression in many bacterial pathogens has been reported to be controlled by quorum sensing and by inactivating the quorum sensing signal molecules such as acylhomoserine lactones (AHLs) of pathogenic bacteria by AHL-degrading bacteria has been observed in these microorganisms. Two major classes of AHL-inactivating enzymes were described as follows: the lactonases which cleave the lactone

ring of the signal molecule and the acylases which cleave the AHL molecule into homoserine lactone and a fatty acid (Fast and Tipton, 2012). Lactonases and acylases can be produced by *Bacillus* spp. and *Pseudomonas* spp., respectively (Dong, et al., 2002; Sio, et al., 2006). Degradation of these signal molecules can reduce the virulence or the virulence factors of these important aquaculture pathogens (Swift, et al., 1999; Wu, et al., 2001).

2.4.1.3 Rendering specific nutrients unavailable

Competition for nutrients can theoretically play an important role in the composition of the microbiota like in the gut. For example, the competition for iron-being an essential element in a variety of metabolic and informational cellular pathways in many organisms- is apparent among important human and animal pathogens because iron is not always available in these microorganisms. In the microbial world, the most widespread and most successful mechanism of high-affinity iron acquisition is the use of siderophores (an iron-chelating compound) because it is capable of exploiting all available iron sources independent of their nature (Miethke and Marahiel, 2007). Siderophore-producing bacteria were observed to survive in nutrient-poor environments (Braun and Braun, 2002). In fish, Gatesoupe (1997) demonstrated that the *Vibrio* strain E has outcompeted the pathogenic *Vibrio splendidus* by making iron available for itself due to siderophore production *in vitro*. When the *Vibrio* strain E was added to turbot larvae it resulted in a reduced mortality after a pathogenic *V. splendidus* challenge.

2.4.1.4 Production of digestive enzymes

Autochthonous bacteria in the gut were suggested that they contribute to their host by enhancing their ability to digest and assimilate nutrients (including non-digestible dietary components) by providing digestive enzymes. This phenomenon is believed to speed-up the digestive process in the host and will eventually result to improved growth. Indeed in several reports, various bacteria that were isolated from the fish digestive system were shown to digest chitin, starch, protein, cellulose and lipids (Bairagi, et al., 2002; Gatesoupe, et al., 1997; MacDonald, et al., 1986). The *Bacillus* species, which are known probiotic bacteria in aquaculture, were reported to be producing exoenzymes such

as proteases, carbohydrolases, and lipases that can break down proteins, carbohydrates and lipids (Arellano-Carbajal and Olmos-Soto, 2002; Wang, 2007; Ziaei-Nejad, et al., 2006). Indeed, when a commercial probiotic *Bacillus* (which contains spores of *B. subtilis, B. licheniformis, B. polymyxa, B. laterosporus* and *B. circulans*) were added to the feed or to the rearing water of *FenneroPenaeus indicus*, specific activities of amylase, total protease and lipase were significantly higher in the *Bacillus* spp.-treated shrimps as compared to non-*Bacillus*-treated control shrimps (Ziaei-Nejad, et al., 2006). The report further showed that the *Bacillus*-fed shrimps resulted in a significantly higher growth and survival. While the importance of digestive enzymes produced by bacteria has been recognized, the application of "introduced" probiotic has yet to be investigated fully. To consider its effectiveness in the gut, these probiotic bacteria should viably persist in the acidic conditions of the digestive tract and should also be able to colonize.

2.4.1.5 Production of beneficial dietary compounds

Bacteria in the gut can produce beneficial dietary compounds such as vitamins as their secondary metabolites. Several microorganisms that are capable of producing vitamin B_{12} were previously isolated in several aquaculture fish species such as in carp, channel catfish, tilapia and rainbow trout (Sugita, et al., 1990; 1991a; Sugita, et al., 1991b). Several studies suggested that the production of SCFA (Clements, 1997) and lipids (Ringø, et al., 1992a; Ringø, et al., 1992b) by intestinal bacteria can significantly contribute to the diet of some fish species.

2.4.1.6 Improvement of the water quality

It was suggested that application of gram-positive bacteria like *Bacillus* spp. were efficient in converting organic matter back to CO_2, hence the build-up of dissolved and particulate organic carbon during culture can be minimized (Verschuere, et al., 2000). In intensive recirculating systems, the application of ammonia-oxidizing bacteria to oxidize ammonia to nitrite and subsequently to nitrate by nitrite-oxidizing bacteria has been practiced (Gross, et al., 2003). In shrimp, the application of *Bacillus* sp. and *Nitrosobacter* sp. to the culture water resulted in a significantly lower ammonia-nitrogen concentration

and particulate matter as compared to the non-treated water. As a result, the survival of the shrimp increased with about 75% (Janeo, et al., 2009).

2.4.1.7 Enhancement of the immune response

Enhancing the immune system is one of the many beneficial effects of probiotics. Its role in modulating the immune system in fish and shellfish has been discussed in several papers (De, et al., 2014; Lazado and Caipang, 2014; Nayak, 2010; Pérez-Sánchez, et al., 2014). Modulation of the immune response by probiotics has been demonstrated in several fish species. In rainbow trout, the administration of lactic acid bacteria (LAB) species like *Lactococcus lactis* ssp. *lactis* CFLP 100, *Leuconostoc mesenteroides* CFLP 196 *and LactoBacillus sakei* CLFP 202 has been shown to enhance the trout resistance to furunculosis by increasing the phagocytic activity of head kidney leucocytes (Balcázar, et al., 2007). Significant increases in phagocytic activity and phagocytic index were also observed in grouper *Epinephelus coioides* after feeding the *Bacillus pumilus or Bacillus clausii* containing diets for 60 days as compared with those fish fed the control diet (Sun, et al., 2010). Phagocytosis is a defense mechanism used by organisms to remove pathogens and cell debris by ingesting them to form into endocytic vesicles called phagosomes. Through phagocytic cells (i.e. macrophages and neutrophils), the phagocytosed foreign particles are destroyed by releasing degradative enzymes (i.e nucleases, proteases, antimicrobial peptides etc.) to eliminate the spread and /or growth of the intruding pathogens (Neumann, et al., 2001). Probiotic application was also demonstrated enhancing the respiratory burst activity (Nikoskelainen, et al., 2003; Xing, et al., 2013), lysozyme production (Das, et al., 2013; Panigrahi, et al., 2004), cytokine production (Biswas, et al., 2013; Panigrahi, et al., 2007), peroxidase (Salinas, et al., 2008) and anti-protease production (Sharifuzzaman and Austin, 2009) in several fish species. Stimulation of the immune system by probiotic bacteria has also been reported in crustaceans. The application of *LactoBacillus plantarum* to white shrimp *L. vannamei* resulted in a significant increase in phenoloxidase (PO) activity, superoxide dismutase (SOD) activity, prophenoloxidase (proPO) and peroxinectin (PE) messenger RNA (mRNA) transcription. The clearance efficiency to pathogenic *V. alginolyticus* and the survival rate *of L. vannamei* after *V. alginolyticus* challenge were also significantly

increased in *L. plantarum*-treated shrimp. Niu, et al. (2014) reported that feeding the gnotobiotic *Artemia* larvae with *Bacillus* sp. LT3 resulted in a significant increase of proPO expression and a significant increase in survival after a *V. campbellii* challenge. Furthermore, another report show the immune enhancing capability of *Bacillus subtilis* strain L10 and G1 to *L. vannamei* wherein the mRNA levels of proPO, PE, lipopolysaccharide (LPS) and β-1,3-glucan –binding protein (LGBP) and serine protein (SP) were significantly upregulated (Zokaeifar, et al., 2012). The shrimps fed the *Bacillus subtilis* strains show significantly higher survival than the control after a *V. harveyi* challenge. In *P. monodon*, the immune enhancing capability of *B. cereus* was observed when the bacterium was added at 0.4% in the feed. The total haemocyte count, phenoloxidase activity, respiratory burst activity, lysozyme activity, plasma protein concentrations and bactericidal activity were significantly higher in the *B. cereus*-fed shrimp than with the control (NavinChandran, et al., 2014). The growth and survival of the shrimp was also significantly higher in shrimp fed the *B. cereus*. From the following studies mentioned, it showed that the increased survival and protection of the animal against pathogenic infection was linked to the stimulation of the host's immune response by probiotics.

2.4.2 LIMITATIONS OF PROBIOTIC APPLICATION IN AQUACULTURE

As described above, probiotic application thus provides beneficial effects to farmed aquatic animals in a number of ways. However, some limitations of probiotic application in aquaculture are still needed to be address to make this approach more effective and sustainable. For example, the application of probiotic needs to be regularly supplemented because the introduced microorganism(s) cannot always maintain themselves predominantly in the target sites such as in the gut or in the rearing water (Nikoskelainen, et al., 2003; Robertson, et al., 2000). Furthermore, probiotic application requires considerable dose to make it more effective hence probiotic treatment such as in ponds needs to be economically evaluated since probiotic application commands added cost to the overall aquaculture production investment. Other equally important factors that also affect the overall efficiency of the probiotic application include: (1) the type of probiotic

strain that will be used (e.g. indigenous vs. exogenous; monospecies vs. multispecies), (2) mode of supplementation, and (3) the environmental conditions (Nayak, 2010).

2.5 *BACILLUS* SPECIES AS PROBIOTICS IN AQUACULTURE

Bacillus species are saprophytic, aerobic or facultatively anaerobic gram-positive or gram-variable (meaning they are positive or negative in the gram stain) spore forming bacteria that are commonly found in soil, water, dust, and air (Nicholson, 2002). The vegetative cells of the *Bacillus* species ranges from 0.5 by 1.2 to 2.5 by 10 μm in diameter and can grow at an optimal temperatures ranging from 25 to 37 °C, although some thermophilic and psychrophilic ones are capable of growing at temperatures as high as 75 °C or as low as 3 °C (Drobniewski, 1993). Some *Bacillus* species can also thrive at extreme acidity or alkalinity, ranging from pH 2 to 10.

Bacillus species are generally considered to be interesting probiotic bacteria for aquaculture purposes. In aquatic animals, there are several reports of *Bacillus* species being isolated from fish (Sugita, et al., 1998), crustaceans (Sugita, et al., 1996) and bivalves (Sugita, et al., 1981) (see further review of Gatesoupe (1999) . *Bacillus* species were also found in the microbiota of the gills, skin and intestinal tract of the shrimp (Sharmila, et al., 1996). The ability of the *Bacillus* species to produce spores considered them as more advantageous over other probiotic species because of its ease of application and long term storage (Hong, et al., 2005). Furthermore, the application of *Bacillus* spores as probiotics allow these bacteria to survive across the acidic conditions of the stomach, which is not always the case with other probiotic bacteria that are introduced in vegetative form.

The production of several antimicrobial compounds to inhibit pathogenic microorganisms is one of the several probiotic characteristics of the *Bacillus* species. Among these antimicrobials compounds that were produced by the *Bacillus* species include the bacteriocin and bacteriocin-like inhibitory substances (e.g. Subtilin, Coagulin) and antibiotics (e.g. Surfactin, Iturins A. C. D. E., called aminocoumacin A, and Bacilysin) which all reported being active against several pathogens (Hyronimus, et al., 1998; Itoh, et al., 1981; Nagai, et al., 1996; Pinchuk, et al., 2001; Urdaci, et al., 2004). In *P. vanamei*

culture, the application of probiotic *Bacillus subtilis* UTM 126 showed antimicrobial activity against pathogenic *Vibrio* species such as *V. alginolyticus, V. parahaemolyticus,* and *V. harveyi*. Feeding the shrimp with *Bacillus subtilis* UTM 125 has resulted in higher survival of the shrimp during a *V. harveyi* challenge test as compared to shrimps fed no *Bacillus* treatment (Balcazar and Rojas-Luna, 2007).

Bacillus species were also reported to produce enzymes that inactivate quorum sensing molecules such as AHLs. AHLs have been used by a range of gram-negative bacterial species as quorum sensing signals to regulate different biological functions, including production of virulence factors (Bassler, et al., 1993). For example, the *Bacillus* sp. 240B1 was reported to produce AiiA enzyme, identified as N-acylhomoserine lactonase, which inactivates AHL by hydrolysing the ester bond of the lactone ring to give acylhomoserine (Dong, et al., 2000). A number of other *Bacillus* species with AHL-inactivating properties were also reported such as the *B. cereus* group (*B. cereus, B. thuringiensis, B. mycoides and B. anthracis*) and these bacteria were found to possess homologues of *aiiA* gene that encodes the AHL lactonase (Dong, et al., 2002; Lee, et al., 2002). Several AHL-degrading *Bacillus* spp. were also isolated from the gut of *P. vannamei* and sea bass *Dicentrarchus labrax* and microalgae cultures and these AHL degrading *Bacillus* spp. were tested for use as a novel method to combat bacterial diseases by disrupting the quorum sensing system in important aquatic pathogens (Defoirdt, et al., 2011b; Pande, et al., 2015). Pande, et al. (2015) reported that treating the rearing water of *M. rosenbergii* larvae with an AHL-degrading *Bacillus* isolate has resulted in a significant larval survival during a pathogenic *V. campbellii* challenge as compared to non-*Bacillus* treated giant river prawn.

Bacillus species were also known to produce several exoenzymes such as proteases, carbohydrolases and lipases, which can break down protein, carbohydrates and lipids, respectively (see further review of Ray, et al. (2012)). For example, in *Fenneropenaeus indicus* postlarvae, the application of *Bacillus* spp. has significantly increased the activity of amylase, total protease, and lipase in the shrimp. The feed conversion ratio, specific growth rate and final production all showed significantly higher in *Bacillus*-treated shrimp as compared to non-*Bacillus*-treated shrimp (Ziaei-Nejad, et al., 2006). The same results were observed in *Litopenaeus vannamei* treated with *Bacillus*

spp. wherein a significant increase in activity of amylase, lipase, cellulase and protease were observed (Wang, 2007). Growth and survival were also significantly higher in the *Bacillus*-treated shrimp as compared with the control.

Several reports also showed the immune stimulating effect of *Bacillus* species towards the host. In *L. vannamei*, feeding the shrimp with *Bacillus subtilis* E20 exhibited significant increases in phenoloxidase activity, phagocytic activity and clearance efficiency of the shrimp as compared to shrimps not treated with *Bacillus* (Tseng, et al., 2009). Feeding the *P. monodon* with *Bacillus* S11 for 90 days resulted in increased phagocytic activity in the hemolymph as well as phenoloxidase and antibacterial activity (Rengpipat, et al., 2000). The growth and survival of the shrimp were also significantly higher in *Bacillus* treated shrimps than with the control. In fish, the application of *B. pumilus* and *B. clausii* in grouper resulted in a significant phagocytic activity and phagocytic index (Sun, et al., 2010); similar results were observed in other fish such as in gilthead seabream *S. aurata* fed with *Bacillus subtilis* (Salinas, et al., 2005) and in major carp *C. catla* fed with *B. circulans* PB7 (Bandyopadhyay and Mohapatra, 2009). *Bacillus* species were also reported to enhance the superoxide dismutase activity (Li, et al., 2007), glutathione peroxidase activity (Shen, et al., 2010) and serum lysozyme activity (Newaj-Fyzul, et al., 2007) in several tested aquatic animals.

Finally, *Bacillus* species were also used in the water quality management of aquaculture and wastewater treatment to remove organic and nitrogen waste substances. For example, adding *B. subtilis* and *B. megaterium* in the rearing water of a high density culture red-parrot fish resulted in a decreased total ammonia nitrogen and chemical oxygen demand in the water (Chen and Chen, 2001). In *L. vannamei* culture, the addition of *Bacillus* species in the rearing water has significantly reduced the ammonia and nitrite concentration in the water as compared to the control (Nimrat, et al., 2012). A similar observation was reported by Wang, et al. (2005) after adding commercial probiotics containing *Bacillus* species in the shrimp pond. Dissolved inorganic nitrogen, chemical oxygen demand and dissolved reactive phosphorous were significantly decreased in the probiotic treated pond as compared with the control. In all the studies mentioned, growth and/or survival of the animals were observed to be significantly higher in the *Bacillus*-treated water than with the control. Furthermore, the application of *Bacillus* species in

wastewater treatments to remove nitrogen waste compounds has been suggested. The most interesting aspect of using *Bacillus* species in wastewater treatment is there phenomenal ability to convert ammonium to gaseous nitrogen under fully aerobic conditions (Yang, et al., 2011). This process has shorten the conventional way of removing nitrogen in wastewater which is normally done by using aerobic autotrophic nitrifiers and subsequently anaerobic heterotrophic denitrifiers to convert nitrogenous compounds to nitrogen gas (Chiu, et al., 2007). The application of both aerobic nitrifiers and anaerobic denitrifers also demands a more complex set-up to house separate aerobic and anaerobic tanks (Khin and Annachhatre, 2004). Indeed, in a previous report, different heterotrophic *Bacillus* strains were found to convert ammonia to N_2 without formation of nitrous oxide under aerobic conditions (Kim, et al., 2005). Hence, the ability of the *Bacillus* species to both nitrify and denitrify makes these microorganisms more attractive candidates in removing these nitrogenous wastes present in the aquaculture systems and wastewater.

2.5.1 Pathogenicity associated with the Bacillus species

The application of any probiotic whether for humans or animals should take into account its safety, for example, if it is for human use, it should not cause disease or harm to the human population and if it is use as a feed additive for animals, it does not increase the risk of inter-species transfer of antibiotic resistance genes to animal pathogens that can cross the species barrier and infect humans through food products (Hong, et al., 2005). Some *Bacillus* species were used as commercial probiotics for humans (table 2.3), there are however several *Bacillus* species reported to be of clinical importance. Among those, the *B. anthracis* and *B. cereus* are known human pathogens. The former requires no further elaboration as they are the cause of the classical anthrax disease (Van Ness, 1971) while the use of *B. cereus* appears to be a cause for concern on a case-to-case basis, considering that some strains were used as human probiotics (table 2.3). Some strains of *B. cereus* causes two types of gastrointestinal disease, the diarrhoeal and the emetic syndromes which are caused by different types of toxins. The emetic disease is caused by a single heat-stable peptide toxin called cereulide which causes vomiting few hours after ingesting the toxin from contaminated food (Agata, et al., 1995).

The toxin is performed in food and has been shown to be toxic to mitochondria by acting as a potassium ionophore (Mikkola, et al., 1999). The toxin is synthesized by the nonribosomal peptide synthase gene cluster *cesHPTABCD* located on a pXO1-like megaplasmid (200-270 kb) (Ehling-Schulz, et al., 2006b). Conversely, the diarrhoeal disease is caused by heat-labile enterotoxins which disrupt the integrity of the plasma membrane of the epithelial cells in the small intestine. The disease is produced by ingesting viable cells or spores of *B. cereus* from contaminated foodstuffs, and producing protein enterotoxins in the small intestine. The three toxins that are the causative agents of the diarrhoeal disease are the pore-forming cytotoxins hemolysin BL (Hbl) (Beecher and Macmillan, 1991), nonhemolytic enterotoxin (Nhe) (Lund and Granum, 1996) and the single protein cytotoxin K (CytK) (Lund, et al., 2000). Hbl is a three-component hymolysin that consists of two lytic components (L1 and L2, encoded by *hbl*D and *hbl*C) and a binding protein B (encoded by *hbl*A). Nhe is also a three-component , but nonhemolytic toxin that is encoded by three genes *nhe*A, *nhe*B and *nhe*C (Ehling-Schulz, et al., 2006a). Immunological assays for the detection of Nhe and Hbl are commercially available but none for CytK and cereulide. However, all the toxin genes mentioned can be detected by PCR assay (Ehling-Schulz, et al., 2006a). *B. cereus* is also a causative agent of nongastrointestinal infections such as wound infections, ophthalmic infections, endocarditis, postoperative meningitis, urinary tract infection and liver infection [see review of Drobniewski (1993)]. Nongastrointestinal infections by *B. cereus* are relatively rare, and the occurrence are normally associated in immunologically compromised patients, neonates, drug addicts and patients with a history of traumatic and surgical wounds or catheters (Drobniewski, 1993). The enzymes that are involved to various pathogenicity of *B. cereus* include phospholipase C, collagenase, cysteine-dependent serine proteinase and various beta-lactamases (Kotiranta, et al., 2000). Furthermore, some strains of *B. licheniformis* were reported to be producing toxins and were implicated causing food poisoning (Salkinoja-Salonen, et al., 1999). Mikkola, et al. (2000) reported that the toxin associated with the food poisoning of *B. licheniformis* strains was identical to lichenysin A.

Table 2.3 Commercial probiotic products containing *Bacillus* spores that were used for humans [Redrafted from *Hong, et al. (2005)*]

Product	Manufacturer	Comments
Bactisubtil®	Originally produced by Marion Merrell Dow Laboratories (Levallois-Perret, France); but also by Hoechst and then Aventis Pharma following merger acquisition. Also cited as being produced by Casella-Med, Cologne, Germany	Capsule carrying 1 x 10^9 spores of *B. cereus* strain IP 5832[b] (ATCC 14893; originally deposited as *B. subtilis*
Bibactyl	Tendiphar Corporation, Ho Chi Minh City, Vietnam	Sachet (1 g) carrying 10^7-10^8 spores of *B. subtilis*
Bidisubtilis	Bidiphar. Binh Dinh Pharmaceutical and Medical Equipment Company, 498 Nguyen Thai Hoc, Qui Nhon, Vietnam	Labelled sachets carrying 1x 10^6 spores of *B. subtilis*
Biosporin®	Biofarm, Dniepropetrovsk, Ukraine	Biosporin® is a mixture of two strains of living antagonistic bacteria *B. subtilis* 2335 (sometimes referred to as *B. subtilis* 3) and *B. licheniformis* 2336 (ratio is 3:1). Originally isolated from animal fodder
	Garars, Russia	There are a number of versions of this product produced in different countries including a recombinant form, Subalin
Biosubtyl	Biophar Company, Da lat, Vietnam	Sachet (1 g) carrying 10^6–10^7 of *B. cereus* spores mixed with tapioca. Product labelled as *B. subtilis*. The strain is closely related by 16S rRNA analysis to IP 5832 used in Bactisubtil®
Biosubtyl DL	IVAC, 18 Le Hong Phong, Da Lat, Vietnam	Sachets (1g) carrying 10^7–10^8 colony forming unit (CFU) of *B. subtilis* and *LactoBacillus acidophilus*
Biosubtyl	Biophar Company, Da lat, Vietnam	Sachet (1 g) carrying 10^6–10^7 of *B. pumilus* spores mixed with tapioca. Product labelled as *B. subtilis*
Biovicerin®	Geyer Medicamentos S. A. Porto Alegre, RS, Brazil http://www.geyermed.com	*B. cereus* strain GM Suspension of 10^6 spores ml^{-1}
Bispan®	Binex Co. Ltd, Busan, S. Korea www.bi-nex.com	Tablet carrying spores (1.7 x 10^7) of *B. polyfermenticus* SCD[d]
Domuvar	BioProgress SpA, Anagni, Italy http://www.giofil.it	Vial carrying 1 x 10^9 spores of *Bacillus clausii* in suspension, labelled as carrying *B. subtilis*. No longer marketed
Enterogermina®	Sanofi Winthrop SpA, Milan, Italy www.automedicazione.it	Vial (5 ml) carrying 1 x 10^6 spores of *B. clausii* in suspension. At least four different strains of *B. clausii* present and product originally labelled as carrying *B. subtilis*

Flora-Balance	Flora-Balance, Montana, USA www.flora-balance.com	Capsules labelled as carrying *Bacillus laterosporus* BOD[d] but containing *BrevoBacillus laterosporus* BOD
Lactipan Plus	Istituto Biochimico Italiano SpA, Milan, Italy	Capsule carrying spores of *Bacillus subtilis* labelled as carrying 2x 10^9 spores of *LactoBacillus sporogenes*[d]
Lactospore	Sabinsa Corp., Piscataway, NJ, USA www.sabinsa.com	Labelled as carrying *LactoBacillus sporogenes*[d] but contains *B. coagulans* 6-15 x 10^9 g^{-1}
Medilac	Hanmi Pharmaceutical Co. Ltd., Beijing, China http://www.hanmi.co.kr	*B. subtilis* strain RO179 (at 10^8 g^{-1}) in combination with *Enterococcus faecium*
Nature's First Food	Nature_s First Law, San Diego, CA, USA http://www.rawfood.com	42 species listed as probiotics including: *B. subtilis, B. polymyxad B. pumilus and B. laterosporus*[d]
Neolactoflorene	Newpharma S.r.l., Milan, Italy	Mixture of lactic acid bacteria inc. *L. acidophilus, B. bifidum and L. sporogenes*[d] *L. sporogenes* at 3.3 x 10^5 CFU g^{-1} whose valid name is *B. coagulans* is mislabelled and is a strain of *B. subtilis*
Pastylbio	Pasteur Institute of Ho Chi Minh City, Vietnam.	Sachets (1g) carrying 10^8 spores of *B. subtilis*
Primal Defense™	Garden of Life®	14 bacterial components including *B. subtilis* and *B. licheniformis*
Subtyl	Mekophar, Pharmaceutical Factory No. 24, Ho Chi Minh City, Vietnam	Capsule carrying 10^6–10^7 spores of a *B. cereus* species termed *B. cereus* var *vietnami*. Product labelled as carrying *B. subtilis*

[d] not officially recognized as *Bacillus* species (www.bacterio.cict.fr)

2.6 POLY-β-HYDROXYBUTYRATE- A NEW MICROBIAL AGENT FOR AQUACULTURE

There is a continuous effort in finding alternative strategies to control diseases in aquaculture and in recent years, the application of the bacterial storage compound, poly-β-hydroxybutyrate (PHB) was identified as a new disease control agent (Defoirdt, et al., 2011a). PHB is an important member of the family of polyhydroxyalkanoates (PHA), a class of microbially-produced polyesters that have potential applications similar to synthetic polymers (Madison and Huisman, 1999). In aquaculture applications, it has been shown that PHB has several beneficial effects to the cultured animals such as improved growth and robustness effects (De Schryver, et al., 2010; Defoirdt, et al., 2007). The observed beneficial effects of PHB to the host were linked to its degradation products (i.e SCFA) (Defoirdt, et al., 2009), its steering effect to the microbial community in the gut (De Schryver, et al., 2011; Najdegerami, et al., 2012) and its immune stimulating effect to the host (Suguna, et al., 2014). The advantage of using PHB for aquaculture is that it can easily be incorporated in formulated feed and it is stable in water (if introduced via bioencapsulation method). With the growing interest of this compound in aquaculture and in several industrial applications, the production cost can be decreased rapidly and can be economically attractive for aquaculture. However, the amount of research conducted on PHB is still limited and in order to maximise the potentials of this compound to aquaculture, more research are required.

2.7 THE POLYHYDROXYALKANOATE FAMILY

Polyhydroxyalkanoates are a family of linear polyesters of 3-, 4-, 5-, & 6-hydroxyalkanoic acids (Figure 2.2) that are synthesized by a wide variety of bacteria through sugars, lipids, alkanes, alkenes, and alkanoic acid fermentation (Philip, et al., 2007). These compounds are found in discrete cytoplasmic inclusion bodies (as amorphous mobile polymer) in bacterial cells (Figure 2.3) and are produced under nutrient-limiting conditions with excess carbon. Each monomer units in PHA contains a side chain (R)-β-hydroxy fatty acids, where the pendant group (R) varies from methyl (C1) to tridecyl (C13) (Madison and Huisman, 1999). The type of alkyl group found at the β-

position of the molecule determines the identity of the monomer (Kadouri, et al., 2005). The pendant (R) group is usually a saturated alkyl group but can also take the form of unsaturated alkyl group, branched alkyl group and substituted alkyl group (Zinn, et al., 2001). The type of bacterium and the growth conditions normally determines the chemical composition and size of PHA, however molecular masses of these PHAs typically ranges from 50,000 to 1,000,000 Da (Lee, 1996; Madison and Huisman, 1999). PHAs could be separated in three classes based on the number of component carbon atoms in their monomer units: short chain length PHA (sclPHA) with carbon number of monomers ranging from C3 to C5; medium chain length PHA (mclPHA) with C6 to C14 and long chain length PHA (lclPHA) with > C14 (Lee, 1996; Luengo, et al., 2003). About 150 different PHA monomers have been identified and this continues to increase due to chemical or physical modifications of the naturally-occurring PHA (Zinn, et al., 2001), or through genetically modified organisms which produced PHA with specialized functions (Escapa, et al., 2011). The most common PHAs are polymers of 3-hydroxyacids wherein PHB, also known as Poly-(R)-3-hydroxybutyrate is the most frequently found member. PHB like other PHAs exhibit thermoplastic and elastomeric properties (Byrom, 1987). PHB homopolymer shows similarities in its physical properties with polypropylene (PP) except that PHB is biodegradable while PP is not (Table 2.4). In general, PHAs are natural, recyclable, insoluble in water, non-toxic, biocompatible, and biodegradable (easily be degraded to carbon dioxide and water). Due to these properties, there has been considerable interest in the commercial use of these polyesters for several practical applications such as in daily life, medicine and agriculture (Table 2.5).

Table 2.4 Chemical and physical properties of polypropylene and PHB [after *Brandl, et al. (1990)*]

Parameter	PHB	PP
Melting point T_m (°C)	171-182	171-186
Glass transition temperature T_g (°C)	5-10	-15
Crystallinity (%)	65-80	65-70
Density (g cm^3)	1.23-1.25	0.905-0.94
Molecular weight M_w (x 10^{-5})	1-8	2.2-7
Molecular weight distribution	2.2-3	5-12
Flexular modulus (GPa)	3.5-5	1.7
Tensile strength (MPa)	40	39
Extension to break (%)	6-8	400
UV resistance	Good	Poor
Solvent resistance	Poor	Good
Oxygen permeability (cm^3 m^{-2} atm^{-1} d^{-1})	45	1700

Table 2.5 Practical applications of PHA *(Brandl, et al., 1990; Philip, et al., 2007; Zhang, et al., 2009)*

Medical applications:

- Surgical pins, sutures, staples and swabs
- Wound dressing
- Blood vessel replacements
- Bone replacements and plates
- Stimulation of bone growth by piezoelectric properties
- Biodegradable carrier for long term dosage of drugs and medicines

Industrial applications:

- Packaging containers, bottles, wrappings, bags and films
- Disposable items such as diapers or feminine hygiene products
- Source of biofuels

Agricultural applications:

- As bacterial inoculants to enhance nitrogen fixation in plants
- Biodegradable carrier for long term dosage of herbicides, fungicides, insecticides or fertilizers

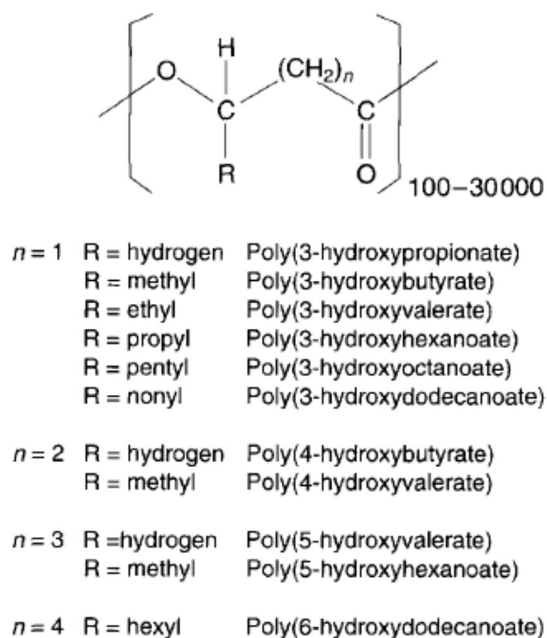

Figure 2.2 General molecular structure of polyhydroxyalkanoates and some representative members

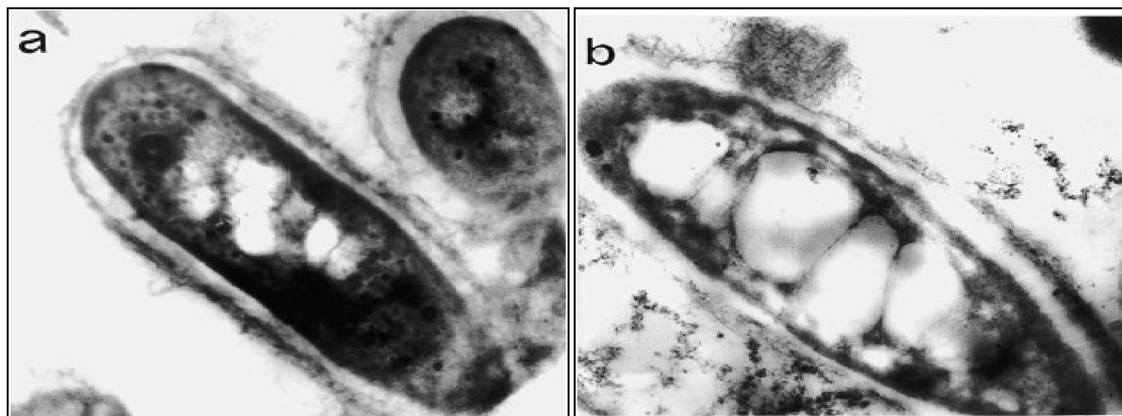

Figure 2.3 Transmission electron microscopy of *Bacillus megaterium* cells cultivated in a bioreactor under optimized culture conditions (a) 24 h and (b) 48 h incubation (x 30,000)[after *Gouda, et al. (2001)*]

2.8 BIOSYNTHESIS OF PHB/PHA BY MICROORGANISMS

Accumulation of PHA is a natural phenomenon in bacteria to store carbon and energy, when nutrient supplies are imbalanced such as the depletion of nitrogen, phosphorus or oxygen while the carbon that is present is in excess (Shang, et al., 2003). However, some papers discussed that microorganisms like *Alcaligenes eutrophus, Alcaligenese latus, Azotobacter vinelandii* UWD and a mutant *Azotobacter vinelandii* were able to accumulate PHAs under non-limiting conditions (Keshavarz and Roy, 2010; Ojumu, et al., 2004). Depending on the type of carbon source, the bacterial cell metabolized the carbon substrates in different pathways (Figure 2.4) (Philip, et al., 2007). Sugars such as glucose and fructose are mostly metabolized via pathway I and yields PHA homopolymer. In this pathway, it involves three enzymatic reactions and is catalysed by three key enzymes. The first reaction consists of the condensation of two acetyl coenzyme A (acetyl-CoA) molecules, from the tricarboxylic acid (TCA) cycle to form acetoacetyl-CoA by the enzyme β-ketoacyl-CoA thiolase (encoded by *PhaA*) (Senior and Dawes, 1971). The second reaction is the reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA by a nicotinamide adenine dinucleotide phosphate-dependent (*R*)-3-ketoacyl-CoA reductase (for PHB synthesis this enzyme is acetoacetyl-CoA reductase) (encoded by *PhaB*). Lastly, the PHA synthase or polymerase enzyme (encoded by *PhaC*) catalyses the polymerization via esterification of (*R*)-3-hydroxybutyryl-CoA into PHA with a concomitant release of CoA (Madison and Huisman, 1999). Synthesis of PHA by bacteria from alkanes, alkenes and alkanoates are processed via pathway II or the fatty acid β-oxidation pathway. In this pathway, the β-oxidation intermediate trans-2-enoyl-CoA is converted to (*R*)-hydroxyacyl-CoA by a (*R*)-specific enoyl-CoA hydratase (encoded by *PhaJ*) (Fukui and Doi, 1997; Fukui, et al., 1998). In pathway III, the intermediates observed in the synthesis were obtained from the fatty acid *de novo* biosynthetic pathway wherein the (*R*)-3-hydroxyacyl intermediates from the fatty acid biosynthetic pathway are converted from their acyl carrier protein form to the CoA form by acyl-ACP-CoA transacylase enzyme (encoded by *PhaG*) (Rehm, et al., 1998). Pathway III has some significant interest because it helps to generate monomers for PHA synthesis from structurally unrelated and simple, inexpensive carbon sources. The

monomeric composition of the biopolymer depends on the metabolic pathways operating in the cell and the external carbon source (Aldor and Keasling, 2003). Fatty acids or sugars that are metabolized via pathway II, III or other pathways produce PHA copolymers (Aldor and Keasling, 2003; Verlinden, et al., 2007).

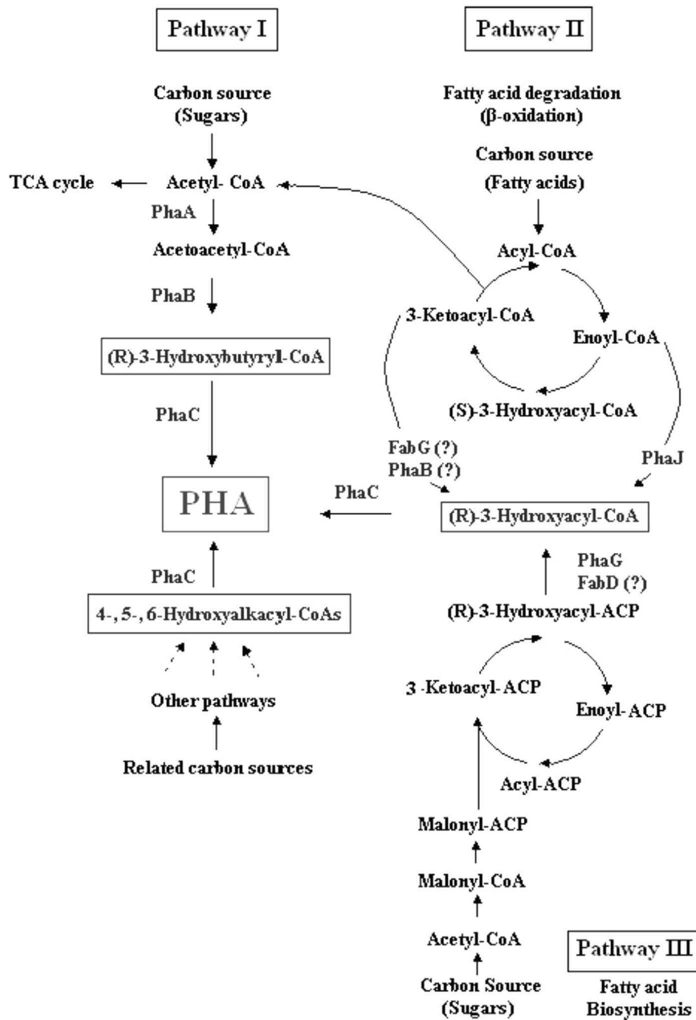

Figure 2.4 Metabolic pathways for PHA biosynthesis in bacteria [after *Philip, et al. (2007)*]

2.9 PHA DETECTION, QUANTIFICATION, PRODUCTION AND RECOVERY

PHA granules inside the bacterial cells can be rapidly detected by staining with Sudan Black B (Wei, et al., 2011b). Other methods like fluorescent staining using acridine orange (Kumar and Prabakaran, 2006), Nile blue A (Ostle and Holt, 1982) or Nile red (Spiekermann, et al., 1999) were also being used for detecting PHA and/or rapid

screening of PHA-accumulating bacteria. The most common method to quantify PHA is through spectrophotometric method wherein PHA is extracted from the bacterial cells through chloroform and the extracted PHA is converted to crotonic acid using concentrated sulphuric acid, and PHA is estimated spectrophotometrically at 235 nm (Law and Slepecky, 1961). The use of gas chromatography (Braunegg, et al., 1978), high performance liquid chromatography (Karr, et al., 1983), ionic chromatography, and enzymatic determination (Hesselmann, et al., 1999) are also some of the methods used for PHA quantification.

Selection of microorganisms for PHA production should consider its high PHA productivity (i.e. can grow efficiently to high cell densities with a high PHA content in a relatively short period of time). Likewise, the PHA yield (defined as gram PHA produced per gram of carbon substrate consumed) should also be high so as not to waste the utilization of substrate to non-PHA materials (Lee, 1996). While these factors are important, the carbon source that will be used should also be low-cost, considering its major contribution in the overall cost of the PHA production. Hence, alternative carbon sources like from waste materials and other by-products have been tested (Castilho, et al., 2009; Koller, et al., 2010; Pandian, et al., 2010). A variety of taxonomical different groups of microorganisms were identified to be producing PHA (Brandl, et al., 1990). Some PHA-producing microorganisms require the limitation of an essential nutrient (i.e N, P, Mg, K, O or S) to stimulate the synthesis of the biopolymer from an excess carbon source (eg. *Alcaligenes eutrophus, Pseudomonas oleverans, Protomonas extorquens* etc.) while some bacteria does not require nutrient limitation (e.g. *Alcaligenes latus*, mutant strain of *Azotobacter vinelandii* and recombinant *E. coli* harbouring the *A. eutrophus* PHA biosynthesis operon) (Lee, 1996). Different fermentation strategies were being employed in these microorganisms to attain maximum PHA production. For example, fed-batch culture with two-step cultivation method is often employed in those bacteria requiring a nutrient limitation wherein cells are first grown to desired concentration without nutrient limitation, after which an essential nutrient is limited to allow efficient PHA synthesis while those bacteria that does not require nutrient limitation strategize on the nutrient feeding itself (i.e. carbon and nitrogen source used). Most bacterial species used for the industrial scale production of PHA belongs to Gram-

negative bacteria such as *Alcaligenes eutrophus, Alcaligenes latus, Azotobacter vinelandii, Pseudomonas oleovorans*, recombinant *Escherichia coli,* and among others. The utilization of Gram-positive bacteria (i.e. *Bacillus* species) as source of PHAs has also been suggested (Valappil, et al., 2007). The lack of LPS in Gram-positive bacteria was suggested to be beneficial considering that LPS may co-purify the PHAs and may have some immunogenic reactions. This aspect is important especially in PHAs that are used for medical applications. *Bacillus* species have been shown to accumulate PHA in the range of 2.2 to as high as 97.3 % on cell dry weight (CDW) and mostly accumulate co-polymers of 3-hydroxybutyrate (Valappil, et al., 2007). The application of recombinant DNA technique has allowed the development of various recombinant bacteria that can produce PHA more efficiently, synthesize PHA from inexpensive carbon sources and/or synthesize unusual and industrially important PHAs. These advances will lower the PHA production cost.

Efficient PHA isolation is considered an important economic factor in the overall PHA production thus industries put more effort in developing efficient methods for PHA recovery. In general, cells containing the PHA are separated from fermentation broth by centrifugation to concentrate cells. Subsequently, the harvested cells are then broken up and various chemicals are added to digest non-PHA materials. Cell lysis by hypochlorite has been used as an excellent method for efficient and cheap release of PHA (Berger, et al., 1989). Solvent-based extraction methods using chloroform, methylene chloride, propylene carbonate, dichloroethane are also being employed. After removing all the cellular components, the polymer is precipitated in cold ethanol or methanol. The method was suggested to attain high PHA purity (> 98%) (Zinn, et al., 2001).

2.10 THE PHB GRANULE

PHB was the first PHA discovered by a French scientist Lemoigne in 1925 in the cytoplasm of *B. megaterium* as a granular inclusion bodies (Macrae and Wilkinson, 1958). PHB and related PHAs are considered storage compounds acting as carbon and energy reserves by both sporulating and non-sporulating bacteria to enhance its survival during periods of starvation (Anderson and Dawes, 1990). In spore-forming bacteria, PHB is

utilized as an energy source to fuel the sporulation process (Valappil, et al., 2007). The diameter of PHB and PHA granules ranges from 200-500 nm in wild type strains, but the size mostly depends on the species and culture conditions used (Jendrossek and Pfeiffer, 2014). Electron microscopical studies suggest that PHB granules *in vivo* are covered by a surface layer that is distinct from the polymer core. According to Griebel, et al. (1968), PHB granule consisted of ~97.7% of PHB, 1.87% protein and trace amounts of lipids (phosphatidic acid and unidentified acetone soluble compound). The report suggests that the PHB granule *in vivo* are surrounded by a lipid (mono)layer into which proteins are embedded. However, the *in vivo* presence of phospholipids in the PHB granule are still being debated in some papers and suggested to investigate its presence or absence in the PHB granules (Jendrossek and Pfeiffer, 2014). Nevertheless, the model that was mostly used in describing the PHB granule structure in several review papers was based on the report of Griebel, et al. (1968) wherein a coil of amorphous polymer chains are surrounded by a layer of phospholipids and proteins (Figure 2.5). PHB granules can vary in terms of physical states: the intracellular native, amorphous state wherein the granules are still present *in vivo* in bacterial cells and the partially crystalline state wherein the granule is already extracted outside the bacterial cell. Once the PHB granules are exposed to chemical (e.g. treatment with alkali or solvents), physical (freezing, pelleting by centrifugation) or biochemical (treatment with enzymes, bioactive compound) stresses, the granules rapidly denature and become resistant to the attack of intracellular PHB depolymerases (Griebel and Merrick, 1971; Merrick, 1965; Merrick, et al., 1965).

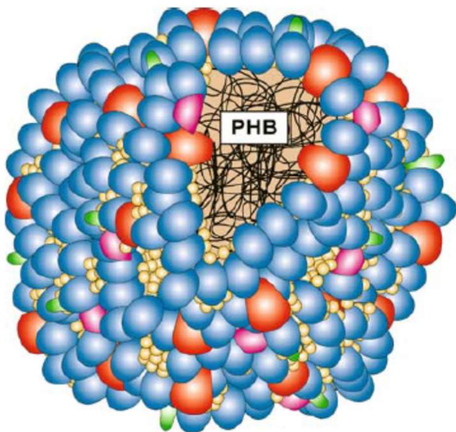

Figure 2.5 Scheme of a PHB granule. The core consists of PHA polymer that is covered by a lipid monolayer (yellow globes) with integrated proteins. The integrated proteins consists of PHA synthase (red globes), PHA depolymerase (magenta globes), phasins (blue globes) [after Pötter and Steinbüchel (2006)]

2.11 PHB GRANULE-ASSOCIATED PROTEINS (PGAPS)

Both *in vitro* and *in vivo* studies show the presence of a considerable number of different PHB/PHA specific proteins in the surface layer of the polyester granules. In *Bacillus cereus* and *Bacillus megaterium*, a dense membrane of about 15-20 nm were observed at the surface of its PHB granule (Lundgren, et al., 1964) while the PHB granules from *Caryophanon latum* cells revealed a densely packed, paracrystalline-like layer of particles with a diameter of ~8 nm (Jendrossek, et al., 2007). It was concluded that these membrane particles represent PGAPs and were thought to mostly or completely cover the granule surface (Figure 2.5) (Jendrossek and Pfeiffer, 2014). These PGAPs were reported playing a major role in the synthesis and degradation of PHA as well as in the formation of the PHA granule (Jendrossek, 2009; Pötter and Steinbüchel, 2005). Four different types of granule-associated proteins were identified and reported in PHA producing bacteria showing prominent function to PHA metabolism: These are (1) PHA synthase, a key enzyme for PHA synthesis; (2) PHA depolymerases, are enzymes that degrade PHAs (3) phasins, are suggested to be the major structural proteins of the membrane surrounding the inclusion and (4) regulator of phasin expression (PhaR) (Table 2.6) (Jendrossek and Pfeiffer, 2014; Pötter and Steinbüchel, 2005). *(For the relevance of the work presented in Chapter 7, an overview on phasins is presented below).*

Table 2.6 Overview on PGAPs and related proteins with prominent function in PHA metabolism in *Bacillus* megaterium and Ralstonia eutropha H16. Protein with asterisk (*) are not expressed in wild-type or are not PHB-bound in vivo. Bold-type gene designations indicate experimental evidence for the binding of the gene product to PHA granules in vivo [redrafted from *Jendrossek and Pfeiffer (2014)*]

Species and/or strain	PHA	Gene	(Putative) Function	Reference
Bacillus megaterium	PHB	***phaC, phaR***	PHB synthase	(McCool and Cannon, 1999; 2001)
		phaZ1	i-PHB deoplymerase	(Chen, et al., 2009)
		phaP	phasin	(McCool and Cannon,

				1999; 2001)
		phaQ	transcriptional regulator of phasin expression	(Lee, et al., 2004; McCool and Cannon, 1999; 2001)
Ralstonia eutropha H16	PHB, scl-PHA	***phaC1***	PHB synthase	(Schubert, et al., 1988; Slater, et al., 1988)
		*phaC2**	PHB synthase (gene not expressed)	(Brigham, et al., 2010; Peplinski, et al., 2010)
		phaP1	Phasin	(Wieczorek, et al., 1995; York, et al., 2001)
		phaP2, phaP3, phaP4	Phasins	(Pfeiffer and Jendrossek, 2011; Pötter, et al., 2004)
		PhaP5	Phasin	(Pfeiffer and Jendrossek, 2011)
		phaP6, phaP7**	Phasins	(Pfeiffer and Jendrossek, 2012)
		PhaM	Granule segregation factor, activator of PHB synthase, phasin	(Pfeiffer and Jendrossek, 2011; 2014; Wahl, et al., 2012)
		PhaR	Transcriptional regulator of phasin expression	(Pötter, et al., 2002; Seo, et al., 2003)
		PhaZa1 (phaZ1)	PHB depolymerase	(Handrick, et al., 2000; Saegusa, et al., 2001)
		phaZa2 (phaZ2)*	PHB depolymerase	(Brigham, et al., 2012; York, et al., 2003)
		phaZa3(phaZ5)*	PHB depolymerase	(Brigham, et al., 2012; York, et al., 2003)
		phaZa4(phaZ4)*	PHB depolymerase	(Schwartz, et al., 2003)

	phaZa5(phaZ3)*	PHB depolymerase	(Brigham, et al., 2012)
	phaZd1(phaZ6)*	PHB depolymerase	(Abe, et al., 2005)
	phaZD2 (PhaZ7)*	PHB depolymerase	(Pohlmann, et al., 2006)
	phaZb (phaY1,phaZ2)*	Oligomer hydrolase	(Kobayashi, et al., 2003; Kobayashi, et al., 2005)
	phaZc (phaY2)*	Oligomer hydrolase	(Kobayashi, et al., 2003; Kobayashi, et al., 2005)

2.11.1 PHASINS

Phasins are considered the major PGAPs that cover most of the PHA granules (Figure 2.5). They are synthesized in large quantities and represent as much as 3-5% of the total protein of PHB accumulating cell granules (Wieczorek, et al., 1995). Due to their amphiphilic nature, these proteins play an important structural function forming an interphase between the hydrophobic content of PHA granules and the hydrophilic cytoplasm content. They stabilize the granule and prevent coalescence between granules. Phasins do not constitute a highly conserved group of proteins and based on their sequence similarities, four types of phasin families have been distinguished, each contain a characteristic domain (Mezzina and Pettinari, 2016). The first family (PF09361) is the most numerous one which includes sequences found in Alpha, Beta and Gamma Proteobacteria such as in *R. eutropha*. The second family (PF09602) are phasins found in the *Bacillus* species, while the third group (PF09650) contains a diverse group of proteins belonging to different Proteobacteria. The last family (PF05597) contains proteins from different Proteobacteria and include phasins belonging to *Pseudomonas* that accumulate mclPHAs. While most phasins to date belong to one of the four families described, there are however few identified phasins that show very little similarity and contain no recognizable phasin related domains and these include phasin from *R. ruber* (GA14), *Synechocytis sp.* PCC 6803 and archael phasins (Cai, et al., 2012; Hauf, et al., 2015).

Aside from the structural role of phasins, many other functions related to PHA accumulation, degradation, granule size, number and distribution have been described for different phasins (Mezzina and Pettinari, 2016). It was observed that the occurrence of phasins in the cells is strictly dependent on PHA synthesis. For example, in *R. eutropha* cell with a mutant defective PHA synthase show undetectable levels of phasin while an increasing levels of PHA accumulation display an increasing levels of intracellular phasin (Wieczorek, et al., 1995; York, et al., 2001). Phasin from *R. eutropha* ($PhaP_{Re}$) has increased the activity of class II PHA synthases PhaC1 and PhaC2 from *P. aeroginosa in vitro*, thereby activating the PHA synthesis (Qi, et al., 2000). The phasin PhaM from *R. eutropha* was described as physiological activator of the PHA synthase in this microorganism (Pfeiffer and Jendrossek, 2014). Aside from its role on PHA synthesis, the importance of phasins on PHB degradation was also reported. In *Rhodospirillum rubrum*, it was observed that the phasin ApdA activates the PHB depolymerase to hydrolyse the PHB granule *in vitro* (Handrick, et al., 2004). In *R. eutropha*, the presence of $PhaP_{Re}$ on the granule surface was also reported to be important for PHB degradation (Kuchta, et al., 2007). The suggested involvement of phasin on PHB degradation could be by providing the PHB depolymerase an access to the surface of the PHB granule or there is a direct interaction of phasin and the PHB depolymerase during PHB utilization (Kuchta, et al., 2007). Phasin also showed a key role in intracellular localization and equal distribution of PHA granules to daughter cells during cell division in *P. putida* (Galán, et al., 2011). The overexpression of phasin in *R. eutropha* has also resulted in the formation of many small granules of PHB in the cell (Pötter, et al., 2002). Furthermore, the protective effect of phasins towards stress was also observed in recombinant *E. coli*. PHB production was shown to cause stress in recombinant *E. coli* (as evidenced by an increased expression of chaperones, sigma factors and other stress related genes) and the presence of $PhaP_{Az}$ (phasin from *Azotobacter* sp. FA8) has dramatically decreased the expression of these stress-related genes unlike in those strain that do not synthesize the phasin (de Almeida, et al., 2011). $PhaP_{Az}$ also showed a protective effect in non-PHB synthesizing *E. coli* under normal and stress conditions, as demonstrated by a reduction in heat shock protein levels, increased growth and higher resistance towards heat shock and superoxide stress by paraquat (de Almeida, et al., 2011). The chaperone-like activity

of phasin was also demonstrated both *in vitro* and *in vivo* using $PhaP_{Az}$ (Mezzina, et al., 2015). The phasin was able to protect citrate synthase (CS) from thermal unfolding followed by aggregation *in vitro*. Furthermore, incubating chemically denatured CS in the presence of $PhaP_{Az}$ has enhanced the rate and levels of CS refolding, suggesting that the phasin helps the protein from returning to its native state. *In vivo* observation also shows that $PhaP_{Az}$ plays role in the inclusion bodies (IBs) (IBs are protein aggregates formed as a result of incorrect protein folding or unfolding) constructions/deconstruction process such that a decrease in number and size of IBs was observe in cells expressing the $PhaP_{Az}$. The phasin was also observed to be binding the aggregated proteins in IBs (Mezzina, et al., 2015).

In summary, aside from the structural role of phasins to the PHA granule, several functions of these proteins related to PHA synthesis, distribution and size of PHA in the cell, and PHA utilization were observed. Its possible role in related stress protection and fitness enhancement in bacteria is an interesting feature to look at on the possible role of phasins on the protective capabilities of PHB in aquatic animals. Hence, the work presented in **Chapter 7** elucidates if phasins has contribution on the protective effect of PHB in cultured aquatic animals such as on *Artemia*.

2.12 APPLICATION OF PHB IN AQUACULTURE

2.12.1 ANTIMICROBIAL ACTIVITY OF PHB

The antimicrobial activity of PHB could be linked to its degradation products (i.e. SCFAs) wherein several studies have shown the inhibitory effect of SCFAs in pathogenic bacteria (Sun, et al., 1998; Van Immerseel, et al., 2003). The ability to degrade extracellular PHA to β-hydroxy SCFAs depends on the secretion of specific PHA depolymerase enzymes, and is widely distributed among bacteria and fungi (Jendrossek, 1998). The main bacteriostatic and/or bactericidal effect of SCFAs is believed to be due to the undissociated form of the acid, which can pass through the bacterial cell wall. Once inside the cell, it is dissociated in the more alkaline cytoplasm and releases proton (H^+) resulting to the lowering of the intracellular pH (Cherrington, et al., 1991). As a consequence, the bacterium has to redirect its energy towards the efflux of excess proton,

thereby exhausting the cell metabolism and leading to lower cell growth or even cell death (Hismiogullari, et al., 2008). The other less direct growth interfering effects associated with SCFAs include the disruption of the bacterial cell wall resulting in leakage, interference with nutrient transport and altered energy or molecule synthesis (Ricke, 2003).

In aquaculture applications, Defoirdt, et al. (2006a) investigated the effects of different concentrations of SCFAs namely formic, acetic, butyric, propionic and valeric acid on the growth of pathogenic *V. campbellii* in Luria Bertani (LB) medium. The study showed that at pH 6, all the fatty acids completely inhibited the growth of the pathogen at a concentration of 100 mM. The results further showed that the growth-inhibitory effect of the fatty acids was pH dependent and that the effect decreases with increasing pH, a similar phenomenon observed in other studies (McHan and Shotts, 1993). Finally, adding 20 mM of the SCFAs to the culture water of the *Vibrio* challenged-gnotobiotic *Artemia* has resulted to a significantly higher survival of the infected nauplii. Furthermore, the antimicrobial activity of β-hydroxybutyrate was also investigated by Defoirdt, et al. (2007). The same with their findings on other SCFAs, the fatty acid was also effective in controlling the growth of the pathogenic *Vibrio campbellii* in LB medium at 100 mM dose at pH 6. Adding the β-hydroxybutyrate in the culture water of the *Vibrio*-challenged gnotobiotic *Artemia* resulted in a significantly increased survival of the infected nauplii. Moreover, in the same study, the application of PHB (the polymer form of β-hydroxybutyrate) was also investigated to determine if the biopolymer has similar antimicrobial effect with the β-hydroxybutyrate. The results showed that adding PHB to the culture water of the gnotobiotic *Artemia* at 100 mg L^{-1} or more offered a preventive and curative protection to the *Artemia* against the pathogenic *Vibrio campbellii*. Interestingly, a complete protection (mortality was not significantly different from unchallenged *Artemia*) was observed in the challenged *Artemia* when PHB was added in the water at 1000 mg L^{-1}. Their findings demonstrated that PHB was about 100 times more efficient than the β-hydroxybutyrate. The authors hypothesized that the PHB particles were (partially) degraded into β-hydroxybutyrate in the *Artemia* gut and that the release of this fatty acid has protected the *Artemia* from the pathogen. Although, it is still remains to be demonstrated if and how PHB is degraded in the *Artemia* gut considering

that the work was conducted in a gnotobiotic system and any microbial degradation could not be possible.

From the following research mentioned above, it can be considered that the β-hydroxybutyrate and other SCFAs can be used as antimicrobial agents to control bacterial infections in aquaculture. However, the only limitation of using these fatty acids is that they are water soluble substances; hence the practical application of these compounds for aquaculture could be a challenge in terms of its effective delivery to the target sites. The addition of SCFAs in formulated feed could be lost in great amounts in the water due to leaching and if it is added directly in the water will require a considerable dose to be effective. Conversely, the application of PHB as a source of SCFAs in the gut suggests a more promising approach. PHB is a water-insoluble compound hence the compound can be easily incorporated in formulated feed and is easily delivered in the gut for degradation.

2.12.2 PHB ON GROWTH AND ROBUSTNESS EFFECTS IN CULTURED AQUATIC ANIMALS

The effects of PHB on the growth, survival and robustness in some cultured aquatic animals were investigated in recent studies. The work of De Schryver, et al. (2010) tested the effects of feeding different levels of PHB in the diet of European sea bass juveniles. PHB was incorporated in the formulated fed in different concentrations at 0, 2, 5, 10 and 100% PHB. Their results showed that fish receiving the 5% PHB attained a significantly higher weight gain than those fish fed the diets with no PHB incorporation. Their results further showed that PHB could be used as an energy source for the fish, a similar observation was reported in gnotobiotic starved *Artemia* (Defoirdt, et al., 2007). Indeed, PHB as a fatty acid is a typical source of energy (Azain, 2004) and β-hydroxybutyrate in the form of ketone bodies has been used as a source of energy in developing *Artemia* (Weltzien, et al., 2000). However, feeding the juvenile sea bass with 100% PHB resulted in an incomparably low weight gain, suggesting that PHB alone could not be used as a sole source of food to support growth in the fish. Furthermore, all the fish receiving the PHB (partial or full replacement in the feed) had a significantly lower pH than those fish fed no PHB addition, suggesting that PHB has led to the increased concentration of (short chain fatty) acids (i.e β-hydroxybutyrate) in the fish gut.

