Promoter: **Prof. dr. ir. Peter Bossier**
Department of Animal Production, Faculty of Bioscience Engineering, Ghent University.
(*Peter.Bossier@UGent.be*)

Co-promoter: **Prof. dr. Hans Nauwynck**
Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University
(*Hans.Nauwynck@UGent.be*)

Dean: **Prof. dr. ir. Herman Van Langenhove**

Rector: **Prof. dr. Paul Van Cauwenberge**

Members of the Examination and Reading Committee

Prof. dr. ir. Jacques Viaene (Chairman)

Department of Agricultural Economics, Faculty of Bioscience Engineering, Ghent University.
(*Jacques.Viaene@UGent.be*)

Prof. dr. Patrick Sorgeloos

Department of Animal Production, Faculty of Bioscience Engineering, Ghent University.
(*Patrick.Sorgeloos@UGent.be*)

Prof. dr. ir. Monica Höfte (Secretary) **(*)**

Department of Crop Protection, Faculty of Bioscience Engineering, Ghent University.
(*Monica.Hofte@UGent.be*)

Prof. dr. Indrani Karunasagar **(*)**
Department of Microbiology, College of Fisheries, Mangalore, India.
(mircen@sancharnet.in)

Prof. dr. Evelyne Bachère **(*)**
University Montpellier 2, France.
(*Evelyne.Bachere@ifremer.fr*)

(*) Members of the Reading Committee

Le Hong Phuoc

Single and dual experimental infection of specific pathogen-free *Litopenaeus vannamei* shrimp with White Spot Syndrome Virus and *Vibrio* species

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title: Enkelvoudige en tweevoudige experimentele infectie van specifiek pathogeenvrije garnaal *Litopenaeus vannamei* met het 'White Spot Syndrome Virus' en *Vibrio* species

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LIST OF ABBREVIATIONS AND UNITS

cm	Centimeter
g	Gram
h	Hour
min	Minute
ml	Milliliter
mm	Millimeter
nm	Nanometer
^{o}C	Defree Cencius
µl	Microliter
µm	Micrometer
α2M	Alpha-2-macroglobulin
AI	Autoinducer
ANOVA	Analysis of Variance
ARC	Artemia Reference Center
ASDD	Abdominal Segment Deformity Disease
BLIS	Bacteriocin-like Substance
CAI	Cholerae Autoinducer
CE	Cuticular Epithelium
CFU	Colony Forming Unit
DAB	3,3-diaminobenzidine
DNA	Deoxyribonucleic Acid
ds-DNA	Double-stranded Deoxyribonucleic Acid
ECPs	Extra Cellular Products
EDTA	Ethylene Diaminetetra-acetic Acid

EUS	Epizootic Ulcerative Syndrome
FAO	Food and Agriculture Organization
FASW	Filtered Autoclaved Seawater
G	Gill
GAV	Gill Associated Virus
GBP	β-1,3-glucan Binding Protein
GST–PAP	Glutathione-S-transferase-PAP
HA/protease	Hemaglutinin/protease
HAI-1	Harveyi Autoinducer-1
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HP	Haematopoietic tissue
hpi	Hours post injection
HPV	Hepatopancreatic Parvovirus
hpvi	Hours post *Vibrio campbellii* injection
IHC	Immuno-histochemistry
IHHNV	Infectious Hypodermal and Hematopoietic Necrosis Virus
IHNV	Infectious Hematopoietic Necrosis Virus
IIF	Indirect Immuno Fluorescent
IMNV	Infectious Myonecrosis Virus
IP	Intra-peritioneal
IPNV	Infectious Pancreatic Necrosis Virus
ISA	Infectious Salmon Anaemia
L	Luminescent
LD_{50}	Lethal Dose 50

LGBP	Lipopolysaccharide- and β-1,3-glucan-binding Protein
LGH	Large Granule Haemocyte
LMG	Laboratory of Microbiology of Ghent University
LO	Lymphoid Organ
LOV	Lymphoid Organ Virus
LPS	Lipopolysaccharides
LSNV	Laem Singh Virus
MA	Marine Agar
MABV-F	Marine Birnavirus-F
MACR	Marine Agar with Congo Red
MAR	Marine Agar containing 100 ppm Rifampicin
MB	Marine Broth
MBP-VP19	Maltose Binding Protein-Viral Protein 19
MBV	Monodon Baculovirus
MBW	Mean Body Weight
MCF	Mucoid Colony Forming
MCMS	Mid-crop Mortality Syndrome
MoV	Mourilyan Virus
MSGS	Monodon Slow Growth Syndrome
NHP	Necrotizing Hepatopancreatitis
NL	Non-luminescent
NMCF	Non-mucoid Colony Forming
NS	Not Significant
NWE	No Water Exchange
OD	Optical Density
OIE	Office International des Epizooties

p	Statistical p-value
PAP	Phagocytosis Activating Protein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCV2	Porcine Circovirus Type 2
Pen	Penaeidin
PGBP	Peptidoglycan-binding Protein
PL	Postlarvae
PMWS	Post-weaning Multisystemic Wasting Syndrome
PO	Phenoloxidase
ppA	Prophenoloxidase Activating enzyme
PPV	Porcine Parvovirus
PRDC	Porcine Respiratory Disease Complex
ProPO	Prophenoloxidase
PRRS	Porcine Reproductive and Respiratory Syndrome
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
PS	Physiological Solution
PVC	Polyvinyl Chloride
PvNV	*Penaeus vannamei* Nodavirus
RIA2	Research Institute for Aquaculture No2
RNA	Ribonucleic Acid
ROIs	Reactive Oxygen Intermediates
RR	Rifampicin resistant
RS	Rifampicin sensitive
rVP	Recombinant Viral Protein
SCRV	*Siniperca chuatsi* Rhabdovirus

SE	Stomach Epithelium
SEMBV	Systemic Ectodermal and Mesodermal Baculovirus
SGH	Small Granule Haemocyte
SID_{50}	Shrimp Infectious Dose with 50% endpoint
SMV	Spawner-isolated Mortality Virus
SNAP	S-nitroso-N-acetylpenicillamine
SPF	Specific Pathogen-Free
SPR	Specific Pathogen-Resistant
ss-DNA	Single-stranded Deoxyribonucleic Acid
ss-RNA	Single-stranded Ribonucleic Acid
TCBS	Thiosulfate Citrate Bile Sucrose
TEM	Transmission Electron Microscopy
TSV	Taura Syndrome Virus
VBNC	Viable But Non-Culturable
VC	*Vibrio campbellii*
VHML	*Vibrio harveyi* Myovirus Like
VP	Viral Protein
WE	Water Exchange
WSSV	White Spot Syndrome Virus
YHLV	Yellow Head Like Virus
YHV	Yellow Head Virus

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CHAPTER

Introduction and thesis outline

World aquaculture production has grown tremendously during the last fifty years (Figure 1.1). In 2004, total world aquaculture production was 59.4 million tons and had a value of US$70.3 billion (FAO, 2006). Although aquaculture has been practiced for millennia, the main expansion of aquaculture occurred only in the last three decades. Approximately, a 9-fold increase in total world aquaculture production was documented between 1980 and 2004 (Figure 1.1).

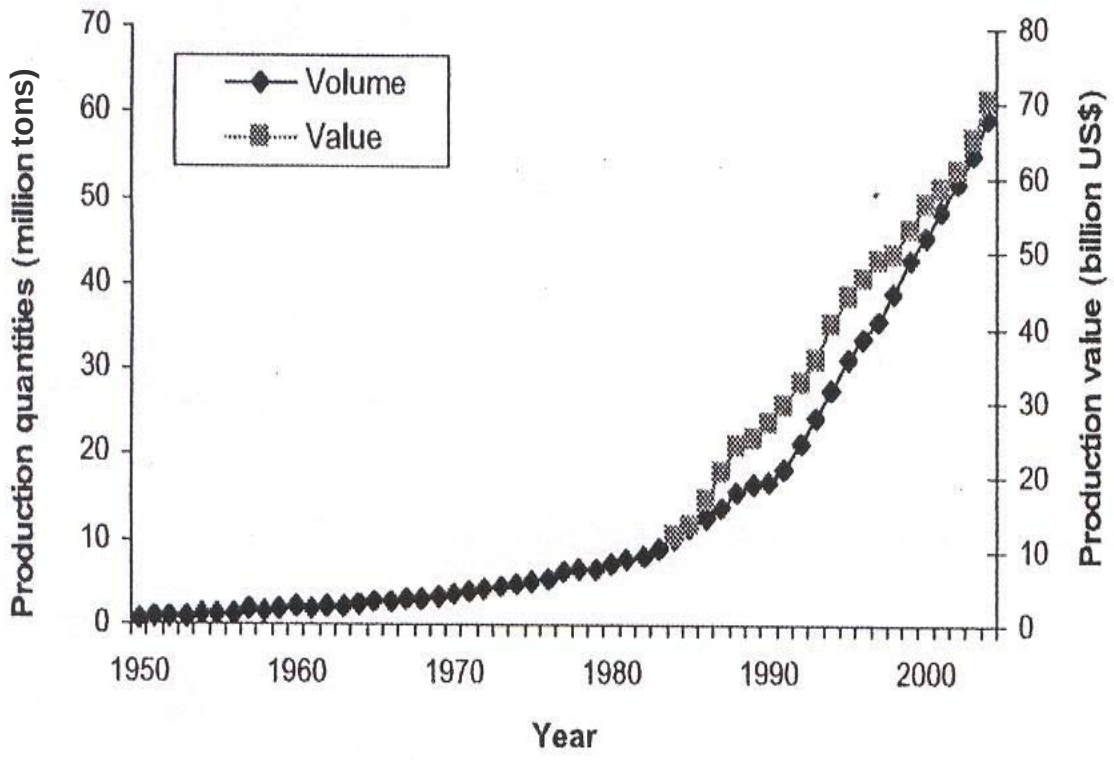

Figure 1.1. Trend in total world aquaculture production and value (including plants) between 1950 and 2004 (FAO, 2006)

Thanks to better knowledge of the biology of selected species, new technology developments and attractive market demands, a number of success stories such as trout, tilapia, catfish, seabass, seabream, Atlantic salmon, and penaeid shrimp species are at the origin of the increased production. However, shrimp success has been very variable i.e. total world shrimp aquaculture production significantly increased from 213,635 tons in 1985 to 928,328 tons in

1995, but then decelecrated reaching a plateau level of approximately 900,000 tons in 1990s (FAO, 2007a). Many factors influence shrimp health status such as the age of shrimp, management conditions, biotic and abiotic stress, and pathogens. Infectious disease is one of the limiting factors in shrimp farming. Shrimp can be threatened by protozoan, fungal, bacterial and viral pathogens, but viral and bacterial diseases cause major troubles in shrimp farming (Lightner, 1996). Shrimp farming also contributes to environmental problems. Shrimp farming itself has got a significant effect on the environment, e.g. loss of mangrove ecosystems, nutrient enrichment and eutrophication of coastal waters, development of antibiotic resistance in marine bacteria and accumulation of chemicals and toxicity to non-target species (Menasveta, 1997; Primavera, 1997). The use and occasional misuse of antibiotics in shrimp farming has led to multiple drug resistant pathogens and sometimes even to rejection of farmed shrimp by importing countries (Baticados and Paclibare, 1992). There is a need for the development of alternative solutions for disease prevention in shrimp aquaculture. Various solutions have been proposed such as the use of probiotics, immunostimulation, "vaccination", specific pathogen-free (SPF) or specific pathogen-resistant (SPR) shrimp. In the past, experimental challenge tests were performed with non-SPF shrimp. The use of non-SPF shrimp in challenge tests might contribute to a lack of reproducibility because of unverified burden of viral and bacterial pathogens. Since SPF shrimp are now readily available, even for commercials settings, it is meaningful to develop and standardize a protocol for *Vibrio* challenge with a SPF-host. This might contribute to the development of solutions for the prevention of shrimp diseases.

The general objective of the present thesis is to develop and standardize a protocol for *Vibrio* challenge in specific pathogen-free shrimp *Litopenaeus vannamei*, and to apply this protocol for the study of vibriosis and dual infection of *Vibrio* spp. and White Spot Syndrome Virus (WSSV) in this host.

The specific objectives and the thesis outline are as follows:

- Chapter 1 gives introduction and thesis outline.

- Chapter 2 reviews the literature concerning shrimp biology, shrimp aquaculture, shrimp diseases, virulence of *Vibrio*, problems faced by the shrimp culture industry and application of disease prevention techniques, crustacean defense system, and polymicrobial diseases.

- Chapter 3 describes the development of a protocol for *Vibrio* challenge in specific pathogen-free shrimp *Litopenaeus vannamei* which is then used to study the dual infection of *Vibrio campbellii* and white spot syndrome virus (WSSV) in this host. The challenge tests were performed in normal as well as stressful conditions such as starvation, salinity or ammonium stress. Compromised shrimp, through damage to the cuticle or by removing a pleopod, were also challenged by bacteria. Many different challenge routes were tested such as immersion, injection and oral infection through the feed.

- Chapter 4 aims to compare the virulence of luminescent and non-luminescent isogenic bacteria towards gnotobiotic *Artemia* and specific pathogen-free shrimp *Litopenaeus vannamei*.

- Chapter 5 describes observations on the use of selective and non-selective culture media for enumerating *Vibrio*.

- Chapter 6 studies the co-infection of *Vibrio campbellii* and white spot syndrome virus (WSSV) in specific pathogen-free shrimp *Litopenaeus vannamei*, including the effect of sonicated *V. campbellii* on the mortality of white spot syndrome virus infected shrimp.

- In Chapter 7, a dose-effect relationship for *Vibrio campbellii* in WSSV-infected shrimp is investigated. The study also includes challenge routes for *Vibrio*.

- In Chapter 8, the results communicated in the various chapters are discussed. The main conclusions are mentioned. Additionally, the perspectives for further research are given.

CHAPTER

Literature study

2.1. Penaeid shrimp culture

2.1.1. Biology of *Litopenaeus vannamei*

Litopenaeus vannamei is a decapod crustacean that belongs to the family Penaeidae. *L. vannamei* is a member of the genus *Penaeus*, which is distinguished by the presence of the teeth on both the upper and lower margin of the rostrum, and by the lack of setae on the body (Wyban and Sweeney, 1991).

The taxonomical position of *Penaeus* (*Litopenaeus*) *vannamei* Boone is as follows (Wyban and Sweeney, 1991):

Phylum: Arthropoda

Class: Cruscacea

Subclass: Malacostraca

Series: Eumalacostraca

Superorder: Eucarida

Order: Decapoda

Suborder: Dendrobrachiata

Infraorder: Penaeoidea

Family: Penaeidae

Genus: Penaeus

Subgenus: Litopenaeus

Species: vannamei

Distribution and life cycle: *L. vannamei* is native to the Pacific coast of Mexico, central, and South America where ocean water temperatures generally remain above 20^{o}C for the whole year (Wyban and Sweeney, 1991). The life cycle of *L. vannamei* with various development stages is shown in Figure 2.1. The shrimp mature, mate, and spawn in the water column just

offshore of the coastal areas at a temperature of 26-28^{o}C and a salinity of about 35 gL^{-1}. The eggs hatch and the larvae develop as a part of the zooplankton. *L. vannamei* postlarvae move inshore and settle to the bottom in shallow estuaries where the nutrition, salinities and temperatures are suitable for growing. After several months in an estuary, juvenile shrimp return to the offshore marine environment, where sexual maturation, mating, and spawning occurs (Rosenberry, 2005).

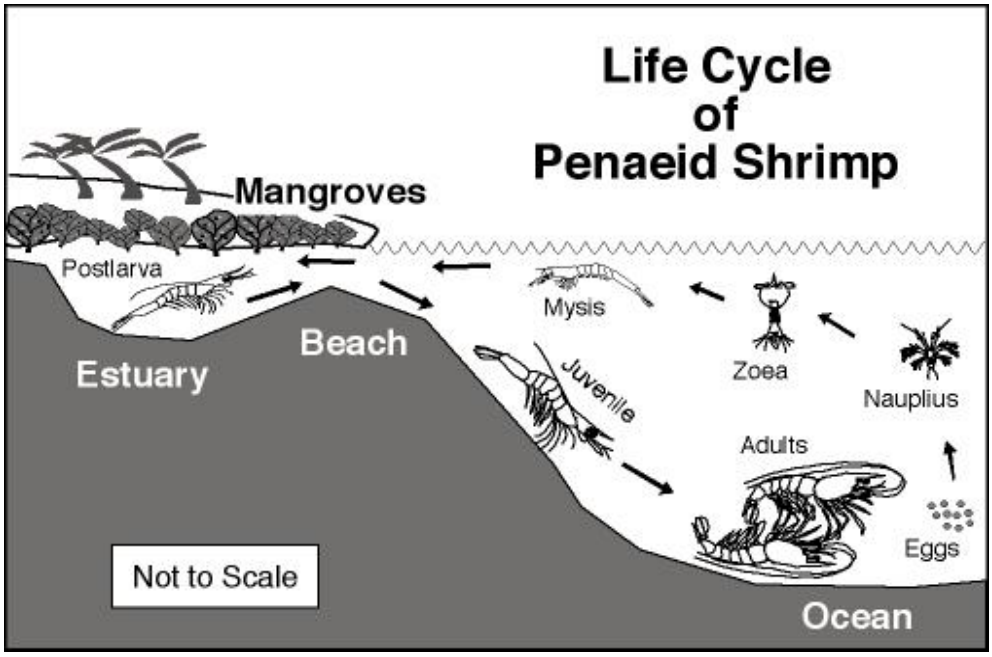

Figure 2.1. Life cycle of penaeid shrimp (Rosenberry, 2005)

Culture characteristics and morphology: *L. vannamei* can adapt well to a wide range of environmental conditions (Jory and Cabrera, 2003). They can be stocked at small size and tolerate salinities from 2 to 40 gL^{-1}, but do grow faster at the lower salinities. *L. vannamei* will die if exposed to water below 15^{o}C or above 33^{o}C for 24 hours or more. Sub-lethal stresses may occur at 15-22^{o}C and 30-33^{o}C. Juvenile shrimp grow well in warm water. Males can reach a total length of 187 mm and females 230 mm (Jory and Cabrera, 2003).

The external anatomy of *L. vannamei* is characterised by a cephalothorax with a characteristic hard rostrum (Figure 2.2). Most internal organs like gills, heart, lymphoid organ,

haepatopancreas, and stomach are located in the cephalothorax while the muscles concentrate in the abdomen. In the head region, antennules and antennae perform sensory functions. In the thorax region, the maxillipeds are the first three pairs of appendages, modified for food handling, and the remaining five pairs are the walking legs (pereiopods). Five pairs of swimming legs (pleopods) are found on the abdomen (Rosenberry, 2005).

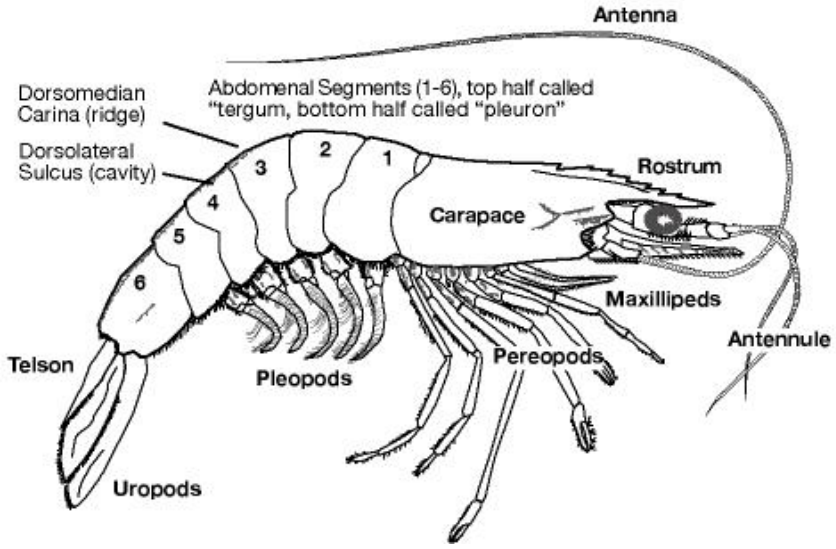

Figure 2.2. External anatomy of *Litopenaeus vannamei* (Rosenberry, 2005)

2.1.2. Shrimp aquaculture

2.1.2.1. History of shrimp farming

Farming of various marine shrimp species has been developed in the last three decades. In the beginning, marine shrimp were cultivated in South-East Asia by farmers who raised them as incidental crops in tidal fish ponds (Jory and Cabrera, 2003). However, marine shrimp farming was launched in the 1930s, when Motosaku Fujinaga, a Japanese researcher succeeded in spawning the Kuruma shrimp (*Marsupenaeus japonicus*). Thereafter, he generously shared his findings by publishing papers on his work. In the early 1970s, many researchers and entrepreneurs in various countries in Asia as well as in Latin America invested in shrimp farming. Over the years, enormous progress in developing shrimp culture

techniques has been made. Shrimp culture evolved from an extensive form, using tidal zones, to a super-intensive one in the 2000s using ponds more inland (Rosenberry, 2004). Currently, there are about 68 countries having shrimp farm operations (FAO, 2007a). Major references on global shrimp farming include Wyban (1992), Wickins and Lee (2002) and Rosenberry (2001, 2005).

2.1.2.2. Mode of shrimp culture

Grow-out shrimp ponds can be different in shape and size. Shrimp culture systems are classified by stocking densities e.g. extensive, semi-intensive, intensive with high stocking density and intensive with very high stocking density (Table 2.1). These intensity forms are currently applied in many countries such as Thailand, Indonesia, Bangladesh, Vietnam, India, Malaysia, Taiwan, Mexico, Ecuador… More intensive farms are located in Taiwan, Japan, and the USA (Jory *et al.*, 2001; Rosenberry, 2001). As densities increase, the technology gets more sophisticated, capital costs go up and production per unit of space increases dramatically (Rosenberry, 2001). Hence, the farmers need to invest more when performing intensive shrimp culture. The mode of culturing highly depends on region-specific conditions. Extensive or traditional farming is normally found in poor countries with low costs of land, labor, capital and energy. Recently, shrimp farms in many countries try to reduce water exchange and hence reduce the volume of water required to produce shrimp. Shrimp farms can produce two, three or more crops per year depending on the growing conditions (Jory and Cabrera, 2003).

Table 2.1. Summary of standard culture methods and facilities employed in shrimp culture (adapted from Jory and Cabrera, 2003)

Parameters	**Intensity**			
	Extensive (low density)	Semi-intensive (medium density)	Intensive (high density)	Intensive (very high density)
Stocking density (PL/m^2)	1-5	5-25	25-120	120-300
Pond/tank area (ha)	Ponds (5-100)	Ponds (1-25)	Ponds/tanks (0.1-5.0)	Tanks (0.1-1.0)
Seedstock source	Wild	Wild and laboratory	Laboratory	Laboratory
Water exchange (% daily)	Tidal (<5%)	Pumping (5-12%)	Pumping (up to 25%)	Pumping (>25%)
Feed	Natural feeds	Natural and formulated feeds	Natural and formulated feeds	Natural and formulated feeds
Management	Minimal	Moderate	High	Very high
Fertilisation	No	Yes	Sometimes	No
Mechanical aeration	Yes	Yes	Yes	Yes
Annual production (kg/ha)	50-500	500-5,000	5,000-20,000	20,000-100,000 (estimated)

PL = Postlarvae

2.1.2.3. World shrimp aquaculture production

Total world shrimp production increased significantly during the last two decades. World shrimp aquaculture production has grown tremendously from a production of 200,000 tons in 1985 to approximately 2.7 millions tons in 2005 (Figure 2.3). This is approximately a 13-fold increase in production in two decades. Between 1985 and 1990, total world shrimp production increased by 218%, and between 1990 and 1995 by 22%. The periods of decreasing world shrimp production are coincident with disease outbreaks reported in many countries. The outbreak of white spot disease in 1992-1993 causing enormous economic loss has had a big affect on total world shrimp production. In the 1990s, the annual growth of shrimp production decelerated reaching a plateau level of around 900,000 tons per year. Until

2000, *Penaeus monodon* was the major species of shrimp culture with production increasing yearly from 1985 to 1995. Due to a white spot syndrome virus (WSSV) outbreak, production of this species decreased dramatically after 1995. As a consequence, many countries shifted from *P. monodon* to the white shrimp (*Litopenaeus vannamei*) as a main species in their shrimp farming. Recently, *L. vannamei* became more important compared to *P. monodon* in terms of contribution to the total world shrimp aquaculture production. *L. vannamei* is now farmed everywhere in Southeast Asia, except for Bangladesh (Rosenberry, 2004). In 2002, both species contributed equally, with about 500,000 tons. However, in 2005 production reached about 1.6 millions tons for *L. vannamei*, whereas only few thousand tons of *P. monodon* was produced. As a result, *L. vannamei* contributed 60% of shrimp aquaculture production, followed by *P. monodon* (27%). The other 13% are other species (Figure 2.4). Nowadays, shrimp production is dominating world crustacean culture. According to the FAO report of the year 2007a, shrimps and prawns take the major share (39.03%) of the total world crustaceans production followed by freshwater crustaceans and crab/sea-spiders with a share of 25.38% and 13.80% respectively (Figure 2.5). Recently, most of the shrimp aquaculture production comes from Asian countires. The lead countries with shrimp aquaculture production are China, Thailand and Vietnam (Table 2.2). In 2005, 68 countries reported penaeid shrimp aquaculture production, 22 countries reported producing *L. vannamei*, while 23 countries were producing *P. monodon*.

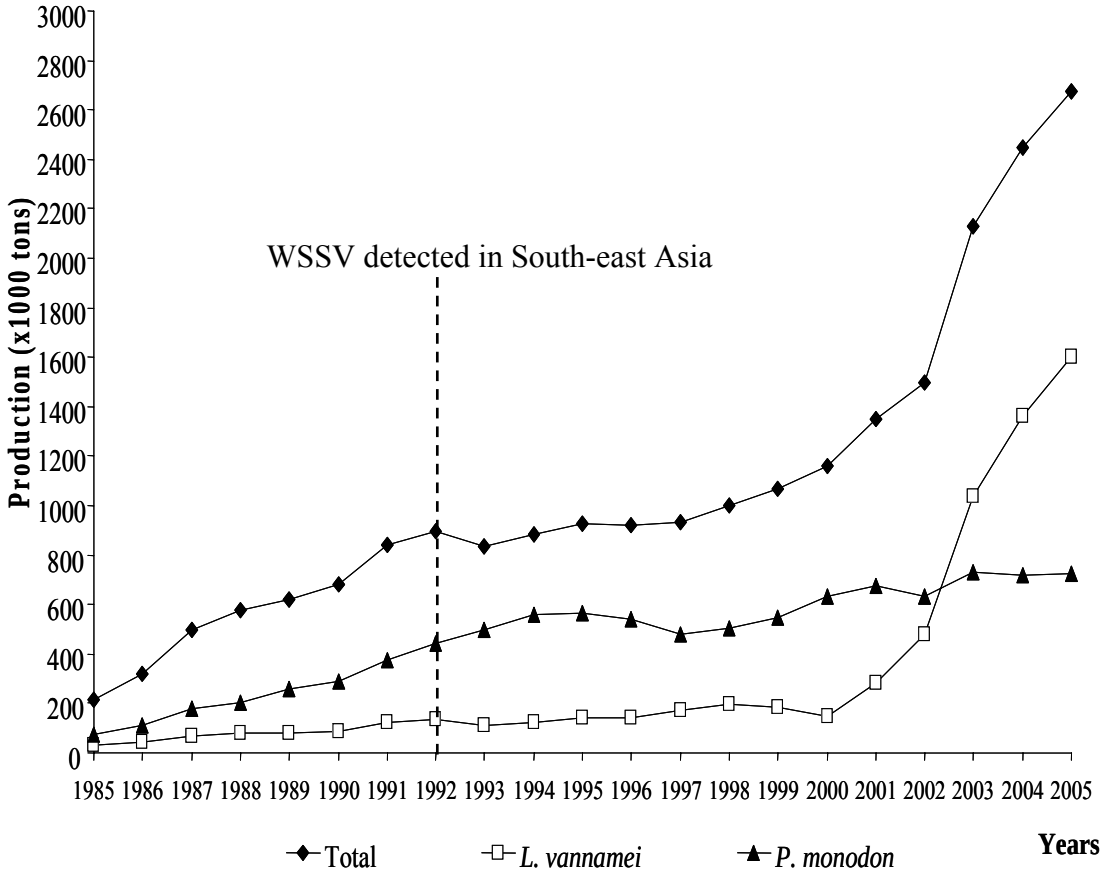

Figure 2.3. Total world shrimp aquaculture production (FAO, 2007a)

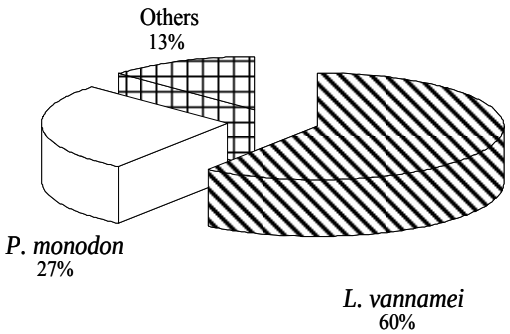

Figure 2.4. Total world shrimp aquaculture production by species in 2005 (FAO, 2007a)

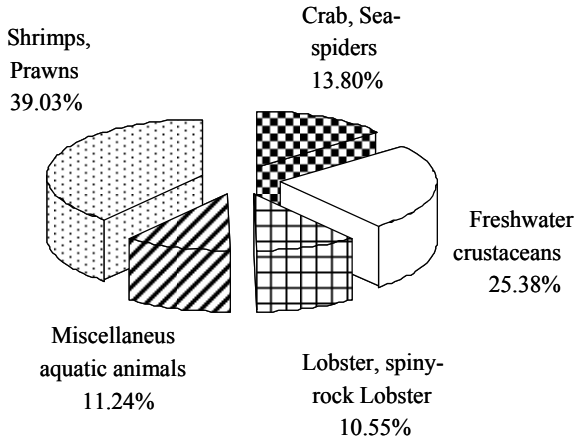

Figure 2.5. Total world crustacean aquaculture production by species in 2005 (FAO, 2007a)

Table 2.2. Top 10 countries with shrimp aquaculture production in 2005 (FAO, 2007a)

No	Country	Production (tons)	Percentage (%)
1	China	1,024,949	41.77
2	Thailand	375,320	15.30
3	Vietnam	327,200	13.34
4	Indonesia	279,539	11.39
5	India	143,170	5.84
6	Mexico	72,279	2.95
7	Brazil	63,134	2.57
8	Bangladesh	63,052	2.57
9	Ecuador	56,300	2.29
10	Myanmar	48,640	1.98

2.2. Penaeid shrimp diseases and preventive measures in shrimp diseases

Protozoa, fungi, bacteria, and virus have been suggested to have an important influence in shrimp culture (Gabriel and Felipe, 2000). Viral diseases have caused hatchery mortalities and considerable grow-out problems in marine shrimp (Owens, 2003). The major taxonomic groups are the families *Nimaviridae*, *Parvoviridae*, *Picornaviridae* and the order *Nidovirales*. Presently, approximately 18 viruses have been reported in penaeid shrimp (Munro and Owens, 2007). The major viral diseases are listed in Table 2.3.

Table 2.3. Major and emerging viral diseases of penaeid shrimp

Abbreviation/Full name	Nucleic acids	Virion size (nm)	References
WSSV (White Spot Syndrome Virus)	ss-DNA	80-120 by 250-380	Lightner and Redman, 1998; van Hulten and Vlak, 2001; Mayo, 2002; Flegel, 2006.
TSV (Taura Syndrome Virus)	ss-RNA	30-32	Brock *et al.*, 1995; Hasson *et al.*, 1995; Mayo, 2002.
YHV/GAV/LOV (Yellow Head Virus/Gill Associated Virus/Lymphoid Organ Virus)	ss-RNA	183-200 by 34-42	Boonyaratpalin *et al.*, 1993; Cowley *et al.*, 2000; Mayo, 2002; Munro and Owens, 2007.
MBV (Monodon Baculovirus)	ds-DNA	75 by 300	Fegan *et al.*, 1991; Lightner *et al.*, 1985; Flegel, 2006.
HPV (Hepatopancreatic Parvovirus)	ds-DNA	22-23	Flegel *et al.*, 1995; Bonami *et al.*, 1995; Flegel *et al.*, 2004; Umesha *et al.*, 2003.
IHHNV (Infectious Hypodermal and Hematopoietic Necrosis)	ss-DNA	22-23	Bonami *et al.*, 1990; Lightner, 1996; Tang *et al.*, 2003.
IMNV (Infectious Myonecrosis Virus)	ds-RNA	40	Poulos *et al.*, 2006.
MoV (Mourilyan Virus)	ss-RNA	85-100	Cowley *et al.*, 2005a,b; Sellars *et al.*, 2006.
LSNV (Laem Singh Virus)	ss-RNA	27	Sritunyalucksana *et al.*, 2006a,b; Flegel, 2006.
ASDD (Abdominal Segment Deformity Diseases)	N/A	N/A	Flegel, 2007.
PvNV (*Penaeus vannamei* Nodavirus)	ds-RNA	22	Tang *et al.*, 2007.

ss = Single-stranded; ds = Double-stranded; N/A = Not available

2.2.1. White Spot Syndrome Virus (WSSV)

Viral diseases have caused hatchery mortalities and considerable grow-out problems in marine shrimp (Owens, 2003). Of all shrimp viruses, white spot syndrome virus (WSSV) has had the largest impact on shrimp culture and remains a major problem up to the present day

(Rosenberry, 2004). Recently, about a quarter of wild-caught shrimp spawners in India are infected with WSSV (FAO, 2007b). WSSV started in 1992 in Taiwan causing massive mortality (Chou *et al.*, 1995). In 1993, WSSV destroyed almost US$1 billion worth of *Fenneropenaeus chinensis* in China. WSSV was spread with devastating consequences when infected shrimp (*Marsupenaeus japonicus*) were imported from China and Korea to Japan. WSSV was further spread to Thailand, India, Bangladesh, (Karunasagar *et al.*, 1997; Mazid and Banu, 2002), and other Asian countries (Park *et al.*, 1998). In 1995 it was detected in the United States of America (Texas and South-Carolina) (Wang *et al.*, 1999a). From 1996 to 1999, WSSV was detected in many countries such as Indonesia, Sri Lanka, Malaysia (Durand *et al.*, 1996; Kasornchandra *et al.*, 1998; Rajan *et al.*, 2000), Vietnam (Bondad-Reantaso *et al.*, 2001), and Peru (Rosenberry, 2001). In 1999, WSSV was reported in the Philippines, Ecuador, Colombia, and Mexico (Magbanua *et al.*, 2000; Hossain *et al.*, 2001; Wu *et al.*, 2001). In 2002, WSSV was also detected in Europe (France) and the Middle East (Iran) (Marks, 2005). WSSV belongs to the family *Nimaviridae* (van Hulten and Vlak, 2001; Vlak *et al.*, 2005). The virus is bacilliform and non-occluded enveloped (Chou *et al.*, 1995; Wongteerasupaya *et al.*, 1995). Intact enveloped virions range between 210 and 380 nm in length and 70-167 nm in maximum width (Flegel and Alday-Sanz, 1998; Rajendran *et al.*, 1999). WSSV can be transmitted horizontally either *per os* by predation on diseased individuals, or by virus particles in the water. Infection by the latter is thought to occur primarily through the gills, but may occur via other body surfaces as well (Chou *et al.*, 1995). The virus is also transmitted vertically from mother to offspring, although it is not clear whether the WSSV virions are present inside the shrimp eggs (Lo *et al.*, 1997; Peng *et al.*, 2001; Tsai *et al.*, 1999).

In cultured shrimp, infected animals show lethargic behavior e.g lack of appetite, anorexia, lethargy, slow movement, and reddish to pink body discoloration (Otta *et al.*, 1999). WSSV-

infected shrimp gather near the pond edge and display clinical signs for 1 or 2 days before the first mortalities occur (Kou *et al.*, 1998). WSSV infection can reach a cumulative mortality of up to 100% within 3-10 days (Lightner, 1996). Characteristic for WSSV infected shrimp are white spots on the exoskeleton from which the disease name is derived. These spots are the result of calcified deposits that range in size from a few mm to 1 cm or more in diameter (Chou *et al.*, 1995). The exact mechanism of white spot formation is not known. Wang *et al.* (1999a) speculated that WSSV infection may induce the dysfunction of the integument resulting in the accumulation of calcium salts within the cuticle and giving rise to white spots. Juvenile shrimp of all ages and sizes are susceptible to WSSV but massive mortality usually occurs at 1 or 2 months after stocking (Kasornchandra *et al.*, 1998). It has also been suggested that exposure of shrimp to stressors increases the risk of white spot disease, since stressors can compromise the shrimp defence system (Takahashi *et al.*, 1995). By histopathology, WSSV infection is characterized by cells with hypertrophied nuclei showing amphophilic intranuclear inclusions and marginated chromatin (Durand *et al.*, 1997; Wang *et al.*, 2000). These intranuclear inclusions are markedly distinct and bigger than the Cowdry A-type inclusions characteristic of the infectious hypodermal and haematopoietic necrosis virus (Wongteerasupaya *et al.*, 1995). Infected nuclei become progressively more basophilic and enlarged (Otta *et al.*, 1999; Takahashi *et al.*, 2000). In the late stages of infection, karyorrhexis and cellular disintegration may occur, leading to the formation of necrotic areas characterized by vacuolization (Kasornchandra *et al.*, 1998; Wang *et al.*, 1999a). Besides shrimp, WSSV infects a wide range of other aquatic crustaceans such as salt, brackish and fresh water crayfishes, crabs and lobsters. In contrast to shrimp, the infection is often not lethal for these species, and therefore they may serve as reservoirs and carriers of the virus (Lo *et al.*, 1996; Wang *et al.*, 1998). Because of its broad host range, WSSV is not only a major threat to shrimp farming, but also to the worldwide marine ecology (Flegel, 1997).

