

Virulence of *Vibrio parahaemolyticus* to shrimp juvenile

Tahera Tamanna Mou

Roll No: MS-180602

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Khulna-9208, Bangladesh

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Virulence of *Vibrio parahaemolyticus* to shrimp juvenile

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Tahera Tamanna Mou

Roll No: MS-180602

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Approved as to style and content by



14.10.19

Dr. Muhammad Abdur Rouf

Professor and Head

Fisheries and Marine Resource Technology Discipline

Khulna University, Khulna, Bangladesh



July, 2019

DECLARATION

The results presented in the thesis with the above mentioned title are entirely of the candidate's own investigations and no part of the results has been accepted for any degree nor it is being concurrently submitted for any degree.

Tahera Tamanna Mou
.....

(Signature of the candidate)

Tahera Tamanna Mou

Roll No: MS-180602

Session: 2018-19

G. R. Banu
.....

(Signature of co-supervisor)

Dr. Ghausiatur Reza Banu

Professor

Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna-9208, Bangladesh

Shikder Saiful Islam
.....

(Signature of the supervisor)

Shikder Saiful Islam

Assistant Professor

Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna-9208, Bangladesh

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Dedicated to

My Beloved parents

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List of Abbreviations and non-English Words

Abbreviation	Elaboration
BFT	Biofloc Technology
CFU	Colony Form Unit
DoF	Department of Fisheries
et al.	And Others
FAO	Food and Agricultural Organization
FCR	Food Conversion Ratio
Fig.	Figure
GDP	Gross Domestic Product
PCR	Polymerase chain reaction
EMS	Early Mortality Syndrome
AHPND	Acute Hepatopancreatic Necrosis Disease
QS	quorum sensing
L	Litre
μ M	Micro Molar
MS	Microsoft
MT	Metric Tonne
TDH	Thermostable Direct Hemolysin
SPF	Specific Pathogen Free
Spp	Species
TBC	Total Bacteria Count
TCBS	Thiosulphate Citrate Bile Salt Sucrose
TVC	Total <i>Vibrio</i> Count

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Tahera Tamanna Mou

Student ID: MS-180602

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Abstract

Shrimp aquaculture in the world is depressed by various diseases, especially bacterial and viral diseases. Using antibiotics in aquaculture found ineffective and results in increased virulence of pathogens and residual effect of drugs. Cinnamaldehyde and indole can provide a good solution of these problems. This experiment was conducted to investigate the virulence of *Vibrio parahaemolyticus* to shrimp juvenile and finding an effect of indole and cinnamaldehyde on the survival of *Penaeus monodon* against *Vibrio parahaemolyticus*. The challenge test was conducted with 10 shrimp juvenile (14.36 ± 3.48 g/juvenile) in 30 L plastic tanks and at $\pm 10^6$ CFU/ml bacterial inoculation, and the indole and cinnamaldehyde concentrations were 100 μ M respectively. The experimental treatments were C1 (juvenile and feed), C2 (juvenile, feed and bacteria), T1 (juvenile, feed and indole), T2 (juvenile, feed and cinnamaldehyde), T3 (juvenile, feed, bacteria and indole), T4 (juvenile, feed, bacteria and cinnamaldehyde) and T5 (juvenile, feed, bacteria, and both indole and cinnamaldehyde). The results of this experiment represented that the survival of *Penaeus monodon* was found highest (86.67%) in C1 compared to C2 and others group, which was lowest (26.67%) in T5. Nevertheless, the probability of cumulative hazard to mortality of shrimp in this experiment was found to higher in T1 and T3 compared to other treatments. Similarly, it was higher in C2 than that of other groups. C1 was found to have lowest hazardous. However, lower probability of cumulative hazards is in T2, T4 and T5. Total bacterial count was found higher $((2.07 \pm 1.31) \times 10^8$ CFU/g) in group T4 and lower in T5 $((3.28 \pm 2.43) \times 10^7$ CFU/g). Moreover, *Vibrio* spp. load was found to be higher in T4 $(1.31 \pm 1.29) \times 10^6$ CFU/g and lower in T2 $((12.07 \pm 6.82) \times 10^4$ CFU/g).

Hence, *Vibrio parahaemolyticus* might have putative virulence activity to shrimp juvenile and cinnamaldehyde and indole might have impact on reducing their virulence activity.



*C*hapter *O*ne

INTRODUCTION

1 Introduction

Fisheries sector represents one of the most productive and dynamic sectors in Bangladesh. Since the independence of the country Bangladesh has achieved remarkable progress in fisheries sector by playing an increasingly important role in the economy for the last few decades. Bangladesh is one of the world's leading fish producing countries with a total production of 4.277 million MT, where aquaculture contributes 56.44% to total production (DoF, 2018). The average growth performance of this sector is almost 5.43% during the last 10 years. Considering this high growth performance Government is trying to develop this sector as one of the promising income generating industry, which eventually ensures to achieve the projected production target of 4.552 million MT by 2020-21 (DoF, 2018). Bangladesh earns a considerable amount of foreign currencies by exporting fish, shrimps and other fisheries products. Bangladesh produced an estimated 2,39,798MT of farmed shrimp in 2015-16, which is about 6.18% of the total fish production in Bangladesh (FRSS, 2017). Not only this sector earns valuable foreign exchange, but also employs significant numbers of rural workers and provides a livelihood for households throughout Bangladesh.

Bangladesh is very much blessed with a suitable geographical position, agro-climatic environment, available culture techniques and proper supply of hatchery produced seeds (Wahab *et al.*, 2012; Ahmed *et al.*, 2008). Shrimp farming concentrated largely in tropical developing countries for export to the west, has experienced spectacular growth over recent decades. Although, this sector has developed a lot in Bangladesh as well as in many other parts of the world, but especially southwest region has been facing serious problems due to microbial diseases. In aquatic environments, diseases in fishes and shrimps are caused by opportunistic pathogens (Lundin, 1996). Therefore, the growth of aquaculture industry is hampered day by day due to unpredictable mortalities, many of which are caused by pathogenic microorganisms. Hence, microbial disease is one of the major problems in shrimp aquaculture (Hossain *et al.*, 2013).

Microbes play very important role in Aquaculture systems, both at hatchery and grow-out levels as water quality and disease incidences are directly affected by microbial activity (Kumar *et al.*, 2006). Bacterial diseases caused by *Vibrio* spp. are generally the major causes

of mortality in shrimp hatcheries, particularly at early larval stage. It results in significant low production of farmed shrimp and financial loss to commercial growers, which accounts around 25% of the total world aquaculture product (Kumar *et al.*, 2016). Although the shrimp hatchery technology has advanced over the decades, the hatchery production is more often hampered by severe mortalities caused mostly by bacteria. Increasing *Vibrio* spp. population in larvae and rearing tank water has been reported to reduce the survival rate of larvae and post larvae (Singh, 1986; Hameed, 1993).