Interestingly, in the same study the amplified DNA from the intestinal matter of the PHB-fed seabass showed a trend of lower band pattern similarity in the Denaturing Gradient Gel Electrophoresis (DGGE) analysis, suggesting that there was a shift in the bacterial community composition in the GIT of the sea bass receiving high levels of PHB. The authors suggested that the observed changes in bacterial community composition were related to the dose effect of PHB and/or its degradation products and such changes in the intestinal bacterial community structure may have some closely related function to the observed beneficial growth effects of PHB in the fish. In another report, the application of PHB on giant freshwater prawn *Macrobrachium rosenbergii* larvae was also investigated and the results showed a significantly higher survival and growth in the PHB-fed prawn larvae than with the control (Nhan, et al., 2010). Moreover, the total bacterial and *Vibrio* spp. counts were found to be significantly lower in the PHB-fed larvae as compared to the control group, indicating that the PHB addition in the diet of the prawn larvae has resulted to the growth-inhibitory towards these potentially pathogenic bacteria. A similar result was also reported in Chinese mitten crab *Eriocheir sinensis* larvae after feeding PHB via *Artemia* bioencapsulation wherein the survival and growth of the crab larvae were also significantly enhanced (Sui, et al., 2012). In the same study, the survival of PHB-fed crab larvae after a *V. anguillarum* challenge was significantly higher as compared to the challenge control group, suggesting a protective effect of PHB in the Chinese mitten crab larvae during infection. This observation was in agreement with the results obtained by Defoirdt, et al. (2007) in gnotobiotic *Artemia*, wherein the PHB-fed *Artemia* was completely protected from pathogenic *V. campbellii* infection. To understand further the protective effect of PHB, Suguna, et al. (2014) determined the effects of PHB-hydroxyvalerate (PHB-HV) (extracted from *B. thuringiensis*) on the immune response of tilapia *Oreochromis mossambicus.* In this study, PHB-HV was added in the formulated feed of the tilapia in different concentrations at 0, 1, 3 and 5%. Their results showed that all the doses with PHB-HV supplementation were effective in stimulating both the specific and nonspecific immune response of the fish. Furthermore, when the tilapia was challenged with virulent *Aeromonas hydrophila*, all the fish receiving the PHB-HV resulted in a significantly higher survival as compared with the challenge control. The authors

suggested that the protective effect of PHB in tilapia was due to the immunostimulating effect of the polymer.

From the research mentioned above, it can be considered that PHB can improve the growth, survival and robustness of the cultured aquatic animals. The effect of PHB on growth is maybe linked to its influence in the microbial community composition in the gut wherein the microorganisms present may have some closely related functions to the overall growth effects in the animal. Likewise, the effects of PHB on improving the survival of the tested aquatic animals could be due to the immunostimulating effect of the polymer to the host, such that an increase resistance could be observed during pathogen infection. The other possibility could be the antimicrobial activity of the PHB monomer wherein the growth of potential pathogens could be inhibited. Furthermore, it has been suggested that applying PHB in amorphous form (i.e. still inside a bacterial cell) seems to increase the efficiency as compared to the application of PHB in crystalline form (i.e. extracted from the bacterial cell) (Halet, et al., 2007). Therefore, in this research the application and possible mode of action of amorphous PHB contained in *Bacillus* cells is investigated.

PART 2

ISOLATION, SCREENING & CHARACTERIZATION OF PHB-ACCUMULATING *BACILLUS* SPP.

CHAPTER 3

ISOLATION AND CHARACTERISATION OF POLY-β-HYDROXYBUTYRATE (PHB) ACCUMULATING *BACILLUS* SPP. OBTAINED FROM PASTEURIZED SHRIMP POND SEDIMENTS

Joseph Leopoldo Q. Laranja[1,2], Gladys Ludevese-Pascual[1], Edgar C. Amar[2], Maarten Aerts[3], Peter Van damme[3], Peter De Schryver[1], Peter Bossier[1]

[1]Laboratory of Aquaculture and *Artemia* Reference Center, Ghent University, 9000 Gent, Belgium
[2]Aquaculture Department, Southeast Asian Fisheries Development Center, 5021 Tigbauan, Iloilo, Philippines
[3]Laboratory of Microbiology, Department of Biochemistry and Microbiology, Ghent University, 9000 Gent, Belgium

ABSTRACT

It has been shown that crystalline poly-β-hydroxybutyrate (PHB) can be used as biocontrol agent in crustacean culture, while *Bacillus* spp. are generally considered to be interesting probiotics for aquaculture purposes. Hence, the aim of this study was to isolate PHB-accumulating *Bacillus* spp. from shrimp pond sediments. Sediment samples from a shrimp pond in the Philippines were pasteurized to obtain a spore forming mixed bacterial culture (mBC). From this mBC, 50 pure bacterial isolates were obtained and were further analysed using matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for bacterial grouping. Furthermore, nine representative isolates from the major MALDI-TOF-based groups were randomly selected for PHB measurement using spectrophotometric method. The bacterial isolates resulted in two major groups. Based on the PHB analysis of the nine representative isolates, the group 1 isolates were higher PHB accumulators than group 2. The PHB accumulation from the representative isolates showed a ranged from 3.0- 28.6% PHB with a total PHB yield of 0.1- 1.1 g L^{-1} on cell dry weight. The *Bacillus* isolate capable of accumulating higher amounts of intracellular PHB was selected and was designated as *Bacillus* sp. JL47.

Keywords: Poly-β-hydroxybutyrate accumulating *Bacillus* spp., amorphous PHB, MALDI-TOF MS, bacterial isolation, characterization

3.1 INTRODUCTION

The aquaculture sector provides roughly half of the aquatic food products consumed by the world's human population (FAO, 2014). In its path of ever continuing growth, increased production has been paralleled by the emergence of pathogenic diseases. The use of antibiotics and other chemotherapeutic agents to control this problem has been proven unsustainable due to the development of antibiotic resistant pathogens (Cabello, 2006). Therefore, there is a need for developing equally effective yet sustainable and environment-friendly strategies to address this problem, such as the application of the biocontrol agents- PHB and probiotics.

PHB is a bacterial storage compound accumulated as a cellular energy and carbon reserve by a large variety of bacteria that include the genera *Alcaligenes*, *Rhizobium*, *Pseudomonas* and *Bacillus* (Anderson and Dawes, 1990). Production of PHB by these microorganisms has drawn much attention in recent years because of its potential application as bioplastic in medicine (i.e. sutures, bone tissue scaffolds made from PHB) as these biopolymers were observed to be biocompatible in various host systems, including in humans; as bacterial inoculants in agriculture (e.g. the use of nitrogen-fixing bacteria able to accumulate PHB are more efficient in enhancing nitrogen fixation in plants) and daily life (biodegradable food packaging materials) (Philip, et al., 2007; Sabir, et al., 2009; Siracusa, et al., 2008; Volova, et al., 2003). In the field of aquaculture, the application of crystalline PHB particles (extracted from the bacterial cells) as a dietary additive or natural food enrichment has been reported to improve growth, survival and robustness (i.e. the animal has higher resistance against stresses both biotic and abiotic resulting in higher survival) of some cultured species including *Artemia* nauplii, European sea bass, Chinese mitten crab and Nile tilapia (De Schryver, et al., 2010; Defoirdt, et al., 2007; Situmorang, et al., 2016; Sui, et al., 2012). The suggested mode of action of PHB is that this bacterial biopolymer is degraded intestinally resulting in the release of its monomer form, 3-hydroxybutyrate. This compound can act antimicrobially by acidifying the cytoplasm of the pathogen and thus the pathogen has to redirect its cellular energy to maintain homeostasis (i.e. use of transporters that catalyze active proton transport), resulting in a decreased virulence (Defoirdt, et al., 2009). Conversely, PHB was also observed to stimulate the immunity of tilapia and gnotobiotic *Artemia* (Baruah, et al., 2015;

Suguna, et al., 2014). The work of Baruah et al., (2015) demonstrated that PHB conferred protection to *Artemia* against pathogenic *Vibrio campbellii* by inducing heat shock protein 70 (Hsp 70) which mediates the enhanced expression of the immune-protective genes (i.e. prophenoloxidase and transglutaminase) in *Artemia* during pathogenic *Vibrio* challenge. The use of amorphous PHB (i.e. PHB still contained inside the bacterial cell) was found even more efficient than using the extracted PHB form (Halet, et al., 2007). Indeed, in the previous work the application of PHB-accumulating bacteria as effective biocontrol agent for giant freshwater prawn larvae culture was demonstrated (Thai, et al., 2014).

The application of probiotic bacteria in cultured aquatic animals as preventive measures for diseases is a promising alternative to chemicals and antibiotics (Hai, 2015; Verschuere, et al., 2000). Among the probiotic bacteria that have been used in aquaculture, the *Bacillus* species are interesting because of their various beneficial probiotic effects such as stimulating the host's immune system, producing enzymes that degrade quorum sensing molecules thereby regulating virulence of pathogenic bacteria, producing antimicrobial compounds (i.e. bacteriocins, bacteriocin-like inhibitory substance such as subtulin, coagulin and antibiotics such as surfactin, iturin, isocoumarin, aminocoumarin A etc.) that inhibit growth of pathogenic microorganisms, producing exoenzymes (i.e. proteases, lipases, carbohydrases) that aid in digestion and absorption of nutrients in the gut and maintaining a healthy balance of commensal microbiota in the gastro-intestinal tract (Maruta, et al., 1996; Pande, et al., 2015; Pinchuk, et al., 2001; Ray, et al., 2012; Tseng, et al., 2009; Urdaci, et al., 2004). Their ability to produce spores makes them more advantageous compared to other probiotic bacteria in terms of long term storage and ease of application (Hong, et al., 2005). Interestingly, *Bacillus* species are capable of accumulating PHB as energy source to fuel the sporulation process (Slepecky and Law, 1961). We hypothesized that selecting *Bacillus* species with high PHB accumulating capacity could be an interesting approach to apply in aquaculture, considering the possible synergy of amorphous PHB and the probiotic effect of *Bacillus*.

This study demonstrated the isolation and screening procedure conducted for selecting *Bacillus* species focusing on superior PHB accumulation capacity of the bacteria as criterion. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

(MALDI-TOF MS) was used for bacterial grouping to rapidly select different PHB accumulating *Bacillus* isolates. From the MALDI-TOF MS based groupings, representative isolates were then selected for PHB quantification. The superior PHB accumulating isolate(s) was selected and identified for further *in vivo* testing which were presented in the succeeding chapters of this study.

3.2 MATERIALS AND METHODS

3.2.1 BACTERIAL ISOLATES

A sediment sample was collected from a *P. monodon* pond in Bacolod (Philippines) and pasteurized (80 °C, 20 min) to obtain a mixed bacterial culture (mBC) consisting of spore forming *Bacillus* species. During the time of sediment collection, the pond was stocked with *P. monodon* and no diseases were observed during and prior to the sampling. Also, the pond was not treated with any probiotic products that could potentially influence the composition of the bacterial collection in the study. A total of 50 isolates was selected based on morphological differences of the colony and purified using the streak plate procedure on Luria Bertani (LB) agar (tryptone (Himedia, 10 g L^{-1}), yeast extract (Himedia, 5 g L^{-1}) and sodium chloride (Sigma-Aldrich, 20 g L^{-1})). The streak plating was done three times to ensure purity of the isolates. All isolates were kept at -80 °C in 20% glycerol as stock cultures.

3.2.2 MALDI-TOF MS ANALYSIS

MALDI-TOF MS was used for rapid dereplication of the bacterial isolates (Ghyselinck, et al., 2011). The bacterial stock cultures were activated as a 1% (v/v) inoculum in LB medium at 28 °C shaken at 100 rounds per minute (rpm) for 16 h and subsequently used to inoculate LB supplemented with 20 g L^{-1} glucose (Sigma-Aldrich). The cultures were grown at 28 °C and 100 rpm agitation for 24 h. One mL of liquid culture was transferred in a 96-well plate (Nunc, Thermo Fisher) and harvested by centrifugation at 2,250 *g* for 10 min set at 4 °C. Media was removed by slow pipetting and cells were washed two times with 500 µL of MilliQ purified water and centrifuged again at 2,250 *g*

for 10 min at 4 °C. The pellets were dissolved in 100 µL MilliQ purified water from which two times 1 µL (technical replicates) of each bacterial suspensions was spotted on a 384 Opti-TOF 123 mm x 81 mm stainless steel MALDI-TOF MS target plate (AB Sciex) and dried at room temperature. Subsequently, the sample spots were overlaid with 1 µL of a 0.5% (w/v) α-cyano-4-hydroxycinnamic acid (α-CHCA) in acetonitrile:water:trifluoroacetic acid solution (50:48:2). For each bacterial stock, two suspensions were prepared as biological replicates. The target plate with the spotted samples was analysed using the 4800 *Plus* MALDITOF/TOF Analyzer (AB Sciex) in the linear positive ion mode. Ions were generated by a 200 Hz tripled UV Nd:YAG laser, accelerated at 20 kV through a grid at 19.34 kV and 1 µs delay time and subsequently separated according to their *m/z* (mass to charge) ratio in a 1.5 m long linear, field-free drift region. Each spectrum was the sum of the ions from 40 laser shots at 50 random positions within the measuring spot and was analysed in the mass range 2-20 kDa. Before acquisition, the TOF was calibrated using adrenocorticotropic hormone (ACTH) fragment 18–39 (m/z 2465.7), insulin (m/z 5734.6), ubiquitin I (m/z 8565.9), cytochrome C (m/z 12361.5) and myoglobin (m/z 16952.3).

After baseline corrections and smoothing, peaks with S/N (signal to noise) values higher than 5 were exported as text files via the DataExplorer™ software and imported in BioNumerics v. 7.5 (Applied Maths) for further analyses. Similarities between the profiles were calculated based on the Pearson Product Moment Correlation and clustered via Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

3.2.3 Quantitative measurement of PHB in selected bacterial isolates

Based on the MALDI-TOF analysis, one representative isolate was randomly selected from each cluster for PHB quantification. The isolates were activated and cultured as described above. After 24 h of culturing, 2 mL aliquots were sampled and frozen immediately at -80 °C until measurement of PHB. PHB was quantified spectrophotometrically following the procedure of Law and Slepecky (1961) with a few modifications. In brief, samples were harvested by centrifugation at 18,000 x *g* for 20 min in dried, pre-weighed 2 mL eppendorf tubes with safe-lock. Supernatant was discarded and pellets were two times washed with distilled water. The bacterial pellets were oven dried at 110 °C until constant weight. The bacterial pellets were lysed in 1.5 mL sodium

hypochlorite (32% active chlorine), vortexed and heated in a water bath for 10 min at 60 °C. The samples were centrifuged at 18,000 *g* for 20 min and sodium hypochlorite was carefully removed. The remaining pellets were washed with distilled water, vortexed and centrifuged at 18,000 x *g* for 20 min. The supernatant was carefully removed and volumes of 750 µL of ethanol and acetone were added. The samples were vortexed, centrifuged at 18,000 *g* for 20 min and the supernatant was carefully removed. The remaining samples were oven-dried at 40 °C overnight. The dried samples were then suspended in 2 mL chloroform to extract the PHB and left overnight in a shaker. Aliquots of the chloroform samples (100 µL) were transferred into clean glass vials and the chloroform was evaporated in a dryer (40 °C). After the extracts were evaporated to dryness, concentrated sulfuric acid (4 mL) was added to the dried sample and maintained at 100 °C in a water bath for 20 min to convert PHB to crotonic acid (Law and Slepecky, 1961). Samples were cooled to room temperature and two-fold diluted with 0.014M sulfuric acid. The crotonic acid was measured using a spectrophotometer (Infinite M200, Tecan Deutschland GmbH, Germany) at 235 nm. The concentration of PHB in the sulfuric acid aliquots was determined based on a standard curve constructed using crystalline PHB (Sigma-Aldrich) as standard. The PHB content in the bacterial cells was then calculated by the formula:

$$\text{PHB in bacterial cells (\% dry weight)} = \left(\text{conc of PHB in the aliquots (mg PHB/mL)} \times 2 \times 4\ \text{mL} \div 0.1\ \text{mL} \times 2\ \text{mL}\right) \div \text{(weight bacterial pellet (mg cell dry weight))} \times 100.$$

3.2.4 *IDENTIFICATION OF ISOLATE JL47 BY 16S RRNA SEQUENCE ANALYSIS*

A single colony of the isolate was picked using a sterilized toothpick and suspended in 0.5 mL sterile saline water and then centrifuged at 9,000 *g* for 10 min. The supernatant was discarded and the pellet was suspended in 0.5 mL of InstaGene Matrix (Bio-Rad USA) and incubated for 30 min. at 56°C and then heated at 100°C for 10 min. After heating, the supernatant was used for PCR. A 1 µl aliquot of the template DNA was added in 20 µl PCR reaction solution. PCR targeting a *c*. 1400 bp fragment of the 16S rRNA gene of the isolates was performed with a PTC-225 Peltier Thermal Cycler (MJ Research, USA) using the universal primer pair 27F and 1492R for bacteria (Lane, 1991)

and the program was set at 94°C for 45 seconds (denaturation), 55°C for 60 seconds (annealing) and 72°C for 60 seconds (extension). Purification of the amplified product was done using a Montage PCR Clean up kit (Millipore). Sequencing reactions were performed using primer pair 518F and 800R (Anzai et al., 1997) and a BigDye Terminator Cycle Sequencing kit (Applied BioSystems, USA) and the BigDye XTerminator Purification kit (Applied BioSystems, USA) was used for purification. Sequencing was performed on an Applied BioSystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) at Macrogen, Seoul, Korea. The sequence of the isolate was deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank) under the accession numbers KJ496325.1. Homology searches were completed with the BLAST server of the National Center for Biotechnology Information for comparison of the nucleotide query sequence against a nucleotide sequence database (blastn).

3.2.5 Statistical Analysis

All values represent means ± standard error of the mean (SEM). All data were analysed using one-way analysis of variance (ANOVA) followed by a post hoc Duncan Multiple Range Test for assessment of significant differences between treatments using SPSS 16.0 software. Data with percentage values were subjected to arcsin transformation prior to statistical analysis but only non-transformed data are presented.

3.3 Results

3.3.1 MALDI-TOF MS analysis

Based on the MALDI-TOF analysis, the 50 isolates could be dereplicated into two major groups containing respectively 37 and 13 isolates. Within group 1 all replicate profiles delineated in six additional subclusters, while no subgroups were observed in group 2 (Table 3.1). Examples of the MALDI-TOF MS profiles from each subcluster from group 1 and 3 examples from group 2 are shown in Figure 3.1. The profiles between groups 1 and 2 are clearly different and in accordance with the low - i.e. 2.1% - Pearson Product Moment Correlation as observed in the dendrogram (Figure 3.2). Between the

subclusters, the correlations are higher (Figure 3.2). Although these MS profiles share most peaks, their correlations are somewhat differed by few unique spectral features such as background (e.g. group 1E), emerging peaks (e.g. groups 1B, 1F) or variations in peak intensities (e.g. groups 1C, 1D) (Figure 3.1). Isolate 26, isolate 18, isolate 7, isolate 47, isolate 30, isolate 16 and isolates 14, 39, and 44 were randomly selected as representatives for dereplicated groups 1A, 1B, 1C, 1D, 1E, 1F and 2, respectively.

Table 3.1 Grouping of the 50 bacterial isolates based on MALDI-TOF MS analysis

MALDI-TOF MS		Isolate
Group	Subgroup	No.
1	A	JL31
1	A	JL20
1	A	JL10
1	A	JL9
1	A	JL27
1	A	JL12
1	A	JL21
1	A	JL6
1	A	JL3
1	A	JL22
1	A	JL32
1	A	JL41
1	A	JL34
1	A	JL42
1	A	JL13
1	A	JL5
1	A	JL40
1	A	JL4
1	A	JL24
1	A	JL2
1	A	JL48

1	A	JL26
1	A	JL38
1	A	JL29
1	B	JL18
1	C	JL7
1	D	JL1
1	D	JL28
1	D	JL47
1	E	JL11
1	E	JL23
1	E	JL49
1	E	JL46
1	E	JL30
1	F	JL16
1	F	JL35
1	F	JL8
2	-	JL14
2	-	JL15
2	-	JL17
2	-	JL19
2	-	JL25
2	-	JL33
2	-	JL36
2	-	JL37
2	-	JL39
2	-	JL43
2	-	JL44
2	-	JL45
2	-	JL50

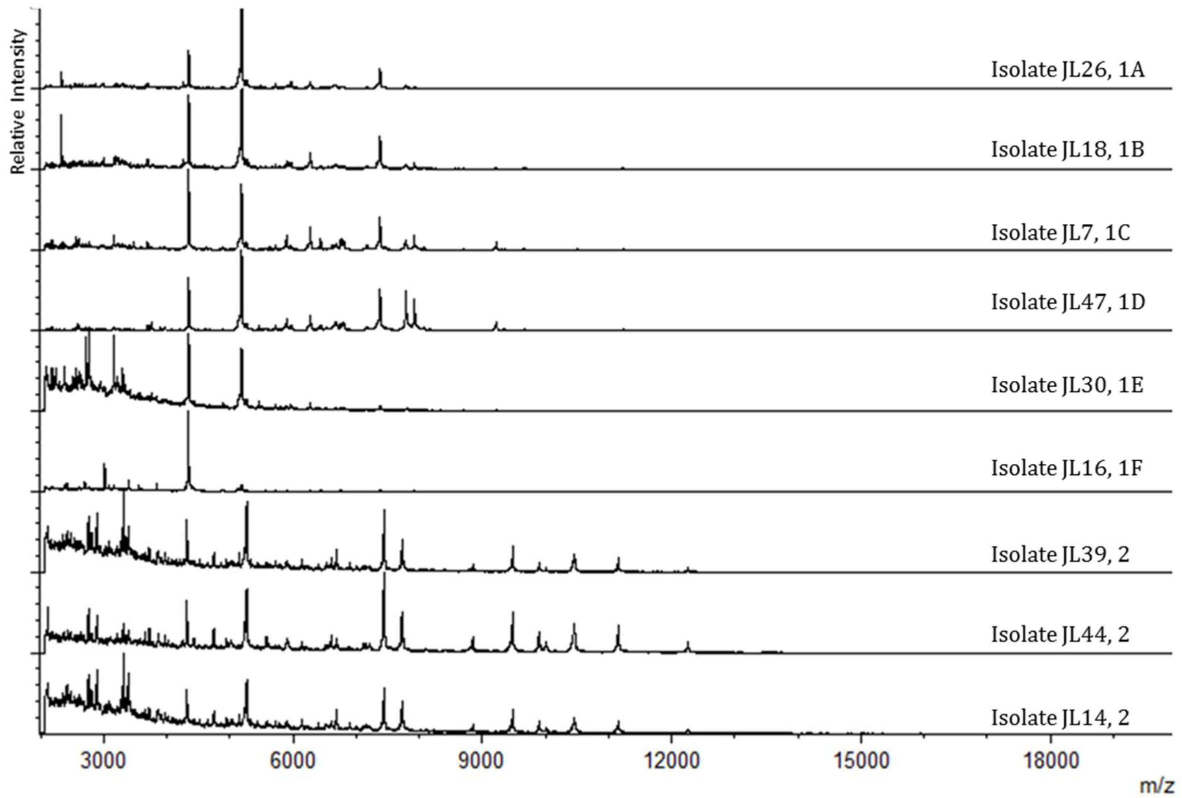

Figure 3.1 MALDI-TOF MS profiles of the bacterial isolates that represent each dereplication cluster. The upper 6 profiles represent isolates randomly selected from groups 1A-1F. The lower 3 profiles represent isolates randomly selected from main group 2. The figure was created with the mMass v5.5.0 software package *(Strohalm, et al., 2010)*

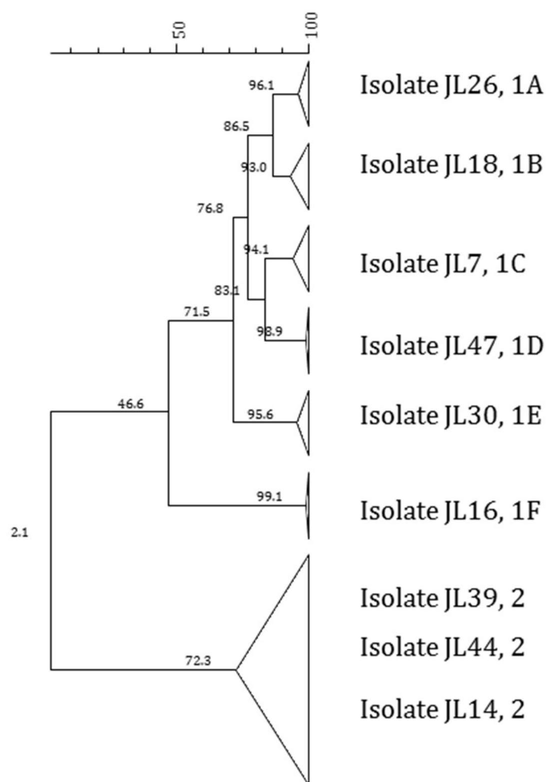

Figure 3.2 UPGMA dendrogram of the 9 representative isolates of the MALDI-TOF dereplicated groups based on their spectral similarity as calculated by the Pearson Product Moment Correlation considering all peaks with S/N-values above 5. Each isolate was represented by 2 biological replicates from which 2 mass spectra (technical replicates) were acquired.

3.3.2 QUANTITATIVE MEASUREMENT OF PHB IN SELECTED BACTERIAL ISOLATES

The measurement of PHB in the representative isolates from each dereplicated group showed a significantly higher PHB accumulation capacity for the isolates from group 1 than for isolates from group 2, except for isolate JL39 which showed no significant difference to some of the isolates in group 1 (Figure 3.3-A). Among the isolates tested isolate JL47, a representative of group 1D, attained the highest accumulation of 28.6% PHB on cell dry weight. Combined with the significantly higher cell dry weight yield of 3.76 g L^{-1} as compared to the other isolates this resulted in the overall highest PHB yield of 1.07 g PHB L^{-1} under the current culture conditions (Figure 3.3).

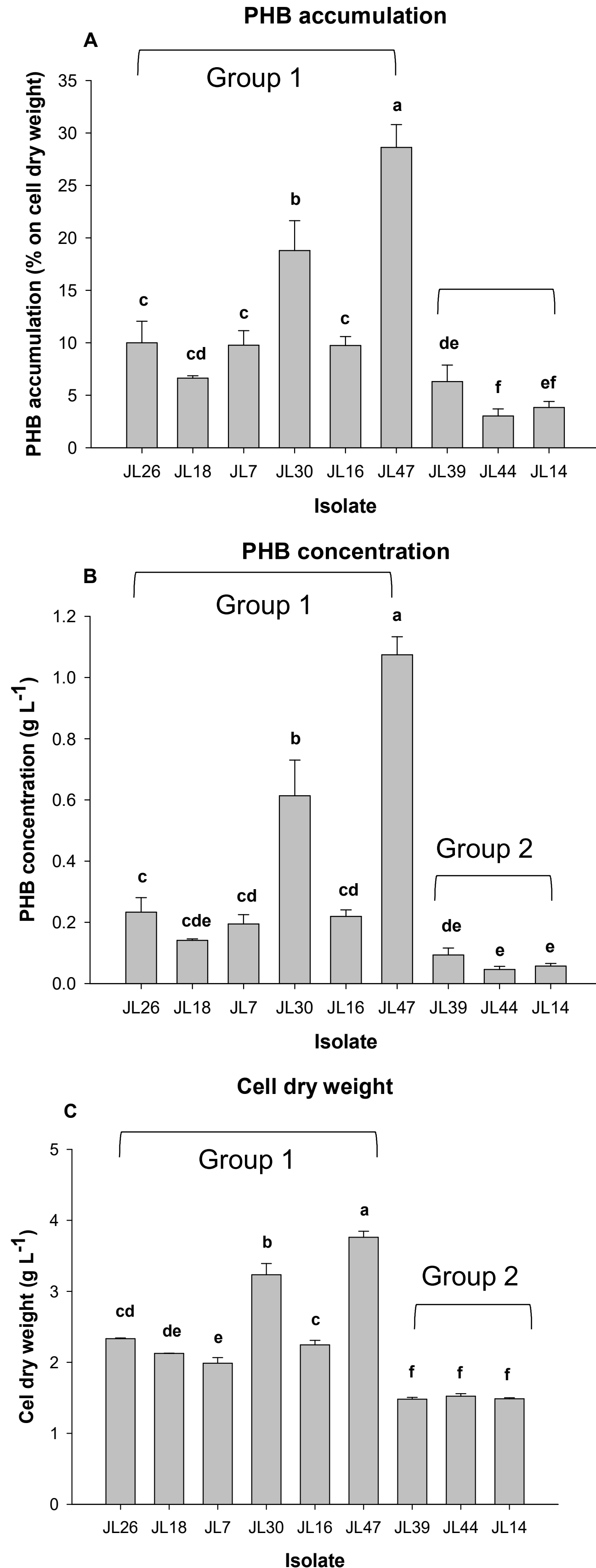

Figure 3.3 PHB accumulation (A), total PHB (B) and cell dry weight (C) concentration of the representative bacterial isolates grown in LB + 20 g L^{-1} glucose for 24 h. Bars indicate means ± SEM (n = 3). Different letters indicate significant differences ($P<0.05$).

Table 3.2 Results of the homology search for the isolate nucleotide query sequences using NCBI BLASTN search tool

Isolate (GenBank accession no.)	Closest match (GenBank accession no.)	Identity similarity (%)
JL47 (KJ496325.1)	*Bacillus cereus* SBTBC-008 (KF601957.1)	99
	Bacillus anthracis JN22 (KF150341.1)	99
	Bacillus thuringiensis AHBR13 (KF241	99

3.4 DISCUSSION

In this research, we were able to demonstrate the rapid isolation and screening of PHB accumulating bacteria from shrimp pond sediments. The selection of beneficial *Bacillus* species in this study was focused on the PHB accumulation capacity of the microorganism. Indeed, while isolating micro-organisms, the number of isolates can easily run high implying the necessity of dereplication – i.e. the recognition of identical isolates at a specific taxonomic level and grouping them accordingly - before proceeding to any further analysis (Ghyselink et al., 2011). Therefore, whole intact cells of the 50 isolates were profiled by MALDI-TOF MS in an attempt to correlate mass spectral grouping with PHB accumulation. Based on these results, the 50 isolates could be categorized into two major groups. Based on the spectral profiles obtained, only a few, low intensity peaks were common between both groups (Figure 3.1), indicating a rather low evolutionary relationship. Most peaks, however, are uniquely present in one respective group explaining the low correlation between these spectra as indicated in Figure 3.1.

To determine which of the isolates accumulate higher amount of amorphous PHB, representatives from each dereplicated (sub) groups were randomly selected and their PHB content was quantified spectrophotometrically. The results show that group 1 isolates seem to accumulate more PHB than group 2 isolates suggesting that there seems a trend on the grouping pattern in the MALDI-TOF MS and the PHB accumulation capacity. It is worth mentioning that the PHB accumulation of all the isolates in subgroup 1D (i.e. JL1, JL28 and JL47) attained a statistically similar PHB accumulation content

(data not shown), suggesting that the PHB accumulation capacity of the isolates in a subgroup may not deviate significantly. From the data, it is tempting to suggest that the PHB content or accumulation capability of the isolates has influenced the MALDI-TOF based-grouping results. One possibility could be the alteration in the protein expression pattern in PHB accumulating bacteria due to physiological changes in these bacteria brought by PHB accumulation. In a previous report, comparison on the proteome expression profiles between the metabolically engineered PHB-producing *E. coli* (XL1-Blue pJC4) and its none PHB producing equivalent (XL1-Blue pJC4Δ*phb*) showed alteration in protein expression levels such as proteins responsible for cell process like heat shock proteins (GRoEL, GroES and Dnak), transport-binding proteins (D-ribose perisplasmic binding protein), and enzymes related to energy metabolism (fructose-bisphosphate aldolase, class II, triosephosphate isomerase, 2-oxo-3-deoxy-6-phosphogluconate aldolase) (Han, et al., 2001). After all, such abundant proteins are perfect candidates to be detected by rough MALDI-TOF MS profiling analyses (Arnold and Reilly, 1999; Ryzhov and Fenselau, 2001). Although, we did not verify in our research if these proteins are actually being detected by the MALDI-TOF MS analysis, it is however very interesting to further investigate each of the peaks detected from PHB accumulating *Bacillus* species and to identify which proteins these peaks represent and so to clarify if indeed MALDI-TOF MS can segregate low and high PHB accumulators based on the profile peaks observed. It should also be mentioned that MALDI-TOF spectrum types might originate from the taxonomic relationship between both groups of isolates and is not necessarily related to their PHB accumulation capacity. Still, the clear correlation between the spectra and PHB accumulation serves as a possible lead for biomarker discovery and might facilitate new screening experiments for environmental PHB producers.

Isolate JL47 from subgroup 1D was the superior PHB accumulating isolate based on the quantitative PHB analysis. The accumulation of PHB in isolate JL47 (28.6% PHB) is comparably low compared to other reported PHB accumulating bacteria like in *Ralstonia eutropha* (80%; (Thai, et al., 2014)) or in other *Bacillus* strains (79% PHB; (Yüksekdağ, et al., 2003)), however this can still be improved by optimizing the culture conditions of isolate JL47 to attained higher PHB production. Indeed, in the next chapters

of this work, higher PHB production of the isolate JL47 were explored and tested in cultured animals *in vivo* (Chapter 4 and Chapter 5). The isolate JL47 was characterized phenotypically and was found to be Gram-positive and spore-forming rods. The isolate is able to grow in different salinities (0-30 ppt) indicating that the isolate can be used in fresh and marine water conditions. The isolate was confirmed to be under the genus *Bacillus* by partially sequencing the 16S rDNA and by sequence similarity searching it is closely related to the *Bacillus cereus* group (Table 3.2)

In summary, in this study PHB-accumulating *Bacillus* species were isolated subsequent to pasteurization of field samples. The novelty of this work is that the *Bacillus* species were not screened in the first place for their probiotic characteristics but for their capacity to accumulate higher amount of PHB. The results suggest that MALDI-TOF MS patterns can be used as a tool to identify bacteria with high PHB accumulating capacity. Further research should confirm this observation. The beneficial effects of the PHB-accumulating *Bacillus* isolates were presented in the succeeding chapters of this work.

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PART 3

APPLICATION

CHAPTER 4

HIGH AMORPHOUS PHB CONTENT IN *BACILLUS* SP. JL47 DISPLAYS BETTER PROTECTIVE EFFECTS IN GNOTOBIOTIC *ARTEMIA* DURING A PATHOGENIC *VIBRIO CAMPBELLII* CHALLENGE

Joseph Leopoldo Q. Laranja[1,2], Gladys L. Ludevese-Pascual[2], Edgar C. Amar[2], Maarten Aerts[3], Peter Vandamme[3], Peter Bossier[1], Peter De Schryver[1]

[1]Laboratory of Aquaculture & *Artemia* Reference Center, Ghent University, Coupure Links 653, 9000 Gent, Belgium
[2]Aquaculture Department, Southeast Asian Fisheries Development Center, 5021 Tigbauan, Iloilo, Philippines
[3]Laboratory of Microbiology, Faculty of Sciences, Ghent University, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium

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ABSTRACT

In this study, the *Bacillus* sp. JL47, a superior PHB-accumulating *Bacillus* isolate identified from our previous experiment (chapter 3), was tested for its protective effects in gnotobiotic *Artemia franciscana* during a pathogenic *Vibrio campbellii* challenge. The survival of the *Artemia* fed the *Bacillus* sp. JL47 at 1 x 10^7 cells mL^{-1} was significantly higher as compared with the challenged control and the survival was almost doubled when the dose was increased to 5 x 10^7 cells mL^{-1}. However, feeding the *Artemia* at 10^6 cells mL^{-1} or lower showed no significant protective effects. Based on these densities, the estimated concentration of amorphous PHB showing a significant protection in *Artemia* was *c.* 2.44 mg L^{-1} and the effects was even better when the amorphous PHB level was increased to 12.19 mg L^{-1}. Furthermore, feeding *Bacillus* sp. JL47 containing 55% amorphous PHB (on CDW) to *Artemia* showed a significantly higher survival in a *Vibrio* challenge relative to *Bacillus* sp. JL47 containing 29% PHB. The data suggest that the amorphous PHB accumulated in the *Bacillus* sp. JL47 strain is a main determinant for the increased survival of challenged *Artemia*.

Keywords: amorphous PHB, *Bacillus*, gnotobiotic *Artemia*, *Vibrio* campbellii, challenge test

4.1 INTRODUCTION

Disease outbreaks are considered to be a major constraint to the growth of many aquaculture species and are responsible to the tremendous economic losses in the aquaculture industry (FAO, 2014). In crustacean culture, infections caused by pathogenic *Vibrios* have resulted in serious losses in the larviculture of lobster, crabs and especially shrimps (Diggles, et al., 2000; Lavilla-Pitogo, et al., 1990; Muroga, et al., 1994). The conventional approach in dealing with these opportunistic bacteria relies heavily on the use of antibacterial compounds that either kill or inhibit their growth. However, the use of such strategy needs to be regulated, if not abandoned because indiscriminate use of antibiotics could lead to the occurrence of multiple antibiotic resistant bacteria that could inflict potential hazard to human health and the environment (Cabello, 2006; Holmström, et al., 2003). Hence, the suggested approach of controlling disease infections in aquaculture has been geared towards the application of equally effective yet sustainable and environment-friendly strategies and among those are the use of probiotics and poly-β-hydroxybutyrate (Defoirdt, et al., 2007).

As described in Chapter 2, PHB is an important member of the family of polyhydroxyalkanoates, a class of microbially-produced polyesters that have potential applications similar to synthetic polymers (Madison and Huisman, 1999). However, only recently PHB has been identified as a potential biocontrol agent for aquaculture. PHB was reported to enhance the growth and survival of some fish species such as European seabass *Dicentrarchus labrax* and Siberian sturgeon *Acipenser baerii* (De Schryver, et al., 2010; Najdegerami, et al., 2012). In Nile tilapia *Oreochromis niloticus* juveniles, feeding the fish with PHB-supplemented diets resulted in an increased resistance of the fish during a pathogenic *Edwardsiella ictaluri* challenge (Situmorang, et al., 2016). PHB was also found to increase the growth and survival of fresh water prawn *Macrobrachium rosenbergii* and Chinese mitten crab *Eriocheir sinensis* larvae (Nhan, et al., 2010; Sui, et al., 2014), increased the resistance of *P. monodon* postlarvae against lethal dose of ammonia and exposure to pathogenic *V. campbellii* (Ludevese-Pascual, et al., 2017), as well as increased the resistance of *Artemia franciscana* against *Vibriosis* (Defoirdt, et al., 2007). In all the studies mentioned, PHB was applied in crystalline form (i.e. PHB extracted from bacterial cells). However, the application of PHB in amorphous state (i.e.

PHB still inside the bacterial cell) was suggested to be more efficient. Indeed, an earlier study has shown that PHB containing bacteria may be more effective than PHB powder to protect *Artemia franciscana* from pathogenic *V. campbellii* infection (Halet, et al., 2007). Hence, the challenge now lies in finding appropriate bacterial culture capable of accumulating a high amount of amorphous PHB. Interestingly, *Bacillus* species are capable of accumulating PHB intracellularly as energy source to fuel the sporulation process (Slepecky and Law, 1961). *Bacillus* species have been shown to accumulate PHB in the range of 2.2 up to as high as 97.3 % on cell dry weight (CDW) under optimal conditions. Interestingly, apart from their capability to accumulate significant amounts of PHB, these bacteria are also known as probiotics in aquaculture. Indeed, several *Bacillus* species were reported to be improving the growth, survival and robustness of various cultured aquatic animals such as finfish, sea cucumbers, oysters and especially shrimps (Karim, et al., 2013; Liu, et al., 2010a; Sun, et al., 2010; Zhao, et al., 2012). Aside from their various beneficial probiotic effects, their ability to produce spores makes them more advantageous over other probiotic species in terms of long-term storage and ease of application (Hong, et al., 2005).

In the previous chapter of this work, the *Bacillus* sp. JL47 was identified as the superior PHB accumulating isolate. For this reason, it was selected to assess the beneficial effects of using *Bacillus* species as a bacterial carrier for amorphous PHB. Hence, the study aimed at determining the protective effects of PHB-accumulating *Bacillus* sp. JL47 in gnotobiotic *Artemia franciscana* during a pathogenic *V. campbellii* challenge. We further investigated if the different levels of amorphous PHB present in *Bacillus* sp. JL47 affect the protective effect of the bacterium in challenged gnotobiotic *Artemia.*

4.2 MATERIALS AND METHODS

4.2.1 BACTERIAL STRAINS AND GROWTH CONDITIONS

Bacillus sp. JL47, a superior PHB-accumulating *Bacillus* isolate identified in chapter 3 was grown in Luria Bertani (LB) (tryptone (Himedia, 10 g L^{-1}), yeast extract (Himedia, 5 g L^{-1}) and sodium chloride (Sigma-Aldrich, 20 g L^{-1})) medium for 16 h and

subsequently inoculated at 1% (v/v) in LB supplemented with 20 g L^{-1} glucose (Sigma-Aldrich). The bacterial culture was grown at 30 °C and 100 rounds per minute (rpm) agitation for 24 and/or 48 h depending on the experiment. *Vibrio campbellii* LMG 21363, a known pathogen in *Artemia* (Defoirdt, et al., 2007), was obtained from the BCCM/LMG Bacteria collection (Ghent, Belgium). *Vibrio campbellii* LMG 21363 and *Aeromonas hydrophila* LVS3 (Verschuere, et al., 1999) were grown in LB medium for ~16 h and subsequently inoculated at 1% (v/v) in LB medium and the bacterial cultures were grown at 30 °C with shaking (100 rpm) for 24 h.

4.2.2 Measurement of PHB in JL47 at 24 and 48 h culture

Bacillus sp. JL47 was activated and cultured as described above. After 24 and 48 h of culturing, 2 mL aliquots were sampled and frozen immediately at -80 °C until measurement of PHB. Measurement of PHB was conducted following the procedure described in chapter 3.

4.2.3 MALDI-TOF MS analysis

Bacillus sp. JL47 was activated and cultured as described above. After 24 and 48 h of culturing, the bacterial cells were used for the MALDI-TOF MS analysis. The analysis was conducted following the procedure described in chapter 3.

4.2.4 Preparation of axenic hatching of *Artemia franciscana*

Axenic hatching of *Artemia* was carried out following the procedure described by Defoirdt, et al. (2007) with a few modifications. In brief, 0.2 g of high-quality *Artemia franciscana* cyst (EG® Type, batch 6940, INVE, Aquaculture, Belgium) were hydrated in 18 mL of tap water for 30 min with moderate aeration. Sterile cysts and nauplii were obtained via a decapsulation process (Marques, et al., 2004) by adding 660 µL of NaOH (32%) and 10 mL of NaOCl (32% active chlorine) to the hydrated cysts suspension. The decapsulation was stopped after 3-4 min by adding 14 mL of $Na_2S_2O_3$ (10 g L^{-1}) and the decapsulated cysts were immediately washed with filtered autoclaved seawater (FASW) on a sterile sieve (50 µm). The cysts were re-suspended in a 50 mL Falcon tube containing 30 mL of FASW and allowed to hatch for 24 h on a rotor (4 min^{-1}) at 30°C with

constant illumination (approximately 27 μE/(m^2.sec)). All manipulations were conducted in a laminar flow hood. Axenic instar II nauplii (4-6 h after hatching) were used in the *in vivo* challenge experiment.

4.2.5 In vivo challenge test

4.2.5.1 Experiment 1: Effects of *Bacillus* sp. JL47 on the survival of gnotobiotic *Artemia* challenged with pathogenic *Vibrio campbellii* LMG 2136

Bacillus sp. JL47, being the superior PHB accumulating isolate identified in chapter 3, was used in an *in vivo Artemia* experiment. It was grown as described above and subsequently inoculated at 1% (v/v) in LB supplemented with 20 g L^{-1} glucose (Sigma-Aldrich). The culture was grown at 30 °C and 100 rpm agitation for 24 h. After culture, the bacterial cells were harvested by centrifugation at 4,500 *x g* for 5 min discarding the supernatant and washing once with sterile seawater and finally re-suspended in sterile seawater prior to feeding.

V. campbellii LMG 21363 was used as a pathogen in the challenge test. The pathogen was activated as described above and subsequently inoculated in LB broth (Sigma-Aldrich). The culture was grown following the culture conditions described above for 24 h. After cultivation, the pathogen was immediately used.

A. hydrophila LVS3 has been used as feed for *Artemia* nauplii (Defoirdt, et al., 2005). The strain was cultured for 24 h as described above. After cultivation, the bacterial cells were harvested by centrifugation at 4,500 x *g* for 5 min discarding the supernatant and washing once with FASW and finally re-suspended in FASW. The bacterial suspension was autoclaved prior to feeding.

The bacterial densities for all bacterial strains (*Bacillus* sp. JL47, *A. hydrophila* LVS3 and *V. campbellii* LMG 21363) used were determined by spectrophotometry assuming that an optical density of 1.0 corresponds to 1.2 x 10^9 cells mL^{-1}, according to the Mc Farland standard (BioMerieux, Marcy L'Etoile, France)

In a first trial, axenic Instar II *Artemia* nauplii were stocked in tubes containing 20 mL FASW at a density of 1 individual mL^{-1}. *Bacillus* sp. JL47 was added to the *Artemia* culture water at 0, 1 x 10^5, 1 x 10^6 and 1 x 10^7 cells mL^{-1} as treatments. All tubes were supplemented with autoclaved *A. hydrophila* strain LVS3 (Verschuere, et al., 1999) at 1

x 10^7 cells mL^{-1} as food. Then, for each treatment half of the tubes was supplemented with the pathogen *V. campbellii* LMG 21363 at 10^6 CFU mL^{-1} six hours after adding *Bacillus* sp. JL47 (n = 4). All tubes were placed on a rotor at 30°C for 48 h with constant illumination (approximately 27 µE/(m^2.sec)) after which survival of the *Artemia* was determined.

In a repeat of this trial cell densities of *Bacillus* sp. JL47 of 0, 5 x 10^5, 5 x 10^6 and 5 x 10^7 cells mL^{-1} were applied.

4.2.5.2 Experiment 2: Effects of *Bacillus* sp. JL47 containing different levels of amorphous PHB on the survival of gnotobiotic *Artemia* challenged with pathogenic V. campbellii LMG 21363

In this experiment, the *Bacillus* sp. JL47 containing different levels of amorphous PHB were tested for its protective effects in *Artemia.* In brief, the *Bacillus* sp. JL47 was activated as described above and subsequently inoculated at 1% (v/v) in LB supplemented with 20 g L^{-1} glucose (Sigma-Aldrich). The culture was grown following the culture conditions described above for 24 and 48 h to attain different levels of amorphous PHB as follows: 29% PHB on CDW (24 h culture) and 55% PHB on CDW (48 h culture). After culture, the bacterial cells were harvested by centrifugation at 4,500 *x g* for 5 min discarding the supernatant and washing once with sterile seawater and finally re-suspended in sterile seawater prior to feeding.

V. campbellii LMG 21363 and *A. hydrophila* strain LVS3 were prepared according to the procedure described above.

In a first trial, axenic Instar II *Artemia* nauplii were stocked in tubes containing 20 mL FASW at a density of 1 individual mL^{-1}. *Bacillus* sp. JL47 containing different levels of amorphous PHB were added to the *Artemia* culture water at 1 x 10^7 cells mL^{-1} as treatments. All tubes were supplemented with autoclaved *A. hydrophila* strain LVS3 at 1 x 10^7 cells mL^{-1} as food. Then, for each treatment half of the tubes was supplemented with the pathogen *V. campbellii* LMG 21363 at 10^6 CFU mL^{-1} six hours after adding *Bacillus* sp. JL47 (n = 4). All tubes were placed on a rotor at 30°C for 48 h with constant illumination (approximately 27 µE/(m^2.sec)) after which survival of the *Artemia* was determined.

In the second experiment, the *Bacillus* sp. JL47 containing 29% or 55% amorphous PHB was fed to *Artemia* at different densities as follows: 1 x 10^6, 1 x 10^7, 1 x 10^8 cells mL^{-1}. All tubes were supplemented with autoclaved *A. hydrophila* strain LVS3 at 1 x 10^7 cells mL^{-1} as food. *Artemia* fed the *Bacillus* sp. JL47 were all supplemented with *V. campbellii* LMG 21363 at 10^6 CFU mL^{-1} six hours after *Bacillus* sp. JL47 addition (n = 4). *Artemia* without *Bacillus* sp. JL47 was also challenged or not challenged with *V. campbellii* and served as references. All tubes were placed on a rotor at 30°C for 48 h with constant illumination (approximately 27 µE/(m^2.sec)) after which survival of the *Artemia* was determined.

4.2.6 Statistical analysis

All values represent means ± standard error of the mean (SEM). The data on survival were analysed using one-way analysis of variance (ANOVA) followed by a post hoc Duncan Multiple Range test for assessment of significant differences between treatments at a 5% significance level ($P<0.05$). Data on CDW and PHB accumulation were analysed using t-test. Data with percentage values were subjected to arcsine transformation prior to statistical analysis but only non-transformed data are presented. A two-way ANOVA followed by Bonferroni post-test was used to compare the main effects of the two factors (cell density, PHB accumulation) and their interaction. Values were considered significantly different if $p<0.05$. t-test, one-way and two-way ANOVA analyses were conducted using SPSS 16.0 software. The Pearson product moment correlation coefficient (r) and the Pearson two-tailed p value were determined using GraphPad Prism version 5.03 for Windows, GraphPad Software.

4.3 Results

4.3.1 Effects of PHB-accumulating Bacillus sp. JL47 on the survival of Artemia challenged with pathogenic V. campbellii LMG 21363

In the first *in vivo* test, challenged *Artemia* fed with an increasing dose of *Bacillus* sp. JL47 showed an increasing trend in survival. However challenged *Artemia* fed the *Bacillus* sp. JL47 at 1 x 10^5 cells mL^{-1} or 1 x 10^6 cells mL^{-1} did not show any significant

difference in survival with the challenged control. Supplementing *Bacillus* sp. JL47 at 1 x 10^7 cells mL^{-1}, however, significantly increased the survival as compared to the challenged control although the survival was still lower as compared to the unchallenged control (
Figure 4.1-A). Based on these densities, the estimated concentration of amorphous PHB showing a significant protective effect to *Artemia* was *c.* 2.44 mg $L^{-}1$ while feeding the *Artemia* lower than this amorphous PHB concentration showed no significant protective effect (Table 4.1). In the second trial, feeding the *Artemia* with *Bacillus* sp. JL47 at 5 x 10^5 cells mL^{-1}, 5 x 10^6 cells mL^{-1}, 5 x 10^7 cells mL^{-1} showed a similar pattern in survival compared to trial 1. Specifically, as in the first trial, no significant difference was observed in *Artemia* fed the *Bacillus* sp. JL47 at 5 x 10^5 cells mL^{-1} and 5 x 10^6 cells mL^{-1} and challenged control, however, the survival result for each of these respective treatments was slightly higher as compared to the same treatments in the first trial. Overall, the highest protective effect of *Bacillus* sp. JL47 in this particular experiment was observed at a concentration of 5 x 10^7 cells mL^{-1} (
Figure 4.1-B). The estimated concentration of amorphous PHB supplied to *Artemia* at this density was *c.* 12.19 mg L^{-1} (Table 4.1).

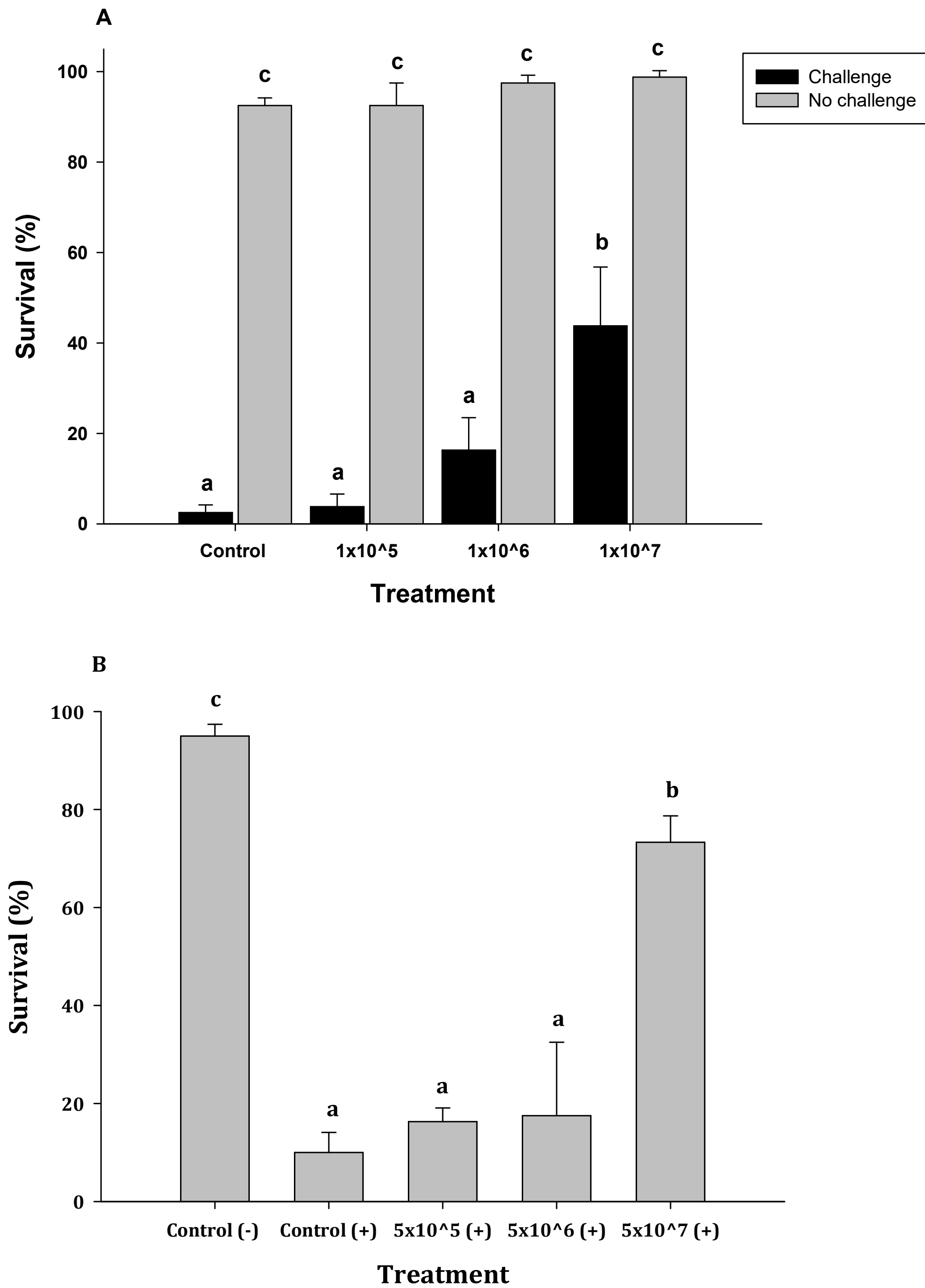


Figure 4.1 Survival of gnotobiotic *Artemia* after 48 h of culture fed different densities of *Bacillus* sp. JL47. *Bacillus* sp. JL47 was cultured for 24 h on LB + 2% glucose. For run B, treatment with plus (+) means challenge with *V. campbellii* at 10^6 cells mL^{-1}. Different letters indicate significant differences ($P<0.05$). ($N=4$, AVERAGE ± SEM)

Table 4.1 Estimated concentration of amorphous PHB given to *Artemia* based on the PHB accumulation of *Bacillus* sp. JL47. (* computed based on the calculated correlation of CFU and CDW in *Bacillus* cells: 10^{12} CFU=0.841 g CDW; *(Deriase and El-Gendy, 2014)*)

Treatment	PHB accumulation (% on CDW)	*Estimated amount of amorphous PHB supplied to *Artemia* (mg L^{-1})
Experiment 1A		
Control	-	-
JL47 1 x 10^5	29	0.02
JL47 1 x 10^6	29	0.24
JL47 1 x 10^7	29	2.44
Experiment 1B		
Control	-	-
JL47 5 x 10^5	29	0.12
JL47 5 x 10^6	29	1.22
JL47 5 x 10^7	29	12.19

4.3.2 ACCUMULATION OF PHB IN JL47 AFTER 24 AND 48 H OF CULTURE ON LB+ 2% GLUCOSE

PHB accumulation of *Bacillus* sp. JL47 grown in LB supplemented with 20 g L^{-1} glucose at 48 h culture was significantly higher as compared to its accumulation at 24 h culture (Figure 4.2). Specifically, the PHB accumulation of *Bacillus* sp. JL47 at 24 and 48 h culture were 29% and 55% amorphous PHB on cell dry weight (CDW), respectively. The CDW of *Bacillus* sp. JL47 was also significantly higher at 48 h culture (3.4 g L^{-1}) than at 24 h culture (2.1 g L^{-1}). The accumulation of amorphous PHB and bacterial biomass of *Bacillus* sp. JL47 at 48 h culture was 1.9 and 1.6-fold higher, respectively than at 24 h culture.

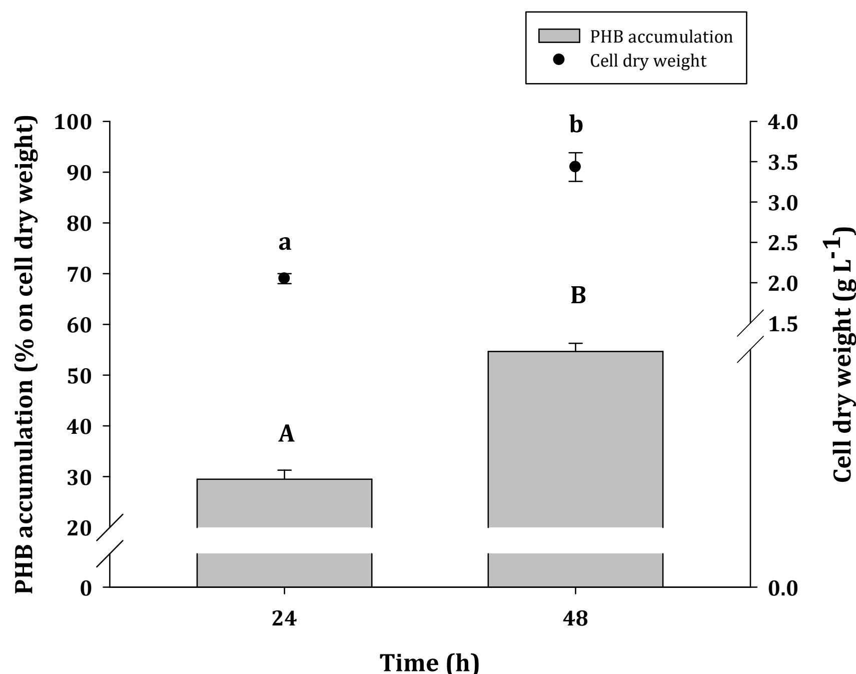


Figure 4.2 PHB accumulation and cell dry weight (CDW) concentration of *Bacillus* sp. JL47 grown in LB + 2% (w/v) glucose at 24 and 48 h culture. Different letters indicate significant differences (P<0.01); small letters: CDW; capital letters: PHB accumulation. (N=3; AVERAGE ± SEM)

4.3.3 MALDI-TOF MS ANALYSIS

Based on the MALDI-TOF MS analysis, the MS profiles of *Bacillus* sp. JL47 grown at 24 and 48 h showed a spectral similarity of 93.5% by Pearson Product Moment correlation (Figure 4.3). Based on the peaks counted, the JL47 grown at 24 h (lower PHB accumulation) revealed 22-35 peaks while at 48 h (higher PHB accumulation) 28-39 peaks where observed (Figure 4.3).

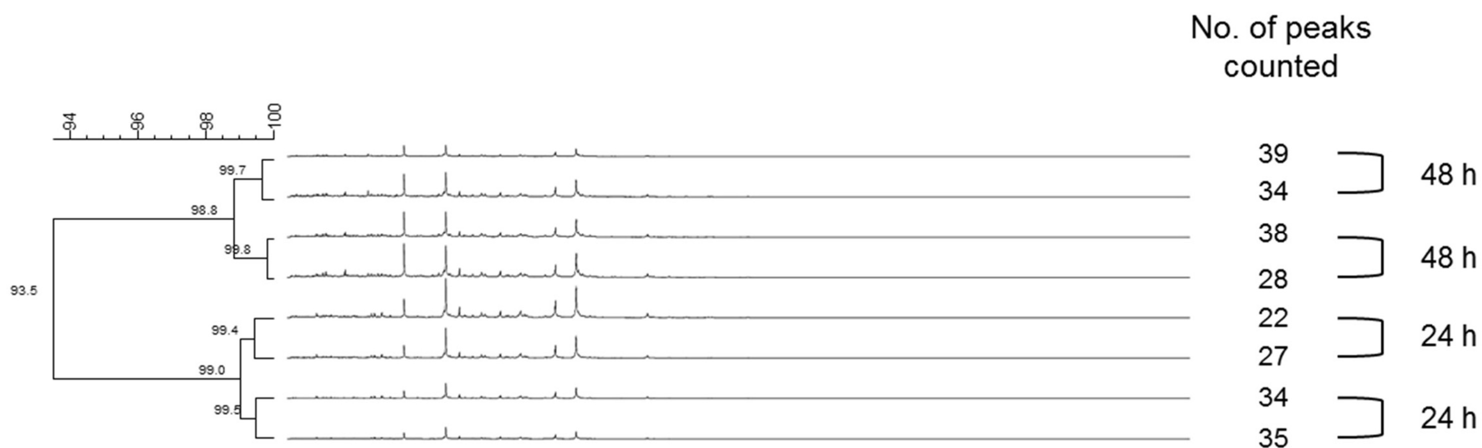


Figure 4.3 UPGMA dendrogram of *Bacillus* sp. JL47 at 24 and 48 h culture based on their spectral similarity values as calculated by the Pearson Product Moment Correlations considering all peaks with S/N-values above 5. The number of peaks for each MS profile sample was also presented. Each isolate is represented by 2 biological replicates from which 2 mass spectra (technical replicates) were acquired.