2.2.2. Vibriosis

Vibriosis is considered as one of the most important bacterial diseases responsible for mortality of cultured shrimp worldwide (Lavilla-Pitogo *et al.*, 1998, Chen *et al.*, 2000). Vibriosis in penaeid shrimps is mainly caused by *Vibrio anguillarum* (Lightner, 1996), *V. alginolyticus* (Selvin and Lipton, 2003), *V. parahaemolyticus* (Alapide-Tendencia and Dureza, 1997), *V. harveyi* (Prayitno and Latchford, 1995), *V. penaeicida* (Saulnier *et al.*, 2000a) *V. campbellii*-like (Hameed, 1995) and occasionally *V. splendidus* (Lavilla Pitogo *et al.*, 1998). *V. harveyi* was considered as the most important species causing vibriosis. Luminous *Vibrio* caused an important disease of hatchery-reared as well as grow-out *Penaeus monodon* shrimp culture (Lavilla-Pitogo *et al.*, 1990). *V. harveyi* luminescent strains have been reported to cause major losses in the shrimp larviculture in the Philippine (Lavilla-Pitogo *et al.*, 1990), Australia (Pizzutto and Hirst, 1995), South America (Álvarez *et al.*, 1998; Robertson *et al.*, 1998) and Mexico (Vandenberghe *et al.*, 1999). The loss in production of *Marsupenaeus japonicus* due to vibriosis has been estimated at approximately 30.8 metric tons per year in Japan. In 1991, most shrimp hatcheries in Java were hit by a disease caused by luminous bacteria with shrimp loss estimated at more than US $ 85 millions (Prayitno and Latchford, 1995). Vibriosis usually occurs during the first month of culture and can cause more than 50% mortality. However, as reported by Saulnier *et al.* (2000b), *Vibrio* may act mostly as an opportunistic agent in secondary infections and outbreaks of vibriosis among shrimp often happen only in combination with stress factors (pH, salinity, ammonia, temperature…) or following primary infections with other pathogens. Horowitz and Horowitz (2001) speculated that absence of primary infection, physical damage or stress results in an enhanced resistance against vibrios. Histopathological studies of infected shrimp larvae with luminescent vibriosis reveal a massive bacterial colonization of the oral region (Lavilla Pitogo *et al.*, 1990), as well as atrophy and necrosis of the hepatopancreas tubules (Robertson *et al.*, 1998). In gross observations, vibriosis is evident as

black or brown cuticular lesions, necrosis, opacity of musculature, black lymphoid organ, and melanization of appendages. By histopathology, necrosis and inflammation of different organs (lymphoid organ, gills, heart, hepatopancreas, etc.) are normally found in diseased shrimp.

2.2.3. Virulence factors associated with *Vibrio harveyi*

2.2.3.1. Association of bacteriophage with virulence in *V. harveyi*

Oakey and Owens (2000) discovered that an infection of *V. harveyi* with bacteriophage resulted in enhanced haemolytic activity to rainbow trout blood and increased pathogenicity to Atlantic salmon and *Artemia.* Later Austin *et al.* (2003) reported that the presence of a bacteriophage enhanced haemolytic activity in bacterial cultures. Munro *et al.* (2003) demonstrated that the presence of the *V. harveyi* myovirus like (VHML) bacteriophage may confer virulence to *V. harveyi* strain 642. *Vibrio harveyi* strains 12, 20, 45 and 645 infected with bacteriophage VHML display an up-regulation of haemolysin, an up-regulation of protein synthesis with some proteins being recognised as the same toxic subunits found in *V. harveyi* strain 642, and an increase in virulence towards larval penaeid shrimp.

2.2.3.2. Quorum-sensing and virulence of *Vibrio*

Bacteria communicate with one another using chemical signaling molecules. They release, detect, and respond to the accumulation of these molecules, which are called autoinducers. Detection of autoinducers allows bacteria to distinguish between low and high cell population density and to control gene expression in response to changes in cells numbers. This process, termed “quorum-sensing”, allows a population of bacteria to co-ordinately control gene expression of the entire community. Many bacterial behaviours are regulated by quorum-sensing, including symbiosis, virulence, antibiotic production, and biofilm formation (Schauder and Bassler, 2001).

Defoirdt *et al.* (2005) reported that the virulence of *V. harveyi* towards the brine shrimp *Artemia franciscana* is regulated by autoinducer 2 quorum-sensing. The quorum sensing-disrupting natural furanone, (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone, was found to block autoinducer 2 quorum-sensing in *V. harveyi* in a concentration-dependent way. *In vivo* challenge tests with gnotobiotic *Artemia* revealed that the natural furanone and a synthetic derivative protected the nauplii from the pathogenic isolates (Defoirdt *et al.*, 2006). Tinh *et al.* (2007) concluded that the growth rate effect of *V. harveyi* BB120 in rotifer *Brachionus plicatilis* cultures was attenuated since all quorum-sensing pathways of this strain were blocked by furanone.

2.2.3.3. Metalloprotease

A metalloprotease gene pap6 was cloned from *V. harveyi* AP6. Pap6 has a potential role in pathogenesis since it was shown to digest a range of host proteins, including gelatin, fibronectin, and type IV collagen. Bacterial proteases produced by pathogens act as toxic factors to their host and have been implicated in virulence and pathogenicity (Miyoshi and Shinoda, 2000). Many bacterial proteases are zinc-containing metalloproteases (Häse and Finkelstein, 1993). Hemagglutinin/protease (HA/protease) produced by *Vibrio cholerae* 01 is a zinc-dependent metalloprotease. *In vitro* studies showed that HA/protease was able to cleave several physiologically important host substrates like mucin, fibronectin and lactoferrin (Teo *et al.*, 2003).

2.2.3.4. Siderophores

Siderophores are secreted molecules produced by bacteria. These molecules have high affinity for Fe^{3+} and scavenge it from the environment for bacterial growth, particularly for replication of the cytochrome systems. Siderophore production is often accompanied by the production of iron-repressible outer-membrane proteins which act as receptors for the iron-

siderophore complex (Crosa, 1989). These systems have been found to be major determinates of virulence in *Vibrio anguillarum* (Owens and Austin, 1996). Testing of 88 *V. harveyi* strains, Owens and Austin (1996) found 78.4% were positive for the production of siderophores. They concluded that siderophores must be considered a virulence factor in fish infecting *V. harveyi* but not in strains infecting invertebrates.

2.2.3.5. Cystein protease

Cystein protease is a major exotoxin of the pathogenic luminous *V. harveyi* 820514. The protease was lethal to *P. monodon* with an LD_{50} value of 0.3 µg protein/g shrimp. Cystein protease can neutralize the blotting ability of shrimp haemolymph. It was concluded that cysteine protease is a major toxin produced by the bacterium (Liu and Lee, 1999).

2.2.3.6. Proteinaceous and bacteriocin-like substance (BLIS)

Two luminous strains of *V. harveyi*, previously demonstrated to be virulent to *Penaeus monodon* larvae were shown to produce proteinaceous exotoxins capable of causing mortality in mice and *P. monodon* at relatively small concentrations. These toxins were isolated from cell-free supernatants of mid-exponential phase broth cultures. Toxin T1 is produced by *V. harveyi* strain 47666-1. Toxin T2 is produced by *V. harveyi* strain 642 (Harris and Owens, 1999). Bacteriocins are heterogeneous antagonists of a proteinaceous nature that are produced by bacteria, and demonstrate inhibitory and/or bactericidal activity against closely related strains. According to Prasad *et al.* (2005), BLIS produced by *V. harveyi* 571 was demonstrated against *Vibrio* sp., *V. fischeri*, *V. gazogenes* and *V. parahaemolyticus*.

2.2.3.7. Chitinase

Chitin is produced by many marine organisms, including zooplankton and several phytoplankton species (Gooday, 1990). *V. harveyi* excreted several chitin-degrading proteins into the culture media when exposed to different types of chitin. Svitil *et al.* (1997) found six

separate chitinase genes in which one gene apparently codes for a chitinase that is cleaved or degraded to produce additional chitinases, resulting in the observed total of 10 excreted chitinases. The presence of chitinases presumably aids the invasion of the pathogen. The great diversity of chitin structures present in the environment necessitates bacteria to produce different forms of chitinase (Svitil *et al.*, 1997).

2.2.3.8. Low molecular weight lipopolysaccharide (LPS)

V. harveyi E2 obtained from an outbreak of disease of *L. vannamei* postlarvae produced a thermostable exotoxin, which was lethal to Dublin Bay shrimp, *Nephrops norvegicus L.*, when injected intramuscularly. This kind of extracellular products (ECPs) were toxic for shrimp and retained the same pathology as the crude ECPs after heating at 100°C for 10 min or digestion with protease K. Western blotting demonstrated that all the ECP preparations contained low molecular weight lipopolysaccharides, which may constitute the lethal toxin produced by *V. harveyi* (Montero and Austin, 1999).

2.2.3.9. Protease, phospholipase

Proteases secreted by *V. parahaemolyticus, V. alginolyticus* and several strains of *V. harveyi* act by destroying enzymatic haemolymph clotting in shrimp (Lee *et al.*, 1999a). Challenging *Artemia franciscana* nauplii with *V. harveyi*, Soto-Rodriguez *et al.* (2003) found significant correlations between naupliar mortality and *in vitro* production of proteases, phospholipases or siderophores (Soto-Rodriguez *et al.*, 2003).

2.2.3.10. Hemolysin

Hemolysins are responsible for the disruption of the erythrocyte membrane or hemolysis. The genes encoding for hemolysins have been reported to be present in several members of the genus including *V. harveyi* (Hirono *et al.*, 1996; Nishibuchi *et al.*, 1990; Zhang *et al.*, 2001).

Zhang and Austin (2000) suggested that hemolysins are one of the pathogenic determinants of several *V. harveyi* strains isolated from a variety of hosts and geographic origins.

2.2.4. Preventive measures in shrimp diseases

Many approaches have been tried to improved shrimp aquaculture production such as probiotics, immunostimulations, vaccination, and the use of specific pathogen-free (SPF) or specific pathogen-resistant (SPR).

2.2.4.1. Probiotics

Probiotics are defined as ‘‘live microorganisms, which when consumed in adequate amounts, confer a health benefit for the host’’ (Reid *et al.*, 2003). Applying different probiotic bacteria, Balcázar *et al.* (2007) found that *V. alginolyticus* UTM 102, *Bacillus subtilis* UTM 126, *Roseobacter gallaeciensis* SLV03, and *Pseudomonas aestumarina* SLV22 are effective probiotics in preventing *V. parahaemolyticus* infection in shrimp. In hatchery and grow-out farming of *Fennerpenaeus indicus* shrimp, Ziaei-Nejad *et al.* (2006) found that feed conversion ratio, specific growth rate, and final production were higher in shrimp receiving a probiotic mixture of 5 *Bacillus* species (*B. subtilis*, *B. licheniformis*, *B. polymyxa*, *B. laterosporus* and *B. circulans*) than in control shrimp which had received no probiotic. Gulliana *et al.* (2004) mentioned that *Vibrio* P62 and *Bacillus* P64, isolated from the hepatopancreas of healthy wild shrimp collected in Manglaralto, Ecuador, had probiotic activity preventing disease in *L. vannamei.*

Bacillus S11 bacterium provided protection in *P. monodon* shrimp when challenged with *V. harveyi*. After a 100-day feeding trial, shrimp in the treatment groups displayed 100% survival after challenge with *V. harveyi*, whereas high mortality was observed in the control group (Rengpipat *et al.*, 1998).

2.2.4.2. Immunostimulation

Peptidoglycans, lipopolysaccharides, and β-glucans found on the surfaces of bacteria and fungi can stimulate the immune system of penaeid shrimp when used together with shrimp feed. Many applications of immunostimulation have been reported in shrimp. Using heat-killed *V. harveyi*, and yeast glucan as immunostimulants, Devaraja *et al.* (1998) showed that these products caused peak immune responses after 48 h of application. Other products like fucoidan, a sulfated polysaccharide extracted from brown algae, protected *M. japonicus* from WSSV challenges (Takahashi *et al.*, 1998).

Ergosan, a seaweed compound containing 1% alginic acid, has been applied for immunostimulation in shrimp culture. Intermoult adult white shrimp (*L. vannamei)* fed with this product for 15 days showed enhancement of the *in vitro* antimicrobial activity in the haemolymph towards pathogenic *Vibrio* isolates. An increase in relative growth was recorded in shrimp fed with Ergosan when compared with control groups (Montero-Rocha *et al.*, 2006). Heat-killed *V. anguillarum* has been confirmed as an effective immunostimulant for postlarval (PL_{30}) *P. monodon* (Azada *et al.*, 2005).

2.2.4.3. Vaccination

The term vaccination in crustaceans is still controversial. The invertebrate defence system is often described as based on innate immunity only, which excludes the possibility of vaccination. According to Van der Braak (2002a), vaccination is a term that should strictly be applied only in long-lasting protection through immunological memory. Defence stimulation in invertebrates is often called “vaccination” but this “vaccination” is not equal to vertebrate vaccination. Therefore, this term will be used between quotation marks when used for shrimp. Very little is known about the shrimp’s immune response to viral infections. Therefore, the potential of shrimp “vaccination” is uncertain. Nonetheless, several studies reveal methods to significantly enhance the invertebrate defence mechanisms. One example

is the so-called "vaccination" against white spot syndrome virus, i.e. VP19 and VP28 being major structural WSSV proteins present in the virion envelope, their recombinant HIS-VP28 and MBP-VP19 fusion proteins were purified and injected into shrimp which were subsequently injection-challenged with WSSV. Witteveldt *et al.* (2004) reported that MBP-VP19 or a mixture of MBP-VP19 and HIS-VP28 significantly slowed down or reduced mortality caused by WSSV.

Using a formalin-inactivated viral preparation derived from WSSV-infected shrimp tissue as vaccination, Singh *et al.* (2005) found a positive effect of this product against WSSV in *F. indicus*. Kuruma shrimp vaccinated with formalin-inactivated WSSV showed some resistance to the virus on day 10 post-vaccination (dpv) but not on 30 dpv. Additional injections with glucan or killed *V. penaeicida* enhanced the efficacy of formalin-inactivated WSSV. Shrimp injected with recombinant proteins of WSSV (rVP26 or rVP28) displayed a higher resistance to WSSV (Namikoshia *et al.*, 2004).

Four genes encoding the WSSV structural proteins VP15, VP28, VP35 and VP281 were inserted individually into DNA vaccine vector pVAX1 to generate recombinant constructs. *P. monodon* shrimp were "immunized" with these DNA constructs and later challenged with WSSV. Since a significant level of protection was offered by the plasmids-encoding VP28 or VP281 till 7 weeks, Rout *et al.* (2007) concluded that the DNA vaccination strategy has potential against WSSV infection in shrimp cultivation.

2.2.4.4. SPF and SPR shrimp

Specific pathogen-free (SPF) shrimp are produced in highly biosecure facilities and are free from specified pathogens such as IHHNV, WSSV, HPV, TSV, YHV, MBV, microsporidians, gregarines, haplosporidians, and metazoan parasites.

Specific pathogen-resistant (SPR) shrimp are selected for their characteristic to be not (or less) susceptible to infection by one or several specific pathogens. The main causes for using SPF and SPR shrimp are their ability to be reared at high density and low salinity (Singh and Lakra, 2008). The risk of introducing exotic pathogens can be reduced significantly by using SPF stocks (Flegel, 2006). The use of shrimp stocks derived from specific pathogen-free (SPF) or high-health sources is highly recommended (Pruder *et al.*, 1995; Lotz, 1997; Fegan and Clifford, 2001) in order to produce a good quality shrimp seed. Recently, most countries in Latin America have begun domestication and genetic selection programmes using pond-reared broodstock in maturation systems. This has been done in an attempt to stabilize predictability and improve the disease resistance and growth performance of their shrimp stocks. Many countries are concentrating on the production of SPR shrimp, selecting the best surviving animals from pond on-growing facilities and on-growing them further in various facilities before transfer to maturation systems. In 2003, SPF broodstocks of the Pacific white shrimp, *L. vannamei* were introduced from Hawaii to Korea for mass production of postlarvae in 2004. Consequent culture results were very satisfactory, i.e. the growth rate was 30% to 50% higher and the frequency of viral outbreaks was much lower in *L. vannamei* than in *F. chinensis* (Jang and Jun, 2005). In Thailand, imported SPF *L. vannamei* broodstocks were first tested in 2001 and yielded high survival and fast growth. The SPF shrimp were tolerant to much higher densities than in *P. monodon* culture and mass mortalities were greatly reduced (Wyban, 2007). Crop value and profits with *L. vannamei* are 2-3 times greater than with *P. monodon*. Reliability of production is also higher with SPF *L. vannamei* (Wyban, 2007). Serious shrimp diseases can come from source waters and/or organisms in source waters (Browdy and Bratvold, 1998). Therefore, by limiting the use of source waters and pretreating these waters before usage and reconditioning effluents before recycling, chances of disease outbreaks are greatly reduced. These precautions combined with the use of

specific pathogen-free (SPF) or specific pathogen-resistant (SPR) shrimp can greatly reduce disease incidence during grow-out.

2.3. Crustacean immunology

Shrimps possess immune systems that are different from vertebrate immune systems. Both humoral and cellular responses are present in shrimp. Haemocytes play an important role in the cellular immune response (Newman and Bullis, 2001).

2.3.1. Haemocytes

Crustaceans possess blood cells that are different from the vertebrates. The haemocytes were described in the haemolymph of crustaceans instead of the red or white blood cells in higher vertebrates. There were many different ideas about the classification of penaeid shrimp haemocytes. Three different cell types in penaeid shrimp haemocytes were characterized by Martine and Graves (1985). Agranular haemocytes are the smallest (12.4 x 7.8 μm), ovoid in shape. This cell type lacks granules and constitutes only 5-10% of the circulating haemocytes. Small-granular haemocytes make up 75% of all haemocytes. They are ovoid and slightly longer and wider (14.8 x 8.3 μm) than agranular haemocytes. This cell type contains 1-40 granules in the cytoplasm. The third cell type is large-granular haemocytes, constituting 10-20% of the haemocytes. They are ovoid and spherical in shape and are about the same size (13.6 x 9.5 μm) as the small-granular haemocytes. Using electron microscopy for identification of different haemocyte types in *P. monodon* shrimp, van de Braak *et al.* (2002a) also found three haemocyte types, namely agranular (hyalinocyte), semi-granular and granular haemocytes. Different cell types in the haemolymph of some shrimp species were quantified by Vargas-Albores *et al*. (2005) (Table 2.4). The function of haemocyte cell types was studied in crayfish and crab (see Table 2.5 for overview of literature sources).

Table 2.4. Total and differential count of shrimp haemocytes (Vargas-Albores *et al*., 2005)

Cell types	***F. californiensis***	***L. vannamei***	***L. stylirostris***
Total count (10^6 cells mL^{-1})	4.0 ± 1.6	9.4 ± 2.1	14.2 ± 5.3
Hyaline (%)	27.4 ± 4.4	25.0 ± 3.9	24.5 ± 2.1
SGH (%)	52.0 ± 6.5	53.0 ± 10.6	51.4 ± 2.5
LGH (%)	20.6 ± 6.1	22.0 ± 8.2	14.1 ± 1.9

SGH = Small-granule haemocyte; LGH = Large-granule haemocyte; *F. californiensis* = *Farfantepenaeus californiensis; L. vannamei* = *Litopenaeus vannamei.* Samples from 10 animals of each species were analyzed in triplicate.

Table 2.5. The function of crayfish and crab haemocytes, as described in literature

Haemocyte types	**Function in immunity**	**References**
Hyaline cell	Phagocytosis	Smith and Söderhäll, 1983; Söderhäll *et al.*, 1986; Thörnqvist *et al.,* 1994.
Semi-granular cell	Encapsulation	Kobayashi *et al.*, 1990.
	Phagocytosis (limited)	Smith and Söderhäll, 1983, Söderhäll *et al.*, 1986; Thörnqvist *et al.*, 1994.
	Storage and release of the proPO system	Johansson and Söderhäll, 1985.
	Cytotoxicity	Söderhäll *et al.*, 1985.
Granular cell	Storage and release of the proPO system	Johansson and Söderhäll, 1985.
	Cytotoxicity	Söderhäll *et al.*, 1985.

2.3.2. Lysozyme

Lysozyme is one of the earliest known antibacterial proteins, ubiquitous in many eukaryotes and prokaryotes, which has been recognized as a molecule involved in non-specific innate immunity (Tyagi *et al.*, 2007). Six types of lysozyme are recognized (Hikima *et al.*, 2003) such as chicken type (c-type), goose type (g-type), plant type, bacterial type lysozyme, T4 phage lysozyme (phage type) and invertebrate type (i-type). The nucleotide sequence of

c-type lysozyme from *L. vannamei*, *P. monodon,* and *M. japonicus* has been reported. The expression of c-type lysozyme from *L. vannamei* and its activity against *V. alginolyticus*, *V. parahaemolyticus* and *V. cholerae* have been studied (de-la-Re-Vega *et al.*, 2004, 2006). The antimicrobial activity of penaeid shrimp lysozyme, as described in literature, is listed in Table 2.6.

Table 2.6. Lysozymes in penaeid shrimp

Species	**Genbank codes**	**Antimicrobial activity**	**References**
Tiger shrimp (*P. monodon*)	B1784440	Against *V. harveyi*, *Micrococcus luteus*	Tyagi *et al.*, 2007
White shrimp (*L. vannamei*)	AF425673	Against *V. alginolyticus*, *V. parahaemolyticus, V. cholerae, Micrococcus luteus*.	de-la-Re-Vega *et al.*, 2004; Hikima *et al.*, 2003
Kuruma shrimp (*M. japonicus*)	AB080238	Against *Vibrio* spp., *V. penaeicida*	Hikima *et al.*, 2003

2.3.3. Phenoloxidase and melanin formation

The prophenoloxidase (proPO) system is considered a constituent of the immune system and forms an important part of an immuno-recognition process of the defence mechanism in invertebrates (Ashida and Brey, 1998; Söderhäll and Cerenius, 1998). According to Lai *et al.* (2005), the proPO system in haemocytes of the white shrimp *L. vannamei* serves an important function in non-self recognition and host immune reactions. In invertebrates, a major innate defense system is the melaninzation of pathogens and damaged tissues. This important process is controlled by the enzyme phenoloxidase (PO) (Cerenius and Söderhäll, 2004).

The proPO is activated to phenoloxidase by a cascade of events which is initiated when β-1,3-glucans, lipopolysaccharides, peptidoglycans (i.e. β GBP, LGBP, and PGBP activities) or other compounds such as endogenous factors produced upon tissue damage bind to the

pattern-recognition proteins. A cascade of serine proteinases will result in the cleavage of the pro-form of the prophenoloxidase-activating enzyme (pro-ppA) into active ppA. Ca^{2+} is required for the conversion of the pro-ppA to ppA that transforms proPO to active phenoloxidase (PO) (Gollas-Galván *et al.*, 1999). ppA will cleave prophenoloxidase into phenoloxidase (Cerenius and Söderhäll, 2004). The major enzyme produced by the activated proPO is PO which is necessary for the melanisation process observed in response to infection, which occurs in cuticular wounds or nodules and capsule formation around invading parasites (Johansson and Söderhäll, 1989a; Söderhäll and Cerenius, 1992). This active enzyme will catalyze the oxygenation of monophenols forming quinones and melanin (Figure 2.6). Melanin is a quite undefined designation of the dark insoluble polymers that, for example, are formed at sites of infection in arthropods (Nappi *et al.*, 1995).

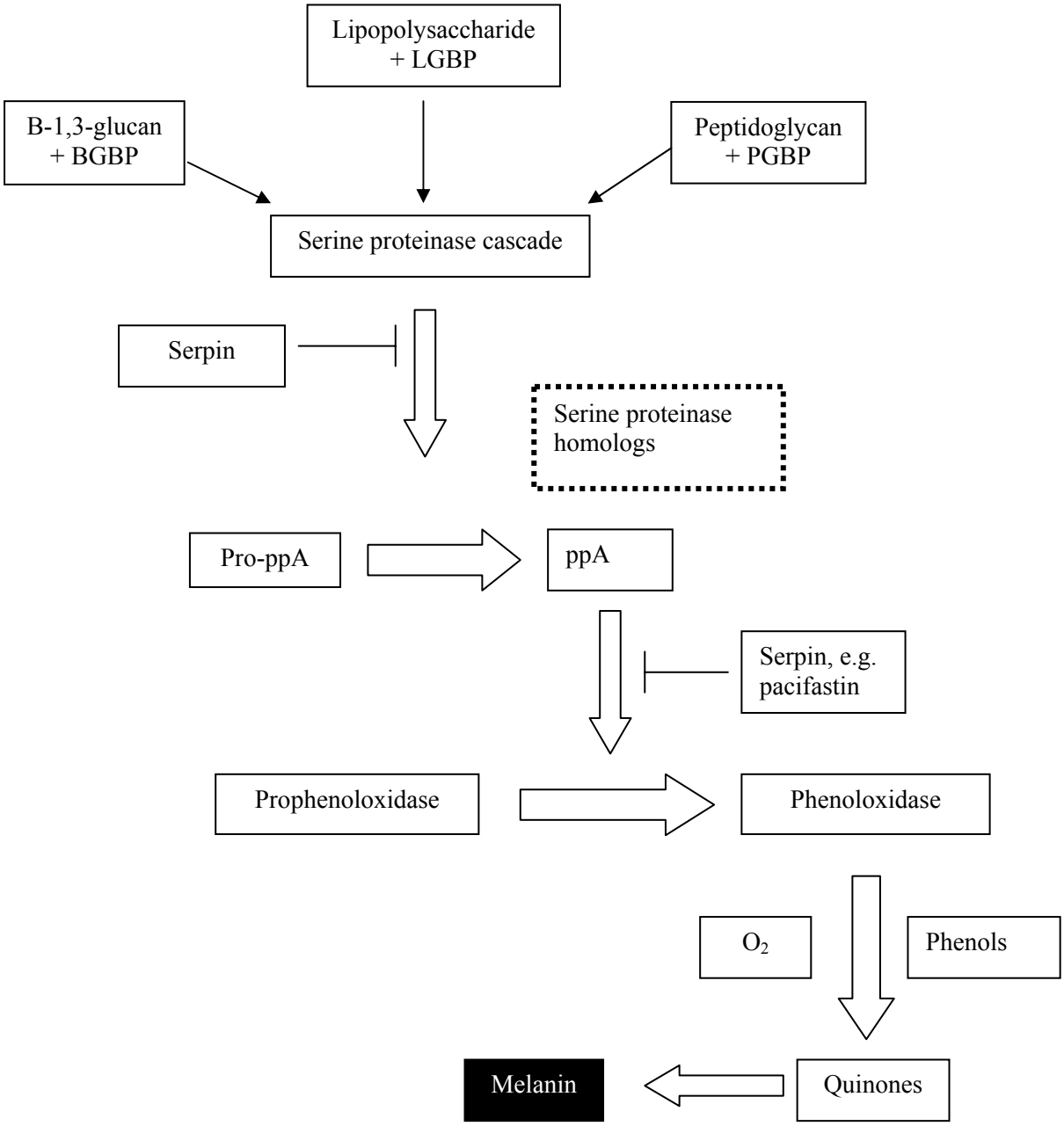

Figure 2.6. Overview of the arthropod prophenoloxidase (proPO)-activating system (Cerenius and Söderhäll, 2004) (GBP = β-1,3-glucan Binding Protein; LGBP = Lipopolysaccharide- and β-1,3-glucan-binding Protein; PGBP = Peptidoglycan-binding Protein ; ppA = Prophenoloxidase Activating enzyme).

2.3.4. Antimicrobial peptides (penaeidin)

Penaeidins are members of a new family of antimicrobial peptides isolated from a crustacean, which present both Gram-positive antibacterial and antifungal activities. The localization of synthesis and storage of penaeidins was studied in the shrimp *L. vannamei*. Penaeidins are

constitutively synthesized and stored in the shrimp haemocytes. It was shown by immunocytochemistry, at both optical and ultrastructural levels, that the peptides are localized in granulocyte cytoplasmic granules (Destoumieux *et al.*, 2000). Five different antimicrobial peptides were found in shrimp. They were named penaeidin 1, 2, 3, 4, and 5. The characteristic and functions of these penaeidins are list in Table 2.7.

Table 2.7. The characteristic and functions of five different penaeidins in penaeid shrimp

Types	Number of AA	Closest species	Antimicrobial activities	References
Pen-1	50	*L. vannamei*	Against bacteria (*Micrococcus luteus*, *Escherichia coli*) and fungi (*Neurospora crassa*, *Fusarium oxysporum*)	Destoumieux *et al.*, 1997.
Pen-2	50	*L. vannamei* *L. stylirostris*	Against bacteria (*Bacillus megaterium*, *Aerococcus viridans*, *Micrococcus luteus*, *Escherichia coli*) and fungi (*Neurospora crassa*, *Fusarium oxysporum*)	Destoumieux *et al.*, 1997; Munoz *et al.*, 2004.
Pen-3	62	*L. stylirostris*	Against bacteria (*Bacillus megaterium*, *Aerococcus viridans*, *Micrococcus luteus* and *Escherichia coli*) and fungi (*Neurospora crassa* and *Fusarium oxysporum*)	Destoumieux *et al.*, 1997; Munoz *et al.*, 2004.
Pen-4	47	*L. stylirostris*	Against bacteria (*Bacillus megaterium*) and Fungi (*Botrytis cinerea*, *Botrytis cinerea*)	Cuthbertson *et al.*, 2005.
Pen-5	55	*P. monodon*	Against bacteria (*Aerococcus viridans*) and filamentous fungi (*Fusarium pisi* and *Fusarium oxysporum*)	Hu *et al.*, 2006; Chen *et al.*, 2004.

AA = Amino acid; Pen = Penaeidin

2.3.5. Clotting

Clotting plays an important role in the rapid sealing of wounds to prevent the entrance of pathogens or the loss of haemolymph. In crustaceans, the clotting reaction is very rapid

(Söderhäll and Cerenius, 1992). A clottable protein was purified from the haemolymph of tiger shrimp (*P. monodon*). The molecular mass of this protein was determined to be 380 kDa. The size and amino acid composition of the clottable protein are similar to those of several other shrimps, prawns, lobster and crayfish, and their N-terminal amino acid sequences are 60-80% identical (Yeh *et al.*, 1998).

2.3.6. Phagocytosis and related mechanisms

Phagocytosis: This phenomenon includes attachment to the foreign particles, and ingestion and destruction of foreign particles (Holmblad and Söderhäll, 1999). Circulating haemocytes are not the only phagocytic cells. Fixed phagocytes have been demonstrated in the gills and heart (Johnson, 1987). The mechanisms for non-self recognition and the subsequent mediation for phagocytosis are not yet fully understood. However, it is known that opsonic factors are involved. These factors or opsonins will mark or coat invading microorganisms and facilitate phagocytosis. Opsonic factors are at least partially involved in the recognition, mediation, and initiation of phagocytosis in at least some crustaceans, as has been demonstrated in *Carcinus maenas* (Thörnqvist *et al.*, 1994).

The phagocytosis activating protein (PAP) gene was isolated from *P. monodon*. It is expressed upon infection with the WSSV. An alpha-2-macroglobulin (α2M) was one of the proteins that interacted with PAP. α2M may facilitate the entry of GST–PAP (glutathione-S-transferase-PAP) into phagocytic cells and increase the survival rate of the shrimp after being infected with WSSV (Chotigeat *et al.*, 2007).

Encapsulation and nodules: encapsulation occurs when a parasite is too large to be ingested by phagocytosis (Holmblad and Söderhäll, 1999). When encapsulation is taking place a capsule of 5 to 30 semi-granular cells is formed around the macroscopic parasite or pathogen. The proPO-cascade seems to regulate this capsule formation (Johansson and Söderhäll,

1989b). Nodule formation that appears similar to capsule formation occurs when the number of invading bacteria is high. Capsules and nodules are always melanized in arthropods (Holmblad and Söderhäll, 1999).

2.3.7. Cytotoxicity

The most important cell-mediated cytotoxicity is the production of reactive oxygen intermediates (ROIs) (Rodríguez and Le Moullac, 2000). During phagocytosis, particles or micro-organisms are internalized into the cell which later forms a digestive vacuole called the phagosome. The elimination of phagocyted particles involves the release of degradative enzymes into the phagosome and the generation of ROIs. ROIs were described in the shore crab *Carcinus maenas* (Bell and Smith, 1993), in *P. monodon* (Song and Hsieh, 1994), and *L. vannamei* (Muñoz, *et al.*, 2000).

2.3.8. Agglutinins and lectins

Agglutinins: Agglutinin molecules remove invading organisms from the haemolymph by aggregating them, preventing the spread of these possible pathogens through the body of the shrimp. The agglutination can either work in a rather non-specific manner or show specificity with certain membrane molecules of invading organisms (Söderhäll *et al.*, 1988). The levels of agglutinins in the haemolymph can differ considerably between individuals, but enhanced titers are found after infection of *P. monodon* (Adam, 1991).

Lectins: agglutinins reacting with specific polysaccharides are called lectins. In *P. monodon* one lectin specific for sialic acid has been described. These lectins are very specific and it is assumed that besides a defence function they also could be involved in the transport of sugars and calcium ions, control of cell re-aggregation, growth, and development (Ratanapo and Chulavatnatol, 1990).

2.4. Polymicrobial diseases

Although little attention has been paid to polymicrobial diseases in aquaculture, this concept of polymicrobial disease is well accepted in human and in terrestrial animal production. According to Brogden (2002), polymicrobial diseases represent the clinical and pathological manifestations induced by multiple microorganisms. In polymicrobial diseases, there are complex interactions among two or more etiologic agents, leading to synergistic clinical symptoms and pathologies often triggering an array of host responses (Brogden and Guthmiller, 2003). The infection in polymicrobial diseases are often called complex infections, dual infections, mixed infections, secondary infections, co-infection, synergistic infections, concurrent infections or polymicrobial infections. The polymicrobial diseases can be induced by polymicrobial infections involving viruses and bacteria, fungi and parasites, different viruses, or as a result of microbe-induced immunosuppression. Co-infection of bacteria and viruses has been studied in *in vitro*. Most findings demonstrated the enhancement of invasiveness of bacterial cells into virus infected cells but more details about the mechanisms of enhanced bacterial invasiveness following viral infection is unknown. Bukholm and Degré (1984) found that the invasiveness of *Salmonella enterica* serova *typhimurium* was significantly increased in HEp-2 cells infected with coxsackie B1 virus, compared with virus-uninfected controls. They suggested that the virus infection might somehow alter the cell membrane such that bacterial adhesion and/or invasiveness are enhanced. Another *in vitro* study on co-infection of *S. flexneri* and virus-infected cells by Bukholm *et al.* (1986) discovered that bacterial invasiveness was significantly enhanced in HEp-2 cell cultures incubated with measles virus prior to *S. flexneri* inoculation. Studying *Yersinia enterocolitica* and *Y. pseudotuberculosis* infection of virus-infected cells *in vitro*, Di Biase *et al.* (2000) speculated that rotavirus-infection enhanced bacterial invasiveness may have resulted from altered membrane permeability and fluidity. They suggested that rotavirus infection may produce receptors, such that bacterial attachment and entry are facilitated.

Conversely, the decrease in viral antigen synthesis observed in the rotavirus and *Yersinia*-infected cells may have been the result of the breakdown of cellular organelles caused by the multiplication of intracellular bacteria.

2.4.1. Polymicrobial diseases in human

Studying co-infection in humans, Sulkowski (2008) found that secondary infection occurred easily in people having been infected with primary pathogens. He concluded that persons at high risk for human immunodeficiency virus (HIV) infection are also likely to be at risk for other infectious pathogens, including hepatitis B virus (HBV) or hepatitis C virus (HCV). Epidemics of influenza result in an increased hospital admission rate for bacterial pneumonia due to pneumococcus, *Haemophilus influenzae* and *Staphylococcus aureus* (Floret, 1997).

2.4.2. Polymicrobial diseases in terrestrial animals

Many researches on co-infection have been done in animals. Recently, porcine reproductive and respiratory syndrome (PRRS) spread worldwide and caused economical losses in many countries (Wesley *et al.*, 2004). Besides, post-weaning multisystemic wasting syndrome (PMWS) in late nursery and early fattening pigs has also caused economic losses in many European as well as Asian countries (Allan and Ellis, 2000). This disease has been described as being associated with porcine circovirus type 2 (PCV2) infections (Allan *et al.*, 1999). Rovira *et al.* (2002) found that a concurrent infection of PCV2 and PRRSV could result in increased PCV2 viral copy numbers, and that PRRSV infections enhanced PCV2 replication. The PCV2 load in serum was higher in pigs inoculated with PRRSV one week before PCV2 challenge, than in pigs inoculated with PCV2 only (Kim *et al.*, 2003). Additionally, field studies showed that PRRSV can be detected often in PMWS affected pigs (Segalés *et al.*, 2002), and under experimental conditions severe disease can be reproduced in pigs concurrently infected with PCV2 and PRRSV (Harms *et al.*, 2001; Rovira *et al.*, 2002; Stockhofe-Zurwieden *et al.*, 2003). PCV2 has been found as widely prevalent in pigs with

porcine respiratory disease complex (PRDC). It was concluded that PCV2 should be considered a major respiratory pathogen (Kim *et al.*, 2003). The co-infection of PCV2 and other co-existing pathogens e.g. porcine parvovirus (PPV), *Pasteurella multocida*, *Mycoplasma hyopneumoniae* was documented in PRDC (Kim *et al.*, 2003).

2.4.3. Polymicrobial diseases in aquatic animals

Polymicrobial diseases have been mentioned in aquaculture. Epizootic Ulcerative Syndrome (EUS) has been described as one of the important polymicrobial diseases in fish. According to Baldock *et al.* (2005), EUS has caused major fish losses in many countries for over three decades and during that time it has been given several colloquial names e.g. mycotic granulomatosis, red spot disease, ulcerative mycosis. EUS was considered as a polymicrobial disease since causative agents were a combination of many causative agents such as viruses (birnavirus, rhabdoviruses, reoviruses and a distinct group of type-C retroviruses), fungi (*Aphanomyces invadans*) and bacteria (*Aeromonas, Vibrio* and *Plesiomonas* spp.). It was speculated that following skin damage by viral infection, the bacteria would precede the fungi in its entry and subsequent activity (Kanchanakhan *et al.*, 1998; Frerichs *et al.*, 1993; Iqbal *et al.*, 1998).

Co-infection of infectious pancreatic necrosis virus (IPNV) and infectious hematopoietic necrosis virus (IHNV), and co-infection of infectious salmon anaemia (ISA) virus and a novel togavirus-like virus, were detected in rainbow trout (*Oncorhynchus mykiss*) (Alonso *et al.*, 2003) and in Atlantic salmon (Kibenge *et al.*, 2000). These kinds of co-infection have caused serious diseases in the fish farms. Co-infection of two viruses has been observed in the mandarin fish (*Siniperca chuatsi*), a widely cultured species in China. Rhabdovirus has been isolated from diseased fish and was called *S. chuatsi* rhabdovirus (SCRV). Based on morphological structure and partial biochemical and biophysical characteristics, it was

concluded that SCRV should be a rhabdovirus member related to the genus *Vesiculovirus* in the Rhabdoviridae (Tao *et al.*, 2007).