Vibrio parahaemolyticus is a gram-negative halophilic bacillus, curved and motile with a single flagellum. It is a facultative anaerobic organism that naturally inhabits estuarine and coastal waters worldwide (Fabbro *et al.*, 2010; Cantet *et al.*, 2013)

Typically, within 24 hours after eating contaminated seafood, *Vibrio parahaemolyticus* causes acute, self-limiting gastroenteritis characterized by diarrhea, abdominal cramps, nausea, vomiting, fever and chills which lasts for 1-3 days. Pathogenic *Vibrio* especially *Vibrio parahaemolyticus* have been a public health concern for seafood consumers and have been the cause of import bans, detentions and rejections in international fish trade (Bakr *et al.*, 2011). *Vibrio parahaemolyticus* has been identified in a variety of seafood including shrimps, crabs, oysters and clams due to its halophilic characteristic and seawater habitat throughout the world including Indian subcontinent (Thakur *et al.*, 2003; Gopal *et al.*, 2005).

Intensive fish farming has promoted the growth of several bacterial diseases, which has led to an increase in the use of antimicrobials (Defoirdt *et al.*, 2011, 2007). Current levels of antimicrobial use in aquaculture worldwide are not easy to determine because different countries have different distribution and registration systems. Because a wide variety of chemicals are currently used in aquaculture production, control measures have been introduced over the years. These include disinfectants (e.g., hydrogen peroxide and malachite green), antibiotics (e.g., sulfonamides and tetracyclines) and anthelmintic agents (e.g., pyrethroid insecticides and avermectins) (Rawn *et al.*, 2009). Nevertheless, BurrIDGE *et al.* (2010) reported that the amount of antibiotics and other compounds used in aquaculture differed significantly between countries. Defoirdt *et al.*, (2011) previously estimated that approximately 500–600 metric tons of antibiotics were used in shrimp farm production in Thailand in 1994; he also emphasized the large variation between different countries, with

antibiotic use ranging from 1 g per metric ton of production in Norway to 700 g per metric ton in Vietnam. Adeniyi *et al.*, (2011) have given an overall review about immunostimulant activity of glucan, chitosan, levamisole and herbal plant products. Various herbal products such as from *Hygrophila spinosa*, *Withania somnifera*, *Zingiber officinale*, *Solanum trilobatum*, *Andrographis paniculata*, *Psoralea corylifolia*, *Eclipta erecta*, *Ocimum sanctum*, *Picrorhiza kurroa*, *Phyllanthus niruri*, *Tinospora cordifolia*, purified Silajit and cod-liver oil have the characteristics of growth promotion, anti-stress, immunostimulation and anti-bacterial. These preparations had a prerogative influence on the *Penaeus* larviculture (Al-Musalam *et al.*, 2014). Five herbs *Acalypha indica*, *H. spinosa*, *P. kurroa*, *T. cordifolia* and *Z. officinale* were selected to screen for the in vitro immunostimulant activity against the shrimp pathogen *Vibrio harveyi* (Rajeswari *et al.* 2012). However, various types of antibiotic are used in aquaculture to control or eradicate vibriosis and other bacterial diseases, it has some hazardous effects on culture species, culture environment and even on human consumers. This is why people are now interested in culturing fish species in a healthy and safe way to avoid health hazard altogether. The aim of this study is to find out an alternative way to improve diseases free aquaculture product rather than using antibiotic.

Objectives of the study

1. To investigate the total bacterial load and *Vibrio parahaemolyticus* load in the gut of *P. monodon* from a controlled challenged test system.
2. To determine the survival of *P. monodon* challenged with *V. parahaemolyticus* and find out the protective effect of cinnamaldehyde and indole against the virulence of this pathogenic bacterium.

Chapter Two

LITERATURE REVIEW

2 Literature review

2.1 Overview of Aquaculture

Global fish production peaked at about 171 million tonnes in 2016, with aquaculture representing 47 percent of the total and 53 percent if non-food uses (including reduction to fishmeal and fish oil) are excluded. The total first sale value of fisheries and aquaculture production in 2016 was estimated at USD 362 billion, of which USD 232 billion was from aquaculture production. The contribution of aquaculture to the global production of capture fisheries and aquaculture combined has risen continuously, reaching 46.8 percent in 2016, up from 25.7 percent in 2000. The share of aquaculture in Asian fish production (excluding China) increased to 40.6 percent in 2016, up from 19.3 percent in 2000 (FAO, 2018).

Fish is the second most valuable agricultural crop in Bangladesh and its production contributes to the livelihoods and employment of millions of people. The culture and consumption of fish therefore has important implications for national income and food security. It is the second largest export industry in Bangladesh and produces 2.5 percent of the global production of shrimp (Ghose, 2014). Hence, Bangladesh is considered as one of the most suitable countries in the world for farming freshwater prawn (*Macrobrachium rosenbergii*) (WFC, 2011). Shrimp and prawn production takes place mainly in south and south-western Bangladesh in converted rice fields known as 'ghers'. These cover a total area of 244,000 ha. The contribution of black tiger shrimp (*Penaeus monodon*) and giant freshwater prawn (*Macrobrachium rosenbergii*) to the Bangladesh national economy is significant and shrimp and prawn are the export commodity with the second highest value after readymade garments, which generating US\$412 million in 2009/2010 (WFC, 2011).

2.2 The impact of diseases on aquaculture

Disease is one of the major constraints of fish and shrimp farming in the country (Mazid *et al.*, 2002). Diseases and parasitic problems could constitute significant economic losses in fish production if not controlled (Okaeme *et al.*, 1987). The most obvious effect of the occurrence of disease is mortality, followed by economic losses.

These impacts seem most problematic in the stressful and crowded conditions of aquaculture, which increasingly dominates seafood production. In comparison, it is often difficult to accurately estimate disease impacts on wild populations. Farmed species often receive

infectious diseases from wild species and export infectious agents to wild species. The movement of exotic infectious agents to new areas continues to be the greatest concern (Lafferty *et al.*, 2015).