4.3.4 Effects of PHB-accumulating *Bacillus* sp. JL47 containing different levels of amorphous PHB on the survival of gnotobiotic *Artemia* challenged with pathogenic *V. campbellii* LMG 21363

The effects of feeding *Bacillus* sp. JL47 containing low (29% PHB on CDW) or high (55% PHB on CDW) amount of amorphous PHB in gnotobiotic *Artemia* was verified. In the first experiment, the results showed a significantly higher survival in *Artemia* fed the *Bacillus* sp. JL47 containing 55% amorphous PHB as compared to those *Artemia* fed the JL47 containing 29% PHB (Figure 4.4). The results further showed that all *Artemia* receiving the *Bacillus* sp. JL47 resulted in a significantly higher survival as compared with the challenged control. In addition challenged *Artemia*, fed *Bacillus* sp. JL47 containing 55% amorphous PHB showed no significant difference with the non-challenged *Artemia,* suggesting a complete protection against *Vibrio* infection. The calculated amorphous PHB concentration offered to *Artemia* from *Bacillus* sp. JL47 containing 29% or 55% PHB can be estimated as 2.44 and 4.63 mg L^{-1}, respectively (Table 4.2)

In the second experiment, *Artemia* nauplii were fed with different densities of *Bacillus* sp. JL47 containing different levels of PHB accumulation (i.e. 29% and 55% amorphous PHB on CDW). The results showed that *Artemia* survival was always higher in *Artemia* fed the *Bacillus* sp. JL47 containing 55% amorphous PHB as compared to *Artemia* fed the JL47 containing 29% PHB (Figure 4.5) ($p=0.003$). Both factors, the cell density ($p=0.000$) and PHB accumulation ($p=0.003$) significantly affected the survival of challenged *Artemia*. However, no interaction was observed between cell density and PHB accumulation ($p=0.132$). The analysis suggests that regardless of cell density, survival of challenged *Artemia* fed the JL47 containing 55% amorphous PHB is always higher.

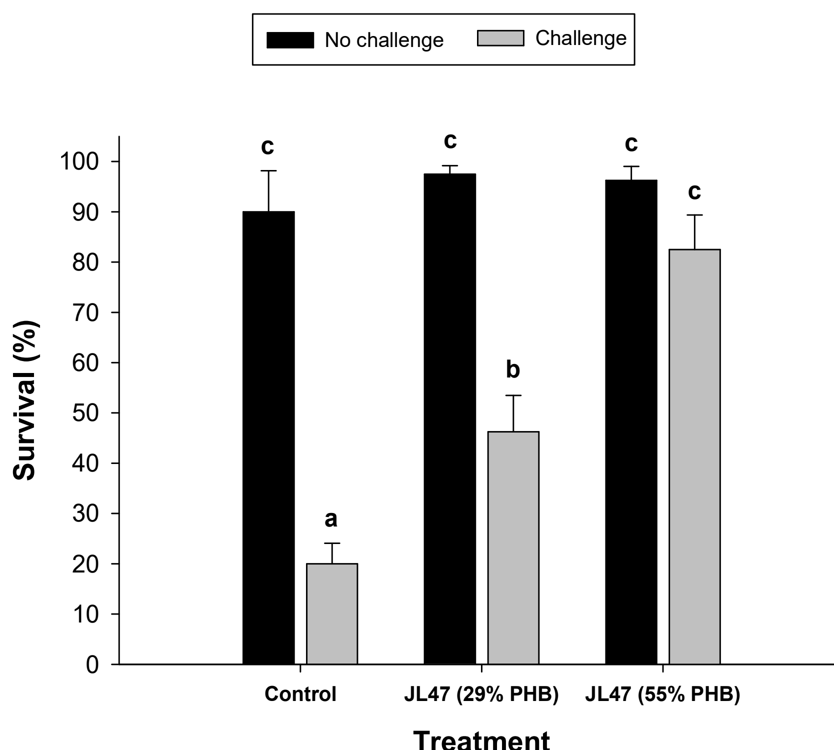
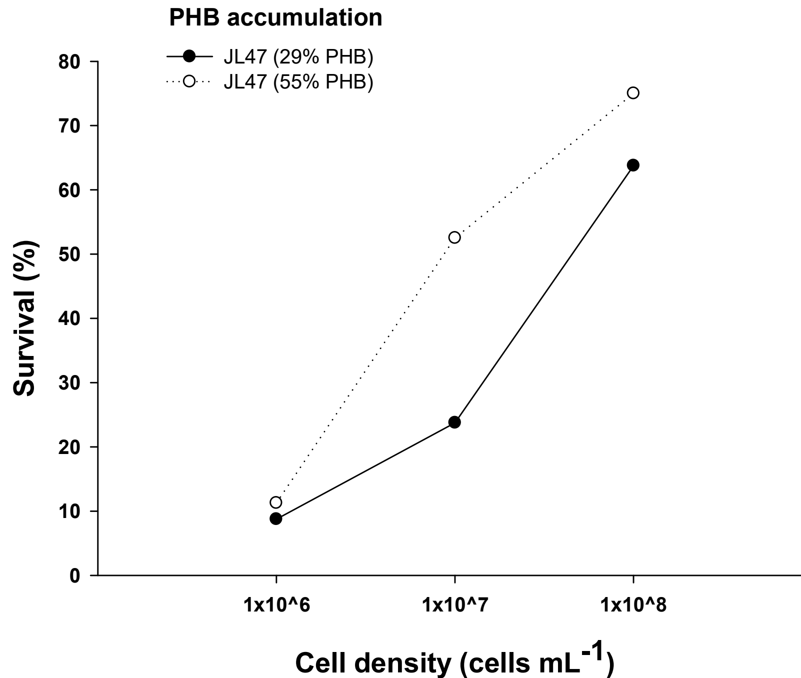


Figure 4.4 Survival of gnotobiotic *Artemia* after 48 h of culture fed the *Bacillus* sp. JL47 (1 x 10^7 cells mL^{-1}) containing different levels of amorphous PHB. *Artemia* were challenged with *V. campbellii* at 10^6 cells mL^{-1}. Different letters indicate significant differences (P<0.05). (N=4; AVERAGE± SEM)

Table 4.2 Estimated concentration of amorphous PHB supplied to *Artemia* based on the PHB accumulation of *Bacillus* sp. JL47. (* computed based on the calculated correlation of CFU and CDW in *Bacillus* cells: 10^{12} CFU=0.841 g CDW; (Deriase and El-Gendy, 2014))

Treatment	PHB accumulation (% on CDW)	*Estimated amount of amorphous PHB supplied to *Artemia* (mg L^{-1})
Experiment 2A		
Control	-	-
JL47 1 x 10^7	29	2.44
JL47 1 x 10^7	55	4.63



Cell density	significant; p=0.000
PHB accumulation	significant; p=0.003
Interaction	not significant; p=0.132

Figure 4.5 Effects of different cell densities and different PHB accumulation of *Bacillus* sp. JL47 on the survival of *Artemia* challenged with pathogenic *V. campbellii*. A two-way ANOVA was applied to analyse if survival was affected by cell density, PHB accumulation level and its interaction.

4.4 Discussion

The beneficial effects of crystalline PHB for different aquaculture animals were reported in several studies (Ludevese-Pascual, et al., 2017; Najdegerami, et al., 2015; Najdegerami, et al., 2012; Nhan, et al., 2010; Situmorang, et al., 2016; Sui, et al., 2012; Sui, et al., 2014). The strategy of applying PHB contained in a bacterial carrier (i.e. *Bacillus* species) has, however not fully been explored. In this study, we demonstrated the beneficial effects of applying PHB in amorphous state using *Bacillus* sp. JL47 strain as a bacterial carrier in challenged gnotobiotic *Artemia franciscana*. The utilization of gnotobiotic *Artemia* is important in this study to elucidate clearly the effects of PHB-accumulating *Bacillus* sp. JL47 in the host considering that any possible microbial

interference (which are naturally present in conventional culture system) are eliminated in this model culture system, and therefore more conclusive results can be obtained.

Indeed, the results show that the PHB-accumulating *Bacillus* sp. JL47 strain can significantly protect the *Artemia* from pathogenic *V. campbellii* infection. This result corresponds to what was found earlier on the effects of PHB-accumulating bacteria on the increased survival of gnotobiotic *Artemia franciscana* and freshwater prawn *Macrobrachium rosenbergii* larvae after challenge with pathogenic *Vibrio* species (Halet, et al., 2007; Thai, et al., 2014). The data show that the *Bacillus* sp. JL47 which can accumulate 29% amorphous PHB on cell dry weight can significantly improve the survival of challenged *Artemia* when fed at 1 x 10^7 cells mL^{-1} and the survival was almost doubled when the dose was increased to 5 x 10^7 cells mL^{-1} (Figure 4.1). Based on these densities used, the amount of amorphous PHB given to *Artemia* can be estimated as 2.44 and 12.19 mg L^{-1}, respectively. These levels are 82-410 times lower compared to the effective level observed in crystalline PHB (Defoirdt, et al., 2007). Halet, et al. (2007) also observed a comparable result using different PHB-accumulating bacterial strain (i.e. *Brachymonas denitirifcans*) wherein the amorphous PHB fed to gnotobiotic *Artemia* through *B. denitrificans* was 100 times lower than the effective dose of the crystalline PHB. Hence, the work of Halet, et al. (2007) and this study strongly support the concept of a higher efficiency of amorphous PHB compared to crystalline PHB. This can be explained by the fact that amorphous PHB particles are much smaller inside the bacterial cell (typically < 0.5 μm in diameter (Anderson and Dawes, 1990); compared to crystalline PHB (~30 μm; (Defoirdt, et al., 2007)) and the particle size may affect the depolymerisation efficiency of the biopolymer. Likewise, the physical state of the biopolymer could be another reason affecting the degradation and utilization of PHB. It was suggested that the degree of crystallinity of the polymer is a crucial factor affecting the degradation process (Tokiwa, et al., 2009). In the amorphous state, the molecules are loosely packed and the hydrolytic enzymes can diffuse faster, thus this makes the amorphous PHB more susceptible to degradation. Indeed, the work of Yu, et al. (2005) demonstrated that the amorphous PHB was hydrolysed 30 times faster than crystalline PHB.

Culturing the *Bacillus* sp. JL47 for 24 h in LB supplemented with 2% (w/v) glucose showed a 29% PHB (on CDW) accumulation and the accumulation was further increased

to 55% PHB (on CDW) at 48 h culture. This condition allows us to further evaluate the protective performance of the *Bacillus* sp. JL47 in *Artemia* when containing different amounts of amorphous PHB. The results showed that the protective effects of *Bacillus* sp. JL47 in *Artemia* was always higher when the strain contains 55% amorphous PHB as compared to only 29% amorphous PHB, regardless of the cell density used (Figure 4.5). Indeed, a significant linear correlation (p=0.03) between the amount of amorphous PHB given to *Artemia* and the increased survival of the challenged *Artemia* (r=0.86) existed. These observations correspond to what was found earlier by Thai, et al. (2014) in giant freshwater prawn larvae wherein the PHB content and the dose of the bacterial carrier (i.e. *Alcaligenes eutrophus*) significantly influenced the survival and growth of the cultured prawn larvae. Interestingly, in all the challenged *Artemia*, only those fed the *Bacillus* sp. JL47 containing 55% amorphous PHB showed no significant difference with the non-challenged *Artemia*, suggesting that with this treatment a complete protection in *Artemia* against *Vibrio campbellii* infection could be obtained (Figure 4.4). Based on the outcome of the different experiments it can be concluded that the amorphous PHB is a key determinant of the protective effect. The suggested mode of action of PHB is that this bacterial biopolymer is degraded intestinally resulting in the release of its monomer form, 3-hydroxybutyrate. This compound can act antimicrobially by acidifying the cytoplasm of the pathogen and thus the pathogen has to redirect its cellular energy (i.e. use of transporters that catalyse active proton transport), to maintain homeostasis, resulting in a decreased virulence (Defoirdt, et al., 2009). Conversely, the compound was also observed to enhance the immunity of some cultured animals such as in *Artemia* nauplii and juvenile tilapia (Baruah, et al., 2015; Suguna, et al., 2014). In addition the monomers can also directly serve as an energy source (Dedkova and Blatter, 2014), facilitating the mounting of such an immune response.

It is however, worth mentioning that apart from the PHB content, other features, such as the difference in cell wall bound and cytosol-born protein fingerprints (see MALDI-TOF analysis), might contribute to the probiotic properties of the *Bacillus* sp. JL47 strain (eg; if some of these proteins would have an immunostimulatory property). Based on the MALDI-TOF MS profiles of the JL47 containing 55% PHB and 29% PHB some spectral dissimilarity was revealed, in which the higher PHB content goes together with more

peaks. These additional peaks might represent protein with an immunostimulatory effect or might be associated with cellular changes resulting in immunostimulaton. Indeed, it has been reported that PHB accumulation in *E. coli* strain leads to the expression of heat shock proteins (i.e. (GRoEL, GroES and Dnak) (Han, et al., 2001) and these proteins are considered immune stimulators (Baruah, et al., 2010; Ferrero, et al., 1995; Wallin, et al., 2002). On the other hand the 2-way ANOVA failed to demonstrate an interactive effect, suggesting that any additional protein does not act in a synergistic way with PHB. This does not exclude an immunostimulatory function for these differential expressed proteins, something that would need further investigation.

The length and/or weight increases of *Artemia,* fed the different concentrations of bacteria (in total), were not analysed in this experiment. However, feeding all the *Artemia* (all treatments) with LVS3 supplies adequate nutrients for growth and survival within the 48 h challenge (as seen in the uniform results in the non-challenge group; Figure 4.1A & Figure 4.4) and the addition of *Bacillus* sp. JL47 in different concentrations and/or different amorphous PHB level are the factors being investigated. Yet, it could still be argued that different doses of bacteria received by *Artemia* may affect growth and that this may modulate the response (e.g. immunity) of the animal during infection. However, we suggests that such effect (e.g. growth) may relate already to the overall beneficial effect caused by feeding PHB-accumulating *Bacillus* sp. JL47 as demonstrated in the subsequent results of our experiment (growth effects in shrimp; chapter 5). Nevertheless, we suggest to use a non-PHB producing *Bacillus* sp. JL47 mutant strain as another control for this experiment to provide stronger claim of the results.

4.5 Conclusion

In conclusion, this study demonstrates the protective effects of the PHB-accumulating *Bacillus* sp. JL47 as effective biocontrol agent for crustacean culture. The protective effect of *Bacillus* sp. JL47 is superior when it contains higher amount of amorphous PHB. Indeed, our data suggest that the amorphous PHB appears to be a main determinant in the protective effects of the *Bacillus* sp. JL47 strain. The possible synergistic effect of amorphous PHB and the other probiotic properties of the *Bacillus* sp.

JL47 are however not ruled out in the perceived beneficial effects of the PHB-accumulating *Bacillus* strain in gnotobiotic *Artemia*.

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CHAPTER 5

EFFECTS OF POLY-β-HYDROXYBUTYRATE-ACCUMULATING *BACILLUS* SPP. ON THE SURVIVAL, GROWTH AND ROBUSTNESS OF *PENAEUS MONODON* (FABRICIUS, 1798) POSTLARVAE

Joseph Leopoldo Q. Laranja[1,2], Gladys L. Ludevese-Pascual[1,2], Edgar C. Amar[2], Patrick Sorgeloos[1], Peter Bossier[1], Peter De Schryver[1]

[1]Laboratory of Aquaculture and *Artemia* Reference Center, Ghent University, 9000 Gent, Belgium
[2]Aquaculture Department, Southeast Asian Fisheries Development Center, 5021 Tigbauan, Iloilo, Philippines

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ABSTRACT

Low larval survival resulting from suboptimal culture conditions and luminous *Vibriosis* poses a major problem for the larviculture of penaeid shrimp. In this study, a poly-β-hydroxybutyrate (PHB) accumulating mixed bacterial culture (mBC; 48.5% PHB on cell dry weight) and two PHB accumulating bacterial isolates, *Bacillus sp.* JL47 (54.7% PHB on cell dry weight) and *Bacillus sp*. JL1 (45.5% PHB on cell dry weight), were obtained from a Philippine shrimp culture pond (chapter 3) and investigated for their capacity to improve growth, survival and robustness of *Penaeus monodon* postlarvae (PL). Shrimp PL1 and shrimp PL30 were provided with the PHB containing bacterial cultures in the feed for 30 days followed by, respectively, a challenge with pathogenic *Vibrio campbellii* and exposure to a lethal dose of ammonia. Prior to the pathogenic challenge or ammonia stress, growth and survival were higher for shrimp receiving the PHB accumulating bacteria as compared to shrimp receiving diets without bacterial additions. After exposure to the pathogenic challenge the shrimp fed PHB accumulating bacteria showed a higher survival as compared to non-treated shrimp, suggesting an increase in robustness for the shrimp. Similar effects were observed when shrimp PL30 were provided with the PHB accumulating bacterial cultures during a challenge with pathogenic *V. campbellii* through the water. The survival of shrimp exposed to lethal ammonia stress showed no significant difference between PHB accumulating bacteria-fed shrimp and non-PHB treated shrimp. The data illustrate that bacilli capable of accumulating PHB can provide beneficial effects to *P. monodon* post-larvae during culture in terms of growth performance, survival and resistance against pathogenic infection.

Keywords:
poly-β-hydroxybutyrate accumulating bacteria, *Bacillus* spp., *Penaeus monodon* postlarvae, growth, survival, robustness

5.1 INTRODUCTION

Outbreaks of luminescent *Vibriosis* are one of the major constraints in the culture of giant tiger prawn, *Penaeus monodon*. This bacterial disease significantly affects the early life stages of the shrimp resulting in major economic losses (Karunasagar, et al., 1994; Lavilla-Pitogo, et al., 1990). The use of antibiotics as prophylactic agents against bacterial diseases has been proven unsustainable and ineffective due to the development of antibiotic resistance in pathogens (Cabello, 2006). Therefore, several alternative strategies have been proposed to address this problem as described in the review of Defoirdt et al. (2007). One of them is the application of the bacterial storage compound poly-β-hydroxybutyrate (PHB). PHB is a compound accumulated as a cellular energy and carbon reserve by a large variety of bacteria that include the genera *Alcaligenes, Pseudomonas, Rhizobium* and *Bacillus* (Anderson and Dawes, 1990; Jiang, et al., 2008; Kaynar and Beyatli, 2009; Paganelli, et al., 2011; Rebah, et al., 2009; Wang, et al., 2012). It is deposited intracellularly in amorphous state in inclusions in the cytoplasm (Amor, et al., 1991; Barnard and Sanders, 1989), and inclusion levels are depending on the nutritional (i.e. carbon source, C/N ratio, etc.) and the environmental conditions (i.e. pH, oxygen, etc.) during growth (Borah, et al., 2002; Du, et al., 2000).

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). It was previously shown that the addition of PHB particles in starved *Artemia* resulted in a prolonged survival of the animal suggesting that PHB particles are (at least partially) degraded in the gut and are used by the animal as an energy source during stressful conditions (Defoirdt, et al., 2007) . By now, the use of PHB as a biocontrol agent for crustacean culture has been tested and found promising to control *Vibriosis* (Defoirdt, et al., 2007; Nhan, et al., 2010; Sui, et al., 2012). In these studies, PHB has been applied in a powdered, crystalline form (i.e. extracted from a bacterial cell). The efficiency of the PHB strategy, however, has been shown to be considerably higher when PHB is supplied in amorphous state (i.e. still contained in a bacterial cell). Indeed, an earlier study has shown that PHB containing bacteria may be more effective than PHB powder to protect *Artemia franciscana* from pathogenic *Vibrio campbellii* infection (Halet,

et al., 2007). The challenge now lies with finding appropriate bacterial cultures capable of accumulating PHB for application in aquaculture settings. In the genus *Bacillus*, PHB is used as an energy source to fuel the sporulation process (Valappil, et al., 2007). PHB is accumulated during or shortly after logarithmic growth with a maximum just prior to the formation of spores. During sporulation, PHB is degraded to fuel the process. Bacilli have been shown to accumulate PHB in the range of 11% up to as high as 79% on cell dry weight (CDW) under optimized conditions (Singh, et al., 2009; Yüksekdağ, et al., 2003). Spore-forming *Bacilli* are commonly found in the gut of fish and shrimp, and *Bacillus megaterium* and *Bacillus pasteurii* originating from the intestinal environment of various fish species have been shown to accumulate PHB up to 23% on CDW (Kaynar and Beyatli, 2009).

Bacillus spp. are generally considered to be interesting probiotic bacteria for aquaculture purposes (Irianto and Austin, 2002). Aside from their various beneficial probiotic effects, their ability to produce spores make them more advantageous over other probiotic species in terms of long term storage and ease of application (Hong, et al., 2005). Therefore, using *Bacillus* species with high PHB accumulating capacity could be an interesting approach to apply in aquaculture, while the possibly combined activity of intracellular PHB and the probiotic activity of *Bacillus* species is an interesting feature to explore. In this study, PHB accumulating *Bacillus* spp. were therefore isolated and tested in *P. monodon* postlarvae (PL) culture. Their effects on survival, growth and robustness against stress of the shrimp PL during culture were determined. This study is the first attempt of using PHB accumulating *Bacillus* spp. in larviculture.

5.2 MATERIALS AND METHODS

5.2.1 PHB ACCUMULATING BACTERIAL CULTURES

The mixed bacterial culture (mBC), consisting of spore forming *Bacillus* species and the two superior PHB accumulating isolate, designated as isolate JL1 and isolate JL47 were all obtained from the research work conducted in chapter 3. The bacterial cultures were kept at -80 °C in 20% glycerol as stock cultures.

5.2.2 IDENTIFICATION OF THE ISOLATES BY 16S RRNA GENE SEQUENCING

Identification of the isolates by 16s rRNA gene sequencing was done following the procedure described in chapter 3. The sequences of the isolates were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank) under the accession numbers KJ496326.1 and KJ496325.1 for isolate JL1 and isolate JL47, respectively. Homology searches were completed with the BLAST server of the National Center for Biotechnology Information (NCBI) for comparison of the nucleotide query sequence against a nucleotide sequence database (blastn). The results of the homology searches are presented in Table 5.1.

5.2.3 FEED PREPARATION

The bacterial stock cultures were activated in LB medium for 16 h and subsequently inoculated at 1% (v/v) in LB added with 20 g L^{-1} glucose (Sigma-Aldrich, Singapore). The cultures were grown at 30°C and 100 rpm agitation for 72 h. After culture, the bacterial cells were harvested by centrifugation at 5000 rpm for 5 minutes, discarding the supernatant and washing with sterile saline (8.5 g L^{-1} NaCl) (repeated 3 times).

A formulated shrimp diet containing 40.5% crude protein and 11.5% crude fat was prepared according to Catacutan (1991). During preparation, the shrimp feed was added with either freshly grown JL1, JL47 or mBC at 4 g wet bacterial weight kg^{-1} feed by spraying the bacterial suspensions gradually into the feeds ensuring a homogenous distribution. A final coating of the feed with cod liver oil (CLO) was used to minimize the leaching of bacteria. The control diet without bacteria was also CLO coated. To avoid excess amounts of lipids in the feed, the fraction of CLO used in the final coating of the bacteria was omitted from the formulation during feed preparation. Feeds sprayed with bacteria and CLO were dried at 30°C for 1 hour and kept at -20°C until needed.

5.2.4 MEASUREMENT OF PHB IN JL1, JL47 AND MBC

At 24, 48 and 72h of culturing JL1, JL47 and mBC, 3 mL aliquots were sampled and frozen immediately at -80°C until measurement of PHB. PHB was quantified spectrophotometrically following the procedure described in chapter 3.

5.2.5 *IN VIVO TESTS*

5.2.5.1 Experiment 1: Effects of PHB accumulating bacterial cultures on *P. monodon* PL1

Survival

P. monodon postlarvae (PL1) were obtained from the Tigbauan Main Station shrimp hatchery of the Southeast Asian Fisheries Development Center- Aquaculture Department, Tigbauan, Iloilo, Philippines and acclimatized to the experimental conditions for a week by stocking the animals in 60 L oval tanks (60 cm length x 40 cm width x 25 cm height) at 20 shrimp L^{-1} with moderate aeration. Temperature ranged from 28-30°C. On a daily basis, the tanks were siphoned and half of the water was replaced with filtered and UV treated seawater. At the start of the experiment, the shrimp from the holding tanks were restocked in 60 L oval tanks at a density of 1 shrimp L^{-1} seawater. During the experimental period of 30 days, the treatments consisted of the formulated shrimp diet added with either JL1, JL47 or mBC (n = 4 per treatment). Shrimps were fed to satiation and excess feed was siphoned out every morning before feeding. Filtered and UV treated seawater was used for a daily water change of 50%. Survival was recorded at the end of the experiment.

Vibrio campbellii LMG 21363 challenge test

After culturing the PL1 for 30 days with the experimental diets, 30 shrimp from each treatment replicate were transferred to a 60 L tank (60 cm length x 40 cm width x 25 cm height). The shrimp were challenged for 24 h with pathogenic *Vibrio campbellii* LMG 21363 at approximately 10^6 CFU per mL of rearing water in combination with a sub-lethal dose of ammonium chloride (75 mg L^{-1}). *V. campbellii* was previously grown for 16 h in LB medium. Shrimp from the control treatment that were challenged acted as positive controls. Shrimp that were reared in the 4 tanks that were kept in parallel to the control treatment were not challenged and acted as negative controls. After 24h of challenge, the water containing the pathogen and ammonium chloride was replaced with filtered and UV treated seawater and the mortality of the shrimp was determined at 7 and 15 days post challenge. During the post-challenge monitoring period, shrimp were fed to satiation using the formulated control diet without PHB accumulating bacteria and excess feed was

siphoned out every morning before feeding. Filtered and UV treated seawater was used for a daily water change of 50%.

5.2.5.2 Experiment 2: Effects of PHB accumulating bacterial cultures on *P. monodon* PL30

Growth and survival

P. monodon postlarvae (PL30) were obtained and acclimatized as described for experiment 1. At the start of the experiment, the shrimp from the holding tanks were restocked in 60 L oval tanks (60 cm length x 40 cm width x 25 cm height) at a density of 1 shrimp L^{-1} seawater. The same feeding treatments and rearing procedure from experiment 1 were applied for 30 days (n = 4 per treatment). The average body weight (ABW) and average body length (ABL) of the shrimp were determined at the beginning and at the end of the experiment. Survival was recorded at the end of the experiment.

Ammonia stress test

After culturing the PL30 for 30 days with the experimental diets, 15 shrimp from 3 replicate tanks per treatment were transferred to 5 L plastic containers (25 cm length x 20 cm width x 10 cm height). A lethal dose of ammonium chloride of about 150 mg L^{-1} was added in each container and survival of the shrimp was determined after 24h and 48h of exposure. The ammonium dose was the LD_{50} concentration for shrimp of that age in seawater as determined prior to the experiment. The concentration of non-ionized ammonia (NH_3) at a pH value of 7.8 in the seawater was estimated according to Armstrong et al. (1978) and was about 1.78 mg L^{-1} ammonia. Moderate aeration was provided and no feed was supplied during the 48h exposure period.

5.2.5.3 Experiment 3: Effects of PHB accumulating bacterial cultures on *P. monodon* PL30 challenged daily with *Vibrio* campbellii LMG 21363

P. monodon postlarvae (PL30) were obtained and acclimatized as described for experiment 1. At the start of the experiment, the shrimp from the holding tanks were restocked in 13.3 L round containers (13 cm radius; 25 cm height) at a density of 2 shrimp L^{-1} seawater (n = 4 per treatment). The rearing water was added daily with either JL1,

JL47 or mBC at 10^6 CFU mL^{-1} harvested at 48h of culture. The shrimp were challenged by daily addition of freshly grown *V. campbellii* LMG 21363 at 10^6 CFU mL^{-1} to the rearing water. Shrimp in rearing water without addition of PHB accumulating bacteria or pathogenic bacteria made up the negative control treatment (n = 4), while shrimp in rearing water without addition of PHB accumulating bacteria but with addition of pathogenic bacteria made up the positive control treatment (n = 4). All shrimp were fed daily with formulated shrimp diet without PHB accumulating bacteria and excess feed was siphoned out every morning prior to feeding. On a daily basis, 90% of the tank water volume was replaced. All bacteria were added after all water management was conducted. Survival of the shrimp was determined at 10 days, 20 days, and 30 days of culture.

5.2.6 Statistical analysis

All values represent means ± standard error of the mean (SEM). The data on growth and survival were analyzed using one-way ANOVA followed by a post hoc Duncan Multiple Range test for assessment of significant differences between treatments at a 5% significance level ($p < 0.05$) using SPSS 16.0 software. Percentage survival was subjected to arcsine transformation prior to statistical analysis. The Pearson product moment correlation coefficient (r) and the Pearson two-tailed p value were determined using GraphPad Prism version 5.03 for Windows, GraphPad Software.

5.3 Results

5.3.1 Accumulation of PHB

All PHB accumulating cultures used in this study attained their highest PHB accumulation at 48h of culture (Figure 5.1). In specific, isolate JL47 attained the highest accumulation of PHB (54.6% on CDW) followed by mBC (48.5% on CDW) and isolate JL1 (45.5% on CDW). Based on these values, the PHB doses in the feed could be calculated as 0.41 g PHB kg^{-1} feed, 0.36 g PHB kg^{-1} feed, and 0.34 g PHB kg^{-1} feed for the JL47, mBC and JL1 treatment, respectively.

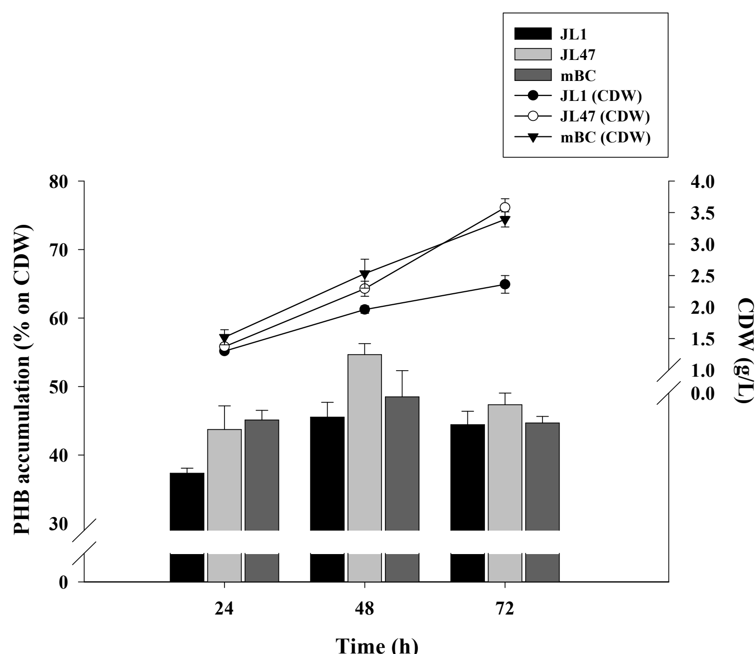


Figure 5.1 PHB accumulation and cell dry weight (CDW) concentration of the PHB accumulating bacterial cultures grown in LB + 2% (w/v) glucose.

5.3.2 GROWTH AND SURVIVAL OF P. MONODON POSTLARVAE

The survival of the PL1 and PL30 in experiment 1 and 2, respectively, showed similar trends (Figure 5.2). A higher survival was observed for shrimp receiving diets containing PHB accumulating bacteria as compared to shrimp fed the control diet. In the PL1 experiment, the survival of the JL47 fed shrimp was significantly higher than that of the control fed shrimp. Survival of JL1 fed and mBC fed shrimp was not significantly higher than of control shrimp. In experiment 2, the survival of PL30 fed with JL47 bacteria or JL1 bacteria was significantly higher than that of the control fed PL30. The survival of mBC-fed shrimp was not significantly higher as compared to the survival of the control shrimp.

The ABW and ABL of the PL30 in experiment 2 after 30 days of culture was significantly higher for the JL47 fed shrimp as compared to the control shrimp (Figure 5.3). The JL1 and mBC fed shrimp did not show a significantly higher ABW than the control fed shrimp. The ABL of mBC fed shrimp, however, was significantly higher than that of the control shrimp but not significantly different from that of JL1 and JL47 fed shrimps.

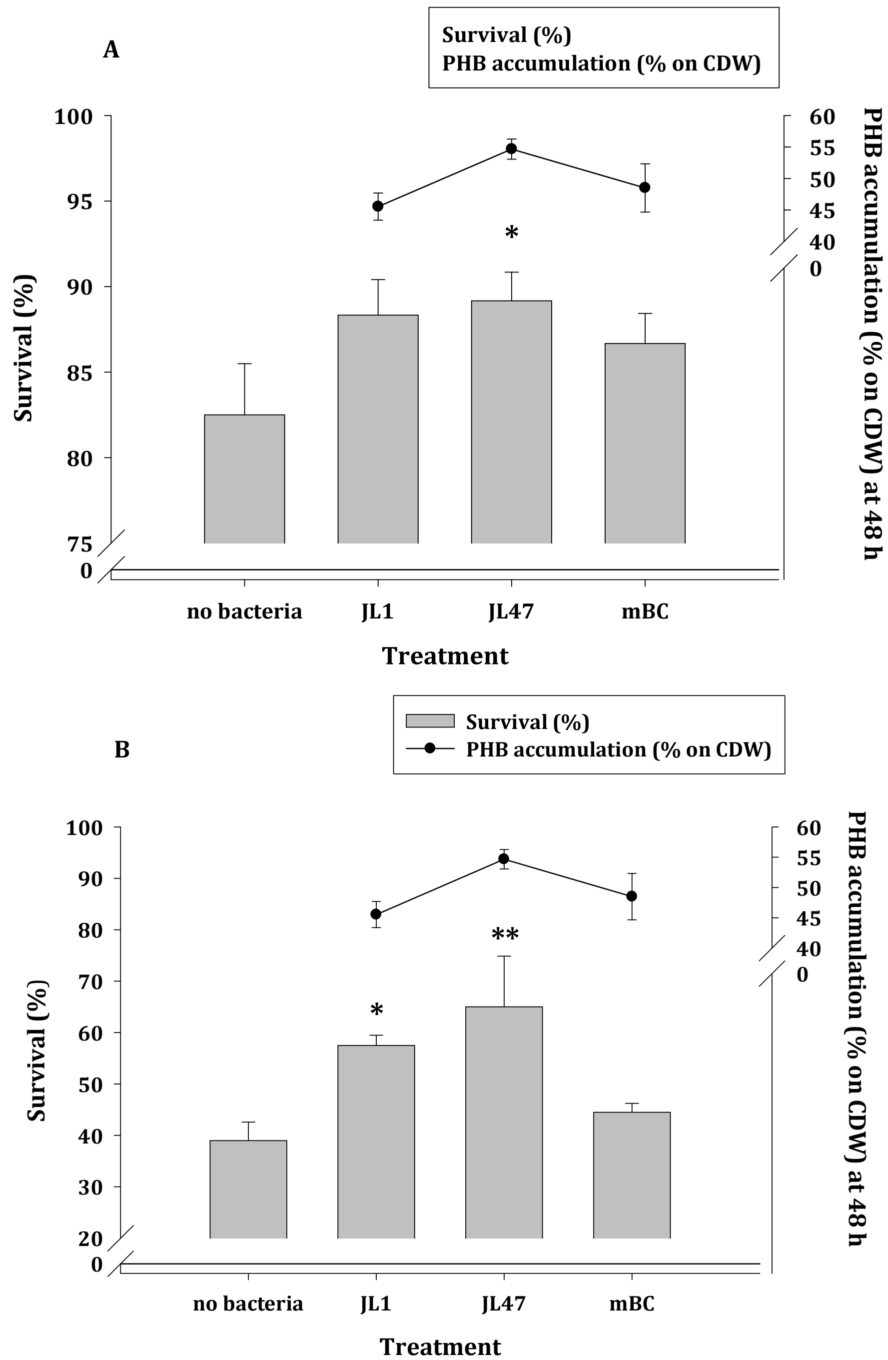


Figure 5.2 Survival of shrimp PL1 in experiment 1 (A) and survival of shrimp PL30 in experiment 2 (B) after 30 days of feeding with shrimp formulated diet added with and without different PHB-accumulating bacterial cultures. The PHB content of the bacterial cultures added to the feed is presented. Bars represent means ± SEM (n = 4). Asterisks (* P < 0.05; ** P < 0.01) indicate a significantly different survival as compared to the control (no bacteria).

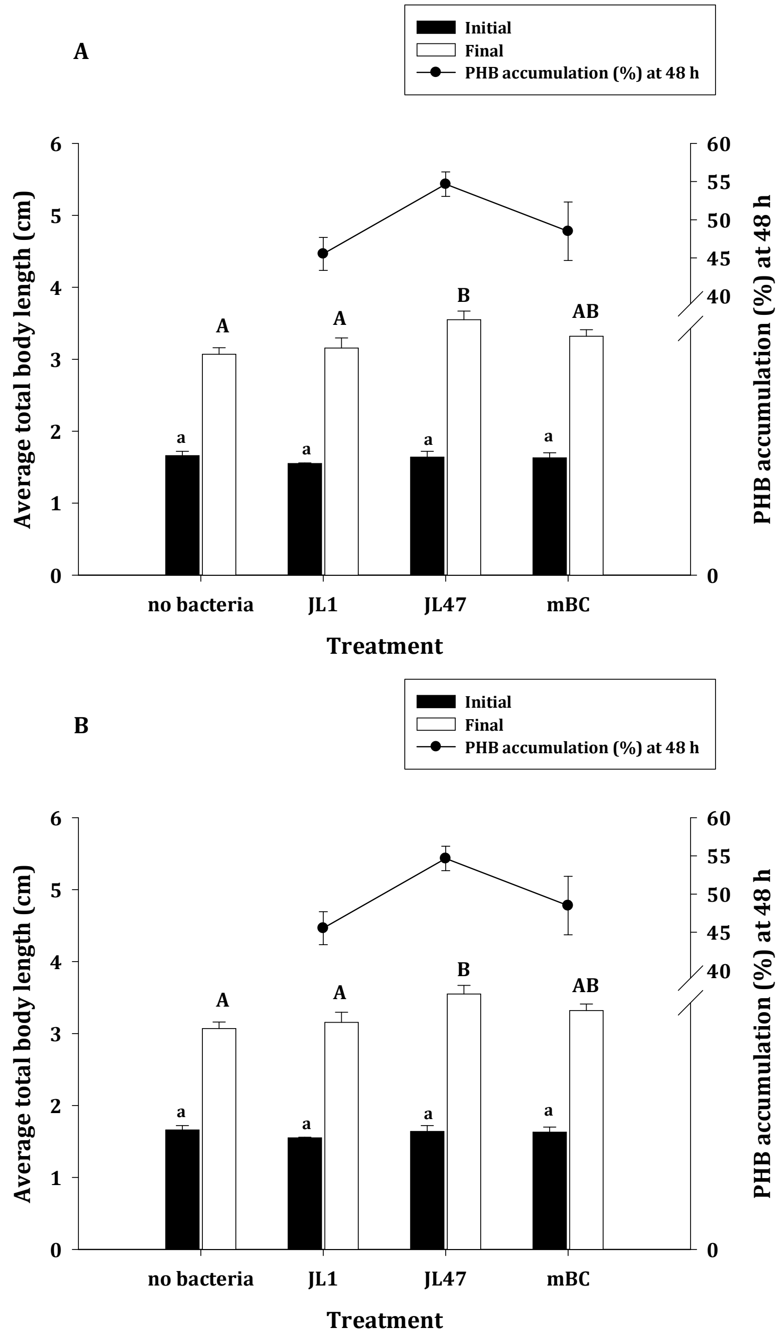
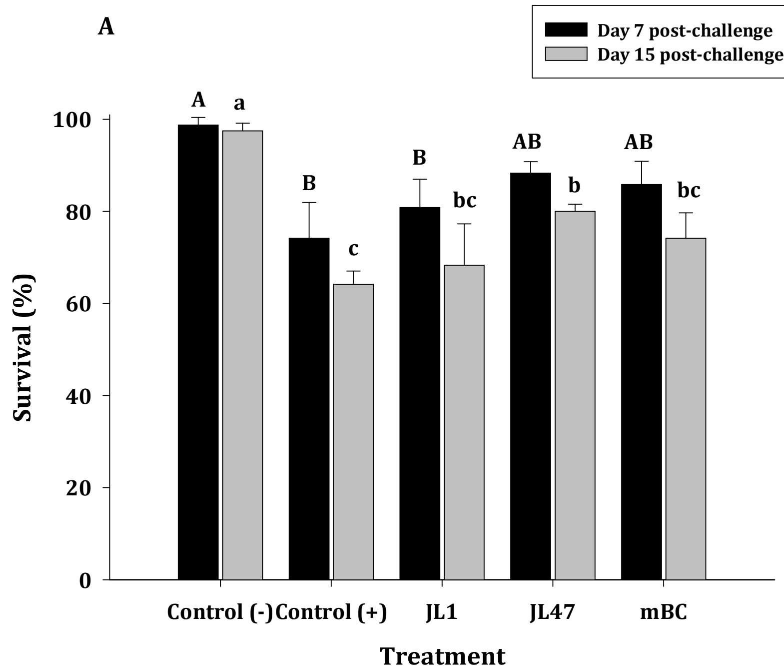


Figure 5.3 Initial and final average body weight (A) and average body length (B) of *P. monodon* PL30 after 30 days of feeding with shrimp formulated diet added with and without different PHB-accumulating bacterial cultures (experiment 2).The PHB content of the bacterial cultures added to the feed is presented. Bars indicate means ± SEM (n = 4). Different letters indicate significant differences (P<0.05) and small and capital letters denote different comparisons in a figure.

5.3.3 *CHALLENGE TESTS*

In the pathogen challenge test from experiment 1, there seemed to be a trend of higher survival for the shrimp fed PHB accumulating bacteria than for the challenged control shrimp after 7 days post-challenge. However, the differences were not significant (Figure 5.4-A). At 15 days post-challenge, the same trend was observed but now the JL47 fed shrimp survived significantly better than the challenged control shrimp. The non-challenged control attained a higher survival than all other treatments at day 7 and day 15 post-challenge, and the difference was only non-significant when compared to the JL47 and mBC fed shrimp at 7 days post-challenge.

In the pathogen challenge test from experiment 3, similar survival trends were observed as in the challenge test from experiment 1 (Figure 5.4-B). The survival after 30 days of culture was significantly higher for the shrimp supplied of PHB accumulating JL47 in the rearing water as compared to the positive control shrimp, while this was not the case for the shrimp from the JL1 and mBC treatments. The survival of shrimp supplied of JL47 was comparable to and not significantly different from the non-challenged negative control shrimp.



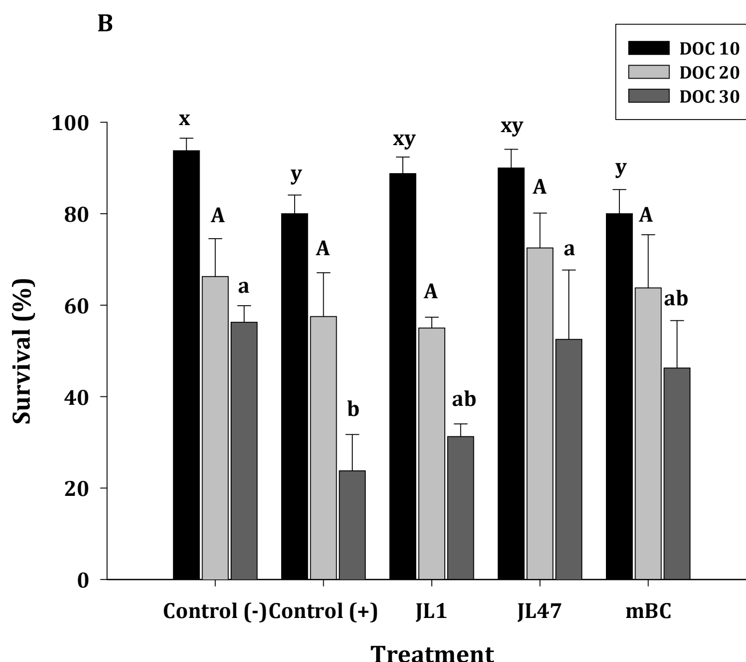


Figure 5.4 (A) Survival of shrimp PL1 fed 30 days with diets supplemented with different PHB accumulating bacterial cultures and subsequently challenged for 24 h with pathogenic *Vibrio campbellii* LMG 21363 and a sub-lethal dose of ammonium chloride (75 mg L^{-1}) (experiment 1). (B) Survival of shrimp PL30 after daily addition of different PHB accumulating bacterial cultures and pathogenic *Vibrio campbellii* LMG 21363 at 10^6 CFU $mL^{-1}$of rearing water for 30 days during culture (experiment 3). Control (-) = non-challenged negative control, Control (+) = challenged positive control. Bars represent means ± SEM (n = 4). Different letters within a day indicate significant differences (P<0.05). DOC = day of culture.

5.3.4 AMMONIA STRESS TEST

The survival of the shrimp after 24 h and 48 h of ammonia challenge (experiment 2) was not significantly different between treatments (Figure 5.5). The lowest survival, however, was consistently observed in the challenged positive control indicating a trend of higher survival in case the shrimp were supplied of PHB accumulating bacteria in the diet.

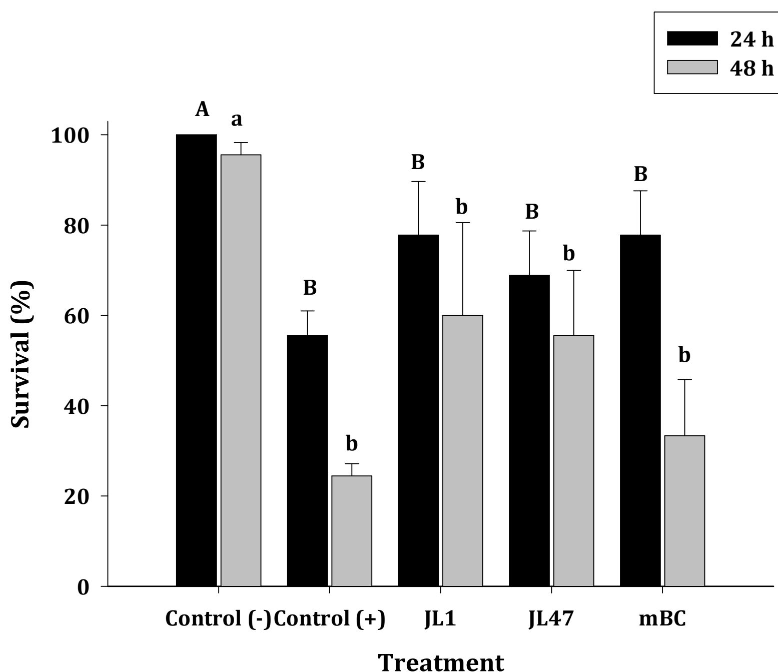


Figure 5.5 Survival of shrimp PL30 fed 30 days with different PHB-accumulating bacterial cultures and subsequently exposed to a lethal dose of ammonium chloride (150 mg L^{-1}) (experiment 2). Bars represent means ± SEM (n = 3). Different letters indicate significant differences (P < 0.05) and small and capital letters denote different comparisons.

Table 5.1 Results of the homology search for the isolate nucleotide query sequences using NCBI BLASTN search tool

Isolate (GenBank accession no.)	Closest match (GenBank accession no.)	Identity similarity (%)
JL47 (KJ496325.1)	*Bacillus cereus* SBTBC-008 (KF601957.1)	99
	Bacillus anthracis JN22 (KF150341.1)	99
	Bacillus thuringiensis AHBR13 (KF241	99
JL1 (KJ496326.1)	*Bacillus cereus* AVP12 (KF527826.1)	99
	Bacillus anthracis Y43 (KF730755.1)	99
	Bacillus thuringiensis GS1 (FJ462697.1)	99

5.4 Discussion

The provision of PHB to *Artemia franciscana* in amorphous form (i.e. contained within a bacterial cell) seems to increase the efficiency as compared to the provision of

PHB in crystalline form (i.e. extracted from the bacterial cell) (Halet, et al., 2007). In this research, we were able to show that PHB accumulating *Bacillus* spp., a genus of bacteria often associated with strong probiotic effects, positively affected the culture efficiency and robustness of *P. monodon* postlarvae. The availability of PHB accumulating spore formers presents a major advance in PHB application as it allows for an efficient and long-term storage of the bacterial precursor of this beneficial compound.

The PHB accumulating bacterial cultures used in this study attained the highest PHB accumulation at 48 h of culture. The PHB content further decreased when bacteria reached their maximum growth (72 h). *Bacilli* are known to accumulate PHB during exponential growth to prepare for spore formation (Valappil, et al., 2007). The stored PHB is then used as an energy source to fuel the actual sporulation process (Benoit, et al., 1990). For this reason, the bacteria to be added in the diets of the experimental animals were harvested at 48 h, aiming to use them at their maximum PHB accumulation state. Furthermore, the results in chapter 4 suggest that the PHB-accumulating *Bacillus* sp. JL47 is more superior when it contains higher amount of amorphous PHB.

Both shrimp PL1 and PL30 receiving PHB accumulating bacilli in the diet showed a higher survival as compared to the control shrimp. This gives a clear indication that the bacilli provided beneficial effects to the shrimp during culture. In previous experiments, increased survival of crustaceans following crystalline PHB supplementation has been related to a decreased presence of opportunistic pathogens such as *Vibrio*s (Nhan, et al., 2010). Potentially, a similar mechanism acted here. Growth was also better for the shrimp supplied of the PHB accumulating bacteria than those receiving the control diet. PHB has been reported to increase the growth performance of other aquatic animals as well (De Schryver, et al., 2010; Nhan, et al., 2010), although the exact mechanisms so far remain unknown. The improved growth of the shrimp PL30 in this study trended according the level of PHB in the diet. A significant correlation ($p = 0.01$) existed between the PHB dose in the diet and the increase in shrimp body weight as compared to the control ($r = 0.99$). These data suggest that the level of PHB in the diet was a main determinant for the growth performance of the shrimp. Several *Bacillus* spp., however, are known to produce exoenzymes, such as proteases, carbohydrolases and lipases, that can break down proteins, carbohydrates and lipids, respectively, and as such contribute to the digestion

and absorption of feed in the gut (Arellano-Carbajal and Olmos-Soto, 2002; Wang, 2007; Ziaei-Nejad, et al., 2006). The activity of the bacilli added to the feed may thus also have contributed to the growth performance of the shrimp (e.g. facilitates digestion and absorption of nutrients). Further experimenting is required to determine the relative contribution of the PHB effect and a probiotic effect on the growth improvement that is achieved by supplying the PHB-accumulating bacilli to the shrimp.

In addition to survival and growth, the application of the PHB-accumulating bacteria affected the robustness of the shrimp as determined by several challenge tests. Two kinds of challenges were selected that are relevant for *P. monodon* larviculture: a pathogenic challenge test and an ammonia stress test. *Vibrio campbellii* is an important pathogen for penaeid shrimp and among the *Penaeus* species *P. monodon* is the one most sensitive and susceptible to this pathogen (Hameed, 1995). In this study, shrimp were challenged with *V. campbellii* in two ways: i) a single exposure to the pathogen in the rearing water in combination with a sub-lethal stressor (ammonium chloride) for 24 h; and ii) a continuous exposure by daily addition of the pathogen in the rearing water for 30 days. In both variants, the survival was higher for the shrimp supplied of the PHB-accumulating bacilli as compared to the shrimp from the challenged control, with a maximum attained for the shrimp supplied of the highest PHB accumulator (JL47). These observations correspond to what was found earlier on the application of PHB in challenge tests with the model organism *Artemia franciscana* (Defoirdt, et al., 2007; Halet, et al., 2007). The suggested mode of action is that PHB is degraded intestinally resulting in a release of its monomer, 3-hydroxybutyrate. This compound can act antimicrobially by acidifying the cytoplasm of the pathogen. The pathogen has to redirect cellular energy (i.e. use of transporters that catalyse active proton transport), to maintain homeostasis, resulting in a decreased virulence (Defoirdt et al., 2009). Alternatively, the PHB may have acted immunostimulatory towards the shrimp (Suguna, et al., 2014). Similar as for the growth and survival results, however, it should also be taken into account that the different *Bacillus* cultures used in these experiments may have had probiotic activities contributing to the improved robustness of the shrimp during challenge. Additional tests will have to show if a probiotic effect of the bacilli also contributed to the observations. In this context,

a set of preliminary *in vitro* experiments did not show a direct antagonistic effect of the bacilli towards the pathogen (data not shown).

Ammonia was applied as a second stressor as it is one of the most frequent stressors in high density larviculture of *P. monodon* (Chin and Chen, 1987). The results indicate that supplementation of the PHB accumulating bacteria may reduce the effects of ammonia stress, although there were no significant differences between the PHB treatments and the control. An explanation can be that PHB and its degradation products have earlier been found to act as an energy source for starved *Artemia* (Defoirdt et al., 2007b). The PHB can thus have contributed in the energy delivery to the shrimps increasing their strength to resist stress.

5.5 CONCLUSION

In conclusion, this study demonstrated the potential of newly isolated PHB-accumulating bacilli as biocontrol agents and feed supplements in *P. monodon* larviculture. In chapter 4, the significance of amorphous PHB as important determinant in the protective effects of the PHB-accumulatiing *Bacillus* sp. JL47 was presented. However, further research is needed to elucidate (clearly) the relative contributions of the stored PHB and the probiotic activity of the bacilli in the survival, growth and robustness effects. This will allow optimizing the application of PHB-accumulating *Bacillus* spp. for shrimp larviculture. In addition, it has to be noted that the method of culturing the PHB bacilli in LB medium for PHB accumulation may not be applicable to hatchery practices because of economical as well as practical reasons. A challenge lies in finding a cheap and easy method to overcome this problem. An integrated technique consisting of a combination of *Artemia* culture to produce live food for early postlarval shrimp and the culture of the PHB accumulating bacilli may offer a solution. The strategy of growing bacteria on the waste metabolites released during *Artemia* hatching has been proposed earlier (Cam, et al., 2009). By optimizing the conditions during *Artemia* hatching, such as C/N ratio in the medium, the PHB-accumulating bacilli can most likely be triggered to accumulate high concentrations of PHB *in situ*.

Acknowledgement

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PART 4

MODE OF ACTION

CHAPTER 6

POLY-β-HYDROXYBUTYRATE-ACCUMULATING *BACILLUS* SP. JL47 STIMULATES THE INNATE IMMUNE RESPONSE OF *PENAEUS MONODON* POSTLARVAE

Joseph Leopoldo Q. Laranja[1,2], Gladys L. Ludevese-Pascual[1], Edgar C. Amar[2], Yufeng Niu[1], Mary Joy Geaga[2], Peter De Schryver[1], Peter Bossier[1]

[1]Laboratory of Aquaculture and *Artemia* Reference Center, Ghent University, 9000 Gent, Belgium
[2]Aquaculture Department, Southeast Asian Fisheries Development Center, 5021 Tigbauan, Iloilo, Philippines

†Redrafted after:
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ABSTRACT

In this experiment, we investigated the effects of the PHB-accumulating isolate on the immune response of *P. monodon* postlarvae before and after the *Vibrio* challenge. Shrimp postlarvae were cultured and fed with *Artemia* nauplii enriched with *Bacillus* sp. JL47. Shrimp receiving the *Artemia* nauplii without JL47 enrichment were used as control. After 15 days of feeding, the shrimp were challenged with pathogenic *V. campbellii* LMG 21363 at 10^6 cells mL^{-1} by immersion. Relative expression of the immune related genes encoding for prophenoloxidase (proPO), transglutaminase (TGase) and heat shock protein 70 (Hsp70) in the shrimp were measured before (0 h) and after (3, 6, 9, 12, 24 h) the *Vibrio* challenge by quantitative real-time PCR using β-actin as the reference gene. The expressions of proPO and TGase were significantly up-regulated ($p<0.05$) within 12 h after challenge in shrimp receiving the *Bacillus* sp. JL47 as compared to the challenged and non-challenged controls. Hsp70 expression was significantly increased ($p<0.05$) at 3 h post-challenge in all challenged shrimp. Interestingly, proPO and TGase genes were significantly up-regulated ($p<0.05$) in *Bacillus* sp. JL47 treated shrimp even before the *Vibrio* challenge was applied. No up-regulation in the Hsp70 gene, however, was observed under these conditions. The data suggest that the protective effect of the PHB-accumulating *Bacillus* sp. JL47 in shrimp was due to its capacity to stimulate the innate immune related genes of the shrimp, specifically the proPO and TGase genes. The application of probiotic *Bacillus* species, capable of accumulating a significant amount of PHB, is suggested as potential immunostimulatory strategy for aquaculture.

Keywords:

Amorphous PHB, probiotic *Bacillus*, prophenoloxidase, transglutaminase, heat shock protein 70, *Penaeus monodon* postlarvae

6.1 INTRODUCTION

Black or giant tiger shrimp *P. monodon* was one of the most economically important crustacean species in the aquaculture industry. However, the culture of this species has consistently been hampered by disease outbreaks, most often caused by bacterial and viral pathogens (Johnson, 1989; Lightner and Redman, 1998). For example, mass mortality at the early larval stages of the animal was reported to result from luminescent *Vibriosis* (Karunasagar, et al., 1994; Lavilla-Pitogo, et al., 1990). Hence, managing their health by enhancing their immunity is of vital importance.

Invertebrates such as shrimp do not have adaptive immunity but have to rely on their innate immune system as the major defense mechanisms to fight invading pathogens. The activation of this innate immune system is initiated upon the recognition of non-self-molecules associated with pathogens, also known as pathogen-associated molecular patterns (PAMPs), by well-defined receptors referred to as pathogen/pattern recognition receptors (PRRs) (Ishii, et al., 2008; Janeway Jr and Medzhitov, 2002). Among the diverse array of immune responses, melanisation (prophenoloxidase (proPO) activation system) has been suggested as one of the most important immune mechanism in many invertebrates (Amparyup, et al., 2013). In this immune reaction, the recognition of microbial PAMPs by appropriate PRRs leads to the activation of a series of serine proteinases and eventually culminates in the proteolytic cleavage of the proPO zymogen to the active phenoloxidase (PO) enzyme. The activation of PO results in the production of quinones and other short-lived intermediates which possess cytotoxic activity towards microorganisms, restrain the invasion of microbial pathogens into the host body cavity as well as participate in wound healing process in damaged tissues (i.e. sclerotisation) (Amparyup, et al., 2013; Cerenius, et al., 2008; Nappi and Christensen, 2005; Zhao, et al., 2007; Zhao, et al., 2011). Finally, in this immune response the production of the polymeric melanin as a more long-lived product is deposited precisely at the site of infections or around the surface of foreign microorganisms to physically encapsulates foreign microorganisms (Amparyup, et al., 2013). Furthermore, clot formation is also another first line of defense in shrimp that is being activated to prevent blood loss and microbial spread at sites of injury. Considering its quickness and efficiency, it is

considered to be an integral part of the overall immune response in crustaceans for survival. In the clotting process, transglutaminase (TGase) is the central enzyme involved in the final step for the stabilization of the blood clot. In shrimp, TGase and clotting proteins have been suggested to be essential molecules in shrimp blood coagulation and immunity (Maningas, et al., 2008). Moreover, heat shock proteins (Hsps) are considered to be crucial mediators in conferring tolerance towards environmental stress (Kregel, 2002). Cells inducing Hsps as a result of stress show resistance to a subsequent stress - a phenomenon called "stress tolerance" (De Maio, 1999). Molecular chaperones like heat shock protein 70 (Hsp70) were reported to repair partially denatured proteins, facilitate the degradation of irreversibly denatured proteins and inhibit protein aggregation and therefore protecting cells from harmful environmental stresses (Parsell and Lindquist, 1993). In addition, Hsp70 facilitates immune responses against many diseases as demonstrated in a wide variety of experimental models *in vitro* and *in vivo* (Joly, et al., 2010; Junprung, et al., 2017; Tsan and Gao, 2009).