Olive flounder, *Paralichthys olivaceus* is one of the most important cultured fish in Korea. Mass mortality of this species showing ascites occurred at many aquaculture farms in Korea. Marine birnavirus (MABV-F) and bacteria (*V. harveyi* and *Edwardsiella tarda*) were isolated from diseased fish. The result of experimental infection showed that MABV-F alone did not cause direct mortality, whereas cumulative mortality reached more than 90% when co-infection with MABV-F and bacteria by IP was performed. It was concluded that the cause of mass mortality of the adult fish was probably due to bacterial infection in fish already infected with MABV-F (Oh *et al.*, 2006).

Although many disease outbreaks in fish have been desbribed as polymicrobial diseases, very little attention has been paid to experimental dual challenge. Lee *et al.* (1999b) performed dual challenged grouper (*Epinephelus* sp.) with infectious pancreatic necrosis virus (IPNV) and *Vibrio carchariae*. They found an increase in mortality in dual treatment. Mass mortalities occurred in fish infected with IPNV for 2 weeks prior to the infection with the bacteria in either immersion or intraperitoneal injection challenges.

In crustaceans, co-infection of yeast and parasite has been described in edible crabs *Cancer pagurus* and velvet swimming crabs *Necora puber* by Stentiford *et al.* (2003). The dinoflagellate parasite *Hematodinium* sp. infected edible and velvet swimming crabs, which were captured and found to be co-infected with a yeast-like organism. They supposed that *Hematodinium* infection in these crabs may increase the likelihood of secondary infections via an indirect suppression of the host immune system.

Shrimp culture has developed in many countries. The increase in culture area and the current mode of culture has facilitated the outbreak of infectious diseases. Recently, many shrimp

farms as well as the hatcheries have faced viral and bacterial infections in which polymicrobial infections have caused significant economic loss. Although co-infection has been found in the shrimp ponds, no attention has been paid to experimental co-infection under laboratory conditions. Co-infection of many *Vibrio* strains was noticed in red disease syndrome of *P. monodon.* The syndrome is characterized by the reddening of the shrimp body. *V. harveyi, V. parahaemolyticus, V. fluvialis* and *Vibrio* sp. were isolated from diseased shrimp in which *V. harveyi* and *V. parahaemolyticus* showed the most dominant species. Performing shrimp challenge tests with the isolated *V. parahaemolyticus* and *V. harveyi*, Alapide-Tendencia and Dureza (1997) concluded that these bacterial strains can produce the characteristic red discoloration in healthy shrimp.

In 1993, 1994 and 1995 the so-called Syndrome 93' caused low yields and survival rates in Mexican shrimp culture. High stocking density and sudden water temperature drop seemed to have an impact on the development of the disease. The haemolymph of diseased shrimps was generally infected with one predominant strain of bacteria, *Vibrio* sp. Other species were found such as *Pseudomonas*, *Flavobacterium* and *Cytophaga*. Numerous pycnotic haemocytes and atypical basophilic granulations were also observed in most tissues, especially the lymphoid organ, heart, connective tissue of the stomach and cuticular epithelium. These basophilic granulations seemed to be an indication of the presence of other pathogens like Yellow Head Virus (YHV) and Taura Syndrome Virus (TSV). Therefore, these viruses and *Vibrio* sp. were postulated as causative agents in Syndrome 93' (Mermoud *et al.*, 1998).

Jimenez *et al.* (2001) discovered a dual infection by infectious cuticular epithelial necrosis virus and a *Chlamydia*-like organism in cultured *L. vannamei* in Ecuador. Shrimp samples were collected from five different nursery ponds. All ponds showed co-infection of the two before mentioned pathogens. The prevalence of infection ranged from 55 to 80%.

Using histopathology and PCR as tools for the detection of HPV, WSSV, IHHNV and MBV, Flegel *et al.* (2004) found dual, triple and quadruple infection in 240 shrimp samples collected from 6 commercial shrimp ponds in Thailand where visible lesions were apparently caused by MBV. Very high prevalence of co-infection was described e.g. 94% of the specimens gave a positive test for at least one of the four viruses. The dual to quadruple infection accounted for 73% of the total samples. Seventy nine percent of triple infection samples included HPV and IHHNV as 2 of the 3 viruses detected.

In 2001-2002, a retardation of *P. monodon* shrimp growth was noted in shrimp ponds in Thailand. This problem received the name of Monodon Slow Growth Syndrome (MSGS). Samples of affected shrimp were screened by histopathology, polymerase chain reaction (PCR) and transmission electron microscopy (TEM) for a wide range of pathogens. It was discovered that several causative agents were involved. Many shrimp specimens had dual or multiple infections with MBV, HPV and IHHNV (Chayaburakul, 2004). After screening shrimp samples from 18 ponds in India, Umesha *et al.* (2006) found that the animals in 7 ponds showed dual infections with WSSV and MBV and in 10 ponds triple infections with HPV, WSSV and MBV could be found.

White spot disease is considered as one of the most important diseases causing high losses in shrimp culture. The infection becomes more serious when the animals are co-infected with other pathogenic agents. Selvin and Lipton (2003) documented the presence of a virulent strain of *V. alginolyticus* in shrimp collected from a pond hit by a WSSV outbreak. Although not all sampled shrimp were infected by both pathogens, it was stated that shrimp weakened by WSSV would succumb to a secondary infection by *V. alginolyticus.* Another case of WSSV and bacteria co-infection was mentioned by Karunasagar *et al.* (1998). Since WSSV infected shrimp showed reddish coloration in addition to the white spots in the cuticle, they concluded that reddish coloration of WSSV infected shrimp could be attributable to

secondary bacterial infection. This idea was supported by Lee *et al.* (1996) who assumed that the primary WSSV infection probably weakened the shrimp and made them more susceptible to bacterial infection. A secondary pathogen (*V. alginolyticus*) from outbreaks of WSSV causing mass mortality in Taiwan could be isolated in cultured tiger shrimp.

Within the yellow head like virus (YHLV), there are two main groups of viruses that have been indicated to cause disease when a dual infection occurs. The two groups of viruses that interact with each other are the YHLV with WSSV and YHLV with spawner-isolated mortality virus (SMV). In 1993, it was first seen as a dual infection with WSSV and YHLV in Thailand in *P. monodon* and it was originally named systemic ectodermal and mesodermal baculovirus (SEMBV) (Wongteerasupaya *et al.*, 1995). Later on, the dual infection of WSSV and YHLV has been reported in India and Taiwan (Mohan *et al.*, 1998; Wang and Chang 2000). According to Wang and Chang (2000), the reason for mass loss in the shrimp culture industry in Taiwan between 1996 and 1999 was not only WSSV, but a dual infection of WSSV and YHLV. They found evidence of co-infection by different viruses in diseased shrimp. *P. monodon* collected for at least 3 years, which were diagnosed as YHV positive, were also co-infected with WSSV.

Two epizootics of cultured shrimp occurred in India in 1994 showing co-infection of YHV and WSSV. The first case of co-infection of these viruses was recorded in tiger shrimp *P. monodon.* The disease was very similar to yellow head virus (YHV) infection with regard to gross clinical signs, host species and size of shrimp affected. However, histopathology showed typical signs of a WSSV infection in shrimp showing gross signs of yellow head syndrome. The second case of co-infection was found in *P. monodon* and *P. indicus*. The symptoms of diseased shrimp were typical of a WSSV infection, based on clinical and histopathology. Moreover, typical inclusions for YHV infection found in the lymphoid organ

and haematopoietic tissue of WSSV-infected shrimp indicated a possible dual infection (Mohan *et al.*, 1998).

Furthermore, WSSV can interact with other pathogens to create different forms of co-infection in the shrimp ponds. Using a single-step multiplex PCR for simultaneous detection of WSSV and IHHNV in penaeid shrimp, Yang *et al.* (2006) detected co-infection of WSSV and IHHNV in cultured *L. vannamei* in China.

In 1994, mid-crop mortality syndrome (MCMS) caused mass mortality in grow-out shrimp in Australia. The two viruses that were implicated as being involved in this syndrome were YHLV and SMV (Anderson and Owens 2001). Owens *et al.* (1998) reported that SMV virulence in MCMS-affected shrimp was enhanced by the presence of other co-infecting viruses such as an enveloped, filiform virus. The co-infecting virus was YHLV, which had first been identified by Spann *et al.* (1995). In recent years, two other viruses have been reported as being present during the MCMS. These viruses are IHHNV (Krabsetsve *et al.*, 2004) which is similar to SMV as it is a non-enveloped DNA virus, and Mourilyan virus (MoV) (Cowley *et al.*, 2005a).

Co-infection has been found not only in shrimp ponds but also in shrimp hatcheries. Multiple viral infections were discovered in shrimp postlarvae in an Indian hatchery. Moribund *P. monodon* postlarvae (PL_8–PL_{10}) were highly infected with monodon baculovirus (MBV), hepatopancreatic parvovirus (HPV), and white spot syndrome virus (WSSV) (Manivannan *et al.*, 2002). Using microscopic observation and PCR for screening of viral infection in 1020 hatchery tanks rearing postlarvae in India, Uma *et al.* (2006) found that 4.5% of the poslarval shrimp samples showed dual infection with HPV and MBV.

CHAPTER

Standardization of a protocol for *Vibrio* challenge in specific pathogen-free (SPF) shrimp (*Litopenaeus vannamei*)

L.H. Phuoc, P. Sorgeloos, P. Bossier

(Submitted)

3.1. Abstract

This study was conducted to develop a standardized protocol for *Vibrio* challenge in specific pathogen-free (SPF) shrimp (*Litopenaeus vannamei*). Shrimp were challenged with *Vibrio campbellii*, *V. harveyi* 642, *V. harveyi* E022, *V. harveyi* E2, and *V. penaeicida* by immersion and injection in normal as well as stressful conditions.

For immersion, shrimp were challenged with 10^3, 10^5, and 10^7 CFU mL^{-1} bacterial cells. During the 5 days of the experiment, shrimp were fed with either *Artemia* nauplii, *Artemia* bio-encapsulated with *V. campbellii*, a piece of shrimp muscle into which a *V. campbellii* suspension was injected, or artificial feed pellets. There was no significant difference in mortality between the control and *Vibrio*-challenged groups maintained under optimal conditions ($p > 0.05$). Then, different stress factors were tested such as starvation or exposure to ammonium salt or to reduced salinities. Starvation, ammonium stress before and during challenge periods, and salinity stress at 5, 10, 20 gL^{-1} did not enhance the susceptibility of shrimp to *Vibrio* challenge by immersion.

In absence of stress, SPF shrimp are not susceptible to the tested *Vibrio* species and strains neither by immersion nor injection. Only under ammonium stress, SPF shrimp appear susceptible to pathogenic *Vibrio* when injected at doses of 10^6 CFU $shrimp^{-1}$.

3.2. Introduction

Shrimp culture has undergone rapid development in many countries especially in Southeast Asia. Shrimp production is frequently and seriously affected by problems linked to environmental pollution and to infectious and non-infectious diseases (Primavera, 1997). Reports on epizootic luminescent bacterial diseases, especially in Asian shrimp farms, have been connected to the expansion of intensive shrimp production (Karunasagar *et al.*, 1994).

Vibrio species are abundant as free-living micro-organisms in the marine environment, e.g. representing nearly 80% of the bacterial population in surface water of the western Pacific Ocean (Tsukamoto *et al.*, 1993). Vibriosis in penaeid shrimps is mainly caused by *V. anguillarum* (Lightner, 1996) , *V. alginolyticus* (Vandenberghe *et al.*, 1998; Selvin and Lipton, 2003), *V. parahaemolyticus* (Alapide-Tendencia and Dureza, 1997), *V. harveyi* (Prayitno and Latchford, 1995; Martin *et al.*, 2004), *V. penaeicida* (Saulnier *et al.*, 2000a), *V. campbellii*-like (Hameed, 1995) and occasionally *V. splendidus* (Lavilla-Pitogo *et al.*, 1998). Leaño *et al.* (1998) isolated and identified 172 representative bacteria from diseased shrimps and found that approximately 90% of the isolates belonging to *Vibrio* species. *V. harveyi* and *V. campbellii* can infect larval, juvenile as well as adult stages of penaeid shrimp (Gómez-Gil *et al.*, 1998b; Lavilla-Pitogo *et al.*, 1998). *V. harveyi* was considered as the most important species causing luminous vibriosis in both hatchery-reared and pond-grown *Penaeus monodon* (Lavilla-Pitogo *et al.*, 1990). In the Philippines, *V. harveyi* concentrations as low as 10^2 cells mL^{-1} did cause total losses in the hatchery rearing of *P. monodon* (Lavilla-Pitogo *et al.*, 1990). Bacterial infections related to *V. harveyi* luminescent strains have also been reported to cause major losses in the shrimp larviculture in Australia (Pizzutto and Hirst, 1995), South America (Álvarez *et al.*, 1998; Robertson *et al.*, 1998) and Mexico (Vandenberghe *et al.*, 1999). The disease usually occurs during the first month of culture and

can cause more than 50% mortality. Luminous *Vibrio*, the causative organism of the disease, is an opportunistic pathogen. Histopathological studies of shrimp infected with luminescent vibriosis reveal a massive bacterial colonization of the oral region (Lavilla-Pitogo *et al.*, 1990), as well as atrophy and necrosis of the hepatopancreatic tubules (Robertson *et al.*, 1998).

Many different infection routes have been documented, e.g. immersion (Roque *et al.*, 1998; Soto-Rodríguez *et al.*, 2006), oral intubation (De la Peña *et al.*, 1995) and injection (Pizarro and Alfaro, 1994; Wang and Chen, 2006). The immersion challenges revealed LD_{50} doses in the range of 10^4 to 10^9 CFU mL^{-1} of pathogenic bacteria. The LD_{50} ranged from 10^3 to 10^7 CFU $shrimp^{-1}$ for injection challenge. De la Peña *et al.* (1995) succeeded in infecting *P. japonicus* with low numbers of a *V. penaeicida* isolate (10^3-10^4 CFU $shrimp^{-1}$) orally administered. However, some of them failed to infect shrimp in unstressed condition or the infection only happened when using a high dose. For instance, Hameed *et al.* (1996) failed to produce infection in *P. indicus* at 3.5 x 10^6 CFU mL^{-1}. With a non-specified strain, *P. monodon*, injected with 3 x 10^3 CFU $shrimp^{-1}$ of *V. harveyi* STD3-69 considered to be a shrimp pathogen, showed no mortality in two consecutive experiments (Merchier, 1998). Hameed (1995) performed the bath challenge in *P. monodon* with very high bacterial density, 10^6-10^9 CFU mL^{-1}.

Different shrimp species were used as experimental animals in previous researches, e.g. *P. monodon* (Prayitno and Latchford, 1995; Lee *et al.*, 1996), *L. vannamei* (Robertson *et al.*, 1998; Wang and Chen, 2005), *P. setiferus*, *P. duorarum*, *P. aztecus* (Lightner and Lewis, 1975), *P. indicus*, *P. semisculatus* (Hameed, 1995), and *P. stylirostris* (Saulnier *et al.*, 2000b).

The effect of salinity and ammonium stress on the susceptibility to *Vibrio* was also studied in shrimp. Testing the effect of salinity on the susceptibility of *L. vannamei* to *V. alginolyticus*, Wang and Chen (2005) found that over the period of 24-96 h the cumulative mortality for shrimp transferred to 5 gL^{-1} and 15 gL^{-1} from 25 gL^{-1} was significantly higher than for shrimp held in 25 gL^{-1} and 35 gL^{-1}. Ambient ammonia decreased the resistance of *L. vannamei* to *V. alginolyticus* infection. Ammonia increased the susceptibility of *L. vannamei* by a reduction in immune response such as a decrease in phagocytic activity and clearance efficiency to *V. alginolyticus* (Liu and Chen, 2004). The shrimp which were exposed to ammonia prior to immersion challenge, showed more frequent and earlier pathological changes than shrimp exposed to bacteria alone (Alday-San *et al.*, 2002).

In all previous reports, the experimental animals were non-SFP shrimp, thus introducing an important variable. With the present availability of SPF shrimps (*Litopenaeus vannamei*) the possibility is offered to revisit the cause of shrimp susceptibility to *Vibrio* under more standardized conditions. For this purpose, a standard challenge was developed with SPF *Litopenaeus vannamei*.

3.3. Materials and Methods

3.3.1. Specific pathogen-free (SPF) shrimp

SPF *L. vannamei* of the Kona-Hawaii (USA) strain (Wyban *et al.*, 1992) were used in this study. Animals are claimed to be free of WSSV, IHHNV, MBV, HPV, TSV, YHV and GAV viral particles, as well as other pathogens (prokaryotes: NHP; protozoa: Microsporidians, Haplosporidians, Gregarines) as verified by PCR and histopathology. Batches of shrimp arrived at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, as postlarvae (PL_{8-12}). They were kept in a recirculation system at a water

temperature of 28-29°C, 34 gL^{-1} salinity, and pH of 7.8-8.1. During the first week, the animals were fed twice daily with *Artemia* nauplii. After one week their diet was shifted to A2 monodon high performance shrimp feed (2.2 mm fraction, INVE Aquaculture NV, Dendermonde, Belgium). The feeding ratio was 2.5% of the mean body weight (MBW) per day. Two days before the start of a new test, the shrimp were transferred to the challenge room for acclimation in 20 L culture tanks.

3.3.2. Preparation of bacterial stocks

Five *Vibrio* strains were used for experimental infection, i.e. *Vibrio campbellii* (LMG21363), *V. harveyi* ACMM642 and *V. penaeicida* (LMG19663) obtained from the Laboratory for Microbiology (Ghent University, Belgium), *V. harveyi* E2 (Montero and Austin, 1999), and *V. harveyi* E022 (Robertson *et al.*, 1998). These strains, previously stored in 20% glycerol at -80°C, were aseptically inoculated in Marine Agar (MA) and incubated for 24 h at 28°C. Single colonies were subsequently transferred and incubated overnight (28°C, 150 rpm) in Marine Broth (MB) 2216 (Difco Laboratories, Detroit, MI, USA). The cultures were then transferred to centrifugation tubes and treated at 2200 g for 15 min. The supernatant was discarded and pellets were washed twice and finally re-suspended in filtered autoclaved seawater (FASW). The bacterial densities were determined spectrophotometrically at an optical density of 550 nm assuming that an optical density of 1.000 corresponds to 1.2 x 10^9 cells mL^{-1}, the McFarland standard (BioMerieux, Marcy L'Etoile, France).

3.3.3. Rifampicin-resistant strains

Rifampicin-resistant bacterial strains were produced as follows: a colony picked from MA plates was cultured for 24 h in 25 mL MB in a 100 mL erlenmeyer. After incubation, 50 µl of the culture was taken by micropipette and transferred to fresh 25 mL MB containing

0.5 mgL^{-1} rifampicin (R3501, Sigma-aldrich). The culture was incubated for 24 h at 28°C. The growth of the bacteria was confirmed by an increase in the turbidity of the culture. In the following days, further sub-cultures were made in MB, increasing the rifampicin concentration gradually from 1, 2, 4, 8, 16, 32, 64 to finally 100 mgL^{-1}. When bacteria cultures were growing well in the final concentration of rifampicin (100 mgL^{-1}) they were once more inoculated on MA plates containing 100 mgL^{-1} rifampicin in order to isolate single colonies. The stock was stored in 20% glycerol at -80°C for long term storage.

3.3.4. Counting of bacterial density

The shrimp samples were washed once with 70% alcohol and twice with FASW. Afterwards, they were homogenized in FASW by stomacher, serial diluted, and plated on MA with 100 mgL^{-1} rifampicin (MAR). The plates were incubated at 28°C for 24 h. For *Vibrio* enumeration of water samples, 10-fold serial dilutions of the samples were made in FASW, and then plated on MAR.

3.3.5. Injection and immersion challenge in SPF shrimp

Seawater (35 gL^{-1}) was disinfected with 30 mgL^{-1} sodium hypochlorite for three days. A strong aeration was provided for 24-48 h to remove residual chlorine. Before starting the experiment, the water was checked for residual sodium hypochlorite by using starch and potassium iodine as color agent (Van Stappen, 1996).

3.3.5.1. Injection challenge

Shrimp were exposed with ammonium chloride at a concentration of 50 mgL^{-1} NH_4^+. The water was refreshed after 12 h of ammonium stress and replaced with fresh seawater containing 20 mgL^{-1} NH_4^+. Subsequently the shrimp were injected with *V. campbellii*. The

bacteria were inoculated with doses of 10^4 or 10^6 CFU shrimp^{-1} by intramuscular injection between the second and third abdominal segment. Control shrimps were injected with an equal volume of FASW. After injection, each group was held in separate 20 L plastic tanks containing 5 L continuously aerated seawater (35 gL^{-1} salinity) at 28^{o}C. Shrimp were fed with formulated shrimp feed (2.2 mm fraction, INVE Aquaculture NV, Dendermonde, Belgium) twice a day. At 48 h post injection, the water was refreshed and replaced with new NH_4Cl-free seawater.

3.3.5.2. Immersion challenge in the absence of ammonium stress

Challenge tests were conducted in duplicate, triplicate or quadruplicate (n-value) with seven to ten shrimps per replicate (s-value) depending on the experiment. After acclimation, shrimp were distributed to PVC oxygenation tanks well aerated with an air stone. All experimental materials were disinfected with sodium hypochlorite. The tanks were covered to prevent the shrimp from jumping out and to prevent cross-contamination by aerosols. The postlarvae were exposed to different concentrations of bacterial cells (10^3, 10^5, 10^7 CFU mL^{-1}). During the experimental period, salinity levels remained constant at 35 gL^{-1}, pH in the range of 7.9 to 8.2 and temperature at 28 ± 1°C. Shrimp were fed twice daily with *Artemia* or commercial shrimp pellets. The mortality was recorded at 6, 12, 24, 36, 48, 72, 96 and 120 h after exposure to the bacteria.

3.3.5.3. Immersion challenge in stress condition

Immersion challenge with starvation condition: The experiments were set up in the same way as described in part 3.3.5.2 but the animals were kept in starvation condition for 1, 2, 3, 4 or 5 days, prior to being challenged with the bacteria.

Immersion challenge with salinity stress: Shrimps were shocked at 10 or 20 gL^{-1} salinity for 30 minutes and then challenged with *V. campbellii*, *V. harveyi* E2 and *V. harveyi* 642 at 10^7 CFU mL^{-1}. In another experiment, shrimps were shocked at 5 or 10 gL^{-1} salinity before and during the challenge with *V. campbellii*, *V. harveyi* E2 and *V. harveyi* 642 at 10^7 CFU mL^{-1}

Immersion challenge with ammonium stress: Shrimp were exposed to continuous or discontinuous ammonium stress. To find out the sub-lethal dose of NH_4Cl for shrimp, juveniles were exposed to different concentrations of ammonium chloride, i.e. 25, 40, 50, 75, 100, 125, 150 mgL^{-1} NH_4^+. The mortality was recorded at 6, 12, 24 h after exposure to ammonium chloride. Tests with postlarvae were performed in the same way as the juveniles, but at concentrations of 10, 20, 30, 40, and 50 mgL^{-1} NH_4^+. The bacterial challenges performed in combination with ammonium stress were conducted in different ways, i.e. shrimp were stressed with 20 mgL^{-1} NH_4^+ for 24 h and subsequently challenged in the absence of NH_4Cl; in other experiments, they were stressed with 50 or 20 mgL^{-1} NH_4^+ for 12h and subsequently replaced in new seawater containing 20 mgL^{-1} NH_4^+ and challenged with bacteria at 10^7 CFU mL^{-1}. Extra details about the various treatments are given in the results section. Shrimp were fed with different feed sources during the challenge periods, i.e. artificial feed, *Artemia* bio-encapsulated with *V. campbellii*, or a piece of shrimp muscle into which *V. campbellii* was injected at a dose of 10^9 CFU $shrimp^{-1}$. For bio-encapsulation with *V. campbellii*, *Artemia* nauplii were suspended for 5 hours in 3 x 10^8 CFU mL^{-1} *V. campbellii* before being fed to shrimp. The shrimp were fed with infected *Artemia* for 3 days, and then artificial feed was used until the end of the experiment. During the challenge period water was either changed or not.

3.3.5.4. Immersion challenge with removing of the pleopod and wounding

In order to facilitate the penetration of *Vibrio* into the shrimp body, shrimps were artificially wounded. To achieve this, a small incision approximately 5 mm long was made throughout

the cuticle and into the muscle of the third abdominal segment by pushing a surgical blade against the carapace until it penetrated. In another treatment, one pleopod of the shrimp was removed by a scissor. After wounding (or removing of the pleopod), shrimps were immediately immersion challenged with 10^8 CFU mL^{-1} of *V. campbellii*.

3.3.6. Decapsulation and challenge test in *Artemia*

The decapsulation method of Sorgeloos *et al.* (1986) was modified by Marques *et al.* (2004) to produce sterile *Artemia* nauplii. After hatching, separate groups of 30 nauplii were transferred to sterile 50 mL falcon tubes containing 30 mL of FASW and challenged with vibrios at 10^4 CFU mL^{-1}. At the start of the experiment the *Artemia* were fed once with 10^7 CFU mL^{-1} autoclaved LVS3 bacteria, an isolate that is known to enhance growth and survival of *Artemia* (Verschuere *et al.*, 1999). Each treatment was carried out in quadruplicate. All manipulations were performed under a laminar flow hood in order to maintain sterility of the cysts and nauplii. After treatment, the tubes were put back on the rotor and kept at 28^{o}C. The mortality was scored at 36 h and 48 h after exposure to the bacteria. In each test, the sterility of the feed and the control treatments (to which no live bacteria were added) were checked at the end of the challenge by taking 100 µl of the feed suspension or *Artemia* culture water and spreading it on fresh MA. The plates were incubated overnight at 28^{o}C. If a control tube was found to be contaminated, the data from the corresponding experiment were not considered and the experiment was repeated.

3.3.7. Statistical analysis: Values of shrimp mortality (%) were ArcSin-transformed to satisfy normal distribution. Differences between treatments were evaluated by performing analysis of variances (ANOVA) using statistical analysis software SPSS (version 13.0 for Windows).

3.4. Results

3.4.1. Injection challenge

3.4.1.1. Ammonium stress in juvenile shrimp

The toxicity of ammonium chloride was tested on juvenile shrimps. Shrimps (mean weight = 4.05 ± 0.33 g) were exposed to different concentrations of ammonium chloride (Table 3.1). The experiment was set up with two replications per treatment. Each treatment contains 10 shrimps (s = 10; n = 2). At 150 mgL^{-1} NH_4^+ (or higher), shrimp died within minutes. At 75 mgL^{-1} NH_4^+, they started to die after 6 h exposure. At 50 mgL^{-1} NH_4^+, mortality raised to 35% after 24 h exposure. After 12 h exposure to 50 mgL^{-1} NH_4^+, no mortality was noticed. Hence, the latter conditions were considered to be sub-lethal for juvenile shrimp.

Table 3.1. Cumulative percent mortality of juvenile *L. vannamei* at different time intervals after exposure to different concentrations of NH_4^+

NH_4^+ (mgL^{-1})	6 h	12 h	24 h	36 h	48 h
0 (control)	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a
20	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	5 ± 7^a
50	0 ± 0^a	0 ± 0^a	35 ± 7^b	35 ± 7^b	100 ± 0^b
75	10 ± 0^a	65 ± 7^b	100 ± 0^c	100 ± 0^c	100 ± 0^b
100	85 ± 21^b	85 ± 21^b	100 ± 0^c	100 ± 0^c	100 ± 0^b

Values within a column with different superscripts were significantly different ($p < 0.05$).

3.4.1.2. Injection challenge under continuous ammonium stress

Since shrimp appear to be more susceptible to pathogenic bacteria when exposed to ammonium stress (Alday-Sanz *et al.*, 2002; Liu and Chen, 2004), we applied injection challenge in animals which were continuously exposed to ammonium stress. Shrimp (mean weight = 5.36 ± 0.56 g), stressed with 50 mgL^{-1} NH_4^+ for 12 h, were transferred to seawater containing 20 mgL^{-1} NH_4^+ and were injected with *Vibrio* at the concentration of 10^4 or 10^6 CFU $shrimp^{-1}$ (s = 10; n = 2). At the end of the experiment, high mortality (75-100%) was

found in the ammonium treatments (with or without *Vibrio* challenge) (Table 3.2). However, low mortality (15-25%) was found in the treatments challenged with bacteria that were not exposed to ammonium stress. Under conditions of ammonium stress, a significant effect of bacterial challenge was found at 24, 36, and 96 hours post injection (hpi) ($p < 0.05$). There was only interaction (Table 3.2) between ammonium and *V. campbellii* on shrimp mortality at 48 hpi ($p < 0.05$). The presence of ammonium resulted in significantly higher mortality at 120 hpi but no challenge nor interaction effect was found. For *V. harveyi* E022, no interaction effects of bacteria and ammonium on shrimp mortality were found but a highly significant effect of ammonium on shrimp mortality was evident at 120 hpi ($p < 0.001$). At 96 hpi, a higher but non-significant mortality was found in treatments with *V. harveyi* E022 as compared to the control. With ammonium stress, a high dose (10^6 CFU shrimp^{-1}) of *V. campbellii* caused higher mortality compared to a low dose (10^4 CFU shrimp^{-1}) at all time points. In contrast, no difference in mortality was found in shrimp challenged with a high or low dose of *V. campbellii* in unstressed condition (Table 3.2). No difference in mortality was found in shrimps challenged with a high and low dose of *V. harveyi* E022 under stressed and unstressed conditions.

Table 3.2. Cumulative percent mortality of *L. vannamei* at different time intervals after challenge with *V. campbellii,* or *V. harveyi* E022 by injection under continuous ammonium stress

Treatments	**12 h**	**24 h**	**36 h**	**48 h**	**72 h**	**96 h**	**120 h**
Experiment 1							
Control (no ammonium)	0 ± 0^{a}	5 ± 7^{ab}	5 ± 7^{ab}	5 ± 7^{a}	5 ± 7^{a}	5 ± 7^{a}	10 ± 0^{a}
Control (ammonium)	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	5 ± 7^{a}	85 ± 7^{b}
VC (10^4, no ammonium)	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	10 ± 14^{ab}	15 ± 7^{a}
VC (10^4, ammonium)	0 ± 0^{a}	5 ± 7^{ab}	5 ± 7^{ab}	5 ± 7^{a}	15 ± 21^{a}	15 ± 21^{ab}	75 ± 7^{b}
VC (10^6, no ammonium)	0 ± 0^{a}	5 ± 7^{ab}	5 ± 7^{ab}	5 ± 7^{a}	5 ± 7^{a}	15 ± 7^{ab}	15 ± 7^{a}
VC (10^6, ammonium)	20 ± 14^{a}	25 ± 7^{b}	25 ± 7^{b}	30 ± 0^{b}	30 ± 0^{b}	50 ± 0^{b}	85 ± 21^{b}
Experiment 2							
Control (no ammonium)	0 ± 0^{a}	5 ± 7^{a}	5 ± 7^{a}	5 ± 7^{a}	5 ± 7^{a}	5 ± 7^{a}	10 ± 0^{a}
Control (ammonium)	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	5 ± 7^{a}	85 ± 7^{b}
E022 (10^4, no ammonium)	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	5 ± 7^{a}	15 ± 7^{a}
E022 (10^4, ammonium)	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	5 ± 7^{a}	10 ± 0^{a}	40 ± 14^{a}	100 ± 0^{b}
E022 (10^6, no ammonium)	5 ± 7^{a}	10 ± 0^{a}	10 ± 0^{a}	10 ± 0^{a}	10 ± 0^{a}	20 ± 14^{a}	25 ± 21^{a}
E022 (10^6, ammonium)	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	5 ± 7^{a}	5 ± 7^{a}	35 ± 21^{a}	95 ± 7^{b}
Two way ANOVA							
VC	NS	(*)	(*)	(**)	NS	(*)	NS
Ammonium	NS	NS	NS	(*)	NS	NS	(**)
VC x ammonium	NS	NS	NS	(*)	NS	NS	NS
E022	NS	NS	NS	NS	NS	NS	NS
Ammonium	NS	(*)	(*)	NS	NS	NS	(***)
E022 x ammonium	NS	NS	NS	NS	NS	NS	NS

VC = *Vibrio campbellii*; E022 = *Vibrio harveyi* E022. All values are means of two replicates. No mortality was observed at 6 hpi; NS = Not significant; (*): Significant at $p < 0.05$; (**): Significant at $p < 0.01$; (***): Significant at $p < 0.001$; Values within a column with different superscripts were significantly different ($p < 0.05$).

3.4.1.3. Injection challenge with discontinuous 2-step ammonium stress

Since high mortality was found in the control group with continuous ammonium stress (Table 3.2), a new experiment was set up starting with the same ammonium pretreatment regime (see part 3.4.1.2). However, at 48 hpi the water was replaced with fresh NH_4Cl-free seawater (s = 10; n = 3). High mortality (56-60%) was only found in the challenged groups (Table 3.3). There was a significant difference in mortality between the control and the *Vibrio* injection with water exchange ($p < 0.05$). No significant difference in mortality was found between the control and the challenged shrimp without water exchange, suggesting that shrimps are more sensitive to *V. campbellii* when stressed.

Table 3.3. Cumulative percent mortality of *L. vannamei* at different time intervals after challenge with *V. campbellii* by injection with 10^6 CFU shrimp^{-1} under discontinuous ammonium stress

Treatments	12 h	24 h	36 h	48 h	72 h	96 h	120 h
Control (WE)	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	3 ± 6^a	7 ± 12^a	10 ± 10^a
Control (NWE)	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	3 ± 6^a	10 ± 10^{ab}	20 ± 10^{ab}
VC (WE)	30 ± 0^b	43 ± 7^b	47 ± 6^b	47 ± 6^b	53 ± 15^b	57 ± 12^c	57 ± 26^b
VC (NWE)	27 ± 12^b	40 ± 10^b	43 ± 12^b	43 ± 12^b	50 ± 17^b	53 ± 21^{bc}	60 ± 12^b

WE = Water exchange, and hence discontinuous ammonium stress at 48 hpi; NWE = No water exchange, hence continuous ammonium stress; VC = *Vibrio campbellii*. No mortality was observed at 6 hpi. All values are means of three replicates (s = 10, n = 3). Values within a column with different superscripts were significantly different ($p < 0.05$).

3.4.1.4. Susceptibility of SPF shrimp (*L. vannamei*) under 2-step discontinuous ammonium stress to virulent *Vibrio* strain

In this experiment, shrimp (mean weight = 9.38 ± 0.54 g) were exposed to 50 mgL^{-1} NH_4^+ for 12 h. Afterward, the water was renewed with seawater containing 20 mgL^{-1} NH_4^+. Subsequently, the shrimp were injected with the *Vibrio* strains (10^6 CFU shrimp^{-1}). At 48 hpi, the water was exchanged and replaced with new NH_4Cl-free seawater. The results showed that among the five strains tested *V. campbellii* was the most virulent strain. In particular, at

48 hpi, *V. campbellii* caused significantly higher mortality than all other strains and only the groups challenged with *V. campbellii* were significant different from the control at 120 hpi ($p < 0.05$) (Table 3.4).

Table 3.4. Cumulative percent mortality of *L. vannamei* at different time intervals after challenge with vibrios by injection with 10^6 CFU shrimp^{-1} under discontinuous ammonium stress

Treatments	12 h	24 h	36 h	48 h	72 h	96 h	120 h
Control	0 ± 0^{a}	0 ± 0^{a}	3 ± 6^{a}	3 ± 6^{a}	7 ± 6^{a}	7 ± 6^{a}	13 ± 12^{a}
V. campbellii	10 ± 10^{a}	20 ± 10^{b}	20 ± 10^{a}	30 ± 0^{b}	33 ± 6^{b}	37 ± 6^{b}	47 ± 6^{b}
V. harveyi 642	10 ± 10^{a}	10 ± 10^{ab}	13 ± 6^{a}	13 ± 6^{a}	13 ± 6^{a}	20 ± 0^{ab}	23 ± 6^{ab}
V. harveyi E2	3 ± 6^{a}	3 ± 6^{ab}	3 ± 6^{a}	7 ± 6^{a}	7 ± 6^{a}	13 ± 12^{a}	23 ± 12^{ab}
V. harveyi E022	3 ± 6^{a}	7 ± 6^{ab}	10 ± 10^{a}	13 ± 6^{a}	20 ± 10^{ab}	23 ± 6^{ab}	27 ± 6^{ab}
V. penaeicida	7 ± 6^{a}	7 ± 6^{ab}	10 ± 0^{a}	13 ± 6^{a}	20 ± 0^{ab}	23 ± 6^{ab}	30 ± 10^{ab}

No mortality was found at 6 hpi; All values are means of three replicates. Values within a column with different superscripts were significantly different ($p < 0.05$).

3.4.2. Immersion challenge in the absence of ammonium stress

Shrimps (mean weight = 0.04 ± 0.01 g) were challenged with *V. campbellii* (experiment 1 and 2) (s = 7; n = 3) or with *V. harveyi* 642 (experiment 3; shrimp mean weight = 2.64 ± 0.61 g) (s = 10; n = 3) at different concentrations (10^3, 10^5 and 10^7 CFU mL^{-1}). During the challenge periods, shrimp were fed twice daily with *Artemia* nauplii (experiment 1, 2) or artifical feed (experiment 3). Very low mortalities (0-14%) were observed in all experiments at 120 h post challenge. There was no significant difference in mortality between the treatments ($p > 0.05$) (results not shown). Generally, *V. harveyi* 642 and *V. campbellii* did not cause any shrimp mortality in the absence of ammonium stress.

3.4.3. Immersion challenge with starvation

Shrimp (mean weight = 0.06 ± 0.01 g) were starved for 1, 2, 3, 4, or 5 days and subsequently challenged with *V. campbellii* and *V. harveyi* E2 at 10^7 CFU mL^{-1} (s = 10; n = 2). In the

group previously starved for 4 or 5 days, shrimp started to die at 24 h after exposure to the bacteria. At the end of the experiment, high mortalities (65-95%) were prevalent in all shrimp groups challenged with *V. campbellii* and *V. harveyi* E2. Since there was also a high mortality in the control (75-95%), no significant differences in mortality were found between the control and the *Vibrio* challenge groups. The same results were obtained at the end of the experiments in which the shrimp were starved for 1, 2, 3, 4 or 5 days before challenge (results not shown).