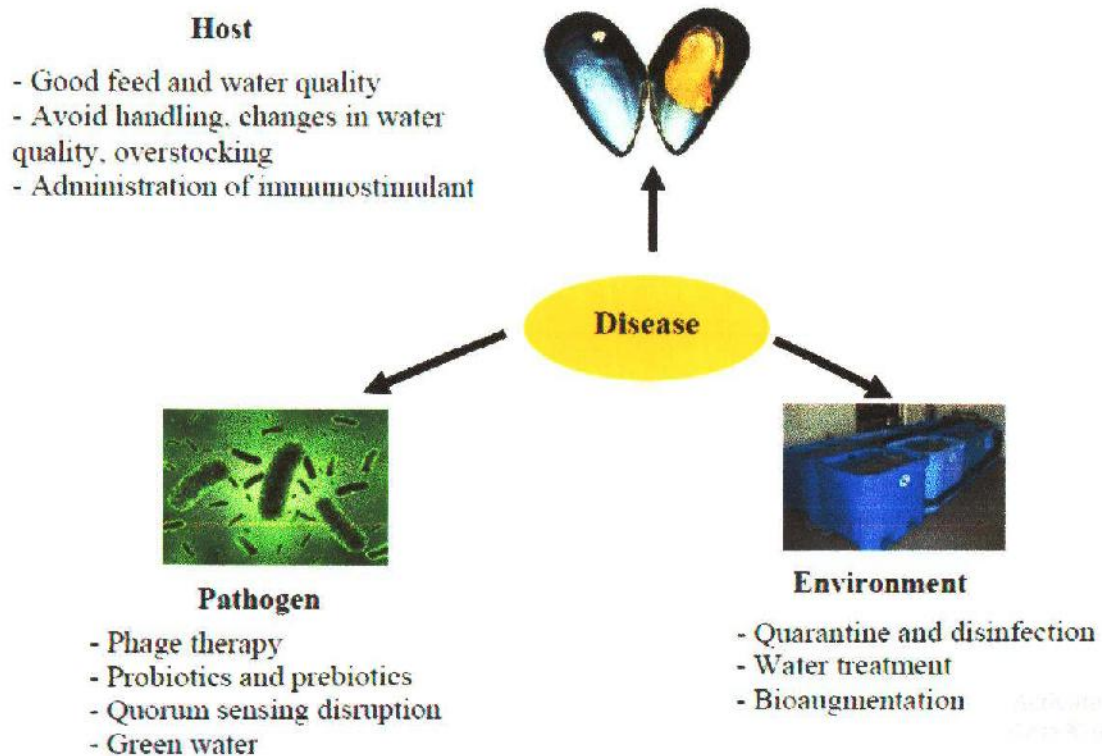


Figure 2.1 Different aspects of host-pathogen-environment relationship and measures that can be taken to prevent or control disease (Defoirdt *et al.*, 2007 and 2011).

It is estimated that about 34% of the diseases in aquaculture species are caused by bacteria, 25% by viruses, 19% by protozoans and 18% by metazoans (Lafferty *et al.*, 2015). Bacterial disease has become a major restraint for the further development and sustainability of the aquaculture industry. Aquatic animals live together in the same environment with its feed, excreta and as well as pathogen leading to independent proliferation, multiplication and transmission of pathogen in to the aquatic animals (Leung and Bates, 2013).

Non-seafood marine species can also have economic value that can be diminished by disease (Lafferty *et al.*, 2015). Infectious diseases that might not normally affect wild hosts can become problematic in aquaculture. Farmed stock is often not co-evolved with local infectious

agents, and this can lead to unpredictable outcomes (Lafferty *et al.*, 2015). The oyster culture industry has moved oyster diseases around the world, and several of these diseases have led to substantial mortality and economic impacts (Sinderman, 1984). The most devastating economic and social impacts have been in shrimp aquaculture for which it was estimated in 1996 that the global direct and indirect costs of emerging diseases had reached \$US 3 billion annually or 40% of the total production capacity of the industry (Israngkura and Sae-Hae, 2002; Arthur *et al.*, 2002; Lundin, 1996). With the development of intensive aquaculture and the appearance of more serious environmental pollution in recent years, aquatic animal disease has become a major problem.

In freshwater aquaculture, apart from many bacterial, viral, fungal, protozoan and other diseases, bacterial septicemia is considered to be one of the most important diseases in the history of Chinese aquaculture. From 1990 to 1992, it occurred widely in almost all major aquaculture provinces and regions in China (Lundin, 1996). However, in marine and brackish water aquaculture, shrimp disease is the major problem.

2.2.1 Diseases caused by pathogenic bacteria

In the natural environment and cultured condition a lot of pathogens are responsible for the diseases of fishes and shellfishes. However, in most of the cases the diseases are caused by the bacteria belonging to *Vibrio* spp. (such as *V. campbellii*, *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, *V. vulnificus*, *V. penaeicida* etc., even other undefined *Vibrio* species), these species also called vibriosis in shrimp (Brock and Lightner, 1990; Ishimaru *et al.*, 1995; Chen *et al.*, 2002). *Vibrio* spp. are widely distributed in the aquatic environment and occupy variety of ecological niches, such as the human and animal gut, the surface of chitinous organisms, most notably copepods, and the coral mucus layer (Sawabe *et al.*, 2007). It is also stated that the members of the family Vibrionaceae contribute 60% of the total bacterial population (Simidu and Tsukamoto, 1985). Shell disease is commonly observed in larvae, post-larvae and adults in prawns. It is variously termed as 'black spot', 'brown spot', 'burnt spot' or chitinolytic bacterial disease. This is caused by the invasion of chitinolytic bacteria, which break down the chitin of the exoskeleton, leading to erosion and melanization (dark brown to black pigmentation) at the site of infection. Several chitinolytic bacteria (Gram negative rods) such as *Vibrio* spp., *Pseudomonas* spp., *Aeromonas* spp., *Spirillum* spp. etc are

involved in the process of exoskeleton breakdown. Filamentous bacteria such as *Leucothrix mucor*, *Thriothrix* spp., *Flexibacter* spp. etc., sometimes cause mortality in prawn larvae subsequent to discoloration of gills and associated secondary infections (Jayaprakash, 2005).