Stimulation of the immune-related genes in the host has been an effective strategy to protect shrimp from diseases. Probiotic bacteria like *Bacillus* species were reported to have immunostimulatory effects in shrimps resulting in improved resistance towards pathogenic diseases (Rengpipat, et al., 2000; Tseng, et al., 2009). Likewise, many products of bacterial origin were also reported to have immunostimulatory effects such as peptidoglycan (PG), lipopolysaccharide (LPS), β-glucans, and other bacterial cell wall components (Murata, et al., 1993; Robertsen, et al., 1990; Skalli, et al., 2013). A recent report shows that the biopolymer PHB-hydroxyvalerate (PHB-HV) extracted from *Bacillus thuringiensis* increased both the specific and non-specific immune mechanisms in fish (Suguna, et al., 2014). Hence, the idea of using *Bacillus* species carrying significant amount of amorphous PHB is suggested in this study. In chapter 5, we demonstrated the protective effects of the PHB-accumulating *Bacillus* sp. JL47 (containing 55% PHB) in *P. monodon* postlarvae against pathogenic *V. campbellii* LMG 21363. In this experiment we aimed at investigating the effects of the PHB-accumulating *Bacillus* isolate JL47 at a molecular level by looking at the *in vivo* expressions of proPO, TGase and HSp70 genes of the *P. monodon* postlarvae before and after a *Vibrio campbellii* challenge.

6.2 MATERIALS AND METHODS

6.2.1 BACTERIAL STRAINS

The PHB-accumulating *Bacillus* sp. JL47 (GenBank: KJ496325.1), was cultured following the procedure described in chapter 5. In brief, JL47 was activated in LB (tryptone (Himedia, India; 10 g L^{-1}), yeast extract (Himedia, India; 5 g L^{-}1) and NaCl (Sigma-Aldrich, Singapore; 20 g L^{-1}) medium for 16 h and subsequently inoculated at 1% (v/v) in LB supplemented with 20 g L^{-1} glucose (Sigma-Aldrich, Singapore). The culture was grown at 30°C and 100 rpm agitation for 48 h. After culture, the bacterial cells were harvested by centrifugation at 5,000 rpm for 5 min, discarding the supernatant and washing with sterile saline (8.5 g L^{-1} NaCl) (repeated 2 times). The bacterial cells were kept at -80°C until used for *Artemia* enrichment.

Pathogenic *Vibrio campbellii* LMG 21363 was used in the challenge experiment. The bacterial preparation and the challenge with pathogenic *V. campbellii* LMG 21363 (except for the addition of sub-lethal dose of ammonium chloride in the water) in shrimp were conducted according to the procedures described in the previous chapter (chapter 5).

6.2.2 ARTEMIA ENRICHMENT

High 5 *Artemia* cysts (INVE Aquaculture, Thailand) were hatched daily in filtered seawater at 2 g cysts L^{-1} for 30 h in a 30-L hatching tank. Vigorous aeration was provided with illumination set at approximately 27 μE/(m^2.sec)). At approximately 30-h incubation, *Artemia* nauplii (*Artemia* instar II) were harvested, washed with filtered seawater and restocked in enrichment tanks at ~5,000 individuals L^{-1} seawater. *Artemia* instar II nauplii were then enriched with *Bacillus* sp. JL47 (containing ~55% amorphous PHB on cell dry weight) at 0.5 g (wet weight) L^{-1}. The enrichment tank was provided with vigorous aeration. The enrichment was done for 6 h, after which the enriched *Artemia* were harvested and rinsed with seawater prior to feeding. Non-enriched *Artemia* nauplii were also maintained and harvested prior to feeding.

6.2.3 SHRIMP CULTURE AND SAMPLING

P. monodon postlarvae (PL5) were obtained from the Tigbauan Main Station shrimp hatchery of the Southeast Asian Fisheries Development Center-Aquaculture Department (SEAFDEC/AQD), Tigbauan, Iloilo Philippines and acclimatized to the experimental conditions for five days by stocking the animals at 15 shrimps L^{-1} in ~13 L capacity round containers (13 cm radius; 25 cm height) containing 10 L filtered and UV-treated seawater with moderate aeration. The overall status of the shrimps was also examined by randomly collecting shrimp samples from the same batch and the shrimp samples were submitted to the Fish Health Section of SEAFDEC/AQD for standard shrimp pathogen analyses to ensure that the shrimps are free from diseases (i.e. WSSV, MBV and IHHNV). During the acclimation period, all shrimps were fed with non-enriched *Artemia* to satiation. On a daily basis, the tanks were siphoned and ~80% of the water was replaced with filtered and UV-treated seawater. After the acclimation period, the experiment was started and the shrimp from each tank were recounted to ensure uniformity of stocking density at 15 shrimps L^{-1}. Shrimps were fed with *Bacillus* sp. JL47-enriched *Artemia* as treatment (N=3). Shrimps fed non-enriched *Artemia* were used as control (N = 3; wherein 3 tanks were used for the challenge test (negative control) while the remaining 3 tanks were not challenged (positive control)) (see Figure 6.1 for the schematic diagram). Feeding of enriched and non-enriched *Artemia* was done twice daily (morning and afternoon) to satiation. Water was changed daily at 80% of the water volume with fresh filtered and UV- treated sea water. Excreta were siphoned out every morning prior to feeding. The immersion challenge with *V. campbellii* LMG 21363 was performed after 15 days of culture by the addition of 10^6 cells mL^1 in the culture water. Five shrimps were sampled per tank just before the challenge (0 h) and 3, 6, 9, 12 and 24 h after the challenge. Shrimp sampled at each time from the same tank were pooled and treated as one replicate in a given treatment. This yielded three biological replicates for each treatment. During the sampling, shrimp were immediately put in eppendorf tubes with RNA later (Ambion, USA) and kept cooled in ice all throughout the sampling. In the lab, shrimps were kept at -80°C until RNA isolation.

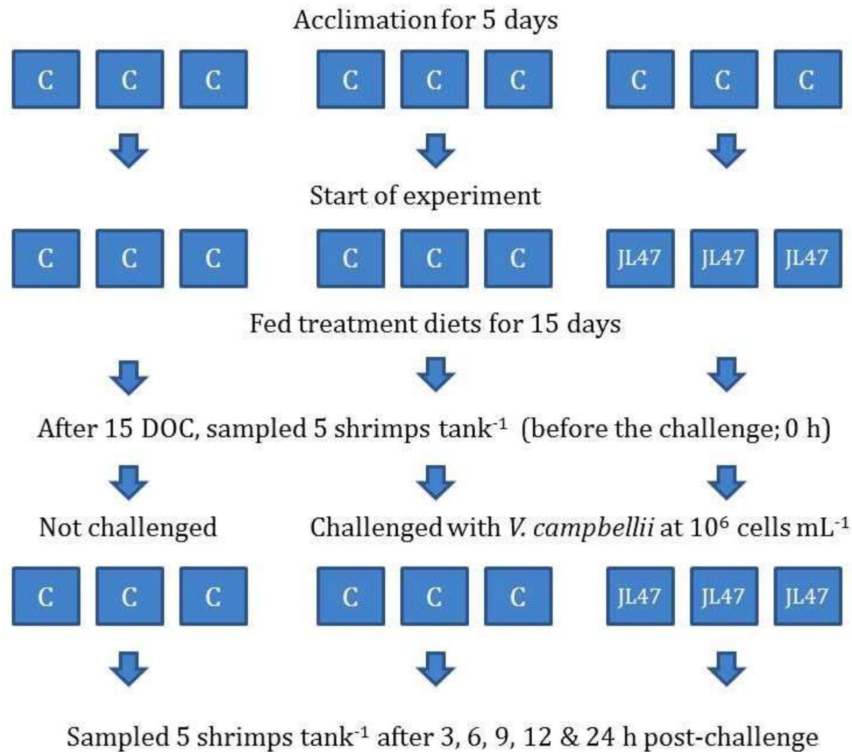

Figure 6.1 Schematic diagram of the acclimation, feeding, challenge test and sampling of the shrimp done during the whole feeding trial. C=shrimps fed the none-enriched *Artemia*; JL47=shrimps fed the *Bacillus* sp. JL47-enriched *Artemia*.

6.2.4 RNA ISOLATION AND REVERSE TRANSCRIPTION

Total RNA was extracted using SV Total RNA isolation system (Promega) following the manufacturer's instructions. RNA was quantified using NanoPhotometer™ UV/Vis (Implen GmbH, Munich, Germany) at 260 and 280 nm and only samples with an RNA absorbance ratio (A_{260}:A_{280}) greater than 1.8 were used for further analysis. First strand complementary DNA (cDNA) was generated using a RevertAid H Minus First Strand cDNA synthesis kit (Thermo Scientific) in a 20 µL reaction volume containing 1 µg total RNA, 5X reaction buffer, 20 U RiboLock RNAse inhibitor, 10 mM dNTP mix, 200 U RevertAid H Minus M-MuLV Reverse Transcriptase . The gene reaction was conducted at 25°C for 5 min followed by 42°C for 60 min and terminated by heating at 70°C for 5 min using Geneamp PCR system 97000 (AB Applied Science, Singapore).

6.2.5 Quantitative real-time PCR analysis

Expression of the three target gene transcripts (proPO , TGase and Hsp70) and internal control (β-actin) was measured by quantitative real-time PCR (qPCR) using the primer pairs reported from previous studies (see Table 1). Primers of proPO could amplify the previously reported proPO gene in *P. monodon* (GenBank accession no. AF099741) (Sritunyalucksana, et al., 1999), primers for TGase could amplify two TGases of *P. monodon* STG I and STG II (GenBank accession no. AY074924, AY771615) (Chen, et al., 2005; Huang, et al., 2004), primers for Hsp70 could amplify heat shock cognate 70 in *P. monodon* (Lo, et al., 2004) and primers for β-actin could amplify the β-actin gene in *P. monodon* (GenBank accession no. DW042525). The qPCR amplifications were carried out using a Corbett Real Time Thermocycler, Rotor Gene 6000 (Sydney, Australia) in a 25 µL reaction volume, containing 2 µL cDNA, 12.5 µL of Maxima SYBR Green qPCR Master mix (Thermo Scientific), 0.4 µL of each primers (20 µM) and 9.7 µL nuclease free water. The qPCR was programmed using a two-step cycling protocol as follows: pre-treatment (2 min at 50°C); initial denaturation (10 min 95°C); 40 cycles of denaturation and annealing/extension (15 sec at 95°C and 60 sec at 60°C); melting curve (72-95°C with heating rate of 0.2°C s^{-1} and continuous fluorescence measurement); and cooling (4°C). Dissociation curve analysis in the qPCR was performed to check for the amplification of untargeted fragments. Each sample was analysed in duplicate. The qPCR was validated by amplifying serial dilutions of cDNA synthesized from 1 µg of RNA isolated from shrimp postlarvae and then amplification efficiency of the target genes and the house-keeping gene was calculated. Relative quantification of the target gene transcripts was done following the Paffl method as described previously (Pfaffl, 2001).

Table 6.1 Primers used for the quantitative real-time PCR analysis of the three immune-related genes and β-actin of *P. monodon* postlarvae.

Gene	Primers	Product size (bp)	Reference
proPO	5'-TGGCACTGGCACTTGATCTA-3' 5'-GCGAAAGAACACAGGGTCTCT-3'	590	(Jiravanichpaisal, et al., 2007)
TGase	5'-TGGGYCTTCGGGCAGTT-3' 5'-CGAAGGGCACGTCGTAC-3'	627	(Jiravanichpaisal, et al., 2007)
Hsp70	5'-AGAAGTCACTCCGTGATGCCAAGA-3' 5'-ACTCCTTGCCGTTGAAGAAGTCCT-3'	123	(Rungrassamee, et al., 2010)
β-actin	5'-GCTTGCTGATCCACATCTGCT-3' 5'-ATCACCATCGGCAACGAGA-3'	320	(Proespraiwong, et al., 2010)

6.2.6 STATISTICAL ANALYSIS

Data for the relative gene expression were log-transformed prior to statistical analysis but only non-transformed data are presented. Normality was tested by means of a Shapiro-Wilk test. Relative gene expression data before the *Vibrio* challenge were analysed using a t-test while the relative gene expression data after the *Vibrio* challenge were subjected to one-way analysis of variances followed by a Duncan's Multiple Range test to determine significant differences among treatments at a 5% significance level ($p<0.05$). All statistical analyses were done using SPSS 16.0 software.

6.3 RESULTS

6.3.1 RELATIVE EXPRESSION OF PROPO GENE IN P. MONODON POSTLARVAE

The proPO is considered as the central enzyme in the proPO system hence the relative expression of the proPO gene in *P. monodon* postlarvae was investigated to determine if the PHB-containing *Bacillus sp.* JL47 will enhance the expression of the proPO gene before and after a pathogenic *V. campbellii* challenge. As shown in Figure

6.2-A, the relative proPO expression of the shrimp that were fed the *Bacillus* sp. JL47-enriched *Artemia* was already two-fold higher (p=0.003) before the *V. campbellii* challenge (0 h) as compared to the shrimp fed the *Artemia* without JL47 addition. After the challenge, the relative expression of the proPO gene was significantly up-regulated in all the challenged shrimp at 12 h post-challenge as compared to the non-challenged control (Figure 6.2-E) and then decreased at 24 h post-challenge. Interestingly, a significantly higher proPO expression level was observed in shrimp fed the *Bacillus* sp. JL47-enriched *Artemia* at 3 h (p<0.05) and 12 h (p<0.05) post-challenged as compared to the challenged control (Figure 6.2-B & E). When compared with the non-challenged control, the proPO mRNA level in the JL47-treated shrimp was 2 (p<0.05), 3.8 (p<0.05) and 5.1-fold (p<0.01) higher at 3, 9 and 12 h post-challenged, respectively. At 24 h post-challenge, the proPO mRNA level was reduced to almost the same level with the non-challenged and challenged control and no significant difference was observed in all treatments (Figure 6.2-F).

6.3.2 RELATIVE EXPRESSION OF TGASE GENE IN P. MONODON POSTLARVAE

The expression pattern of the TGase gene in shrimp fed the *Bacillus* sp. JL47-enriched *Artemia* was similar with the expression pattern of the proPO gene. Before the *Vibrio* challenge, TGase expression was also significantly higher (p<0.039) in JL47-treated shrimp as compared to the control shrimp (Figure 6.3-A). After the challenge, the relative expression of the TGase gene was significantly up-regulated at 9 h post-challenge in all the challenged shrimp as compared to the non-challenged control (Figure 6.3-D) and then decreased at 24 h post-challenge. Interestingly, a significantly higher TGase expression level was observed in shrimp fed the *Bacillus* sp. JL47-enriched *Artemia* at 3 h (p<0.05) and 9 h (p<0.05) post-challenged as compared to the challenged control (Figure 6.3-B & D). When compared with the non-challenged control, the TGase expression level in the JL47-treated shrimp was 1.5 (p<0.05), 2.6 (p<0.05) and 1.9-fold (p<0.05) higher at 3, 9 and 12 h post-challenge, respectively. At 24 h post-challenge, the TGase expression level showed no significant difference in all treatments (Figure 6.3-F).

6.3.3 RELATIVE EXPRESSION OF HSP70 GENE IN P. MONODON POSTLARVAE

The Hsp70 gene expression showed a different pattern from the proPO and TGase genes. Before the challenge no significant difference was observed between the JL47-treated shrimp and control shrimp (Figure 6.4-A). It was only at 3 h post-challenge that Hsp70 expression showed a significant up-regulation and the increases were observed in both the challenged JL47-treated and challenged control shrimp (Figure 6.4-B). At 6, 9, 12, and 24 h post-challenge, the Hsp70 expression level in all treatments showed no significant difference (Figure 6.4 C-F).

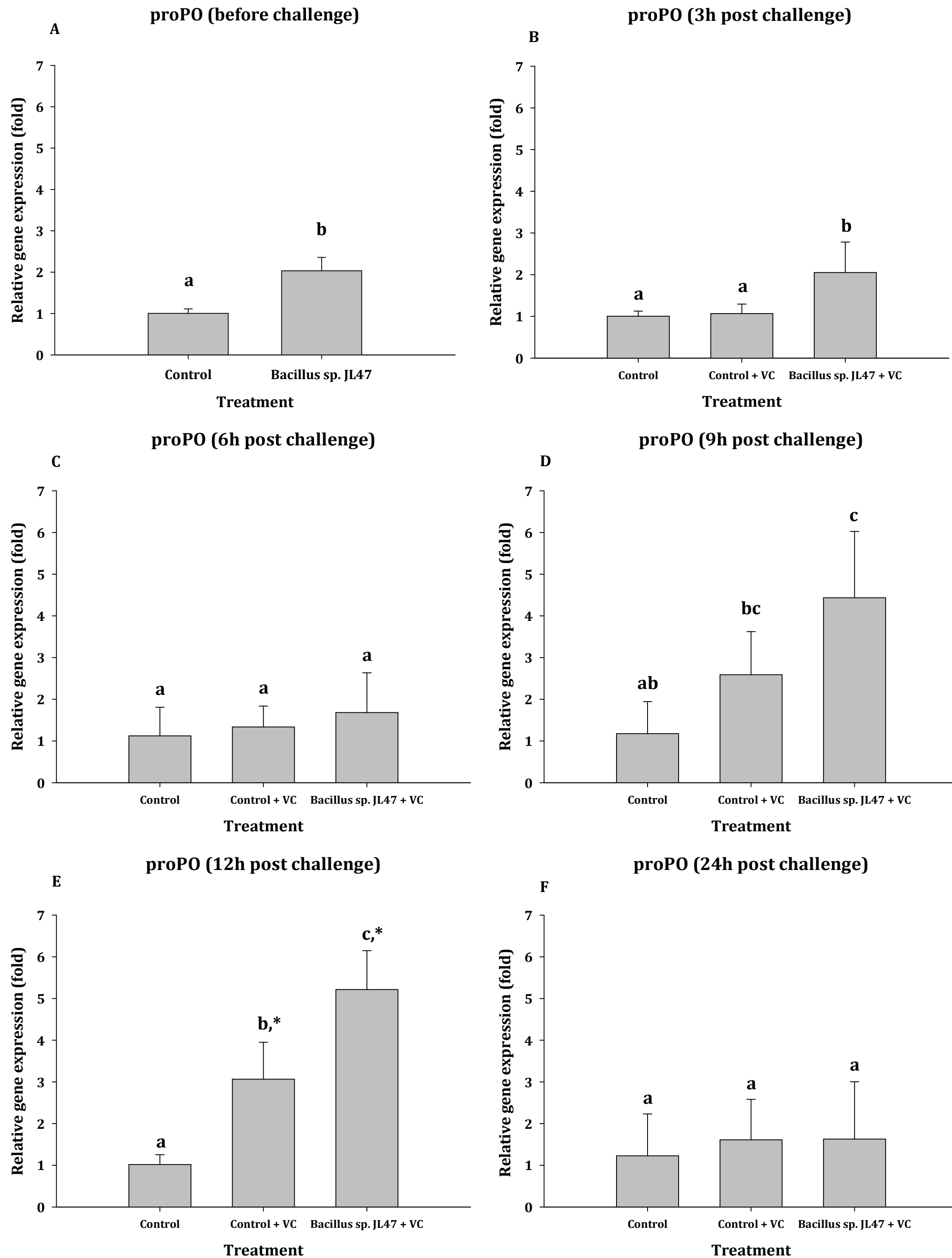

Figure 6.2 mRNA expression level of the proPO gene (relative to β-actin) in *P. monodon* postlarvae after V. campbelllii (VC) challenge. Control shrimps were fed with non-enriched *Artemia* nauplii while treated shrimps were fed with *Bacillus* sp. JL47-enriched *Artemia* nauplii. Values represent means ± S.D. (n=3). Different letters in a figure indicate significant differences at p<0.05. Asterisk (*) indicates significant difference with non-challenged control at p<0.01.

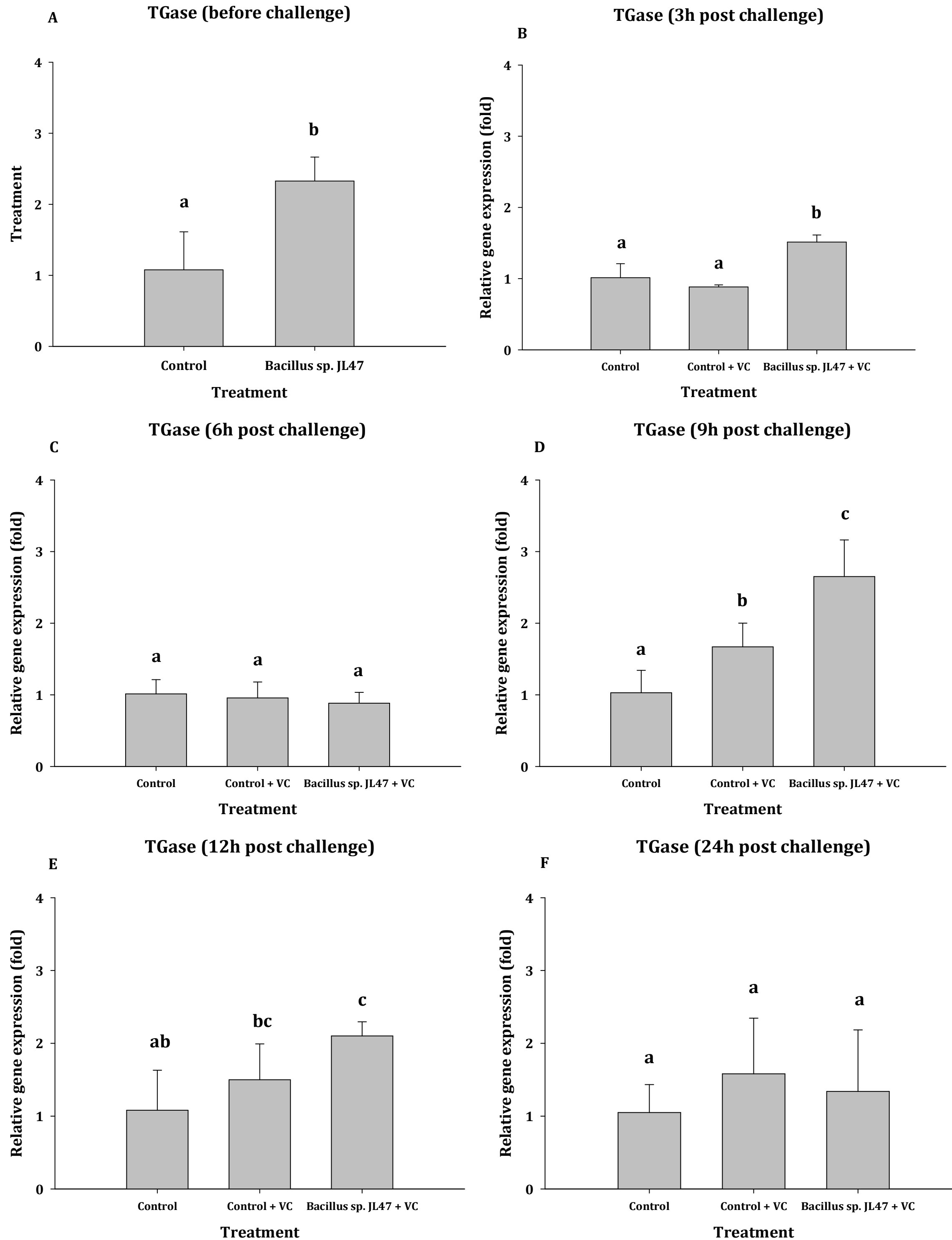

Figure 6.3 mRNA expression level of the TGase gene (relative to β-actin) in *P. monodon* postlarvae before and after V. campbellIii (VC) challenge. Control shrimps were fed with none-enriched *Artemia* nauplii while treated shrimps were fed with *Bacillus* sp. JL47-enriched *Artemia* nauplii. Values represent means ± S.D. (n=3). Different letters in a figure indicate significant differences at p<0.05.

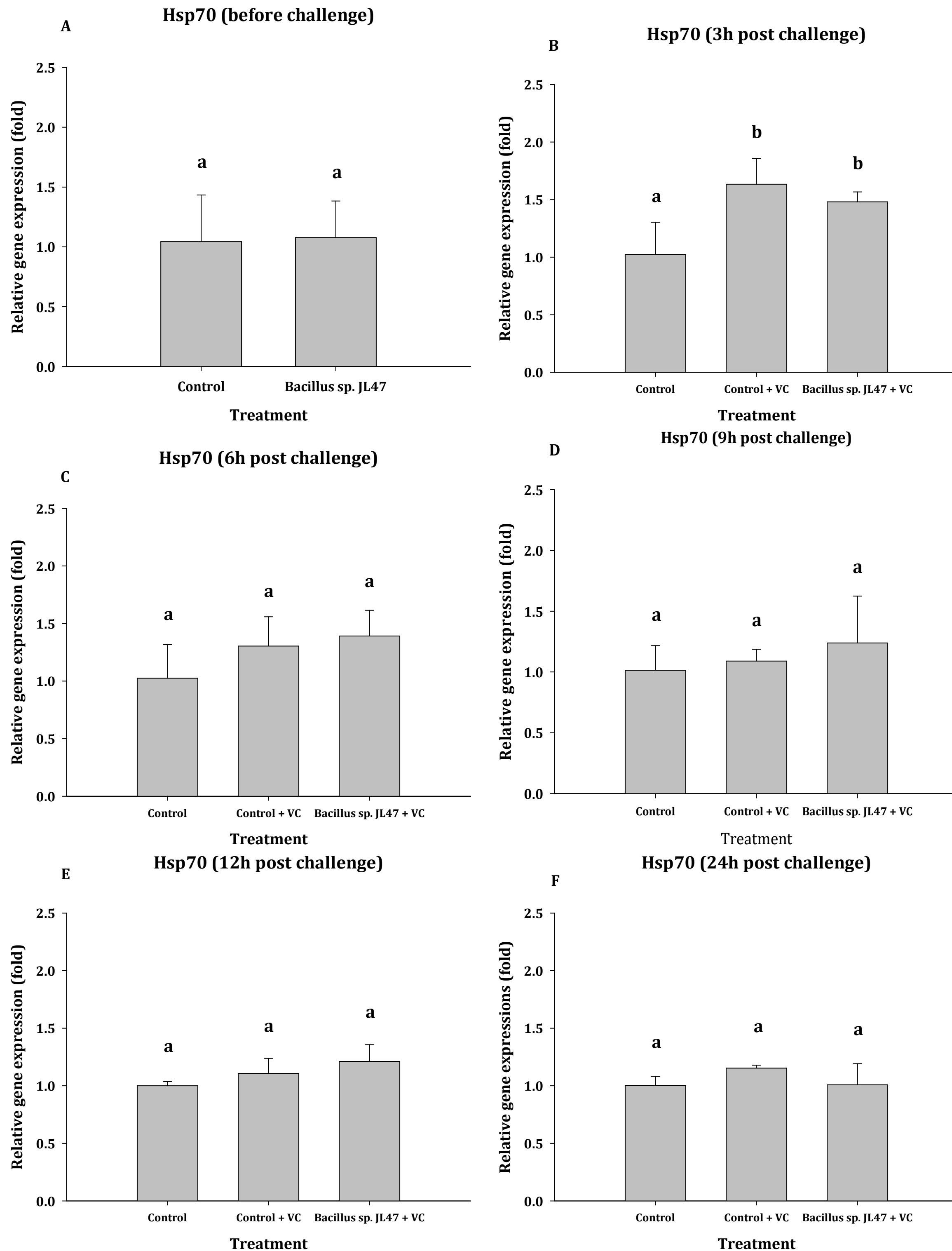

Figure 6.4 mRNA expression level of the Hsp70 gene (relative to β-actin) in *P. monodon* postlarvae before and after V. campbellliii (VC) challenge. Control shrimps were fed with non-enriched *Artemia* nauplii while treated shrimps were fed with *Bacillus* sp. JL47-enriched *Artemia* nauplii. Values represent means ± S.D. (n=3). Different letters in a figure indicate significant differences at p<0.05.

6.4 DISCUSSION

In this research, an immersion technique was used to establish a pathogenic *V. campbellii* exposure in *P. monodon* postlarvae and to study the immune response of the animals when treated with or without PHB-accumulating *Bacillus* sp. JL47. We were able to demonstrate that supplementation of PHB-accumulating *Bacillus* sp. JL47 significantly enhanced the immunity of *P. monodon* postlarvae before and after a V*. campbellii* challenge.

The proPO system plays an important role in the defense reaction of *P. monodon* through the conversion of proPO zymogen to active PO for quinone production and subsequently melanin formation (Amparyup, et al., 2013). Our data show a significant up-regulation of the proPO gene in all the challenged shrimp within 12 h of pathogenic *Vibrio* challenge. In previous reports, the expression of proPO was also up-regulated in juvenile *Litopenaeus vannamei* within 12 h of *Vibrio* species injection (Wang, et al., 2010; Yeh, et al., 2009). Taken together, the data are illustrative for the proPO system being an important immune response against *Vibrio* species. Interestingly, however, a significantly higher expression of the proPO gene was observed in *Bacillus* sp. JL47-treated shrimps as compared to the challenged shrimp that were not fed with JL47. The data suggest that feeding the *P. monodon* postlarvae with PHB-accumulating *Bacillus* sp. JL47 significantly enhances the expression of the proPO gene of the shrimp and is much higher than the induction caused by the *Vibrio* infection alone (as observed in the challenged control). The significantly higher level of proPO transcript detected in the JL47-treated shrimp suggests a stronger defense reaction of the animal against the pathogenic *Vibrio* by enhancing the shrimp's proPO system. Interestingly, a significantly higher expression of the proPO was also observed even before the animals were challenged. We suggest an immune priming effect (i.e. an increase in innate immune activity in the host after first exposure to an immunomodulating agent) by the PHB-accumulating JL47 to the proPO system of *P. monodon*.

Aside from the proPO, the expression of the TGase gene was also investigated and a pattern similar to the one of the proPO gene was observed in shrimp fed the PHB-accumulating JL47. Tgase is a key enzyme in the coagulation process in shrimp

(Maningas, et al., 2008), a conserved defense mechanism in crustaceans for preventing blood loss and controlling spread of pathogens. Our data suggests that *Bacillus* sp. JL47 enhanced this mechanism by stimulating TGase production, thus promoting a stronger protection during the *Vibrio* challenge. Interestingly, Tgase expression before the *Vibrio* challenge was also significantly higher than the unchallenged control. An explanation can be a priming effect similar to what has been described for the proPO gene.

Previous studies have highlighted the use of immunostimulants- i.e. substances that stimulate the host's immune responses- to increase the ability of the immune system of the animal to fight infections and diseases (Bricknell and Dalmo, 2005). A number of microbially-derived products such as β-glucan, peptidoglycan, lipopolysaccharide and muramyl dipeptide were reported to possess immunostimulatory properties and have been tested for its effectiveness as immunostimulants in aquaculture (Itami, et al., 1998; Kodama, et al., 1993; Olsen, et al., 2012; Takahashi, et al., 2000). Interestingly, PHB, a compound that is produced by a wide variety of microorganisms as an intracellular energy and carbon storage was suggested to be a promising immunostimulant for aquaculture. A recent report showed that supplementation of PHB-HV (the biopolymer was extracted from *Bacillus thuringiensis*) in the diet of unchallenged tilapia *Oreochromis mossambicus* resulted in the enhancement of both the specific and non-specific immune response of the fish such as enhanced antibody response, lysozyme activity, serum peroxidases activity and serum protease activity (Suguna, et al., 2014). In juvenile Pacific white shrimp *Litopenaeus vannamei*, the compound significantly induces the intestinal immune enzyme activities such as total –antioxidant capacity, inducible nitric oxide synthase and lysozyme (Duan, et al., 2017). Furthermore, in the same study the intestinal Hsp 70, Toll and immune deficiency genes were also up-regulated in the shrimp fed the diet with PHB. Our data show that the shrimp fed the PHB-containing JL47 demonstrated an enhanced expression of proPO and TGase genes before and after the *Vibrio* challenge. It is tempting to suggest that the enhanced expression of these two important immune-defense genes in *P. monodon* postlarvae was prompted by the amorphous PHB present in JL47, considering that in our previous *in vivo* experiment, the amorphous PHB appears to be the main determinant in the observed protective effects of JL47 in *Vibrio*-challenged gnotobiotic *Artemia* (chapter 4). Furthermore, the difference in the enhanced expression

of the shrimps can be (partly) related to nutritional factor considering that that the However, to clearly link immunostimulation with PHB content, further experimentation is required by comparing the immunostimulating effects of JL47 containing low and high amorphous PHB in the animal. In addition it will be necessary to completely characterize the *Bacillus* JL47, in order to establish e.g. differential expression in microbe-associated molecular patterns (MAMPs). Conversely, short chain fatty acids (SCFAs) such as butyrate have been suggested to be modulators of mammalian immunity (Meijer, et al., 2010). SCFAs have been reported to modulate the anti-inflammatory (Cox, et al., 2009), regulation of autophagy (Tang, et al., 2011), regulation of T-cell differentiation (Furusawa, et al., 2013) and stimulation of heat shock protein production (Ren, et al., 2001). Likewise, the immune binding effects of SCFA on G-protein coupled receptors such as GPR43 which are highly expressed in the neutrophils, macrophages, and monocytes in mice were also reported (Brestoff and Artis, 2013). PHB, which is a SCFA polymer, could probably be acting through its monomer form β-hydroxybutyrate in stimulating the host's immunity considering that the compound is biologically degraded into β-hydroxybutyrate or PHB oligomers in the gastrointestinal tract of organisms (Defoirdt, et al., 2007). Furthermore, since the PHB was delivered inside a bacterial cell, we cannot also disregard the possibility that other components of the bacterial cell (aside from amorphous PHB) elicited an immune response of the shrimp. *Bacillus* species are known enhancers of immunity in several cultured crustaceans (Rengpipat, et al., 2000; Tseng, et al., 2009; Zokaeifar, et al., 2012) and the suggested mechanism by which *Bacillus* species activate the immune system of the host is through the recognition of non-self-molecules (MAMPs) present in the cell wall of *Bacillus* (i.e. peptidoglycan) by appropriate recognition receptors in the host. Indeed, peptidoglycan which is present in the cell wall of Gram-positive bacteria was suggested to be an immunostimulating compound reported to enhance the immunity of several cultured shrimp species. Oral administration of peptidoglycan (PG) in *P. monodon* fed at 180 mg kg^{-1} feed resulted in enhanced PO, superoxide anion and bactericidal activity of the shrimp (Purivirojkul, et al., 2006). In another study, PG derived from the cell wall of *Bifidobacterium thermopilum* increased the expression of proPO and TGase genes, clotting protein and other immune-related genes of the kuruma shrimp *P. japonicus* (Fagutao, et al., 2008).

Another factor that was investigated in this study was Hsp70, a known molecular chaperone that plays an important role in repairing and refolding denatured proteins during heat shock stress (Morimoto, 1998). Hsps are also reported to respond to other factors such as oxidative stress, heavy metals, xenobiotic stresses and pathogen infection (Moseley, 2000). In this study, up-regulation of the Hsp70 gene could be clearly observed during the stressful condition caused by the pathogenic *Vibrio campbellii.* A similar observation was reported for juvenile *P. monodon* where Hsp70 and other Hsps were upregulated in case of a pathogenic *Vibrio harveyi* injection (Rungrassamee, et al., 2010). A recent report suggested that Hsp70 and Hsp90 mediated the immunity of the shrimp *P. vanamei* against *V. parahaemolyticus*$_{\text{AHPND}}$ strain (the causative agent of acute hepatopancreatic necrosis disease) and that this Hsp mediation resulted to the tolerance of the shrimp against the disease (Junprung, et al., 2017). Interestingly, the work of Baruah *et al.* (Baruah, et al., 2015) demonstrated that PHB enhances Hsp70 protein production in both challenged and non-challenged *Artemia* and also enhances the phenoloxidase activity in challenged *Artemia*. The study argued that PHB induced the production of Hsp70 protein and that the induction of this stress protein conferred enhanced immunity of *Artemia*. Our data show an up-regulation of Hsp70 gene at 3 h after the *Vibrio* challenge but did not clearly demonstrate that Hsp70 expression was stimulated by PHB-accumulating JL47 (as no significant increase in Hsp70 transcript was observed before the *Vibrio* challenge). In this study, Hsp70 was only measured at the mRNA level and not at the protein level, the possibility that we did not hit the timing of the Hsp70 up-regulation due to the short half-life of Hsp70 mRNA in cells (which could even be shorter in cells containing Hsp70 protein) (De Maio, 1999; Theodorakis, et al., 1999) could be possible. Indeed, the work of Baruah *et al.* (Baruah, et al., 2015) demonstrated that induction of Hsp70 by PHB in *Artemia* was clearly observed only at the protein level but not at the mRNA level. Nevertheless, the immune enhancing capability of PHB through the involvement of Hsp70 (or other Hsps) in the host necessitate further investigation.

In total, we conclude that the PHB-accumulating *Bacillus* sp. JL47 enhanced the defense system of the shrimp before and after the pathogenic *Vibrio* exposure by stimulating the innate immune response of the animal, specifically the proPO and clotting

system. The application of *Bacillus* species containing a significant amount of amorphous PHB could be a promising immunostimulating strategy for aquaculture. In this study, we only investigated the immune related genes that are responsible for controlling, eliminating the pathogen as well as for defending the host from (further) damage due to infection. However, it is also worth investigating other immune related genes that are responsible for detecting the pathogens i.e. receptor proteins (e.g. LPS receptor proteins, beta-glucan binding receptor proteins, Toll-like receptor proteins, peptidoglycan binding receptor proteins). Converesely, to clearly establish that the enhanced immunity in the shrimp is prompted by amorphous PHB, by the *Bacillus* cells alone, or its combination, further experimentation is required by comparing the immune response of the animal exposed to JL47 with different amorphous PHB level (i.e. low and high PHB content and/or with and without PHB). A complete characterization of MAMPs in JL47 with low and high PHB or with PHB and without PHB content *Bacillus* would facilitate further interpretation of the immune response. However, a non-PHB producing *Bacillus* sp. JL47 mutant strain should be established first to facilitate such experiments. Furthermore, we did not determine the size of the shrimp at the end of the experiment, leaving the possibility that differences in the growth of the animals occurred (as a consequence of *Artemia* being offered with different energy or nutritional content). The latter might lead to differences in immune responses (although all shrimps were fed *ad libitum* in all treatments). Hence, unequivocal interpretation of the current results would profit from an additional control treatment in which *Artemia* enriched with a non-PHB producing *Bacillus* sp. JL47 mutant strain would be used.

6.5 CONCLUSION

In the previous chapter (chapter 5), we demonstrated the protective effects of the PHB-accumulating *Bacillus* sp. JL47 in *P. monodon* postlarvae against pathogenic *Vibrio campbellii* LMG 21363 infection by either adding the isolate in the feed or in the water. In this study, we showed that the PHB-accumulating *Bacillus* sp. JL47 significantly enhanced the important immune defense genes of the shrimp, and more specifically, its clotting and proPO system before and after the pathogenic *Vibrio* exposure. The

application of *Bacillus* species containing significant amount of amorphous PHB could be a promising strategy to stimulate the immunity of cultured animals considering the possible synergistic effects of the amorphous PHB and the other cellular components of the probiotic *Bacillus* as immunostimulating agents. Finally, the study concludes that the protective effect of PHB-accumulating *Bacillus* sp. JL47 in shrimp was associated with its capacity to stimulate the immune system of the animal.

ACKNOWLEDGEMENT

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CHAPTER 7

MOLECULAR CLONING OF PHASIN PROTEIN FROM *BACILLUS* SP. JL47 AND ITS EFFECTS ON THE SURVIVAL OF GNOTOBIOTIC *ARTEMIA* DURING A *VIBRIO CAMPBELLII* CHALLENGE

Joseph Leopoldo Q. Laranja[1,2], Dung Viet Nguyen[1], Gladys L. Ludevese-Pascual[1], Edgar C. Amar[2], Kartik Baruah[1], Peter De Schryver[1], Peter Bossier[1]

[1]Laboratory of Aquaculture and *Artemia* Reference Center, Ghent University, 9000 Gent, Belgium
[2]Aquaculture Department, Southeast Asian Fisheries Development Center, 5021 Tigbauan, Iloilo, Philippines

ABSTRACT

The application of amorphous PHB (i.e. the polymer is still inside the bacterial cell) as a biocontrol agent has been shown to be more efficient as compared when the compound is applied in extracted form (i.e. crystalline PHB). Inside the bacterial cell, the amorphous PHB granules are mostly covered by phasin proteins. These proteins play an important structural function forming the interphase between the hydrophobic content of the PHA granules and the hydrophilic cytoplasm content. Aside from their role as part of the PHA granule cover, the proteins were also reported to have protective effects in stressed cells and demonstrated chaperone-like function in both *in vitro* and *in vivo* studies. Hence, in this study, we investigated further if the phasin protein can provide a protective effect in gnotobiotic *Artemia franciscana* during a pathogenic *Vibrio campbellii* challenge. Briefly, the gene that encodes the phasin protein from the PHB-accumulating *Bacillus* sp. JL47 was cloned and sequenced. The protein has 174 amino acids with a 20.42 kDa size. By multiple sequence alignment, the protein is 99% identical to the phasin protein found in *Bacillus cereus* and *Bacillus thuringiensis.* Furthermore, the phasin protein was overproduced in non-pathogenic *E. coli* cells by means of an arabinose induction and was fed to gnotobiotic *Artemia* challenged with pathogenic *V. campbellii*. The results showed no protective effects in the *Vibrio*-challenged *Artemia* receiving the recombinant *E. coli* containing overproduced phasin.

Keywords: phasin, PHB granule-associated protein, challenge test

7.1 INTRODUCTION

Poly-β-hydroxybutyrate (PHB) is a bacterial polymer accumulated as a cellular energy and carbon reserve by several bacteria including the *Bacillus* species. It has been suggested that this compound is a promising biocontrol agent for aquaculture. Several studies demonstrated beneficial effects of applying crystalline PHB (extracted from the bacterial cell) as a dietary additive or natural food enrichment. Crystalline PHB has been shown to improve growth, survival and robustness of some cultured species including *Artemia* nauplii, European sea bass, Chinese mitten crab and Nile tilapia (De Schryver, et al., 2010; Defoirdt, et al., 2007; Situmorang, et al., 2016; Sui, et al., 2012). However, a previous study (Halet, et al., 2007) and our study (chapter 4 and 5) suggest that the use of amorphous PHB (i.e. PHB still contained inside the bacterial cell) could be more efficient than crystalline PHB. This could be due to the difference in particle size between the amorphous PHB (< 0.5 μm inside the bacterial cell) and the crystalline PHB (~ 30 μm). This difference in size might e.g. affect depolymerisation speed. Alternatively, uncharacterized bacterial components might be present in the bacterial carrier containing the amorphous PHB and these components may have contributed to the beneficial effects mediated amorphous PHB application.

Inside the bacterial cell, the surface of the PHB granules are covered mostly, if not completely by PHB granule-associated proteins (PGAPs) (Jendrossek and Pfeiffer, 2014). Among these PGAPs, the phasin proteins are considered to be the major component covering most of the PHA granules. These proteins play an important structural function forming the interphase between the hydrophobic content of the PHA granules and the hydrophilic cytoplasm content (Pötter and Steinbüchel, 2006). Aside from their function as granule structural proteins, several studies also demonstrated the important role of phasins in PHA synthesis (York, et al., 2001), PHA degradation (Kuchta, et al., 2007) as well as their effects on PHA granule formation (i.e. size and number) and distribution inside the bacterial cell (Wieczorek, et al., 1995). Interestingly, a previous report showed that the presence of recombinant phasin ($PhaP_{Az}$; the protein was cloned from *Azotobacter* sp. FA-8) in *E. coli* cells reduced the expression of stress-related genes such as *ibpA and dnaK* and that the phasin protein protected the bacteria against heat

shock and superoxide stress by paraquat (de Almeida, et al., 2011). In another study, both *in vitro* and *in vivo* experiments demonstrated chaperone-like functions of $PhaP_{AZ}$ in recombinant *E. coli*. Taken together, the results from the different studies suggest that phasins may have general protective properties against stresses (de Almeida, et al., 2011; Mezzina, et al., 2015).

In chapter 4, we suggested that amorphous PHB was the main determinant in the observed protective effects of the PHB-accumulating *Bacillus* sp. JL47 in *Artemia*. Hence, it is worth investigating the possible contribution of phasin, being a surface protein covering the PHB granule, to the protective effects displayed by amorphous PHB . In this study, we investigated the effects of phasin in gnotobiotic *Artemia* exposed to bacterial infection. Briefly, the gene that encodes the phasin protein from the PHB-accumulating *Bacillus* sp. JL47 was cloned and the recombinant phasin protein was subsequently overproduced in non-pathogenic *E. coli* cells. Phasin overproducing *E. coli* cells was then fed to *Artemia* and challenged with pathogenic *V. campbellii*. The survival of *Artemia* was determined.

7.2 MATERIALS AND METHODS

7.2.1 BACTERIA STRAIN AND GROWTH CONDITIONS

Bacillus sp. JL47 was grown in LB agar and subsequently inoculated in LB broth and grown at 30 °C and 100 rpm agitation for 24 h.

For the *in vivo* challenge test, the pathogenic *Vibrio campbellii* LMG 21363 was grown following the procedures described in chapter 4. *Aeromonas hydrophila* LVS3 was prepared following the methods described in chapter 4.

7.2.2 BACTERIAL DNA PREPARATION, PCR AMPLIFICATION AND CLONING OF THE BACILLUS SP. JL47 PHASIN GENE

A 100 µL of the *Bacillus* sp. JL47 culture sample was centrifuged at 4000 x g for 5 min and the culture medium was removed by careful pipetting. 100 µL lysing buffer (0.025 N NaOH; 0.0125 % sodium dodecyl sulfate) was then added to the bacterial pellet and

was mixed by slow pipetting. The mixture was heated at 90°C in a water bath for 5 min and was put back on ice and precipitated by centrifugation. The mixture was prepared as DNA template for the amplification of the phasin gene in JL47.

The phasin gene of *Bacillus* sp. JL47 was amplified by polymerase chain reaction (PCR) and cloned into the TOPO® cloning vector using a pBAD Directional TOPO® Expression kit (InvitrogenTM, Merelbeke, Belgium) according to manufacturer's recommendations. The PCR reaction was performed in a 50 µL reaction mixture containing 34 µL nuclease-free water, 10 µL of 5x Phusion® HF buffer, 1 µL of 10 mM dNTP mix, 1 µL of each primers (25 µM): Phasinforward 5'-CACCATGGAAACTAAACCATACGAATTAGTC-3'; Phasinreverse 5'-CTTGATGGAAGTAAATAGATTTTTTGAGTTTTCCTC-3', 2 µL bacterial template and 1 µL Phusion® polymerase (Thermo Fisher Scientific, Belgium) mixed in this order. PCR conditions were set as follows: initial denaturation at 98°C for 30 sec followed by 35 cycles of 98°C for 15 sec (denaturation), 65°C for 20 sec (annealing) and 72°C for 30 sec (extension) followed by 72°C for 10 min. The amplification of the appropriate 522 base pairs (bp) fragment of the phasin gene was verified by electrophoresis (Figure 7.1-a). Prior to the ligation step, the PCR product was cleaned using a Wizard® SV Gel and PCR Clean-up system (Promega, Begium) according to the manufacturer's recommendation. It was then ligated into the TOPO cloning vector and transformed into One Shot TOP10 (non-pathogenic *E. coli*) cells, which were grown on LB agar containing 50 µg mL-1 Kanamycin at 37°C. Grown colonies were picked individually and were then verified if the gene was inserted successfully by performing an electrophoresis for each bacterial colony using the following primers: TrxFusforward 5'- TTCCTCGACGCTAACCTG-3´ (forward primer provided in the kit that allows the amplification of the insert in the sense orientation) and Phasinreverse 5'-CTTGATGGAAGTAAATAGATTTTTTGAGTTTTCCTC-3' (the reverse primer used in targeting the phasin gene). Successful *E. coli* transformants containing the phasin gene from *Bacillus* sp. JL47 (Figure 7.1-b) were labelled as *E. coli* JL47 PhaP and were kept at -80°C in 20% glycerol as stock cultures.

7.2.3 PHASIN GENE SEQUENCING AND ANALYSES

For the gene sequencing analysis, the recombinant *E. coli* $JL47_{PhaP}$ strain was grown in LB broth containing 50 µg mL^{-1} Kanamycin for 24 h at 37°C. Bacterial plasmid was purified using Wizard® Plus SVMinipreps DNA Purification System (Promega, Belgium) according to manufacturer's instruction. The purified plasmid was supplemented either with the $TrxFus_{forward}$ primer (5'- TTCCTCGACGCTAACCTG-3´) or the $pBAD_{reverse}$ primer (5'-GATTTAATCTGTATCAGG-3') for sequencing and the plasmid sample was submitted to LGC Genomics GmbH (Berlin, Germany) for sequencing. The sequence was assembled using Vector NTI 10.3.0 software (Invitrogen Corporation) and confirmed if the phasin gene was in frame with the N-terminal His-Patch thioredoxin peptide and the C-terminal V5 epitope and polyhistidine (6xHis) tag). The inserted sequence was obtained and the amino acid translation of the sequence as well as the molecular weight was determined using ApE software (A plasmid Editor V2.0.47) (USA). Homology searches were conducted with the BLAST server of the National Center for Biotechnology Information for comparison of the amino acid query sequence against an amino acid sequence database (blastp).

Homology comparison between the recombinant phasin from *Bacillus* sp. JL47 ($PhaP_{JL47}$) and the phasins from other *Bacillus* species was also determined using Clustal Omega multiple-alignment method (Sievers, et al., 2011) (Figure 7.4-B & C).

7.2.4 INDUCTION OF PHASIN PROTEIN IN E. COLI $JL47_{PHAP}$ STRAIN

The recombinant *E. coli* $JL47_{PhaP}$ strain was grown at 37°C for 24 h on LB agar with 50 µg mL^{-1} Kanamycin and subsequently cultured in LB broth with 50 µg mL^{-1} Kanamycin to log phase. Overproduction of the phasin protein in *E. coli* $JL47_{PhaP}$ was stimulated by adding different doses of L-arabinose (0 (non-induced), 0.2, 2, 4%) for a fixed time of 4 h.

7.2.5 SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) ANALYSIS

The induced phasin protein in *E. coli* JL47$_{PhaP}$ strain was further resolved by SDS-PAGE. Briefly, 200 µL of the bacterial culture (OD_{550}=0.7) was centrifuged at 4,000 x *g* for 10 min and the supernatant was removed. The bacterial pellet was resuspended in 50 µL demi water and 50 µL SDS sample buffer (containing 95% (v/v) Laemmli sample buffer (Bio-Rad Laboratories, Inc., USA) and 5% (v/v) Betamercaptoethanol (Bio-Rad Laboratories, Inc., USA)) and was mixed carefully by slow pipetting. The sample mixture was heated at 95°C for 5 min and was spun down. 10 µL of the sample mixture was carefully loaded to each lane of the polyacrylamide gel using Mini-PROTEAN® TGX^{TM} PrecastGel (Bio-Rad Laboratories, Inc., USA) installed in a tank with ~ 700 mL 5x Tris electrophoresis buffer (Tris, 15 g L^{-1}; glycine, 72 g L^{-1}; SDS, 5 g L^{-1}) . The electrophoresis was run at 90V for 15 min and 150V for 45 min. Subsequently, the gel was then slowly removed and stained with Coomassie Biosafe (BioRad Laboratories, Inc., USA) for 1 h. The gel was de-stained by washing 3x for 15 min with demi-water. Bands were documented on a white background using a ChemiDoc^{TM} MP Imaging System (Bio-Rad Laboratories, Inc., USA). Based on qualitative observation of the band thickness, 4% arabinose was used in overproducing phasin protein in *E. coli* for the *in vivo* challenge test in *Artemia* (Figure 7.5).

7.2.6 AXENIC HATCHING OF ARTEMIA FRANCISCANA AND IN VIVO CHALLENGE TEST

Axenic hatching of *Artemia* as well as the challenge test were carried following the procedures described in chapter 4. During the *in vivo* challenge the gnotobiotic *Artemia* were fed with either of the following: LVS3 (control), arabinose-induced *E. coli* containing overproduced phasin protein (treatment 2) and non-induced *E. coli* JL47$_{Phap}$ (treatment 3). In all treatments ~10^7 cells mL^{-1} were supplemented Both the arabinose-induced (4% arabinose) and non-induced (0% arabinose) *E. coli* cells were prepared by culturing the *E. coli* JL47$_{PhaP}$ following the procedures described above. The non-induced and the arabinose-induced *E. coli* JL47$_{PhaP}$ cells were harvested by centrifugation at 4,000 *x g* for 5 min, discarding the supernatant and washing once with sterile seawater and finally re-suspended in sterile seawater prior to feeding. The *Artemia* were challenged with

pathogenic *V. campbellii* at 10^6 cells mL^{-1} 2, 6 and 24 h after receiving the treatments. Survival of the *Artemia* was determined 48 h after exposure.

7.3 RESULTS AND DISCUSSIONS

7.3.1 SEQUENCE ANALYSIS OF PHASIN IN BACILLUS SP. JL47

The cloned phasin gene has 522 bp with 174 deduced amino acids (aa) (Figure 7.2). The phasin protein has a calculated molecular mass of 20.42 kDa. Based on homology searches using standard protein BLAST, the amino acid sequence of the $PhaP_{\textit{Bacillus}spJL47}$ showed a high degree of identity to the phasin protein found in *Bacillus cereus* (WP_000448576.1) and *Bacillus thuringiensis* (WP_000448588.1) with a 99% identical sequence (Figure 7.3 & Figure 7.4-A). The amino acid sequence of $PhaP_{\textit{Bacillus}spJL47}$ was also compared to other phasins found in other *Bacillus* species and the results showed different levels of identity for these phasins ranging from 98%-27% (Figure 7.3 & Figure 7.4). Phasin found in *Bacillus megaterium, Bacillus halodurans and Bacillus azotoformans* showed the lowest level of identity to $PhaP_{\textit{Bacillus}spJL47}$. Furthermore, comparing the amino acid sequence of $PhaP_{\textit{Bacillus}spJL47}$ to other phasins outside the *Bacillus* genus such as those from *R. eutropha* revealed no significant similarity (data comparison not shown). Indeed, it has been reported that phasins do not constitute a highly conserved group of proteins. Based on the Pfam database (Bateman, et al., 2004), there are four reported phasin related families, each containing a specific domain. The PF090602 (PhaP_Bmeg) family corresponds to phasins found in *Bacillus* species, such as those in *B. megaterium*, while PF09361 (Phasin_2), belongs to phasins found in Alpha, Beta and Gamma proteobacteria, such as the most studied phasin (PhaP1) from *R. eutropha*. The third family (PF09650; PHA_gran_rgn) contains a diverse group of (structurally) uncharacterized proteins belonging to different proteobacteria and the last family (PF05597; Phasin) contains proteins from different proteobacteria, including all characterized phasins belonging to the *Pseudomonas* species that accumulate medium-chain length PHAs, such as PhaF and PhaI from *P. putida* (Mezzina and Pettinari, 2016).

7.3.2 INDUCTION OF PHASIN PROTEIN IN E. COLI JL47$_{PHAP}$ STRAIN

The protein was induced using different levels of L-arabinose at a fixed induction time of 4 h. The size of the recombinant protein was increased to 36.42 kDa from the expected size of the phasin which is 20.42 kDa due to the integration of thioredoxin in the N-terminal tag (13 kDa) and polyhistidine (6xHis) in the C-terminal tag (3 kDa) in the TOPO® cloning vector (Figure 7.5). Furthermore, by visual comparison of the bands using different dose of L-arabinose showed a stronger band at 2 and 4% as compared with 0.2% arabinose (Figure 7.5). Thus, in the in vivo challenge experiment, 4% L-arabinose was used in overproducing the phasin protein in the recombinant *E. coli* strain.

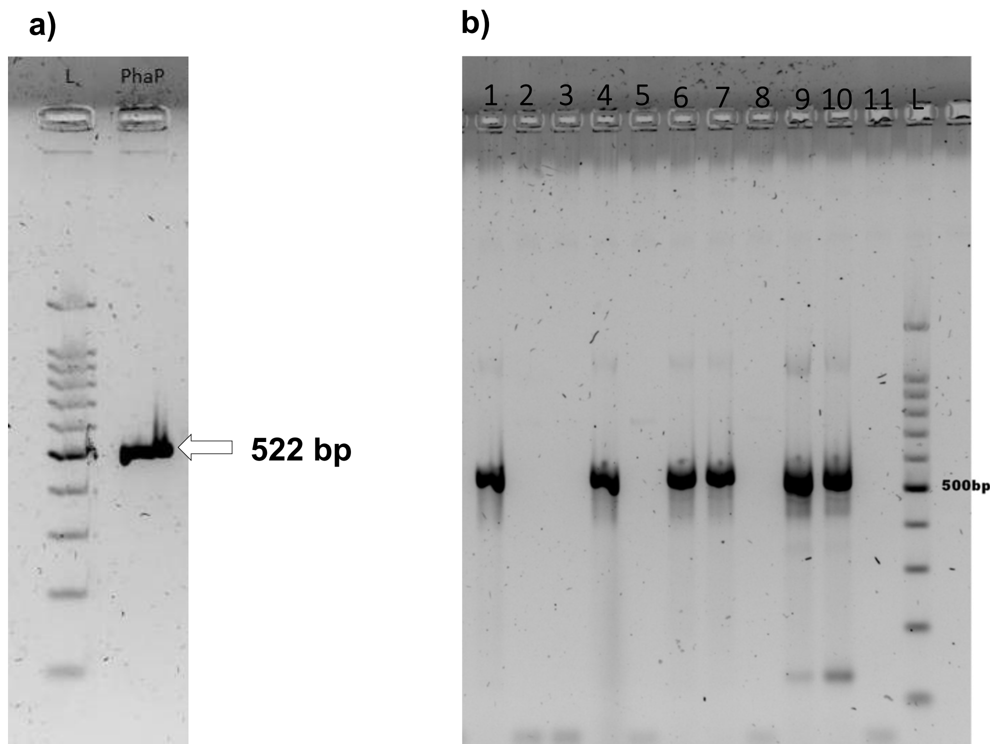

Figure 7.1 Agarose gel electrophoresis of the PCR amplified phasin gene of (a) *Bacillus* sp. JL47. L- ladder; PhaP- phasin gene; (b) Lanes 1,4 6, 7, 9 & 10 are successful *E. coli* transformants showing the amplified recombinant phasin gene; lanes 2, 3, 5 8, 11 are unsuccessful *E. coli* transformants showing no amplified phasin gene.

```
  1   M  E  T  K  P  Y  E  L  V  D  A  F  W  K  N  W  S  Q  S  L
  1   ATGGAAACTAAACCATACGAATTAGTCGATGCATTTTGGAAAAACTGGTCTCAATCTCTT
 21   S  L  F  S  S  A  G  K  Q  L  E  Q  L  T  L  E  T  L  K  Q
 61   TCCCTTTTCTCTTCAGCTGGGAAACAATTAGAGCAACTTACTTTAGAAACATTAAAACAA
 41   Q  Q  D  A  L  H  K  L  T  S  G  V  D  E  L  E  K  E  L  Q
121   CAACAAGACGCTTTGCATAAATTAACATCAGGAGTAGATGAACTGGAAAAAGAACTGCAA
 61   Q  F  T  T  Q  F  N  N  Q  Y  T  D  Y  V  K  Q  L  T  G  N
181   CAATTTACTACTCAATTCAATAATCAATATACAGATTACGTGAAGCAATTAACTGGAAAC
 81   S  L  N  D  Q  I  N  E  W  Q  D  K  W  K  E  L  S  A  H  M
241   TCCTTAAATGATCAAATTAACGAGTGGCAAGACAAGTGGAAAGAACTTTCTGCTCATATG
101   Q  Q  L  T  V  S  P  T  K  T  S  L  S  I  L  T  Q  T  S  G
301   CAACAGCTAACTGTTTCTCCTACAAAAACATCTTTGTCTATCCTTACTCAAACAAGCGGT
121   Q  F  E  E  T  T  K  Q  F  I  E  Q  Q  Q  L  Q  R  E  E  A
361   CAATTTGAAGAAACAACGAAGCAATTTATTGAACAACAACAATTACAACGTGAAGAGGCT
141   Q  K  Q  L  E  G  F  L  E  D  F  K  T  K  Q  L  E  L  V  K
421   CAAAAACAGTTAGAAGGTTTTTTGGAAGATTTCAAGACAAAACAGTTGGAACTCGTAAAA
161   K  F  E  E  N  S  K  N  L  F  T  S  I  K
481   AAGTTCGAGGAAAACTCAAAAAATCTATTTACTTCCATCAAG
```

Figure 7.2 Nucleotide (black; 522 bp) and deduced amino acid (blue; 174 aa) sequences of PhaP from *Bacillus* sp. JL47.