3.4.4. Immersion challenge with salinity stress

To verify whether the mortality was enhanced by salinity stress, the following four experiments were performed in duplicate (s = 10; n = 2): shrimps (mean weight = 0.06 ± 0.01 g) were transferred to lower salinity (20 or 10 gL^{-1}) for 30 min (experiment 1, 2), followed by an immersion challenge with *V. harveyi* 642 and *V. campbellii*, in the other treatments (experiments 3 and 4), shrimps (mean weight = 0.09 ± 0.01 g) were continuously stressed at 5 and 10 gL^{-1} salinity and challenged with the above mentioned bacterial strains. Very low mortality was found in all experiments except for experiment 3 (35% mortality in the treatment with *V. harveyi* 642). However, the mortality in the control group exposed to a salinity of 5 gL^{-1} was also high (25%). There was no significant difference in mortality between *Vibrio*-challenged and control groups in all experiments (results not shown).

3.4.5. Ammonium stress in postlarvae

In preliminary experiment (s = 10; n = 2), it was established that 10% mortality occurred after 12 h exposure to 50 mgL^{-1} NH_4^+ while no mortality was observed at 40 mgL^{-1}. The test appeared reproducible (Table 3.5), and 12 h exposure to 40 mgL^{-1} NH_4^+ was considered as sub-lethal for postlarvae.

Table 3.5. Cumulative percent mortality of postlarvae *L. vannamei* at different time intervals after exposure to different concentration of NH_4^+

NH_4^+ (mgL^{-1})	6 h	12 h	24 h	36 h	48 h
0 (control)	0 ± 0	0 ± 0[a]	0 ± 0[a]	0 ± 0[a]	0 ± 0[a]
30	0 ± 0	0 ± 0[a]	5 ± 7[a]	10 ± 0[a]	20 ± 7[b]
40	0 ± 0	0 ± 0[a]	15 ± 7[ab]	25 ± 7[a]	35 ± 7[b]
50	0 ± 0	10 ± 0[a]	40 ± 0[b]	75 ± 7[b]	100 ± 0[c]

Values are means of two replicates. Values within a column with different superscripts were significantly different ($p < 0.05$).

3.4.6. Immersion challenge of postlarvae under ammonium stress and different feed sources

3.4.6.1. Immersion challenge under discontinuous ammonium stress

Shrimps (mean weight = 1.42 ± 0.27 g) were exposed to 20 mgL^{-1} NH_4^+ for 24 h. The water was renewed with fresh NH_4Cl-free seawater (s = 10; n = 3). Subsequently, the shrimps were challenged with bacteria at 10^7 $CFUmL^{-1}$. No mortality was found up to 36 h post immersion. At the end of the experiment, very low mortalities (3-17%) were noticed. 17% mortality was found in the *V. harveyi* E2 challenge group (Table 3.6). However, no significant difference in mortality was found between the control and the *Vibrio* challenge groups.

Table 3.6. Cumulative percent mortality of *L. vannamei* at different time intervals after immersion challenge with *V. campbellii*, *V. harveyi* 642, and *V. harveyi* E2 under discontinuous ammonium stress

Treatments	48 h	72 h	96 h	120 h
Control	0 ± 0[a]	3 ± 6[a]	3 ± 7[a]	7 ± 7[a]
V. campbellii	0 ± 0[a]	0 ± 0[a]	3 ± 7[a]	3 ± 7[a]
V. harveyi E2	3 ± 6[a]	7 ± 6[a]	10 ± 10[a]	17 ± 6[a]
V. harveyi 642	0 ± 0[a]	3 ± 6[a]	3 ± 7[a]	7 ± 6[a]

All values are means of three replicates per experiment. No moratlity was observed at 6, 12, 24, and 36 h post-chalenge. Values within a column with different superscripts were significantly different ($p < 0.05$).

3.4.6.2. Immersion challenge under continuous ammonium stress

Three experiments were set up with continuous ammonium stress. Shrimp were exposed to 20 mgL^{-1} NH_4^+ for 24 h. The water was replaced with seawater containing 20 mgL^{-1} NH_4^+. Subsequently, they were challenged with the vibrios. There was no significant difference in mortality between any of the *Vibrio*-challenged and control groups (results not shown). In two other experiments, shrimp were stressed with higher concentrations of NH_4^+ (50 mgL^{-1}). After 12 h exposure to NH_4Cl, the water was exchanged and replaced with new seawater containing 20 mgL^{-1} NH_4^+. Subsequently, shrimp were challenged by immersion but no mortality differences were recorded.

3.4.6.3. Immersion challenge with extra addition of bacteria through the feed

During the challenge periods, shrimps were fed with many different feed sources, i.e. artificial feed, *Artemia* nauplii, *Artemia* bio-encapsulated with *V. campbellii* and a piece of shrimp muscle into which *V. campbellii* was injected. However, none of these treatments resulted in increased mortalities upon challenge (results not shown).

3.4.6.4. Immersion challenge with removing of the pleopod and wounding

After wounding (or removing of the pleopod), shrimps (MBW = 2.41 ± 0.65 g) were immediately challenged by immersion with 10^8 CFU mL^{-1} of *V. campbellii* (s = 5, n = 4). Bacteria were added once to the tanks and remained there during the experimental period. No mortality was observed during 5 days experiment (results not shown).

3.4.7. Bacterial density in water and surviving shrimp

Shrimps stressed in 20 mgL^{-1} NH_4^+ for 24 h. Subsequently, they were transferred to the tank with fresh seawater containing 20 mgL^{-1} NH_4^+ and challenged with rifamycin-resistant

V. campbellii at 10^7 CFU mL^{-1}. The water was collected daily and plated on MAR. Bacterial density was high in the first 2 days (10^5-10^7 CFUmL^{-1}). In the following days, the bacterial density dropped and reached 10^2 CFU mL^{-1} at the end of experiment. 9.7 x 10^3 CFU $shrimp^{-1}$ were detected in surviving shrimp at 120 h post-challenge.

3.4.8. Virulence of five *Vibrio* strains towards *Artemia* nauplii

The virulence of five *Vibrio* strains was also tested in gnotobiotic *Artemia*. The lowest survival was found in *Artemia* challenged with *V. harveyi* E2 (40-48%). *V. penaeicida* caused very low mortality in *Artemia* up to 48 h after challenge (Table 3.7). There was a significant difference in mortality between *Artemia* challenged with *V. harveyi* E2, *V. harveyi* E022, *V. campbellii* and the control ($p < 0.05$). *V. harveyi* E2 appeared to be the most virulent strain.

Table 3.7. Percentage survival of *Artemia* after 48 h challenge with *V. harveyi* and *V. campbellii*

Treatments	Experiment 1	Experiment 2
Control	93 ± 3^{c}	94 ± 3^{d}
V. harveyi E2	48 ± 8^{a}	41 ± 5^{a}
V. harveyi E022	64 ± 7^{ab}	68 ± 9^{b}
V. campbellii	64 ± 6^{ab}	70 ± 5^{bc}
V. harveyi 642	73 ± 3^{b}	80 ± 3^{c}
V. penaeicida	88 ± 7^{c}	93 ± 3^{d}

All values are means of four replicates. Values within a column with different superscripts were significantly different ($p < 0.05$).

3.5. Discussion

Many challenge tests have been described in literature as tools to determine the causative agents or to study the pathogenesis of vibriosis in shrimp. The challenge tests considered many different important variables, e.g. shrimp species, strains and age, bacterial strains, infection doses, challenge routes, stress conditions. However, the experimental animals were non-SPF nor from a specific genetic stock. As many factors can determine the outcome of a challenge, it is important to reduce random environmental interference. The use of SPF shrimp can be paramount in that objective, reducing the possibility that the uncontrolled health status of the shrimp is interfering with the challenge outcome. In this study, we aimed to develop an appropriate protocol for *Vibrio* challenge in SPF shrimp Kona strain *L. vannamei*, facilitating a more standardized study on vibriosis.

Since SPF shrimp appeared to resist *Vibrio* challenge, we combined the injection challenge with ammonium stress. A significant increase in *Vibrio*-induced mortality was observed in the ammonium stressed groups, revealing that ammonium stress enhanced the susceptibility of *L. vannamei* to *V. campbellii* (Table 3.2). Liu and Chen (2004) claimed that ammonia increased the susceptibility of *L. vannamei* to *V. alginolyticus* by a reduction in the immune response as a result of decreased phagocytic activity and clearance efficiency of *V. alginolyticus*. Alday-Sanz *et al.* (2002) observed that shrimp which were exposed to ammonia prior to the immersion challenge, showed more frequent and earlier pathological changes than shrimp exposed to the bacteria alone. In this study, high mortality occurred between 12 and 48 h after injection with 10^6 CFU shrimp^{-1} *V. campbellii*, similar to the observations of Nash *et al.* (1992). They reported that shrimp injected with bacteria are weak in the first two days but can recover within 3-5 days. The injection dose depends on the virulence of bacterial strains, the species, and the age of experimental animals.

Since injection challenge showed positive results, immersion challenge was also verified. However, water concentrations of 10^7 CFU mL^{-1} *V. campbellii* did not result in increased mortalities. As *Vibrio* spp. are known to be opportunistic pathogen, stressors such as starvation, salinity shocks, ammonium exposure, wounding or physical damage can be an appropriate stressors since these factors can be referred as a primary risk in the development of vibriosis. Brock and Lightner (1990) stated that infection of shrimp with *Vibrio* species is associated with injury, stress or disease associated with other pathogens.

Immersion challenge in starvation condition did not show any significant effect since most shrimp died before the end of the experiments. Shrimp displayed cannibalism after a few days of starvation, especially when kept in small tanks and after several days of starvation. Liu and Chen (2004) documented that the susceptibility of *L. vannamei* to *V. alginolyticus* correlated well with reductions in immune parameters and clearance efficiency when the shrimp were transferred from high to low salinity. In our study, very low mortalities were observed when shrimp were challenged with continuous and discontinuous salinity stress: i.e. even when shrimp were transferred from 35 to 5gL^{-1} salinity no enhancement was noticed in susceptibility to *V. campbellii*, *V. harveyi* E2 nor *V. harveyi* 642. A difference between our results and previous findings can be found in the age of the animals (older animals may better resist to disease), the shrimp species, or the SPF status of the shrimp. We were using SPF shrimp which might better resist to a *Vibrio* challenge. Horowitz and Horowitz (2001) also postulated that absence of primary infection, physical damage or stress might result in an enhanced resistance against vibrios.

Our immersion challenges were performed with different ammonium regimes. Shrimp challenged under discontinuous or continuous ammonium stress did not show any increased mortalities. High bacterial density in the water was found in the first two days of immersion

challenge but by the end of the experiment these levels had dropped significantly. Similar observations were made by Rengpipat *et al.* (2000) who documented bacterial density drops from 10^7 CFU mL^{-1} to 10^3 CFU mL^{-1} within 4 days after adding *V. harveyi* to the culture tanks. Apparently, Bacteria can be eliminated quickly by the shrimp's haemocytes, i.e. the concentration of live bacteria in the haemolymph can decrease by 97% from 5 min to 2 h after injection (van de Braak *et al.*, 2002b).

In our study, low bacterial densities were found in the shrimp muscle at the end of the immersion challenge. This proves that bacteria that penetrate into the shrimp body via the gills, the mouth, wounds, etc. can be eliminated by the shrimp. As reported by Saulnier *et al.* (2000b), vibrios may act as opportunistic agents in secondary infections. The strains have more chance to overwhelm the immune system and become virulent when the environment is sub-optimal in nature, an outbreak of shrimp vibriosis often happens in combination with stress factors (pH, salinity, temperature, ammonia,…) or following a primary infection. It is possible that the lacking of primary infection in our study was the cause of no mortality when exposing the animals to an immersion challenge. Selvin and Lipton (2003) concluded that shrimp weakened by white spot syndrome virus (WSSV) would succumb to a secondary infection by *V. alginolyticus*.

In conclusion, SPF shrimp are not susceptible to *Vibrio* challenge, neither by injection nor immersion in the absence of stress. Only when exposed to ammonium stress, SPF shrimp became very susceptible to vibrios injection. Under the described experimental conditions SPF shrimp become susceptible to infection by opportunistic pathogenic *Vibrio* through injection of 10^6 CFU $shrimp^{-1}$ only after an initial ammonium stress of 50 mgL^{-1} during 12 h, followed by a continuous ammonium exposure to 20 mgL^{-1}.

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CHAPTER

Virulence of luminescent and non-luminescent isogenic vibrios towards gnotobiotic *Artemia franciscana* larvae and specific pathogen-free (SPF) *Litopenaeus vannamei* shrimp

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L.H. Phuoc, P. Sorgeloos, P. Bossier

(Submitted)

4.1. Abstract

This study was conducted to test the virulence of luminescent (L) and non-luminescent (NL) isogenic strains of *Vibrio campbellii* LMG21363, *Vibrio harveyi* BB120 (wild type) and quorum-sensing mutant strains derived from the wild type such as *Vibrio harveyi* BB152, BB170, MM30, and BB886. The NL strains were derived from the rifampicin-resistant luminescent strains. The latter were prepared by sub-culturing bacteria with increasing rifampicin concentrations in Marine Broth. The NL strains could be obtained by culturing luminescent strains in the dark under static condition. The autoinducers 1, 2, 3 (AI1, AI2, AI3) were detected in L as well as NL cultures of *V. campbellii* and *V. harveyi* BB120. The virulence of the L and NL strains was tested in gnotobiotic *Artemia franciscana* larvae challenged with 10^4 CFU mL^{-1} of bacteria. All luminescent isogenic tested strains showed higher virulence compared to NL ones. The virulence of L and NL *V. campbellii* and *V. harveyi* BB120 was also tested in specific pathogen-free (SPF) juvenile shrimp upon intramuscular injection with 10^6 CFU of bacteria. In contrast with *Artemia*, there was no significant difference in mortality between the groups challenged with L and NL strains ($p > 0.05$). The relevance of the NL state is discussed.

4.2. Introduction

Vibriosis is considered as one of the most important infectious diseases in the hatchery as well as in grow-out shrimp ponds. *Vibrios* are commonly present in various marine and brackish habitats. The rapid increase in shrimp culture area is facilitating disease outbreak. *V. harveyi* is a luminescent bacterium most notably associated with disease in cultured shrimp worldwide (Liu and Lee, 1999). Mass mortalities of *Penaeus monodon* and larvae or juveniles associated with luminous vibrios have been observed in hatcheries or farms in Indonesia (Sunaryanto and Mariam, 1986), Philippines (Lavilla-Pitogo, 1990), India (Karunasagar *et al.*, 1994), Taiwan (Liu *et al.*, 1996; Song and Lee, 1993), Thailand (Jiravanichpaisal *et al.*, 1994), Australia (Pizzutto and Hirst, 1995) and China (Vandenberghe *et al.*, 1998).

It is known that bioluminescence is regulated by quorum-sensing, a process that allows bacteria to communicate via the secretion of chemical signaling molecules, called autoinducers (Miller and Bassler, 2001). The light intensity emitted by luminous bacteria depends on bacterial density. Indirect evidence suggests that toxin production is regulated by quorum-sensing signal molecules (Manefield *et al.*, 2000). Quorum-sensing in *V. harveyi* utilizes three cell signaling systems that function in parallel to regulate positively bioluminescence (Bassler *et al.*, 1993, 1994; Henke and Bassler, 2004a), metalloprotease (Mok *et al.*, 2003), siderophore, and exopolysaccharide production (Lilly and Bassler, 2000) and to regulate negatively type III secretion (Henke and Bassler, 2004b) in a cell population density-dependent manner. The LuxM/N system relies on N-(3-hydroxybutanoyl)-L-homoserine lactone (3-hydroxy-C4-HSL), which is synthesized by LuxM (Cao and Meighen, 1989; Bassler *et al.*, 1993). The LuxS/PQ system utilizes the signal molecule 3A-methyl-5,6-dihydro-furo(2,3-D)(1,3,2) dioxaborole- 2,2,6,6A-tetraol (termed AI-2). The unborated AI-2

precursor is synthesized by LuxS (Surette *et al.*, 1999; Schauder *et al.*, 2001; Chen *et al.*, 2002). The CqsA/S system utilizes the signal molecule CAI-1, which is dependent on CqsA for its synthesis. CAI-1 is (S)-3- hydroxytridecan-4-one, a new type of bacterial autoinducer (Higgins *et al.*, 2007). These three signal molecules are distinct from each other and work synergistically in gene regulation (Milton, 2006).

Phenotypic variation has been defined as a process of reversible, high-frequency phenotypic switching that is mediated by DNA mutations, reorganization or modification (Saunders *et al.*, 2003). Phenotypic variation is used by several bacterial species to generate population diversity that increases bacterial fitness under certain circumstances and is important in niche adaptation, including immune evasion. Although phenotypic variation, or antigenic variation, has primarily been associated with host-pathogen interactions, a number of reports describe phenotypic variation in a broader context. These reports show that phenotypic variation is also involved in the production of exo-enzymes, production of secondary metabolites and affects colonization behaviour and biocontrol activity of rhizosphere bacteria (Chabeaud *et al.*, 2001; Chancey *et al.*, 2002; van den Broek *et al.*, 2003). Phenotypic variation was found in *Comamonas testosteroni* (Bossier and Verstraete, 1996), *Pseudomonas aeruginosa* (Drenkard and Ausubel, 2002), *Escherichia coli* (Kussell *et al.*, 2005), *Candida parapsilosis* (Laffey, 2005), and *Vibrio anguillarum* (Fujiwara-Nagata and Eguchi, 2003).

The aim of this study is to compare the virulence of luminescent and non-luminescent isogenic *Vibrio* towards gnotobiotic *Artemia* and specific pathogen-free (SPF) juveniles shrimp *Litopenaeus vannamei*.

4.3. Materials and Methods

4.3.1. Preparation of bacterial stocks[1]

The bacterial strains (Table 4.1), previously stored in 20% glycerol at -80^{o}C, were aseptically inoculated in Marine Agar (MA). The plates were incubated for 24 h at 28^{o}C. Single colonies were subsequently transferred and grown in Marine Broth (MB) 2216 (Difco Laboratories, USA) by incubation overnight in the shaker (28^{o}C, 150 rpm). The culture was then transferred to centrifugation tubes and centrifuged at 2200 g for 15 min. The supernatant was discarded and pellets were washed twice and finally re-suspended in filtered autoclaved seawater (FASW). The bacterial densities were determined spectrophotometrically at an optical density (OD) of 550 nm assuming that an OD of 1.0 corresponds to 1.2 x 10^{9} cells mL^{-1}, the McFarland standard (Bio Merieux, France).

[1] This section has been described in chapter 3 (section 3.3.2)

Table 4.1. Bacterial strains used in this study

Strain	Relevant features	References
LVS3	Isolate that enhances growth and survival of *Artemia*	Verschuere *et al*., 1999
Vibrio campbellii LMG21363	Pathogen for shrimp and *Artemia*, luminescent	Soto-Rodriguez *et al*., 2003
V. campbellii LMG21363 (RR-L)	Rifampicin resistant, luminescent	This study
V. campbellii LMG21363 (RR-NL)	Rifampicin resistant, non-luminescent	This study
Vibrio harveyi BB120	Wild type, luminescent	Bassler *et al*., 1997
V. harveyi BB120 (RR-L)	Rifampicin resistant, luminescent	This study
V. harveyi BB120 (RR-NL)	Rifampicin resistant, non-luminescent	This study
V. harveyi BB152	Mutation in *LuxM* (AI-1 synthase)	Bassler *et al*., 1994
V. harveyi BB152 (RR-L)	Rifampicin resistant, luminescent	This study
V. harveyi BB152 (RR-NL)	Rifampicin resistant, non-luminescent	This study
V. harveyi BB170	Mutation in *LuxN* (AI-1 receptor)	Bassler *et al*., 1994
V. harveyi BB170 (RR-L)	Rifampicin resistant, luminescent	This study
V. harveyi BB170 (RR-NL)	Rifampicin resistant, non-luminescent	This study
V. harveyi MM30	Mutation in *LuxS* (AI-2 synthase)	Surette *et al*.,1999
V. harveyi MM30 (RR-L)	Rifampicin resistant, luminescent	This study
V. harveyi MM30 (RR-NL)	Rifampicin resistant, non-luminescent	This study
V. harveyi BB886	Mutation in *LuxP* (AI-2 receptor)	Bassler *et al*.,1994
V. harveyi BB886 (RR-L)	Rifampicin resistant, luminescent	This study
V. harveyi BB886 (RR-NL)	Rifampicin resistant, non-luminescent	This study
V. harveyi JAF375	Mutation in *luxN*: Cm^R*luxQ* :Kan^R, luminescent	Freeman and Bassler, 1999a
V. harveyi JMH597	Mutation in *luxN*: Tn5 *cqsS*: Cm^R, luminescent	Henke and Bassler, 2004b
V. harveyi JMH612	Mutation in *luxPQ*: Tn5 *cqsS*: Cm^R, Luminescent	Henke and Bassler (2004b)

RR = Rifampicin resistant; L = Luminescent; NL = Non-luminescent

4.3.2. Qualitative detection of autoinducers and measurement of luminescence

Cell-free culture fluids of the strains collected in stationary phase were tested for the presence of *V. harveyi* BB120 and *V. campbellii* autoinducers by the assay developed by Defoirdt *et al.* (2007) using the double mutants JAF375 (sensor HAI-1$^-$, sensor AI-2$^-$, sensor CAI-1$^+$), JMH597 (sensor HAI-1$^-$, sensor AI-2$^+$, sensor CAI-1$^-$) and JMH612 (sensor HAI-1$^+$, sensor AI-2$^-$, sensor CAI-1$^-$) as reporters for CAI-1, AI-2 and HAI-1, respectively. The reporter strains were grown at 28^{o}C with shaking (100 min^{-1}) in MB to an OD600 of approximately 1 and diluted 1:5000 in fresh medium. Of the diluted reporter cultures, 50 µL was mixed with 50 µL of cell-free culture fluids in 3-mL test tubes. Cell-free culture fluids of *V. harveyi* BB120 were used as a positive control; fresh medium was used as a negative control. The test tubes were incubated at 28^{o}C and luminescence was measured every hour with a Lumac Biocounter M2500 luminometer (Lumac b.v) until the luminescence with fresh medium was minimal (after 3-4 h).

4.3.3. Rifampicin-resistant strains[2]

Rifampicin-resistant (RR) *V. campbellii* and *V. harveyi* BB120 was produced as follows: a colony picked from MA plates was cultured for 24 h in 25 mL MB in a 100 mL erlenmeyer. After incubation, 50 µL of the culture was taken by micropipette and transferred to 25 mL of fresh MB containing 0.5 mgL^{-1} rifampicin (R3501, Sigma-aldrich). The culture was incubated for 24 h at 28^{o}C. The growth of bacteria was monitored by the turbidity of the culture. In the following days, further sub-cultures were made in MB, increasing the rifampicin concentration gradually from 1, 2, 4, 8, 16, 32, 64 to finally 100 mgL^{-1}. When bacteria cultures were growing well in the final concentration of rifampicin (100 mgL^{-1}), they were inoculated on MA plates containing 100 mgL^{-1} rifampicin (MAR) for obtaining single colonies. The stock was stored in 20% glycerol at -80°C for long term storage.

[2] This section has been described in chapter 3 (section 3.3.3)

4.3.4. Production of non-luminescent bacteria and staining of luminescent and non-luminescent colonies by Congo red

The NL bacteria were produced by culturing rifampicin-resistant luminescent strains in static condition in an erlenmeyer. Therefore, the stock was plated on MA. The plates were incubated for 24 h at 28^{o}C. One single colony was picked up and inoculated in a 500 mL erlenmeyer containing 50 mL MB. The culture was kept in static condition for 3 days or longer depending on the bacterial strain. Subsequently, the broth was discarded and the cells attached to the bottom of erlenmeyer were collected by adding 5 mL of FASW and shaking the erlenmeyer. These cells were plated on MA and incubated for 24 h at 28^{o}C to check for the presence of NL colonies. Totally dark colonies were picked up and inoculated on MAR to verify for this phenotype, ensuring that dark colonies were not accidental contaminants. In case no NL colonies were detected, fresh MB was added to the erlenmeyer and the above described procedure was repeated until a NL strain was found. For staining of L and NL colonies, the strains were inoculated in Marine Agar with Congo red (MACR). The medium was prepared with 37.4 g MB, 50 g sucrose, 15 g agar and 0.8 g Congo red L^{-1}. Congo red was prepared as a stock solution, autoclaved at 121^{o}C for 15 min and then added to the culture medium when it had cooled to 55^{o}C. Plates inoculated with L or NL strains were incubated for 24 h at 28^{o}C. Strains that produce extracellular slime develop black colonies (Freeman *et al.*, 1989).

4.3.5. Restoration of luminescence

Culturing of NL-RR strains in the supernatant of rifampicin-sensitive *V. harveyi* BB120:

It was tried to restore the luminescence of NL strains by culturing the NL-RR strains in filtered sterilized supernatant from the culture of rifampicin-sensitive *V. harveyi* BB120 (wild type). The samples were collected at 30 min, 1, 2, 3, 6, 12, 24 h and plated on MAR. The plates were incubated for 24 h at 28^{o}C for checking of L-RR colonies.

Co-culture of L-RS and NL-RR strains: The NL-RR strains were co-cultured with the original isogenic L-RS strains in a 250 mL erlenmeyer containing 50 mL MB. The samples were collected at 3, 6, 9, 12, 24, 48 h after inoculation and plated on MAR for detecting of L-RR colonies.

Passing of NL-RR strains through the hosts: It was tried to restore the luminescence of NL-RR strains by passing the strains through *Artemia* and juvenile *L. vannamei* shrimp. *Artemia* were challenged by immersion with 10^4 CFU mL^{-1} of NL-RR strains. Twenty-four hours after the challenge, infected *Artemia* were collected and homogenized by the stomacher to re-isolate strains on MAR. The NL strains were re-isolated and passed through the host for 3 times. Every passage cycle, the strains were plated on MAR for detecting L colonies. The same procedure was applied in *L. vannamei* shrimp but shrimp were challenged with NL-RR strains by injection. More details about the challenge is described further in the in the Materials and Methods section.

Restoration of luminescence by chemicals: Two chemical products, H_2O_2 and S-nitroso-N-acetylpenicillamine (SNAP), were used in this study. The NL strains were prepared in FASW; the initial bacterial concentration was 5 x 10^6 CFU mL^{-1} and different concentrations of chemical products were added: 20, 30, 40, 50, 60, 70, 80, 90 and 100 mgL^{-1} of H_2O_2 or 0.01, 0.1, 0.5, 1, 2, 3, 4, 5 , 10, 20, 30, 40, 50 mgL^{-1} of SNAP. The samples were collected at 10, 20, 30 min, 1, 2, 3, 4, 5, 6, 12, 24 h and after plating on MAR were checked for L colonies.

4.3.6. Decapsulation and challenge test in *Artemia*[3]

All experiments were performed with high quality hatching cysts of *Artemia franciscana* (EG® Type, batch 6940, INVE Aquaculture, Baasrode, Belgium). The modified decapsulation

[3] This section has been described in chapter 3 (section 3.3.6)

method of Sorgeloos *et al.* (1986) was chosen in order to obtain sterile *Artemia* nauplii. The protocol used was previously optimized by Marques *et al.* (2004). After hatching, groups of 30 nauplii (instar II) were transferred to sterile 50 mL falcon tubes containing 30 mL of FASW. They were challenged with vibrios at 10^4 CFU mL^{-1}. *Artemia* were fed once with autoclaved LVS3 (equivalent to 10^7 CFU mL^{-1}) at the beginning of the experiment. The challenge protocol was adapted from the protocol described by Defoirdt *et al.* (2005). Each treatment was carried out in quadruplicate. All manipulations were performed under a laminar flow hood in order to maintain sterility of the cysts and nauplii. Finally, the tubes were put back on the rotor and kept at 28°C. The mortality was scored at 36 h and 48 h after exposure to bacteria. In each test, the sterility of the feed and the control treatments (to which no live bacteria were added) were checked at the end of the challenge by taking 100 µl of the feed suspension or *Artemia* culture water and spreading it on fresh MA. The plates were incubated for 48 h at 28°C. If a control tube was found to be contaminated, the data from the corresponding experiment were not considered and the experiment was repeated.

4.3.7. Specific pathogen-free (SPF) shrimp[4]

SPF *L. vannamei* of the Kona-Hawaii (USA) strain (Wyban *et al.*, 1992) were used in this study. Shrimp were imported from Molokai Sea Farms, Hawaii (USA). Animals were certified to be free of WSSV, IHHNV, MBV, HPV, Taura syndrome virus (TSV), Yellow head virus (YHV) and Gill-associated virus (GAV), as well as other pathogens (fungi, protozoa) as verified by PCR and histopathology. Batches of shrimp arrived at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, as postlarvae (PL_{8-12}). They were kept in a recirculation system at a water temperature of 28 - 29°C, 34 gL^{-1} salinity, and pH of 7.8 - 8.1. During the first week, the animals were fed twice daily with *Artemia* nauplii. After one week their diet was shifted to A2 monodon high performance shrimp feed

[4] This section has been described in chapter 3 (section 3.3.1)

(2.2 mm fraction, INVE Aquaculture NV, Dendermonde, Belgium). The feeding ratio was 2.5% of the mean body weight (MBW) per day. Before starting the experiments, shrimp were transferred to the challenge room where they were acclimated for 2 days in PVC tanks.

4.3.8. Injection challenge in SPF shrimp

Challenge tests were conducted in quadruplicate with ten shrimps per replicate. After acclimation, shrimp were distributed to PVC tanks in which air stons and air tubes were installed. Those experimental materials were disinfected with sodium hypochlorite. The tanks were covered to prevent the shrimp from jumping out and to prevent cross-contamination by aerosols. Salinity was maintained at 35 gL^{-1}, pH = 7.9 to 8.2 and temperature at 28 ± 1°C during the experimental periods. Shrimp were shocked with ammonium chloride at the concentration of 50 mgL^{-1} NH_4^+. The water was refreshed after 12 h of ammonium stress and replaced with new seawater containing 20 mgL^{-1} NH_4^+ (Phuoc *et al.*, submitted). Subsequently the shrimp were injected with 10^6 CFU of *V. campbellii* or 5 x 10^6 CFU of *V. harveyi* BB120. The bacteria were inoculated into shrimps by intramuscular injection between the second and third abdominal segment. Control shrimps were injected with an equal volume of FASW. After injection, each group was held in a separate 20 L plastic tank containing 5 L seawater (35 gL^{-1} salinity) at 28^oC with aeration. Shrimp were fed with formulated shrimp feed twice a day up to 5 days. Forty-eight hours post injection, the water was refreshed and replaced with new NH_4Cl-free seawater.

4.3.9. Statistical analysis

Values of shrimp mortality (%) were ArcSin-transformed to satisfy the requirement for a normal distribution. Differences between treatments were evaluated by performing analysis of variances (ANOVA) using statistical analysis software SPSS (version 13.0 for Windows).

4.4. Results

4.4.1. The luminescence and quorum-sensing molecules of luminescent and non-luminescent strains

The luminescence of NL strains was very low compared to that of L strains (Table 4.2). The NL strains derived from *V. campbellii* and *V. harveyi* BB120 were completely dark since the obtained values was not significantly different from the control. The quorum-sensing molecules (AI1, AI2, AI3) were detected not only in L but also in NL *V. harveyi* BB120 as well as in NL *V. campbellii*. The NL *V. campbellii* colonies seem to be more mucoid and less round shaped on MA compared to the L strains. The two types of colonies stained differently *in situ* with Congo red. The NL colonies were black (Figure 4.1a) while L colonies were red (Figure 4.1b) on Marine agar with Congo red (MACR). The L and NL of *V. harveyi* BB120 and quorum-sensing mutant strains did not show any difference in color on MACR.

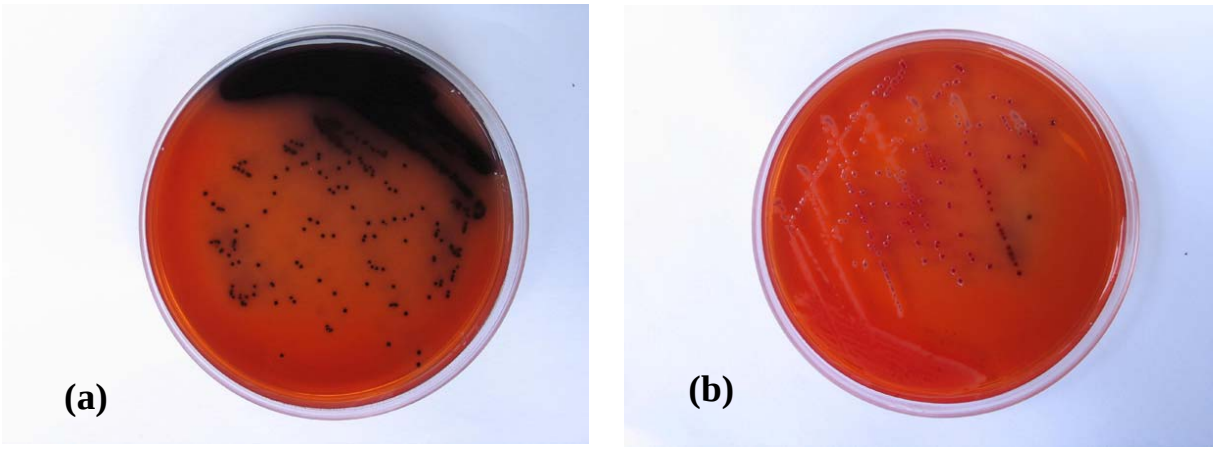

Figure 4.1. Non-luminescent (a) and luminescent (b) colonies of *V. campbellii* on Marine Agar with Congo red

Table 4.2. Luminescence and quorum-sensing molecules of luminescent and non-luminescent *V. campbellii* and *V. harveyi* BB120 strains

Parameters	*V. harveyi* BB120		*V. campbellii*		Control
	RR-L	RR-NL	RR-L	RR-NL	
Lu	5.6×10^7	18	2.5×10^7	10.3	8.7
AI1	+	+	+	+	-
AI2	+	+	+	+	-
AI3	+	+	+	+	-

Lu = Luminescence; NL = Non-luminescent ; RR = rifampicin resistant; AI = Autoinducer.

4.4.2. Relative percentage mortality of *Artemia* after 36 h challenge with luminescent and non-luminescent *V. campbellii*

The relative percentage mortality (RPM) was calculated by the following formula:

$$\text{RPM (\%)} = \frac{\text{Percentage mortality caused by non-luminescent isogenic strain}}{\text{Percentage mortality caused by luminescent isogenic strain}} \times 100$$

RPM caused by *V. campbellii* was 66 and 70% in experiment 1 and 2 respectively, meaning that luminescent *V. campbellii* caused higher mortality compared to the non-luminescent isogenic strain.

4.4.3. Relative percentage mortality of *Artemia* after 48 h challenge with luminescent and non-luminescent *Vibrio harveyi* BB120 and quorum-sensing mutant strains

In two experiments, all luminescent isogenic strains showed higher virulence compared to NL ones (Table 4.3). The RPM of the *Artemia* challenge with the wild type *V. harveyi* BB120 and quorum-sensing mutant strains ranged from 48 to 88% in experiment 1 and 53 to 81% in

experiment 2. The mortality in the control treatment was 18 and 13% in experiment 1 and 2 respectively.

Table 4.3. Relative percentage mortality of *Artemia* (mean ± SD) after 48 h challenged with luminescent and non-luminescent *V. harveyi* BB120 and isogenic mutants.

Treatments	Experiment 1	Experiment 2
V. harveyi BB120 (NL)	48 ± 7	53 ± 10
V. harveyi BB120 (L)		
V. harveyi BB152 (NL)	65 ± 7	71 ± 6
V. harveyi BB152 (L)		
V. harveyi BB170 (NL)	57 ± 7	57 ± 7
V. harveyi BB170 (L)		
V. harveyi MM30 (NL)	61 ± 7	70 ± 18
V. harveyi MM30 (L)		
V. harveyi BB886 (NL)	88 ± 16	81 ± 12
V. harveyi BB886 (L)		

L = Luminescent; NL = Non-luminescent; Values are means of four replicates.

4.4.4. Mortality of SPF *L. vannamei* after challenge with luminescent and non-luminescent *V. campbellii*

There was no significant difference in mortality between the group challenged with luminescent and non-luminescent *V. campbellii*. Most of shrimp died at around 24 h post injection (Table 4.4). Cumulative mortality of luminescent and non-luminescent injection groups was more or less the same at 24, 48, 72, 96, and 120 hpi. At the end of the experiment, cumulative mortality in the *V. campbellii* injection groups ranged from 53 to 59% in experiment 1 and 38-40% in experiment 2.

Table 4.4. Cumulative shrimp mortality (%) after challenge with luminescent and non-luminescent *Vibrio campbellii*

Treatments	6 h	12 h	24 h	36 h	48 h	72 h	96 h	120 h
Experiment 1								
Control	0 ± 0	0 ± 0^{a}	0 ± 0^{a}	3 ± 6^{a}	3 ± 6^{a}	9 ± 6^{a}	9 ± 6^{a}	13 ± 10^{a}
VC (RS)	3 ± 6	41 ± 12^{b}	53 ± 19^{b}	56 ± 16^{b}	56 ± 16^{b}	56 ± 16^{b}	56 ± 16^{b}	59 ± 16^{b}
VC (NL)	0 ± 0	38 ± 10^{b}	50 ± 18^{b}	50 ± 18^{b}	50 ± 18^{b}	50 ± 18^{b}	53 ± 21^{b}	53 ± 21^{b}
VC (L)	0 ± 0	34 ± 6^{b}	53 ± 12^{b}	56 ± 16^{b}	56 ± 16^{b}	56 ± 16^{b}	56 ± 16^{b}	56 ± 15^{b}
Experiment 2								
Control	0 ± 0	0 ± 0^{a}	0 ± 0^{a}	31 ± 6^{a}	3 ± 6^{a}	6 ± 7^{a}	6 ± 7^{a}	6 ± 7^{a}
VC (RS)	3 ± 6	16 ± 12^{a}	22 ± 16^{a}	28 ± 12^{a}	34 ± 16^{ab}	38 ± 18^{a}	38 ± 18^{b}	41 ± 21^{ab}
VC (NL)	0 ± 0	16 ± 12^{a}	9 ± 12^{a}	22 ± 19^{a}	25 ± 14^{ab}	31 ± 16^{a}	31 ± 16^{ab}	34 ± 12^{ab}
VC (L)	3 ± 6	13 ± 10^{a}	19 ± 16^{a}	25 ± 14^{a}	31 ± 16^{b}	34 ± 19^{a}	38 ± 14^{b}	38 ± 14^{b}

VC = *V. campbellii*; L = Luminescent; NL = Non-luminescent; RS = Rifampicin sensitive; Values within a column with different superscripts were significantly different ($p < 0.05$).