2.2.1.1 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a gram-negative bacterium that occurs naturally in the estuarine environment. This human pathogen is frequently found in seawater, sediments, plankton, finfish and shellfishes (Udyavara *et al.*, 2016) and 30 different marine species, including eel, crab, clams, oysters, lobsters, scallops, sardines, shrimp, and squid (Fishbein *et al.*, 1974) and can cause acute gastroenteritis characterized by diarrhea, vomiting and abdominal cramps through consumption of and abdominal cramps through consumption of contaminated raw fish or shellfish (Rippey, 1994). It forms part of the indigenous microflora of aquatic habitats of various salinity (Colwell *et al.*, 1984) and are the major causative agents for some of the most serious diseases in fish, shellfish and penaeid shrimp (Lee *et al.*, 1996). *V. parahaemolyticus* is a marine bacterium that can cause seafood borne gastroenteritis and traveler's diarrhea in humans, after consumption of contaminated raw or partially cooked fish or shell fish, particularly oysters or exposure to a marine environment (Khan *et al.*, 2002). *Vibrio parahaemolyticus* occurs in marine coastal waters and is a commonly found inhabitant of estuarine environments (including water, sediment, suspended particles, plankton, fish and shellfish) worldwide; it is a leading cause of seafood-associated bacterial gastroenteritis globally (Levin, 2006), although not all strains of this species are pathogenic (Nishibuchi and Kaper 1995). In addition to its status as a human pathogen, there are some reports of *V. parahaemolyticus* being an important shrimp pathogen (Ruangpan and Kitao 1991; Alapide-Tendencia and Dureza 1997; Sudheesh and Xu 2001). Ruangpan and Kitao (1991) isolated the species (96 out of 205 isolates were *V. parahaemolyticus*) from diseased black tiger shrimp, *Penaeus monodon*, cultured in Thailand from 1988 to 1990. Vandenberghe *et al.* (1999) suggested that *V. parahaemolyticus* infections typically occur in juveniles and adults of *Litopenaeus vannamei*. *Vibrio parahaemolyticus* has also been associated with the mass mortality of molluscs, such as abalone (*Haliotis diversicolor supertexta*) (Cai *et al.* 2006b) and there are some reports of outbreaks of mass mortality caused by *V. parahaemolyticus* on the south coast of China (Liu *et al.* 2000). The survival of *V. parahaemolyticus* in seafoods

subjected to chilling, freezing, heating, drying and smoking (Vanderzant and Nickelson, 1972; Karunasagar *et al.*, 1986) has been reported. This organism is known to be completely destroyed in cooked foods, especially in South Asian countries cooked with plenty of spices. Illness due to *V. parahaemolyticus* was reported in many countries like China, Indonesia, Vietnam, Australia, Great Britain, France, Germany, Italy, Canada and United States (CDC, 1998; CDC, 1999), but it is most prevalent food borne pathogen in many Asian countries, where seafood is often consumed (Pan *et al.*, 1997).

Acute hepatopancreatic necrosis disease (AHPND) is a severe, newly emergent penaeid shrimp disease caused by *Vibrio parahaemolyticus* that has already led to tremendous losses in the cultured shrimp industry. Until now, its disease-causing mechanism has remained unclear. Here we show that an AHPND-causing strain of *V. parahaemolyticus* contains a 70-kbp plasmid (pVA1) with a post segregational killing system, and that the ability to cause disease is abolished by the natural absence or experimental deletion of the plasmid-encoded homologs of the *Photobacterium* insect-related (Pir) toxins PirA and PirB. We determined the crystal structure of the *V. parahaemolyticus* PirA and PirB (PirAvp and PirBvp) proteins and found that the overall structural topology of PirAvp/PirBvp is very similar to that of the *Bacillus* Cry insecticidal toxin-like proteins, despite the low sequence identity (<10%). This structural similarity suggests that the putative PirABvp heterodimer might emulate the functional domains of the Cry protein, and in particular its poreforming activity. The gene organization of pVA1 further suggested that pirABvp may be lost or acquired by horizontal gene transfer via transposition or homologous recombination (Lee *et al.*, 2015).

The ecosystem disturbance caused by the current practice of disinfecting ponds to remove potential pathogens or their carriers prior to stocking shrimp postlarvae most probably does more harm than good. The increase in nutrient availability after disinfection combined with a destabilized and impoverished microbial community (and a consequent lack of competition) favors fast-growing bacteria (such as many pathogenic *Vibrio* spp.) in recolonizing the environment (Attramadal *et al.*, 2012). Considering that EMS/AHPND most probably is caused by a *Vibrio*, this practice is thus more likely to stimulate proliferation of the EMS/AHPND causing agent in the pond than counteract it. In fact, important lessons can be learned from the outbreaks of luminescent vibriosis in the early 1990s. This disease was

caused by *Vibrio harveyi* and closely related species all belonging to the *Harveyi* clade of vibrios (and therefore closely related to the causative agent of EMS/AHPND) (Defoirdt *et al.*, 2007).

TDH was the first recognized virulence factor for *V. parahaemolyticus* and has been used as an important marker for identifying virulent strains (Okuda *et al.*, 1997; Cook *et al.*, 2002). TDH was produced from the *tdh2* gene rather than the *tdh1* gene. The nucleotide homologies of *tdh2* with *tdh1*, *tdh3* and *tdh4* were 97%, 98.6% and 98.6%, respectively (Nishibuchi and Kaper 1985; Nishibuchi and Kaper, 1995).

2.3 Use of antibiotics in aquaculture

Antibiotics play a major role in combating many diseases of cultured aquatic organisms but their undesirable consequences such as persistence of antibiotic residues in farm and development of antibiotic-resistant bacteria discourage the use of these chemotherapeutic agents (Chaithanya *et al.*, 1999; Jernberg *et al.*, 2010). In addition, use of excessive antibiotics in pond has an adverse effect on the aquatic environment. Many drugs used in the culture ponds to control diseases, may be taken up by and accumulated in the animals after resolving in the water, thus affecting the seafood safety (Biao and Kaijin, 2007).

2.3.1 Alternatives to antibiotics

2.3.2 Immunostimulant

An immunostimulant is defined as a substance which enhances the innate or non-specific immune response by interacting directly with cells of the immune system and activating them. An alternative approach has been the application of various compounds to boost or stimulate the immune system of fishes and shrimp. Such compounds are known as the immunostimulants. In recent year, the established beneficial effects of immunostimulants in many living systems promote their application for disease management in aquaculture practices. Use of immunostimulants in culture systems has become powerful measure to control diseases in aquaculture ponds (Mastan, 2015). Immunostimulants are dietary additives that enhance the innate (non-specific) defense mechanisms and increase resistance to specific pathogens. Immunostimulants can be grouped under different agents based on the source, such

as bacterial preparations, polysaccharides, animal or plant extracts, nutritional factors and cytokines (Sakai, 1999).