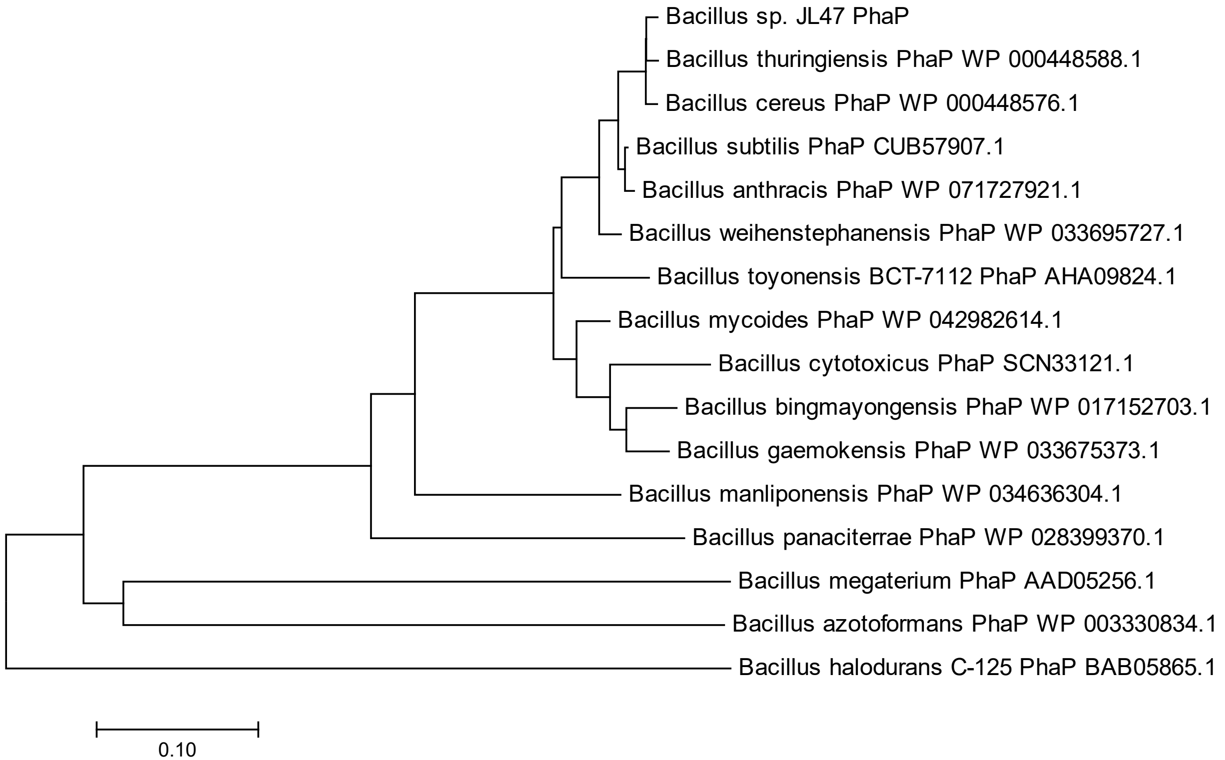

Figure 7.3 Phylogenetic tree of phasin protein found in different *Bacillus* species including the phasin protein found in *Bacillus* sp. JL47. The tree was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000) and are in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA7 (Kumar, et al., 2016).

A

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Bacillus_sp._JL47_PhaP	340	340	100%	2e-125	100%	Query_80682
☐	Bacillus_cereus_PhaP_WP_000448576.1	337	337	100%	2e-124	99%	Query_80683
☐	Bacillus_thuringiensis_PhaP_WP_000448588.1_	337	337	100%	4e-124	99%	Query_80684
☐	Bacillus_subtilis_PhaP_CUB57907.1	332	332	100%	3e-122	98%	Query_80685
☐	Bacillus_anthracis_PhaP_WP_071727921.1	330	330	100%	3e-121	97%	Query_80686
☐	Bacillus_weihenstephanensis_PhaP_WP_033695727.1	326	326	99%	8e-120	96%	Query_80687
☐	Bacillus_mycoides_PhaP_WP_042982614.1	311	311	100%	8e-114	91%	Query_80688
☐	Bacillus_toyonensis_BCT-7112_PhaP_AHA09824.1	306	306	100%	4e-112	91%	Query_80690
☐	Bacillus_bingmayongensis_PhaP_WP_017152703.1	307	307	100%	3e-112	89%	Query_80689
☐	Bacillus_gaemokensis_PhaP_WP_033675373.1	300	300	100%	1e-109	87%	Query_80691
☐	Bacillus_cytotoxicus_PhaP_SCN33121.1	298	298	100%	1e-108	87%	Query_80692
☐	Bacillus_manliponensis_PhaP_WP_034636304.1	250	250	100%	5e-90	75%	Query_80693
☐	Bacillus_panaciterrae_PhaP_WP_028399370.1	229	229	100%	2e-81	63%	Query_80694
☐	Bacillus_halodurans_C-125_PhaP_BAB05865.1	18.5	52.0	62%	0.70	34%	Query_80697
☐	Bacillus_megaterium_PhaP_AAD05256.1	68.9	85.9	95%	8e-19	27%	Query_80695
☐	Bacillus_azotoformans_PhaP_WP_003330834.1	59.3	59.3	90%	5e-15	27%	Query_80696