Similar to *V. campbellii*, no significant difference in mortality was observed between the groups challenged with L and NL *V. harveyi* BB120. At the end of the experiment, cumulative mortality in shrimp challenged with NL strain ranged from 33 to 40% and approximately 40% mortality was observed in shrimp challenged with L strain (Table 4.5).

Table 4.5. Cumulative shrimp mortality (%) after challenge with luminescent and non-luminescent *Vibrio harveyi* BB120

Treatments	12 h	24 h	36 h	48 h	72 h	96 h	120 h
Experiment 1							
Control	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
BB120 (RS)	17 ± 6^{b}	23 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}	40 ± 10^{b}	40 ± 10^{b}	40 ± 10^{b}
BB120 (NL)	10 ± 0^{b}	23 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}	40 ± 0^{b}	40 ± 0^{b}	40 ± 0^{b}
BB120 (L)	13 ± 6^{b}	33 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}	37 ± 6^{b}
Experiment 2							
Control	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	3 ± 5^{a}	3 ± 5^{a}	3 ± 5^{a}
BB120 (RS)	13 ± 6^{a}	30 ± 0^{a}	37 ± 6^{a}	37 ± 6^{ab}	37 ± 6^{a}	37 ± 6^{b}	37 ± 6^{ab}
BB120 (NL)	10 ± 0^{a}	23 ± 6^{a}	30 ± 10^{a}	30 ± 10^{ab}	33 ± 15^{a}	33 ± 15^{ab}	33 ± 15^{ab}
BB120 (L)	13 ± 6^{a}	30 ± 0^{a}	37 ± 6^{a}	37 ± 6^{b}	37 ± 6^{a}	37 ± 6^{b}	37 ± 6^{b}

No mortality was observed at 6 hpi; Values within a column with different superscripts were significantly different ($p < 0.05$).

4.4.5. Switching from non-luminescent to luminescent

As mentioned in Materials and Methods, many different ways were tried to make the NL strains switch back to luminescence but none of the described procedures was successful in generating L strains from NL strains.

4.5. Discussion

In this study, the non-luminescent (NL) strains were produced by sub-culturing luminescent (L) strains in dark under static condition. It might be that a contact with the hydrophilic glass surface under static conditions helps L to switch to NL. A different switching speed from luminescent to non-luminescent was observed in *V. campbellii* and *V. harveyi*. After three days of culturing luminescent *V. campbellii* in static condition, the NL cells were detected. On the other hand, more than two months was needed before NL *V. harveyi* BB120 and quorum-sensing mutant strains started to appear (results not shown). A difference in

morphology of L and NL *V. campbellii* colonies was noticed as the NL colonies were less round shaped on MA compared to the L ones. The difference in colony appearance was substantiated by the different staining on MACR. On the other hand, staining of *V. harveyi* BB120 and quorum-sensing mutant strains with Congo red did not reveal the difference as seen in *V. campbellii*. In this study, three simultaneous phenotypic changes are described in *V. campbellii*, namely a change in staining on MACR, a switch from L to NL, and a switch from virulent to less virulent state. The latter two phenotypic changes were also obtained in *V. harveyi* BB120. This strongly argues against the idea that NL were obtained by a spontaneous mutation in the luciferase gene. Rather a phenotypic shift is likely to be responsible. Phenotypic variation has been described in other species under identical culture conditions (Bossier and Verstraete, 1996; Drenkard and Ausubel, 2002; Kussell *et al.*, 2005). In *Comamonas testosteroni,* Bossier and Verstraete (1996) found two types of colonies on Luria-Bertani (LB) agar plates, namely colonies with mucoid appearance and colonies with a non-mucoid appearance. Also in that study, in absence of agitation and in contact with a glass surface, a culture with predominantly nonmucoid-colony forming (NMCF) cells very rapidly shifted to a culture dominated by mucoid-colony-forming (MCF) cells. A difference in morphology was also mentioned. Working with *Pseudomonas aeruginosa* resistant against kanamycin, Drenkard and Ausubel (2002) found approximately 30% of the resistant variants exhibited a rough colony phenotype compared with the wild type.

The quorum-sensing system of *V. harveyi* has been described by Bassler *et al.* (1993), Henke and Bassler (2004a). In this study, it was verified whether quorum-sensing would be involved in the phenotype switch. Two types of experiments were performed to substantiate this hypothesis. It was found that *V. harveyi* strains with mutation in the AI1 and AI2 quorum-sensing pathway were still able to switch to NL strains. In addition, the production of quorum-sensing molecules in the NL strains was measured. It was verified that the quorum-

sensing molecules (AI1, AI2, AI3) were still present in cultures of NL *V. harveyi* BB120 and *V. campbellii* (Table 4.2). Hence, the results did not reveal any support for quorum-sensing molecule production being drastically reduced in NL strains. Neither does quorum-sensing abolishment prevents L strains from becoming dark under static culture conditions. Hence, it is more likely that another mechanism is regulating this phenomenon.

The difference in virulence between the L and the NL of *V. campbellii* was demonstrable in *Artemia* but not clear in shrimp. In *Artemia*, the relative percentage mortality was less than 100% in all NL tested strains, indicating a reduced virulence of the non-luminescent isogenic strains. In the shrimp experiments the *Vibrio* was injected intramuscularly. Hence under these experimental circumstances the *Vibrio* does not need to colonize the gut and overcome barriers such as the mucosal layer, to finally infect otherwise sterile tissue. In *Artemia*, we assume that infection can only happen through the gut (Verschuere *et al.*, 2000). Hence the reduced virulence of NL *V. campbellii* in *Artemia* might be caused by its reduced capability to infect the gut.

Bossier and Verstraete (1996) reported the forward and backward shift between NMCF cells and MCF cells could be the result of a genetic switch mechanism. According to Van den Broek *et al.* (2005), colony phase variation is a regulatory mechanism at the DNA level which usually results in high frequency, reversible switches between different phenotype of colonies. Mutational events can be involved in the mechanism of phenotypic variation (Massey and Buckling, 2002). Environment can regulate the rates of mutation at specific sites. Other mechanisms include elevated genome-wide mutation rates, environmentally regulated genome-wide mutation rates and elevated site-specific mutations.

In the laboratory conditions, the switching between two phenotypes can be controlled by chemicals. For instance, the mucoid colonies can be switched to non-mucoid colonies by

H_2O_2 (Bossier and Verstraete, 1996). It was hypothesised that under stress of reactive oxygen, NL strains would switch to L. In this study, luminescence could not be restored by treatment with H_2O_2 or SNAP (NO generator). Co-culture of L and NL and passing the NL strains through the host were also not successful in generating NL strains.

In conclusion, luminescent *Vibrio* strains can switch to non-luminescence by being cultured in static condition. Concomitant with the loss of luminescence, the NL strain stains less on MACR and becomes less virulent.

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CHAPTER

5

Effect of washing vibrios in two different salt solutions on their enumeration on selective and non-selective media

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L. H. Phuoc, P. Sorgeloos, P. Bossier

(Submitted)

5.1. Abstract

This study was conducted to investigate the effect of washing *Vibrio* cells with filtered autoclaved seawater (FASW) or physiological solution (PS) on their enumeration on selective and non-selective media. Four different bacterial strains were tested, namely *Vibrio campbellii* LMG21363, *Vibrio harveyi* BB120, *Vibrio harveyi* JAF483, and *Vibrio harveyi* JAF548. The latter two strains are isogenic strains of *V. harveyi* BB120 affected in the quorum-sensing signaling pathway resulting into very low luminescence or constitutive luminescence. The strains were first cultured in Marine Broth (MB) for 24 h at 28°C. Subsequently, the cells were centrifuged and washed twice with FASW or PS. Finally, the washed cells were plated both on a non-selective medium (Marine Agar, MA) and on a selective medium (Thiosulfate Citrate Bile Sucrose, TCBS) for enumeration. Bacterial densities in MA were higher than in TCBS regardless whether the cells were washed with FASW or PS. The cells washed with FASW showed significantly higher numbers of colonies in TCBS compared to cells washed with PS (between 10 and 300-fold) ($p < 0.01$). There was a significant interaction between the factors "washing solution" and "culture media" on the colony forming ability of all tested strains ($p < 0.01$). The results of this study suggest that washing *Vibrio* cells in physiological solution can channel them into an injured state probably because of an osmotic shock in the washing step, preventing their correct enumeration especially on selective medium.

5.2. Introduction

Viable plate count is one of the most common methods for enumerating bacteria. However, large differences between bacterial counts performed by plating and by microscopy have been noticed (Besnard *et al.*, 2000). Even under laboratory conditions, enumeration of bacteria by direct plate counting on different media may result in apparently different bacterial densities (Cerdà-Cuéllar *et al.*, 2001). Under certain conditions, some bacterial strains loose the ability to form colonies on selective or non-selective media. This phenomenon occurs when the cells are injured or bacteria enter the viable but non-culturable (VBNC) state. Injury in bacteria is defined as an increased sensitivity to components of growth media that are normally not inhibitory (Mackey, 2000). The injured state is transient, resulting from cumulative cellular damage, and can be reversed under appropriate conditions to enable the injured cells to resume growth (Bogosian and Bourneuf, 2001). The bacterial cells for instance can be injured by hyperosmotic stress as described by Braux *et al*. (1997). When culturing *Escherichia coli* O157:H7 and *Staphylococcus aureus* in brain heart infusion (BHI) containing different concentrations of NaCl, Hajmeer *et al*. (2006) found that more cell injury occurred as the NaCl concentration increased from 5% to 10%.

VBNC is defined as a state in which bacteria fail to grow on routine bacteriological media on which they would normally grow and develop into colonies (Oliver, 2005). The VBNC state in pathogenic bacteria was first noticed by Xu *et al.* (1982). They observed that *Escherichia coli* and *Vibrio cholerae* cells remained viable but quickly lost their ability to form colonies on culture media after they were re-suspended in artificial seawater. The VBNC state is mostly described for Gram-negative bacteria but Byrd *et al.* (1991) described the loss of colony forming abilities in two Gram-positive bacteria namely *Micrococcus flavus* and *Streptococcus faecalis*. The VBNC state is supposed to be a survival strategy in response to

environmental stress. VBNC bacteria apparently retain metabolic activity (Nilsson *et al.*, 1991) and virulence (Colwell *et al.*, 1996).

Vibrio species represent nearly 80% of the bacterial population in surface water of the western Pacific Ocean (Tsukamoto *et al.*, 1993). However, the distributions of certain coastal *Vibrio* populations are influenced by environmental factors including salinity (Motes *et al.*, 1998), temperature (Jiang and Fu, 2001; Motes *et al.*, 1998), and in some cases the abundance of host organisms (Lee and Ruby, 1994). Many studies of coastal vibrios used culture dependent enumeration techniques and hence it remains unknown whether the observed dynamics reflect shifts in physiology to a viable-but-non-culturable state or represent fluctuations in cell density.

The aim of this study was to verify the effect of washing *Vibrio* cells with either filtered autoclaved seawater (35 gL^{-1}) or physiological solution (8.5 gL^{-1}) on their enumeration on Marine Agar and on *Vibrio* selective medium Thiosulfate Citrate Bile Sucrose (TCBS) agar.

5.3. Materials and Methods

5.3.1. Bacterial strains

The bacterial strains used in this study were described in Table 5.1. These strains were chosen to test whether the wild type (*V. harveyi* BB120), quorum-sensing mutant strains (*V. harveyi* JAF483 and *V. harveyi* JAF 548), and *V. campbellii* react differently to osmotic stress.

Table 5.1. Bacterial strains used in this study

Strains	Relevant features	References
Vibrio campbellii LMG21363	Luminescent	Soto-Rodriguez *et al.* (2003)
Vibrio harveyi BB120	Wild type, luminescent	Bassler *et al.* (1997)
Vibrio harveyi JAF483	Mutation in *LuxO* D47A linked to Kan^R, luminescent	Freeman and Bassler (1999a)
Vibrio harveyi JAF548	Mutation in *LuxO* (encoding LuxO, a protein in the AHL and AI-2 signal transduction), non-luminescent	Freeman and Bassler (1999a)

5.3.2. Experimental set up

Bacterial strains previously stored in 20% glycerol at -80°C, were aseptically inoculated on Marine Agar (MA). The plates were incubated for 24 h at 28°C. Single colonies were subsequently transferred and grown in an 250 mL erlenmeyer containing 100 mL Marine Broth (MB) 2216 (Difco Laboratories, USA). The culture was incubated overnight on the shaker (28°C, 150 rpm). Subsequently, they were centrifuged at 2200 g for 15 min. The pellets were re-suspended either in FASW or in physiological solution. The cells were washed twice with the respective solutions and collected by centrifugation at 2200 g for 15 min. Finally, the pellets were re-suspended in the same solutions. Serial 10 fold dilutions were prepared in FASW or PS. For enumeration, 50 µL of each dilution was placed in triplicate on MA and TCBS. The suspensions were spread over the surface of the medium using a spiral plater. The plates were incubated for 24 h at 28°C so that colonies were formed. Colonies on a plate containing between 30 and 300 colonies were counted.

5.3.3. Statistical analysis

Differences between treatments were evaluated by performing analysis of variances (ANOVA) using statistical analysis software SPSS (version 13.0 for Windows)

5.4. Results

In general, the count of CFU mL^{-1} on TCBS was lower than on MA (Table 5.2, 5.3, 5.4). There was a significant difference (about 300-fold) in colony forming ability on TCBS when *V. campbellii* were washed with physiological solution (Table 5.2). When washed with FASW the difference was two-fold. Similar findings were obtained with *V. harveyi* BB120 and quorum-sensing mutant strains. Washing *V. harveyi* cells with FASW resulted in (3-5)-fold higher colony counts on MA compared to TCBS (Table 5.3, 5.4). Moreover, washing *V. harveyi* with physiological solution affected the colony forming ability of vibrios on TCBS more dramatically, resulting in (30-200)-fold lower count compared to MA. The results are ideal for a 2-way variance analysis allowing for determining the effect of the washing, the effect of the plating medium and the interaction between these two factors. The washing with PS resulted in a significant reduction in colony forming ability of all tested strains on both media ($p < 0.01$). Significant differences in CFU on TCBS and MA were also observed independent from the type of washing. There was a significant interaction ($p < 0.01$) between the two parameters, indicating that the reduction in CFU was significant more severe when PS-washed cells were plated on TCBS (Table 5.2, 5.3, 5.4). There was no apparent link between genotype or phenotype and the reduction in colony forming ability by washing cells in physiological water.

Table 5.2. Effects of washing *V. campbellii* cells in FASW and PS on the enumeration on MA and TCBS (in 10^5 CFU mL^{-1})

Media	**Washing solution**	
	FASW	PS
MA	1481 ± 40^{c}	773 ± 82^{b}
TCBS	778 ± 49^{b}	2.5 ± 1^{a}
Two-way ANOVA		
Media (MA, TCBS)	(**)	
Washing solution (FASW, PS)	(**)	
Media * washing solution	(**)	

MA = Marine Agar; TCBS = Thiosulfate Citrate Bile Sucrose; FASW = Filtered Autoclaved Seawater; PS = Physiological Solution. Values (mean ± SD) of different treatments with different superscripts were significantly different. (**) = significant difference with $p < 0.01$.

Table 5.3. Effects of washing *V. harveyi* BB120, *V. harveyi* JAF483 and *V. harveyi* JAF548 cells in FASW and PS on the enumeration on MA and TCBS (in 10^5 CFU mL^{-1}) (experiment 1)

Strains	***V. harveyi* BB120**		***V. harveyi* JAF483**		***V. harveyi* JAF548**	
Factors	FASW	PS	FASW	PS	FASW	PS
MA	1946 ± 97^{c}	1896 ± 109^{c}	1836 ± 23^{d}	1722 ± 53^{c}	2188 ± 122^{d}	1759 ± 55^{c}
TCBS	620 ± 72^{b}	27 ± 11^{a}	403 ± 31^{b}	10 ± 2^{a}	276 ± 29^{b}	9 ± 3^{a}
Two-way ANOVA						
Media (MA, TCBS)	(**)		(**)		(**)	
Washing solution (FASW, PS)	(**)		(**)		(**)	
Media * Washing solution	(**)		(**)		(**)	

MA = Marine Agar; TCBS = Thiosulfate Citrate Bile Sucrose; FASW = Filtered Autoclaved Seawater; PS = Physiological Solution. Values (mean ± SD) of different treatments in the same strain with different superscripts were significantly different. (**) = significant difference with $p < 0.01$.

Table 5.4. Effects of washing *V. harveyi* BB120, *V. harveyi* JAF483 and *V. harveyi* JAF548 cells in FASW and PS on the enumeration on MA and TCBS (in 10^5 CFU mL^{-1}) (experiment 2)

Strains	***V. harveyi* BB120**		***V. harveyi* JAF483**		***V. harveyi* JAF548**	
Factors	FASW	PS	FASW	PS	FASW	PS
MA	1429 ± 55^{c}	1606 ± 70^{d}	976 ± 33^{d}	838 ± 19^{c}	1542 ± 61^{c}	1488 ± 124^{c}
TCBS	517 ± 29^{b}	47 ± 13^{a}	341 ± 52^{b}	21 ± 4^{a}	292 ± 19^{b}	19 ± 1.2^{a}
Two-way ANOVA						
Media (MA, TCBS)	(**)		(**)		(**)	
Washing solution (FASW, PS)	(**)		(**)		(**)	
Media * Washing solution	(**)		(**)		(**)	

MA = Marine Agar; TCBS = Thiosulfate Citrate Bile Sucrose; FASW = Filtered Autoclaved Seawater; PS = Physiological Solution. Values (mean ± SD) of different treatments in the same strain with different superscripts were significantly different. (**) = significant difference with $p < 0.01$

5.5. Discussion

The results of this study reveal that the salinity of the cell washing medium has a big effect on the growing ability of vibrios in selective media. A significant ($p < 0.01$) drop, 30 to 200-fold, in the counts was recorded when *Vibrio* cells grown in Marine Broth and washed in physiological solution were plated on TCBS. This was the results of a strong interaction between the two tested parameters (namely salt content of the washing solution and plating media) on the colony forming ability of all tested strains. This combined effect was mainly due to a strong reduction in growing ability as a consequence of the osmotic shock the *Vibrio* cells received by washing the cells in physiological solution (8.5 g NaCl L^{-1}) after being grown in MB (35 g NaCl L^{-1}).

A reduced growing ability can be the result of cell injury or the transition of cells into the VBNC state. Entrance into the VBNC state is supposed to be triggered by the specific metabolic program in response to stress and results into cells that no longer form colonies in either a non-selective or a selective medium, but that can still be stained with direct viable count methods. Injured cells (by stress) normally retain their ability to form colonies in a non-selective medium but show reduced growing ability on a selective medium.

As reported by Braux *et al*. (1997) and Mackey (2000), the bacterial cells can be injured by hyperosmotic stress. According to Jiang and Chai (1996), the plate count difference between non-selective and selective media represents the number of injured cells that have been exposed to a sublethal physical or chemical environment (heat, refrigeration, freezing). High counts on Trypticase Soy Agar indicated that most of the injured cells could recover. Lee *et al.* (2006) reported the effect of salinity and pH on the growth of *V. parahaemolyticus* on TCBS. The authors concluded that PBS-3% NaCl (pH 6.6) was the most effective solution

for viable cell during dilution for direct plate counting on TCBS and minimizing the difference in cell numbers between TCBS and non-selective nutrient agar. Different media can result in different plate count results. In a comparative test with four different media for *V. vulnificus* namely cellobiose-colistin (CC) agar, *Vibrio vulnificus* medium (VVM), a modification of VVM agar (VVMc agar), and TCBS, Cerdà-Cuéllar *et al.* (2001) found that VVMc agar was the less stressing medium, giving the highest plating efficiency. With the four different *Vibrio* strains the reduction in CFU on MA was minimal after the imposed osmotic shock, suggesting that the strong reduction as detected by TCBS is due to cell injury, rather than transition into VBNC.

The plating ability of the wild type (*V. harveyi* BB120) and other two quorum-sensing mutants strains derived from this strain were tested. *V. haveryi* JAF483 contains a point mutation in *luxO* (*luxO* D47A) that renders the LuxO protein incapable of phosphorelay. This mutation in *luxO* completely abolishes the density-dependent expression of the *luxCDABEGH* operon resulting in constitutive light production. *V. harveyi* JAF548 contains the *luxO* D47E mutation. In this strain LuxO is blocked in the P-LuxO-like form resulting in a constitutively low expression of e.g. the lux operon. JAF548 produces almost no light (Freeman and Bassler, 1999b). The plating efficiency of these two strains is similar to the wild type *V. harveyi* BB120. LuxR is directly regulated by LuxO (Myamoto *el at*., 2003), but mRNA from LuxR homologues, namely VanT (from *V. anguillarum*), is also regulated by RpoS, a general stress response regulator. So in *V. anguillarum* it is suggested that RpoS works together with quorum-sensing system to modulate VanT (Weber *et al*., 2008). Hence the *V. harveyi* JAF548 strain, blocked in the P-luxO-like form and expressing little light because of low LuxR levels, might be more sensitive to stress. However, the reduction in plating efficiency on TCBS in combination with the washing was comparable among 3

strains, suggesting that low LuxR concentrations are not necessary rendering cells more stress sensitive.

In conclusion, washing vibrios with physiological solution resulted in physiological stress and affected the growth of vibrios on TCBS and this phenomenon might not be related to luminescence and quorum-sensing. Based on literature data and our data, TCBS should be used with great care for enumeration of *Vibrio*. Especially *Vibrio* cells that are to be enumerated on a selective medium should be handled in an isotonic way.

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CHAPTER

6

Increased susceptibility of White Spot Syndrome Virus-infected *Litopenaeus vannamei* to *Vibrio campbellii*

L.H. Phuoc, M. Corteel, H.J. Nauwynck, M.B. Pensaert, V. Alday-Sanz, W. Van den Broeck, P. Sorgeloos, P. Bossier

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6.1. Abstract

The concept of polymicrobial disease is well recognized in human and veterinary medicine but has received very little attention in the field of aquaculture. This study was conducted to investigate the synergistic effect of white spot syndrome virus (WSSV) and *Vibrio campbellii* on the development of diseases in specific pathogen-free (SPF) shrimp *Litopenaeus vannamei*. The juvenile shrimp were first injected with *WSSV* at a dose of 30 SID_{50} $shrimp^{-1}$ (SID_{50} = Shrimp Infectious Dose with 50% endpoint) and 24 h later with 10^6 CFU of *V. campbellii* $shrimp^{-1}$. Controls receiving just one of the pathogens or blank inocula were included. In the treatment with WSSV only, shrimp started to die at 48-108 hours post injection (hpi) and cumulative mortality reached 100% at 268-336 hpi. Shrimp in the dual treatment died very quickly after *V. campbellii* injection, 100% cumulative mortality was obtained at 72-96 hpi. When WSSV injected shrimp were given sonicated *V. campbellii* instead of live *V. campbellii*, no synergistic effect was observed. Density of *V. campbellii* in the haemolymph of co-infected moribund shrimp collected 10 h after *V. campbellii* injection was significantly higher than in shrimp injected with *V. campbellii* only ($p<0.01$). However, there was no difference in WSSV replication between shrimp inoculated with WSSV only compared to dually inoculated ones. This study revealed that prior infection with WSSV enhances the multiplication and disease inducing capacity of *V. campbellii* in shrimp.

6.2. Introduction

Infectious diseases are a major constraint to shrimp production in many countries. The rapid increase in cultured areas since the 1980's facilitated the spreading and outbreaks of a high number of pathogens, viruses in particular. Since its emergence in 1992 (Chou *et al.* 1995), white spot syndrome virus (WSSV) has been one of the major disease problems in shrimp culture around the world (Lightner, 2003; Rosenberry, 2004; Sanchez-Martinez *et al.*, 2007; Escobedo-Bonilla *et al.*, 2008). In cultured penaeid shrimp, WSSV infections can cause a cumulative mortality up to 100% within 3-10 days (Lightner, 1996). Infected shrimp show lethargic behaviour, loss of appetite, reddish discoloration and white spots in the exoskeleton composed of calcified deposits (Chou *et al.*, 1995). Reports have described both acute and chronic WSSV infections which caused different rates of mortality in shrimp ponds (Sudha *et al.*, 1998) and under experimental conditions (Wang *et al.*, 1999b; Rahman, 2008 accepted). WSSV not only infects all shrimp species, but also a wide range of other decapod crustaceans (Lightner *et al.*, 1998).

Species of *Vibrio* are well known in penaeid shrimp culture as causative agents of vibriosis. This important disease is mainly caused by *V. anguillarum, V. alginolyticus, V. parahaemolyticus, V. harveyi, V. penaeicida, V. campbellii*, both in hatcheries and grow-out ponds (Lightner, 1988a; Lavilla-Pitogo *et al.*, 1990). Infections with luminescent *V. harveyi* strains have been reported to cause major losses in shrimp larviculture in Australia (Pizzutto and Hirst, 1995), South America (Álvarez *et al.*, 1998; Robertson *et al.*, 1998) and Mexico (Vandenberghe *et al.*, 1999). Vibriosis usually occurs during the first month of culture and can cause more than 50% mortality. So far, it is not clear whether *Vibrio* spp. are opportunistic or primary pathogens. According to Saulnier *et al*. (2000a), *Vibrio* spp. may act as opportunistic agents in secondary infections or be true pathogens. Since low number of

V. penaeicida bacterial cells were shown to produce the disease in *P. japonicus* and *P. stylirostris*, Saulnier *et al*. (2000b) reported that *V. penaeicida* could act as a primary pathogen. However, pathogenic vibrios were also isolated from apparently healthy penaeid shrimp (Gómez-Gil *et al.*, 1998a, Vandenberghe *et al*., 1998). These observations lead researchers to consider *Vibrio* spp. are opportunistic pathogens. Horowitz and Horowitz (2001) postulated that if shrimp are not suffering from primary infections, physical damage or stress, their resistance against vibrios is adequate to prevent disease. This idea was further supported by Alday-Sanz *et al*. (2002) who showed that shrimp, when exposed to ammonia prior to immersion challenge with *Vibrio*, suffered more frequent and earlier pathological changes than shrimp exposed to the bacteria alone.

Only a few cases of polymicrobial disease have been described in shrimp aquaculture. In 2001-2002, a retardation of the *Penaeus monodon* growth rate was noted in shrimp ponds in Thailand. This problem was named Monodon Slow Growth Syndrome. Samples of affected shrimp were screened by histopathology, polymerase chain reaction (PCR) and transmission electron microscopy for a wide range of pathogens. It was discovered that several causative agents were involved. Many shrimp specimens had dual or multiple infections with monodon baculovirus (MBV), heptopancreatic parvovirus (HPV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Chayaburakul *et al*., 2004). After screening shrimp samples from 18 ponds in India, Umesha *et al.* (2006) found that the animals in 7 ponds showed dual infections with WSSV and MBV and in 10 ponds triple infections with HPV, WSSV and MBV could be found. Selvin *and* Lipton (2003) demonstrated the presence of a virulent strain of *V. alginolyticus* in shrimp from a pond hit by a WSSV outbreak. Although both pathogens could not be isolated from all sampled shrimp, it was stated that shrimp weakened by WSSV would succumb to a secondary infection by *V. alginolyticus.*

Vibrio is known to be one of the dominant species of bacteria living in shrimp ponds (Hisbi *et al.*, 2000). As a known facultative pathogenic bacterium, it is probable that *Vibrio* regularly co-infects shrimp with WSSV in the field.

The objective of this study was to reproduce a co-infection of shrimp with WSSV and *Vibrio* under laboratory conditions using standardized challenge protocols and to investigate the existence of any synergistic effect. More specifically, the question was raised whether a WSSV infection already present in specific pathogen-free *Litopenaeus vannamei* shrimp would allow *Vibrio* to cause faster and higher mortality rates than the virus or bacteria administered separately.

6.3. Materials and Methods

6.3.1. Viral and bacterial stocks

6.3.1.1. Viral stock: A Vietnamese WSSV isolate was used in this study. This isolate was studied before and was shown to be significantly less virulent than 2 other isolates from Thailand (Rahman *et al.*, 2007a; Rahman *et al.*, 2007b). The original WSSV isolate from naturally infected *P. monodon* was passaged once into crayfish (*Cherax quadricarinatus*). Crayfish gill suspension containing WSSV was received from Research Institute for Aquaculture No2, Vietnam. The isolate was amplified in SPF *L. vannamei* juveniles. The virus stock was titrated *in vivo* by intramuscular route and the virus titer was $10^{5,8}$ shrimp infection doses 50% endpoint per mL (SID_{50} mL^{-1}) as determined by indirect immuno-fluorescence (IIF) and 1-step PCR (Escobedo-Bonilla *et al.*, 2005). A dose of 30 SID_{50} was prepared in a volume of 50 µL by diluting the stock with phosphate buffered saline (PBS). As a control inoculum (mock), PBS alone was used.

6.3.1.2. Bacterial stock[5]: *Vibrio campbellii* (LMG21363) was obtained from the Laboratory of Microbiology (Ghent University, Belgium). The strain, previously stored in 20% glycerol at -80°C, was aseptically inoculated in Marine Agar (MA). The plates were incubated for 24 h at 28°C. Single colonies were subsequently transferred and grown in Marine Broth (MB) 2216 (Difco Laboratories, USA) by incubation overnight (28°C, 150 rpm). The culture was then transferred to centrifugation tubes and centrifuged at 2200 g for 15 min. The supernatant was discarded and pellets were washed twice and finally re-suspended in filtered autoclaved seawater (FASW). The bacterial densities were determined spectrophotometrically at an optical density of 550 nm assuming that an optical density of 1.0 corresponds to 1.2 x 10^9 cells mL^{-1}, according to the McFarland standard (Bio Merieux, France). A bacterial dose of 10^6 CFU $100\mu L^{-1}$ was prepared. As a control, FASW was used.

6.3.2. Experimental animals and conditions

Specific pathogen-free (SPF) *L. vannamei* of the Kona-Hawaii (USA) strain (Wyban *et al.*, 1992) were used in this study. Shrimp were imported from Molokai Sea Farms, Hawaii (USA). Animals were certified to be free of WSSV, IHHNV, MBV, HPV, Taura syndrome virus (TSV), Yellow head virus (YHV) and Gill-associated virus (GAV), as well as other pathogens (fungi, protozoa) as verified by PCR and histopathology. Batches of shrimp arrived at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, as postlarvae (PL_{8-12}). They were kept in a recirculation system at a water temperature of 28-29°C, 34 gL^{-1} salinity, and pH of 7.8-8.1. During the first week, the animals were fed twice daily with *Artemia* nauplii. After one week their diet was shifted to A2 monodon high performance shrimp feed (2.2 mm fraction, INVE Aquaculture NV, Belgium). The feeding ratio was 2.5 % of the mean body weight (MBW) day^{-1}. For the infection test, shrimp were taken randomly from the population and gradually acclimatised to

[5] This section has been described in chapter 3 (section 3.3.2)

a salinity of 15 gL^{-1} over four days. Acclimatised shrimp were transported to the facilities of the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, where the infection experiments were performed under biosafety conditions. One day before and during the entire period after the inoculation, shrimp were housed individually in covered 10 L aquaria, filled with artificial seawater prepared with distilled water at a salinity of 15 gL^{-1}, provided with constant aeration and maintained at 27 ± 1°C in a thermostatically controlled room. Feeding was skipped for 24 h prior to the injection, and resumed 6 hours post injection (hpi). During the experiments, shrimp were fed 3 pellets of feed every 12 h.

6.3.3. Rifampicin-resistant *V. campbellii* [6]

Rifampicin-resistant (RR) *V. campbellii* was produced as follows: a colony picked from MA plates was cultured for 24 h in 25 mL MB in a 100 mL erlenmeyer. After incubation, 50 µL of the culture was taken by micropipette and transferred to 25 mL of fresh MB containing 0.5 mgL^{-1} rifampicin (R3501, Sigma-aldrich). The culture was incubated for 24 h at 28°C. The growth of bacteria was monitored by the turbidity of the culture. In the following days, further sub-cultures were made in MB, increasing the rifampicin concentration gradually from 1, 2, 4, 8, 16, 32, 64 to finally 100 mgL^{-1}. When bacteria cultures were growing well in the final concentration of rifampicin (100 mgL^{-1}), they were inoculated on MA plates containing 100 mgL^{-1} rifampicin for obtaining single colonies. The stock was stored in 20% glycerol at -80°C for long term storage.

6.3.4. Immunohistochemistry (IHC) and quantification of WSSV-infected cells

Shrimp samples were collected and fixed in Davidson's fixative for 36 h and kept in 50% ethanol afterwards. Samples were processed as described by Bell and Lightner (1988). Paraffin-embedded tissue sections were cut at 5 µm and placed onto Silane-coated slides (A3648, Sigma-Aldrich). Sections were deparaffinised and rehydrated. The endogenous

[6] This section has been described in chapter 3 (section 3.3.3)

peroxidase was blocked by incubating the slides for 30 min at room temperature in a solution of 1% sodium azide and 0.02% hydrogen peroxidase in Tris buffer (pH 7.4). Sections were incubated for 1 h at 37°C with 2 μg mL^{-1} of monoclonal antibody 8B7 (Diagxotics Inc, USA) raised against WSSV envelope protein VP28 (Poulos *et al.* 2001). Sections were washed in Tris buffer (pH 7.6) and incubated for 1 h at 37°C with a 1:200 dilution of biotinylated sheep anti-mouse IgG antibodies (RPN1001, Amersham Biosciences). Afterwards they were washed, incubated for 30 min at room temperature with 1:200 dilution of streptavidine-biotinylated horseradish peroxidase complex (RPN1051 Amersham Biosciences) and washed again. Color was developed with 0.01% of 3, 3'-diaminobenzidine (D8001 Sigma-Aldrich). Sections were counterstained with Gill's hemaluin and washed in water, dehydrated and mounted. WSSV-infected cells were counted using light microscopy (Leica DM RBE) at a 400x magnification in 5 fields in gills and lymphoid organs and in 2-3 fields in haematopoietic tissue. These counts were converted to the number of WSSV-infected cell mm^{-2}. Both WSSV-infected and uninfected cells in stomach epithelium and cuticular epithelium were counted in 5 fields and the average percentage (%) of infected cells was calculated.

6.3.5. Enumeration of *V. campbellii*

Rifampicin-resistant *V. campbellii* were enumerated on MA with 100 mgL^{-1} rifampicin (MAR). Haemolymph was obtained from the ventral part of the haemocoel of the second abdominal segment, using a 25 gauge needle and a 1 mL syringe rinsed with cold modified Alsever's solution (AS; 19.3 mM Na citrate, 239.8 mM NaCl, 182.5 mM glucose, 6.2 mM EDTA (ethylene diaminetetra-acetic acid); pH 7.2) as an anticoagulant (Rodriguez *et al.*, 1995). Directly after sampling, haemolymph was serially diluted in FASW and plated on MAR. The plates were incubated at 28°C for 24 h. As RR *V. campbellii* are luminous

bacteria, all colonies were checked for luminescence before counting to ensure that no contamination had occurred during the sampling procedure.

6.3.6. Destruction of bacterial cells by sonication

A bath sonicator and glass beads were used for destruction of the bacterial cells. For this purpose, *V. campbellii* were up-scaled for 12 h in an erlenmeyer containing 25 mL of MB. Harvesting of bacteria was done by centrifugation for 15 min at 2200 g. The cells were washed twice with FASW and finally re-suspended in the same solution. Six mL of bacterial suspension with a density of 10^7 CFU mL^{-1} was transferred to a 50 mL falcon tube containing glass beads and was vortexed for 3 min. Then, it was kept in -80°C for 20 min. Subsequently, it was thawed in the sonicator for 3 min, vortexed for 3 min, sonicated for 6 min, and placed back in -80°C for 20 min. The freeze and thaw cycle was repeated for 6 times. After each cycle, samples were collected and plated on MA to check whether the bacteria had been killed by the procedure. The supernatant was prepared by culturing of *V. campbellii* in MB for 24 h. The cultures were then transferred to the falcon tube and centrifuged for 15 min at 2200 g. The supernatant was collected and filtered through 0.2 µm filter paper.

6.3.7. Experimental design

Experiment 1: Dose effect of *V. campbellii* on mortality of *L. vannamei*

The aim of this experiment was to find the highest possible dose of *V. campbellii* that does not cause significant mortalities in *L. vannamei*. This sub-lethal dose was used for the co-infection experiments. Juvenile shrimp (MBW = 12.47 ± 0.89 g) were injected (100 µL) with different doses of *V. campbellii* containing 10^3, 10^4, 10^5, 10^6, or 10^7 CFU per shrimp respectively. As control inoculum, shrimp were injected with the same volume of FASW. Each treatment was done with two replicates of 10 shrimp and the experiment lasted 5 days. Every 6 h, shrimp were monitored for disease symptoms and moribund/dead were collected.

Experiment 2: Effect of sonicated *V. campbellii* and supernatant from *V. campbellii* cultures on mortality of *L. vannamei*

This experiment was conducted to test whether toxic products produced by *V. campbellii*, both extra-or intracellular, could cause shrimp mortality. The experiment was set up with treatments as outlined in Table 6.1. Shrimp (MBW = 1.1 ± 0.17 g) were injected either with supernatant or sonicated *V. campbellii* and with MB or FASW as a control. Each treatment was done with three replicates of 10 shrimp. Every 6 h, they were monitored for disease symptoms and moribund/dead shrimp were removed. The experiment was terminated after 5 days.

Table 6.1. Design of experiment 2 to examine the effect of sonicated *V. campbellii* (VC) and supernatant from *V. campbellii* on mortality of *L. vannamei*

Treatments	Number of shrimp	FASW	MB	Sonicated VC	Supernatant
Control 1	3 x 10	+	-	-	-
Control 2	3 x 10	-	+	-	-
Sonicated VC	3 x 10	-	-	+	-
Supernatant	3 x 10	-	-	-	+

VC = *V. campbellii*; FASW = Filtered autoclaved seawater; MB = Marine broth; SID_{50} = Shrimp infectious dose with 50% endpoint; - = mock inoculation.

Experiment 3: Effect of sonicated *V. campbellii* on mortality of WSSV-infected *L. vannamei*

This experiment tested the effect of sonicated *V. campbellii* on the mortality rate of WSSV-infected shrimp. Groups of 6 shrimp (MBW = 5.17 ± 0.9 g) were either injected with WSSV (treatment 1, 4 and 5) or mock inoculated (treatment 2, 3, and 6) (Table 6.2). At 24 hpi, shrimp were either injected with 10^6 CFU of *V. campbellii* (treatment 2 and 4), negative inocula (treatment 1 and 6) or sonicated *V. campbellii* (treatment 3 and 5). After each

injection procedure, shrimp were placed in their individual 10 L aquarium. Every 12 h, they were monitored for disease symptoms and moribund/dead shrimp were collected. Every 5 days, 75% of water was replaced with new seawater (15gL^{-1} salinity) to minimise ammonia build-up. The experiment was terminated when mortality reached 100% in the WSSV only group.