2.3.3 Probiotics

Probiotics are organisms and substances which contribute to intestinal microbial balance (Fuller, 1989). Generally probiotic strains have been isolated from indigenous and exogenous microbiota of aquatic animals. The use of probiotics in the culture of aquatic organisms is increasing with demand for more environment friendly aquaculture practices (Gatesoupe, 1999). In this context, microbial intervention can play a vital role in aquaculture production, and effective probiotic treatments may provide broad spectrum and greater nonspecific disease protection (Rengpipat *et al.*, 2000; Paningrahi and Azad, 2007). Intake of probiotics could improve intestinal microbial balance, leading to enhanced absorption of microbial balance, leading to enhanced absorption of food, increased activity of digestive enzyme and reduced pathogenic problems in the gastrointestinal tract (Balcazar, 2003; Balcazar *et al.*, 2006). Enhancements of colonization resistance and/or direct inhibitory effects against pathogens are important factors where probiotics have reduced the incidence and duration of diseases. Probiotic strains have been shown to inhibit pathogenic bacteria both in vitro and in vivo through several different mechanisms (Balcazar *et al.*, 2006). Probiotics have a very important role to play in the degradation of organic matter thereby significantly reducing the sludge and slime formation. As a result, water quality would improve by reducing the disease (Krishnaprakash *et al.*, 2009). *A. salmonicida* and *Y. ruckeri* was seen to increase the phagocytic activity, respiratory burst as well as serum and gut mucosal lysozyme activity (Kim and Austin, 2006).

Parameswaran and coworkers (2006) developed cell lines from embryonic, kidney and spleen cells derived from fish. It thus seems possible to develop a cell line for the epithelial cells of the fish gut to study the invasive capacity of the pathogens after a probiotic treatment. Ideally, the results obtained would be combined with information on the gene activity in the pathogen. For example, hemolysin production by pathogenic *Vibrios* may be interesting to study as it is an important factor in the virulence (San Luis and Hedreyda, 2006).

2.3.4 Prebiotics

Dietary supplementation of different feed additives e.g. immunostimulants, probiotics and prebiotics usually in small quantities for the purpose of fortifying it with certain nutrients have been found to be beneficial for improving immune status, feed efficiency and growth performance of crustaceans and finfishes (Ganguly *et al.*, 2013). A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon. Prebiotics in fish and shellfish have the effects on growth, feed conversion, gut microbiota, cell damage/morphology, resistance against pathogenic bacteria and innate immune parameters such as alternative complement activity (ACH50), lysozyme activity, natural haemagglutination activity, respiratory burst, superoxide dismutase activity and phagocytic activity (Ringo. *et al.*, 2010).

2.3.5 Bioflocs

Biofloc system is developed to improve environmental control over production (Hargreaves, 2013). Biofloc technology (BFT) is a method with filtration directly from water. It has traditionally been used in outdoor ponds that contain naturally photoautotrophic algae that provides the water with oxygen and becomes an additional food source to herbivorous and omnivorous species. BFT have shown to reduce feeding costs, maintain water quality and to help to control bacterial infections in the pond. To maintain a better water stability, BFT could be applied indoors, then sunlight becomes restrained and instead of algae, heterotrophic bacteria will dominate. Biofloc technology (BFT) can contribute as an important environmental advantage when feeding aquaculture. The main principle of BFT is that microbes accumulates of excess feed and, if added, carbohydrates (Granberg, 2016).

2.3.6 r/k strategy

According to the ecological theory of r/K selection, an unstable environment containing high nutrient levels per individual selects for organisms with the capacity to exploit nutrients and increase population size, termed r-strategists whereas stable environment where the resources per individual are scarce will select for slow-growing organisms, termed K- strategists. Most bacterial diseases in aquaculture (and especially larviculture) are caused by opportunistic pathogens that are ubiquitous in the marine environment and that are capable of quickly

increasing their population size in the aquaculture environment: i.e., r-strategists (Vadstein *et al.*, 2013; De Schryver and Vadstein, 2014). Disinfection practices can favor r-strategists because they decrease the competition between bacteria (by decreasing the bacterial density), and this also holds for feeding regimes that result in large fluctuations in nutrient levels in the rearing water (Blancheton *et al.*, 2013). Slow growth conditions can be imposed by controlled microbial colonization of the (disinfected) inflow water with K-strategists (Skjermo *et al.*, 1997) and by avoiding large fluctuations in nutrient levels in the water.

2.3.7 Indole

The pathogenicity of bacteria is determined by the production of different virulence factors (gene products that enable them to infect their host), and quorum sensing (bacterial cell-to-cell communication) controls virulence factor production in many pathogenic bacteria. Various chemical compounds and enzymes have been reported to modulate quorum sensing, thereby protecting a host from infection by pathogenic bacteria. Indole signaling is one of the mechanisms that controls the virulence factors in several bacteria, including some *Vibrio* sp. Therefore, the interference with indole signaling could be another possibility to reduce the production of virulence factors, hence reducing the occurrence of diseases. Indole and some indole analogues have been reported to alter indole signaling in several bacterial species (Gupta, 2016). Recently, indole is gaining attention as a putative quorum sensing signal molecule. Indole is produced by many bacteria, and some other bacteria that do not produce indole are also able to respond to extracellular indole. Indole has been reported to regulate motility, biofilm formation and antibiotic resistance (Lee *et al.*, 2007a). In addition to indole, some indole derivatives, have also been reported to change bacterial behaviour. Therefore, interference with indole signaling might be an interesting novel strategy to control bacterial infections (Li *et al.*, 2014; Melander *et al.*, 2014).

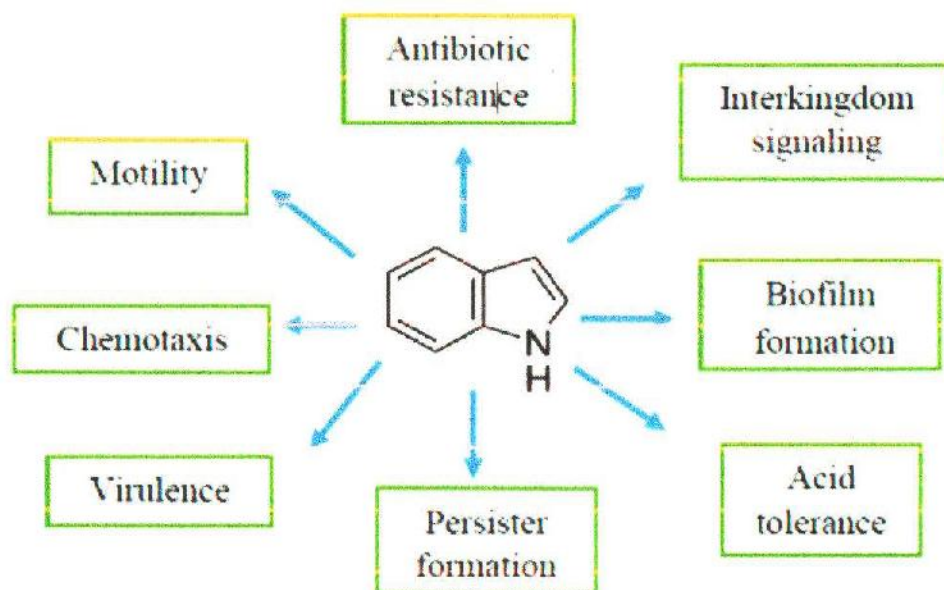


Figure 2.2 Role of indole signaling (Melander *et al.*, 2014).