B

```
Bacillus_panaciterrae_PhaP_WP_028399370.1           MENKPYEMVESFWSNWSHSLSLLTSAGKQMEQFTLETMKQQQEAFHKLTEGMEAMEQEIK    60
Bacillus_manliponensis_PhaP_WP_034636304.1          METKPYELVDAFWKNWSNSLSLFTSAGKQFEELTLETMKQQQEALNKLTEGMDEFEKEIQ    60
Bacillus_cytotoxicus_PhaP_SCN33121.1                METKPYELIDAFWKNWSHSLSLFSSAGKQLEQLTLEILKQQQDALHKLTAGVDELEKELQ    60
Bacillus_bingmayongensis_PhaP_WP_017152703.1        METKPYELVDAFWKNWSHSLSLFSSAGKQLEQLTLETLKQQQDALHKLTAGVDELEKELQ    60
Bacillus_gaemokensis_PhaP_WP_033675373.1            METKPYEIVDAFWKNWSHSLSLFSSAGKQLEQLTLETLKQQQDALHKLTAGADELEKELQ    60
Bacillus_toyonensis_BCT-7112_PhaP_AHA09824.1        METKPYELVDAFWKNWSQSLSLFSSAGKQLEQLTLETLKQQQDALHKLTSGVNELEKELQ    60
Bacillus_mycoides_PhaP_WP_042982614.1               METKPYELVDAFWKNWSQSLSLFSSAGKQLEQLTLETLKQQQDALHKLTAGVDELEKELQ    60
Bacillus_weihenstephanensis_PhaP_WP_033695727.1     METKPYELVDAFWKNWSQSLSLFSSAGKQLEQLTLETLKQQQDALHKLTSGVDELEKELQ    60
Bacillus_sp._JL47_PhaP                              METKPYELVDAFWKNWSQSLSLFSSAGKQLEQLTLETLKQQQDALHKLTSGVDELEKELQ    60
Bacillus_cereus_PhaP_WP_000448576.1                 METKPYELVDAFWKNWSQSLSLFSSAGKQLEQLTLETLKQQQDALHKLTAGVDELEKELQ    60
Bacillus_thuringiensis_PhaP_WP_000448588.1_         METKPYELVDAFWKNWSQSLSLFSSAGKQLEQLTLETLKQQQDALHKLTSGVDELEKELQ    60
Bacillus_subtilis_PhaP_CUB57907.1                   METKPYELVDAFWKNWSQSLSLFSSAGKQLEQLTLETLKQQQDALHKLTSGVDELEKELQ    60
Bacillus_anthracis_PhaP_WP_071727921.1              METKPYELVDAFWKNWSQSLSLFSSAGKQLEQLTLETLKQQQDALHKLTSGVDELEKELQ    60
                                                    **.****:::.**.***.****::*****.*::***  :****.*::*** * : :*:*::

Bacillus_panaciterrae_PhaP_WP_028399370.1           QYASQMSTQYTDYMKQLTGGQFSAQIDEWQEKWNDLSQQMQQASVSPAKASLSVLSQTSG    120
Bacillus_manliponensis_PhaP_WP_034636304.1          QFNNQVNNQYADYMKQFAGNSLNKQIDEWQSKWTEISSQMHQLTVSPSKTSLTLLTQTSG    120
Bacillus_cytotoxicus_PhaP_SCN33121.1                QFTAQWNSQYTEYVNQLTGNSLNDQIHEWQEKWNELSNHIHQLTVSPTKTSLSILTQTSG    120
Bacillus_bingmayongensis_PhaP_WP_017152703.1        QFTAQFNSQYTEYVKQFTGNSLNEQINEWNEKWNELSNHIHQLTVSPTKTSLSLLTQTSG    120
Bacillus_gaemokensis_PhaP_WP_033675373.1            QFTAQLNSQYTEYVKQFTGNALNEQINEWQGKWNELSNHIHQLTVSPTKTSLSILTQTSG    120
Bacillus_toyonensis_BCT-7112_PhaP_AHA09824.1        QFTAQFNNQYTDYVKQLTGNSLNDQINEWQGKWNELSTQMHQLTVSPTKTSLSILTQTSG    120
Bacillus_mycoides_PhaP_WP_042982614.1               QFTAQLNSQYTDYVKQYTGNSLNDQINEWQEKWNELSNHIHQLTVSPTKTSLSILTQTSG    120
Bacillus_weihenstephanensis_PhaP_WP_033695727.1     QLTAQFNNQYTDYVKQLTGNSLNDQINEWQHKWKELSAHMHQLTVSPTKTSLSILTQTSG    120
Bacillus_sp._JL47_PhaP                              QFTTQFNNQYTDYVKQLTGNSLNDQINEWQDKWKELSAHMQQLTVSPTKTSLSILTQTSG    120
Bacillus_cereus_PhaP_WP_000448576.1                 QFTTQFNNQYTDYVKQLTGNSLNDQINEWQDKWKELSAHMQQLTVSPTKTSLSILTQTSG    120
Bacillus_thuringiensis_PhaP_WP_000448588.1_         QFTTQFNNQYTDYVKQLTGNSLNDQINEWQDKWKEFSAHMQQLTVSPTKTSLSILTQTSG    120
Bacillus_subtilis_PhaP_CUB57907.1                   QFTTQFNNQYTDYVKQLTGNSLNDQINEWQEKWKELSAHMQQLTVSPTKTSLSILTQTSG    120
Bacillus_anthracis_PhaP_WP_071727921.1              QFTAQFNNQYTDYVKQLAGNSLNDQINEWQEKWKELSAHMQQLTVSPTKTSLSILTQTSG    120
                                                    *    * ..**::*::* :*. :. **.**: **.::* :::* :***:*:**::*:****

Bacillus_panaciterrae_PhaP_WP_028399370.1           QFEETLKQFISQHQQQREEVQKQMENFLQELKSMQLDLVKKVEESSKHVFPFAK    174
Bacillus_manliponensis_PhaP_WP_034636304.1          QFEEAIRQMITQQQSQREEVQKQMESFLEEFKSMQLDLVKKFEENSKNLFTSMK    174
Bacillus_cytotoxicus_PhaP_SCN33121.1                QFEETTKQFIEQHQLQREELQKQLDDFLMEFKSTQLELVKKFEENSKNLFTSIK    174
Bacillus_bingmayongensis_PhaP_WP_017152703.1        QFEETTKQFIEQQQIQRDEVQKQLEGFLEEFKSTQLELVKKFEENSKNLFTSIK    174
Bacillus_gaemokensis_PhaP_WP_033675373.1            QFEETTKQFIEQQQLQRDEVQKQLESFLEEFKSTQLELAKKFEENSKNLFTSIK    174
Bacillus_toyonensis_BCT-7112_PhaP_AHA09824.1        QFEETTKHFIEQQQSQREEVQKQLEVFLGEFKSKQLELAKQFEENSKNLFTSIK    174
Bacillus_mycoides_PhaP_WP_042982614.1               QFGETTKQFIEQQQLQREEAQKQLEVFLEEFKSKQLELAKKFEENSKNLFTSIK    174
Bacillus_weihenstephanensis_PhaP_WP_033695727.1     QFEETTKQFIEQQQLQREEAQKQLEGFLEEFKSKQLELAKKFEENSKNLFTSIN    174
Bacillus_sp._JL47_PhaP                              QFEETTKQFIEQQQLQREEAQKQLEGFLEDFKTKQLELVKKFEENSKNLFTSIK    174
Bacillus_cereus_PhaP_WP_000448576.1                 QFEETTKQFIEQQQLQREEAQKQLEGFLEDFKTKQLELAKKFEENSKNLFTSIK    174
Bacillus_thuringiensis_PhaP_WP_000448588.1_         QFEETTKQFIEQQQLQREEAQKQLEGFLEDFKTKQLELAKKFEENSKNLFTSIK    174
Bacillus_subtilis_PhaP_CUB57907.1                   QFEETTKQFIEQQQLQREEAQKQLEGFLEEFKLKQLELAKKFEENSKNLFTSIK    174
Bacillus_anthracis_PhaP_WP_071727921.1              QFEETTKQFIEQQQLQREEAQKQLEGFLEEFKLKQLELAKKFEENSKNLFTSIK    174
                                                    ** *: :::* *:* **:* ***:: ** ::*  **:*.*:.**.**::*    :
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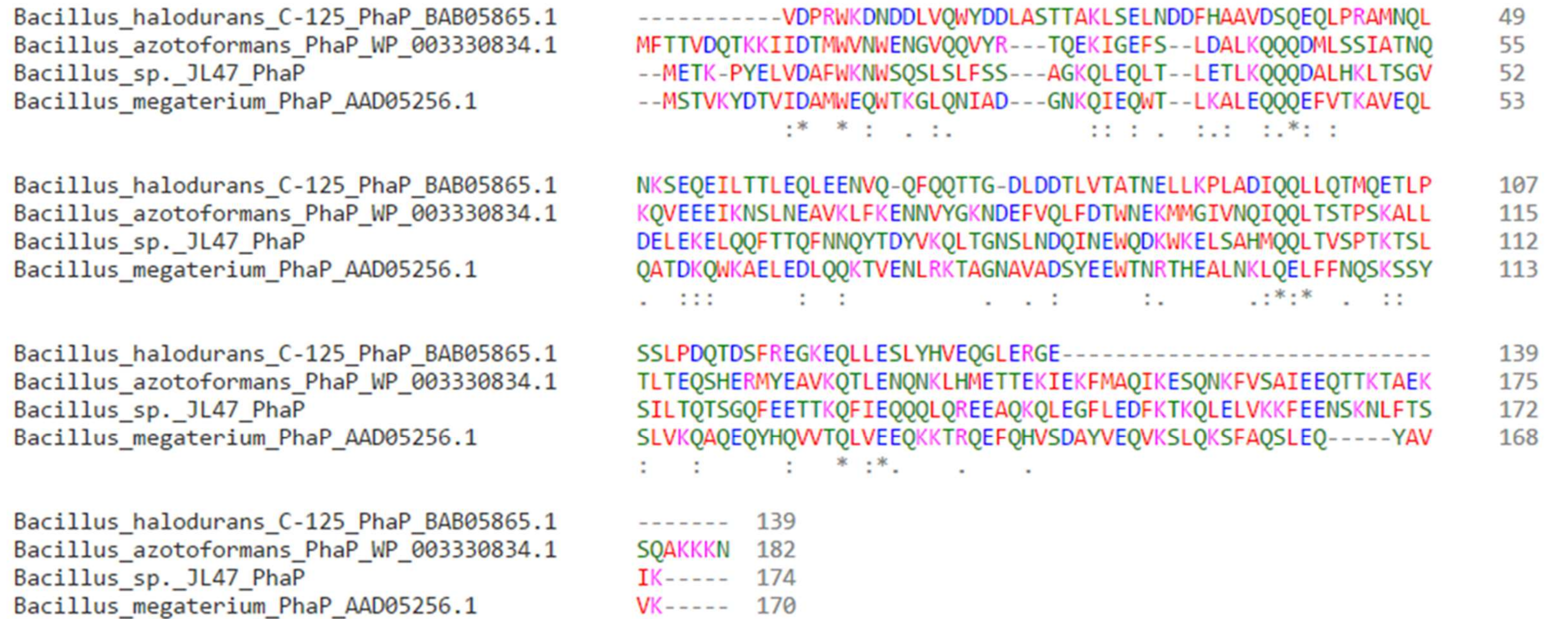


Figure 7.4 (A) Sequence comparison of the phasin (PhaP) protein from *Bacillus* sp. JL47 and the phasin protein from different *Bacillus* species. (B) Phasin sequences that are 99-63% identical to PhaP from *Bacillus* sp. JL47; (C) Phasin sequences that are 34-27% identical to PhaP from *Bacillus* sp. JL47. Identical (*) and similar (. and :) residues identified by the Clustal Omega program are indicated. Missing amino acids are marked by dashes.

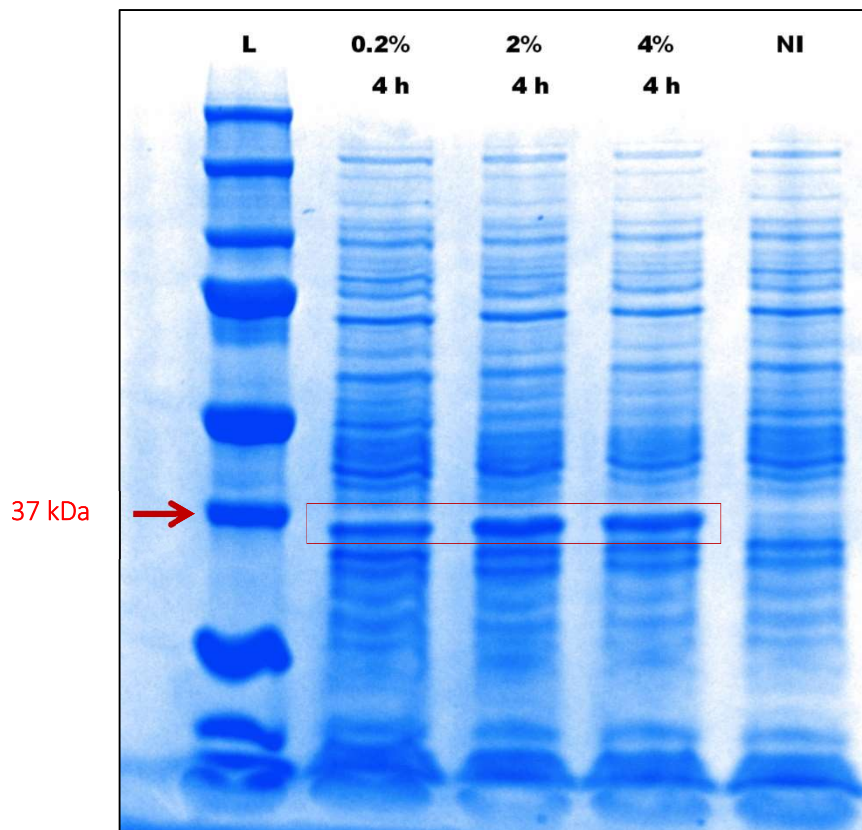
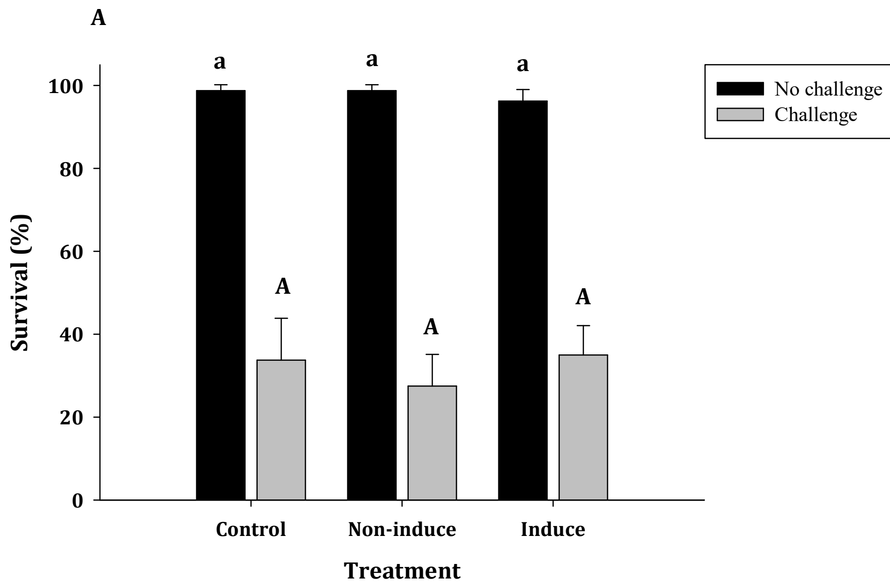


Figure 7.5 Overproduction of the phasin protein in *E. coli* JL47$_{PhaP}$ using different doses of L-arabinose at 0 (non-induced), 0.2, 2 and 4%. Protein extract from the *E. coli* JL47$_{PhaP}$ induced with L-arabinose was resolved by SDS-PAGE gel stained with Coomassie Biosafe. The total size of the recombinant protein is 36.42 kDa (20.42 kDA phasin, 13 kDa N-terminal Thioredoxin tag and 3kDa C-terminal polyhistidine tag). L= ladder; NI= non-induced

7.3.3 In vivo challenge test

In this experiment, we investigated if the phasin protein can protect the *Artemia* against pathogenic *Vibrio campbellii* infection. The recombinant *E. coli* JL47$_{PhaP}$ strain containing the overproduced phasin protein was fed to the gnotobiotically-cultured *Artemia franciscana* nauplii and subsequently challenged with pathogenic *V. campbellii* 2 h, 6 h and 24 h after feeding. In all the challenge experiments, none of the treatments showed a protection (Figure 7.6). Hence, our data suggest that the phasin protein cannot provide protection in *Artemia* during *Vibrio* infection. Hence it can also be concluded that the protein did not contribute to the protective effects displayed by amorphous PHB in the

previous experiments conducted in *Artemia* nauplii (chapter 4) and *P. monodon* postlarvae (Laranja, et al., 2014). Nevertheless, another approach could also be conducted to clearly elucidate the possible contribution of phasin in the protective effect of PHB and that is to purify the protein and then artificially bind/coat the protein to the surface of the extracted PHB granules. It could then be verified if PHB coated/bound with phasin would display a better activity than with crystalline PHB only. Indeed, inside the bacterial cell, phasins were reported to have important roles in the degradation as well as affecting the size of the polymer (Kuchta, et al., 2007; Wieczorek, et al., 1995) and these aspects could be considered important factors influencing the efficiency and activity of the PHB granule. In this study, the phasin protein was over-expressed with a histidine tag hence the phasin protein could be purified by metal chelate affinity chromatography procedure (Franken, et al., 2000; Hochuli, et al., 1987). As for the artificial binding of the protein to the PHB granule, the technique needs to be technically verified although the putative artificial binding of several proteins to the hydrophobic surface of the polymer granule was suggested in previous studies (Jendrossek and Pfeiffer, 2014; Liebergesell, et al., 1992).



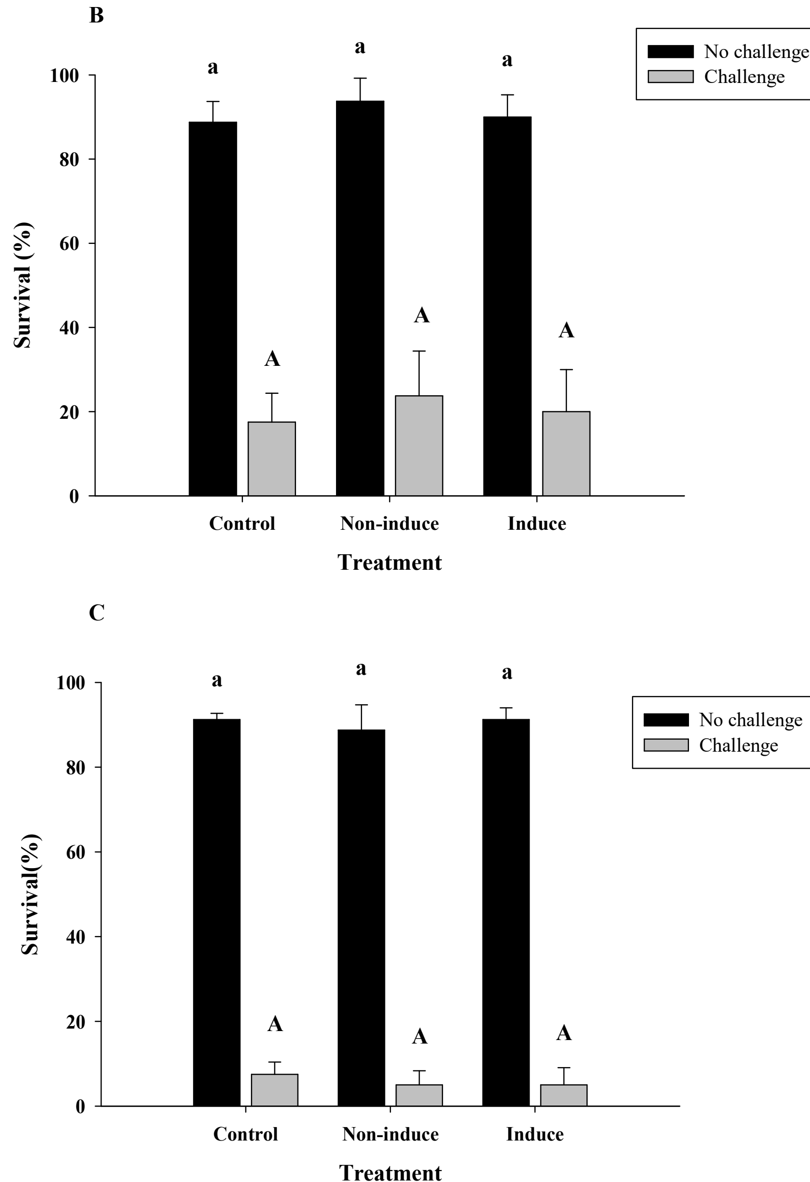


Figure 7.6 Survival of challenged and none-challenged *Artemia* fed the induced and none-induced *E. coli* JL47$_{PhaP}$. *Artemia* were challenged either 2 h (A), 6 h (B) or 24 h (C) after receiving the treatments.

7.3.4. BIOACTIVITY OF E. COLI INTRACELLULAR PHASIN PROTEIN

The overexpressed phasin protein inside the *E. coli* cells used in this study may be present in a non-functional form and as such be responsible for the non-protective effect of the recombinant phasin in the *Artemia* experiment. Indeed, in recombinant

bacteria such as in *E. coli*, the overexpression of plasmid-encoded genes triggers stress responses and often results in misfolding and aggregation of the encoded proteins which are insoluble, non-functional and formed as inclusion bodies (Georgiou and Valax, 1999). In such conditions, normally the cell has conserved mechanisms using molecular chaperones to repair misfolding and aggregation (Carrió and Villaverde, 2003). To mitigate the problem of inclusion body formation, folding modulators are being co-expressed in recombinant protein production (Georgiou and Valax, 1996; Martínez-Alonso, et al., 2010; Thomas, et al., 1997).

In the case of phasin proteins, recent reports from *in vitro* and *in vivo* studies showed that phasins have chaperone-like activity (Mezzina, et al., 2015). In their *in vivo* work, they observed that the phasin from *Azotobacter* sp. FA8 was able to bind to aggregated proteins in inclusion bodies, decreasing also the number and size of inclusion bodies. In this study, it would be worthwhile investigating if the phasin from *Bacillus* sp. JL47 also has some chaperone-like function and even self-chaperoning activity (Lissin, et al., 1990), considering that chaperones may also need other chaperones for folding (Moparthi, et al., 2014). Likewise, it is also worthwhile determining if the overexpression of the phasin from *Bacillus* sp. JL47 resulted to the presence of inclusion bodies in the *E. coli* cells. Inclusion bodies can easily be observed by optical microscopy and by transmission electron microscopy (Bowden, et al., 1991; Carrio, et al., 1998) where it can be seen as ovoid and cylindrical shapes with different smoothed surfaces in the cytoplasm of the bacteria.

Furthermore, to determine if the phasin is bioactive inside the *E. coli* cells, a similar experiment can be done as in the work of Mezzinna, et al. (2015) (this time using the phasin from *Bacillus* sp. JL47) wherein the subcellular localization of the phasin inside the *E. coli* would be analyzed using flourescence microscopy technique. Phasin can be labelled with green fluorescent protein (GFP) and then the capacity of the labelled phasin to bind to PHB granules *in vivo* can be determined by staining the PHB with nile red. PHB granules (red) showing a GFP-labelled phasin (green) co-localization indicates interaction of the phasin with the PHB granule *in vivo*. Likewise, the putative interaction of phasins with inclusion bodies inside the *E.coli* cells could also be investigated by using *E. coli* cells producing an insoluble protein that can cause formation of inclusion bodies. Effects

of phasin on inclusion bodies production would demonstrate the activity of the recombinant phasin inside the *E coli* cells.

7.4 CONCLUSION

This study is the first to determine the effects of phasin on cultured aquatic animals exposed to bacterial infection. The data suggests that phasin protein has no protective effects in gnotobiotic *Artemia* against pathogenic *Vibrio* infection using the current conditions. However, to elucidate further the contribution of the phasin protein in the protective effects of PHB, it is suggested to further purify the phasin protein and coat/bind the protein to the extracted PHB and compare if PHB coated/bound with phasin could display a better activity than with crystalline PHB only. If recombinant *E. coli* overproducing phasin is used, the bioactivity of the phasin needs to be verified first.

ACKNOWLEDGEMENT

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PART 5

CHAPTER 8

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

8.1 INTRODUCTION

The aquaculture sector is considered as the fastest growing food-producing sector in the world, with a huge potential to grow considerably larger in the future. However, its development and expansion has always been hindered by diseases (Chapter 1), thereby affecting its worldwide production and trade. Hence, measures to combat diseases for aquatic farmed animals have been the primary focus for research for the further expansion of the aquaculture sector. In this research, PHB-accumulating *Bacillus* spp. was explored and tested as new biological control agents for aquaculture. The strategy of using amorphous PHB where the compound is still contained in (live) bacteria that are often associated with strong probiotic effects (e.g. *Bacillus* spp.) may further advanced the capabilities of both PHB and the probiotic strategy as important disease-controlling measures for aquaculture.

This PhD research demonstrated the potentials of PHB-accumulating *Bacillus* spp. as new biological control agents for aquaculture. To understand its beneficial effects and mode of action, *Bacillus* spp. with superior PHB accumulation capabilities were isolated, identified and then tested in selected crustacean species wherein their mode of action(s) where studied *in vivo*. The PHB-accumulating *Bacillus* strains were obtained in the culture environment of the animal (i.e. *P. monodon* pond sediments) with the aim of using autochthonous PHB-accumulating *Bacillus* strains for *P. monodon* culture. Unlike in the previous studies where PHB is applied in crystalline form, or in amorphous state but in lyophilized form (where the viability of the bacterium is often not considered), in this study we assessed if the application of PHB-accumulating *Bacillus* spp. containing a significant amount of intracellular amorphous PHB can induce similar or significantly different beneficial effects to the cultured animal. Based on the results presented in the previous chapters (Chapter 4, Chapter 5 & Chapter 6) it is clear that the application of PHB-containing *Bacillus* spp. is useful and very promising for aquaculture. However, there are still several gaps that need to be clarified to optimally apply the strategy. In this chapter, we presented our results and discussed them in line with existing literature and suggest future research and practice opportunities.

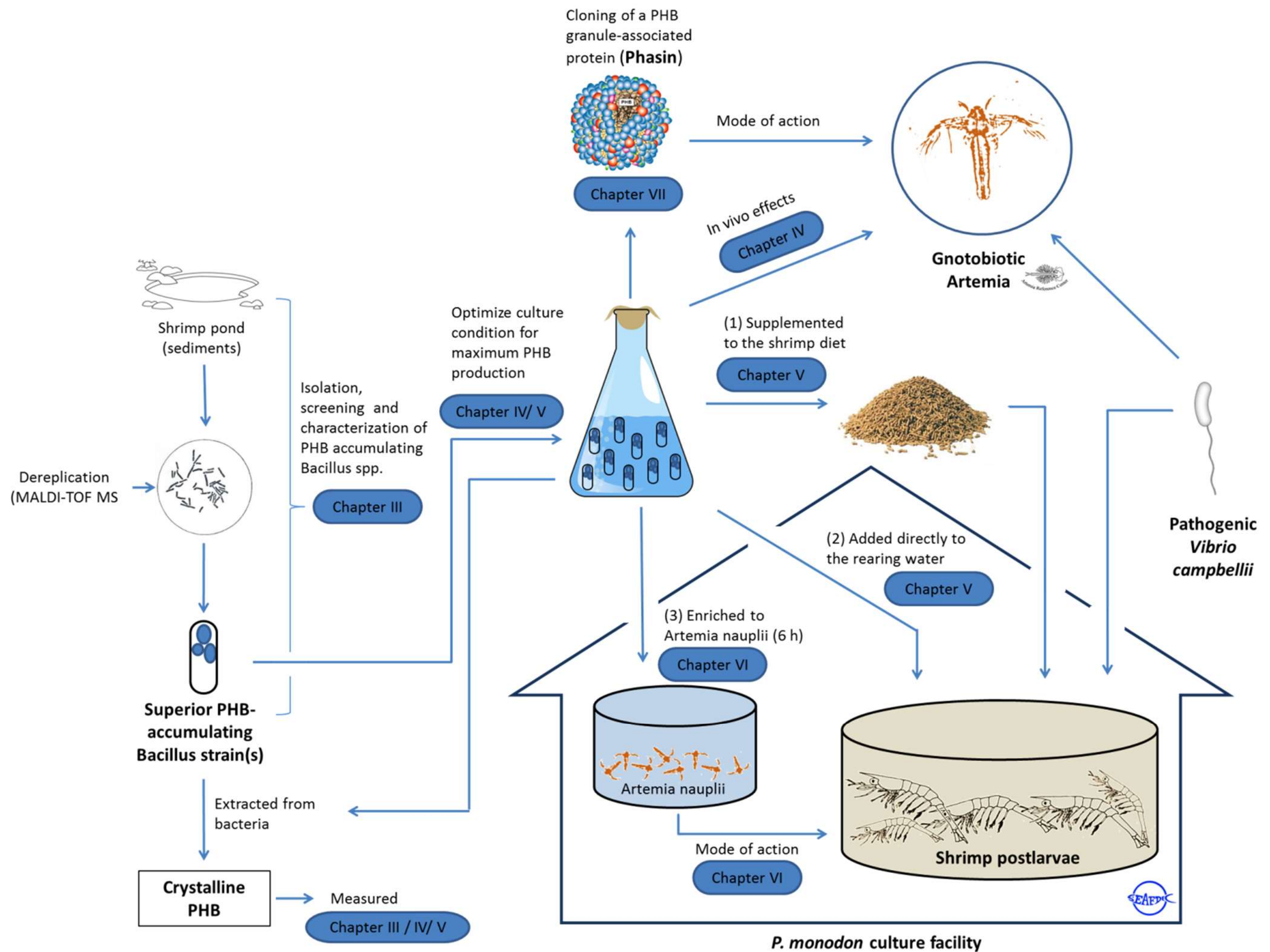

Figure 8.1 Schematic representation of the experiments conducted for the establishment, application and mode of action of PHB-accumulating *Bacillus* spp. for crustacean (larvi)culture.

8.2 MAIN ACCOMPLISHMENTS

The whole work of this research is schematically presented in Figure 8.1. The research has provided several developments on the establishment, application and mode of action of PHB-accumulating *Bacillus* species as biological control agents for crustacean larviculture. Some of the major accomplishments made in this work are as follows:

- The study has demonstrated for the first time the strategy for isolating and rapid screening of PHB-accumulating *Bacillus* species from pasteurized shrimp pond sediments (Chapter 3). This study showed that the utilization of matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) technique as a tool for dereplication (i.e. the recognition of identical isolates at a specific taxonomic level and grouping them accordingly) has allowed short-listing of acquired bacterial isolates for screening and selection of superior PHB accumulators. The study suggests that MALDI-TOF MS patterns can be used as a tool to identify bacteria with high PHB accumulation capability, however further research should be conducted to confirm this observation. Furthermore, the superior PHB-accumulating *Bacillus* isolate identified in this study was *Bacillus* sp. JL47 and has the capability to accumulate amorphous PHB at 28.6% PHB on cell dry weight after 24 h of culture in LB medium with 2% glucose. Further experimentation however revealed that the isolate can accumulate as high as 55% PHB on cell dry weight after 48 h of culture using LB medium with 2% glucose.
- Under the fully controlled axenic culture system, the study has demonstrated the protective effects of the superior PHB accumulator *Bacillus* sp. JL47 strain in gnotobiotic *Artemia* against pathogenic *Vibrio campbellii* infection (Chapter 4). The study also showed that the protective effect of *Bacillus* sp. JL47 was superior when the bacterium contains high amount of amorphous PHB, irrespective of the cell density used. Specifically, the protective effect of *Bacillus* sp. JL47 in *Artemia* was superior when it contains 55% PHB as compared when it contains only 29% PHB. The data suggests that the amorphous PHB present in the *Bacillus* cell is a main determinant in the protective effects of the PHB-accumulating *Bacillus* sp. JL47 on *Artemia*. The novelty of this work is that the *Bacillus* strain was not screened in the first place for its probiotic characteristics but for its capacity to accumulate higher amount of PHB.
- The study has demonstrated for the first time the beneficial effects of using PHB-accumulating *Bacillus* spp. in *Penaeus monodon* postlarvae cultured in tanks (Chapter 5). Shrimps fed the PHB-accumulating *Bacillus* spp. showed an improved survival, enhanced growth performance and higher resistance against pathogenic

Vibrio campbellii LMG 21363, with a maximum attained for the shrimp supplied of the highest PHB accumulator (i.e. *Bacillus* sp. JL47). The study showed that supplementation of the PHB-accumulating *Bacillus* sp. JL47 in the shrimp feed at 4 g kg^{-1} wet weight (approximately 0.41 g PHB kg^{-1}) protected the shrimps from *V. campbellii* infection. Likewise, adding the PHB-accumulating *Bacillus* sp. JL47 in the rearing water at 10^6 cells mL^{-1} day^{-1} also protected the shrimps from the pathogen (wherein the pathogen was also added daily in the rearing water at 10^6 cells mL^{-1}).

- The study has demonstrated the immune-enhancing effect of PHB-accumulating *Bacillus* sp. JL47 in *P. monodon* postlarvae (Chapter 6). Shrimps fed the *Artemia* enriched with the PHB-accumulating *Bacillus* sp. JL47 at 0.5 g L^{-1} (wet weight) showed an up-regulation in the expression of prophenoloxidase and transglutaminase in the shrimp before and after the *Vibrio campbellii* challenge. The data suggest that the protective effect of the PHB-accumulating *Bacillus* sp. JL47 demonstrated in chapter 5 can be attributed to the immune-enhancing effects of the PHB-accumulating *Bacillus* sp. JL47 in the shrimp.
- The study has investigated for the first time the effects of phasin protein (i.e. a PHB-granule associated protein covering the surface of the amorphous PHB) on the survival of gnotobiotic *Artemia* challenged with pathogenic *V. campbellii* LMG 21363. The results however confirmed no protective effect of the phasin protein to *Artemia* against pathogenic *Vibrio campbellii* infection (Chapter 7).

8.3 ESTABLISHMENT OF PHB-ACCUMULATING *BACILLUS* SPP. AS BIOLOGICAL CONTROL AGENTS FOR CRUSTACEAN CULTURE

8.3.1 SOURCE OF PHB-ACCUMULATING BACILLUS SPP.

In this study, the acquisition of bacterial isolates as candidates for screening PHB accumulating *Bacillus* spp. was obtained from shrimp pond sediments so that the isolates that will be acquired for screening are indigenous to the shrimp environment. Indeed, it can be considered logical to obtain potential microorganisms of interest from the host or from the environment of the host in which the microorganism(s) will be applied and

supposed to exert their beneficial effects. Furthermore, pond sediments can be considered as a suitable place to look for bacteria with superior PHB accumulation capability considering the stressful and unstable conditions present in the pond environment (i.e. inconsistent nutrient availability, physical and chemical changes) which may allow bacteria to develop strategies for survival such as the accumulation of reserve materials (i.e. PHA). Indeed, it was observed that PHA accumulation and degradation is one strategy by which bacteria can improve their establishment, proliferation and survival in competitive environments like the soil (Kadouri, et al., 2005) or in natural oligotrophic aquatic environments (López, et al., 1995). For the genus *Bacillus*, PHA can be used as a carbon and energy reserve for sporulation process (Valappil, et al., 2007). In these bacteria PHA synthesis began during or shortly after logarithmic growth and maximize just prior to the formation of spores. This biopolymer is then degraded during the process of sporulation and disappears with the concomitant appearance of mature spores during late stationary phase (Kominek and Halvorson, 1965).

8.3.2 ISOLATION, DEREPLICATION AND SELECTION OF SUPERIOR PHB-ACCUMULATING BACILLUS STRAINS

Prior to the isolation step, the shrimp pond sediments were first pasteurized to obtain spore-forming bacteria. Furthermore, the study utilized the application of MALDI-TOF MS as a derelication step i.e. the process of recognizing identical isolates at a specific taxonomic level and grouping them accordingly (Ghyselinck, et al., 2011). Dereplication can be considered an important step especially in large-scale isolation and screening studies considering that during the isolation process the number of isolates can easily run high, and with a dereplication step, a subsequent selection of representatives from the dereplicated groups reduces the number of isolates to be analysed in further downstream analyses. Thus, this also lessens the intensity of work, time and cost incurred in the whole screening process. MALDI-TOF MS has been suggested as an efficient tool for dereplication because of its simplicity in the preparation of cell samples, sensitivity, and rapidity of the technique and also the whole process in analysing per sample is inexpensive (Neville, et al., 2011). In the concept of dereplication, other powerful techniques can also be used such as repetitive element sequence based Polymerase

Chain Reaction (repPCR) (De Clerck and De Vos, 2002), randomly amplified polymorphic DNA (RAPD) (Martín-Platero, et al., 2009) and fatty acid methyl ester (FAME) (Coorevits, et al., 2008) analyses however these techniques can be considered time consuming, laborious and expensive. In this study, whole intact cells of the isolates were used in the MALDI-TOF MS analysis. The work of Ryzhov and Fenselau (2001) showed that proteins that are being detected by the MALDI-TOF MS using whole intact cells of *E. coli* K12 are mostly matched by mass to proteins found inside the bacterial cell (i.e. cytosol born), except for one protein that is considered to be a major outer-membrane lipoprotein precursor. The study showed that almost half of the peaks detected in the MALDI spectra are matched by mass to ribosomal proteins, while other peaks matched to DNA binding proteins HU and cold-shock proteins. The authors discussed that due to the organic solvents and acidic conditions used in the analysis, the thin cell walls of the vegetative bacteria are lysed, resulting in the release of the cytoplasmic proteins for detection in the MALDI spectra. Indeed, from the 50 isolates analysed in this study (chapter 3), two major groups were observed and within the first group, 6 sub-groups were further shown after the MALDI-TOF MS-based grouping. An interesting observation from our data showed that there seemed a trend in the grouping pattern generated by MALDI-TOF MS and PHB accumulation capacity of the isolates. We suspected that the proteins detected by the MALDI-TOF MS are probably distinct proteins related to PHB accumulation knowing that prior to the MALDI-TOF MS analysis all the isolates were grown in a culture medium with extra carbon and such culture condition can induce accumulation of PHB in bacteria. However, to confirm this observation further study should be conducted by investigating each of these peaks detected and identify which proteins these peaks represent because it could also be that the MS grouping is just a result from the taxonomic relationship between both groups and not necessarily related to their PHB accumulation capability. Nevertheless, the indication that there is a clear correlation between the spectra and PHB accumulation suggests for a possible lead for biomarker discovery that might be used to facilitate new screening studies related to environmental PHB producers. Indeed, it has been suggested that MALDI-TOF MS can be used in three possible approaches, among which: (1) it can be used to detect specific biomarker proteins that is indicative for specific genotype or phenotypic properties

(Fagerquist, et al., 2005; Wolters, et al., 2011); (2) it can be used to rank or identify organisms (Claydon, et al., 1996); and (3) it can be used to compare and rule out specific species or strain (Dickinson, et al., 2004).

Furthermore, the accumulated amorphous PHB in the bacterial cell can be quantified by spectrophotometric method wherein the compound is extracted from the bacterial cell through chloroform and is further converted to crotonic acid using a concentrated sulphuric acid. The converted PHB can then be estimated spectrophotometrically at 235 nm through a PHB-crotonic standard (Law and Slepecky, 1961). In this study, the method was employed for quantifying the amorphous PHB in the PHB-accumulating bacterial isolates.

Our results in chapter 3 showed that there seems a varying accumulation capability of PHB between (sub) groups of isolates and that group 1 isolates are higher accumulators than group 2. The accumulation capacity using the representative isolates showed a ranged from 3.0-28.6% PHB with a total PHB yield of 0.1-1.1 g L^{-1} on cell dry weight. The novelty of this work is that the *Bacillus* species were not screened in the first place for their probiotic characteristics but for their capacity to accumulate higher amount of PHB. From here, the identified superior PHB-accumulating *Bacillus* strains were then tested in selected crustacean species and determined their beneficial effects and mode of action *in vivo*.

8.3.3 In vivo tests to demonstrate the beneficial effects of PHB-accumulating Bacillus spp. in selected crustacean species

On one end, the purpose of the study is to screen and select for *Bacillus* species with superior accumulating capability, but also more importantly, it should also be tested if the superior PHB-accumulating *Bacillus* spp. identified could provide beneficial effects to cultured aquatic animals. In this research, the initial *in vivo* test was conducted in a gnotobiotic culture system using *Artemia* as a model organism for crustacean species. The use of gnotobiotic systems (i.e. animals are cultured in axenic conditions or with a known microflora (Marques, et al., 2006)) can be considered an excellent tool to understand better the mechanisms involved in host-microbial interaction, knowing that in this system the arbitrary influences (i.e. unknown microorganisms) present are

excluded. In aquaculture environments, the concentration of bacteria can easily reach as high as 10^4 CFU mL^{-1} such as in the larval and postlarval rearing tanks of *P. monodon* (Otta, et al., 2001), 10^5 CFU mL^{-1} in the culture water of giant fresh water prawn *M. rosenbergii* larvae (Kennedy, et al., 2006) or even 10^5-10^6 CFU mL^{-1} in microbially matured rearing water (Attramadal, et al., 2016). Such conditions permit the stochastic inflow of microorganisms which could result in variations of intestinal microbiota in the cultured animal (Fjellheim, et al., 2007). A thorough understanding of mechanisms involved behind the host and microbe interaction needs to define first the animal functioning in the absence of all-microorganisms and then evaluate the effects of a single or defined population of microbes or compound (Gordon and Pesti, 1971) because it would be difficult to establish a clear mechanistic investigation in such conditions when other factors (i.e. microbial composition) are not known and controlled. For the purpose of studying host-microbial interactions for aquaculture, several gnotobiotic model animals have been developed such as gnotobiotic zebrafish, gnotobiotic seabass and gnotobiotic tilapia (Dierckens, et al., 2009; Rawls, et al., 2004; Situmorang, et al., 2014). In the crustacean species, the gnotobiotic *Artemia* has been used as a model crustacean organism in several host-microbial interaction studies (Marques, et al., 2006). And so, in this research, we employed the application of gnotobiotic *Artemia* to initially investigate the protective effect of the superior PHB-accumulating *Bacillus* strain in the animal during *Vibrio* infection and more importantly, to understand the possible contribution of amorphous PHB as a (separate) factor influencing the probiotic effect of the PHB-accumulating *Bacillus* strain. Indeed, based on our results, the superior PHB-accumulating *Bacillus* sp. JL47 able to protect the *Artemia* from *Vibrio* infection and further showed that the amorphous PHB is a key determinant in this effect (Chapter 4). While we have successfully established that there is indeed a significant difference in the protective effect of the *Bacillus* sp. JL47 when the bacterium contains a high amount of amorphous PHB, it is still worth knowing to compare the protective effect of a wild-type PHB-accumulating *Bacillus* sp. JL47 versus a non-PHB producing *Bacillus* sp. JL47 (non-PHB synthesizing mutant) in *Artemia* to clearly illustrate the contribution of amorphous PHB in the protective effect of this *Bacillus* strain. Similarly, by full characterization of both the wild-type PHB-producing *Bacillus* and non-PHB producing *Bacillus* mutant will give

information on differential proteins (e.g. if these proteins are immunostimulatory) that may have influenced the probiotic characteristic of the *Bacillus* strain. Furthermore, in chapter 7, we also used the gnotobiotic *Artemia* to determine if the recombinant phasin protein could be responsible for the observed protective effect of amorphous PHB in the animal, however our data seemed to show that phasin protein has no protective effect in *Artemia* during *Vibrio* infection.

Conversely, the gathered information obtained from the application of PHB-accumulating *Bacillus* in gnotobiotic *Artemia* also need to be validated in the actual, non-bacteria-free environment. With this experiment, it can provide and evaluate further the perceived mode of action of the biocontrol agent in relevance to actual culture conditions. Indeed, in chapter 5 the different superior PHB-accumulating *Bacillus* spp. was tested in *P. monodon* postlarvae culture in nursery tanks. Our results from the gnotobiotic *Artemia* experiment are in parallel to our results in the *P. monodon* postlarvae culture. Shrimps fed the PHB-accumulating *Bacillus* spp. showed an improved survival, enhanced growth performance and higher resistance against pathogenic *Vibrio campbellii* LMG 21363*,* with a maximum attained for the shrimp supplied of the highest PHB accumulator (i.e. *Bacillus* sp. JL47).

8.4 THE MODE OF ACTION OF PHB-ACCUMULATING *BACILLUS* SPP.

8.4.1 ENHANCES SURVIVAL AND ROBUSTNESS

In this research, the survival and robustness-enhancing effects of PHB-accumulating *Bacillus* spp. in the host were demonstrated in two crustacean species using gnotobiotic *Artemia franciscana* in controlled axenic culture system (Chapter 4) and in *Penaeus monodon* postlarvae in actual culture system in nursery tanks (Chapter 5).

In the gnotobiotic *Artemia in vivo* experiment (Chapter 4), the survival and robustness enhancing effects of the PHB-accumulating *Bacillus* sp. JL47 only became apparent upon challenge with *V. campbellii* LMG 21363, since in the non-challenged groups, there were no significant differences in survival of the *Artemia* fed with or without PHB-accumulating *Bacillus* sp. JL47. The experiment further showed that the protective effects of the PHB-accumulating *Bacillus* sp. JL47 on the gnotobiotic *Artemia* is dose dependent since feeding lower than 1 x 10^7 cells mL^{-1} to *Artemia* showed no significant

difference with the challenged control. Interestingly, the study further showed that the survival and robustness-enhancing effects of *Bacillus* sp. JL47 was superior when the bacterium contains higher amounts of amorphous PHB, irrespective of the cell density used. The data suggest that the amorphous PHB present in the *Bacillus* sp. JL47 is a main determinant in the survival and robustness-enhancing effects of the *Bacillus* sp. JL47 strain. Conversely, the different PHB-accumulating *Bacillus* strains were also tested in the different stages of *P. monodon* postlarvae culture using PL1 and PL30 shrimps (Chapter 5). Unlike in the gnotobiotic *Artemia* experiment, the survival and robustness enhancing effects of the different PHB-accumulating *Bacillus* spp. used was already observed in the unchallenged animal wherein a significantly higher survival was observed in shrimps fed the diet containing PHB-accumulating *Bacillus* strains as compared with the control. The difference in the results between the two animals tested can be attributed to the longer culture and feeding period of the shrimp postlarvae on the PHB-accumulating *Bacillus* strains (i.e. 30 days) as compared to *Artemia* nauplii which were cultured for only 2 days. Interestingly, similar with the challenged gnotobiotic *Artemia*, the survival of the *P. monodon* postlarvae challenged with pathogenic *Vibrio campbellii* (either by one-time challenge or the daily exposure of the shrimp to the pathogen) was higher for the shrimp fed with the PHB-accumulating *Bacillus* spp., with a maximum attained for the shrimp supplied of the highest PHB accumulator (i.e. *Bacillus* sp. JL47).

Here, we proposed the possible settings by which the PHB-accumulating *Bacillus* spp. enhances the survival and robustness in the cultured animal based on the results of this research as well as with existing literatures (refer Figure 8.2 below).

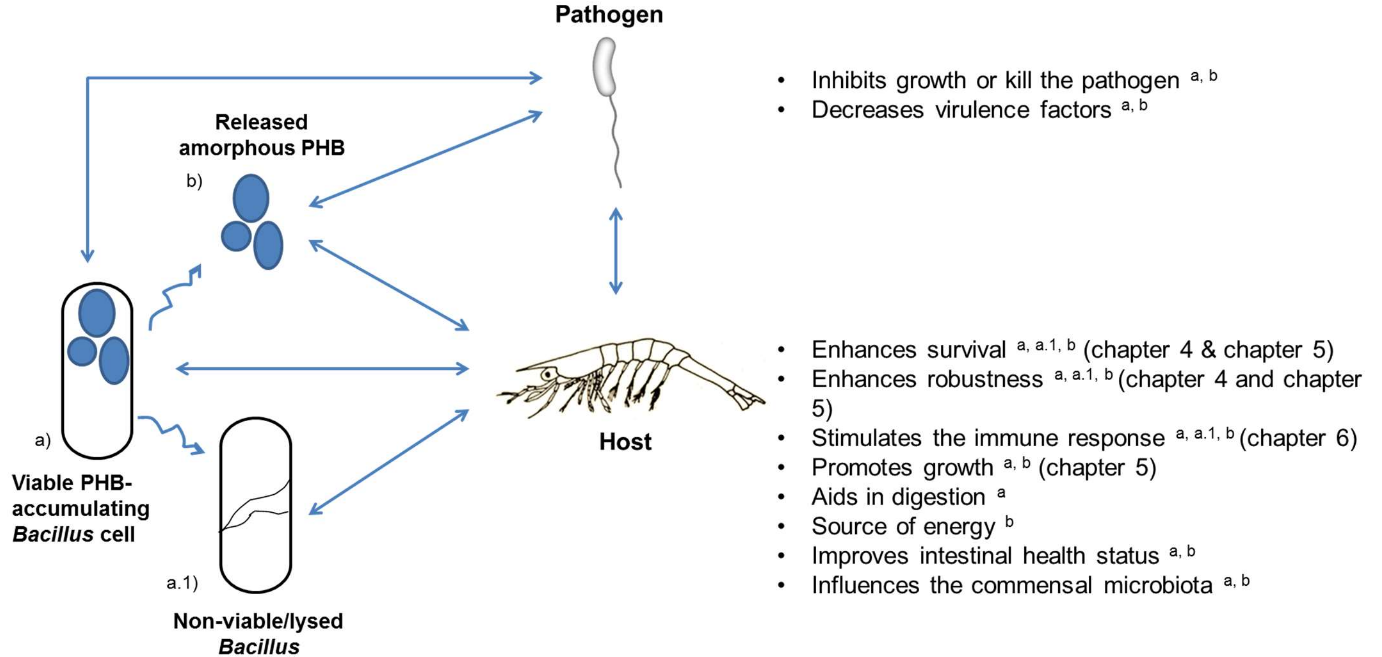

Figure 8.2 Schematic diagram of the (possible) settings by which PHB-accumulating *Bacillus* spp. acts on the host and/or on the pathogen: (a) as viable probiotic *Bacillus*; (a.1) as nonviable/lysed *Bacillus*; (b) through the amorphous PHB.

8.4.1.1 Effects of the intracellular amorphous PHB

Based on the results obtained in the *Artemia* experiment in chapter 4 and in *P. monodon* postlarvae culture in chapter 5, the survival and robustness-enhancing effects observed in the *Vibrio*-challenged animals is consistently higher for the *Bacillus* strains containing the higher amount of amorphous PHB, being it be compared with the same strain (chapter 4) or with different *Bacillus* strains (chapter 5). This observation suggests that amorphous PHB is an important factor influencing the beneficial effects of PHB-accumulating *Bacillus* strains in the host.

Since we used live PHB-accumulating *Bacillus* in our experiments, it can be expected that (some) PHB-accumulating *Bacillus* cells are broken down as it passes through the digestive tract of the animal and thereby the amorphous PHB is exposed and/or released into the gut environment (Pessi, et al., 1999). In shrimp, its digestive

process seems quite similar with fish except that the hepatopancreas is the main secretory organ of digestive enzymes (e.g. proteases, lipases and amylases) while the stomach serves as a chewing structures (i.e. various lamellae, appendages, and calcified parts including gastric mill) where food particles are grinded, disintegrated and filtered (Ceccaldi, 1997; National Research Council, 2011). Such conditions may result in non-viable and/or broken down bacteria. As a consequence, amorphous PHB could be exposed/released where it could be degraded to its degradation intermediate (i.e. β-hydroxybutyrate) either by PHB-degrading bacteria which are known to be present in the gut of cultured aquatic animals including shrimp (Liu, et al., 2010b) or could be (partially) degraded by the animal without the aid of any PHB degraders (Defoirdt, et al., 2007). PHB through its degradation product β-hydroxybutyrate can provide survival and robustness enhancing effects to the animal in three possible ways: (1) by serving as energy source to the animal; (2) by inhibiting the growth or decreasing the virulence of the pathogen; and (3) by enhancing the immune response of the animal.

PHB as source of energy

PHB as a fatty acid is considered as a typical source of energy (Azain, 2004). Previous study shows that the β-hydroxybutyrate in the form of ketone bodies was used as energy source in developing *Artemia* (Weltzien, et al., 2000). Furthermore, in a more recent finding, the lipid-saving effects of dietary PHB (either in the amorphous or crystalline form) was also demonstrated in gnotobiotic *Artemia* wherein the whole body lipid content of the starved *Artemia* was increased (Ludevese, 2016). The research of Situmorang (2015) demonstrated in detail the compartment distribution of PHB in Nile tilapia and suggested the PHB's metabolic fate in the fish. The authors hypothesized that PHB is degraded and absorbed in the intestines resulting in the transport of free fatty acids (FA), consisting of long and short-chain fatty acids (LCFA and SCFA) in the blood. LCFA are transported to the kidney and spleen and then directly enters into the systemic circulation while SCFA are transported to the liver where they are either esterified into triglycerides and converted to cholesterol or phospholipids, or oxidised into ketone bodies. These ketone bodies are further circulated in the blood and are utilized by the heart, brain and muscle as their energy source. Therefore, the capability of PHB and/or

its degradation intermediate β-hydroxybutyrate to serve as alternative or additional energy source for the animal could be beneficial especially during stressful conditions such as molting, reproduction and handling of pathogens. These conditions are considered energy-demanding and therefore with PHB as additional energy source, the animal may have a better chance of surviving from such stressful circumstances. In our shrimp experiment in chapter 5, we applied the PHB-accumulating *Bacillus* spp. in the postlarval stages (PL1 and PL30) of *P. monodon* where molting could be frequently expected, the contribution of PHB as additional energy source may have contributed to the observed significant increase in the survival of the animals.

Anti-pathogenic effects of PHB

The survival-enhancing effects of amorphous PHB to the animal can also be attributed to the anti-pathogenic properties of its monomer form β-hydroxybutyrate. One strong indication on the release of β-hydroxybutyrate from PHB degradation is the lowering of the gut pH and this was clearly observed by De Schryver, et al. (2010) in juvenile seabass after feeding the fish with PHB-supplemented diet. This compound can acidify the cytoplasm of the pathogen and as a consequence the pathogen has to redirect its cellular energy (i.e. use of transporters that catalyse active proton transport), to maintain homeostasis, resulting to either lower cell growth, decrease in virulence, or even cell death (Defoirdt, et al., 2009). The work of Defoirdt, et al. (2007) demonstrated the growth inhibitory effect of β-hydroxybutyrate on the pathogen *V. campbellii* LMG 21363 at a dose of 100 mM at pH 6. Likewise, a recent study showed that PHB through its degradation intermediate β-hydroxybutyrate, effectively shut down the phenotypic expression of virulence factors of the *Vibrio* PUGSK8 specifically the biofilm formation, luminescence, motility behaviour, haemolysin production and the *N*-acyl-homoserine lactone (AHL)-mediated quorum sensing (Kiran, et al., 2016). Indeed the same authors further demonstrated the anti-adhesive activity of PHB using a microtitter plate assay wherein the biofilm formation of pathogenic *Vibrio*s spp. was inhibited after coating the wells with PHB (Kiran, et al., 2014).

Enhancing the immune responses

Lastly, the exposed amorphous PHB can also stimulate the immune response of the animal resulting to an enhanced survival and robustness effects. In previous studies, the immune enhancing effects of PHB-HV extracted from *Bacillus thuringiensis* was observed wherein both the specific and none-specific immune responses of the tilapia *Oreochromis mossambicus* were stimulated after feeding the fish with diet supplemented with PHB (Suguna, et al., 2014). Furthermore, the work of Baruah, et al. (2015) demonstrated an immune enhancing effect of PHB in *Artemia* and proposes a mechanism by which PHB enhances the immune system of the animal through Hsp70 biosynthesis. The authors suggest that PHB particles are partially degraded into different monomeric, dimeric and/or oligomeric forms in the *Artemia* gut and that the released fatty acid (through non-ionic diffusion) might have caused cellular acidification. The lowering of the cellular pH might have created a (mild) stress conditions that could have potentially driven the production of inducible Hsp70 in the intestinal epithelial cells of *Artemia*. The induction of this stress protein may then have conferred enhanced immunity of the animal by regulating the expression of proPO, Tgase and ftn (ferritin) immune genes in the *Artemia* larvae. Although, to date, the exact mechanism by which Hsp proteins modulate the immune response is still vague. Previous reports only described that Hsps are highly expressed in response to pathogen infection (Zhou, et al., 2010) and that induction of these Hsp proteins (e.g. Hsp70) can increase the tolerance of the animal (e.g. *Artemia*) against *Vibrio* insults (Baruah, et al., 2010; Sung, et al., 2007). Also, still in *Artemia,* a parallel increase in expression of important immune defense genes such as proPO, Tgase and Hsp protein production was observed (Baruah, et al., 2015) Interestingly, recent studies further showed that silencing these Hsp proteins (e.g Hsp70 and Hsp90) resulted in a decreased PO activity in shrimp as well as decreased tolerance to *Vibrio* infection (Junprung, et al., 2017). In *Artemia*, a low protection against *Vibrio* infection and heat stress was observed when Hsp70 was silenced (Iryani, et al., 2017). These studies show that Hsp proteins have important roles in the stimulation of the expression of important immune defense genes.

Conversely, PHB through its degradation intermediate β-hydroxybutyrate could also have stimulated the immune response of the animal. SCFA, such as butyrate have been suggested to be modulators of mammalian immunity (Meijer, et al., 2010). It was suggested that SCFA regulates the mammalian immune system by activating the GPR43, a G protein-coupled receptor that recognizes endogenous SCFA ligands, including acetate, propionate and butyrate (Brown, et al., 2003). This free fatty acid receptor is observed to be highly expressed in neutrophils, macrophages and monocytes (Le Poul, et al., 2003). Indeed, previous studies show that the SCFA derived from commensal bacteria promote neutrophil chemotaxis in mice (Sina, et al., 2009; Vinolo, et al., 2011), however no studies have been made on the immune enhancing effects of β-hydroxybutyrate in any aquaculture organisms. Nevertheless, the immune enhancing effects of β-hydroxybutyrate as a degradation product of PHB should be further investigated in aquaculture organisms considering that β-hydroxybutyrate and butyrate differs too much and it cannot be simply assumed that it will induce similar immune modulation as described above.

Our results in chapter 6 showed a significant increase in the expression of proPO and TGase genes in the *Bacillus* sp. JL47-fed *P. monodon* postlarvae after the *Vibrio campbellii* challenge. The enhanced expression of these two important immune-defense genes in *P. monodon* post larvae can be linked to the amorphous PHB present in JL47, considering that in our *in vivo* experiment conducted in chapter 4, the amorphous PHB appears to be the main determinant in the observed protective effects of JL47 in *Vibrio*-challenged gnotobiotic *Artemia*. However, to clearly link immunostimulation with PHB content, further experimentation is required by comparing the immunostimulating effects of JL47 containing low and high amorphous PHB in the animal. Furthermore, our results in chapter 6 also showed a slight (2-fold) but statistically significant increase in the expression of proPO and Tgase genes (except for Hsp70) in the shrimp before the challenge test. We suggest that an immune priming effect (i.e. an increase in innate immune activity in the host after exposure to an immunomodulating agent) by the PHB-containing *Bacillus* sp. JL47 to these two immune-related genes in *P. monodon* may have occurred. Interestingly, the research of Nguyen (2016) also showed that feeding the mussel larvae with amorphous PHB resulted in a significant activity of phenoloxidase in

the animal even without the addition of the pathogen. In the same study, the phenoloxidase activity was further heightened when the animals were fed with amorphous PHB and subsequently challenged with pathogenic *Vibrio coralliilyticus*. The observed immune priming effect in the shrimp after feeding the PHB-containing *Bacillus* sp. JL47 may suggest further investigation, considering that in general the immune priming effect in invertebrates is even not clear to date. This can be done by conducting kinetic gene expression studies in amorphous PHB-fed shrimps before and after a *Vibrio* infection in comparison with non-amorphous PHB-treated shrimps.

8.4.1.2 Acting as viable probiotic *Bacillus* spp.

Aside from the contribution of amorphous PHB, it is also plausible that the robustness enhancing effects observed in our experimental animals was (as well) contributed by the *Bacillus* strains acting as probiotics, considering that we administered live *Bacillus* in the tested animals. Indeed, several studies showed the survival and robustness enhancing effects of applying probiotic *Bacillus* in several cultured crustacean species including *P. monodon* (NavinChandran, et al., 2014; Rengpipat, et al., 2003; Shen, et al., 2010; Silva, et al., 2012). In general, *Bacillus* spp. are widely used as probiotics in aquaculture because of their various beneficial effects such as stimulating the host's immune system, producing enzymes that degrade quorum sensing molecules, producing antimicrobial compounds that inhibit the growth of pathogens, producing exoenzymes (i.e. proteases, lipases, carbohydrases) that aid in digestion and absorption of nutrients in the gut, influencing healthy balance of commensal microbiota in the GIT and even improving the quality of the culture water by removing the nitrogenous wastes present (Kim, et al., 2005; Maruta, et al., 1996; Pande, et al., 2015; Pinchuk, et al., 2001; Ray, et al., 2012; Tseng, et al., 2009; Urdaci, et al., 2004). All of the above mentioned (beneficial) effects of probiotic *Bacillus* spp. can result in enhancing the survival and/or robustness of the animal and depending on the strains used the probiotic *Bacillus* can act directly to the host, to the pathogen and/or to the commensal microbiota in the GIT. In applying probiotic microorganism one has to consider that the applied probiotic strain needs to persist in the gut of the animal in order to elicit stronger effects, that is why attachment and colonization of the treated probiotic microorganism is an important factor

to consider in selecting probiotics (Verschuere, et al., 2000; Vine, et al., 2006). Previous studies suggested that *Bacillus* treatment successfully colonized the digestive tract of the shrimp (Ziaei-Nejad, et al., 2006) while in another study *Bacillus* treatment can even replace the presence of *Vibrio* in the shrimp gut (Rengpipat, et al., 1998). Interestingly, it was also described that bacteria capable of accumulating reserve materials (i.e. PHB) have higher chances of surviving and proliferating in its environment as compared to non-PHB producing strains (Castro-Sowinski, et al., 2010). For example, a previous study showed that a wild-type PHB-accumulating *Bacillus megaterium* strain has increased its density and attained higher survival in a soil microcosm as compared to its non-PHB accumulating *B. megaterium* equivalent (López, et al., 1998). Taken together, there is a greater chance that the PHB-accumulating *Bacillus* spp. administered in our cultured animals have successfully adhered and proliferated in the gut, considering the beneficial effects observed in our cultured animals. However, this hypothesis requires further investigation if indeed PHB-accumulating *Bacillus* spp. can effectively adhere and have higher chances of colonizing the shrimp gut. Advance techniques such as fluorescent in situ hybridization (FISH) can be employed to monitor the colonization and proliferation of PHB-containing *Bacillus* species in the gut environment.

Furthermore, an interesting finding in this research was that the protective effect of the PHB-accumulating *Bacillus* sp. JL47 seemed to be superior when the *Bacillus* strain contains higher amorphous PHB (Chapter 4). We hypothesized that apart from amorphous PHB, other unidentified characteristics such as differences in expressed proteins or cell wall bound proteins in JL47 might have contributed the enhanced probiotic properties of *Bacillus* sp. JL47 strain (e.g. if some of these proteins would have an immunostimulatory property). Therefore, it is suggested to completely characterize the *Bacillus* JL47, in order to establish e.g. differential expression in microbe-associated molecular patterns (MAMPs) (e.g. peptidoglycan) or other expressed proteins (e.g. heat shock proteins) that may have enhanced as a result of the PHB accumulation in the *Bacillus* strain.

8.4.1.3 Non-viable/dead PHB-accumulating *Bacillus* cells

It is also important to mention that not all live PHB-accumulating *Bacillus* cells that are given to the animal will end up in the gut as viable and intact considering that these bacteria will pass through the digestive tract of the animal where the conditions could be hostile causing the bacterial cells to be non-viable or damaged. Indeed, as described above, shrimps have chewing structures located in the stomach wherein food particles are grinded and filtered while the food particles are partially hydrolysed by digestive enzymes delivered from the hepatopancreas (National Research Council, 2011). Hence, there will be a possibility that an unknown amount of dead cells are being administered together with the viable cells that survived in the digestion process. However, even at this state, it was suggested that dead probiotic cells can still modulate the immune response of the host (Adams, 2010). Indeed, examining the cellular innate immune parameters of Gilthead seabream using heat-inactivated probiotic bacteria showed significant increase in respiratory burst and cytotoxic activities of Gilthead seabream head-kidney leucocytes *in vitro* (Salinas, et al., 2006). Thanh Tung, et al. (2010) reported that using heat-killed *L. plantarum* probiotic on Kuruma shrimp *Marsupenaeus japonicas* larvae and postlarvae resulted in increased survival, growth and stress resistance. Hence, there is a possibility that feeding non-viable *Bacillus* cells containing amorphous PHB can still provide beneficial effects to the animal such as by modulating the animal's immune response. Indeed, the work of Halet, et al. (2007) showed that feeding the gnotobiotic *Artemia* with a PHB-accumulating bacteria strain (PHB2) prepared as pasteurized or frozen and thawed provide similar protective effects with the viable (untreated) form in the *Vibrio*-challenged *Artemia*.

Furthermore, another contribution of feeding bacterial cells is that dead probiotic cells can also be a source of nutrients to the animal considering that bacteria can contain 25-49% protein, 2.5-11% carbohydrate, 2.5-9% lipid and 4.7-14% ash (Brown, et al., 1996). This aspect can be relevant especially when the PHB-accumulating *Bacillus* spp. is delivered via bioencapsulation through *Artemia* enrichment. According to Thai (2015) , a calculated increase of about 15% in protein,7% in lipid, 10% in carbohydrates and 20% in ash can be observed in the *Artemia* biomass when enriched with PHB-accumulating

bacteria. Hence, apart from amorphous PHB which can be a source of energy, the extra nutrients from whole bacterial cells may also contribute to the metabolic activity of the fed animal.

8.4.2 GROWTH PROMOTING EFFECTS

The growth promoting effects of PHB-accumulating *Bacillus* spp. was also demonstrated in this research in *P. monodon* postlarvae (PL30) culture (Chapter 5). The data showed that the improved growth in our shrimp patterned according to the level of PHB in the diet wherein a significant correlation between the PHB dose and the increase in shrimp body weight existed. The study suggests that the PHB in the diet was a main determinant for the growth performance of the shrimp.

Indeed, the growth promoting effects of PHB was also demonstrated in several cultured species including juvenile European seabass *Dicentrarchus labrax* (De Schryver, et al., 2010), Siberian sturgeon *Acipenser baerii* fingerlings (Najdegerami, et al., 2012), Pacific white shrimp *Litopenaeus vannamei* (Duan, et al., 2017), giant freshwater prawn *Macrobrachium rosenbergii* larvae (Nhan, et al., 2010) and Chinese mitten crab *Eriocheir sinensis* larvae (Sui, et al., 2012). However, application of PHB in some cultured species such as on tilapia juveniles (Situmorang, et al., 2016), mussel larvae (Van Hung, et al., 2015) and *P. monodon* mysis to postlarvae (Ludevese-Pascual, et al., 2017) did not show significant growth effects. Findings from all of these studies seemed to show that the growth promoting effects of PHB differs between PHB doses, developmental stages, and probably between species. The work of Ludevese-Pascual, et al. (2017) showed no significant growth effect of applying crystalline PHB in *P. monodon* mysis to postlarvae, while in our study feeding the *P. monodon* postlarvae (PL30) with diet supplemented with amorphous PHB contained in bacilli showed a significant growth effect. The difference in the results of the two researches using the same shrimp species could possibly be attributed to difference in the developmental stage of the shrimp used wherein different degradation capacities (enzymatic and/or microbial activity) towards PHB may have occurred in these two different shrimp stages or it could also simply be due to the degradation efficiency of the amorphous PHB as compared with crystalline PHB. The previous work of Ludevese (2016) on *P. monodon* larvae used crystalline PHB as

enrichment to *Artemia* or as a substratum in the culture tank and the PHB in these forms are sometimes difficult to degrade by the animal due to its level of crystallinity (Tokiwa, et al., 2009), our study however supplied the PHB in amorphous (native) form where the compound was expected to be easily depolymerized (Yu, et al., 2005).

Nevertheless, in general, the growth enhancing effects of PHB can be related to its ability to influence the microbial community in the gut. The work of De Schryver, et al. (2010) in juvenile seabass and of Najdegerami, et al. (2012) in juvenile Siberian sturgeon showed that fish fed with PHB resulted to a change in the microbial composition in the gut and that this change in the microbial community composition seemed to be closely associated with the observed increased fish growth. The shift towards more beneficial microbes in the gut which have the capability to help in the digestion and absorption of nutrients may have enhanced the growth of the animal, considering that commensal microbiota were shown to be affecting a wide range of biological process in the gut including nutrient processing and absorption (Rawls, et al., 2004). The growth promoting effect of PHB could also be related to its ability to improve the intestinal health status of the animal. The work of Silva, et al. (2016) demonstrated that feeding the Pacific white shrimp *L. vannamei* with shrimp diet supplemented with PHB or sodium butyrate increases the intestinal length and villi width of the animal, thus with PHB, it improved the integrity and absorptive capacity of the mucosal membrane.

Furthermore, since we applied the amorphous PHB through a bacterial carrier using probiont *Bacillus* strains, we suggested that apart from the amorphous PHB contributing the growth-promoting effects in the animal, the application of the PHB-accumulating *Bacillus* strains acting as viable cells may have also contributed in the growth enhancement of the shrimps. It has been observed that *Bacillus* species are capable of producing exoenzymes such as proteases, carbohydrolases and lipases that can break down proteins, carbohydrates and lipids, respectively, and as such can contribute to the digestion and absorption of feed in the gut (Arellano-Carbajal and Olmos-Soto, 2002; Priest, 1977). Although, we did not determine this in our research, several studies on shrimp including *P. monodon* have demonstrated that feeding the animals with *Bacillus* species enhanced the digestive enzyme activities in the gut and improved the

growth performance of the animal after the *Bacillus* treatment (Nimrat, et al., 2013; Ziaei-Nejad, et al., 2006; Zokaeifar, et al., 2012).

Taken together, the growth promoting effect of PHB-accumulating *Bacillus* spp. through the possible synergistic effects of amorphous PHB and probiotic *Bacillus* is indeed promising for crustacean culture. However, the observed inconsistencies on the growth effect of PHB in the different stages of the shrimp (e.g. the larval stages vs postlarval stages) or in different aquaculture species suggests further investigation to optimize the application of amorphous PHB in cultured aquatic animals.

8.5 ADVANTAGES OF USING PHB-ACCUMULATING *BACILLUS* SPP. AS BIOLOGICAL CONTROL AGENTS

8.5.1 POTENTIAL SYNERGISTIC EFFECTS OF THE AMORPHOUS PHB AND THE PROBIOTIC BACILLUS

As described above, the application of *Bacillus* spp. containing significant amount of amorphous PHB can have several mode of actions (refer Figure 8.2). This strategy is considered more advantageous and efficient considering that the combination of these actions maximizes the chances of success in controlling diseases. As discussed in the review of this study (Chapter 2), the concept of disease occurrence in aquaculture is in concert with the complex interaction of the cultured animal (host), the culture environment and the pathogen. Hence, a biological agent that could target more than one of these intertwining factors is a desirable strategy for attaining a successful shrimp/fish culture. The mode of action of amorphous PHB is described above and on top of that, depending on the *Bacillus* strain that is used as a PHB carrier, the *Bacillus* strain can either act through the pathogen, to the host animal and/or to the culture environment (see review on chapter 2.5 on *Bacillus* species as probiotics), and thus could add up to the whole (beneficial) effects. Our preliminary investigation showed that the PHB-accumulating *Bacillus* sp. JL47 cannot produce inhibitory compounds that can deter growth of the pathogenic *V. campbellii* (non-published results). Furthermore, later findings revealed that the *Bacillus* strain has the capacity to degrade quorum sensing molecules such as

acyl homoserine lactone (AHL) (non-published results). In this study, more of the work was conducted on the effects of the PHB-accumulating *Bacillus* strains in the host (e.g. immune-enhancing effect, growth and survival) but taking into account that this *Bacillus* strain (JL47) can also directly act on the pathogen by decreasing its virulence through quorum quenching presents a perfect example of a multiple mode of action brought by a PHB-accumulating *Bacillus* strain.

8.5.2 EFFICIENCY OF AMORPHOUS PHB AS COMPARED WITH CRYSTALLINE PHB

While it has already been proven that crystalline PHB (i.e. extracted form) can be used as a biocontrol agent for aquaculture, previous findings (Halet, et al., 2007) including this research (Chapter 4 and 5) showed that that the application of amorphous PHB contained in a whole cell (e.g. probiotic *Bacillus*) can be considered more efficient than using crystalline PHB. The effectiveness of using amorphous PHB compared with crystalline PHB could be due to the efficient degradation of amorphous PHB than with crystalline PHB. This difference can be due to (1) the (bio)physical state and (2) the smaller particle size of the amorphous PHB inside the bacterial cell as compared with crystalline PHB. It was suggested that crystallinity of the compound affects its degradability such that an increasing crystallinity reduces the degradability of the biopolymer (Tokiwa, et al., 2009). Indeed, it has been demonstrated that amorphous PHB can be hydrolysed 30 times faster than crystalline PHB because the hydrolytic enzymes can diffuse faster in the amorphous phase than in crystalline phase (Yu, et al., 2005). Furthermore, the smaller particle size of amorphous PHB inside the bacterial cell (<0.5 µm in diameter (Anderson and Dawes, 1990)) as compared to crystalline PHB (*ca.* 30 µm (Defoirdt, et al., 2007)) could have an effect on the depolymerisation efficiency of the compound such that the smaller particle amorphous PHB can be more susceptible to enzymatic and microbial degradation as compared to crystalline PHB (Anderson and Dawes, 1990). Nevertheless, the effectiveness of crystalline PHB can be improved through the addition of PHB degrading bacteria where PHB can be depolymerized by extracellular enzymes secreted by the PHB-degrading bacteria. The work of Liu, et al. (2010b) demonstrated that the protective effect of feeding the gnotobiotic *Artemia* with PHB and a PHB-degrading bacterium resulted in an improved survival than when feeding

the *Artemia* with crystalline PHB alone. The advantage of using amorphous PHB added as intracellular PHB does not anymore requires the aid of PHB degraders to effectively depolymerize the compound.

Moreover, it is also worth mentioning that supplementation of crystalline PHB in the feed at a higher dose can be economical unfeasible considering that the prevailing price of PHB (*c.* 4 € kg^{-1}) is about 4 times higher than a kg of fish feed (Johnson, et al., 2010). Therefore, the use of bacterial cells that contain amorphous PHB can be a best alternative for crystalline PHB. As described above, the higher efficiency of amorphous PHB required only a smaller amount to be used to obtain a similar significant effect with crystalline PHB. In our gnotobiotic *Artemia* experiment (Chapter 4), the amorphous PHB used was 82-410 times lower than the suggested effective level of crystalline PHB for *Artemia* (Defoirdt, et al., 2007), in our shrimp culture experiment (Chapter 5) the amorphous PHB added in the feed was 73-88 times lower than the suggested crystalline PHB level observed in shrimp (Duan, et al., 2017), in chapter 6 of this study, the enrichment of amorphous PHB in *Artemia* as feed for *P. monodon* postlarvae was 2 and 100 times lower compared to the enrichment of crystalline PHB in *Artemia* for *P. monodon* (Ludevese-Pascual, et al., 2017) and *M. rosenbergii* (Nhan, et al., 2010) (post)larvae, respectively. Moreover, based from the data of Mudliar, et al. (2008) it can be calculated that the chemicals used for the extraction process for producing pure PHB requires 30% of the total operation cost while with amorphous PHB, the extraction process is already omitted. Taken together, the price of PHB still contained within the bacterial cells is considered to be 40% lower than that of pure extracted (crystalline) PHB (De Schryver, 2010).

8.5.3 APPLICATION OF SPORES AS PRECURSOR OF SUPERIOR PHB-ACCUMULATING BACILLUS PROBIOTICS

The application of *Bacillus* spores as precursor of vegetative PHB-accumulating *Bacillus* can be an interesting strategy. The spores can be added directly during feed preparation and since spores are heat stable, it can withstand the high temperature applied during feed drying. Upon ingestion, the spores can be used partly as feed by the animals or it can also germinate within the GIT allowing the growth of the bacteria and

accumulate PHB which can then be available to the host. However, this strategy needs to be demonstrated to completely realize its significance. Furthermore, spore application will allow long storage of the PHB-accumulating probiotic precursor in the feed without compromising its viability while this can always be not the case for vegetative cells formulated in feed (Aly, et al., 2008).

Another interesting approach is adding spores of PHB-accumulating *Bacillus* in the water which can have a bioremediation effect. Spores can germinate in a nutrient-rich condition allowing the growth of the bacteria while utilizing the organic and nitrogen waste present in the culture water. The spores can as well end up in the GIT of the cultured animal, germinate and may further provide beneficial effects to the host. However, the significance of this strategy needs to be demonstrated in further research.

8.5.4 Incorporation of PHB-accumulating Bacillus spp. in the biofloc technology

The Biofloc technology (BFT) is a technique by which the growth of heterotrophic bacterial biomass is stimulated through the addition of extra carbon in the (aquaculture) system resulting in microbial protein production and uptake of inorganic nitrogen (Avnimelech, 1999). This technology brings major added value to aquaculture by providing nutritional value and water quality control. It has been described that *c.* 2-20% of the organic fraction in the bioflocs is believed to be living microbial cells while *c.* 70% of the total floc weight is total organic matter (Wilén, et al., 2003). The heterotrophic microbes present in the floc is an interesting avenue to incorporate the application of PHB-accumulating bacteria (e.g. *Bacillus* spp.) such that these heterotrophic microorganisms could be “trained” to accumulate significant amounts of PHB through C/N ratio manipulation (Avnimelech, 1999). Indeed, a previous study demonstrated that around 0.9%-16% PHB of dry matter can be produced in bioflocs depending on the C/N ratio and carbon substrate used (De Schryver and Verstraete, 2009). Detection of PHB in bioflocs suggests that PHB-accumulating microorganisms are therefore present. According to Anand, et al. (2014), the major microorganisms present in the bioflocs include fungus, *Vibrio, LactoBacillus* and *Bacillus* species wherein the density of the

Bacillus spp. could reach as high as 10^6 CFU mL^{-1}. The PHB-accumulating *Bacillus* strains identified in this research were obtained from shrimp pond sediments and since bioflocs are normally prepared in ponds or are initially inoculated with pond sediments (Avnimelech, 1999), it can be conceivable that PHB-accumulating *Bacillus* species are among the major actors present in bioflocs and probably are among the "normal residents" in any biofloc system. Further understanding on their (i.e. PHB-accumulating *Bacillus* spp.) presence and integration (e.g. aggregation mechanism) in the biofloc system will unravel further their full potential.

Alternatively, bioflocs production can also be directly stimulated with specific PHB-accumulating *Bacillus* strain(s) e.g. if these strain(s) are known high PHB accumulators and that were proven to have probiotic effects in cultured animals (e.g. *Bacillus* sp. JL47). The research of Crab (2010) showed that inoculating the bioflocs with probiotic *Bacillus* resulted in (5x) reduction of *Vibrio* load in the shrimp tank rearing water. The inoculation of probiotic *Bacillus* strain in this case demonstrated that biofloc can be manipulated to act as a biocontrol strategy and not only as an additional nutrient source and/or a bioremediation strategy. Furthermore, inoculation of PHB-accumulating *Bacillus* strains with immunostimulating characteristic is an interesting feature to investigate wherein the biofloc is manipulated to contain immunostimulating probiotic bacteria or bacterial compounds. Enhancement of the immunity of the cultured animal through biofloc containing immunostimulating bacteria and bacterial products may provide broad resistance to diseases. Nevertheless, the strategy of inoculating the biofloc system with desired microorganisms to produce a multi-functional biofloc needs further investigation in order to confirm these beneficial effects and to optimize the potential of the strategy.

8.6 Exploring the importance of phasin (PhaP) protein

Phasins constitute the major components of the PHA-granule associated proteins (PGAPs) that stabilizes PHA granules and has several functions in PHA synthesis, PHA localization, granule size and PHA depolymerisation (Kuchta, et al., 2007; Pötter and Steinbüchel, 2006; Wieczorek, et al., 1995; York, et al., 2001). Furthermore, the protective characteristic of phasins towards stress was also observed. In a previous

study, it was demonstrated that the presence of recombinant phasin ($PhaP_{Az}$) in *E. coli* cells reduced the expression of stress-related genes (i.e. *ibpA and dnaK*) and that the phasin protein protected the bacteria against heat shock and superoxide stress by paraquat (de Almeida, et al., 2011). In another study, both *in vitro* and *in vivo* experiments demonstrated chaperone-like functions of $PhaP_{AZ}$ in recombinant *E. coli* (Mezzina, et al., 2015). From these studies, it was suggested that phasins may have general protective properties against stresses (de Almeida, et al., 2011; Mezzina, et al., 2015). In this research, we have tested further if the phasin protein cloned from the Phap gene of PHB-accumulating *Bacillus* sp. JL47 ($PhaP_{\textit{Bacillus}\text{spJL47}}$) has a direct protective effect in the gnotobiotic *Artemia* challenged with pathogenic *V. campbellii* LMG 21363. Our results however showed no protective effects in *Artemia* receiving the *E. coli* containing the overproduced $PhaP_{\textit{Bacillus}\text{spJL47}}$. Thus, we suggest that under our experimental conditions used the protein did not directly contribute to the protective effects displayed by amorphous in our gnotobiotic *Artemia* (Chapter 4) and shrimp culture experiment (Chapter 5). Conversely, the contribution of phasins in the protective effect of PHB in cultured (aquatic) animals can still be determined in a different approach wherein the protein can be purified and then artificially bind/coat to the surface of the extracted PHB granules. The protective performance of a crystalline PHB coated with and without phasin can then be compared.