Table 6.2. Design of experiment 3 to examine the effect of sonicated *V. campbellii* (VC) on mortality of WSSV-infected *L. vannamei*

Treatments	Number of shrimp	WSSV	VC	Sonicated VC
WSSV	6	30 SID_{50}	-	-
VC	6	-	10^6 CFU $shrimp^{-1}$	-
Sonicated VC	6	-	-	10^6 CFU $shrimp^{-1}$
WSSV + VC	6	30 SID_{50}	10^6 CFU $shrimp^{-1}$	-
WSSV + sonicated VC	6	30 SID_{50}	-	10^6 CFU $shrimp^{-1}$
Negative control	6	-	-	-

WSSV = White spot syndrome virus; VC = *V. campbellii*; SID_{50} = Shrimp infectious dose with 50% endpoint; CFU = Colony forming unit; - = mock inoculation.

Experiment 4: Co-infection of *L. vannamei* with WSSV and *V. campbellii* (1st run)

The experiment was set up with 5 treatments to investigate the synergistic effect of WSSV and *V. campbellii* on the mortality rate of *L. vannamei* (Table 6.3). At 0 hpi, groups of 6 shrimp (MBW = 16.8 ± 2.1 g) were either injected with WSSV (treatment 1 and 3) or mock inoculated (treatment 2, 4, and 5). After 24 h, all shrimp were either injected with 10^6 CFU (treatment 2 and 3) or 10^7 CFU of *V. campbellii* (treatment 5) or mock inoculated (treatment 1 and 4). After each injection procedure, shrimp were placed in their individual 10 L aquarium. Every 12 h, they were monitored for disease symptoms and moribund/dead shrimp

were removed. Temperature and ammonium were checked daily. Every 5 days, 75% of water was replaced with new seawater (15 gL^{-1} salinity) to minimise ammonia build-up.

Table 6.3. Design of experiment 4 to examine the synergistic effect of WSSV and *V. campbellii* (VC) on mortality of *L. vannamei* (1st run)

Treatments	Number of shrimp	WSSV	VC
WSSV	6	30 SID_{50}	-
VC	6	-	10^6 CFU $shrimp^{-1}$
WSSV + VC	6	30 SID_{50}	10^6 CFU $shrimp^{-1}$
Negative control	6	-	-
Positive control	6	-	10^7 CFU $shrimp^{-1}$

WSSV = White spot syndrome virus; VC = *V. campbellii*; SID_{50} = Shrimp infectious dose with 50% endpoint; CFU = Colony forming unit; - = mock inoculation.

Experiment 5: Co-infection of *L. vannamei* with WSSV and *V. campbellii* (2nd run) and quantification of WSSV

In this experiment, we aimed at repeating experiment 4, and at the same time collect samples that would allow for the quantification of WSSV. The experiment was identical to experiment 4, except that the positive control group was omitted. Moribund shrimp (MBW =21.5 ± 2.4g) were collected at different time points for quantification of WSSV-infected cells by IHC.

Experiment 6: Quantification of WSSV and *V. campbellii* in co-infected *L. vannamei*

This experiment was set up to collect moribund shrimp for quantification of WSSV and *V. campbellii.* The experimental design was identical to experiment 5 but shrimp were injected with 10^5 CFU of *V. campbellii* and not with 10^6 CFU as in previous experiments. This dose was chosen as it did not cause any mortality when injected alone but could lead to a very clear acceleration of mortality in co-infections with WSSV. In this experiment, RR

V. campbellii were used to avoid contamination during re-isolation. A test confirmed that the selection process had not altered the virulence of this strain. Each treatment started with 12 shrimp (MBW = 4.35 ± 0.72 g). Ten hours post *V. campbellii* injection (hpvi), 6 shrimp were collected from each treatment for quantification of the 2 pathogens.

6.3.8. Statistical analysis: Differences between treatments were evaluated by performing t-test analysis using statistical analysis software SPSS (version 13.0 for Windows). Values in percentages (WSSV-infected cells in stomach epithelium) were ArcSin-transformed to satisfy normal distribution.

6.4. Results

6.4.1. Experiment 1: Dose effect of *Vibrio campbellii* on mortality of *Litopenaeus vannamei*

In treatments with lower than 10^6 CFU of *V. campbellii* shrimp^{-1}, no mortality was observed (Figure 6.1). When 10^6 CFU was administered, 5% cumulative mortality was recorded during the 5-day experimental period. On the other hand, shrimp died very quickly after injection with 10^7 CFU of *V. campbellii* and 100% cumulative mortality was found at 12 hpi. From these results it was concluded that up to a dose of 10^6 CFU shrimp^{-1}, *V. campbellii* on its own was not capable of causing any significant mortality. Therefore, this sub-lethal dose was chosen for the following co-infection experiments of WSSV and *Vibrio*.

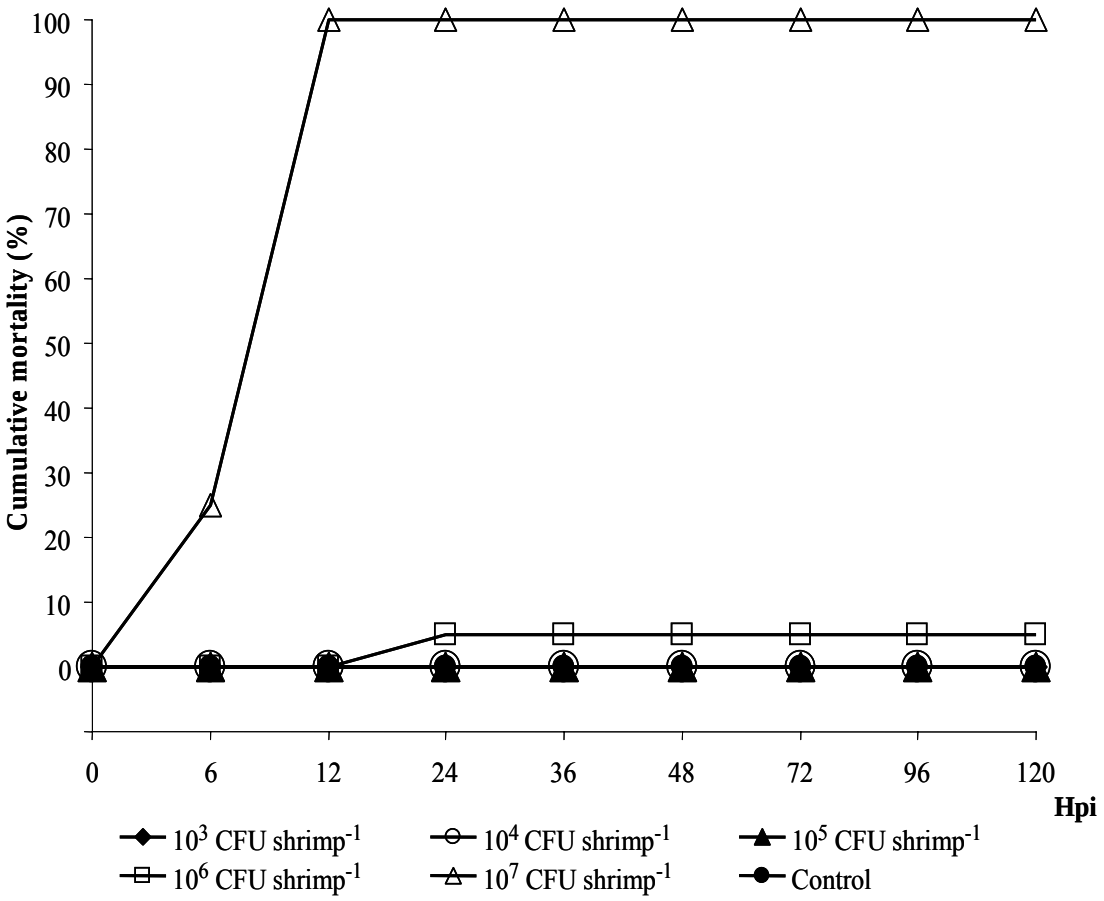

Figure 6.1. Cumulative mortality caused by different doses of *V. campbellii* injected in *L. vannamei* (experiment 1)

6.4.2. Experiment 2: Effect of sonicated *V. campbellii* and supernatant from *V. campbellii* cultures on mortality of *L. vannamei*

No mortality was observed when shrimp were injected with supernatant, sonicated *V. campbellii*, MB or FASW. It was, therefore concluded that no toxic products from *V. campbellii* which could influence shrimp mortality were present in the inocula.

6.4.3. Experiment 3: Effect of sonicated *V. campbellii* on mortality of WSSV-infected *L. vannamei*

Mortalities in the treatments with WSSV only and in the dual treatment with WSSV and sonicated *V. campbellii* evolved in a similar, slow manner (Figure 6.2). Cumulative mortality

reached 100% at 132 hpi and 144 hpi, respectively. At 84 hpi, when cumulative mortality was only 16.7% in both groups, all shrimp had already died in the dual treatment of WSSV and live *V. campbellii*. These results clearly demonstrated that injection of sonicated *V. campbellii* in combination with WSSV did not result in an increase of shrimp mortality.

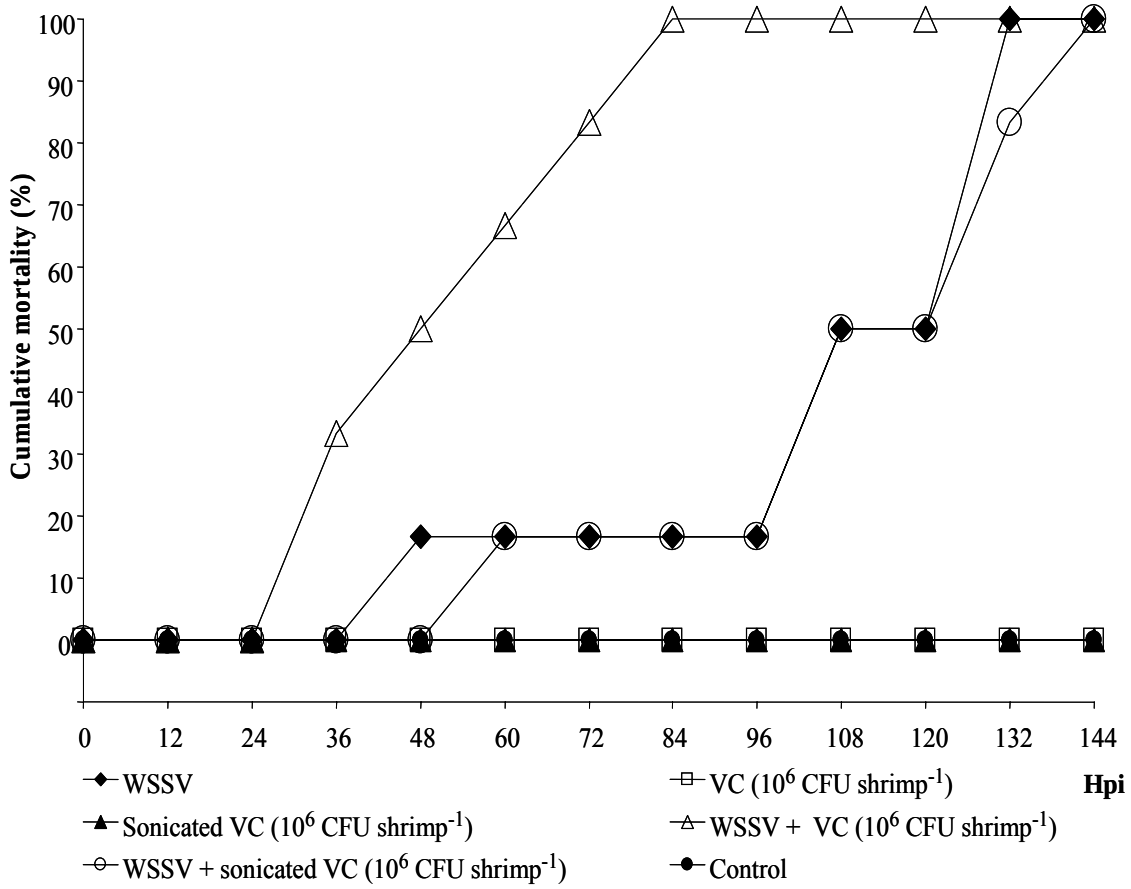

Figure 6.2. Cumulative shrimp mortality after challenge with WSSV and sonicated *V. campbellii* (experiment 3)

6.4.4. Experiment 4: Co-infection of *L. vannamei* with WSSV and *V. campbellii* (1st run)

Shrimp injected with WSSV only started to die at 72 hpi and cumulative mortality reached 100% at 252 hpi (Figure 6.3). In the treatment with 10^6 CFU of *Vibrio* only, one shrimp died within 12 hpvi and cumulative mortality reached 16.7% at the end of the experiment. As in experiment 1, shrimp died quickly after injection with 10^7 CFU of *V. campbellii*. In the

treatment with co-infection of WSSV and *V. campbellii*, shrimp started to die at 36 hpi and 66.7% mortality occurred within 48 hpi. These data indicated that the accelerated mortality caused by co-infection as observed in experiment 3 could be reproduced.

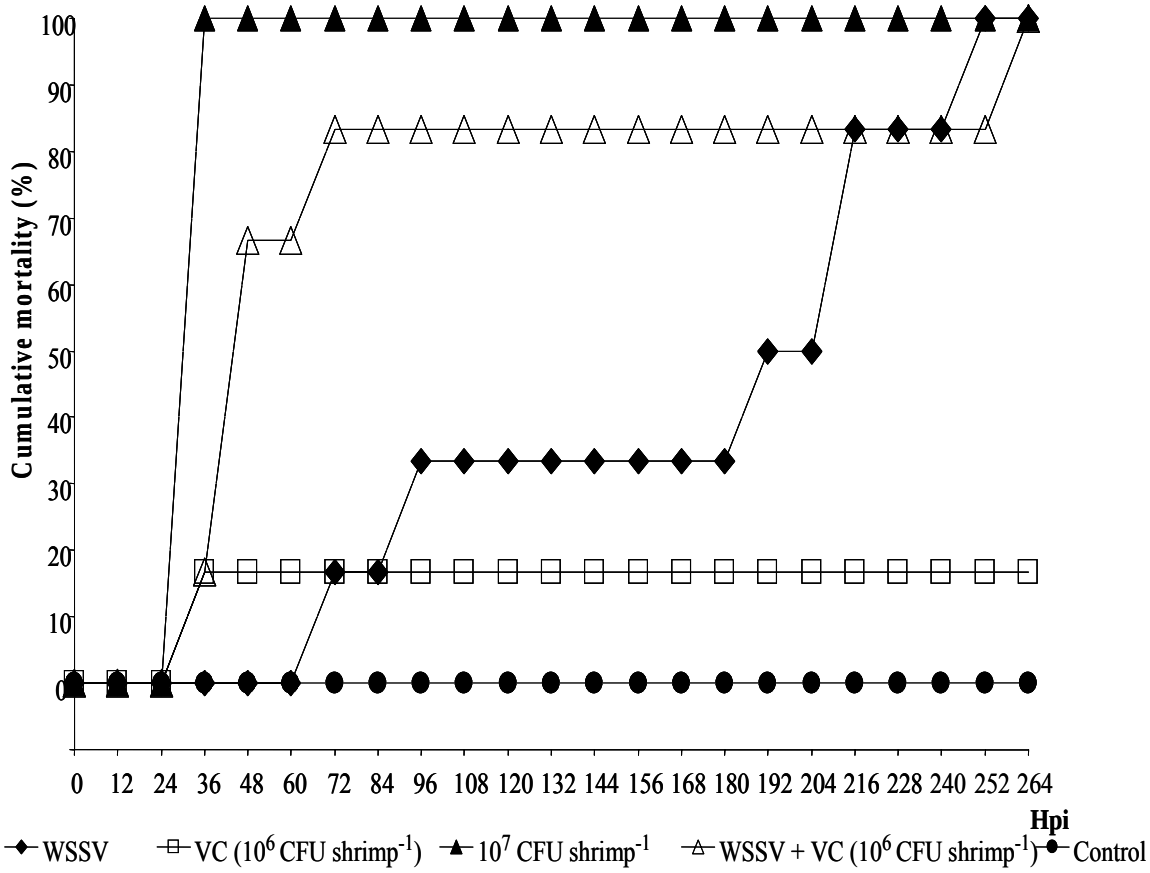

Figure 6.3. Cumulative shrimp mortality after challenge with WSSV and *V. campbellii* (1st run, experiment 4)

6.4.5. Experiment 5: Co-infection of *L. vannamei* with WSSV and *V. campbellii* (2nd run)

Shrimp injected with WSSV only started to die at 108 hpi and cumulative mortality reached 100% at 336 hpi (Figure 6.4). Shrimp injected with *Vibrio* only showed exactly the same mortality rate as in the previous experiment (16.6%). WSSV and *V. campbellii* co-infected animals died quickly after the challenge with *V. campbellii*, with mortality reaching 66.7% by 48 hpi and 100% by 96 hpi. Again, the observations showed that the results of the 1st run of experiment 4 could be reproduced.

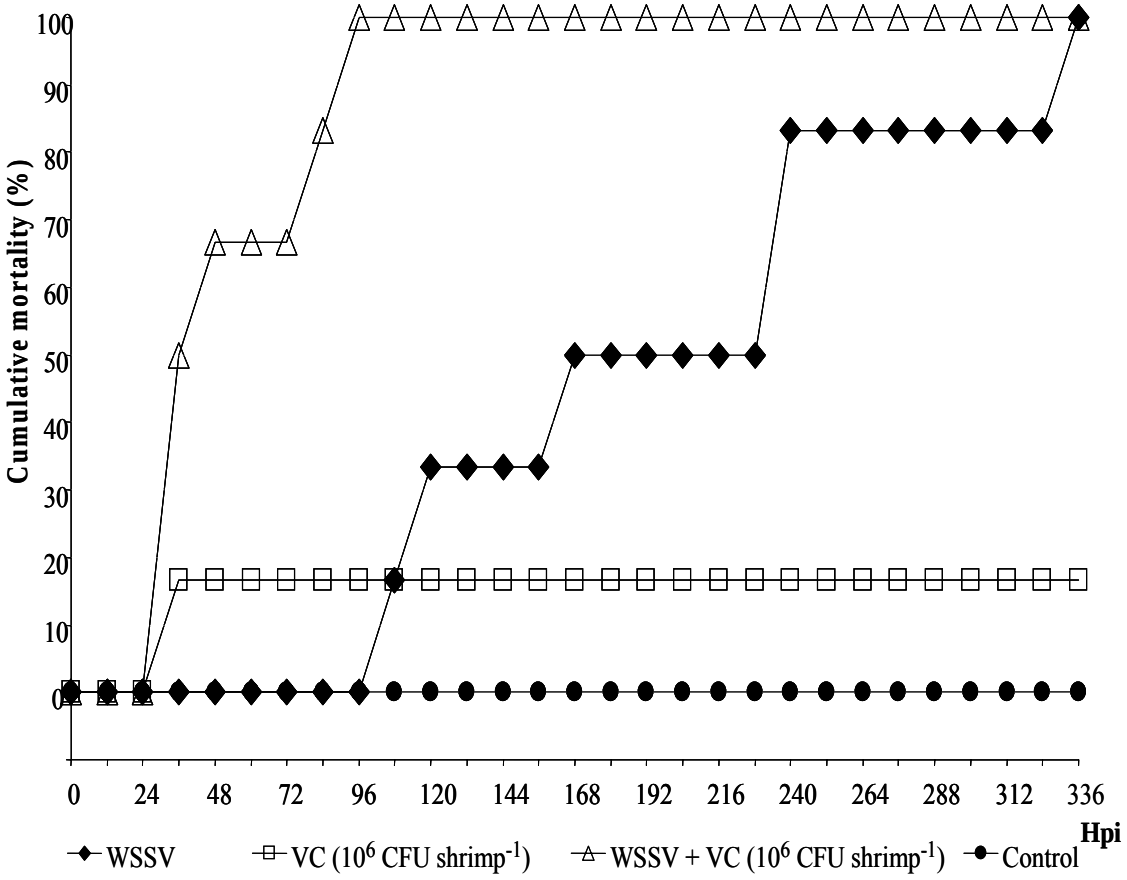

Figure 6.4. Cumulative shrimp mortality after challenge with WSSV and *V. campbellii* (2nd run, experiment 5).

6.4.6. Experiment 5 and 6: Quantification of WSSV and *V. campbellii* in co-infected *L. vannamei*

In experiment 5, shrimp injected with WSSV only died between 119 and 334 hpi. Dually infected animals started to die much earlier, between 29 and 96 hpi. These moribund shrimp were collected for quantification of WSSV. Gills, stomach and cuticular epithelium and haematopoietic tissue were screened for WSSV-infected cells. In the treatment with WSSV only, all shrimp were found to be infected in all organs (Table 6.4). Overall, less WSSV-infected cells were counted in shrimp of the dual treatment and not in all organs.

In experiment 6, shrimp were collected at 10 hpvi for quantification of WSSV and *V. campbellii* (Table 6.5). Dual infections did not appear to cause any change in the number of

WSSV-infected cells. Positive cells were found in all organs, but no significant difference was found between groups which were administered both pathogens or WSSV alone ($p < 0.01$). The number of WSSV-infected cells in haematopoietic tissue (15-568 cells mm^{-2}) was higher than that in gills (55-241 cells mm^{-2}) and lymphoid organs (3-165 cells mm^{-2}) (Table 6.5). In the stomach epithelium, 2-29% of cells were infected. The number of *V. campbellii* isolated from bacteria-only injected shrimp was lower than 100 CFU mL^{-1}. In contrast, a very high density of *V. campbellii* (1.8×10^6 CFU mL^{-1}) was observed in the haemolymph of shrimp in the dual treatment with WSSV and *Vibrio*.

Table 6.4. Quantification of WSSV-infected cells in gills (G), stomach and cuticular epithelium (SE and CE) and haematopoietic tissue (HP) of shrimp collected at time of death (experiment 5)

Treatments	Shrimp	Hpi	G (cells mm^{-2})	SE (%)	CE (%)	HP (cells mm^{-2})
WSSV	1	119	68	34	19	50
	2	119	132	21	17	190
	3	170	52	16	19	80
	4	232	9	7	8	13
	5	237	32	13	24	168
	6	334	39	15	19	118
WSSV + VC	1	29	0	0	0	0
	2	35	10	0.8	0.5	3
	3	37	0	0	0	0
	4	41	1	0	0	0
	5	83	199	12.4	14.9	115
	6	96	85	23.6	18.9	30

WSSV = White spot syndrome virus; VC = *V. campbellii*; G = Gills; SE = Stomach epithelium; CE = Cuticular epithelium; HP = Haematopoietic tissue.

Table 6.5. Quantification of WSSV-infected cells (mean ± SD) and *V. campbellii* (CFU mL^{-1} of haemolymph) in gills (G), stomach epithelium (SE), lymphoid organ (LO) and haematopoietic tissue (HP) of shrimp collected 10 h after *V. campbellii* injection (experiment 6; shrimp in dual treatment were moribund).

Treatments	WSSV-infected cells in organs				VC (CFU mL^{-1})
	G (cells mm^{-2})	SE (%)	LO (cells mm^{-2})	HP (cells mm^{-2})	
WSSV	167 ± 58^{a} (55 - 221)	17 ± 10^{a} (2 - 29)	83 ± 56^{a} (12 - 138)	315 ± 194^{a} (70 - 568)	-
VC	-	-	-	-	43 ± 61^{a} (0 - 157)
WSSV + VC	184 ± 40^{a} (130 - 241)	14 ± 5^{a} (4 - 19)	90 ± 67^{a} (3 - 165)	208 ± 168^{a} (15 - 503)	183721 ± 73177^{b} (113000 - 314600)

WSSV = White spot syndrome virus; VC = *V. campbellii*; G = Gills; SE = Stomach epithelium; LO= Lymphoid organ; HP = Haematopoietic tissue; Numbers between brackets are minimum and maximum values of 6 shrimp. Numbers of infected cells in the same tissue or CFU mL^{-1} with different superscripts were significantly different between the two treatments ($p < 0.01$).

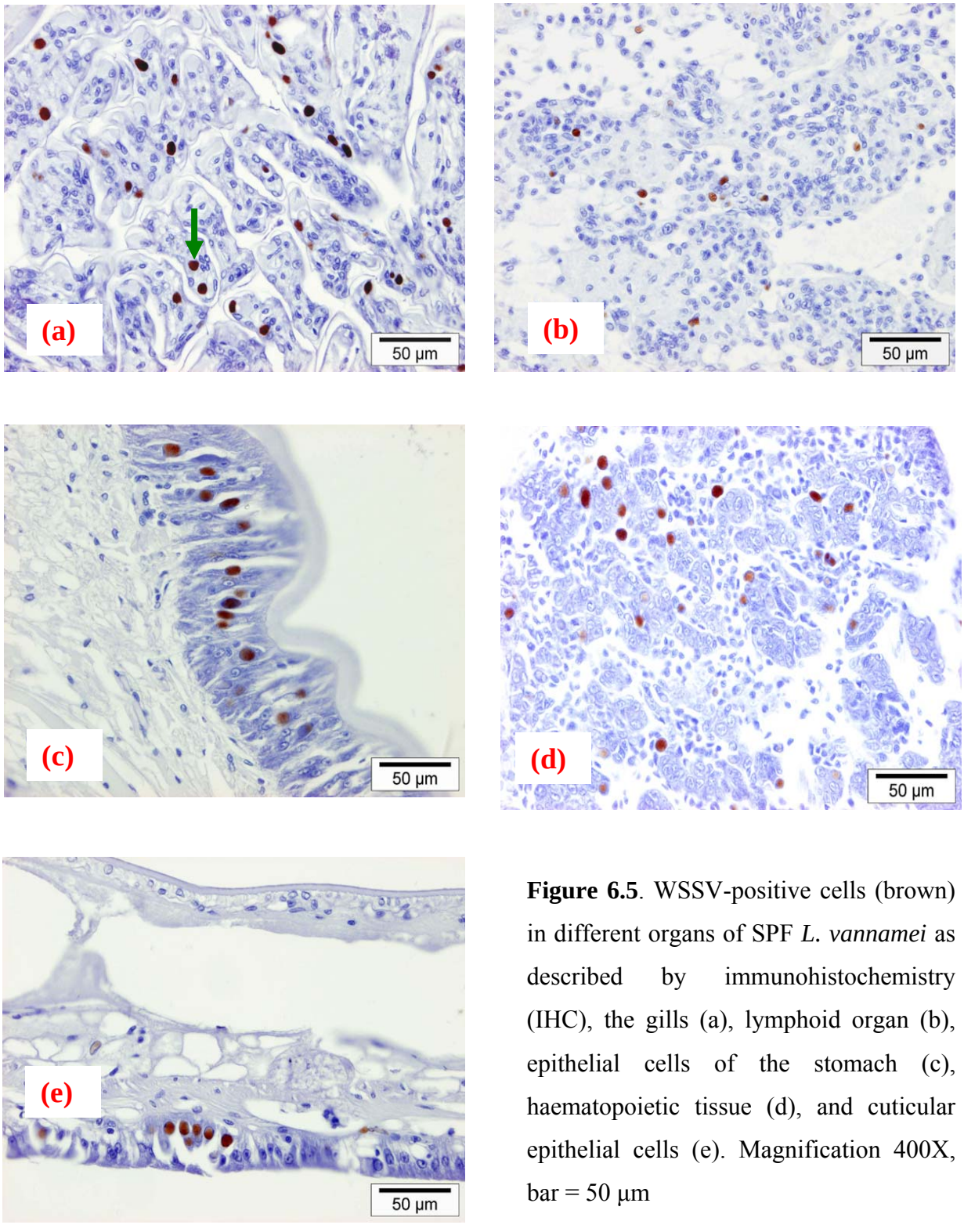

Figure 6.5. WSSV-positive cells (brown) in different organs of SPF *L. vannamei* as described by immunohistochemistry (IHC), the gills (a), lymphoid organ (b), epithelial cells of the stomach (c), haematopoietic tissue (d), and cuticular epithelial cells (e). Magnification 400X, bar = 50 µm

6.5. Discussion

The WSSV Viet strain used in the current study caused a rather slow mortality compared to other strains reported in literature (Lightner, 1996; Sanchez-Martinez *et al.*, 2007; Escobedo-Bonilla *et al.*, 2008). Shrimp injected with only WSSV started to die at 48-108 hpi and cumulative mortality reached 100% at 132-336 hpi. This outcome is not completely in concurrence with previous observations but still within the reported range. Working with the same WSSV strain, injected dose, and the same shrimp species but from a different batch, Rahman *et al.* (2008, accepted) documented an onset of mortality at 36-60 hpi and 100% of cumulative mortality at 204-348 hpi. It is interesting to note that the WSSV induced mortality rate is hardly dependent on the injected dose. Using the same virus batch, Rahman *et al*. (2007a) injected 10000 SID_{50}, obtaining 15-25% cumulative mortality after 36-48 hpi and 100% mortality after 168-196 hpi. All moribund shrimp injected with WSSV only in experiment 5 were positive for WSSV on IHC staining, confirming that all shrimp died due to the WSSV infection.

The current study supported the hypothesis that *V. campbellii* used in this study does not cause primary disease in healthy juvenile shrimp, unless high doses were administered. Shrimp injected with *V. campbellii* alone in doses of 10^5 CFU or lower did not present any signs of mortality. However, the surviving shrimp showed a black spot at the site of *V. campbellii* injection. This observation has also been described by Sarathi *et al.* (2007), who saw haemocytic infiltration and melanization at the injection site upon injections with *Vibrio alginolyticus* in *Fenneropenaeus indicus* shrimp. In the present study, cumulative mortality reached 16.7% when shrimp were injected with 10^6 CFU of *V. campbellii* (Figure 6.2, 6.3). Because shrimp died within hours after injection with 10^7 CFU of *V. campbellii,* it was first considered that shrimp might die as a consequence of toxins produced by the bacteria.

However, since no mortality was observed when shrimp were challenged with the supernatant of *V. campbellii* cultures or with sonicated *V. campbellii*, this possibility was ruled out and it was concluded that live bacteria were required to cause the disease in shrimp. In dual treatments of WSSV and *V. campbellii*, a clear acceleration of the mortality of WSSV-infected shrimp was noticed shortly after inoculation with bacteria. Only 1 out of 18 co-infected shrimp survived longer than 96 hpi. A similar observation has been published by Pakingking *et al.* (2003). They reported that the mortality rate of flounder fish (*Paralichthys olivaceus*) increased by secondary infection with *Streptococcus iniae* or *Edwardsiella tarda* one week after infection with marine birnavirus (MABV). In the same fish species, Oh *et al.* (2006) also found an increase in mortality of fish co-infected with marine birnavirus (MABV) and *Vibrio harveyi* or *Edwardsiella tarda.*

To find out the underlying cause for the rapid mass mortality of the shrimp in the dual treatment, quantification of WSSV in different organs and *V. campbellii* in the shrimp's haemolymph was done. It has been established that gills, stomach and cuticular epithelium, haematopoietic tissue and lymphoid organ are major target organs of WSSV replication (Chang *et al.*, 1996). Escobedo-Bonilla *et al.* (2007) and Rahman *et al.* (2008, accepted) also selected these organs for enumerating WSSV-infected cells. In the present study, all observed organs of moribund shrimp collected at 10 hpvi (34 hpi) were positive with WSSV. However, the obtained counts were different from the ones obtained by Rahman *et al.* (2008, accepted) who reported lower numbers of WSSV-infected cells. This difference might be contributed to the use of shrimp from a different batch and different size and age stage. The shrimp in the present study were 4.5 times smaller than the ones used by Rahman *et al.* (2008, accepted). Additionally, moribund shrimp were collected in this study instead of euthanized shrimp as mentioned in their study. The most important finding, however, was that the quantification did not reveal any significant difference in the number of WSSV-infected cells between the

single and dual treatments. This observation showed that injection of *V. campbellii* did not result in any increase of WSSV replication. Therefore it is very unlikely that WSSV was responsible for the accelerated mortality. As antibody staining methods for *V. campbellii* are not readily available, re-isolation was chosen as means to quantify *V. campbellii* in the shrimp's haemolymph. This procedure was facilitated by the use of a rifampicin-resistant strain of *V. campbellii*. Plating of haemolymph samples is one of the few ways to estimate the amount of bacteria replicating inside the body of shrimp and has been used with success in previous studies (Mermound *et al.*, 1998; van de Braak *et al.*, 2002c). In contrast to the viral load, the *V. campbellii* load showed a highly significant difference between single and dual treatments. At 10 hpvi, the amount of *V. campbellii* in the haemolymph of moribund shrimp inoculated with both WSSV and *V. campbellii* was more than 10^3 times higher than that in shrimp that received bacteria only. Apparently, low densities of bacteria could be eliminated quickly by shrimp that were not coping with a WSSV infection. Van de Braak *et al.* (2002b) also recorded that the concentration of live bacteria in haemolymph of shrimp decreased by 97% within 2 h after injection. This was attributed to the host's defense system, more specifically, the clearing mechanism of the shrimp's haemocytes.

Sizeable individual variations in numbers of WSSV-infected cells and *V. campbellii* in the haemolymph at certain time points were observed in dual and single treatments. Individual variations in the load of WSSV in shrimp were reported earlier (Tan *et al.*, 2001; Durand and Lightner 2002) and can be explained by differences in viral replication, defense response of the host and/or susceptibility among individuals of the same species. Differences in bacterial density between individual shrimp can also reflect the variable health status of shrimp. It appears that stronger shrimp can eliminate bacteria very fast, while weaker ones do it slowly or are not resistant against the bacteria and finally die.

To explain the findings of the current study, it is postulated that the bacterial clearing capacity of shrimp can be severely undermined by a WSSV infection, even in the early stages of the viral disease. Consequently, *V. campbellii* is allowed to multiply unchecked in the shrimp's body, rapidly leading to death. Previous research has documented negative effects, both of WSSV and *V. campbellii*, on vital physiological processes. Jiravanichpaisal *et al.* (2006) reported that granular haemocytes of WSSV-infected crayfish had lost their capacity to induce melanisation. WSSV inhibits the prophenoloxidase (proPO) system upstream of phenoloxidase or simply consumes the native substrate for the enzyme so that no activity can occur. Scholnick *et al.* (2006) found that *L. vannamei* injected with *V. campbellii* decreased oxygen uptake by 27% after 4 h. This phenomenon persisted 24 h after *Vibrio* injection. The inhibition of melanisation due to WSSV might be directly linked with improved dissemination of *V. campbellii* inside shrimp. The combination with a reduced oxygen level caused by the *Vibrio* can result in an acute threat to the survival of shrimp.

In conclusion, an injection with live *V. campbellii* 24 h after a WSSV injection clearly accelerated mortality in juvenile SPF *L. vannamei* shrimp. Such accelerated mortality was not observed when shrimps were injected with very high dose of WSSV (Rahman *et al*., 2007a). The presence of *V. campbellii* did not result in any increase of WSSV replication, but the density of *V. campbellii* in haemolymph increased spectacularly, resulting in much faster mortality of dually inoculated shrimp. The combination of these findings strongly argues for a synergistic effect in which a low non-lethal WSSV load allows for a rapid *Vibrio* multiplication. All research done on *Vibrio* infection of shrimp can no longer deny this phenomenon, as viral load at the start of the experiment has a dramatic effect on the outcome. The use of SPF shrimp is paramount, or the quantification of the viral load should be taken into account.

Seeing their wide distribution, it is probable that co-infections of WSSV and *Vibrio* can occur regularly in the field. However, detailed research is required to elucidate the importance and the exact mechanism of polymicrobial infections in shrimp ponds.

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CHAPTER

7

Effect of dose and challenge routes of *Vibrio* spp. on co-infection with White Spot Syndrome Virus in *Litopenaeus vannamei*

L.H. Phuoc, M. Corteel, H.J. Nauwynck, M.B. Pensaert, V. Alday-Sanz, W. Van den Broeck, P. Sorgeloos, P. Bossier

(Submitted)

7.1. Abstract

This study was conducted to investigate the effect of dose, species of *Vibrio* bacteria, and challenge route on the outcome of co-infection with white spot syndrome virus (WSSV) in specific pathogen-free (SPF) *Litopenaeus vannamei* shrimp. Two experiments were carried out challenging experimentally WSSV-infected shrimp with *V. campbellii* either by injection or by immersion. Juvenile shrimp were first injected with 30 SID_{50} of WSSV and 24 h later with 10^3, 10^4, 10^5 or 10^6 CFU shrimp^{-1} of *V. campbellii*. Controls receiving only *V. campbellii* and negative inocula did not die during the experiment, except the ones that received 10^6 CFU shrimp^{-1} (35-65%). In WSSV-inoculated shrimp, the 50% and 100% cumulative mortality were reached at 96-108 and 144-360 hpi. WSSV-infected shrimp died much faster when injected with at least 10^4 CFU of *V. campbellii* with the 50% and 100% cumulative mortality reached at 36-48 and 48-96 hours post injection (hpi) of virus. The density of *V. campbellii* in haemolymph of co-infected moribund shrimp collected 6 h after *V. campbellii* injection was significantly higher than that in shrimp injected with *V. campbellii* only. There was no difference in the number of WSSV-infected cells between shrimp inoculated with WSSV only, compared to dually inoculated ones. Shrimp which were first injected with WSSV and 24 h (or 48 h) later exposed to 10^6, 10^7, or 10^8 CFU mL^{-1} of *V. campbellii* by immersion did not show any accelerated mortality. When WSSV-infected shrimp were challenged with another *Vibrio* species, *V. harveyi* BB120, no accelerated mortality was noted in WSSV-infected shrimp injected with 10^6 CFU shrimp^{-1} of *V. harveyi* BB120.

In conclusion, it can be stated that the synergistic effect between WSSV and *Vibrio* is influenced by the dose, species and infection route of inoculation of the *Vibrio* bacteria.

7.2. Introduction

Infectious diseases especially caused by bacterial and viral pathogens are serious loss factors in shrimp farming (Lightner, 1996). One of the viruses considered to be particularly problematic in shrimp culture around the world is the white spot syndrome virus (WSSV), which belongs to the genus *Whispovirus* in the family *Nimaviridae* (Mayo, 2002). WSSV is found in almost all shrimp producing countries and lethal to all commercially cultivated penaeid shrimp species (Wang *et al.*, 2000; Sanchez-Martinez *et al.*, 2007; Escobedo-Bonilla *et al.*, 2008). White spot syndrome disease is characterized by the presence on the inner surface of the exoskeleton of white spots from which the name is derived (Lo *et al.*, 1996). Other clinical signs include anorexia, lethargy and reddish discoloration of the body (Wang *et al.*, 1999b).