Some indole producing *Vibrio* species are shown below in Table though the role of indole in their virulence and indole signaling mechanisms are not depicted. However, some reports indicated that indole signaling has an impact on the economically important aquaculture pathogen *V. anguillarum*.

2.3.8 Cinnamaldehyde

Cinnamaldehyde, which is extracted from cinnamon, is a natural compound with activity against bacteria and a modulatory immune function. However, the antibacterial activity and immunostimulation of cinnamaldehyde in fish has not been well investigated due to the compound's poor water solubility. Cinnamaldehyde exhibits antimicrobial activity against aquatic pathogens, even antibiotic-resistant bacterial strains and immunostimulating effects to protect the host's defenses against pathogen infection in bacteria-infected fish (Faikoh *et al.*, 2014). A recent investigation demonstrated that essential oil extracted from *Cinnamomum fragrans* can significantly control bacterial loads and enhance *Penaeus monodon* larvae survival rates, which suggests a novel alternative to antibiotics in shrimp hatchery culture and implies that cinnamaldehyde may be developed as an antibacterial agent against aquatic pathogens in aquaculture (Taguchi *et al.*, 2013; Randrianarivelo *et al.*, 2010). Hence, a study

to demonstrate the antimicrobial activity and immunomodulatory function of cinnamaldehyde for disease resistance in fish is imperative (Faikoh *et al.*, 2014).

Table 2.1 Indole producing *Vibrio* species (Defoirdt, 2013; Lee and Lee, 2010).

<i>Vibrio</i> species	Indole production
<i>V. alginolyticus</i>	+
<i>V. cholera</i>	+
<i>V. harveyi</i>	+
<i>V. fluvialis</i>	+
<i>V. hollisae</i>	+
<i>V. mediterranei</i>	+
<i>V. nigripulchritudo</i>	+
<i>V. orientalis</i>	+
<i>V. parahaemolyticus</i>	+
<i>V. splendidus</i>	+
<i>V. tapetis</i>	+
<i>V. tubiashii</i>	+
<i>V. vulnificus</i>	+/-

In general, cinnamaldehyde should be given to aquatic animals through oral administration or direct mixture with feed ingredients. Replacing antibiotics with cinnamaldehyde as an antimicrobial agent may be economically beneficial (Banerjee, 2012).

For most marine aquaculture species, one of the main bottlenecks is the stable production of high quality juveniles. The high and unpredictable mortality in the first weeks after hatching of marine fish larvae remains a challenging problem that needs to be solved. The severity of the problem differs between species, but cannot be considered adequately solved for any species. Both scientific evidence and experience in hatcheries for a variety of fish, shrimp and shellfish species are accumulating as support for the hypothesis that detrimental fish-microbe interactions are the cause of these problems (Vadstein *et al.*, 2013).

Recently, Schwenteit *et al.* (2011) reported that inactivation of the AHL synthase of *A. salmonicida* sub sp. *achromogenes* resulted in reduced mortality of Arctic charr (*Salvelinus alpinus*). The results of our study subsequently showed that addition of the QS inhibitors C14-HSL and cinnamaldehyde blocked the virulence of both *A. hydrophila* and *A. salmonicida*, further substantiating the role of QS in mortality of burbot larvae caused by these pathogens. Long-chain AHLs have been reported before to disrupt different virulence factors regulated by QS in *Aeromonas* spp. Meanwhile, cinnamaldehyde has been shown to disrupt QS in vibrios and protect brine shrimp (*A. franciscana*) from *V. harveyi* (Brackman *et al.*, 2008). All of these results indicate that the use of QS inhibitors would be a valid strategy to fight disease caused by *Aeromonas* spp. in freshwater fish larvae.

2.3.9 Quorum sensing

Quorum sensing is a gene regulatory mechanism in which bacteria monitor one another's presence by the production, release and detection of small signalling molecules (Camilli and Bassler, 2006). A higher detection of signalling molecules indicates a higher presence of pathogens, which is the sign to become virulent. As such, the virulence of a pathogenic population can be regulated as a function of pathogen density making chances for successful infection considerably higher. Three types of QS systems exist depending on the type of signaling molecules: acylatedhomoserine lactones (AHLs), peptides, or autoinducer 1 (AI-1) and autoinducer 2 (AI-2).

For many aquaculture pathogens, the expression of the virulence depends on the ability to communicate with other members of the population by means of quorum sensing (QS) (Bruhn *et al.*, 2005). A novel and environmental-friendly approach in solving this problem is through the disruption of bacterial communication or quorum sensing (QS). In this communication scheme, bacteria regulate their own gene expression by producing, releasing, and sensing chemical signals from the environment. There seems to be a link between QS and diseases through the regulation of certain phenotypes and the induction of virulence factors responsible for pathogen–host association. Several findings have reported that numerous aquatic organisms such as micro-algae, macroalgae, invertebrates, or even other bacteria have the potential to disrupt QS. The mechanism of action varies from degradation of signals through enzymatic or chemical inactivation to antagonistic as well as agonistic activities. This review

focuses on the existing marine organisms that are able to interfere with QS with potential application for aquaculture as bacterial control (Natrash *et al.*, 2011a)

Recently, the disruption of bacterial quorum sensing has been proposed as a new anti-infective strategy and several techniques that could be used to disrupt quorum sensing have been investigated. These techniques comprise (1) the inhibition of signal molecule biosynthesis, (2) the application of QS antagonists (including naturally occurring as well as synthetic halogenated furanones, antagonistic QS molecules and undefined exudates of higher plants and algae), (3) the chemical inactivation of QS signals by oxidized halogen antimicrobials, (4) signal molecule biodegradation by bacterial lactonases and by bacterial and eukaryotic acylases and (5) the application of QS agonists to trigger host responses. Although the technique seems very promising, a lot remains unknown about QS inhibition and it will take a while before practical application comes within reach (Tinh *et al.*, 2008).