Furthermore, phasins being an amphiphilic protein (i.e. compounds containing hydrophilic and hydrophobic ends) can be an interesting compound to investigate considering that these type of compounds are mostly used as (bio) surfactants. Biosurfactants are surface-active agents that have been used in food industry, cosmetics and as emulsifiers of hydrocarbons and also used in bioremediation application (e.g. removal of heavy metals, aids in oil-spill degradation, etc.). Interestingly, biosurfactants were also suggested to have biological applications among which are as antimicrobial agents (i.e. antibacterial, antifungal and/or antiviral activities), immunoregulators and immunomodulators (Cameotra and Makkar, 2004). Previous study shows that the phasin from *A. hydrophila* 4AK4 was identified to be an excellent biosurfactant (Wei, et al., 2011a). Furthermore, the repressor protein PhaR also a PHA granule associated protein from *A. hydrophila* 4AK4 was also identified to be an excellent biosurfactant and was

identified to have a strong bactericidal capacity (Ma, et al., 2013). Hence, in the relevance of this work, investigating the antimicrobial property as well as the immunological effects of phasins (or other PGAPs) in purified form is suggested. The probability that there is a difference in the effectiveness of the protein when delivered in purified form as compared when it is delivered through the *E. coli* cells is imaginable.

8.7 General conclusions

The aquaculture industry has been dependent to synthetic antimicrobials as disease control agents. However due to its unsustainability and further threats to the human health, considerable efforts have been made to look for a more sustainable, environment-friendly, yet equally effective alternatives. The application of PHB-accumulating *Bacillus* spp. as biocontrol agents for crustacean (larvi) culture was explored in this research, and indeed its beneficial effects were demonstrated and its interesting potentials were explained. From this research, the following conclusions are the drawn:

- MALDI-TOF MS can be used as a tool in the process of rapid isolation and characterization of superior PHB-accumulating *Bacillus* species from shrimp pond sediments.
- *Bacillus* spp. with superior PHB accumulation capability can improve the growth, survival and robustness of *P. monodon* postlarvae.
- The amorphous PHB present in probiotic *Bacillus* is an important determinant in the observed beneficial effects of PHB-accumulating (probiotic) *Bacillus* species as effective biocontrol agents.
- In this research, the PHB-accumulating *Bacillus* sp. JL47 can stimulate the innate immune response of the shrimp resulting to enhance robustness and survival and also enhance the growth of the animal. Likewise, the PHB-accumulating *Bacillus* sp. JL47 can also interfere in the QS activity of the pathogen through its AHL degradation capability. With this information, in general, the application of PHB-accumulating *Bacillus* species as biocontrol agents is a very interesting strategy

because apart from the amorphous PHB that acts itself as a biocontrol agent, the bacterial carrier could also provide other beneficial effects to the cultured animal.

- Using the current experimental conditions, the recombinant phasin protein from the *Bacillus* sp. JL47 did not provide a direct protective effect in the gnotobiotic *Artemia* during a pathogenic *V. cambellii* LMG 21363 challenge.

8.8 FUTURE PERSPECTIVES

In this research, we have demonstrated the beneficial effects of using PHB-accumulating *Bacillus* spp. as new biocontrol agents for crustacean (larvi) culture. Further research on the following aspects is however suggested to attain full understanding on PHB-accumulating *Bacillus* species as biocontrol agents and to explore the full potential of PHB-accumulating *Bacillus* species as a biocontrol strategy.

1. In our results in chapter 4, we have established that the amorphous PHB present in the *Bacillus* is a key determinant in the beneficial effects brought by PHB-accumulating *Bacillus* to the cultured animal. However, it is still worth verifying this observation by conducting the following experiments to clearly delineating the contribution of the stored amorphous PHB and the probiotic activity of the *Bacillus* in the survival, growth and robustness effects of the animal. The following experiments are therefore suggested:
    - Develop a non-PHB synthesizing *Bacillus* mutant (by silencing/deactivating the genes responsible for PHB synthesis) from wild-type PHB-accumulating probiotic *Bacillus* strain (e.g. *Bacillus* sp. JL47) and compare their beneficial effects in terms of survival, growth and robustness effects in the host. The usage of such a non-PHB synthesizing *Bacillus* mutant would be a good control to be used in the *Artemia* (Chapter 4) and shrimp (Chapter 6) experiments and would facilitate unequivocal interpretation of the results obtained in this research.

- In chapter 6, we have demonstrated the immune-enhancing effect of the PHB-accumulating *Bacillus* sp. JL47 in shrimp. It is also worth knowing if the immune response of the shrimps fed the PHB-accumulating *Bacillus* sp. JL47 differs when the bacterium contains low or high amorphous PHB or bacterium with or without PHB. Furthermore, a complete characterization of MAMPs or other expressed proteins in JL47 with low and high PHB content and on wild-type strain versus PHB-mutant strain (without PHB) is suggested to further facilitate the interpretation of the immune response (e.g. through MALDI-TOF MS). This experiment will further investigate if the amorphous PHB present affects the probiotic capability of the *Bacillus* strain.
- Investigating other immune related genes like those responsible for the detection of the pathogen i.e. receptor proteins (e.g. LPS receptor proteins, beta-glucan binding receptor proteins, Toll-like receptor proteins, peptidoglycan binding receptor proteins) can also be done.
- Previous studies suggest that bacteria capable of accumulating reserve materials (i.e. PHB) have greater advantage for survival and proliferation in their environment. It is therefore interesting to investigate the colonization potential of PHB-accumulating *Bacillus* spp. containing high amount of amorphous PHB in the gut of cultured aquatic animals. This can be visually monitored by advanced tools like fluorescence in situ hybridization (FISH).

2. Determine the safety, economic cost and benefit and industrial application of PHB-accumulating *Bacillus* spp. as biocontrol agents for aquaculture. To address this aspect, the following research is suggested:
    - In this research, we have identified *Bacillus* sp. JL47 as the superior PHB-accumulating *Bacillus* strain. However, its identity falls in the *Bacillus cereus* group which can be considered harmful to human health if the strain is capable of producing enterotoxins and/or capable of transferring antibiotic resistance. Hence, to safely use the *Bacillus* sp. JL47 strain as probiotics for aquaculture, the suggested guidelines administered by the European Food Safety Authority (EFSA) through the Panel on Additives and Products

or Substances used in Animal Feed (FEEDAP) should be met, among which is the sequencing of the full genome of the strain (including chromosomes and plasmids) and search for genes coding for enterotoxins and cereulide synthase (i.e. non haemolytic enterotoxin (nhe), hemolysin BL (hbl), cytotoxin K (cytK), cereulide (ces)). If there is evidence of homology, the non-functionality of the genes (e.g. mutation/deletion) should be demonstrated (FEEDAP, 2014).

- Search for other PHB-accumulating *Bacillus* strains with superior PHB-accumulating capability that are known non-harmful to humans and/or that has been approved to be used as probiotics for aquaculture (e.g. *B. subtilis*, *B. cereus* var. *toyoi*, *B. lichenoformis*; refer (Balcázar, et al., 2006)). Likewise, aside from having a superior PHB accumulating capability, the selection of *Bacillus* spp. having more than one probiotic characteristics (e.g. QS molecule degraders, produce antimicrobial compounds, immunostimulatory, etc.) are also interesting criteria to look at. Other possibility could be introducing the PHB synthesis genes from superior PHB accumulating bacteria (e.g. *A. eutrophus*) to *Bacillus* strains that are already approved for use as probiotics in aquaculture.
- Optimize the culture condition (such as C/N ratio) of *Bacillus* sp. JL47 for maximum PHB production and integrating the utilization of cheap carbon sources (e.g. agricultural wastes, waste metabolites from *Artemia* hatching) for a more economically feasible production of PHB-accumulating *Bacillus* probiotic.
- For industrial and practical application purposes, the applicability of *Bacillus* spores as precursor of superior PHB-accumulating *Bacillus* strain is worth investigating. Hence, comparing the beneficial effects of using spores and the vegetative form of a superior PHB-accumulating *Bacillus* strain applied for shrimp culture should be established first. As discussed above, the following considerations will be determined such as if applying spores to the nutrient-rich culture water will have some bioremediation effects and if the

spores will end up germinating in the GIT and demonstrate beneficial effects to the animal.

3. Other interesting findings from this research that needs further investigation:
    - The results in chapter 3 suggest that MALDI-TOF MS patterns can be used as a tool to identify bacteria with high PHB accumulating capacity. Further research should confirm this observation by doing proteomic analysis such as investigating abundant proteins that are commonly detected in high PHB-accumulating *Bacillus* which could possibly be used as biomarker protein(s) to delineate high, low and non-PHB accumulating bacteria.
    - In chapter 7, using the current experimental conditions, the phasin protein did not show any protection to the *Vibrio*-challenged gnotobiotic *Artemia*. However, another approach for experimentation can also be done to clearly elucidate the contribution of phasin protein to the effects of amorphous PHB. First, is to purify the protein since it was produced by a His-tag. It should also be possible to recoat the crystalline PHB with phasin and then verify if the crystalline PHB supplemented/ coated with phasin would display a better activity than with only crystalline PHB. Furthermore, the antimicrobial and/or immunostimulating capability of using purified phasin (without PHB) can also be investigated considering that amphiphilic proteins such as phasin where known to have these characteristics including some other reported biosurfactants (Ma, et al., 2013). If the recombinant *E. coli* containing the overproduced phasin is used, the bioactivity of the phasin needs to be verified first.

REFERENCES

Abe, T., Kobayashi, T., Saito, T., 2005. Properties of a novel intracellular poly (3-hydroxybutyrate) depolymerase with high specific activity (PhaZd) in *Wautersia eutropha* H16. Journal of Bacteriology. 187, 6982-6990.

Abrams, S.A., Griffin, I.J., Hawthorne, K.M., Liang, L., Gunn, S.K., Darlington, G., Ellis, K.J., 2005. A combination of prebiotic short-and long-chain inulin-type fructans enhances calcium absorption and bone mineralization in young adlescents. American Journal of Clinical Nutrition. 82, 471-476.

Adams, C.A., 2010. The probiotic paradox: live and dead cells are biological response modifiers. Nutrition Research Reviews. 23, 37-46.

Adams, J.R., Bonami, J.R., (eds.) 1991: Atlas of invertebrate viruses. CRC press, Boca Raton, FL. p 684.

Agata, N., Ohta, M., Mori, M., Isobe, M., 1995. A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. FEMS Microbiology Letters. 129, 17-19.

Aguilar, M., Sáez, J., Lloréns, M., Soler, A., Ortuno, J., Meseguer, V., Fuentes, A., 2005. Improvement of coagulation–flocculation process using anionic polyacrylamide as coagulant aid. Chemosphere. 58, 47-56.

Aguirre-Guzmán, G., Mejia Ruíz, H., Ascencio, F., 2004. A review of extracellular virulence product of *Vibrio* species important in diseases of cultivated shrimp. Aquaculture Research. 35, 1395-1404.

Aguirre-Guzmán, G., Sánchez-Martínez, J.G., Pérez-Castañeda, R., Palacios-Monzón, A., Trujillo-Rodríguez, T., La Cruz-Hernández, D., Ivan, N., 2010. Pathogenicity and infection route of *Vibrio parahaemolyticus* in American white shrimp, *Litopenaeus vannamei*. Journal of the World Aquaculture Society. 41, 464-470.

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

Akinbowale, O.L., Peng, H., Barton, M., 2006. Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia. Journal of Applied Microbiology. 100, 1103-1113.

Aldor, I.S., Keasling, J.D., 2003. Process design for microbial plastic factories: metabolic engineering of polyhydroxyalkanoates. Current Opinion in Biotechnology. 14, 475-483.

Aly, S.M., Abd-El-Rahman, A.M., John, G., Mohamed, M.F., 2008. Characterization of some bacteria isolated from *Oreochromis niloticus* and their potential use as probiotics. Aquaculture. 277, 1-6.

Amor, S.R., Rayment, T., Sanders, J.K.M., 1991. Poly(β-hydroxybutyrate) *in vivo*: NMR and X-ray characterization of the elastomeric state. Macromolecules. 24, 4583-4588.

Amparyup, P., Charoensapsri, W., Tassanakajon, A., 2013. Prophenoloxidase system and its role in shrimp immune responses against major pathogens. Fish & Shellfish Immunology. 34, 990-1001.

Anand, P.S., Kohli, M., Kumar, S., Sundaray, J., Roy, S.D., Venkateshwarlu, G., Sinha, A., Pailan, G., 2014. Effect of dietary supplementation of biofloc on growth performance and digestive enzyme activities in *Penaeus monodon*. Aquaculture. 418, 108-115.

Anderson, A.J., Dawes, E.A., 1990. Occurence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiological Reviews. 54, 450-472.

Angulo, F., 1999. Use of antimicrobial agents in aquaculture: potential for public health impact. Public Health Service. Department of Health & Human Services, CDC. Atlanta, USA.p. 18.

Arellano-Carbajal, F., Olmos-Soto, J., 2002. Thermostable α-1,4- and α-1,6-glucosidase enzymes from *Bacillus* sp. Isolated from a marine environment. World Journal of Microbiology & Biotechnology. 18, 791-795.

Armstrong, D.A., Chippendale, D., Knight, A.W., Colt, J.E., 1978. Interaction of ionized and un-ionized ammonia on short-term survival and growth of prawn larvae, *Macrobrachium rosenbergii*. The Biological Bulletin. 154, 15-31.

Arnold, R.J., Reilly, J.P., 1999. Observation of *Escherichia coli* Ribosomal Proteins and Their Posttranslational Modifications by Mass Spectrometry. Analytical Biochemistry. 269, 105-112.

Ashley, P.J., 2007. Fish welfare: current issues in aquaculture. Applied Animal Behaviour Science. 104, 199-235.

Attramadal, K.J., Minniti, G., Øie, G., Kjørsvik, E., Østensen, M.-A., Bakke, I., Vadstein, O., 2016. Microbial maturation of intake water at different carrying capacities affects microbial control in rearing tanks for marine fish larvae. Aquaculture. 457, 68-72.

Austin, B., Baudet, E., Stobie, M., 1992. Inhibition of bacterial fish pathogens by *Tetraselmis suecica*. Journal of Fish Diseases. 15, 55-61.

Austin, B., Pride, A., Rhodie, G., 2003. Association of a bacteriophage with virulence in *Vibrio harveyi*. Journal of Fish Diseases. 26, 55-58.

Avendaño-Herrera, R., Núñez, S., Barja, J.L., Toranzo, A.E., 2008. Evolution of drug resistance and minimum inhibitory concentration to enrofloxacin in *Tenacibaculum maritimum* strains isolated in fish farms. Aquaculture International. 16, 1-11.

Avnimelech, Y., 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture. 176, 227-235.

Azain, M., 2004. Role of fatty acids in adipocyte growth and development. Journal of Animal Science. 82, 916-924.

Bairagi, A., Ghosh, K.S., Sen, S.K., Ray, A.K., 2002. Enzyme producing bacterial flora isolated from fish digestive tracts. Aquaculture International. 10, 109-121.

Balcazar, J.L., Rojas-Luna, T., 2007. Inhibitory Activity of Probiotic *Bacillus subtilis* UTM 126 Against *Vibrio* Species Confers Protection Against *Vibriosis* in Juvenile Shrimp (*Litopenaeus vannamei*). Current Microbiology. 55 (5), 409-412.

Balcázar, J.L., De Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., Muzquiz, J.L., 2006. The role of probiotics in aquaculture. Veterinary Microbiology. 114, 173-186.

Balcázar, J.L., De Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Gironés, O., Muzquiz, J.L., 2007. Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout (*Oncorhynchus mykiss*). FEMS Immunology and Medical Microbiology. 51, 185-193.

Bandyopadhyay, P., Mohapatra, P.K.D., 2009. Effect of a probiotic bacterium *Bacillus circulans* PB7 in the formulated diets: on growth, nutritional quality and immunity of *Catla catla* (Ham.). Fish Physiology and Biochemistry. 35, 467-478.

Barnard, G.N., Sanders, J.K.M., 1989. The Poly-β-hydroxybutyrate Granule *in Vivo*. The Journal of Biological Chemistry. 264, 3286-3291.

Baruah, K., 2012. Induced heat shock protein production protects *Artemia* against *Vibriosis*, PhD thesis, Ghent University, Ghent, Belgium, pp. 209.

Baruah, K., Ranjan, J., Sorgeloos, P., Bossier, P., 2010. Efficacy of heterologous and homologous heat shock protein 70s as protective agents to *Artemia franciscana* challenged with *Vibrio campbellii*. Fish & Shellfish Immunology. 29, 733-739.

Baruah, K., Ranjan, J., Sorgeloos, P., MacRae, T.H., Bossier, P., 2011. Priming the prophenoloxidase system of *Artemia franciscana* by heat shock proteins protects against *Vibrio campbellii* challenge. Fish & Shellfish Immunology. 31, 134-141.

Baruah, K., Norouzitallab, P., Roberts, R.J., Sorgeloos, P., Bossier, P., 2012. A novel heat-shock protein inducer triggers heat shock protein 70 production and protects *Artemia franciscana* nauplii against abiotic stressors. Aquaculture. 334, 152-158.

Baruah, K., Huy, T.T., Norouzitallab, P., Niu, Y., Gupta, S.K., De Schryver, P., Bossier, P., 2015. Probing the protective mechanism of poly-ß-hydroxybutyrate against *Vibriosis* by using gnotobiotic *Artemia franciscana* and *Vibrio campbellii* as host-pathogen model. Scientific reports. 5, 9427.

Bassler, B.L., Wright, M., Showalter, R.E., Silverman, M.R., 1993. Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. Molecular Microbiology. 9, 773-786.

Bateman, A., Coin, L., Durbin, R., Finn, R.D., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Moxon, S., Sonnhammer, E.L., 2004. The Pfam protein families database. Nucleic Acids Research. 32, D138-D141.

Beecher, D.J., Macmillan, J., 1991. Characterization of the components of hemolysin BL from *Bacillus cereus*. Infection and Immunity. 59, 1778-1784.

Benoit, T.G., Wilson, G.R., Baugh, C.L., 1990. Fermentation during growth and sporulation of *Bacillus thuringiensis* HD-1. Letters in Applied Microbiology. 10, 15-18.

Berger, E., Ramsay, B., Ramsay, J., Chavarie, C., Braunegg, G., 1989. PHB recovery by hypochlorite digestion of non-PHB biomass. Biotechnology techniques. 3, 227-232.

Biswas, G., Korenaga, H., Nagamine, R., Takayama, H., Kawahara, S., Takeda, S., Kikuchi, Y., Dashnyam, B., Kono, T., Sakai, M., 2013. Cytokine responses in the Japanese pufferfish (*Takifugu rubripes*) head kidney cells induced with heat-killed probiotics isolated from the Mongolian dairy products. Fish & Shellfish Immunology. 34, 1170-1177.

Bonami, J.R., Hasson, K.W., Mari, J., Poulos, B.T., Lightner, D.V., 1997. Taura syndrome of marine penaeid shrimp: characterization of the viral agent. Journal of General Virology. 78, 313-319.

Boonyaratpalin, S., Supamattaya, K., Kasornchandra, J., Direkbusaracom, S., Aekpanithanpong, U., Chantanachooklin, C., 1993. Non-Occluded Baculo-like Virus, the Causative Agent of Yellow Head Disease in the Black Tiger Shrimp (*Penaeus monodon*). Fish Pathology. 28, 103-109.

Borah, B., Thakur, P.S., Nigam, J.N., 2002. The influence of nutritional and environmental conditions on the accumulation of poly-β-hydroxybutyrate in *Bacillus mycoides* RLJ B-017. Journal of Applied Microbiology. 92, 776-783.

Bowden, G.A., Paredes, A.M., Georgiou, G., 1991. Structure and morphology of protein inclusion bodies in *Escherichia coli*. Nature Biotechnology. 9, 725-730.

Brandl, H., Gross, R.A., Lenz, R.W., Fuller, R.C., 1990. Plastics from bacteria and for bacteria: poly (β-hydroxyalkanoates) as natural, biocompatible, and biodegradable polyesters, Microbial Bioproducts. Springer, pp. 77-93.

Braun, V., Braun, M., 2002. Active transport of iron and siderophore antibiotics. Current Opinion in Microbiology. 5, 194-201.

Braunegg, G., Sonnleitner, B., Lafferty, R., 1978. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. European journal of applied microbiology and biotechnology. 6, 29-37.

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

Bricknell, I., Dalmo, R.A., 2005. The use of immunostimulants in fish larval aquaculture. Fish & Shellfish Immunology. 19, 457-472.

Brigham, C.J., Reimer, E.N., Rha, C., Sinskey, A.J., 2012. Examination of PHB depolymerases in *Ralstonia eutropha*: further elucidation of the roles of enzymes in PHB homeostasis. AMB express. 2, 1.

Brigham, C.J., Budde, C.F., Holder, J.W., Zeng, Q., Mahan, A.E., Rha, C., Sinskey, A.J., 2010. Elucidation of β-oxidation pathways in *Ralstonia eutropha* H16 by examination of global gene expression. Journal of Bacteriology. 192, 5454-5464.

Broughton, E.I., Walker, D.G., 2009. Prevalence of antibiotic-resistant *Salmonella* in fish in Guangdong, China. Foodborne pathogens and disease. 6, 519-521.

Browdy, C., Hargreaves, J., Hoang, T., Avnimelech, Y., 2014. Proceedings of the Biofloc Technology and Shrimp Disease Workshop. Dec 9-10, 2013, Ho Chi Minh City, Vietnam. The Aquaculture Engineering Society, Copper Hill, VA, USA. www.aesweb.org/shrimp_health.php

Browdy, C.L., 1998. Recent developments in penaeid broodstock and seed production technologies: improving the outlook for superior captive stocks. Aquaculture. 164, 3-21.

Brown, A.J., Goldsworthy, S.M., Barnes, A.A., Eilert, M.M., Tcheang, L., Daniels, D., Muir, A.I., Wigglesworth, M.J., Kinghorn, I., Fraser, N.J., 2003. The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. Journal of Biological Chemistry. 278, 11312-11319.

Brown, C., Bolin, C., 2000. Emerging infectious diseases of animals: an overview. In Emerging diseases of animals, ASM Press, Washington, DC, USA. 1-5.

Brown, M.R., Barrett, S.M., Volkman, J.K., Nearhos, S.P., Nell, J.A., Allan, G.L., 1996. Biochemical composition of new yeasts and bacteria evaluated as food for bivalve aquaculture. Aquaculture. 143, 341-360.

Burrells, C., Williams, P., Forno, P., 2001. Dietary nucleotides: a novel supplement in fish feeds: 1. Effects on resistance to disease in salmonids. Aquaculture. 199, 159-169.

Byrom, D., 1987. Polymer synthesis by microorganisms: technology and economics. Trends in Biotechnology. 5, 246-250.

Cabello, F.C., 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environmental Microbiology. 8, 1137-1144.

Cahill, M.M., 1990. Bacterial flora of fishes: A review. Microbial Ecology. 19, 21-41.

Cahu, C., Infante, J.Z., Peres, A., Quazuguel, P., Le Gall, M., 1998. Algal addition in sea bass (*Dicentrarchus labrax*) larvae rearing: effect on digestive enzymes. Aquaculture. 161, 479-489.

Cai, S., Cai, L., Liu, H., Liu, X., Han, J., Zhou, J., Xiang, H., 2012. Identification of the haloarchaeal phasin (PhaP) that functions in polyhydroxyalkanoate accumulation and granule formation in *Haloferax mediterranei*. Applied and Environmental Microbiology. 78, 1946-1952.

Cam, D.T.V., Nhan, D.T., Ceuppens, S., Hao, N.V., Dierckens, K., Wille, M., Sorgeloos, P., Bossier, P., 2009. Effect of N-acyl homoserine lactone-degrading enrichment cultures on *Macrobrachium rosenbergii* larviculture. Aquaculture. 294, 5-13.

Cameotra, S.S., Makkar, R.S., 2004. Recent applications of biosurfactants as biological and immunological molecules. Current opinion in microbiology. 7, 262-266.

Camilli, A., Bassler, B.L., 2006. Bacterial small-molecule signaling pathways. Science. 311, 1113-1116.

Carrio, M., Corchero, J., Villaverde, A., 1998. Dynamics of *in vivo* protein aggregation: building inclusion bodies in recombinant bacteria. FEMS Microbiology Letters. 169, 9-15.

Carrió, M., Villaverde, A., 2003. Role of molecular chaperones in inclusion body formation. FEBS Letters. 537, 215-221.

Castilho, L.R., Mitchell, D.A., Freire, D.M., 2009. Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. Bioresource Technology. 100, 5996-6009.

Castro-Sowinski, S., Burdman, S., Matan, O., Okon, Y., 2010. Natural functions of bacterial polyhydroxyalkanoates. In Chen GGQ (ed) Plastics from bacteria: natural functions and application. Microbiology monographs, 14 Springer, Heidelberg, Berlin. pp 39-61.

Catacutan, M.R., 1991. Apparent digestibility of diets with various carbohydrate levels and the growth response of *Penaeus monodon*. Aquaculture. 95, 89-96.

Ceccaldi, H., 1997. Anatomy and physiology of the digestive system. Crustacean nutrition. 6, 261-291.

Cerenius, L., Lee, B.L., Söderhäll, K., 2008. The proPO-system: pros and cons for its role in invertebrate immunity. Trends in Immunology. 29, 263-271.

Chávez-Crooker, P., Obreque-Contreras, J., 2010. Bioremediation of aquaculture wastes. Current Opinion in Biotechnology. 21, 313-317.

Chen, C.-C., Chen, S.-N., 2001. Water quality management with *Bacillus* spp. in the high-density culture of red-parrot fish *Cichlasoma citrinellum* × *C. synspilum*. North American Journal of Aquaculture. 63, 66-73.

Chen, G.-Q., Wu, Q., 2005. The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials. 26, 6565-6578.

Chen, H.-J., Pan, S.-C., Shaw, G.-C., 2009. Identification and characterization of a novel intracellular poly (3-hydroxybutyrate) depolymerase from *Bacillus megaterium*. Applied and Environmental Microbiology. 75, 5290-5299.

Chen, M.-Y., Hu, K.-Y., Huang, C.-C., Song, Y.-L., 2005. More than one type of transglutaminase in invertebrates? A second type of transglutaminase is involved

in shrimp coagulation. Developmental & Comparative Immunology. 29, 1003-1016.

Cherrington, C.A., Hinton, M., Pearson, G., Chopra, I., 1991. Short-chain organic acids at pH 5.0 kill *Escherichia coli* and *Salmonella* spp. without causing membrane perturbation. Journal of Applied Bacteriology. 70, 161-165.

Chin, T.-S., Chen, J.-C., 1987. Acute toxicity of ammonia to larvae of the tiger prawn, *Penaeus monodon*. Aquaculture. 66, 247-253.

Chiu, Y.-C., Lee, L.-L., Chang, C.-N., Chao, A.C., 2007. Control of carbon and ammonium ratio for simultaneous nitrification and denitrification in a sequencing batch bioreactor. International Biodeterioration & Biodegradation. 59, 1-7.

Clark, J.R., March, J.B., 2006. Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials. Trends in Biotechnology. 24, 212-218.

Claydon, M.A., Davey, S.N., Edwards-Jones, V., Gordon, D.B., 1996. The rapid identification of intact microorganisms using mass spectrometry. Nature Biotechnology. 14, 1584-1586.

Clegg, J.S., Jackson, S.A., Van Hoa, N., Sorgeloos, P., 2000. Thermal resistance, developmental rate and heat shock proteins in *Artemia franciscana*, from San Francisco Bay and southern Vietnam. Journal of Experimental Marine Biology and Ecology. 252, 85-96.

Clements, K.D., 1997. Fermentation and gastrointestinal microorganisms in fishes. In: Mackie, R.I., Withe, B.A., Isaacson, R.E. (Eds.) Gastrointestinal Microbiology, Vol. 1, Gastrointestinal Ecosystems and Fermentations. Chapman & Hall Microbiology Series, International Thomson Publishing, New York, USA. pp. 156–198.

Colorni, A., 1989. Fusariosis in the shrimp *Penaeus semisulcatus* cultured in Israel. Mycopathologia. 108, 145-147.

Coorevits, A., De Jonghe, V., Vandroemme, J., Reekmans, R., Heyrman, J., Messens, W., De Vos, P., Heyndrickx, M., 2008. Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms. Systematic and Applied Microbiology. 31, 126-140.

Cox, M.A., Jackson, J., Stanton, M., Rojas-Triana, A., Bober, L., Laverty, M., Yang, X., Zhu, F., Liu, J., Wang, S., 2009. Short-chain fatty acids act as antiinflammatory mediators by regulating prostaglandin E (2) and cytokines. World J Gastroenterol. 15, 5549-5557.

Crab, R., 2010. Bioflocs technology: an integrated system for the removal of nutrients and simultaneous production of feed in aquaculture. PhD thesis, Ghent University, Belgium, pp. 176.

Das, A., Saha, D., Pal, J., 2009. Antimicrobial resistance and *in vitro* gene transfer in bacteria isolated from the ulcers of EUS-affected fish in India. Letters in Applied Microbiology. 49, 497-502.

Das, A., Nakhro, K., Chowdhury, S., Kamilya, D., 2013. Effects of potential probiotic *Bacillus amyloliquifaciens* FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*). Fish & Shellfish Immunology. 35, 1547-1553.

de Almeida, A., Catone, M.V., Rhodius, V.A., Gross, C.A., Pettinari, M.J., 2011. Unexpected stress-reducing effect of PhaP, a poly (3-hydroxybutyrate) granule-

associated protein, in *Escherichia coli*. Applied and Environmental Microbiology. 77, 6622-6629.

De, B.C., Meena, D., Behera, B., Das, P., Mohapatra, P.D., Sharma, A., 2014. Probiotics in fish and shellfish culture: immunomodulatory and ecophysiological responses. Fish Physiology and Biochemistry. 40, 921-971.

De Clerck, E., De Vos, P., 2002. Study of the bacterial load in a gelatine production process focussed on *Bacillus* and related endosporeforming genera. Systematic and Applied Microbiology. 25, 611-617.

de la Peña, L.D., Cabillon, N.A.R., Catedral, D.D., Amar, E.C., Usero, R.C., Monotilla, W.D., Calpe, A.T., Fernandez, D.D., Saloma, C.P., 2015. Acute hepatopancreatic necrosis disease (AHPND) outbreaks in *Penaeus vannamei* and *P. monodon* cultured in the Philippines. Diseases of Aquatic Organisms. 116, 251-254.

De Maio, A., 1999. Heat shock proteins: facts, thoughts, and dreams. Shock. 11, 1-12.

De Schryver, P., 2010. Poly-beta-hydroxybutyrate as a microbial agent in aquaculture. PhD thesis, Ghent University, Ghent, Belgium, pp. 237.

De Schryver, P., Verstraete, W., 2009. Nitrogen removal from aquaculture pond water by heterotrophic nitrogen assimilation in lab-scale sequencing batch reactors. Bioresource Technology. 100, 1162-1167.

De Schryver, P., Defoirdt, T., Sorgeloos, P., 2014. Early mortality syndrome outbreaks: a microbial management issue in shrimp farming? PLoS Pathog. 10, e1003919.

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

De Schryver, P., Dierckens, K., Bahn Thi, Q.Q., Amalia, R., Marzorati, M., Bossier, P., Boon, N., Verstraete, W., 2011. Convergent dynamics of the juvenile European sea bass gut microbiota induced by poly-β-hydroxybutyrate. Environmental Microbiology. 13, 1042-1051.

Dedkova, E.N., Blatter, L.A., 2014. Role of β-hydroxybutyrate, its polymer poly-β-hydroxybutyrate and inorganic polyphosphate in mammalian health and disease. Frontiers in physiology. 5, 260.

Defoirdt, T., Sorgeloos, P., Bossier, P., 2011a. Alternatives to antibiotics for the control of bacterial disease in aquaculture. Current Opinion in Microbiology. 14, 251-258.

Defoirdt, T., Boon, N., Bossier, P., Verstraete, W., 2004. Disruption of bacterial quorum sensing: an unexplored strategy to fight infections in aquaculture. Aquaculture. 240, 69-88.

Defoirdt, T., Bossier, P., Sorgeloos, P., Verstraete, W., 2005. The impact of mutations in the quorum sensing systems of *Aeromonas hydrophila*, *Vibrio anguillarum* and *Vibrio harveyi* on their virulence towards gnotobiotically cultured *Artemia franciscana*. Environmental Microbiology. 7, 1239-1247.

Defoirdt, T., Halet, D., Sorgeloos, P., Bossier, P., Verstraete, W., 2006a. Short-chain fatty acids protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*. Aquaculture. 261, 804-808.

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P., 2007. Alternatives to antibiotics to control bacterial infections: luminescent *Vibriosis* in aquaculture as an example. Trends in Biotechnology. 25, 472-479.

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P., 2009. Short-chain fatty acids and poly-β-hydroxyalkanoates:(New) Biocontrol agents for a sustainable animal production. Biotechnology Advances. 27, 680-685.

Defoirdt, T., Crab, R., Wood, T.K., Sorgeloos, P., Verstraete, W., Bossier, P., 2006b. Quorum sensing-disrupting brominated furanones protect the gnotobiotic brine shrimp *Artemia franciscana* from pathogenic *Vibrio harveyi*, *Vibrio campbellii*, and *Vibrio parahaemolyticus* isolates. Applied and Environmental Microbiology. 72, 6419-6423.

Defoirdt, T., Thanh, L.D., Van Delsen, B., De Schryver, P., Sorgeloos, P., Boon, N., Bossier, P., 2011b. N-acylhomoserine lactone-degrading *Bacillus* strains isolated from aquaculture animals. Aquaculture. 311, 258-260.

Deriase, S.F., El-Gendy, N.S., 2014. Mathematical correlation between microbial biomass and total viable count for different bacterial strains used in biotreatment of oil pollution. Biosciences Biotechnololgy Research Asia. 11, 61-65.

Diana, J.S., 2009. Aquaculture production and biodiversity conservation. Bioscience. 59, 27-38.

Dickinson, D.N., La Duc, M.T., Haskins, W.E., Gornushkin, I., Winefordner, J.D., Powell, D.H., Venkateswaran, K., 2004. Species differentiation of a diverse suite of *Bacillus* spores by mass spectrometry-based protein profiling. Applied and Environmental Microbiology. 70, 475-482.

Dierckens, K., Rekecki, A., Laureau, S., Sorgeloos, P., Boon, N., Van Den Broeck, W., Bossier, P., 2009. Development of a bacterial challenge test for gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. Environmental Microbiology. 11, 526-533.

Diggles, B., Moss, G., Carson, J., Anderson, C., 2000. Luminous *Vibriosis* in rock lobster *Jasus verreauxi* (Decapoda: Palinuridae) *phyllosoma* larvae associated with infection by *Vibrio harveyi*. Diseases of Aquatic Organisms. 43, 127-137.

Dong, Y.-H., Xu, J.-L., Li, X.-Z., Zhang, L.-H., 2000. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. Proceedings of the National Academy of Sciences. 97, 3526-3531.

Dong, Y.-H., Gusti, A.R., Zhang, Q., Xu, J.-L., Zhang, L.-H., 2002. Identification of quorum-quenching N-acyl homoserine lactonases from *Bacillus* species. Applied and Environmental Microbiology. 68, 1754-1759.

Drobniewski, F.A., 1993. *Bacillus cereus* and related species. Clinical Microbiology Reviews. 6, 324-338.

Du, G.-C., Chen, J., Gao, H.-J., Chen, Y.-G., Lun, S.-Y., 2000. Effects of environmental conditions on cell growth and poly-β-hydroxybutyrate accumulation in *Alcaligenes eutrophus*. World Journal of Microbiology & Biotechnology. 16, 9-13.

Duan, Y., Zhang, Y., Dong, H., Zheng, X., Wang, Y., Li, H., Liu, Q., Zhang, J., 2017. Effect of dietary poly-β-hydroxybutyrate (PHB) on growth performance, intestinal health status and body composition of Pacific white shrimp *Litopenaeus vannamei* (Boone, 1931). Fish & Shellfish Immunology. 60, 520-528.

Duckworth, D.H., 1976. " Who discovered bacteriophage?". Bacteriological Reviews. 40, 793.

Dung, T.T., Haesebrouck, F., Tuan, N.A., Sorgeloos, P., Baele, M., Decostere, A., 2008. Antimicrobial susceptibility pattern of *Edwardsiella ictaluri* isolates from natural outbreaks of bacillary necrosis of *Pangasianodon hypophthalmus* in Vietnam. Microbial Drug Resistance. 14, 311-316.

Dykova, I., Lom, J., Fajer, E., 1988. A new haplosporean infecting the hepatopancreas in the penaeid shrimp, *Penaeus vannamei*. Journal of Fish Diseases. 11, 15-22.

Ehling-Schulz, M., Guinebretiere, M.-H., Monthán, A., Berge, O., Fricker, M., Svensson, B., 2006a. Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. FEMS Microbiology Letters. 260, 232-240.

Ehling-Schulz, M., Fricker, M., Grallert, H., Rieck, P., Wagner, M., Scherer, S., 2006b. Cereulide synthetase gene cluster from emetic *Bacillus cereus*: structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pXO1. BMC Microbiology. 6, 1.

Ellis, A.E., (ed) 1988. Fish vaccination. Academic press, London. pp. 255.

Escapa, I.F., Morales, V., Martino, V.P., Pollet, E., Avérous, L., García, J.L., Prieto, M.A., 2011. Disruption of β-oxidation pathway in *Pseudomonas putida* KT2442 to produce new functionalized PHAs with thioester groups. Applied Microbiology and Biotechnology. 89, 1583-1598.

Fagerquist, C.K., Miller, W.G., Harden, L.A., Bates, A.H., Vensel, W.H., Wang, G., Mandrell, R.E., 2005. Genomic and proteomic identification of a DNA-binding protein used in the “fingerprinting” of *Campylobacter* species and strains by MALDI-TOF-MS protein biomarker analysis. Analytical Chemistry. 77, 4897-4907.

Fagutao, F.F., Yasuike, M., Caipang, C.M., Kondo, H., Hirono, I., Takahashi, Y., Aoki, T., 2008. Gene expression profile of hemocytes of kuruma shrimp, *Marsupenaeus japonicus* following peptidoglycan stimulation. Marine Biotechnology. 10, 731-740.

FAO, 2006. State of the world aquacuture 2006. FAO Fisheries Department, Food and Agriculture Organization of the United Nations, Rome, Italy, pp. 134.

FAO, 2014. The state of world fisheries and aquaculture 2014, Rome, pp. 223.

FAO, 2016. Global aquaculture production 1950-2013.

Fast, W., Tipton, P.A., 2012. The enzymes of bacterial census and censorship. Trends in Biochemical Sciences. 37, 7-14.

Feder, M.E., Hofmann, G.E., 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annual Review of Physiology. 61, 243-282.

FEEDAP, P.E., 2014. Guidance on the assessment of the toxigenic potential of *Bacillus* species used in animal nutrition. EFSA Journal. 12, 3665.

Fernández-Alarcón, C., Miranda, C., Singer, R., Lopez, Y., Rojas, R., Bello, H., Dominguez, M., González-Rocha, G., 2010. Detection of the floR Gene in a Diversity of Florfenicol Resistant Gram-Negative Bacilli from Freshwater Salmon Farms in Chile. Zoonoses and public health. 57, 181-188.

Ferrero, R.L., Thiberge, J.-M., Kansau, I., Wuscher, N., Huerre, M., Labigne, A., 1995. The GroES homolog of *Helicobacter pylori* confers protective immunity against mucosal infection in mice. Proceedings of the National Academy of Sciences. 92, 6499-6503.

Fjellheim, A.J., Playfoot, K.J., Skjermo, J., Vadstein, O., 2007. Vibrionaceae dominates the microflora antagonistic towards *Listonella anguillarum* in the intestine of cultured Atlantic cod (*Gadus morhua* L.) larvae. Aquaculture. 269, 98-106.

Flegel, T.W., 1995. Progress in characterization and control of yellow-head virus of *Penaeus monodon*. Swimming through troubled water, Proceeding of the special session on shrimp farming, Aquaculture'95, Baton Rouge, Louisiana, USA.

Flegel, T.W., Lio-Po, G., 2009. Current status of viral diseases in Asian shrimp aquaculture, Israeli Journal of Aquaculture-Bamidgeh. Society of Israeli Aquaculture and Marine Biotechnology (SIAMB), pp. 229-239.

Fooks, L.J., Gibson, G.R., 2002. *In vitro* investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens. FEMS Microbiology Ecology. 39, 67-75.

Forst, S., Dowds, B., Boemare, N., Stackebrandt, E., 1997. *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. Annual Reviews in Microbiology. 51, 47-72.

Franken, K.L., Hiemstra, H.S., van Meijgaarden, K.E., Subronto, Y., Den Hartigh, J., Ottenhoff, T.H., Drijfhout, J.W., 2000. Purification of his-tagged proteins by immobilized chelate affinity chromatography: the benefits from the use of organic solvent. Protein expression and purification. 18, 95-99.

Fukui, T., Doi, Y., 1997. Cloning and analysis of the poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) biosynthesis genes of *Aeromonas caviae*. Journal of Bacteriology. 179, 4821-4830.

Fukui, T., Shiomi, N., Doi, Y., 1998. Expression and characterization of (R)-specific enoyl coenzyme A hydratase involved in polyhydroxyalkanoate biosynthesis by *Aeromonas caviae*. Journal of Bacteriology. 180, 667-673.

Fuller, R., 1989. Probiotics in man and animals. Journal of Applied Bacteriology. 66, 365-378.

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., Kato, T., 2013. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 504, 446-450.

Galán, B., Dinjaski, N., Maestro, B., De Eugenio, L., Escapa, I., Sanz, J., García, J., Prieto, M., 2011. Nucleoid-associated PhaF phasin drives intracellular location and segregation of polyhydroxyalkanoate granules in *Pseudomonas putida* KT2442. Molecular Microbiology. 79, 402-418.

Galina, J., Yin, G., Ardo, L., Jeney, Z., 2009. The use of immunostimulating herbs in fish. An overview of research. Fish Physiology and Biochemistry. 35, 669-676.

Garriques, D., Arevalo, G., 1995. An evaluation of the production and use of a live bacterial isolate to manipulate the microbial flora in the commercial production of *Penaeus vannamei* postlarvae in Ecuador, Swimming through troubled water. Proceedings of the special session on shrimp farming, Aquaculture, pp. 53-59.

Gatesoupe, F.-J., 1997. Siderophore production and probiotic effect of *Vibrio* sp. associated with turbot larvae, *Scophthalmus maximus*. Aquatic Living Resources. 10, 239-246.

Gatesoupe, F.-J., Infante, J.-L.Z., Cahu, C., Quazuguel, P., 1997. Early weaning of seabass larvae, *Dicentrarchus labrax*: the effect on microbiota, with particular attention to iron supply and exoenzymes. Aquaculture. 158, 117-127.

Gatesoupe, F.J., 1999. The use of probiotics in aquaculture. Aquaculture. 180, 147-165.
Georgiou, G., Valax, P., 1996. Expression of correctly folded proteins in *Escherichia coli*. Current Opinion in Biotechnology. 7, 190-197.
Georgiou, G., Valax, P., 1999. [3] Isolating inclusion bodies from bacteria. Methods in Enzymology. 309, 48-58.
Ghyselinck, J., Van Hoorde, K., Hoste, B., Heylen, K., De Vos, P., 2011. Evaluation of MALDI-TOF MS as a tool for high-throughput dereplication. Journal of Microbiological Methods. 86, 327-336.
Gibson, G.R., Roberfroid, M.B., 1995. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. Journal of Nutrition. 125, 1401-1412.
Gibson, G.R., Beatty, E.R., Wang, X., Cummings, J.H., 1995. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterology. 108, 975-982.
Gibson, L., Woodworth, J., George, A., 1998. Probiotic activity of *Aeromonas* media on the Pacific oyster, *Crassostrea gigas*, when challenged with *Vibrio tubiashii*. Aquaculture. 169, 111-120.
Gildberg, A., Mikkelsen, H., Sandaker, E., Ringø, E., 1997. Probiotic effect of lactic acid bacteria in the feed on growth and survival of fry of Atlantic cod (*Gadus morhua*), Asia-Pacific Conference on Science and Management of Coastal Environment. Springer, pp. 279-285.
Glenn, G., Roberfroid, M., 1995. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J. nutr. 125, 1401-1412.
Gopalakannan, A., Arul, V., 2011. Inhibitory activity of probiotic *Enterococcus faecium* MC13 against *Aeromonas hydrophila* confers protection against hemorrhagic septicemia in common carp *Cyprinus carpio*. Aquaculture International. 19, 973-985.
Gordon, H.A., Pesti, L., 1971. The gnotobiotic animal as a tool in the study of host microbial relationships. Bacteriological Reviews. 35, 390.
Gouda, M.K., Swellam, A.E., Omar, S.H., 2001. Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. Microbiological Research. 156, 201-207.
Gram, L., Melchiorsen, J., Spanggaard, B., Huber, I., Nielsen, T.F., 1999. Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a Possible Probiotic Treatment of Fish. Applied and Environmental Microbiology. 65, 969-973.
Griebel, R., Merrick, J., 1971. Metabolism of poly-β-hydroxybutyrate: effect of mild alkaline extraction on native poly-β-hydroxybutyrate granules. Journal of Bacteriology. 108, 782-789.
Griebel, R., Smith, Z., Merrick, J., 1968. Metabolism of poly (β-hydroxybutyrate). I. Purification, composition, and properties of native poly (β-hydroxybutyrate) granules from *Bacillus megaterium*. Biochemistry. 7, 3676-3681.
Gross, A., Nemirovsky, A., Zilberg, D., Khaimov, A., Brenner, A., Snir, E., Ronen, Z., Nejidat, A., 2003. Soil nitrifying enrichments as biofilter starters in intensive recirculating saline water aquaculture. Aquaculture. 223, 51-62.
Gudding, R., Lillehaug, A., Evensen, Ø., 1999. Recent developments in fish vaccinology. Veterinary Immunology and Immunopathology. 72, 203-212.

Hai, N., 2015. The use of probiotics in aquaculture. Journal of Applied Microbiology. 119, 917-935.

Halet, D., Defoirdt, T., Van Damme, P., Vervaeren, H., Forrez, I., Van de Wiele, T., Boon, N., Sorgeloos, P., Bossier, P., Verstraete, W., 2007. Poly-b-hydroxybutyrate-accumulating bacteria protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*. FEMS Microbiology Ecology. 60, 363-369.

Hameed, A.S.S., 1995. Susceptibility of Three *Penaeus* Species to a *Vibrio campbellii*-like Bacterium. Journal of the World Aquaculture Society. 26, 315-319.

Han, J.E., Tang, K.F., Tran, L.H., Lightner, D.V., 2015. *Photorhabdus* insect-related (Pir) toxin-like genes in a plasmid of *Vibrio parahaemolyticus*, the causative agent of acute hepatopancreatic necrosis disease (AHPND) of shrimp. Dis. Aquat. Org. 113, 33-40.

Han, M.-J., Yoon, S.S., Lee, S.Y., 2001. Proteome analysis of metabolically engineered *Escherichia coli* producing poly (3-Hydroxybutyrate). Journal of Bacteriology. 183, 301-308.

Handrick, R., Reinhardt, S., Jendrossek, D., 2000. Mobilization of poly (3-hydroxybutyrate) in *Ralstonia eutropha*. Journal of Bacteriology. 182, 5916-5918.

Handrick, R., Technow, U., Reichart, T., Reinhardt, S., Sander, T., Jendrossek, D., 2004. The activator of the *Rhodospirillum rubrum* PHB depolymerase is a polypeptide that is extremely resistant to high temperature (121 C) and other physical or chemical stresses. FEMS Microbiology Letters. 230, 265-274.

Haq, I.U., Chaudhry, W.N., Akhtar, M.N., Andleeb, S., Qadri, I., 2012. Bacteriophages and their implications on future biotechnology: a review. Virology Journal. 9, 1-8.

Hargreaves, J.A., 1998. Nitrogen biogeochemistry of aquaculture ponds. Aquaculture. 166, 181-212.

Hauf, W., Watzer, B., Roos, N., Klotz, A., Forchhammer, K., 2015. Photoautotrophic PHB granule formation: Identification and characterization of the cyanobacterial Phasin in *Synechocystis* sp. PCC 6803. Applied and Environmental Microbiology, AEM. 00604-00615.

Häusler, T., 2006. Viruses vs. superbugs: a solution to the antibiotics crisis? Palgrave Macmillan, New York, USA. pp. 256.

Head, M., Oleszkiewicz, J., 2004. Bioaugmentation for nitrification at cold temperatures. Water Research. 38, 523-530.

Hesselmann, R.P., Fleischmann, T., Hany, R., Zehnder, A.J., 1999. Determination of polyhydroxyalkanoates in activated sludge by ion chromatographic and enzymatic methods. Journal of Microbiological Methods. 35, 111-119.

Heuer, O.E., Kruse, H., Grave, K., Collignon, P., Karunasagar, I., Angulo, F.J., 2009. Human health consequences of use of antimicrobial agents in aquaculture. Clinical Infectious Diseases. 49, 1248-1253.

Hismiogullari, S., Hismiogullari, A., Sahin, F., Oner, E.T., Yenice, S., Karasartova, D., 2008. Investigation of antibacterial and cytotoxic effects of organic acids including ascorbic acid, lactic acid and acetic acids on mammalian cells. J Anim Vet Adv. 7, 681-684.

Hochuli, E., Döbeli, H., Schacher, A., 1987. New metal chelate adsorbent selective for proteins and peptides containing neighbouring histidine residues. Journal of Chromatography A. 411, 177-184.

Holmström, K., Gräslund, S., Wahlström, A., Poungshompoo, S., Bengtsson, B.-E., Kautsky, N., 2003. Antibiotic use in shrimp farming and implications for environmental impacts and human health. International Journal of Food Science & Technology. 38, 255-266.

Hong, H.A., Duc, L.H., Cutting, S.M., 2005. The use of bacterial spore formers as probiotics. FEMS Microbiology Reviews. 29, 813-835.

Hu, B., 2014. Priming the immune system of *Litopenaeus vannamei* with bacterial Heat Shock Protein 70 homologue DnaK against *Vibrio campbellii* and White Spot Syndrome Virus (WSSV) infection Animal Production. PhD thesis, Ghent University, Ghent, Belgium, pp. 176.

Huang, C.-C., Sritunyalucksana, K., Söderhäll, K., Song, Y.-L., 2004. Molecular cloning and characterization of tiger shrimp (*Penaeus monodon*) transglutaminase. Developmental & Comparative Immunology. 28, 279-294.

Hyronimus, B., Le Marrec, C., Urdaci, M., 1998. Coagulin, a bacteriocin-like-inhibitory substance produced by *Bacillus coagulans* I. Journal of Applied Microbiology. 85, 42-50.

Inal, J.M., 2003. Phage therapy: a reappraisal of bacteriophages as antibiotics. Archivum Immunologiae et Therapiae Experimentalis. 51, 237-244.

Irianto, A., Austin, B., 2002. Probiotics in aquaculture. Journal of Fish Diseases. 25, 633-642.

Iryani, M.T.M., MacRae, T.H., Panchakshari, S., Tan, J., Bossier, P., Wahid, M.E.A., Sung, Y.Y., 2017. Knockdown of heat shock protein 70 (Hsp70) by RNAi reduces the tolerance of *Artemia franciscana* nauplii to heat and bacterial infection. Journal of Experimental Marine Biology and Ecology. 487, 106-112.

Ishida, Y., Ahmed, A.M., Mahfouz, N.B., Kimura, T., El-Khodery, S.A., Moawad, A.A., Shimamoto, T., 2010. Molecular analysis of antimicrobial resistance in gram-negative bacteria isolated from fish farms in Egypt. Journal of Veterinary Medical Science. 72, 727-734.

Ishii, K.J., Koyama, S., Nakagawa, A., Coban, C., Akira, S., 2008. Host innate immune receptors and beyond: making sense of microbial infections. Cell host & microbe. 3, 352-363.

Itami, T., Asano, M., Tokushige, K., Kubono, K., Nakagawa, A., Takeno, N., Nishimura, H., Maeda, M., Kondo, M., Takahashi, Y., 1998. Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. Aquaculture. 164, 277-288.

Itoh, J., Omoto, S., Shomura, T., Nishizawa, N., Miyado, S., Yuda, Y., Shibata, U., Inouye, S., 1981. Amicoumacin-A, a new antibiotic with strong antiinflammatory and antiulcer activity. The Journal of antibiotics. 34, 611-613.

Janeo, R.L., Corre, V.L., Sakata, T., 2009. Water quality and phytoplankton stability in response to application frequency of bioaugmentation agent in shrimp ponds. Aquacultural Engineering. 40, 120-125.

Janeway Jr, C.A., Medzhitov, R., 2002. Innate immune recognition. Annual Review of Immunology. 20, 197-216.

Jayaprakash, N., Pai, S., Philip, R., Singh, I., 2006. Isolation of a pathogenic strain of *Vibrio alginolyticus* from necrotic larvae of *Macrobrachium rosenbergii* (de Man). Journal of Fish Diseases. 29, 187-191.

Jendrossek, D., 1998. Microbial degradation of polyesters: a review on extracellular poly (hydroxyalkanoic acid) depolymerases. Polymer Degradation and Stability. 59, 317-325.

Jendrossek, D., 2009. Polyhydroxyalkanoate granules are complex subcellular organelles (carbonosomes). Journal of Bacteriology. 191, 3195-3202.

Jendrossek, D., Pfeiffer, D., 2014. New insights in the formation of polyhydroxyalkanoate granules (carbonosomes) and novel functions of poly (3-hydroxybutyrate). Environmental Microbiology. 16, 2357-2373.

Jendrossek, D., Selchow, O., Hoppert, M., 2007. Poly (3-hydroxybutyrate) granules at the early stages of formation are localized close to the cytoplasmic membrane in *Caryophanon latum*. Applied and Environmental Microbiology. 73, 586-593.

Jiang, Y., Song, X., Gong, L., Li, P., Dai, C., Shao, W., 2008. High poly(β-hydroxybutyrate) production by *Pseudomonas fluorescens* A2a5 from inexpensive substrates. Enzyme and Microbial Technology. 42, 167-172.

Jiravanichpaisal, P., Puanglarp, N., Petkon, S., Donnuea, S., Soderhall, I., Soderhall, K., 2007. Expression of immune-related genes in larval stages of the giant tiger shrimp, *Penaeus monodon*. Fish & Shellfish Immunology. 23, 815-824.

Johnson, K., Kleerebezem, R., van Loosdrecht, M.C., 2010. Influence of the C/N ratio on the performance of polyhydroxybutyrate (PHB) producing sequencing batch reactors at short SRTs. Water Research. 44, 2141-2152.

Johnson, S., 1989. Handbook of shrimp diseases. Texas A & M Sea Grant College Program, Texas, USA. pp. 25.

Joly, A.-L., Wettstein, G., Mignot, G., Ghiringhelli, F., Garrido, C., 2010. Dual role of heat shock proteins as regulators of apoptosis and innate immunity. Journal of Innate Immunity. 2, 238-247.

Junprung, W., Supungul, P., Tassanakajon, A., 2017. HSP70 and HSP90 are involved in shrimp *Penaeus vannamei* tolerance to AHPND-causing strain of *Vibrio parahaemolyticus* after non-lethal heat shock. Fish & Shellfish Immunology. 60, 237-246.

Kadouri, D., Jurkevitch, E., Okon, Y., Castro-Sowinski, S., 2005. Ecological and agricultural significance of bacterial polyhydroxyalkanoates. Critical Reviews in Microbiology. 31, 55-67.

Karim, M., Zhao, W., Rowley, D., Nelson, D., Gomez-Chiarri, M., 2013. Probiotic Strains for Shellfish Aquaculture: Protection of Eastern Oyster, *Crassostrea virginica*, Larvae and Juveniles Against Bacterial Challenge. Journal of Shellfish Research. 32, 401-408.

Karr, D.B., Waters, J.K., Emerich, D.W., 1983. Analysis of poly-β-hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography and UV detection. Applied and Environmental Microbiology. 46, 1339-1344.

Karunasagar, I., Pai, R., Malathi, G.R., Karunasagar, I., 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. Aquaculture. 128, 203-209.

Kautsky, N., Rönnbäck, P., Tedengren, M., Troell, M., 2000. Ecosystem perspectives on management of disease in shrimp pond farming. Aquaculture. 191, 145-161.

Kaynar, P., Beyatli, Y., 2009. Determination of poly-β-hydroxybutyrate production by *Bacillus spp.* isolated from the intestines of various fishes. Fisheries Science. 75, 439-443.

Kennedy, B., Venugopal, M., Karunasagar, I., Karunasagar, I., 2006. Bacterial flora associated with the giant freshwater prawn *Macrobrachium rosenbergii*, in the hatchery system. Aquaculture. 261, 1156-1167.

Keshavarz, T., Roy, I., 2010. Polyhydroxyalkanoates: bioplastics with a green agenda. Current Opinion in Microbiology. 13, 321-326.

Khin, T., Annachhatre, A.P., 2004. Novel microbial nitrogen removal processes. Biotechnology Advances. 22, 519-532.

Kim, J.K., Park, K.J., Cho, K.S., Nam, S.-W., Park, T.-J., Bajpai, R., 2005. Aerobic nitrification–denitrification by heterotrophic *Bacillus* strains. Bioresource Technology. 96, 1897-1906.

Kiran, G.S., Priyadharshini, S., Dobson, A.D., Gnanamani, E., Selvin, J., 2016. Degradation intermediates of polyhydroxy butyrate inhibits phenotypic expression of virulence factors and biofilm formation in luminescent *Vibrio* sp. PUGSK8. npj Biofilms and Microbiomes. 2, 16002.

Kiran, G.S., Lipton, A.N., Priyadharshini, S., Anitha, K., Suárez, L.E.C., Arasu, M.V., Choi, K.C., Selvin, J., Al-Dhabi, N.A., 2014. Antiadhesive activity of poly-hydroxy butyrate biopolymer from a marine *Brevibacterium casei* MSI04 against shrimp pathogenic *Vibrios*. Microbial Cell Factories. 13, 114.

Kobayashi, T., Shiraki, M., Abe, T., Sugiyama, A., Saito, T., 2003. Purification and properties of an intracellular 3-hydroxybutyrate-oligomer hydrolase (PhaZ2) in *Ralstonia eutropha* H16 and its identification as a novel intracellular poly (3-hydroxybutyrate) depolymerase. Journal of Bacteriology. 185, 3485-3490.

Kobayashi, T., Uchino, K., Abe, T., Yamazaki, Y., Saito, T., 2005. Novel intracellular 3-hydroxybutyrate-oligomer hydrolase in *Wautersia eutropha* H16. Journal of Bacteriology. 187, 5129-5135.

Kodama, H., Hirota, Y., Mukamoto, M., Baba, T., Azuma, I., 1993. Activation of rainbow trout (*Oncorhynchus mykiss*) phagocytes by muramyl dipeptide. Developmental & Comparative Immunology. 17, 129-140.

Koller, M., Atlić, A., Dias, M., Reiterer, A., Braunegg, G., 2010. Microbial PHA production from waste raw materials, Plastics from bacteria. Springer, pp. 85-119.

Kominek, L.A., Halvorson, H.O., 1965. Metabolism of poly-β-hydroxybutyrate and acetoin in *Bacillus cereus*. Journal of Bacteriology. 90, 1251-1259.

Kotiranta, A., Lounatmaa, K., Haapasalo, M., 2000. Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes and Infection. 2, 189-198.

Kregel, K.C., 2002. Invited review: heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. Journal of Applied Physiology. 92, 2177-2186.

Krishnani, K.K., Parimala, V., Gupta, B., Azad, I., Meng, X., Abraham, M., 2006. Bagasse-assisted bioremediation of ammonia from shrimp farm wastewater. Water Environment Research, 938-950.

Krol, R.M., Hawkins, W.E., Overstreet, R.M., 1991. Rickettsial and mollicute infections in hepatopancreatic cells of cultured Pacific white shrimp (*Penaeus vannamei*). Journal of Invertebrate Pathology. 57, 362-370.

Kuchta, K., Chi, L., Fuchs, H., Pötter, M., Steinbüchel, A., 2007. Studies on the influence of phasins on accumulation and degradation of PHB and nanostructure of PHB granules in *Ralstonia eutropha* H16. Biomacromolecules. 8, 657-662.

Kumar, B.S., Prabakaran, G., 2006. Production of PHB (bioplastics) using bio-effluent as substrate by *Alcaligens eutrophus*. Indian Journal of Biotechnology. 5, 76-79.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular biology and evolution, msw054.

Laranja, J.L.Q., Ludevese-Pascual, G.L., Amar, E.C., Sorgeloos, P., Bossier, P., De Schryver, P., 2014. Poly-β-hydroxybutyrate (PHB) accumulating *Bacillus* spp. improve the survival, growth and robustness of *Penaeus monodon* (Fabricius,1798) postlarvae. Veterinary Microbiology. 173, 310-317.

Lavilla-Pitogo, C.R., de la Pena, L., 1998. Bacterial Diseases in Shrimp (*Penaeus monodon*) Culture in the Philippines. Fish Pathology. 33, 405-411.

Lavilla-Pitogo, C.R., Baticados, M.C.L., Cruz-Lacierda, E.R., de la Pena, L.D., 1990. Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. Aquaculture. 91, 1-13.

Law, J.H., Slepecky, R.A., 1961. Assay of poly-beta-hydroxybutyric acid. Journal of Bacteriology. 82, 33-36.

Lazado, C.C., Caipang, C.M.A., 2014. Mucosal immunity and probiotics in fish. Fish & Shellfish Immunology. 39, 78-89.

Le Poul, E., Loison, C., Struyf, S., Springael, J.-Y., Lannoy, V., Decobecq, M.-E., Brezillon, S., Dupriez, V., Vassart, G., Van Damme, J., 2003. Functional characterization of human receptors for short chain fatty acids and their role in polymorphonuclear cell activation. Journal of Biological Chemistry. 278, 25481-25489.

Lee, C.-T., Chen, I.-T., Yang, Y.-T., Ko, T.-P., Huang, Y.-T., Huang, J.-Y., Huang, M.-F., Lin, S.-J., Chen, C.-Y., Lin, S.-S., 2015. The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. Proceedings of the National Academy of Sciences. 112, 10798-10803.

Lee, K.-K., Yu, S.-R., Chen, F.-R., Yang, T.-I., Liu, P.-C., 1996a. News & notes: virulence of *Vibrio alginolyticus* isolated from diseased tiger prawn, *Penaeus monodon*. Current Microbiology. 32, 229-231.

Lee, K.K., Yu, S.R., Yang, T.I., Liu, P.C., Chen, F.R., 1996b. Isolation and characterization of *Vibrio alginolyticus* isolated from diseased kuruma prawn, *Penaeus japonicus*. Letters in Applied Microbiology. 22, 111-114.

Lee, S.J., Park, S.-Y., Lee, J.-J., Yum, D.-Y., Koo, B.-T., Lee, J.-K., 2002. Genes encoding the N-acyl homoserine lactone-degrading enzyme are widespread in many subspecies of *Bacillus thuringiensis*. Applied and Environmental Microbiology. 68, 3919-3924.

Lee, S.Y., 1996. Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends in Biotechnology. 14, 431-438.

Lee, T.-R., Lin, J.-S., Wang, S.-S., Shaw, G.-C., 2004. PhaQ, a new class of poly-β-hydroxybutyrate (PHB)-responsive repressor, regulates phaQ and phaP (phasin) expression in *Bacillus megaterium* through interaction with PHB. Journal of Bacteriology. 186, 3015-3021.

Lemos, M., Dopazo, C., Toranzo, A.E., Barja, J., 1991. Competitive dominance of antibiotic-producing marine bacteria in mixed cultures. Journal of Applied Bacteriology. 71, 228-232.

Leyva-Madrigal, K.Y., Luna-González, A., Escobedo-Bonilla, C.M., Fierro-Coronado, J.A., Maldonado-Mendoza, I.E., 2011. Screening for potential probiotic bacteria to reduce prevalence of WSSV and IHHNV in whiteleg shrimp (*Litopenaeus vannamei*) under experimental conditions. Aquaculture. 322, 16-22.

Li, K., Zheng, T., Tian, Y., Xi, F., Yuan, J., Zhang, G., Hong, H., 2007. Beneficial effects of *Bacillus licheniformis* on the intestinal microflora and immunity of the white shrimp, *Litopenaeus vannamei*. Biotechnology Letters. 29, 525-530.

Liebergesell, M., Schmidt, B., Steinbüchel, A., 1992. Isolation and identification of granule-associated proteins relevant for poly (3-hydroxyalkanoic acid) biosynthesis in *Chromatium vinosum* D. FEMS microbiology letters. 99, 227-232.

Lightner, D., Sindermann, C., 1988. Larval mycosis of penaeid shrimp. Disease diagnosis and control in North American marine aquaculture. Ed. 2. 58-63.

Lightner, D., Redman, R., 1998. Shrimp diseases and current diagnostic methods. Aquaculture. 164, 201-220.

Lightner, D., Redman, R., Bell, T., Brock, J., 1983a. Detection of IHHN virus in *Penaeus stylirostris* and *P. vannamei* imported into Hawaii. Journal of the World Mariculture Society. 14, 212-225.

Lightner, D., Redman, R., Williams, R., Mohney, L., Clerx, J., Bell, T., Brock, J., 1985. Recent advances in penaeid virus disease investigations. Journal of the World Mariculture Society. 16, 267-274.

Lightner, D.V., 1985. A review of the diseases of cultured penaeid shrimps and prawns with emphasis on recent discoveries and developments. In Taki Y.., Primavera J.H. and Llobrera J.A. (Eds.) Proceedings of the First International Conference on the Culture of Penaeid Prawns/Shrimps, 4-7 December 1984, SEAFDEC, Iloilo City, Philippines, pp. 79-103.

Lightner, D.V., 1996. A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp. World Aquaculture Society, Baton Rouge, LA, USA. pp. 1-72.

Lightner, D.V., Redman, R., 1985. A parvo-like virus disease of penaeid shrimp. Journal of Invertebrate Pathology. 45, 47-53.

Lightner, D.V., Redman, R., Bell, T., 1983b. Observations on the geographic distribution, pathogenesis and morphology of the baculovirus from *Penaeus monodon* Fabricius. Aquaculture. 32, 209-233.

Lightner, D.V., Redman, R., Pantoja, C., Noble, B., Tran, L., 2012. Early mortality syndrome affects shrimp in Asia. Global Aquaculture Advocate. 15, 40.

Lissin, N., Venyaminov, S.Y., Girshovich, A., 1990. (Mg–ATP)-dependent self-assembly of molecular chaperone GroEL. Nature. 348, 339-342.

Liu, K.-F., Chiu, C.-H., Shiu, Y.-L., Cheng, W., Liu, C.-H., 2010a. Effects of the probiotic, *Bacillus subtilis* E20, on the survival, development, stress tolerance, and immune

status of white shrimp, *Litopenaeus vannamei* larvae. Fish & Shellfish Immunology. 28, 837-844.

Liu, K.F., Liu, W.-J., Kou, G.-H., Lo, C.-F., 2009. Shrimp white spot syndrome-from pathology to pathogenomics. Fish Pathology. 44, 55-58.

Liu, L., Xiao, J., Xia, X., Pan, Y., Yan, S., Wang, Y., 2015. Draft genome sequence of *Vibrio owensii* strain SH-14, which causes shrimp acute hepatopancreatic necrosis disease. Genome announcements. 3, e01395-01315.

Liu, P.-C., Lee, K.-K., Yii, K.-C., Kou, G.-H., Chen, S.-N., 1996a. News & Notes: Isolation of *Vibrio harveyi* from Diseased Kuruma Prawns *Penaeus japonicus*. Current Microbiology. 33, 129-132.

Liu, P., Lee, K., Chen, S., 1996b. Susceptibility of different isolates of *Vibrio harveyi* to antibiotics. Microbios. 91, 175-180.

Liu, Y., De Schryver, P., Van Delsen, B., Maignien, L., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P., Defoirdt, T., 2010b. PHB-degrading bacteria isolated from the gastrointestinal tract of aquatic animals as protective actors against luminescent *Vibriosis*. FEMS Microbiology Ecology. 74, 196-204.

Lo, W.-Y., Liu, K.-F., Liao, I.-C., Song, Y.-L., 2004. Cloning and molecular characterization of heat shock cognate 70 from tiger shrimp (*Penaeus monodon*). Cell Stress & Chaperones. 9, 332-343.

López, N., Ruiz, J., Mendez, B., 1998. Survival of poly-3-hydroxybutyrate-producing bacteria in soil microcosms. World Journal of Microbiology and Biotechnology. 14, 681-684.

López, N.I., Floccari, M.E., Steinbüchel, A., García, A.F., Méndez, B.S., 1995. Effect of poly (3-hydroxybutyrate)(PHB) content on the starvation-survival of bacteria in natural waters. FEMS Microbiology Ecology. 16, 95-101.

Lu, Y., Loh, P., 1992. Some biological properties of a rhabdovirus isolated from penaeid shrimps. Archives of Virology. 127, 339-343.

Ludevese-Pascual, G., Laranja, J., Amar, E., Sorgeloos, P., Bossier, P., De Schryver, P., 2017. Poly-beta-hydroxybutyrate-enriched *Artemia* sp. for giant tiger prawn *Penaeus monodon* larviculture. Aquaculture Nutrition. 23, 422-429.

Ludevese, G., 2016. Application and mode of action of the poly-β-hydroxybutyrate (PHB) in *Penaeus* culture. PhD thesis, Ghent University, Ghent, Belgium, pp. 301.

Luengo, J.M., García, B., Sandoval, A., Naharro, G., Olivera, E.R., 2003. Bioplastics from microorganisms. Current Opinion in Microbiology. 6, 251-260.

Lund, T., Granum, P.E., 1996. Characterisation of a non-haemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. FEMS Microbiology Letters. 141, 151-156.

Lund, T., De Buyser, M.L., Granum, P.E., 2000. A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. Molecular Microbiology. 38, 254-261.

Lundgren, D., Pfister, R., Merrick, J., 1964. Structure of poly-β-hydroxybutyric acid granules. Microbiology. 34, 441-446.

Lundin, C.G., 1996. Global attempts to address shrimp disease. In 2. Asia Pacific Marine Biotechnology Conference and 3. Asia-Pacific Conference on Algal Biotechnology, Phuket, Thailand.

Ma, H.-K., Liu, M.-M., Li, S.-Y., Wu, Q., Chen, J.-C., Chen, G.-Q., 2013. Application of polyhydroxyalkanoate (PHA) synthesis regulatory protein PhaR as a bio-surfactant and bactericidal agent. Journal of biotechnology. 166, 34-41.

MacArthur, R.H., Wilson, E.O., 2015. Theory of Island Biogeography.(MPB-1). Princeton University Press, pp. 216.

MacDonald, N., Stark, J., Austin, B., 1986. Bacterial microflora in the gastro-intestinal tract of Dover sole (*Solea solea* L.), with emphasis on the possible role of bacteria in the nutrition of the host. FEMS Microbiology Letters. 35, 107-111.

Macrae, R., Wilkinson, J., 1958. Poly-β-hyroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. Microbiology. 19, 210-222.

Madison, L.L., Huisman, G.W., 1999. Metabolic engineering of poly (3-hydroxyalkanoates): from DNA to plastic. Microbiology and Molecular Biology Reviews. 63, 21-53.

Maningas, M.B.B., Kondo, H., Hirono, I., Saito-Taki, T., Aoki, T., 2008. Essential function of transglutaminase and clotting protein in shrimp immunity. Molecular Immunology. 45, 1269-1275.

Marques, A., François, J.-M., Dhont, J., Bossier, P., Sorgeloos, P., 2004. Influence of yeast quality on performance of gnotobiotically grown *Artemia*. Journal of Experimental Marine Biology and Ecology. 310, 247-264.

Marques, A., Ollevier, F., Verstraete, W., Sorgeloos, P., Bossier, P., 2006. Gnotobiotically grown aquatic animals: opportunities to investigate host–microbe interactions. Journal of Applied Microbiology. 100, 903-918.

Martín-Platero, A.M., Maqueda, M., Valdivia, E., Purswani, J., Martínez-Bueno, M., 2009. Polyphasic study of microbial communities of two Spanish farmhouse goats' milk cheeses from Sierra de Aracena. Food microbiology. 26, 294-304.

Martin, G.G., Rubin, N., Swanson, E., 2004. *Vibrio parahaemolyticus* and *V. harveyi* cause detachment of the epithelium from the midgut trunk of the penaeid shrimp, *Sicyonia ingentis*. Diseases of Aquatic Organisms. 60, 21-29.

Martínez-Alonso, M., García-Fruitós, E., Ferrer-Miralles, N., Rinas, U., Villaverde, A., 2010. Side effects of chaperone gene co-expression in recombinant protein production. Microbial Cell Factories. 9, 64.

Maruta, K., Miyazaki, H., Masuda, S., Takahashi, M., Marubashi, T., Tadano, Y., Takahashi, H., 1996. Exclusion of intestinal pathogens by continuous feeding with *Bacillus subtilis* C-3102 and its influence on the intestinal microflora in broilers. Animal Science and Technology. 67, 273-280.

McCool, G.J., Cannon, M.C., 1999. Polyhydroxyalkanoate inclusion body-associated proteins and coding region in *Bacillus megaterium*. Journal of Bacteriology. 181, 585-592.

McCool, G.J., Cannon, M.C., 2001. PhaC and PhaR are required for polyhydroxyalkanoic acid synthase activity in *Bacillus megaterium*. Journal of Bacteriology. 183, 4235-4243.

McHan, F., Shotts, E.B., 1993. Effect of short-chain fatty acids on the growth of *Salmonella typhimurium* in an *in vitro* system. Avian Diseases. 37, 396-398.

McIntosh, D., Cunningham, M., Ji, B., Fekete, F.A., Parry, E.M., Clark, S.E., Zalinger, Z.B., Gilg, I.C., Danner, G.R., Johnson, K.A., 2008. Transferable, multiple antibiotic

and mercury resistance in Atlantic Canadian isolates of *Aeromonas salmonicida* subsp. *salmonicida* is associated with carriage of an IncA/C plasmid similar to the *Salmonella enterica* plasmid pSN254. Journal of Antimicrobial Chemotherapy. 61, 1221-1228.

Meena, D., Das, P., Kumar, S., Mandal, S., Prusty, A., Singh, S., Akhtar, M., Behera, B., Kumar, K., Pal, A., Mukherjee, S., 2013. Beta-glucan: an ideal immunostimulant in aquaculture (a review). Fish Physiology and Biochemistry. 39, 431-457.

Meijer, K., de Vos, P., Priebe, M.G., 2010. Butyrate and other short-chain fatty acids as modulators of immunity: what relevance for health? Current Opinion in Clinical Nutrition & Metabolic Care. 13, 715-721.

Merrick, J., 1965. Effect of polymyxin B, tyrocidine, gramicidin D, and other antibiotics on the enzymatic hydrolysis of poly-β-hydroxybutyrate. Journal of Bacteriology. 90, 965-969.

Merrick, J., Lundgren, D., Pfister, R., 1965. Morphological changes in poly-β-hydroxybutyrate granules associated with decreased susceptibility to enzymatic hydrolysis. Journal of Bacteriology. 89, 234-239.

Mezzina, M.P., Pettinari, M.J., 2016. Phasins, the multifacetic polyhydroxyalkanoate granule associated proteins. Applied and Environmental Microbiology, AEM. 01161-01116.

Mezzina, M.P., Wetzler, D.E., Almeida, A., Dinjaski, N., Prieto, M.A., Pettinari, M.J., 2015. A phasin with extra talents: a polyhydroxyalkanoate granule-associated protein has chaperone activity. Environmental Microbiology. 17, 1765-1776.