Amongst the bacterial pathogens, *Vibrio* species are reputed for causing vibriosis in penaeid shrimp. This important disease is known to affect hatchery-reared *Penaeus monodon* as well as juvenile shrimp in grow-out cultures and adults (Lavilla-Pitogo *et al.*, 1990) and is mostly caused by *V. anguillarum, V. alginolyticus, V. parahaemolyticus, V. harveyi, V. penaeicida, V. campbellii*. *Vibrio* spp. can act as primary pathogens in pond waters with increased *Vibrio* populations (Vandenberghe *et al.*, 1998; Saulnier *et al.*, 2000a) but often act as opportunistic agents in secondary infections (Saulnier *et al.*, 2000b). Most outbreaks of shrimp vibriosis happen either in combination with physical stress factors or following primary infections with other pathogens (Sung *et al.*, 2001). In experimental studies, shrimp exposed to salinity or ammonium stress prior to challenge, showed higher susceptibility to vibrios (Liu and Chen, 2004). It has also been indicated that a primary WSSV infection may weaken shrimp, increasing their susceptibility to bacterial infections (Selvin and Lipton, 2003). The influence of all these factors on the susceptibility to *Vibrio* could explain the highly variable mortality

in shrimp, ranging from a few individual shrimp to 100% of the population (Lightner, 1988b).

Under field conditions, animals are often infected with more than one pathogen. Bacteria-bacteria co-infections have been demonstrated in *P. monodon* displaying red disease syndrome. After performing challenge tests with a combination of *V. parahaemolyticus* and *V. harveyi* isolated from diseased shrimp, Alapide-Tendencia and Dureza (1997) concluded that these bacterial strains can reproduce the syndrome in healthy shrimp. Virus-virus co-infection was reported in *P. monodon* shrimp postlarvae postlarvae (PL_8-PL_{10}) in an India hatchery. These shrimp were heavily infected with monodon baculovirus (MBV), hepatopancreatic parvovirus (HPV) and WSSV (Manivannan, 2002). Yang *et al.* (2006) reported co-infection of infectious hypodermal and haematopoietic necrosis virus (IHHNV) and WSSV in cultured *L. vannamei*. Using histopathology and PCR, Flegel *et al.* (2004) found a very high prevalence of dual, triple and quadruple infections with HPV, WSSV, IHHNV and MBV in commercial shrimp ponds in Thailand. While 94% of the sampled shrimp gave a positive test for at least one of the four viruses, dual to quadruple infections accounted for 73% of the total samples.

Selvin and Lipton (2003) demonstrated the presence of a virulent strain of *V. alginolyticus* in shrimp from a pond hit by a WSSV outbreak. Although not all sampled shrimp were infected by both pathogens, it was stated that shrimp weakened by WSSV would succumb to a secondary infection by *Vibrio*. In other investigations, *V. alginolyticus.*, *V. vulnificus*, *V. parahaemolyticus*, *V. damsela*, *Vibrio* sp. were detected in healthy shrimp without gross signs of disease (Gomez-Gil *et al.*, 1998a). Flegel *et al*. (2004) found WSSV in the shrimp without gross or histological signs of disease.

From all these data, it seems plausible that co-infections occur regularly in shrimp ponds. In a previous paper, a dual WSSV-*Vibrio* infection protocol has been described (Phuoc *et al.*, accepted 2008). The aim of this study was to test whether the outcome of the experimental co-infection of WSSV and *V. campbellii* is influenced by (1) the dose of *V. campbellii*, (2) the bacterial species and (3) the challenge route of the *Vibrio* component.

7.3. Materials and Methods

7.3.1. Viral and bacterial stocks

7.3.1.1. Viral stock[7]: A Vietnamese WSSV isolate was used in this study. This isolate has been studied before and was shown to be significantly less virulent than 2 other isolates from Thailand (Rahman *et al*; 2007a; 2007b). The original WSSV isolate from naturally infected *P. monodon* was passaged once into crayfish (*Cherax quadricarinatus*). Crayfish gill suspension containing WSSV was received from Research Institute for Aquaculture No2, Vietnam. The isolate was amplified in SPF *L. vannamei* juveniles. The resulting virus stock was titrated *in vivo* by intramuscular route and the virus titer was $10^{5,8}$ shrimp infection doses 50% endpoint per mL (SID_{50} mL^{-1}) as determined by indirect immuno-fluorescence (IIF) and 1-step PCR (Escobedo-Bonilla *et al.*, 2005). A dose of 30 SID_{50} was prepared in a volume of 50 µL by diluting the stock with phosphate buffered saline (PBS). As a control inoculum (mock), PBS alone was used.

7.3.1.2. Bacterial stock[8]: Two bacterial strains were used in this study. *Vibrio campbellii* (LMG21363) was obtained from the Laboratory of Microbiology (Ghent University, Belgium) and *Vibrio harveyi* BB120 (Bassler *et al.*, 1997) was obtained from the Laboratory for Microbial Ecology and Technology (Ghent University, Belgium). The strains, previously

[7] This section has been described in chapter 6 (section 6.3.1.1)
[8] This section has been described in chapter 3 (section 3.3.2)

stored in 20% glycerol at -80°C, were aseptically inoculated on Marine Agar (MA). The plates were incubated for 24 h at 28°C. Single colonies were subsequently transferred and grown in Marine Broth (MB) 2216 (Difco Laboratories, USA) by overnight incubation (28°C, 150 rpm). The culture was then transferred to centrifugation tubes and centrifuged at 2200 g for 15min. The supernatant was discarded and pellets were washed twice and finally re-suspended in filtered autoclaved seawater (FASW). The bacterial densities were determined spectrophotometrically at an optical density of 550 nm assuming that an optical density of 1.0 corresponds to 1.2 x 10^9 cells mL^{-1}, the McFarland standard (Bio Merieux, France). For injection, 100µL of bacterial suspension was injected. As a control, FASW was used.

7.3.2. Experimental animals and conditions

Specific pathogen-free (SPF) *L. vannamei* were imported from SyAqua Siam Co., Ltd. Bangkok 10110, Thailand. Animals were certified to be free of Taura Syndrome Virus (TSV), WSSV, Yellow Head Virus (YHV) and IHHNV by the Thai Department of Fisheries. [9]Batches of shrimp arrived at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, as postlarvae (PL_{8-12}). They were kept in a recirculation system at a water temperature of 28°C, 35 gL^{-1} salinity, and pH of 7.8-8.1. During the first week, the animals were fed twice daily with *Artemia* nauplii. After one week their diet was shifted to A2 monodon high performance shrimp feed (2.2 mm fraction, INVE Aquaculture NV, Dendermonde, Belgium). The feeding ratio was 2.5% of the mean body weight (MBW) per day. For this study, shrimp were taken randomly from the population and gradually acclimatized to a salinity of 15 gL^{-1} over four days. Acclimatized shrimp were transported to the facilities of the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University, where the infection experiments were performed under biosafety conditions. One day before and during the entire period after the inoculation, shrimp were housed individually

[9] This part has been mentioned in chapter 6 (section 6.3.2)

in covered 10 L aquaria, filled with artificial seawater prepared with distilled water at a salinity of 15 gL^{-1}, provided with constant aeration and maintained at 27 ± 1°C by air heaters. Feeding was stopped 24 h prior to the injection, and resumed 6 hours post injection (hpi). During the experiments, shrimp were fed 3 pellets of feed every 12 h.

7.3.3. Rifampicin-resistant *Vibrio campbellii*[10]

In some experiments (2 and 3), *V. campbellii* had to be quantified by re-isolation and enumeration. To facilitate this procedure, rifampicin-resistant (RR) *V. campbellii* were used instead of rifampicin-sensitive (RS) *V. campbellii*. (RR) *V. campbellii* was produced as follows: a colony picked from MA plates was cultured for 24 h in 25 mL MB in a 100 mL erlenmeyer. After incubation, 50 µL of the culture was taken by micropipette and transferred to 25 mL of fresh MB containing 0.5 mgL^{-1} rifampicin (R3501, Sigma-aldrich). The culture was incubated for 24 h at 28°C. The growth of bacteria was monitored by the turbidity of the culture. In the following days, further sub-cultures were made in MB, increasing the rifampicin concentration gradually from 1, 2, 4, 8, 16, 32, 64 to finally 100 mgL^{-1}. When bacteria cultures were growing well in the final concentration of rifampicin (100 mgL^{-1}), they were inoculated on MA plates containing 100 mgL^{-1} rifampicin for obtaining single colonies. The stock was stored in 20% glycerol at -80°C for long term storage. An *in vivo* challenge test confirmed that the selection process had not altered the virulence of this strain (data not shown).

7.3.4. Immunohistochemistry and quantification of WSSV-infected cells[11]

Shrimp samples were collected and fixed in Davidson's fixative for 36 h and kept in 50% ethanol afterwards. Samples were processed as described by Bell and Lightner (1988). Paraffin-embedded tissue sections were cut at 5 µm and placed onto Silane-coated slides

[10] This section has been described in chapter 3 (section 3.3.3)
[11] This section has been described in chapter 6 (section 6.3.4)

(A3648, Sigma-Aldrich). Sections were deparaffinized and rehydrated. The endogenous peroxidase was blocked by incubating the slides for 30 min at room temperature in a solution of 1% sodium azide and 0.02% hydrogen peroxidase in Tris buffer pH 7.4. Sections were incubated for 1 h at 37°C with 2 μg mL^{-1} of monoclonal antibody 8B7 (Diagxotics Inc, USA) raised against WSSV envelope protein VP28 (Poulos *et al.*, 2001). Sections were washed in Tris buffer (pH 7.6) and incubated for 1 h at 37°C with a 1:200 dilution of biotinylated sheep anti-mouse IgG antibodies (RPN1001, Amersham Biosciences). Afterwards, they were washed, incubated for 30 min at room temperature with 1:200 dilution of streptavidine-biotinylated horseradish peroxidase complex (RPN1051 Amersham Biosciences) and washed again. Color was developed with 0.01% of 3, 3'-diaminobenzidine (D8001 Sigma-Aldrich). Sections were counterstained with Gill's hemaluin and washed in water, dehydrated and mounted. WSSV-infected cells were counted using light microscopy (Leica DM RBE) at a 400x magnification in 5 fields in gills and lymphoid organs and in 2-3 fields in haematopoietic tissue. These counts were converted to the number of WSSV-infected cell mm^{-2}. Both WSSV-infected and uninfected cells in stomach epithelium were counted in 5 fields and the average percentage (%) of infected cells was calculated.

7.3.5. Enumeration of *V. campbellii* [12]

Rifampicin-resistant *V. campbellii* were enumerated on MA with 100 mgL^{-1} rifampicin (MAR). Haemolymph was obtained from the ventral part of the haemocoel of the second abdominal segment, using a 25 gauge needle and a 1 mL syringe rinsed with ice-cold modified Alsever's solution (AS; 19.3 mM Na citrate, 239.8 mM NaCl, 182.5 mM glucose, 6.2 mM ethylene diaminetetra-acetic acid (EDTA); pH 7.2) as an anticoagulant (Rodríguez *et al.*, 1995). Directly after sampling, haemolymph was serially diluted in FASW and plated on MAR. The plates were incubated at 28°C for 24 h.

[12] This section has been described in chapter 6 (section 6.3.5)

7.3.6. Experimental design

Experiment 1: Dose effect of *V. campbellii* on mortality of WSSV-infected *L. vannamei* (1st run)

This experiment was conducted to test the clinical outcome of WSSV infections combined with different doses of *V. campbellii* (Table 7.1). At 0 h, groups of 6 shrimp (MBW = 3.88 ± 0.60 g) were either injected with 30 SID_{50} of WSSV (treatment 1, 6, 7, 8, and 9) or mock inoculated (treatment 2, 3, 4, 5, and 10). After 24 h, shrimp were either injected with different doses of *V. campbellii* (treatment 2, 3, 4, 5, 6, 7, 8, and 9), or mock inoculated (treatment 1 and 10). After each injection procedure, shrimp were placed in their individual 10 L aquarium. Every 12 h, they were monitored for disease symptoms and moribund/dead shrimp were collected. Every 5 days, 75% of water was replaced with new seawater (15 gL^{-1} salinity) to minimize ammonia build-up.

Table 7.1. Design of experiment 1 to examine the dose effect of *V. campbellii* on mortality of WSSV-infected *L. vannamei*

	Treatments	WSSV injection	*V. campbellii* injection	Number of shrimp
1	WSSV	30 SID_{50}	-	6
2	VC	-	10^3 CFU $shrimp^{-1}$	6
3	VC	-	10^4 CFU $shrimp^{-1}$	6
4	VC	-	10^5 CFU $shrimp^{-1}$	6
5	VC	-	10^6 CFU $shrimp^{-1}$	6
6	WSSV + VC	30 SID_{50}	10^3 CFU $shrimp^{-1}$	6
7	WSSV + VC	30 SID_{50}	10^4 CFU $shrimp^{-1}$	6
8	WSSV + VC	30 SID_{50}	10^5 CFU $shrimp^{-1}$	6
9	WSSV + VC	30 SID_{50}	10^6 CFU $shrimp^{-1}$	6
10	Control	-	-	6

WSSV = White spot syndrome virus; VC = *Vibrio campbellii*; SID_{50} = Shrimp infectious dose with 50% endpoint; CFU = Colony forming unit; - = mock inoculation.

Experiment 2: Dose effect of *V. campbellii* on mortality of WSSV-infected *L. vannamei* (2nd run)

In this experiment, we aimed to repeat experiment 1. The experiment was identical to the first experiment except for a very slight difference in shrimp size (MBW = 4.59 ± 0.8 g).

Enumeration of V. campbellii: Forty two extra shrimp from treatments with only *V. campbellii* and dual injection were prepared for enumeration of *V. campbellii*. These shrimp were injected with 10^5 CFU of *V. campbellii.* This dose was chosen as it did not cause any mortality when injected alone but could lead to a very clear acceleration of mortality in co-infections with WSSV. Three shrimp from each treatment were collected at 1, 2, 3, 4, 6, 8, and 10 hours post *V. campbellii* injection (hpvi).

Experiment 3: Co-infection of *L. vannamei* with WSSV and *V. harveyi* BB120

This experiment aimed to investigate whether co-infection also occurs with another *Vibrio* strain. The experiment was set up with 6 treatments (Table 7.2). At 0 h, groups of 6 shrimp (MBW = 5.17 ± 0.94 g) were either injected with 30 SID_{50} of WSSV (treatment 1, 4, and 5) or mock inoculated (treatment 2, 3, and 6). After 24 h, all shrimp were either injected with 10^6 CFU of *V. campbellii* (treatment 2 and 4) or 10^6 CFU of *V. harveyi* BB120 (treatment 3 and 5) or mock inoculated (treatment 1 and 6). Shrimp were kept in the same conditions as described in experiment 1.

Quantification of WSSV and V. campbellii: Eighteen extra shrimp treated either with a single injection of 10^5 CFU *V. campbellii* (or WSSV) or a dual injection were sampled at 6 hpvi for quantification of WSSV and *V. campbellii*. After taking the haemolymph, shrimp were fixed in Davidson's fixative for quantification of WSSV-infected cells by IHC.

Table 7.2. Design of experiment 3 to examine the synergistic effect of WSSV and *V. harveyi* BB120 on mortality of *L. vannamei*

	Treatments	WSSV (injection)	VC (injection)	BB120 (injection)	Number of shrimp
1	WSSV	30 SID_{50}	-	-	6
2	VC	-	10^6 CFU $shrimp^{-1}$	-	6
3	BB120	-	-	10^6 CFU $shrimp^{-1}$	6
4	WSSV + VC	30 SID_{50}	10^6 CFU $shrimp^{-1}$	-	6
5	WSSV + BB120	30 SID_{50}	-	10^6 CFU $shrimp^{-1}$	6
6	Control	-	-	-	6

WSSV = White spot syndrome virus; VC = *Vibrio campbellii*; BB120 = *Vibrio harveyi* BB120; SID_{50} = Shrimp infectious dose with 50% endpoint; CFU = Colony forming unit; - = mock inoculation.

Experiment 4. Immersion challenge of WSSV-infected *L. vannamei* with different doses of *V. campbellii*

The aim of this experiment was to test whether immersion challenge, instead of intramuscular injection, with different doses of *V. campbellii* would also result in accelerated mortality of WSSV-infected *L. vannamei*. Twenty-four or 48 h after WSSV injection, shrimp (MBW = 2.41± 0.65 g) were immersed in artificial sea water containing 10^6, 10^7 or 10^8 CFU mL^{-1} of *V. campbellii* (Table 7.3). Bacteria were added once to the tanks and remained there during the experimental period. Shrimp were kept in the same conditions as described in experiment 1.

Table 7.3. Design of experiment 4 to examine the effect of immersion challenge with different doses of *V. campbellii* on mortality of WSSV-infected *L. vannamei*

	Treatments	WSSV injection	*V. campbellii* immersion	Number of shrimp
1	WSSV	30 SID_{50}	-	6
2	VC	-	10^6 CFU mL^{-1}	6
3	VC	-	10^7 CFU mL^{-1}	6
4	VC	-	10^8 CFU mL^{-1}	6
5	WSSV (24 h) + VC	30 SID_{50}	10^6 CFU mL^{-1}	6
6	WSSV (24 h) + VC	30 SID_{50}	10^7 CFU mL^{-1}	6
7	WSSV (24 h)+ VC	30 SID_{50}	10^8 CFU mL^{-1}	6
8	WSSV (48 h) + VC	30 SID_{50}	10^6 CFU mL^{-1}	6
9	WSSV (48 h) + VC	30 SID_{50}	10^7 CFU mL^{-1}	6
10	WSSV (48 h) + VC	30 SID_{50}	10^8 CFU mL^{-1}	6
11	Control	-	-	6

WSSV = White spot syndrome virus; VC = *Vibrio campbellii*; SID_{50} = Shrimp infectious dose with 50% endpoint; CFU = Colony forming unit; - = mock inoculation.

7.3.7. Statistical analysis: Differences between treatments were evaluated by performing t-test analysis using statistical analysis software SPSS (version 13.0 for Windows). Values in percentages (WSSV-infected cells in stomach epithelium) were ArcSin-transformed to satisfy the requirement for a normal distribution.

7.4. Results

7.4.1. Experiment 1: Dose effect of *V. campbellii* on mortality of WSSV-infected *L. vannamei* (1st run)

Shrimp injected with WSSV only started to die at 48 hpi. At 156 hpi, cumulative mortality had reached 83%. No mortality was observed when shrimp were injected with 10^3, 10^4, or 10^5

CFU shrimp^{-1} of *V. campbellii* but a higher dose (10^6 CFU shrimp^{-1}) resulted in 66.7% cumulative mortality. WSSV-infected shrimp which also had been injected with bacteria typically died at earlier time points than the shrimp in the single treatments. Shrimp in dual treatments receiving bacterial doses of 10^4 CFU or more started to die at 36 hpi and cumulative mortality reached 100 % at 60-96 hpi (Figure 7.1). At 96 hpi, 100% mortality was obtained in dual treatments with WSSV and *V. campbellii* while only 50% was found in shrimp injected with only WSSV. Shrimp injected with WSSV and 10^3 *V. campbellii* did not display an acceleration in their mortality rate.

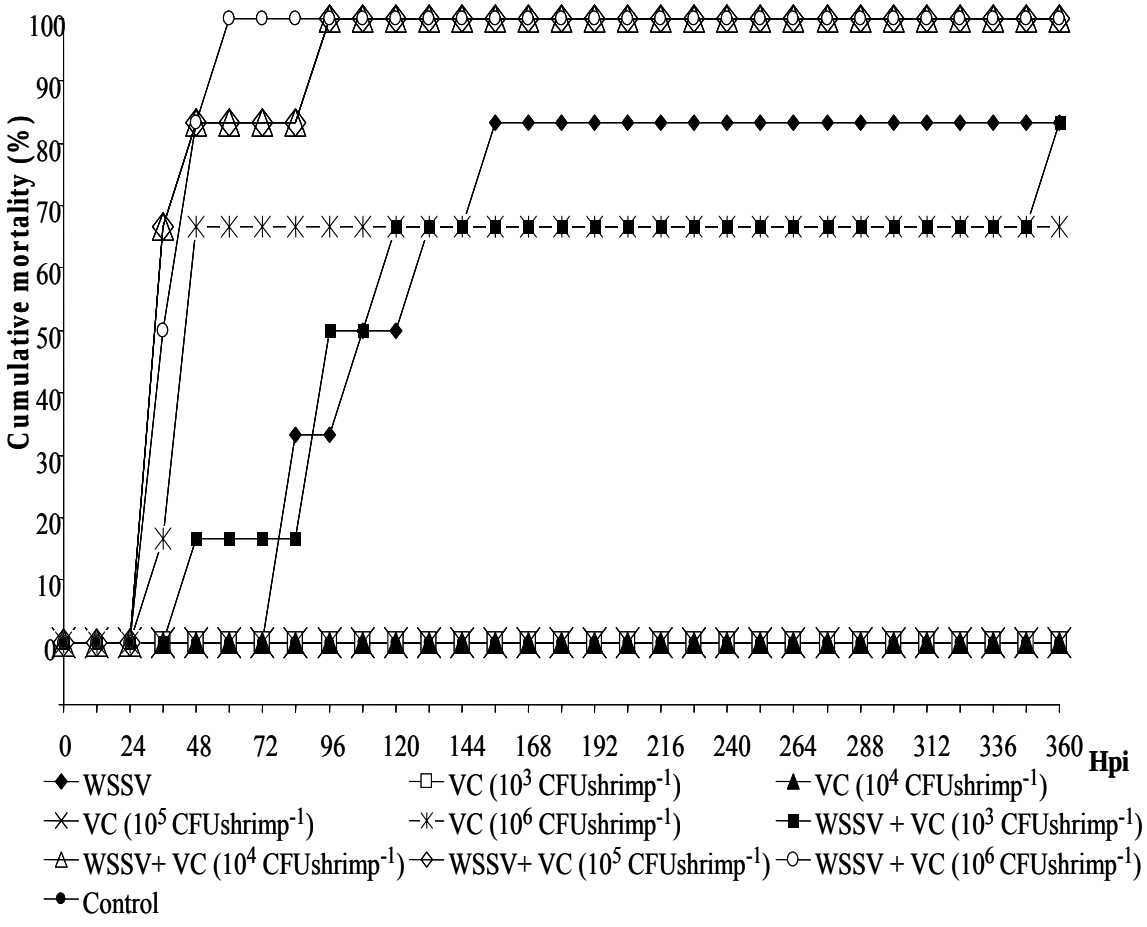

Figure 7.1. Cumulative shrimp mortality (%) after challenge with WSSV and different doses of *V. campbellii* (1st run)

7.4.2. Experiment 2: Dose effect of *V. campbellii* on mortality of WSSV-infected *L. vannamei* (2nd run)

The mortality patterns in the various groups were similar to those in experiment 1 (Figure 7.2). Mortality in the group with WSSV injection only started at 48 hpi and reached 100% at 204 hpi. No mortality was observed in the groups injected with 10^5 CFU or lower quantities of *V. campbellii*. Injection of 10^6 CFU of *V. campbellii* killed approximately 35% of the shrimp. In dual treatments with WSSV and *V. campbellii*, 80-100% mortality was recorded at 96 hpi, while for the shrimp injected with WSSV alone, this was only 33% at that time point. Once again, injections with 10^3 CFU of *V. campbellii* did not have any effect on the mortality in co-infection with WSSV.

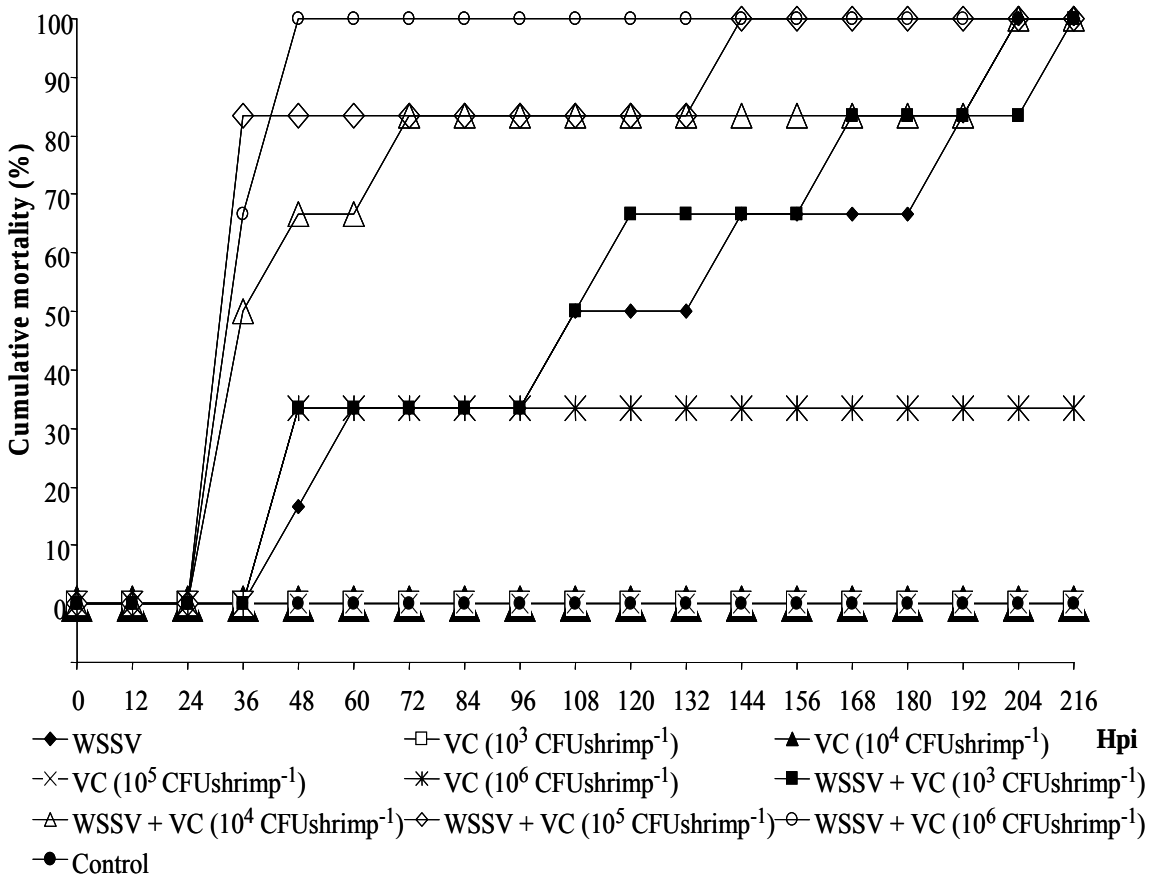

Figure 7.2. Cumulative shrimp mortality (%) after challenge with WSSV and different doses of *V. campbellii* (2nd run)

Enumeration of V. campbellii in the shrimp's haemolymph: In the treatment with *V. campbellii* only, the bacterial density was 126 ± 14 CFU mL^{-1} at 1 h after *Vibrio* injection, and decreased gradually in the following hours. In the haemolymph of dually infected shrimp, the amount of *V. campbellii* was significantly higher than that in shrimp injected with *V. campbellii* only from 3 hpvi onwards (Table 7.4). In the dual treatment, the bacterial density increased spectacularly from 356 ± 204 CFU mL^{-1} haemolymph (1 hpvi) to 8500 ± 4237 (10 hpvi).

Table 7.4. Bacterial density in the haemolymph of euthanized shrimp (CFU mL^{-1} haemolymph; mean ± SD) collected at 1, 2, 3, 4, 6, 8 and 10 hpvi

Time points (hours)	**Treatments**	
	V. campbellii (CFU mL^{-1} of haemolymph)	**WSSV + *V. campbellii*** (CFU mL^{-1} of haemolymph)
1	126 ± 14 (110-135)	356 ± 204 (120-480)
2	90 ± 67 (48-167)	384 ± 205 (192-600)
3	83 ± 55 (28-138)	1037 ± 842 (360-1980)
4	36 ± 26 (20-66)	4466 ± 5193 (250-10267)
6	8 ± 13 (0-23)	5975 ± 6597 (817-17786)
8	8 ± 13 (0-27)	8236 ± 8683 (220-13175)
10	26 ± 45 (0-79)	8500 ± 4237 (3617-11209)

Numbers between brackets are minimum and maximum values of 3 shrimp.

7.4.3. Experiment 3: Co-infection of *L. vannamei* with WSSV and *V. harveyi* BB120

Shrimp injected with WSSV only started to die at 72 hpi. No mortality was observed when shrimp were injected with 10^6 *V. harveyi* BB120 only. As in previous experiments, dually inoculated shrimp with WSSV and *V. campbellii* died quickly after *V. campbellii* injection and reached 100% mortality at 60 hpi.

In contrast with the results obtained with *V. campbellii*, the injection of 10^6 *V. harveyi* BB120 did not accelerate mortality of WSSV-infected shrimp (Figure 7.3).

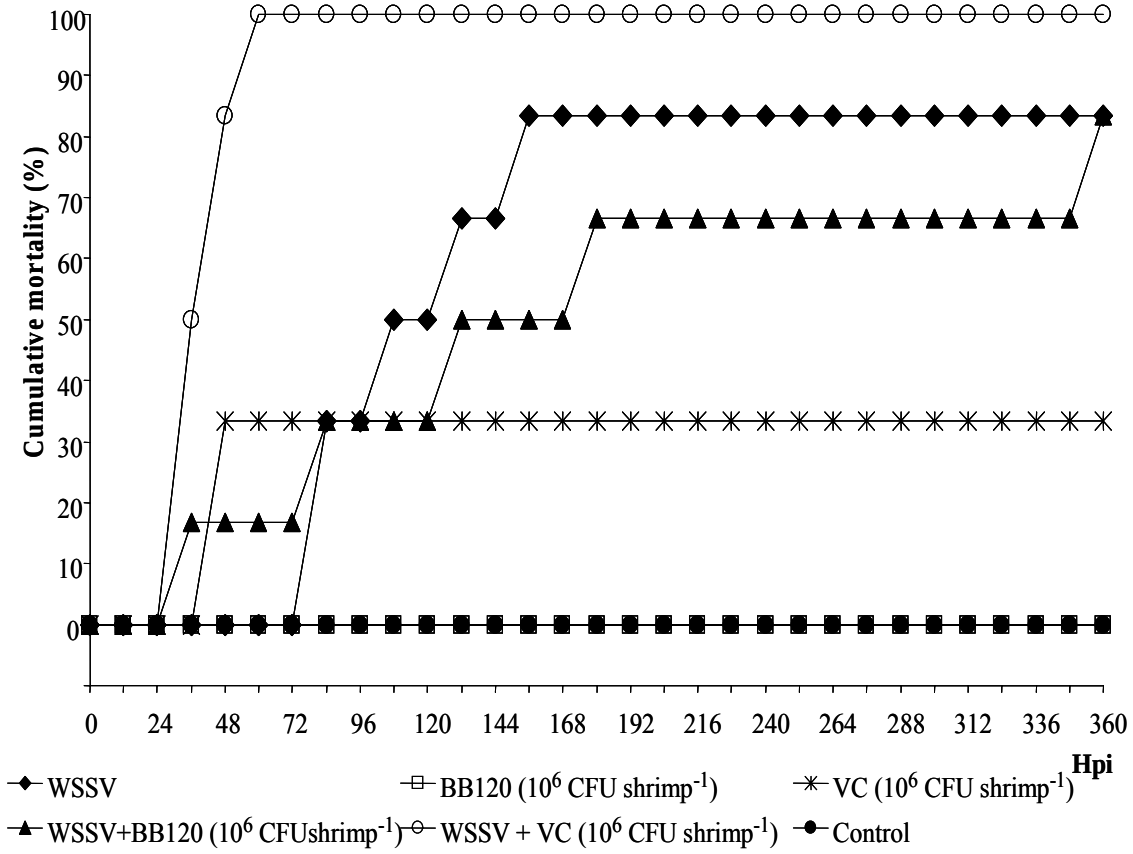

Figure 7.3. Cumulative shrimp mortality (%) after challenge with WSSV and *V. harveyi* BB120

Quantification of WSSV and V. campbellii: Moribund shrimp in dual treatment of this experiment were collected at 6 hpvi for quantification of WSSV and *V. campbellii*. At the same time, shrimp only treated with WSSV or *V. campbellii* were also collected. No significant difference in the number of WSSV-positive cells was found between groups which were administered both pathogens or WSSV alone ($p < 0.01$). The number of WSSV-infected cells in the haematopoietic tissue (10-443 cells mm^{-2}) was higher than that in the gills (12-374 cells mm^{-2}) and the lymphoid organs (2-185 cells mm^{-2}) (Table 7.5). In the stomach epithelium, 1-23% of cells were infected. The number of *V. campbellii* isolated from

shrimp injected with only bacteria was lower than 300 CFU mL^{-1}. In contrast, a very high density of *V. campbellii* (0.8 x 10^5 CFU mL^{-1}) was observed in the haemolymph of shrimp in the dual treatment (Table 7.5).

Table 7.5. Quantification of WSSV-infected cells and *V. campbellii* (mean ± SD) in gills (G), stomach epithelium (SE), lymphoid organ (LO) and haematopoietic tissue (HP) of shrimp collected 6 h after *V. campbellii* injection (shrimp in dual treatment were moribund)

Treatments	WSSV-infected cells in organs				VC (CFU mL^{-1} of haemolymph)
	G (cells mm^{-2})	SE (%)	LO (cells mm^{-2})	HP (cells mm^{-2})	
WSSV	189 ± 130^a (12 - 374)	14 ± 9^a (1 - 22)	59 ± 72^a (2 -185)	210 ± 154^a (10 - 443)	-
VC	-	-	-	-	231 ± 445^a (0 -1131)
WSSV + VC	183 ± 51^a (127 - 251)	15 ± 6^a (6 - 23)	39 ± 18^a (21 - 60)	143 ± 86^a (10 - 220)	83430 ± 66871^b (13800 - 172125)

G = Gills; SE = Stomach epithelium; HP = Haematopoietic tissue; LO = Lymphoid organ. Numbers between brackets are minimum and maximum values of 6 shrimp. Numbers of infected cells in the same tissue or CFU mL^{-1} with different superscripts were significantly different between the two treatments ($p < 0.01$).

7.4.4. Experiment 4. Immersion challenge of WSSV-infected *L. vannamei* with different doses of *V. campbellii*

In this experiment, mortalities were only obtained in shrimp injected with WSSV. Shrimp injected with WSSV only started to die at 48-60 hpi and reached 100% cumulative at 144-168 hpi. Immersion challenge with 10^6, 10^7 or 10^8 CFU mL^{-1} of *V. campbellii* only did not cause any mortality in *L. vannamei* juveniles (Figure 7.4). Shrimp injected with WSSV and challenged by immersion 24 h or 48 h later with different doses of *V. campbellii*, did not show any significant acceleration in mortality compared to single WSSV treatment

(Figure 7.4 & 7.5) The first dead shrimp was observed at 48 h, the time at which WSSV-infected shrimp were challenged with 10^6 or 10^8 CFU of *V. campbellii* (Figure 7.4).