Pande *et al.* (2013a) reported that Vibriosis outbreaks caused by *Vibrio harveyi* and related species are amongst the major obstacles for the further expansion of giant freshwater prawn (*Macrobrachium rosenbergii*) larviculture. *Vibrio harveyi* regulates virulence gene expression through quorum sensing, bacterial cell-to-cell communication, and consequently, quorum sensing disruption has been suggested as an alternative strategy to control infections caused by these bacteria. Previous studies have shown that quorum sensing-disrupting compounds are able to disrupt quorum sensing in *Vibrio harveyi*. In this study, they demonstrated that the quorum sensing-disrupting compounds cinnamaldehyde, (Z)-4-bromo-5-(bromomethylene)-2(5H)-furanone and (Z)-4-((5-(bromomethylene)-2-oxo-2,5-dihydrothiophen-3-yl)methoxy)-4-oxobutanoic acid increased the survival of giant freshwater prawn larvae when challenged to pathogenic *Vibrio harveyi*. Their *in vivo* challenge test showed that cinnamaldehyde and the thiophenone can protect the larvae from *Vibrio harveyi* infection when dosed to the culture water at 1 μ M and 10 μ M, whereas the brominated furanone offered protection at 1 μ M but resulted in complete mortality at 10 μ M. Although there were significant differences in survival between challenged larvae with and without addition of quorum sensing-disrupting compounds, there were no differences in growth. Moreover, Brominated furanones have been reported before to protect brine shrimp larvae from pathogenic *V. harveyi* (Defoirdt *et al.*, 2006) and rainbow trout (*Oncorhynchus mykiss*) from *Vibrio anguillarum* and the thiophenone

compound has been reported before to protect brine shrimp larvae from *V. harveyi* (Defoirdt *et al.*, 2012). As far as we know, this study provides the first evidence of a protective effect of this kind of compounds in a commercial crustacean species.

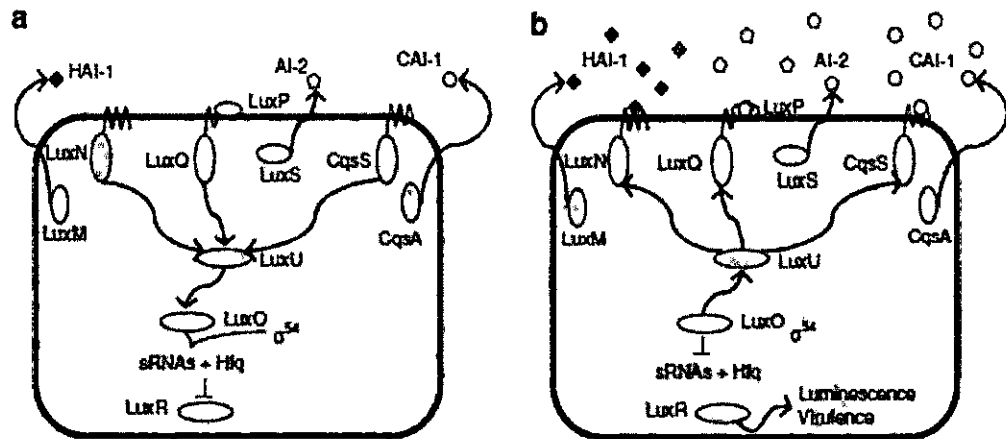


Figure 2.3 *Vibrio harveyi* quorum sensing system (Defoirdt and Sorgeloos, 2012)

According to another study of Pande *et al.* (2013b), *Vibrio campbellii*, one of the major pathogens of aquatic organisms, regulates virulence gene expression by a three channel quorum sensing system. They showed that although they use a common signal transduction cascade, the signal molecules have a different impact on the virulence of the bacterium towards different hosts, i.e. the brine shrimp *Artemia franciscana* and the commercially important giant freshwater prawn *Macrobrachium rosenbergii*. These results suggest that the use of multiple types of signal molecules to regulate virulence gene expression is one of the features that allow bacteria to infect different hosts. Their findings emphasize that it is highly important to study the efficacy of quorum sensing inhibitors as novel biocontrol agents under conditions that are as close as possible to the clinical situation.

The impact of QS on the virulence of both *Aeromonas* species towards burbot larvae was investigated by using *A. hydrophila* AH-1N QS mutants and by using QS inhibitors. Compared to the wild type, higher survival of burbot larvae was seen when challenged to QS mutants with either an impaired AHL synthase or an impaired AHL receptor. Moreover, the addition of the dominant QS molecule in *Aeromonas* spp., C4-HSL, could restore the virulence of the AHL negative mutant.

Chapter Three

MATERIALS AND METHODS

3 Materials and methods

This experiment was conducted in the water chemistry, and fish pathology laboratory of Fisheries and Marine Resource Technology Discipline, Khulna University. Shrimp juveniles were collected from a nursery of a shrimp farmer at Bagerhat district. The animals were carried to the laboratory with proper aeration and stocked in 30 L plastic tanks for pathogen challenge test according to experimental design (Table 3.1). The study continued from July to September 2018.

3.1 Experimental design

The bacteria used for the challenge tests were *Vibrio parahaemolyticus* that was isolated from an infected shrimp farm at Bagerhat district. For each test the shrimp juvenile were stocked in 30 L plastic tanks at the stocking density of 10 per tank with 3 replicates each. All challenge tests ran for 10 days. Since the dead shrimps needed to be stored at -20°C for further laboratory analysis to determine the total bacterial load as well as load of total *Vibrio* spp., we lost few of the samples from treatment T5 during the experiment. The challenge test was continued for 10 days. The survival of shrimp juvenile was determined daily by counting the live animal after each two (02) hours. The bacterial load of dead animals were checked on nutrient and TCBS agar media. All nutrient and TCBS plates were incubated at 28-30°C for 24-48 hours.

Table 3.1 Experimental layout of challenge test of *P. monodon* against *V. parahaemolyticus* under the treatment of cinnamaldehyde and indole.

No of tank	Shrimp Juvenile	Feed	<i>Vibrio</i> spp. ($\times 10^6$)	Indole 100 μ M	Cinnamaldehyde 100 μ M
C1	+	+	—	—	—
C2	+	+	+	—	—
T1	+	+	—	+	—
T2	+	+	—	—	+
T3	+	+	+	+	—
T4	+	+	+	—	+
T5	+	+	+	+	+

3.2 Tank setup

A total of 21 plastic tanks with the volume of 30 liter water holding capacity was set up randomly at water chemistry laboratory. The tanks were washed with chlorine water to disinfect it and tanks were filled with 25 liter 10 ppt saline water. Salinity adjustment was done with 120 ppt brine water. During the study period up to 20% water exchange was done daily according the turbidity and residual feed of the tank water.

3.3 Feeding

The animals were fed 5 % of the body weight, and the feeding frequency was three (03) times a day at 30%, 30% and 40% of the total feed.

3.4 Laboratory requirements

3.4.1 Laboratory equipment's

Autoclave machine(ALP), centrifuge machine (Eppendorf AG 22331 Hamburg), electric balance (OHAUS CORPORATION), spirit lamp and burning spirit, Petri dish, biological dissection box, vortex mixer (Thermolyne), bacteria isolating loops, glass rods (Glassco), oven (Mettler), incubator (SANYO), refrigerator (RANGS), bio-safety cabinet (LABCONCO), micro-pipette (Thermo scientific), tips and tips box were used for laboratory as well as challenge test experiment.