Miethke, M., Marahiel, M.A., 2007. Siderophore-based iron acquisition and pathogen control. Microbiology and Molecular Biology Reviews. 71, 413-451.

Mikkola, R., Saris, N.E.L., Grigoriev, P.A., Andersson, M.A., Salkinoja-Salonen, M.S., 1999. Ionophoretic properties and mitochondrial effects of cereulide. European Journal of Biochemistry. 263, 112-117.

Mikkola, R., Kolari, M., Andersson, M.A., Helin, J., Salkinoja-Salonen, M.S., 2000. Toxic lactonic lipopeptide from food poisoning isolates of *Bacillus licheniformis*. European Journal of Biochemistry. 267, 4068-4074.

Momoyama, K., Sano, T., 1989. Developmental stages of kuruma shrimp, *Penaeus japonicus* Bate, susceptible to baculoviral mid-gut gland necrosis (BMN) virus. Journal of Fish Diseases. 12, 585-589.

Moparthi, S.B., Sjölander, D., Villebeck, L., Jonsson, B.-H., Hammarström, P., Carlsson, U., 2014. Transient conformational remodeling of folding proteins by GroES—individually and in concert with GroEL. Journal of chemical biology. 7, 1-15.

Moret, Y., 2006. 'Trans-generational immune priming': specific enhancement of the antimicrobial immune response in the mealworm beetle, *Tenebrio molitor*. Proceedings of the Royal Society of London B: Biological Sciences. 273, 1399-1405.

Morimoto, R.I., 1998. Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes & Development. 12, 3788-3796.

Morya, V., Choi, W., Kim, E.-k., 2014. Isolation and characterization of *Pseudoalteromonas* sp. from fermented Korean food, as an antagonist to *Vibrio harveyi*. Applied Microbiology and Biotechnology. 98, 1389-1395.

Moseley, P., 2000. Stress proteins and the immune response. Immunopharmacology. 48, 299-302.

Mudliar, S., Vaidya, A., Kumar, M.S., Dahikar, S., Chakrabarti, T., 2008. Techno-economic evaluation of PHB production from activated sludge. Clean Technologies and Environmental Policy. 10, 255-262.

Murata, K., Inose, T., Hisano, T., Abe, S., Yonemoto, Y., Yamashita, T., Takagi, M., Sakaguchi, K., Kimura, A., Imanaka, T., 1993. Bacterial alginate lyase: enzymology, genetics and application. Journal of fermentation and bioengineering. 76, 427-437.

Muroga, K., Sszuik, K., Ishimaru, K., Nogami, K., 1994. *Vibriosis* of swimming crab *Portunus trituberculatus* in larviculture. Journal of the World Aquaculture Society. 25, 50-54.

Nagai, S., Okimura, K., Kaizawa, N., Ohki, K., Kanamoto, S., 1996. Study on surfactin, a cyclic depsipeptide. II. Synthesis of surfactin B2 produced by *Bacillus natto* KMD 2311. Chemical and Pharmaceutical Bulletin. 44, 5-10.

Najdegerami, E., Bakhshi, F., Tokmechi, A., Shiri Harzevili, A., Sorgeloos, P., Bossier, P., 2017. Dietary effects of poly-β-hydroxybutyrate on the growth performance, digestive enzyme activity, body composition, mineral uptake and bacterial challenge of rainbow trout fry (*Oncorhynchus mykiss*). Aquaculture Nutrition. 23, 246-254.

Najdegerami, E.H., Tran, T.N., Defoirdt, T., Marzorati, M., Sorgeloos, P., Boon, N., Bossier, P., 2012. Effects of poly-β-hydroxybutyrate (PHB) on Siberian sturgeon (*Acipenser baerii*) fingerlings performance and its gastrointestinal tract microbial community. FEMS Microbiology Ecology. 79, 25-33.

Nakai, T., Park, S.C., 2002. Bacteriophage therapy of infectious diseases in aquaculture. Research in Microbiology. 153, 13-18.

Nappi, A., Christensen, B., 2005. Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. Insect Biochemistry and Molecular Biology. 35, 443-459.

National Research Council, 2011. Nutrient requirements of fish and shrimp. National academies press, Washington, USA. pp. 392

NavinChandran, M., Iyapparaj, P., Moovendhan, S., Ramasubburayan, R., Prakash, S., Immanuel, G., Palavesam, A., 2014. Influence of probiotic bacterium *Bacillus cereus* isolated from the gut of wild shrimp *Penaeus monodon* in turn as a potent growth promoter and immune enhancer in *P. monodon*. Fish & Shellfish Immunology. 36, 38-45.

Nayak, S., 2010. Probiotics and immunity: a fish perspective. Fish & Shellfish Immunology. 29, 2-14.

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., 2000. Effect of aquaculture on world fish supplies. Nature. 405, 1017.

Nei, M., Kumar, S., 2000. Molecular evolution and phylogenetics. Oxford university press, USA. pp. 352.

Neumann, N.F., Stafford, J.L., Barreda, D., Ainsworth, A.J., Belosevic, M., 2001. Antimicrobial mechanisms of fish phagocytes and their role in host defense. Developmental & Comparative Immunology. 25, 807-825.

Neville, S.A., LeCordier, A., Ziochos, H., Chater, M.J., Gosbell, I.B., Maley, M.W., van Hal, S.J., 2011. The Utility of Matrix Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF MS) following introduction for routine laboratory bacterial identification. Journal of Clinical Microbiology. 49, 2980-2984.

New, M.B., 2005. Freshwater prawn farming: global status, recent research and a glance at the future. Aquaculture Research. 36, 210-230.

Newaj-Fyzul, A., Adesiyun, A., Mutani, A., Ramsubhag, A., Brunt, J., Austin, B., 2007. *Bacillus subtilis* AB1 controls *Aeromonas* infection in rainbow trout (*Oncorhynchus mykiss*, Walbaum). Journal of Applied Microbiology. 103, 1699-1706.

Nguyen, V.H., 2016. The role of poly-β-hydroxybutyrate as protection against *Vibrio* infections in blue mussel larvae. PhD thesis, Ghent University, Ghent, Belgium. pp. 196.

Nhan, D.T., Wille, M., De Schryver, P., Defoirdt, T., Bossier, P., Sorgeloos, P., 2010. The effect of poly-β-hydroxybutyrate on larviculture of the giant freshwater prawn *Macrobrachium rosenbergii*. Aquaculture. 302, 76-81.

Nicholson, W., 2002. Roles of *Bacillus* endospores in the environment. Cellular and Molecular Life Sciences CMLS. 59, 410-416.

Nikoskelainen, S., Ouwehand, A.C., Bylund, G., Salminen, S., Lilius, E.M., 2003. Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*LactoBacillus rhamnosus*). Fish and Shellfish Immunology. 15, 443-453.

Nimrat, S., Suksawat, S., Boonthai, T., Vuthiphandchai, V., 2012. Potential *Bacillus* probiotics enhance bacterial numbers, water quality and growth during early development of white shrimp (*Litopenaeus vannamei*). Veterinary Microbiology. 159, 443-450.

Nimrat, S., Tanutpongpalin, P., Sritunyalucksana, K., Boonthai, T., Vuthiphandchai, V., 2013. Enhancement of growth performance, digestive enzyme activities and disease resistance in black tiger shrimp (*Penaeus monodon*) postlarvae by potential probiotics. Aquaculture International. 21, 655-666.

Niu, Y., Defoirdt, T., Baruah, K., Van de Wiele, T., Dong, S., Bossier, P., 2014. *Bacillus* sp. LT3 improves the survival of gnotobiotic brine shrimp (*Artemia franciscana*) larvae challenged with *Vibrio campbellii* by enhancing the innate immune response and by decreasing the activity of shrimp-associated *Vibrios*. Veterinary Microbiology. 173, 279-288.

Nunan, L., Lightner, D., Pantoja, C., Gomez-Jimenez, S., 2014. Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. Diseases of Aquatic Organisms. 111, 81-86.

Ojumu, T., Yu, J., Solomon, B., 2004. Production of polyhydroxyalkanoates, a bacterial biodegradable polymers. African Journal of Biotechnology. 3, 18-24.

Olafsen, J.A., 2001. Interactions between fish larvae and bacteria in marine aquaculture. Aquaculture. 200, 223-247.

Olsen, R.E., Vecino, J.L.G., Wadsworth, S., Song, S.K., Ringø, E., 2012. Use of immunostimulants and nucleotides in aquaculture: a review. Journal of Marine Science Research & Development. 1, 2.

Ostle, A.G., Holt, J., 1982. Nile blue A as a fluorescent stain for poly-beta-hydroxybutyrate. Applied and Environmental Microbiology. 44, 238-241.

Otta, S., Karunasagar, I., Karunasagar, I., 2001. Bacteriological study of shrimp, *Penaeus monodon* Fabricius, hatcheries in India. Journal of Applied Ichthyology. 17, 59-63.

Overstreet, R.M., 1973. Parasites of some penaeid shrimps with emphasis on reared hosts. Aquaculture. 2, 105-140.

Paganelli, F.L., Lemos, E.G.d.M., Alves, L.M.C., 2011. Polyhydroxybutyrate in *Rhizobium* and *Bradyrhizobium*: quantification and phbC gene expression. World Journal of Microbiology & Biotechnology. 27, 773-778.

Pande, G.S.J., Natrah, F.M.I., Flandez, A.V.B., Kumar, U., Niu, Y., Bossier, P., Defoirdt, T., 2015. Isolation of AHL-degrading bacteria from micro-algal cultures and their impact on algal growth and on virulence of *Vibrio campbellii* to prawn larvae. Applied Microbiology and Biotechnology. 99, 10805-10813.

Pandian, S.R., Deepak, V., Kalishwaralal, K., Rameshkumar, N., Jeyaraj, M., Gurunathan, S., 2010. Optimization and fed-batch production of PHB utilizing dairy waste and sea water as nutrient sources by *Bacillus megaterium* SRKP-3. Bioresource Technology. 101, 705-711.

Panigrahi, A., Kiron, V., Kobayashi, T., Puangkaew, J., Satoh, S., Sugita, H., 2004. Immune responses in rainbow trout *Oncorhynchus mykiss* induced by a potential probiotic bacteria *LactoBacillus rhamnosus* JCM 1136. Veterinary Immunology and Immunopathology. 102, 379-388.

Panigrahi, A., Azad, I., Das, B., Dandpat, J., Das, G., Behera, S., Mishra, S., 2009. Probiotic induced immunomodulation: investigation into the cellular and molecular mechanism involved. Research Journal of Biotechnology. 4, 7-13.

Panigrahi, A., Kiron, V., Satoh, S., Hirono, I., Kobayashi, T., Sugita, H., Puangkaew, J., Aoki, T., 2007. Immune modulation and expression of cytokine genes in rainbow trout *Oncorhynchus mykiss* upon probiotic feeding. Developmental & Comparative Immunology. 31, 372-382.

Parsell, D., Lindquist, S., 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annual Review of Genetics. 27, 437-496.

Pasharawipas, T., Flegel, T., 1994. A specific DNA probe to identify the intermediate host of a common microsporidian parasite of *Penaeus merguiensis* and *P. monodon*. Asian Fisheries Science. 7, 157-167.

Patel, S., Goyal, A., 2012. The current trends and future perspectives of prebiotics research: a review. 3 Biotech. 2, 115-125.

Pauly, D., Christensen, V., Guenette, S., Pitcher, T.J., Sumaila, U.R., Walters, C.J., Watson, R., Zeller, D., 2002. Towards sustainability in world fisheries. Nature. 418, 689-695.

Peplinski, K., Ehrenreich, A., Döring, C., Bömeke, M., Reinecke, F., Hutmacher, C., Steinbüchel, A., 2010. Genome-wide transcriptome analyses of the ‘Knallgas’ bacterium *Ralstonia eutropha* H16 with regard to polyhydroxyalkanoate metabolism. Microbiology. 156, 2136-2152.

Pérez-Sánchez, T., Ruiz-Zarzuela, I., Blas, I., Balcázar, J.L., 2014. Probiotics in aquaculture: a current assessment. Reviews in Aquaculture. 6, 133-146.

Pessi, T., Sütas, Y., Saxelin, M., Kallioinen, H., Isolauri, E., 1999. Antiproliferative effects of homogenates derived from five strains of candidate probiotic bacteria. Applied and Environmental Microbiology. 65, 4725-4728.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Research. 29, e45-e45.

Pfeiffer, D., Jendrossek, D., 2011. Interaction between poly (3-hydroxybutyrate) granule-associated proteins as revealed by two-hybrid analysis and identification of a new phasin in *Ralstonia eutropha* H16. Microbiology. 157, 2795-2807.

Pfeiffer, D., Jendrossek, D., 2012. Localization of poly (3-hydroxybutyrate)(PHB) granule-associated proteins during PHB granule formation and identification of two new phasins, PhaP6 and PhaP7, in *Ralstonia eutropha* H16. Journal of Bacteriology. 194, 5909-5921.

Pfeiffer, D., Jendrossek, D., 2014. PhaM is the physiological activator of poly (3-hydroxybutyrate)(PHB) synthase (PhaC1) in *Ralstonia eutropha*. Applied and Environmental Microbiology. 80, 555-563.

Philip, S., Keshavarz, T., Roy, I., 2007. Polyhydroxyalkanoates: biodegradable polymers with a range of applications. Journal of Chemical Technology and Biotechnology. 82, 233-247.

Piard, J., Desmazeaud, M., 1992. Inhibiting factors produced by lactic acid bacteria. 2. Bacteriocins and other antibacterial substances. Le lait. 72, 113-142.

Pierre, F., Perrin, P., Champ, M., Bornet, F., Meflah, K., Menanteau, J., 1997. Short-chain fructo-oligosaccharides reduce the occurrence of colon tumors and develop gut-associated lymphoid tissue in Min mice. Cancer Research. 57, 225-228.

Pinchuk, I.V., Bressollier, P., Verneuil, B., Fenet, B., Sorokulova, I.B., Mégraud, F., Urdaci, M.C., 2001. In Vitro Anti-Helicobacter pyloriActivity of the Probiotic Strain *Bacillus subtilis* 3 Is Due to Secretion of Antibiotics. Antimicrobial Agents and Chemotherapy. 45, 3156-3161.

Pirarat, N., Ponpornpisit, A., Rodkhum, C., Chansue, N., 2008. *LactoBacillus rhamnosus* GG, a potential human-derived probiotic candidate for Tilapia (*Oreochromis niloticus*) culture, Proceedings, the 15th congress of FAVA, Bangkok, Thailand, pp. 27-30.

Pohlmann, A., Fricke, W.F., Reinecke, F., Kusian, B., Liesegang, H., Cramm, R., Eitinger, T., Ewering, C., Pötter, M., Schwartz, E., 2006. Genome sequence of the bioplastic-producing “Knallgas” bacterium *Ralstonia eutropha* H16. Nature Biotechnology. 24, 1257-1262.

Pötter, M., Steinbüchel, A., 2005. Poly (3-hydroxybutyrate) granule-associated proteins: impacts on poly (3-hydroxybutyrate) synthesis and degradation. Biomacromolecules. 6, 552-560.

Pötter, M., Steinbüchel, A., 2006. Biogenesis and structure of polyhydroxyalkanoate granules, Inclusions in prokaryotes. Springer, pp. 109-136.

Pötter, M., Madkour, M.H., Mayer, F., Steinbüchel, A., 2002. Regulation of phasin expression and polyhydroxyalkanoate (PHA) granule formation in *Ralstonia eutropha* H16. Microbiology. 148, 2413-2426.

Pötter, M., Müller, H., Reinecke, F., Wieczorek, R., Fricke, F., Bowien, B., Friedrich, B., Steinbüchel, A., 2004. The complex structure of polyhydroxybutyrate (PHB) granules: four orthologous and paralogous phasins occur in *Ralstonia eutropha*. Microbiology. 150, 2301-2311.

Priest, F.G., 1977. Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriological Reviews. 41, 711.

Primavera, J.H., 1997. Socio-economic impacts of shrimp culture. Aquaculture Research. 28, 815-827.

Proespraiwong, P., Tassanakajon, A., Rimphanitchayakit, V., 2010. Chitinases from the black tiger shrimp *Penaeus monodon*: phylogenetics, expression and activities. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 156, 86-96.

Purivirojkul, W., Areechon, N., Srisapoome, P., 2006. The Effect of peptidoglycan on Immune response in black tiger shrimp (*Penaeus monodon* Fabricius). Kasetsart J.(Nat. Sci.). 40, 181-187.

Qi, Q., Steinbüchel, A., Rehm, B., 2000. *In vitro* synthesis of poly (3-hydroxydecanoate): purification and enzymatic characterization of type II polyhydroxyalkanoate synthases PhaC1 and PhaC2 from *Pseudomonas aeruginosa*. Applied Microbiology and Biotechnology. 54, 37-43.

Queiroz, J.F., Boyd, C.E., 1998. Effects of a bacterial inoculum in channel catfish ponds. Journal of the World Aquaculture Society. 29, 67-73.

Rawls, J.F., Samuel, B.S., Gordon, J.I., 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proceedings of the National Academy of Sciences of the United States of America. 101, 4596-4601.

Ray, A.K., Ghosh, K., Ringø, E., 2012. Enzyme-producing bacteria isolated from fish gut: a review. Aquaculture Nutrition. 18, 465-492.

Rebah, B.F., Prévost, D., Tyagi, R.D., Belbahri, L., 2009. Poly-β-hydroxybutyrate Production by Fast-Growing Rhizobia Cultivated in Sludge and in Industrial Wastewater. Applied Biochemistry and Biotechnology. 158, 155-163.

Rehm, B.H., Krüger, N., Steinbüchel, A., 1998. A New Metabolic Link between Fatty Acid de NovoSynthesis and Polyhydroxyalkanoic Acid Synthesis. The PHAG gene from *Pseudomonas putida* KT2440 encodes a 3-hydroxyacyl-acyl carrier protein-coenzyme a transferase. Journal of Biological Chemistry. 273, 24044-24051.

Remminghorst, U., Rehm, B.H., 2006. Bacterial alginates: from biosynthesis to applications. Biotechnology Letters. 28, 1701-1712.

Ren, H., Musch, M.W., Kojima, K., Boone, D., Ma, A., Chang, E.B., 2001. Short-chain fatty acids induce intestinal epithelial heat shock protein 25 expression in rats and IEC 18 cells. Gastroenterology. 121, 631-639.

Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S., Menasveta, P., 1998. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture. 167, 301-313.

Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., Menasaveta, P., 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). Aquaculture. 191, 271-288.

Rengpipat, S., Tunyanun, A., Fast, A.W., Piyatiratitivorakul, S., Menasveta, P., 2003. Enhanced growth and resistance to *Vibrio* challenge in pond-reared black tiger shrimp *Penaeus monodon* fed a *Bacillus* probiotic. Diseases of Aquatic Organisms. 55, 169-173.

Ricke, S., 2003. Perspectives on the use of organic acids and short chain fatty acids as antimicrobials. Poultry Science. 82, 632-639.

Ringo, E., Vadstein, O., 1998. Colonization of *Vibrio pelagius* and *Aeromonas caviae* in early developing turbot (*Scophthalmus maximus* L.) larvae. Journal of Applied Microbiology. 84, 227-233.

Ringø, E., Jøstensen, J.P., Olsen, R.E., 1992a. Production of eicosapentaenoic acid by freshwater *Vibrio*. Lipids. 27, 564-566.

Ringø, E., Sinclair, P.D., Birkbeck, H., Barbour, A., 1992b. Production of eicosapentaenoic acid (20: 5 n-3) by *Vibrio pelagius* isolated from turbot (*Scophthalmus maximus* (L.)) larvae. Applied and Environmental Microbiology. 58, 3777-3778.

Ringø, E., Olsen, R., Gifstad, T., Dalmo, R., Amlund, H., Hemre, G.I., Bakke, A., 2010. Prebiotics in aquaculture: a review. Aquaculture Nutrition. 16, 117-136.

Robertsen, B., Rørstad, G., Engstad, R., Raa, J., 1990. Enhancement of non-specific disease resistance in Atlantic salmon, *Salmo salar* L., by a glucan from *Saccharomyces cerevisiae* cell walls. Journal of Fish Diseases. 13, 391-400.

Robertson, P.A.W., O'Dowd, C., Burrels, C., William, P., Austin, B., 2000. Use of *Carnobacterium* sp. as a probiotic for atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). Aquaculture. 185, 235-243.

Rottmann, R., Francis-Floyd, R., Durborow, R., 1992. The role of stress in fish disease. Southern Regional Aquaculture Center, publication no. 474.

Rowley, A.F., Pope, E.C., 2012. Vaccines and crustacean aquaculture—a mechanistic exploration. Aquaculture. 334, 1-11.

Rungrassamee, W., Leelatanawit, R., Jiravanichpaisal, P., Klinbunga, S., Karoonuthaisiri, N., 2010. Expression and distribution of three heat shock protein genes under heat shock stress and under exposure to *Vibrio harveyi* in *Penaeus monodon*. Developmental & Comparative Immunology. 34, 1082-1089.

Ryzhov, V., Fenselau, C., 2001. Characterization of the protein subset desorbed by MALDI from whole bacterial cells. Analytical Chemistry. 73, 746-750.

Sabir, M.I., Xu, X., Li, L., 2009. A review on biodegradable polymeric materials for bone tissue engineering applications. Journal of Materials Science. 44, 5713-5724.

Sae-Oui, D., Tunsutapanich, A., Ruangpan, L., 1987. *Vibrio harveyi* a causative agent of white shrimp nauplii, *Penaeus merguiensis*. Thai Fisheries Gazette. 40, 177-182.

Saegusa, H., Shiraki, M., Kanai, C., Saito, T., 2001. Cloning of an intracellular poly [d (−)-3-hydroxybutyrate] depolymerase gene from *Ralstonia eutropha* H16 and characterization of the gene product. Journal of Bacteriology. 183, 94-100.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology and evolution. 4, 406-425.

Sakai, M., 1999. Current research status of fish immunostimulants. Aquaculture. 172, 63-92.

Sakai, M., Kamiya, H., Ishii, S., Atsuta, S., Kobayashi, M., 1992. The immunostimulating effects of chitin in rainbow trout, *Oncorhynchus mykiss*. Diseases in Asian aquaculture. 1, 413-417.

Salah Mesalhy, A., Yousef, A., Ahlam, A., Moahmed Fathi, M., 2008. Studies on *B. subtilis* and, *LactoBacillus acidophilus*, as potential probiotics, on the immuneresponse and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. Fish & Shellfish Immunology. 25, 128-136.

Salinas, I., Cuesta, A., Esteban, M.Á., Meseguer, J., 2005. Dietary administration of *LactoBacillus delbrüeckii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses. Fish & Shellfish Immunology. 19, 67-77.

Salinas, I., Díaz-Rosales, P., Cuesta, A., Meseguer, J., Chabrillón, M., Morinigo, M.A., Esteban, M.A., 2006. Effect of heat-inactivated fish and non-fish derived probiotics on the innate immune parameters of a teleost fish (*Sparus aurata* L.). Veterinary Immunology and Immunopathology. 111, 279-286.

Salinas, I., Abelli, L., Bertoni, F., Picchietti, S., Roque, A., Furones, D., Cuesta, A., Meseguer, J., Esteban, M.Á., 2008. Monospecies and multispecies probiotic formulations produce different systemic and local immunostimulatory effects in the gilthead seabream (*Sparus aurata* L.). Fish & Shellfish Immunology. 25, 114-123.

Salkinoja-Salonen, M., Vuorio, R., Andersson, M., Kämpfer, P., Andersson, M., Honkanen-Buzalski, T., Scoging, A., 1999. Toxigenic strains of *Bacillus licheniformis* related to food poisoning. Applied and Environmental Microbiology. 65, 4637-4645.

Saulnier, D., Haffner, P., Goarant, C., Levy, P., Ansquer, D., 2000. Experimental infection models for shrimp *Vibriosis* studies: a review. Aquaculture. 191, 133-144.

Schmitt, E., Gehrmann, M., Brunet, M., Multhoff, G., Garrido, C., 2007. Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. Journal of Leukocyte Biology. 81, 15-27.

Scholz, U., Diaz, G.G., Ricque, D., Cruz Suarez, L.E., Vargas Albores, F., Latchford, J., 1999. Enhancement of *Vibriosis* resistance in juvenile *Penaeus vanamei* by supplementation of diets with different yeast products. Aquaculture. 176, 271-283.

Schroeter, J., Klaenhammer, T., 2009. Genomics of lactic acid bacteria. FEMS Microbiology Letters. 292, 1-6.

Schubert, P., Steinbüchel, A., Schlegel, H.G., 1988. Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. Journal of Bacteriology. 170, 5837-5847.

Schwartz, E., Henne, A., Cramm, R., Eitinger, T., Friedrich, B., Gottschalk, G., 2003. Complete nucleotide sequence of pHG1: a *Ralstonia eutropha* H16 megaplasmid encoding key enzymes of H 2-based lithoautotrophy and anaerobiosis. Journal of Molecular Biology. 332, 369-383.

Senapin, S., Phewsaiya, K., Briggs, M., Flegel, T.W., 2007. Outbreaks of infectious myonecrosis virus (IMNV) in Indonesia confirmed by genome sequencing and use of an alternative RT-PCR detection method. Aquaculture. 266, 32-38.

Senior, P., Dawes, E., 1971. Poly-β-hydroxybutyrate biosynthesis and the regulation of glucose metabolism in *Azotobacter beijerinckii*. Biochemical Journal. 125, 55-66.

Seo, M.-C., Shin, H.-D., Lee, Y.-H., 2003. Functional role of granule-associated genes, phaP and phaR, in poly-β-hydroxybutyrate biosynthesis in recombinant *E. coli* harboring phbCAB operon. Biotechnology Letters. 25, 1243-1249.

Shan, H., Obbard, J., 2001. Ammonia removal from prawn aquaculture water using immobilized nitrifying bacteria. Applied Microbiology and Biotechnology. 57, 791-798.

Shang, L., Jiang, M., Chang, H.N., 2003. Poly (3-hydroxybutyrate) synthesis in fed-batch culture of *Ralstonia eutropha* with phosphate limitation under different glucose concentrations. Biotechnology Letters. 25, 1415-1419.

Sharifuzzaman, S., Austin, B., 2009. Influence of probiotic feeding duration on disease resistance and immune parameters in rainbow trout. Fish & Shellfish Immunology. 27, 440-445.

Sharmila, R., Abraham, T.J., Sundararaj, V., 1996. Bacterial flora of semi-intensive pond-reared *Penaeus indicus* (H. Milne Edwards) and the environment. Journal of aquaculture in the tropics. Calcutta. 11, 193-203.

Shen, W.Y., Fu, L.L., Li, W.F., Zhu, Y.R., 2010. Effect of dietary supplementation with *Bacillus subtilis* on the growth, performance, immune response and antioxidant activities of the shrimp (*Litopenaeus vannamei*). Aquaculture Research. 41, 1691-1698.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular systems biology. 7, 539.

Silva, B., Jesus, G., Seiffert, W., Vieira, F., Mouriño, J., Jatobá, A., Nolasco-Soria, H., 2016. The effects of dietary supplementation with butyrate and polyhydroxybutyrate on the digestive capacity and intestinal morphology of Pacific White Shrimp (*Litopenaeus vannamei*). Marine and Freshwater Behaviour and Physiology, 1-12.

Silva, E.F., Soares, M.A., Calazans, N.F., Vogeley, J.L., do Valle, B.C., Soares, R., Peixoto, S., 2012. Effect of probiotic (*Bacillus* spp.) addition during larvae and postlarvae culture of the white shrimp *Litopenaeus vannamei*. Aquaculture Research. 44, 13-21.

Sina, C., Gavrilova, O., Förster, M., Till, A., Derer, S., Hildebrand, F., Raabe, B., Chalaris, A., Scheller, J., Rehmann, A., 2009. G protein-coupled receptor 43 is essential for neutrophil recruitment during intestinal inflammation. The Journal of Immunology. 183, 7514-7522.

Singh, M., Patel, S., Kalia, V., 2009. *Bacillus* subtilis as potential producer for polyhydroxyalkanoates. Microbial Cell Factories. 8, 38.

Sio, C.F., Otten, L.G., Cool, R.H., Diggle, S.P., Braun, P.G., Bos, R., Daykin, M., Cámara, M., Williams, P., Quax, W.J., 2006. Quorum quenching by an N-acyl-homoserine lactone acylase from *Pseudomonas aeruginosa* PAO1. Infection and Immunity. 74, 1673-1682.

Siracusa, V., Rocculi, P., Romani, S., Dalla Rosa, M., 2008. Biodegradable polymers for food packaging: a review. Trends in Food Science & Technology. 19, 634-643.

Sirikharin, R., Taengchaiyaphum, S., Sanguanrut, P., Chi, T.D., Mavichak, R., Proespraiwong, P., Nuangsaeng, B., Thitamadee, S., Flegel, T.W., Sritunyalucksana, K., 2015. Characterization and PCR detection of binary, Pir-like toxins from *Vibrio parahaemolyticus* isolates that cause acute hepatopancreatic necrosis disease (AHPND) in shrimp. PloS one. 10, e0126987.

Situmorang, M.L., 2015. Application of poly-β-hydroxybutyrate in growth and health promotion of Nile tilapia *Oreochromis niloticus* culture. PhD thesis, Ghent University, Ghent, Belgium. pp. 217.

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

Situmorang, M.L., Dierckens, K., Mlingi, F.T., Van Delsen, B., Bossier, P., 2014. Development of a bacterial challenge test for gnotobiotic Nile tilapia *Oreochromis niloticus* larvae. Diseases of Aquatic Organisms. 109, 23-33.

Skalli, A., Castillo, M., Andree, K.B., Tort, L., Furones, D., Gisbert, E., 2013. The LPS derived from the cell walls of the Gram-negative bacteria *Pantoea agglomerans* stimulates growth and immune status of rainbow trout (*Oncorhynchus mykiss*) juveniles. Aquaculture. 416, 272-279.

Slater, S.C., Voige, W., Dennis, D., 1988. Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-beta-hydroxybutyrate biosynthetic pathway. Journal of Bacteriology. 170, 4431-4436.

Slepecky, R.A., Law, J.H., 1961. Synthesis and degradation of poly-β-hydroxybutyric acid in connection with sporulation of *Bacillus megaterium*. Journal of Bacteriology. 82, 37-42.

Smith, P., Christofilogiannis, P., 2007. Application of normalised resistance interpretation to the detection of multiple low-level resistance in strains of *Vibrio anguillarum* obtained from Greek fish farms. Aquaculture. 272, 223-230.

Snieszko, S., 1973. Diseases of fishes and their control in the US. The Two Lakes Fifth Fishery Management Training Course Report, Jansen, London, pp. 55-66.

Song, S.K., Beck, B.R., Kim, D., Park, J., Kim, J., Kim, H.D., Ringø, E., 2014. Prebiotics as immunostimulants in aquaculture: a review. Fish & Shellfish Immunology. 40, 40-48.

Soto-Rodriguez, S., Roque, A., Lizarraga-Partida, M., Guerra-Flores, A., Gomez-Gil, B., 2003. Virulence of luminous vibrios to *Artemia franciscana* nauplii. Diseases of Aquatic Organisms. 53, 231-240.

Spaargaren, D.H., 1998. Cultivation of Tiger Prawns, *Penaeus monodon* Fabricius, 1798 (Decapoda, Natantia) in Hainan, PR China. Crustaceana. 71, 144-157.

Spiekermann, P., Rehm, B.H., Kalscheuer, R., Baumeister, D., Steinbüchel, A., 1999. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Archives of Microbiology. 171, 73-80.

Sritunyalucksana, K., Cerenius, L., Soderhall, K., 1999. Molecular cloning and characterization of prophenoloxidase in the black tiger shrimp, *Penaeus monodon*. Developmental & Comparative Immunology. 23, 179-186.

Sriurairatana, S., Boonyawiwat, V., Gangnonngiw, W., Laosutthipong, C., Hiranchan, J., Flegel, T.W., 2014. White feces syndrome of shrimp arises from transformation, sloughing and aggregation of hepatopancreatic microvilli into vermiform bodies superficially resembling gregarines. PloS one. 9, e99170.

Stentiford, G., Neil, D., Peeler, E., Shields, J., Small, H., Flegel, T., Vlak, J., Jones, B., Morado, F., Moss, S., 2012. Disease will limit future food supply from the global crustacean fishery and aquaculture sectors. Journal of Invertebrate Pathology. 110, 141-157.

Strohalm, M., Kavan, D., Novák, P., Volný, M., Havlíček, V., 2010. mMass 3: A Cross-Platform Software Environment for Precise Analysis of Mass Spectrometric Data. Analytical Chemistry. 82, 4648-4651.

Subasinghe, R., Bondad-Reantaso, M., McGladdery, S., 2001. Aquaculture development, health and wealth. *In* R. P. Subasinghe, P. Bueno, M. J. Phillips, C. Hough, S. E. McGladdery, and J. R. Arthur (eds.), Aquaculture in the third millennium. Technical proceedings of the Conference on Aquaculture in the Third Millennium. NACA, Bangkok, Thailand, and FAO, Rome, Italy. p. 167-191.

Subasinghe, R.P., (Ed) 2001. Aquaculture in the third millennium: technical proceedings of the Conference on Aquaculture in the Third Millennium. Network of Aquaculture Centres in Asia-Pacific, Dept. of Fisheries. FAO Aquaculture Newsletter 25, Rome, Italy. pp. 24.

Subasinghe, R.P., Arthur, J.R., Shariff, M., 1996. Health management in Asian aquaculture, Proceedings of the Regional Expert Consulation on Aquaculture Health Management in Asia and the Pacific FAO Fisheries Technical Paper. No. 360. Rome, FAO, Serdang Malaysia pp. 142.

Sudheesh, P., Xu, H.-S., 2001. Pathogenicity of *Vibrio parahaemolyticus* in tiger prawn *Penaeus monodon* Fabricius: possible role of extracellular proteases. Aquaculture. 196, 37-46.

Sugita, H., Miyajima, C., Deguchi, Y., 1990. The vitamin B12-producing ability of intestinal bacteria isolated from tilapia [*Tilapia nilotica*] and channel catfish [*Ictalurus punctatus*]. Bulletin of the Japanese Society of Scientific Fisheries (Japan). 56, 4. 701.

Sugita, H., Miyajima, C., Deguchi, Y., 1991a. The vitamin B 12-producing ability of the intestinal microflora of freshwater fish. Aquaculture. 92, 267-276.

Sugita, H., Tanaami, H., Kobashi, T., Deguchi, Y., 1981. Bacterial flora of coastal bivalves. Bulletin of the Japanese Society of Scientific Fisheries.

Sugita, H., Takahashi, J., Miyajima, C., Deguchi, Y., 1991b. Vitamin B12-producing ability of the intestinal microflora of rainbow trout (*Oncorhynchus mykiss*). Agricultural and Biological Chemistry. 55, 893-894.

Sugita, H., Matsuo, N., Shibuya, K., Deguchi, Y., 1996. Production of antibacterial substances by intestinal bacteria isolated from coastal crab and fish species. Journal of marine biotechnology. 4, 220-223.

Sugita, H., Hirose, Y., Matsuo, N., Deguchi, Y., 1998. Production of the antibacterial substance by *Bacillus* sp. strain NM 12, an intestinal bacterium of Japanese coastal fish. Aquaculture. 165, 269-280.

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

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

Sui, L., Liu, Y., Sun, H., Wille, M., Bossier, P., De Schryver, P., 2014. The effect of poly-β-hydroxybutyrate on the performance of Chinese mitten crab (*Eriocheir sinensis* Milne-Edwards) zoea larvae. Aquaculture Research. 45, 558-565.

Summerfelt, S.T., 2003. Ozonation and UV irradiation—an introduction and examples of current applications. Aquacultural Engineering. 28, 21-36.

Sun, C.Q., O'Connor, C.J., Turner, S.J., Lewis, G.D., Stanley, R.A., Roberton, A.M., 1998. The effect of pH on the inhibition of bacterial growth by physiological concentrations of butyric acid: implications for neonates fed on suckled milk. Chemico-Biological Interactions. 113, 117-131.

Sun, Y.-Z., Yang, H.-L., Ma, R.-L., Lin, W.-Y., 2010. Probiotic applications of two dominant gut *Bacillus* strains with antagonistic activity improved the growth performance and immune responses of grouper *Epinephelus coioides*. Fish & Shellfish Immunology. 29, 803-809.

Sung, H.-H., Hsu, S.-F., Chen, C.-K., Ting, Y.-Y., Chao, W.-L., 2001. Relationships between disease outbreak in cultured tiger shrimp (*Penaeus monodon*) and the composition of *Vibrio* communities in pond water and shrimp hepatopancreas during cultivation. Aquaculture. 192, 101-110.

Sung, Y.Y., MacRae, T.H., Sorgeloos, P., Bossier, P., 2011. Stress response for disease control in aquaculture. Reviews in Aquaculture. 3, 120-137.

Sung, Y.Y., Van Damme, E.J., Sorgeloos, P., Bossier, P., 2007. Non-lethal heat shock protects gnotobiotic *Artemia franciscana* larvae against virulent *Vibrios*. Fish & Shellfish Immunology. 22, 318-326.

Swift, S., Lynch, M.J., Fish, L., Kirke, D.F., Tomás, J.M., Stewart, G.S., Williams, P., 1999. Quorum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. Infection and Immunity. 67, 5192-5199.

Takahashi, Y., Kondo, M., Itami, T., Honda, T., Inagawa, H., Nishizawa, T., Soma, G.-I., Yokomizo, Y., 2000. Enhancement of disease resistance against penaeid acute viraemia and induction of virus-inactivating activity in haemolymph of kuruma shrimp, *Penaeus japonicus*, by oral administration of *Pantoea agglomerans* lipopolysaccharide (LPS). Fish & Shellfish Immunology. 10, 555-558.

Tang, Y., Chen, Y., Jiang, H., Nie, D., 2011. Short-chain fatty acids induced autophagy serves as an adaptive strategy for retarding mitochondria-mediated apoptotic cell death. Cell Death and Differentiation. 18, 602-618.

Tendencia, E.A., de la Peña, L.D., 2001. Antibiotic resistance of bacteria from shrimp ponds. Aquaculture. 195, 193-204.

Tendencia, E.A., dela Peña, M., 2003. Investigation of some components of the greenwater system which makes it effective in the initial control of luminous bacteria. Aquaculture. 218, 115-119.

Tendencia, E.A., dela Peña, M.R., Choresca, C.H., 2005. Efficiency of *Chlorella* sp. and *Tilapia hornorum* in controlling the growth of luminous bacteria in a simulated shrimp culture environment. Aquaculture. 249, 55-62.

Teo, J.W., Suwanto, A., Poh, C.L., 2000. Novel β-lactamase genes from two environmental isolates of *Vibrio harveyi*. Antimicrobial Agents and Chemotherapy. 44, 1309-1314.

Thai, T.-Q., 2015. Application of poly-β-hydroxybutyrate accumulating bacteria in crustacean larviculture. PhD thesis, Ghent University, Ghent, Belgium. pp. 224.

Thai, T.Q., Wille, M., Garcia-Gonzalez, L., Sorgeloos, P., Bossier, P., De Schryver, P., 2014. Poly-ß-hydroxybutyrate content and dose of the bacterial carrier for *Artemia*

enrichment determine the performance of giant freshwater prawn larvae. Applied Microbiology and Biotechnology. 98, 5205-5215.

Thanh Tung, H., Koshio, S., Ferdinand Traifalgar, R., Ishikawa, M., Yokoyama, S., 2010. Effects of Dietary Heat-killed *LactoBacillus plantarum* on Larval and Post-larval Kuruma Shrimp, *Marsupenaeus japonicus* Bate. Journal of the World Aquaculture Society. 41, 16-27.

Theodorakis, N.G., Drujan, D., De Maio, A., 1999. Thermotolerant cells show an attenuated expression of Hsp70 after heat shock. Journal of Biological Chemistry. 274, 12081-12086.

Thitamadee, S., Prachumwat, A., Srisala, J., Jaroenlak, P., Salachan, P.V., Sritunyalucksana, K., Flegel, T.W., Itsathitphaisarn, O., 2016. Review of current disease threats for cultivated penaeid shrimp in Asia. Aquaculture. 452, 69-87.

Thomas, J.G., Ayling, A., Baneyx, F., 1997. Molecular chaperones, folding catalysts, and the recovery of active recombinant proteins from *E. coli*. Applied Biochemistry and Biotechnology. 66, 197-238.

Tinh, N.T.N., Linh, N.D., Wood, T.K., Dierckens, K., Sorgeloos, P., Bossier, P., 2007. Interference with the quorum sensing systems in a *Vibrio harveyi* strain alters the growth rate of gnotobiotically cultured rotifer *Brachionus plicatilis*. Journal of Applied Microbiology. 103, 194-203.

Tokiwa, Y., Calabia, B.P., Ugwu, C.U., Aiba, S., 2009. Biodegradability of plastics. International Journal of Molecular Sciences. 10, 3722-3742.

Tourtip, S., Wongtripop, S., Stentiford, G.D., Bateman, K.S., Sriurairatana, S., Chavadej, J., Sritunyalucksana, K., Withyachumnarnkul, B., 2009. *Enterocytozoon hepatopenaei* sp. nov. (Microsporida: Enterocytozoonidae), a parasite of the black tiger shrimp *Penaeus monodon* (Decapoda: Penaeidae): Fine structure and phylogenetic relationships. Journal of Invertebrate Pathology. 102, 21-29.

Tovar-Ramírez, D., Infante, J.Z., Cahu, C., Gatesoupe, F.J., Vázquez-Juárez, R., 2004. Influence of dietary live yeast on European sea bass (*Dicentrarchus labrax*) larval development. Aquaculture. 234, 415-427.

Tran, L., Nunan, L., Redman, R.M., Mohney, L.L., Pantoja, C.R., Fitzsimmons, K., Lightner, D.V., 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Diseases of Aquatic Organisms. 105, 45-55.

Tsan, M.-F., Gao, B., 2009. Heat shock proteins and immune system. Journal of Leukocyte Biology. 85, 905-910.

Tseng, D.-Y., Ho, P.-L., Huang, S.-Y., Cheng, S.-C., Shiu, Y.-L., Chiu, C.-S., Liu, C.-H., 2009. Enhancement of immunity and disease resistance in the white shrimp, *Litopenaeus vannamei*, by the probiotic, *Bacillus subtilis* E20. Fish & Shellfish Immunology. 26, 339-344.

Tsing, A., Bonami, J., 1987. A new viral disease of the tiger shrimp, *Penaeus japonicus* Bate. Journal of Fish Diseases. 10, 139-141.

Urdaci, M., Pinchuk, I., Ricca, E., Henriques, A., Cutting, S., 2004. Antimicrobial activity of *Bacillus* probiotics. Bacterial spore formers: probiotics and emerging applications, 171-182.

Utari, H.B., Senapin, S., Jaengsanong, C., Flegel, T.W., Kruatrachue, M., 2012. A haplosporidian parasite associated with high mortality and slow growth in *Penaeus (Litopenaeus) vannamei* cultured in Indonesia. Aquaculture. 366–367, 85-89.

Valappil, S.P., Boccaccini, A., Bucke, C., Roy, I., 2007. Polyhydroxyalkanoates in Gram-positive bacteria: insights from the genera *Bacillus* and *Streptomyces*. Antonie Van Leeuwenhoek. 91, 1-17.

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

Van Immerseel, F., De Buck, J., Pasmans, F., Velge, P., Bottreau, E., Fievez, V., Haesebrouck, F., Ducatelle, R., 2003. Invasion of *Salmonella enteritidis* in avian intestinal epithelial cells *in vitro* is influenced by short-chain fatty acids. International Journal of Food Microbiology. 85, 237-248.

Van Ness, G.B., 1971. Ecology of anthrax. Science. 172, 1303-1307.

Verlinden, R.A., Hill, D.J., Kenward, M., Williams, C.D., Radecka, I., 2007. Bacterial synthesis of biodegradable polyhydroxyalkanoates. Journal of Applied Microbiology. 102, 1437-1449.

Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiology and Molecular Biology Reviews. 64, 655-671.

Verschuere, L., Rombaut, G., Huys, G., Dhont, J., Sorgeloos, P., Verstraete, W., 1999. Microbial Control of the Culture of Artemia Juveniles through Preemptive Colonization by Selected Bacterial Strains. Applied and Environmental Microbiology. 65, 2527-2533.

Vine, N.G., Leukes, W.D., Kaiser, H., 2006. Probiotics in marine larviculture. FEMS Microbiology Reviews. 30, 404-427.

Vinolo, M.A., Ferguson, G.J., Kulkarni, S., Damoulakis, G., Anderson, K., Bohlooly-Y, M., Stephens, L., Hawkins, P.T., Curi, R., 2011. SCFAs induce mouse neutrophil chemotaxis through the GPR43 receptor. PloS one. 6, e21205.

Volman, J.J., Ramakers, J.D., Plat, J., 2008. Dietary modulation of immune function by β-glucans. Physiology & Behavior. 94, 276-284.

Volova, T., Shishatskaya, E., Sevastianov, V., Efremov, S., Mogilnaya, O., 2003. Results of biomedical investigations of PHB and PHB/PHV fibers. Biochemical Engineering Journal. 16, 125-133.

Wahl, A., Schuth, N., Pfeiffer, D., Nussberger, S., Jendrossek, D., 2012. PHB granules are attached to the nucleoid via PhaM in *Ralstonia eutropha*. BMC Microbiology. 12, 262.

Wallin, R.P., Lundqvist, A., Moré, S.H., von Bonin, A., Kiessling, R., Ljunggren, H.-G., 2002. Heat-shock proteins as activators of the innate immune system. Trends in Immunology. 23, 130-135.

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

Wang, K.H.-C., Tseng, C.-W., Lin, H.-Y., Chen, I.-T., Chen, Y.-H., Chen, Y.-M., Chen, T.-Y., Yang, H.-L., 2010. RNAi knock-down of the *Litopenaeus vannamei* Toll gene

(LvToll) significantly increases mortality and reduces bacterial clearance after challenge with *Vibrio harveyi.* Developmental & Comparative Immunology. 34, 49-58.

Wang, Y.-B., 2007. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. Aquaculture. 269, 259-264.

Wang, Y.B., Xu, Z.R., Xia, M.S., 2005. The effectiveness of commercial probiotics in northern white shrimp *Penaeus vannamei* ponds. Fisheries Science. 71, 1036-1041.

Waterfield, N., George Kamita, S., Hammock, B.D., Ffrench-Constant, R., 2005. The *Photorhabdus* Pir toxins are similar to a developmentally regulated insect protein but show no juvenile hormone esterase activity. FEMS Microbiology Letters. 245, 47-52.

Waters, C.M., Bassler, B.L., 2005. Quorum sensing: cell-to-cell communication in bacteria. Annual Review of Cell and Developmental Biology. 21, 319-346.

Wedemeyer, G.A., Nelson, N.C., Yasutake, W.T., 1979. Physiological and biochemical aspects of ozone toxicity to rainbow trout (*Salmo gairdneri*). Journal of the Fisheries Board of Canada. 36, 605-614.

Wei, D.-X., Chen, C.-B., Fang, G., Li, S.-Y., Chen, G.-Q., 2011a. Application of polyhydroxyalkanoate binding protein PhaP as a bio-surfactant. Applied Microbiology and Biotechnology. 91, 1037-1047.

Wei, Y.-H., Chen, W.-C., Huang, C.-K., Wu, H.-S., Sun, Y.-M., Lo, C.-W., Janarthanan, O.-M., 2011b. Screening and Evaluation of Polyhydroxybutyrate-Producing Strains from Indigenous Isolate *Cupriavidus taiwanensis* Strains. International Journal of Molecular Sciences. 12, 252-265.

Welch, W.J., 1993. How cells respond to stress: during emergencies,cells produce stress proteins that repair damage, inquiry into how they work offers promise for coping with infection, autoimmune disease and even cancer. Scientific American. 268, 56-56.

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

Wieczorek, R., Pries, A., Steinbüchel, A., Mayer, F., 1995. Analysis of a 24-kilodalton protein associated with the polyhydroxyalkanoic acid granules in *Alcaligenes eutrophus*. Journal of Bacteriology. 177, 2425-2435.

Wilén, B.-M., Jin, B., Lant, P., 2003. The influence of key chemical constituents in activated sludge on surface and flocculating properties. Water Research. 37, 2127-2139.

Wolters, M., Rohde, H., Maier, T., Belmar-Campos, C., Franke, G., Scherpe, S., Aepfelbacher, M., Christner, M., 2011. MALDI-TOF MS fingerprinting allows for discrimination of major methicillin-resistant *Staphylococcus aureus* lineages. International Journal of Medical Microbiology. 301, 64-68.

Wu, H., Song, Z., Givskov, M., Doring, G., Worlitzsch, D., Mathee, K., Rygaard, J., Høiby, N., 2001. *Pseudomonas aeruginosa* mutations in lasI and rhlI quorum sensing systems result in milder chronic lung infection. Microbiology. 147, 1105-1113.

Xing, C.-F., Hu, H.-H., Huang, J.-B., Fang, H.-C., Kai, Y.-H., Wu, Y.-C., Chi, S.-C., 2013. Diet supplementation of *Pediococcus pentosaceus* in cobia (*Rachycentron canadum*) enhances growth rate, respiratory burst and resistance against photobacteriosis. Fish & Shellfish Immunology. 35, 1122-1128.
Yang, X.-P., Wang, S.-M., Zhang, D.-W., Zhou, L.-X., 2011. Isolation and nitrogen removal characteristics of an aerobic heterotrophic nitrifying–denitrifying bacterium, *Bacillus subtilis* A1. Bioresource Technology. 102, 854-862.
Yang, Y.-T., Chen, I.-T., Lee, C.-T., Chen, C.-Y., Lin, S.-S., Hor, L.-I., Tseng, T.-C., Huang, Y.-T., Sritunyalucksana, K., Thitamadee, S., 2014. Draft genome sequences of four strains of *Vibrio parahaemolyticus*, three of which cause early mortality syndrome/acute hepatopancreatic necrosis disease in shrimp in China and Thailand. Genome announcements. 2, e00816-00814.
Yeh, M.-S., Lai, C.-Y., Liu, C.-H., Kuo, C.-M., Cheng, W., 2009. A second proPO present in white shrimp *Litopenaeus vannamei* and expression of the proPOs during a *Vibrio alginolyticus* injection, molt stage, and oral sodium alginate ingestion. Fish & Shellfish Immunology. 26, 49-55.
York, G.M., Junker, B.H., Stubbe, J., Sinskey, A.J., 2001. Accumulation of the PhaP phasin of *Ralstonia eutropha* is dependent on production of polyhydroxybutyrate in cells. Journal of Bacteriology. 183, 4217-4226.
York, G.M., Lupberger, J., Tian, J., Lawrence, A.G., Stubbe, J., Sinskey, A.J., 2003. *Ralstonia eutropha* H16 encodes two and possibly three intracellular poly [d-(−)-3-hydroxybutyrate] depolymerase genes. Journal of Bacteriology. 185, 3788-3794.
Young, J.C., Agashe, V.R., Siegers, K., Hartl, F.U., 2004. Pathways of chaperone-mediated protein folding in the cytosol. Nature Reviews Molecular Cell Biology. 5, 781-791.
Yu, J., Plackett, D., Chen, L.X., 2005. Kinetics and mechanism of the monomeric products from abiotic hydrolysis of poly [(R)-3-hydroxybutyrate] under acidic and alkaline conditions. Polymer Degradation and Stability. 89, 289-299.
Yüksekdağ, Z.N., Aslım, B., Beyatlı, Y., Mercan, N., 2003. Effect of carbon and nitrogen sources and incubation times on poly-beta-hydroxybutyrate (PHB) synthesis by *Bacillus subtilis* 25 and *Bacillus megaterium* 12. African Journal of Biotechnology. 3, 63-66.
Zhang, Q., Liu, Q., Liu, S., Yang, H., Liu, S., Zhu, L., Yang, B., Jin, J., Ding, L., Wang, X., Liang, Y., Wang, Q., Huang, J., 2014. A new nodavirus is associated with covert mortality disease of shrimp. Journal of General Virology. 95, 2700-2709.
Zhang, X., Luo, R., Wang, Z., Deng, Y., Chen, G.-Q., 2009. Application of (R)-3-hydroxyalkanoate methyl esters derived from microbial polyhydroxyalkanoates as novel biofuels. Biomacromolecules. 10, 707-711.
Zhao, P., Li, J., Wang, Y., Jiang, H., 2007. Broad-spectrum antimicrobial activity of the reactive compounds generated in vitro by *Manduca sexta* phenoloxidase. Insect Biochemistry and Molecular Biology. 37, 952-959.
Zhao, P., Lu, Z., Strand, M.R., Jiang, H., 2011. Antiviral, anti-parasitic, and cytotoxic effects of 5, 6-dihydroxyindole (DHI), a reactive compound generated by phenoloxidase during insect immune response. Insect Biochemistry and Molecular Biology. 41, 645-652.

Zhao, Y., Zhang, W., Xu, W., Mai, K., Zhang, Y., Liufu, Z., 2012. Effects of potential probiotic *Bacillus subtilis* T13 on growth, immunity and disease resistance against *Vibrio splendidus* infection in juvenile sea cucumber *Apostichopus japonicus*. Fish & Shellfish Immunology. 32, 750-755.

Zhou, J., Wang, W.-N., He, W.-Y., Zheng, Y., Wang, L., Xin, Y., Liu, Y., Wang, A.-L., 2010. Expression of HSP60 and HSP70 in white shrimp*, Litopenaeus vannamei* in response to bacterial challenge. Journal of Invertebrate Pathology. 103, 170-178.

Ziaei-Nejad, S., Rezaei, M.H., Takami, G.A., Lovett, D.L., Mirvaghefi, A.-R., Shakouri, M., 2006. The effect of *Bacillus* spp. bacteria used as probiotics on digestive enzyme activity, survival and growth in the Indian white shrimp *Fenneropenaeus indicus*. Aquaculture. 252, 516-524.

Zinn, M., Witholt, B., Egli, T., 2001. Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. Advanced Drug Delivery Reviews. 53, 5-21.

Zokaeifar, H., Balcázar, J.L., Saad, C.R., Kamarudin, M.S., Sijam, K., Arshad, A., Nejat, N., 2012. Effects of *Bacillus subtilis* on the growth performance, digestive enzymes, immune gene expression and disease resistance of white shrimp*, Litopenaeus vannamei*. Fish & Shellfish Immunology. 33, 683-689.

APPENDIX A

SUMMARY/ SAMENVATTING

SUMMARY

The aquaculture sector has been recognized as an important food-producing sector that is expected to contribute significantly to the world's food supply in the next decades, however, its development and expansion has always been hampered by diseases thereby affecting its worldwide production (chapter 1). Several sustainable disease-controlling measures have been explored and developed to mitigate disease problems in aquaculture (chapter 2) however, even with these developments the industry is still continuously confronted not only by existing diseases but with new/emerging diseases as well. Hence, the search for new equally effective and sustainable approach is still important. In this research, PHB-accumulating *Bacillus* spp. was explored and tested as new biological control agents for aquaculture. With the undoubted beneficial effects of crystalline PHB particles (i.e. extracted form of the biopolymer), the approach of using amorphous PHB where the compound is still contained in (live) bacteria that are often associated with strong probiotic effects (e.g. *Bacillus* spp.) is an interesting strategy to explore. This approach may further advanced the potentials of both PHB and the probiotic strategy as important disease-controlling measures for aquaculture.

In this PhD research, the study has demonstrated for the first time the isolation and rapid screening of superior PHB-accumulating *Bacillus* species from pasteurized shrimp pond sediments (chapter 3). This study has demonstrated the application of matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF) technique as a tool for dereplication (i.e. bacterial grouping) which facilitated the short-listing of acquired bacterial isolates for rapid screening and selection of superior PHB accumulators. The study suggests that MALDI-TOF MS patterns can be used as a tool to identify bacteria with high PHB accumulation capability, however further research should be done to confirm this observation. The superior PHB-accumulating *Bacillus* isolate identified in this study was *Bacillus* sp. JL47 which can accumulate amorphous PHB at 28.6% PHB on CDW after 24 h in LB medium with 2% glucose. However, further experimentation revealed that the isolate can also accumulate PHB as high as 55% PHB on CDW after 48 h of culture using LB medium with 2% glucose.

Furthermore, two *in vivo* experiments were conducted to determine the beneficial effects of the PHB-accumulating *Bacillus* species in cultured crustaceans. The first *in vivo*

work was conducted under a fully controlled axenic culture system using gnotobiotically-cultured *Artemia franciscana*. The results showed that the superior PHB accumulator *Bacillus* sp. JL47 strain protected the gnotobiotic *Artemia* against pathogenic *V. campbellii* LMG 21363 infection when fed at 10^7 cells mL^{-1} or higher, while no protective effect was observed to *Artemia* when fed at 10^6 cells mL^{-1} or lower (chapter 4). The study further showed that the protective effect of *Bacillus* sp. JL47 was superior when the bacterium contains high amount of amorphous PHB, irrespective of the cell density used. Specifically, feeding *Bacillus* sp. JL47 containing 55% amorphous PHB (on CDW) to *Artemia* showed a significantly higher survival in a *Vibrio* challenge relative to *Bacillus* containing 29% PHB. The data suggest that the amorphous PHB accumulated in the *Bacillus* sp. JL47 strain is a main determinant for the increased survival of challenged *Artemia*. In the second *in vivo* work, different PHB-accumulating *Bacillus* spp. were tested in *P. monodon* postlarvae in actual nursery culture (chapter 5). The results showed that shrimps fed the PHB-accumulating *Bacillus* spp. improved the survival, growth performance and resistance of the shrimp against pathogenic *V. campbellii*, with a maximum attained for the shrimp supplied of the highest PHB accumulator (i.e. *Bacillus* sp. JL47). The study showed that supplementation of the PHB-accumulating *Bacillus* sp. JL47 in the shrimp feed at 4 g kg^{-1} wet weight (approximately 0.41 g PHB kg^{-1}) protected the shrimps from *V. campbellii* infection. Likewise, adding the PHB-accumulating *Bacillus* sp. JL47 in the rearing water at 10^6 cells mL^{-1} day^{-1} also protected the shrimps from the daily exposure to the pathogen at 10^6 cells mL^{-1}.

Moreover, to understand further the protective effect of the PHB-accumulating *Bacillus* sp. JL47 in shrimp *in vivo*, the effects of the PHB-accumulating *Bacillus* sp. JL47 strain on the expression of proPO, TGase and HSP70 of *P. monodon* postlarvae before and after the *V. campbellii* challenge was investigated (chapter 6). The results showed that proPO and TGase genes were significantly up-regulated ($p<0.05$) within 12 h after challenge in shrimp receiving the *Bacillus* sp. JL47 as compared to the challenged and non-challenged controls. Hsp70 expression was significantly increased ($p<0.05$) at 3 h post-challenge in all challenged shrimp. Interestingly, proPO and TGase genes were significantly up-regulated ($p<0.05$) in *Bacillus* sp. JL47 treated shrimp even before the *Vibrio* challenge was applied. No up-regulation in the Hsp70 gene, however, was

observed under these conditions. The data suggest that the protective effect of the PHB-accumulating *Bacillus* sp. JL47 in shrimp observed in the previous experiment (chapter 5) can be attributed to its capacity to stimulate the innate immune related genes of the shrimp, specifically the proPO and TGase genes.

Furthermore, this research also investigated the effects of phasin- a major PHB granule-associated protein that is present at the surface of the amorphous PHB inside the bacterial cell- on the survival of gnotobiotic *Artemia* challenged with pathogenic *Vibrio campbellii* (chapter 7). In this study, the gene that encodes the phasin protein from the PHB-accumulating *Bacillus* sp. JL47 was cloned, sequenced and over-produced in non-pathogenic *E. coli* by means of arabinose induction. The phasin protein which is 99% related to the phasin protein found in *B. cereus* and *B. thuringiensis* has 174 amino acids and has a size of 20.42 kDa. Furthermore, feeding the gnotobiotic *Artemia* with *E. coli* containing the overproduced phasin protein showed no protective effect to the animal after a pathogenic *Vibrio campbellii* challenge. The result suggest that the protein did not contribute to the protective effects displayed by the amorphous PHB in the previous experiments conducted in *Artemia* nauplii (chapter 4) and *P. monodon* postlarvae (chapter 5).

In summary, the application of PHB-accumulating *Bacillus* species as (new) biocontrol agents for aquaculture is highly promising. As presented and discussed in chapter 8, the application of *Bacillus* species containing significant amount of amorphous PHB can have several mode of actions, thus, making this strategy more advantageous and more efficient in controlling diseases in aquaculture.

SAMENVATTING

De aquacultuursector wordt erkend als een belangrijke voedsel producerende sector waarvan verwacht wordt dat die aanzienlijk zal bijdragen tot de wereldwijde voedselvoorziening in de komende decennia, hoewel de ontwikkeling en uitbreiding gehinderd worden door ziektes (hoofdstuk 1). Verschillende duurzame ziektebeperkende maatregelen werden onderzocht en ontwikkeld om ziektes in aquacultuur te voorkomen (hoodstuk 2). Desalniettemin blijft de industrie continue geconfronteerd met niet alleen bestaande ziektes maar ook met nieuwe opkomende ziektes. Vandaar dat de zoektocht naar nieuwe, effectieve en duurzame behandelingen nog steeds belangrijk is. In dit onderzoek, werden PHB-accumulerende *Bacillus* spp. onderzocht en getest als nieuw biologisch controlemiddel in de aquacultuur. In de wetenschap dat kristallijne PHB deeltjes (bv. geëxtraheerde vorm van de biopolymeer) een gunstig effect hebben, werd hier de mogelijkheid onderzocht dat de applicatie van amorfe PHB, zoals intracellulair vervat in (levende) *Bacillus* spp die vaak geassocieerd worden met sterke probiotische effecten, een interessante strategie zou zijn. Deze benadering kan het potentieel van een probiotische strategie versterken.

In dit doctoraatsonderzoek werd voor het eerst de isolatie en het snelle screenen van superieure PHB-accumulerende *Bacillus* soorten uit gepasteurizeerd sediment van garnaalvijvers aangetoond (hoofdstuk 3). Dit onderzoek heeft aangetoond dat de toepassing van de “matrix assisted laser desorption/ionization time-of-flight mass spectrometry” (MALDI-TOF) techniek als methode voor dereplicatie (dwz bacteriële groepering) van verworven bacteriële isolaten een snelle screening en selectie van superieure PHB accumulatoren toelaat. Dit onderzoek suggereert dat MALDI-TOF MS patronen gebruikt kunnen worden als hulpmiddel om bacteriën te identifceren met hoge PHB accumulerende mogelijkheden, hoewel verder onderzoek nodig is om deze vaststelling te bevestigen. Een superieure PHB-accumulerende *Bacillus* isolaat werd geïdentifieerd in dit onderzoek (*Bacillus* sp. JL47). Het kan 28.6% amorphe PHB accumuleren (uitgedrukt op cellulair drooggewicht, CDW) na 24u in een LB medium met 2% glucose. Optimalistie toonde aan dat de isolaat PHB kan accumuleren tot 55% PHB op het CDW.

Verder werden er 2 *in vivo* experimenten uitgevoerd om de gunstige effecten aan te tonen van de PHB-accumulerende *Bacillus*-soort bij gekweekte schaaldieren. Het eerste *in vivo* werk werd uitgevoerd met gnotobiotisch gekweekte *Artemia franciscana*. De resultaten toonden aan dat *Bacillus* sp. JL47 de gnotobiotische *Artemia* beschermde tegen pathogene *V. campbellii* LMG 21363 infectie wanneer 10^7 cellen ml^{-1} of hoger vervoederd werden, terwijl geen beschermend effect werd vastgesteld bij lagere voedselhoeveelheden (hoofdstuk 4). Het onderzoek toonde verder ook aan dat het beschermende effect van *Bacillus* sp. JL47 superieur was wanneer de cellen een hoge dosis amorfe PHB bevatten, ongeacht de gebruikte celdichtheid. Specifiek, *Bacillus* sp. JL47 met 55% amorfe PHB (op CDW) geeft een significant hogere overleving bij *Artemia* in een *Vibrio* blootstellingstest ten opzichte van *Bacillus* met 29% PHB. De data tonen aan dat de geaccumuleerde amorfe PHB in de *Bacillus* sp. JL47 een belangrijke factor is voor de verhoogde overleving van “challenged” *Artemia*. In het 2^e *in vivo* werk, werden verschillende PHB-accumulerende *Bacillus* spp. getest op *P. monodon* postlarven in een pilootinstallatie (hoofdstuk 5). De resultaten toonden aan dat garnalen gevoed met PHB-accumulerende *Bacillus* spp. een betere overleving en groeiprestatie hebben. Ze induceren ook een verhoogde resistentie van de garnaal tegen de pathogene *V. campbellii*. Het onderzoek toonde aan dat de toevoeging van de PHB-accumulerende *Bacuillis* sp. JL47 in garnalenvoeding aan 4 g kg^{-1} versgewicht (ongeveer 0.41 g PHB kg^{-1}) de garnalen beschermden tegen *V. campbellii* infectie. Het toevoegen van PHB-accumulerende *Bacullis* sp. JL47 in het kweekwater aan 10^6 cells ml^{-1} dag^{-1} beschermde de garnalen eveneens tegen de pathogeen (dagelijks toegevoegd aan het kweekwater met 10^6 cellen ml^{-1}).

Om immunologische effecten van de PHB-accumulerende *Bacillus* sp. JL47 op garnalen te verstaan, werden de effecten van de PHB-accumulerende *Bacillus* sp. JL47 stam op de expressie van proPO, Tgase en Hsp70 van *P. monodon* postlarven onderzocht (voor en na de *V. campbellii* “challenge”) (hoofdstuk 6). De resultaten toonden aan dat proPO en Tgase genen een significante verhoogde expressie ($p<0.05$) vertoonden binnen 12 h na toevoeging van *Bacillus* sp. JL47 in vergelijking met de controles. Hsp70 expressie was beduidend hoger ($p<0.05$) 3 u na de blootstelling in alle blootgestelde garnalen.

Interessant is dat de proPO en Tgase genen een significant verhoogde expressie vertoonden ($p<0.05$) in *Bacillus* sp. JL47 behandelde garnalen, voorafgaand aan een Vibrio “challenge”. De data suggereren dat het bechermende karakter van PHB-accumulerende *Bacillus* sp. JL47 in garnalen zoals vastgesteld in vorige experminenten (hoofdstuk 5), kon toegewezen worden aan zijn capaciteit om de aangeboren immuun gerelateerde genen van garnalen te stimuleren, specifiek de proPO en TGase genen.

Verder werd in dit onderzoek ook de effecten van phasin, een belangrijk PHB granule-geassocieerd eiwit die aanwezig is in de oppervlakte van het amorfe PHB in de bacteriële cel, op de overleving van gnotobiotische *Artemia* blootgesteld aan een pathogene Vibrio *campbellii* onderzocht (hoofdstuk 7). In dit onderzoek, werd het gen dat voor het phasin eiwit van de PHB-accumuleerde *Bacillus* sp. JL47 codeert, gecloond, gesequeneerd en overgeproduceerd in een niet pathogene *E.coli* door middel van een arabinose inductie. Het phasin eiwit dat 99% verwant is aan het phasin eiwit dat terug te vinden is in *B. cereus* en *B. thurungiensis,* heeft 174 aminozuren en heeft de grootte van 20.4 KDa. Verder vertoonde gnotobiotische *Artemia* gevoed met *E. coli* met overgeproduceerd phasin eiwit geen beschermd effect na een pathogene *Vibrio campbellii* blootstelling. Het resultaat laat veronderstellen dat het eiwit niet bijdroeg tot de beschermde effecten die aangetoond werden bij de amorfe PHB toevoeging in eerdere experimenten uitgevoerd in *Artemia* nauplii (hoofdstuk 4) en de *P. monodon* postlarven in hoofdstuk 5.

Samenvattend, de toepassing van PHB-accumulerende *Bacillus* soorten als (nieuwe) biologisch controlemiddel in de aquacultuur is veelbelovend. Zoals voorgesteld en besproken in hoofstuk 8, kunnen de voordelige effecten van de toepassing van *Bacillus* soorten met een hoog gehalte aan amorfe PHB gebaseerd zijn op verschillende “mode of actions”, wat hun toepassing in de aquacultuur aantrekkelijk maakt.

APPENDIX B

CURRICULUM VITAE

Curriculum Vitae

Personal information

Joseph Leopoldo Q. Laranja, Jr.

Address: SEAFDEC Compd., A-4 Carp St. Buyuan, 5021 Tigbauan, Iloilo, Philippines
Email: jllaranja@seafdec.org.ph/joseph.laranja@ugent.be/jlqlaranja@gmail.com
Phone: +63 33 511-9170
Age: 36 (October 16, 1980)
Nationality: Filipino
Civil status: Married

Education

Doctor of Philosophy in Applied Biological Sciences *(candidate)*
Laboratory of Aquaculture and *Artemia* Reference Center
Ghent University, Ghent, Belgium
Thesis: "*Amorphous poly-β-hydroxybutyrate (PHB)-accumulating Bacillus spp. as biocontrol agents in crustacean culture"- promotors: prof. dr. ir. Peter Bossier; dr.ir. Peter De Schryver; dr. Edgar Amar*

Master of Science in Aquaculture *(w/ distinction)*
(2006-2007)
Laboratory of Aquaculture and *Artemia* Reference Center
Ghent University, Ghent, Belgium
Thesis: -*"In vitro determination of the prebiotic potential of dietary carbohydrates in fish"- promotors: prof.dr.ir. Frans Ollevier; dr.ir. Eugene Rurangwa (Katholieke Universetiet Leuven)*

Complementary Studies in Aquaculture *(w/ distinction)*
(2005-2006)
Laboratory of Aquaculture and *Artemia* Reference Center
Ghent University, Ghent, Belgium

Bachelor of Science in Biology
(1996-2000)
Mindanao State University-Iligan Institute of Technology
Tibanga, Iligan City, Philippines
Thesis: *Community structure of seagrasses in Samburon, Linamon, Lanao del Norte, Philippines*

Secondary Education *(w/ honors)*
Our Lady of Peace High School

Malabang, Lanao del Sur, Philippines

Professional experience

Researcher
July 2016-present

Associate Researcher
2008-Jul 2016

Southeast Asian Fisheries Development Center, Aquaculture Department, Buyuan, 5021 Tigbauan, Iloilo, Philippines

Principal investigator/Project leader of the ff. projects:
- Effects of PHB-accumulating *Bacillus* spp. on the water quality and thermal stress response of *Penaeus monodon* postlarvae during culture (2016-2017)
- Utilization of artificial illumination in floating net cages on the nursery culture of pompano *Trachinotus blochii*:effectson growth and survival of pompano and its added economic value (2017)
- The application and mode of action probiotic *Bacillus* in the larviculture of *Penaeus* (2011-2015)
- The effects of dietary tryptophan on the antagonistic behaviour of mud crab *Scylla serrata* (2010-2012)
- Nursery production of mud crab *Scylla serrata* in brackishwater ponds (2009-2012)

Co-proponent
- Effects of thraustochytrid on growth, survival and reproductive performance of hatchery-bred abalone, *Haliotis asinina* (2017)

Technical Officer
April 2003-2005

Philippine Business for Social Progress (PBSP), Catbalogan, Samar, Philippines

Tasks:
- Prepare proposals for different livelihood projects (i.e. marine fish culture in cages) for the local fish farmers in the area
- Assists in the implementation of different livelihood projects (milkfish culture, snapper culture, mud crab culture, mussel culture)
- Conduct trainings related to the different projects implemented

Technical Assistant
Oct 2000-2002
Southeast Asian Fisheries Development Center, Aquaculture Department, Buyuan, 5021 Tigbauan, Iloilo, Philippines

Tasks:

- Assist the study leader in conducting experiments (data collection, data analyses, maintenance of fish stocks)
- Assist in the preparation of reports and presentations

Grants/Awards received

- PhD scholarship grant - Special research fund (BOF), Ghent University , Ghent, Belgium (2011-2017)
- Research grant- International Foundation for Science (IFS), Stockholm, Sweden (2012-2015)
- Master scholarship grant- Vlaamse Interuniversitaire Raad (VLIR) (October 2005-2007)
- FWO Travel grant, Ghent University, Gent, Belgium- oral and poster presenter during the World Aquaculture Society (WAS) Conference in Las Vegas, Nevada, USA (Feb 22-26, 2016)
- Best Research Award for Young Scientist (Merck, Philippines; 2nd placer) (2011)

Publication (A1)

Laranja, J.L.Q., Ludevese-Pascual, G.L., Amar, E.C., Sorgeloos, P., Bossier, P., De Schryver, P., 2014. Poly-β-hydroxybutyrate (PHB) accumulating Bacillus spp. improve the survival, growth and robustness of Penaeus monodon (Fabricius, 1798) postlarvae. Veterinary Microbiology. 173, 310-317

- Ludevese-Pascual, G., **Laranja, J.L.Q.**, Amar, E., Sorgeloos, P., Bossier, P., De Schryver, P., 2017. Poly-beta-hydroxybutyrate-enriched *Artemia* sp. for giant tiger prawn *Penaeus monodon* larviculture. Aquaculture Nutrition 23(2):422-429

- **Laranja, J.L.Q.**, De Schryver, P., Ludevese-Pascual, G., Amar, E., Aerts, M., Vandaame, P., Bossier, P. High amorphous Poly-beta-hydroxybutyrate (PHB) content in a probiotic *Bacillus* strain displays better protective effects in gnotobiotic *Artemia* during a pathogenic *Vibrio campbellii* challenge. *Submitted to Aquaculture (under review)*

- Ludevese-Pascual, G., **Laranja, J.L.Q.**, Amar, E., Bossier, P., De Schryver, P. Application of poly-ß-hydroxybutyrate (PHB)-based biodegradable plastic as artificial substratum in *Litopenaeus vannamei* culture. *Submitted to Journal of Polymers and the Environment (under review)*

- Ludevese-Pascual, G., De Schryver, P., Amar, E.,**Laranja, J.L.Q.**, Bossier, P. The effects of Poly-β-hydroxybutyrate (PHB) supplementation on the lipid and fatty acid composition of crustaceans: *Artemia* sp. as model species. *Submitted to Aquaculture (under review)*

- Ludevese-Pascual, G., Ahmed, F., De Troch, M., Amar, E., **Laranja, J.L.Q.,** Bode, S., Boekcx, P., Bossier, P., De Schryver, P. Determination of poly-β-hydroxybutyrate (PHB) assimilation by crustaceans using stable 13C isotope tracing. *Submitted to Animal Feed Science and Technology (under review)*

- **Laranja, J.L.Q.**, Ludevese-Pascual, G., Amar, E., Sorgeloos, P., Bossier, P., De Schryver, P., 2014. Poly-β-hydroxybutyrate accumulating *Bacillus* spp. improve the survival, growth and robustness of *Penaeus monodon (*Fabricius, 1798) postlarvae. Veterinary Microbiology 173: 310-317

- **Laranja, J.L.Q.**, Quinitio, E.T., Catacutan, M.R., Coloso, R.M., 2010. Effects of dietary L-tryptophan on the agonistic behavior, growth and survival of juvenile mud crab *Scylla serrata*. Aquaculture 310: 84-90

- Rurangwa, E., **Laranja, J.L.**, Houdt, R.V., Delaedt, Y., Geraylou, Z., De Wiele, T.V., Loo, J.V., Craeyveld, V.V., Courtin, C.M., Delcour, J.A., Ollevier, F., 2009. Selected nondigestible carbohydrates and prebiotics support the growth of probiotic fish bacteria mono-cultures *in vitro*. Journal of Applied Microbiology 106: 932-940.

Oral/ Poster presentation

- World Aquaculture Society (WAS) Conference, Las Vegas, Nevada, USA (Feb 22-26, 2016). Poly-β-hydroxybutyrate-accumulating *Bacillus* sp. JL47 protects *Penaeus monodon* postlarvae against pathogenic *Vibrio campbellii* by enhancing the innate immune response. **Laranja, J.L.Q.,** De Schryver, P., Amar, E., Geaga, M.J.,Niu, Y., Bossier, P. (oral presentation)

- World Aquaculture Society (WAS) Conference, Las Vegas, Nevada, USA (Feb 22-26, 2016). Isolation, screening and application of Poly-β-hydroxybutyrate-accumulating *Bacillus* spp. for crustacean culture. **Laranja, JL.Q**., Ludevese-Pascual, G., Amar, E., Bossier, P., De Schryver, P. (poster presentation)

- European Aquaculture Society (EAS) Conference, Trondheim, Norway (August 9-12, 2013). Poly-β-hydroxybutyrate accumulating *Bacillus* spp. improve the growth, survival and robustness of *Penaeus monodon* postlarvae. **Laranja, J.L.Q.**, Ludevese-Pascual, G., Amar, E., Sorgeloos, P., Bossier, P., De Schryver, P. (oral presentation)

- International Symposium on Fish Nutrition and Feeding, Cairns, Queensland, Australia (May 25-30, 2014). The application of the bacterial storage compound poly-β-hydroxybutyrate as a feed additive in aquaculture. Bossier, P., Najdegerami, E., Sui, L., The Nhan, D., **Laranja, J.L.Q.**, Situmorang, M., Quoc Thai, T., Hung V.N., Nevejan, N., Defoirdt, T., De Schryver, P. (oral presentation)

- 20th Annual Convention and Scientific Meeting of the Philippine Society for Microbiology, Inc. , St. Paul University, Iloilo, Philippines (October 2012). Selected nondigestible carbohydrates and prebiotics support the gowth of fish probiotic bacteria and yeasts monocultures *in vitro*. **Laranja, J.L.Q.**, Rurangwa, E., Ollevier, F. (oral presentation)

- 20th Annual Convention and Scientific Meeting of the Philippine Society for Microbiology, Inc. , St. Paul University, Iloilo, Philippines (October 2012). Isolation, screening and identification of PHB accumulating bacteria from shrimp pond sediments. Nacionales, T., **Laranja, J.L.Q.**, Olaguer I. (oral presentation)

PART VI

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Finally, I want to praise & thank God for this earthly achievement. To God be the Glory!

Joseph
30 Sept 2017; 11:34 PM
Hoogpoort 33F, Ghent, Belgium

The secret things belong unto the LORD our God: but those things which are revealed belong unto us and to our children for ever, that we may do all the words of this law (Deuteronomy 29:29, Bible KJV).