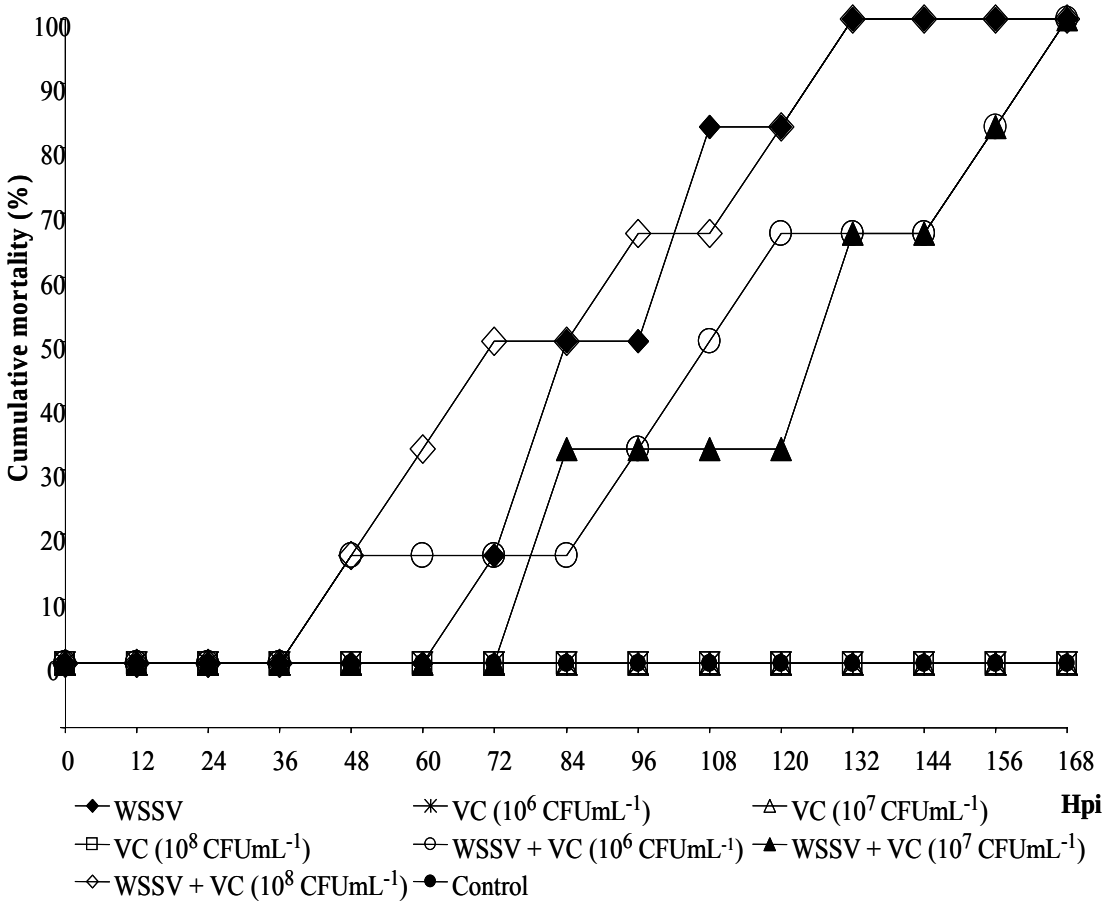

Figure 7.4. Cumulative shrimp mortality (%) after immersion in *V. campbellii* 24 h after WSSV injection

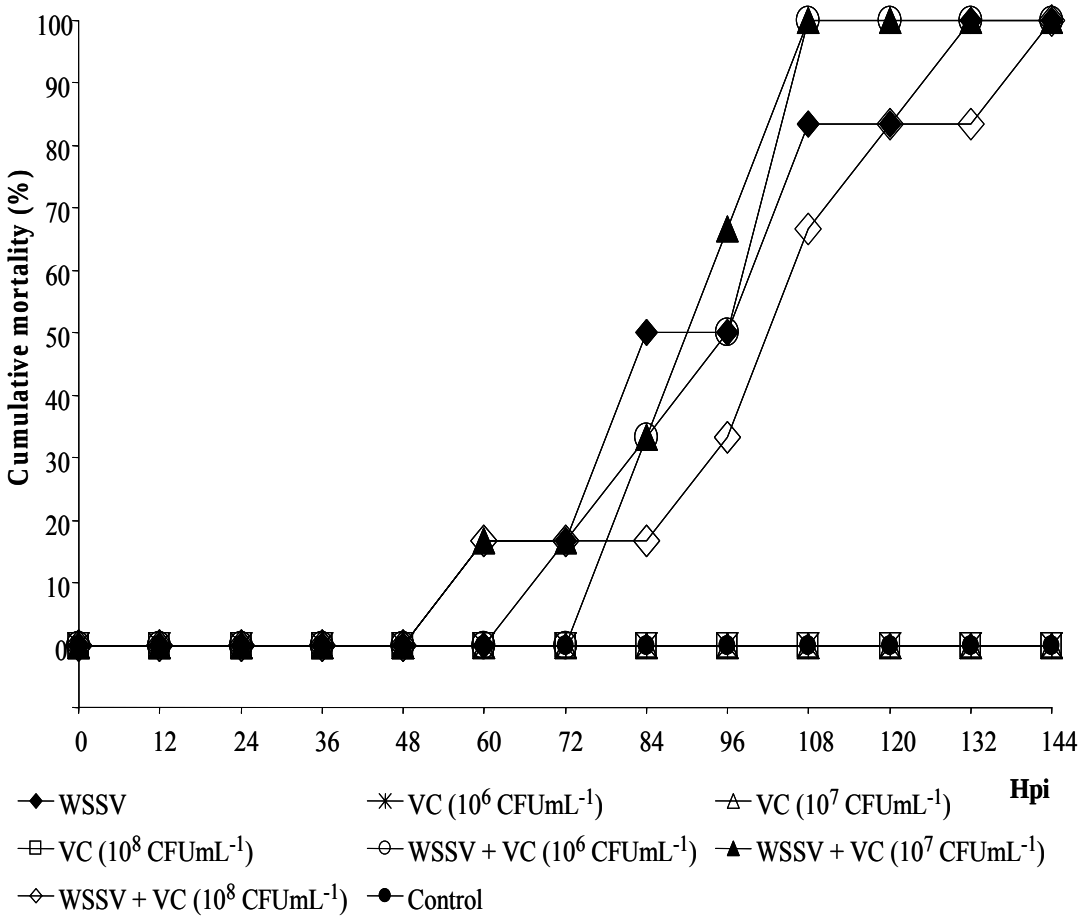

Figure 7.5. Cumulative shrimp mortality (%) after immersion in *V. campbellii* 48 h after WSSV injection

7.5. Discussion

Previous experiments revealed an acceleration in the mortality rate of *L. vannamei* shrimp when dually infected with WSSV and *V. campbellii* (Phuoc *et al.*, accepted 2008). The current study was conducted to determine the threshold dose of *V. campbellii* which can still produce accelerated mortality upon injection in WSSV-compromised shrimp. As in our previous study, shrimp injected with WSSV only started to die between 48 and 84 hpi and cumulative mortality reached 100% at 144-336 hpi. When only *V. campbellii* was injected, a dose as high as 10^6 CFU was needed to kill 30-60% of the experimental animals. A clear acceleration in the mortality rate of WSSV-infected shrimp was observed after inoculation

with *V. campbellii*. Shrimp receiving these dual inoculations started to die at 36-48 hpi and cumulative mortality reached 100% at 48-96 hpi. Dual inoculations only resulted in faster mortality when at least 10^4 CFU of *V. campbellii* was administered.

In this study, the lethal *Vibrio* dose for WSSV-compromised shrimp was a 100-fold lower than for WSSV-free shrimp. Lee *et al.* (1999b) reported an increase in mortality of grouper (*Epinephelus* sp.) when challenged with *Vibrio carchariae* by immersion or injection after being already infected with infectious pancreatic necrosis virus for 2 weeks. This kind of virus-bacteria interaction was also described by Pakingking *et al.* (2003) and Oh *et al.* (2006). So far, virus-virus or virus-bacteria co-infections in shrimp ponds have been described (Mohan *et al.* 1998; Selvin and Lipton, 2003; Umesha *et al.*, 2006) but no experimental studies on co-infections in shrimp have been published. The present study is the first to substantiate the existence of a synergistic effect on mortality of shrimp caused by dual infection under experimental conditions.

In an attempt to uncover the reason behind the accelerated mortality due to dual infection, WSSV and *V. campbellii* were quantified inside the shrimp. By immunohistochemistry, WSSV-infected cells were detected in gills, lymphoid organ, haematopoietic tissue and stomach epithelium. These organs were identified as major target organs for replication of WSSV (Chang *et al.*, 1996) and were also previously selected by Escobedo-Bonilla *et al.* (2007) and Rahman *et al.* (2008, accepted) for enumeration of WSSV-infected cells. The quantification results showed some variation between the organs, but did not show any significant difference in the number of WSSV-infected cells between the single and dual infection treatments. As monoclonal antibodies for *V. campbellii* were not available, re-isolation was chosen as alternative method to quantify *V. campbellii* in shrimp's haemolymph. Plating methods have been applied with success in previous studies

(Mermound *et al.*, 1998; Van de Braak *et al.*, 2002c). Normally, shrimp possess a fast clearing mechanism to eliminate bacteria from their body. Martin *et al.* (1996) reported that radio-labeled *Bacillus subtilis* injected into the haemolymph of the Ridgeback shrimp *Sicyonia ingentis* were cleared rapidly as bacteria were phagocytized and degraded by haemocytes within the first hour after injection. In a similar study by van de Braak *et al.* (2002b), the concentration of live bacteria in the haemolymph decreased by 97% in between 5 min and 2 h after injection. The present findings are in accordance with these earlier reports. Very low *V. campbellii* densities were detected in the haemolymph of shrimp only challenged with *V. campbellii*. It appears that these shrimp had sufficient clearing capacity and managed to eliminate most of the *V. campbellii* shortly after injection. On the other hand, the density of *V. campbellii* was much more elevated in the haemolymph of euthanized co-injected shrimp than in shrimp injected with *V. campbellii* only. The amount of *V. campbellii* in the haemolymph of co-injected shrimp was already 3 times higher 1 h after injection and continued to increase up to 10 hpvi (Table 7.3). Additionally, plating countings were done from haemolymph samples of moribund shrimp collected at 6 hpvi (30 hpi of WSSV). Here, the difference was even more spectacular. It is therefore postulated that the bacterial clearing capacity of shrimp is severely compromised by a WSSV infection.

The hypothesis that WSSV undermines this mechanism is supported by the findings of Selvin and Lipton (2003) who reported that a primary WSSV infection probably weakened shrimp and made them more susceptible to bacterial infection. Moreover, Mathew *et al.* (2007) found significant reductions in the number of circulating haemocytes and in the activities of phenoloxidase, glutathione-dependent antioxidant enzymes and antiperoxidative enzymes of WSSV-infected shrimp. The authors supposed that the drop in circulating haemocytes of WSSV-infected shrimp allowed the proliferation of *V. campbellii* inside shrimp since haemocytes are responsible for encapsulation and phagocytosis of bacteria. Wongprasert *et*

al. (2003) and van de Braak *et al.* (2002c) also found a significant drop in the number of circulating haemocytes of *P. monodon* shrimp after WSSV injection.

In this study, the absence of a significant difference in WSSV-infected cells between single and dually infected shrimp and the rapid proliferation of *V. campbellii* in dually infected shrimp, strongly suggested that dually infected shrimp died because of *Vibrio* proliferation. Using the dual infection strategy, the virulence of another bacterial strain, *V. harveyi* BB120, was tested. A first test showed that the wild-type *V. harveyi* BB120 was not able to cause any mortality in single-injected shrimp at a dose of 10^6 CFU. Since the same dose in WSSV-compromised shrimp did not cause any accelerated mortality, the clinical outcome of co-injection must be strain dependent. The dual infection protocol using a weakly virulent WSSV strain, as it is used in the present study, might be an elegant tool to determine the virulence of *Vibrio* strains as secondary pathogens.

Next to the susceptibility to *V. campbellii* infection by intramuscular route, infection by immersion route was also evaluated in WSSV-compromised shrimp. No co-infection was observed when WSSV-compromised shrimp were challenged by immersion with different doses of *V. campbellii*, even with doses as high as 10^8 CFU mL^{-1}. Results were the same when *Vibrio* was added 24 or 48 h after WSSV injection. This outcome illustrates a weakness of the dual infection protocol presented in this study: both WSSV and *Vibrio* need to be injected to achieve the synergistic effect. Although this protocol might provide data on the virulence of *Vibrio* strains, it does not take into account that under natural conditions, bacteria need to overcome certain protective barriers before they can invade shrimp. The results obtained here confirm those described by Pakingking *et al.* (2003) who could not find any significant difference in cumulative mortality of fish between the control group and groups co-challenged with marine birnavirus (MABV-F) by injection and *V. harveyi* or *E. tarda* by

immersion. In contrast, when co-infection was established by injecting virus and bacteria, cumulative mortality reached more than 90%.

The fact that immersion challenge with *Vibrio* in WSSV-compromised shrimp did not result in co-infection raises questions about the possible mechanisms involved in co-infection in the field as observed by many researchers. With the current knowledge, it is supposed that outbreaks of bacterial disease in shrimp ponds are the result of complex interactions. The status of the hosts aside, occurrence of bacterial disease is determined by 2 main factors: physical stressors and other pathogens which make the way. The former comprise fluctuations in the water (salinity, temperature, pH, alkalinity), deteriorating water quality, high stocking densities, injuries and cannibalism (Kautsky *et al.*, 2000; Fegan and Clifford, 2001; Kiran *et al.*, 2002). The latter can be a myriad of primary and secondary pathogens. Our results proved that WSSV can damage some internal tissues, but that damage is apparently not sufficient to allow *Vibrio* infection from the water. It is possible that the shrimp's immune system was not undermined enough by WSSV to prevent it from responding efficiently to the bacterial challenge. Moreover, shrimp are fully covered by cuticle except for the midgut epithelium, which seems to be refractory to WSSV infection. This possible entry route for the bacteria would then not be directly weakened by the viral replication, while the cuticular epithelia are not accessible. The absence of multiple stress factors in experimental designs could explain the difficulty to experimentally infect shrimp with *Vibrio* by immersion challenge in contrast with field conditions. Since no mortality was observed when shrimp were co-injected with WSSV and 10^3 CFU of *V. campbellii*, we hypothesise that the amount of bacteria able to penetrate into the shrimp's body remained below this threshold level.

In conclusion, the clinical outcome of WSSV and *V. campbellii* co-infection in SPF *L. vannamei* shrimp depends on the bacterial dose, strain and challenge route. An intramuscular dose of 10^4 CFU shrimp^{-1} of *V. campbellii* can be considered as the threshold to enhance the mortality rate of WSSV-compromised shrimp. Injection of *V. campbellii* did not cause any increase of WSSV replication, but in WSSV-compromised shrimp, the density of *V. campbellii* in the haemolymph increased spectacularly when compared to only *Vibrio*-challenged animals. Immersion challenge with *V. campbellii* did not result in any mortality, not even in WSSV-infected shrimp. Not all *Vibrio* strains are pathogenic, as demonstrated by the lack of mortality caused by co-challenge with strain BB120. This last observation opens the possibility to determine the virulence of *Vibrio* strains using the dual infection model.

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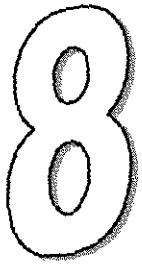
CHAPTER



General discussion, conclusion and future research

8.1. General discussion

In recent years, *Litopenaeus vannamei* became a predominant species in shrimp culture in many countries around the world, especially in Southeast Asia. This booming of penaeid shrimp culture has been accompanied by an increased incidence of infectious diseases. Infections with virus, bacteria, protozoa, and fungi have a devastating effect on shrimp culture (Gabriel and Felipe, 2000). Besides white spot syndrome virus, one of the most important diseases in penaeid shrimp culture in the world, *Vibrio* species cause a big problem in shrimp culture resulting in low survival in both hatcheries and grow-out ponds (Saulnier, 2000b). In the Philippines, virulent *V. harveyi* strains have caused 100% losses in the larval production of *Penaeus monodon* (Lavilla Pitogo *et al.*, 1990). In 2006, a septicemic vibriosis due to *Vibrio nigripulchritudo* was identified in *Litopenaeus stylirostris* shrimp. This new disease, "Summer syndrome", was responsible for major losses in intensive shrimp farms in New Caledonia (Goarant *et al.*, 2006).

In general terms, the outcome of the host-pathogen interaction depends on the condition of the host and the virulence of the pathogen. Many factors are involved in determining the condition of the host such as abiotic, biotic stress, the age of the animals, management conditions, burden of pathogens (Figure 8.1). These factors can influence the susceptibility of the shrimp to pathogens. Since vibrios are mainly opportunistic bacteria (Saulnier *et al.* 2000b), although pathogenic *Vibrio* seems to exist as well, it is assumed that stress factors such as pH, salinity, ammonia play an important role in the outbreak of vibriosis. According to Lemonnier *et al.* (2006), temperatures higher than 23-24 °C seem to be necessary for disease development. They found the first dead shrimp of the outbreak during a period of increasing or decreasing temperature. The feed input and feed quality also have an effect on the host-microbial interaction. The increase of feed input, the percentage of nitrogen in the

feed, and the limitation of water exchange can induce an increase in environmental waste production resulting in eutrophication of water and the outbreaks of mortality (Lemonnier and Faninoz, 2006). The susceptibility of shrimp to *Vibrio* also depends on the age of animals. The older animals might better resist to *Vibrio* infection. Ambient ammonia decreased the resistance of the shrimp to *Vibrio* infection (Liu and Chen, 2004). Crowding (high stocking densities) in the shrimp ponds can increase the risk of transmission of the disease within the pond by cannibalism (Fegan and Clifford, 2001). Hence, it is likely that under field conditions disease outbreak in general and vibriosis in particular is the result of many interacting factors. Also in every disease outbreak a different combination of factors might be responsible. Under laboratory conditions the multitude of interfering factors might be less complex.

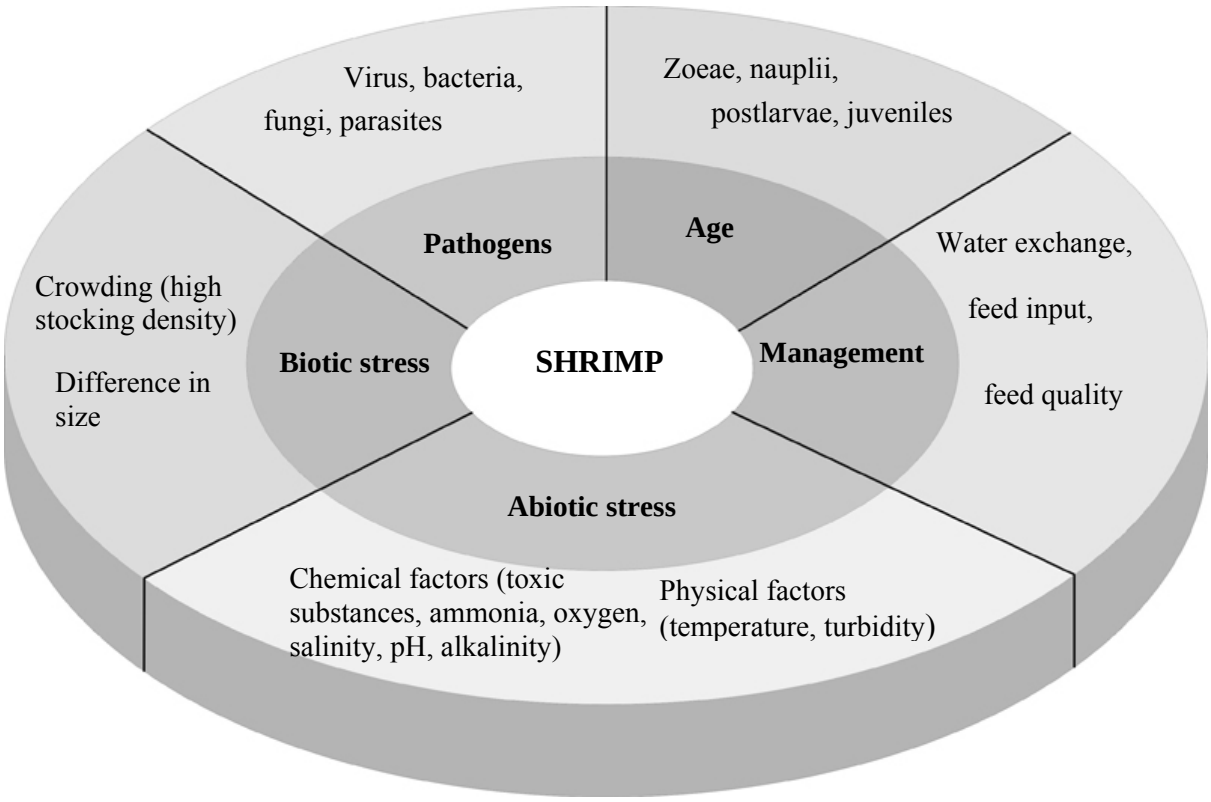


Figure 8.1. Factors influencing the susceptibility of shrimp to pathogens

Beside biotic and abiotic stresses, it is assumed that the burden of pathogens can compromise the host and facilitate disease outbreaks. Recently, many shrimp farms in many countries are using SPF instead of non-SPF shrimp. SPF and SPR shrimp are more able to tolerate high density and low salinity, driving their rapid introduction in commercial shrimp farming (Singh and Lakra, 2008). The use of SPF shrimp can reduce the risk associated with vibriosis and the risk of introducing exotic pathogens (Flegel, 2006). The harvest of *L. vannamei* in Korea has been very satisfactory since SPF broodstocks of *L. vannamei* were introduced from Hawaii in 2004. The growth rate was 30% to 50% higher compared to *Fenneropenaeus chinensis* and the frequency of viral outbreaks was much lower than in *F. chinensis* (Jang and Jun, 2005). Thailand, one of the top ten countries in shrimp aquaculture production, is successful with using SFP shrimp. Wyban (2007) reported that a key factor of Thailand's success with *L. vannamei* is their controlled broodstock imports to ensure sufficient supplies of true SPF *L. vannamei* broodstock. Thailand's revolution in shrimp farming largely benefited from the domestication and breeding of SPF *L. vannamei* by US companies.

In laboratory conditions, a lack of interaction of many stress factors with the host and pathogens can result in a different disease outcome as compared to the field conditions. Yet, even under laboratory conditions, a standardized challenge protocol is necessary for studying shrimp diseases. The *Vibrio* challenge in SPF shrimp in this study allows for rigorous verification of the effect of several factors on the outcome of a challenge. A standard protocol for *Vibrio* challenge also facilitates the study on vibriosis.

Although poorly supported by hard evidence, it is anticipated that pathogen burden can be the source of large differences in the outcome of a challenge test (with e.g. vibrios) between laboratories, jeopardizing sound interpretation of the results. Many data presented in literature on the outcome of a *Vibrio* challenge are obtained with non-SPF shrimp. The results

on the dual infection with WSSV and *Vibrio* presented in chapter V and VI and the single infection with *Vibrio* indicate that conclusions in relation to virulence of *Vibrio* species should only be made in the full awareness that no other pathogens interfere with the outcome of the *Vibrio* challenge assay. In this study, many parameters were tested for their influence on the outcome of a *Vibrio* challenge in SPF shrimp. For instance, stress factors (starvation, salinity, ammonium), WSSV burden, different bacterial strains (namely *V. campbellii* LMG21363, *V. harveyi* 642, *V. harveyi* E022, *V. harveyi* E2, and *V. penaeicida*) were taken into consideration, as well as the challenge route. The immersion challenge in unstressed shrimp did not cause mortality. Moreover, removal of a pleopod and physical damage to the cuticle concurrent with the immersion challenge with a very high dose of *V. campbellii* (10^8 CFU mL^{-1}) also did not result in mortalities. It can be concluded from this study that SPF shrimp are very resistant to *Vibrio* by immersion or injection even if they are stressed. Under ammonium stress, SPF shrimp appear susceptible to pathogenic *Vibrio* when injected with at least 10^6 CFU $shrimp^{-1}$. Among the five tested strains, *V. campbellii* showed the highest virulence.

Another factor, namely WSSV burden, was used as interfering factor in testing the susceptibility of SPF shrimp to *Vibrio*. The aim of this study was to investigate whether an accelerated mortality can be obtained when WSSV-compromised shrimp is challenged with *V. campbellii*. It needs to be stressed here that this dual challenge can only be performed with a low virulent WSSV strain. Highly virulent WSSV strains kill the shrimp swiftly (within 36-84 hpi) (Escobedo-Bonilla *et al*., 2006; Rahman *et al*., accepted) undoing the possibility of seeing an effect of a *Vibrio* challenge. Shrimp injected with only *V. campbellii* showed no mortality when the injected dose was 10^5 CFU *V. campbellii* $shrimp^{-1}$ or lower. However, WSSV-infected shrimp appear to be severally compromised and become more susceptible to *Vibrio*. Very clear differences in mortality between single and dual treatments were observed

in the time period between 12-48 hours post *V. campbellii* injection (hpvi). Shrimp in the dual injection treatments died very quickly after being injected with *V. campbellii*. Since sonicated and dead *V. campbellii* did not cause any increase in mortality of WSSV-infected shrimp, the possibility of an increased sensitivity to a toxin was excluded. Rather, in WSSV-compromised shrimp, the rapid replication of *V. campbellii* is likely to be the cause of the accelerated mortality. This was verified by quantifying WSSV-infected cells in different shrimp's organs and enumerating *V. campbellii* in the shrimp's haemolymph. In the treatment with *V. campbellii* injection only, bacterial densities in the haemolymph dropped rapidly within the first 3 hpvi. The density continuously decreased in the following hours and a very low density of *V. campbellii* was detected in the shrimp's haemolymph 10 hpvi (26 CFU mL^{-1}). On the other hand, the *V. campbellii* load in the haemolymph of dual inoculated shrimp dramatically increased from 1 to 10 hpvi. At the latter time point, the *V. campbellii* load in the dual inoculated shrimp's haemolymph was more than 10^2 times higher than that in shrimp which received bacteria only. It seems that bacteria were cleared quickly from the haemolymph of shrimp injected with *V. campbelli* only. WSSV-compromised shrimp fail to clear *V. campbellii* but rather are conducive to their rapid proliferation. According to Wongprasert *et al.* (2003), the total haemocyte count of *P. monodon* shrimp significantly drops 12 to 36 hours post WSSV injection. Hence it is likely that post WSSV infection the haemocyte count drops dramatically, which as such is not a life-threatening situation, at least in the early phase, paving the way for a rapid proliferation of *Vibrio* when a virulent strain is capable of invading the body (here experimentally simulated by injecting *Vibrio*). Surely, it would be interesting to support this model with immunological data.

Although co-infection of *V. campbellii* and WSSV is causing accelerated mortality, at least 10^4 CFU of *V. campbellii* need to be injected in WSSV-compromised shrimp in order to obtain accelerated mortality. Immersion challenge with 10^8 CFU mL^{-1} of *V. campbellii* did not show any co-infection in WSSV-compromised shrimp. Hence, it seems that even in

WSSV-compromised shrimp, 10^4 CFU of *Vibrio* cannot penetrate into the host causing disease. In fact, our results proved that WSSV can damage some internal tissues, but that damage is still not sufficient to allow for *Vibrio* infection by immersion. This result raises the question on the minimal amount of multiple stress factors (biotic and abiotic) that are needed in the field to cause the frequently observed outbreaks of vibriosis. Perhaps under field conditions many stress factors, absent under laboratory conditions, contribute to the possibility of *Vibrio* infection. The absence of mortality in the immersion challenge with *V. campbellii* might relate to the SPF status of the shrimp, the virulence of the *Vibrio* strains and/or a lacking of interaction of stress factors, pathogens and the host.

Another bacterial strain, *V. harveyi* BB120, was also tested for dual infection with WSSV. In contrast with *V. campbellii*, shrimp injected with 10^6 CFU shrimp^{-1} of *V. harveyi* BB120 did not show any increase in mortality in WSSV-compromised shrimp while clear co-infection was obtained when using the same dose of *V. campbellii* in co-infection experiments. It is suggested that more bacterial strains need to be tested to verify whether co-infection of WSSV and bacteria is strain dependent. Also, further research can establish whether co-infection with WSSV and *Vibrio* using SPF shrimp can help in determining the virulence of *Vibrio* strains towards shrimp.

This study also reveals that a loss of virulence due to phenotypic switch can occur in *Vibrio* when cultured under specific laboratory conditions. At least in *V. campbellii* this phenotype switch is concomitant with the loss of the luminescence and the production of different type of exopolymers. It remains to be established whether this phenomenon is relevant in the field.

8.2. Conclusions

- SPF shrimp become susceptible to opportunistic pathogenic *Vibrio* through injection of 10^6 CFU shrimp^{-1} after an initial ammonium stress of 50 mgL^{-1} during 12 h, followed by a continuous ammonium exposure to 20 mgL^{-1}.

- An injection with live *V. campbellii* 24 h after a WSSV injection clearly increased mortality in WSSV-compromised SPF *L. vannamei* shrimp. The clinical outcome of co-infection with WSSV and *V. campbellii* in SPF shrimp depends on the dose of *V. campbellii* and challenge routes. 10^4 CFU shrimp^{-1} of *V. campbellii* can be considered as a threshold dose to produce co-infection in WSSV-compromised shrimp. In contrast with injection, immersion challenge with *V. campbellii* did not result in any co-infection in WSSV-compromised shrimp. Not all *Vibrio* strains are as virulent as demonstrated by the lack of mortality by co-challenge with strain BB120 in WSSV-compromised shrimp.

- In the laboratory conditions, luminescent strains can switch to non-luminescent strains as a result of culturing them in static conditions. A decrease in virulence is associated with the loss of luminescence. Osmotic stress can result in a drop in colony forming ability of *Vibrio* on selective medium. For enumerating *Vibrio* strains on selective media, field samples should be handled in an isotonic way.

8.3. Future research

The study raises the following questions which could be dealt with in future research

(1) Under what conditions can *Vibrio* infect the host (compromised or not with WSSV)?

(2) What is the infection route for *Vibrio* and WSSV as immersion challenge does not seem to produce mortality?

(3) In which way shrimp become compromised by WSSV infection facilitating the development of *Vibrio*?

(4) What are the immunological parameters that need to be tested in order to back up the observations made in this study?

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SUMMARY

SAMENVATTING

SUMMARY

Shrimp farming has developed and expanded in many countries worldwide. However, disease outbreaks are regularly jeopardising crops. Infectious diseases, especially white spot syndrome virus (WSSV) and vibriosis, have caused huge economic loss in shrimp culture in many countries. The availability of specific pathogen-free (SPF) is now facilitating the development of standardized protocols for pathogen challenge, as established recently for WSSV. Hence, a protocol for *Vibrio* challenge in SPF shrimp was standardized. Shrimp from postlarve to juvenile were challenged with five different *Vibrio* strains namely *V. campbellii*, *V. harveyi* 642, *V. harveyi* E022, *V. harveyi* E2 and *V. penaeicida*. Many stress factors were applied such as starvation, salinity and ammonium stress. In absence of stress, SPF shrimp were not susceptible to *Vibrio* neither by immersion nor by injection. Only under ammonium stress, SPF shrimp became susceptible to infection by *Vibrio* through injection of 10^6 CFU shrimp^{-1} after an initial ammonium stress of 50 mgL^{-1} during 12 h, followed by a continuous ammonium exposure to 20 mgL^{-1}.

Another factor, namely WSSV burden, was used as interfering factor in testing the susceptibility of SPF shrimp to *Vibrio*. Shrimp were first injected with 30 SID_{50} (SID_{50} = Shrimp Infectious Dose with 50% endpoint) of WSSV and 24 h later with 10^6 CFU shrimp^{-1} of *V. campbellii*. Shrimp injected with only WSSV started to die at 60 hours post injection (hpi) and cumulative mortality reached 100% at 168-336 hpi. On the other hand, shrimp in the dual treatment died earlier (36 hpi) and cumulative mortality reached 100% at 72-96 hpi. In WSSV-compromised shrimp, the proliferation capacity of *V. campbellii* is enhanced considerably. The density of *V. campbellii* in the haemolymph of WSSV-compromised shrimp collected 6 and 10 hours after *V. campbellii* injection was between 10^2 and 10^3-fold

higher than in shrimp injected with *V. campbellii* only ($p < 0.01$). In contrast, there was no difference in WSSV replication between shrimp inoculated with WSSV only compared to dually inoculated groups. A dose of 10^4 CFU shrimp^{-1} of *V. campbellii* can be considered as the threshold needed for accelerated mortality of WSSV-compromised shrimp. Shrimp which were first injected with WSSV and 24 h (or 48 h) later exposed to 10^6, 10^7, or 10^8 CFU mL^{-1} of *V. campbellii* by immersion did not show any accelerated mortality. It was verified that the clinical outcome of WSSV and *V. campbellii* co-infection in SPF *L. vannamei* shrimp depends on the bacterial dose, strain and challenge route.

In the laboratory conditions, osmotic stress can result in a drop in colony forming ability of *Vibrio* on selective medium (Thiosulfate Citrate Bile Sucrose, TCBS), a phenomenon that results in erroneous enumerations. Non-luminescent (NL) vibrios can be produced by culturing luminescent (L) strains in the dark under static conditions. The difference in colony appearance of L and NL isogenic *V. campbellii* was substantiated by the different staining on Marine Agar with Congo red (MACR). The virulence of the L and NL isogenic *V. campbellii* and *V. harveyi* BB120 were tested in gnotobiotic *Artemia franciscana* larvae challenged with 10^4 CFU mL^{-1} of bacteria. There was a significant difference in mortality between the L and NL challenged groups ($p < 0.05$). The NL-strains showed less virulent compared to L ones. In contrast with *Artemia*, there was no significant difference in mortality between the juvenile shrimp groups challenged by injection with L and NL strains. The phenomenon of phenotype switching in *Vibrio* warrants further research.

In conclusion, the outcome from this study revealed that SPF shrimp were not susceptible to *Vibrio* neither by immersion nor by injection in absence of stress. However, they can be made more susceptible by abiotic stress (NH_3) and biotic stress (WSSV burden). This study is the first to experimentally reproduce synergistic effect between a viral and a bacterial pathogen

in shrimp. The results corroborate findings of polymicrobial diseases in the field. Luminescent strains can switch to non-luminescence by being cultured in static condition. Concomitant with the loss of luminescence, the NL strain stains less on MACR and becomes less virulent.

SAMENVATTING

De kweek van garnalen heeft zich ontwikkeld en uitgebreid in vele landen over de hele wereld. Niettemin wordt de oogst vaak in gevaar gebracht door het uitbreken van ziekten. Infectieziekten, vooral 'white spot syndrome virus' (WSSV) en vibriosis, hebben in vele landen enorme economische verliezen in de garnalenkweek veroorzaakt. De beschikbaarheid van specifieke pathogeenvrije garnalen (SPF) maakt het nu gemakkelijker om gestandardiseerde protocols te ontwikkelen voor de blootstelling aan pathogenen, zoals recent nog uitgewerkt voor WSSV. Daarom werd een protocol gestandardiseerd voor blootstelling aan *Vibrio* in SPF-garnalen. Garnalen, van het postlarvale tot het juveniele stadium, werden blootgesteld aan vijf verschillende *Vibrio*-stammen, namelijk *V. campbellii, V. harveyi* 642, *V. harveyi* E022, *V. harveyi* E2 en *V. penaeicida.* Talrijke stressfactoren werden toegepast, zoals uithongering, en saliniteits- en ammoniumstress. Bij afwezigheid van stress waren SPF-garnalen niet vatbaar voor *Vibrio*, noch door immersie, noch door injectie. Alleen bij ammoniumstress werd SPF-garnaal vatbaar voor infectie door *Vibrio* door injectie van 10^6 CFU per garnaal, na een initiële ammoniumstress van 50 mgL^{-1} gedurende 12 u, gevolgd door een continue blootstelling aan ammonium aan 20 mgL^{-1}.

Een andere factor, namelijk de belasting met WSSV, werd gebruikt als een interfererende factor bij het testen van de vatbaarheid van SPF-garnalen voor *Vibrio*. Garnalen werden eerst geïnjecteerd met 30 SID_{50} (SID_{50} = garnaal-infectiedosis met 50% eindpunt) van WSSV, en 24 u later met 10^6 CFU per garnaal van *V. campbellii*. Bij garnalen, alleen geïnjecteerd met WSSV, begon sterfte op te treden 60 uur na de injectie (hpi), en de cumulatieve mortaliteit liep op tot 100 % bij 168-336 hpi. Anderzijds trad er bij de tweevoudige behandeling vroeger sterfte op (36 hpi), en de cumulatieve mortaliteit bereikte 100 % bij 72-96 hpi. Bij garnaal

besmet met WSSV was de groeicapaciteit van *V. campbelli* aanzienlijk toegenomen. De densiteit van *V. campbellii* in de hemolymfe van met WSSV besmette garnalen, verzameld 6 en 10 u na injectie met *V. campbellii*, was 10^2 tot 10^3 maal hoger dan in garnaal geïnjecteerd met *V. campbellii* alleen ($p < 0,01$). Er was daarentegen geen verschil in WSSV-replicatie tusssen garnaal besmet met WSSV alleen, en groepen blootgesteld aan tweevoudige besmetting. Een dosis *V. campbellii* van 10^4 CFU per garnaal kan beschouwd worden als de drempel nodig voor een versnelde mortaliteit van garnaal besmet met WSSV. Garnaal, eerst geïnjecteerd met WSSV, en 24 u (of 48 u) later blootgesteld aan 10^6, 10^7 of 10^8 CFU mL^{-1} van *V. campbellii* door middel van immersie, vertoonde geen versnelde mortaliteit. Er werd vastgesteld dat het klinisch resultaat van co-infectie met WSSV en *V. campbellii* in SPF *L. vannamei* afhangt van de bacteriële dosis en stam, en van de wijze van blootstelling.

In laboratoriumomstandigheden kan osmotische stress leiden tot een afname in de mogelijkheid van *Vibrio* tot kolonievorming op een selectief medium (Thiosulfate Citrate Bile Sucrose, TCBS), een fenomeen dat resulteert in foutieve tellingen. Niet-luminescente (NL) *Vibrio* kan geproduceerd worden door het kweken van luminescente (L) stammen in het donker bij statische omstandigheden. Het verchil in kolonie-type tussen L en NL isogene *V. campbellii* ging gepaard met een verschillende kleuring op mariene agar met Kongorood (MACR). De virulentie van de L en NL isogene *V. campbellii* en *V. harveyi* BB120 werd getest in gnotobiotische *A. franciscana*-larven, blootgesteld aan 10^4 CFU mL^{-1} aan bacteriën. Er was een significant verschil in mortaliteit tussen de aan L en NL blootgestelde groepen ($p < 0.05$). De NL-stammen vertoonden minder virulentie dan de L-stammen. In tegenstelling met *Artemia* was er geen significant verschil in mortaliteit tussen de juveniele groepen garnalen, blootgesteld aan L- en NL-stammen door middel van injectie. Het fenomeen van wisseling van fenotype bij *Vibrio* vereist verder onderzoek.

Tot besluit bracht dit onderzoek aan het licht dat SPF-garnalen niet gevoelig waren voor *Vibrio*, noch door immersie noch door injectie, bij afwezigheid van stress. Nochtans kunnen ze gevoeliger gemaakt worden door abiotische (NH_3) en biotische (WSSV-lading) stress. In deze studie wordt voor het eerst een synergistisch effect tussen een virale en een bacteriële pathogeen in garnaal geproduceerd. De resultaten bevestigen de bevindingen van polymicrobiële ziekten in het veld. Luminescente stammen kunnen overgaan tot non-luminescentie wanneer ze gekweekt worden in statische omstandigheden. Samen met het verlies van luminescentie, treedt er minder kleuring op bij de NL-stam op MACR, en worden ze minder virulent.

CURRICULUM VITAE

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Le Hong Phuoc was born in Long an Town, Vietnam on December 20th, 1971. In 1995, he obtained the diploma "Veterinary Medicine Doctor" at the University of Agriculture and Forestry, Vietnam. From 1995 to 1999, he worked as a researcher at the Department of Experimental Biology, Research Institute for Aquaculture No2 (RIA2) in Ho Chi Minh City, Vietnam. From 1996-1999, he was involved in one project entitled "Epidemiology of White Spot Syndrome Virus in Vietnam". In 1999, he was granted a scholarship by Vlaamse Interuniversitaire Raad (VLIR) in Belgium and enrolled in a two-year international course program "Master of Science in Aquaculture" at the Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University. He graduated with great distinction. From 2001 to 2003, he continued working at RIA2 as a Vice Director of Southern Monitoring Center for Aquaculture Environment and Epidemic (MCE). He was involved in projects on fish and shrimp diseases. He attended several workshops and seminars on aquatic animal diseases in Vietnam, Thailand, and USA. In May 2004, he started a PhD program at the ARC, Faculty of Bioscience Engineering, Ghent University. His PhD study was funded by Vietnamese government. Le Hong Phuoc is author of one accepted and four submitted articles in international peer-reviewed journals. During the PhD study, he has participated in several meetings and symposia relating to aquaculture and fish and shellfish larviculture.

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10th PhD Symposium on Applied Biological Sciences. Ghent University, Belgium, September 29, 2004.

Larvi'05-Fish and Shellfish Larliculture Symposium. Ghent University, Belgium, September 5-8, 2005.

Conference on *Vibrio* 2005. Ghent University, Belgium, November 7-8, 2005.

Conference on VLIZ Young Scientists' Day Friday, 2 March 2007-Provinciehuis Boeverbos, Koning Leopold III-laan 41, B-8200 St-Andries Brugge.

The Asian-Pacific Aquaculture conference from 5-8, August 2007 in Hanoi, Vietnam.

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