3.4.2 Reagents and chemicals

Peptone water (HiMedia), absolute alcohol (Merck), yeast extract (HiMedia), ethyl alcohol (70% and 100%), distilled water, TCBS agar media (HiMedia), nutrient agar media (HiMedia) were used for laboratory analysis in this experiment.

3.5 Laboratory work

3.5.1 Sterilization of equipment's

Test tubes, petri dishes (ANUMBRA), conical flask, plastic tips and other glassware was sterilized by autoclaving (VS-1221, Vision Scientific., LTD) at a temperature of 121°C and a pressure of 15 pound per square inch for 15 minutes. Petri dishes, isolating loop, etc. were subjected to dry heat sterilization at 170°C for 1 hour in an electric oven.

3.5.2 Preparation of stock solution from shrimp samples

The death shrimp samples were collected from the challenge test experiment after every two hours of monitoring and subsequently stored at -20°C. To prepare stock solution from collected shrimp sample, it was weighed by an electric balance aseptically (HR-200). Samples weight ranged from 0.178g to 0.52g. The weighed samples were then taken in a sterilize vortex mixer with 5 ml alkaline saline peptone water for proper mixing. The mixed samples were taken in autoclaved eppendorf tubes for centrifugation at 3000 rpm for 4 minutes (Centrifuge (5415 D)). After centrifugation the supernatant was collected in an autoclaved falcon tube, followed by serial dilutions before inoculating on the agar media.

3.5.3 Preparation of media

Media were prepared by compounding the required individual ingredients or more conveniently, by adding distilled water to a dehydrated product which contain all the ingredients. All Media were available commercially in powdered form.

3.5.4 Preparation of thiosulfate citrate bile salt-sucrose (TCBS) agar media

TCBS agar was prepared by adding 89.0 g TCBS powder in 1 L demineralized water. The mixture was subsequently heated to boiling point using a magnetic stirrer. Plates were immediately poured (15 ml per plate) under laminar flow and left to solidify for 30 minutes. The plates were stored in plastic bags at room temperature for further use, or samples were inoculated immediately.

3.5.5 Preparation of nutrient agar (NA) media

Nutrient agar media was prepared by adding 20 g agar powder in 1 liter distilled water, mixing it properly by dissolving it, followed by autoclaving at 121°C for 15 minutes. After autoclaving the agar media was kept under bio-safety cabinet and 20 ml was transferred in each petri-dishes. After pouring into the plates it was kept under the laminar flow to solidify and used for bacterial inoculation.

3.5.6 Inoculation and incubation

0.5ml of diluted solution was poured on solid media aseptically by sterile micropipette. After this spreading was done by glass L-shaped rod until proper mixing and then properly covered the Petri dish with aluminum foil and incubated for 24-48 hours at 30°C. After incubation the bacterial colony grown in the plates in between 10-300 were counted and recorded.

3.6 Data collection and analysis

The data were recorded during the experiment and collected data were accumulated, and interpreted according to the objectives. Collected data were stored, explored and analyzed using Microsoft Excel, SPSS 16.0 version and Microsoft Word program to present results and discussion. Bacterial load was analyzed by Microsoft excell 10.0 and the effect of different bacterial load on mortality of shrimp juvenile was determined by Poisson Regression, and the survival and hazard functions were determined through Kaplan Meier survival test.

*C*hapter *F*our

RESULTS

4 Result

4.1 Virulence of *Vibrio parahaemolyticus* to shrimp juvenile

This experiment was conducted as an approach of microbial community management and finding an alternative to antibiotics to control aquaculture diseases, especially focusing on tiger shrimp (*Penaeus monodon*) using different chemical compound application.

4.1.1 In vivo challenge test

During the 10 days challenge test, the survival of *Penaeus monodon* in C2 (36.67%) was found lower compared to the control group C1 (86.67%) (Fig. 4.1). However, the lowest survival (26.67%) was observed in T5 in which *Vibrio parahaemolyticus*, indole and cinnamaldehyde were applied together. Survival rate was 70% in T3, followed by 66.67% in T1 and 63.33% in T2 (Figure 4.1). That means that indole and cinnamaldehyde individually protect the shrimp compared to the positive control group C2. However, their combination might not have any effect on controlling the *V. parahaemolyticus* or together they may be toxic to shrimp.

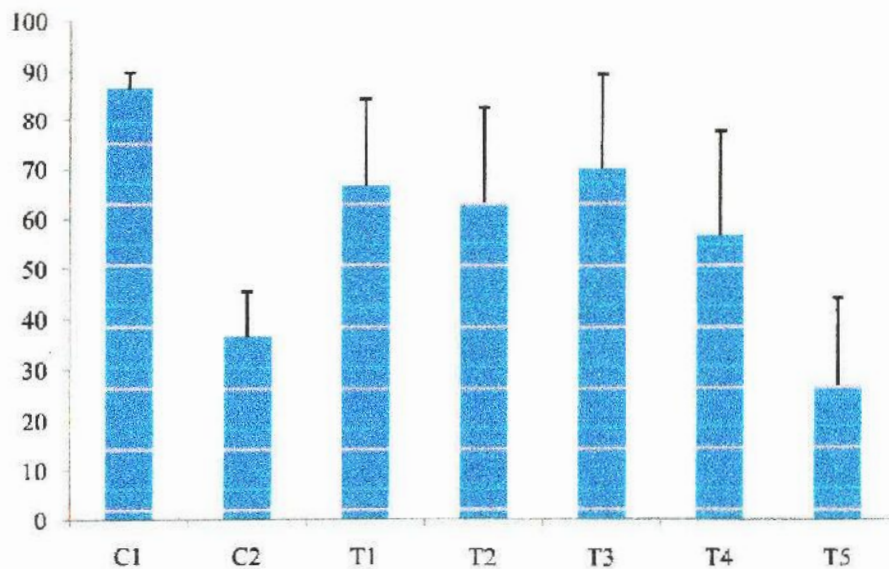


Figure 4.1 Survival of *P. monodon* from the challenge test experiment with *Vibrio parahaemolyticus*, indole and cinnamaldehyde. The error bars represent the standard deviations of three replicates.

4.1.2 Survival function

During 10 days challenge test, the cumulative probability of survival of *Penaeus monodon* in C1 was found 100% at first 24 hours, after which it fall to about 85% and continued up to the end of the experiment. However, it was 100% for up to 140 hours in the case of C2, which then reduced to 85% and further fell down to less than 60% at about 170 hours. This indicated that *V. parahaemolyticus* was not so virulent at the beginning of the challenge test. At the end of the experiment, the survival function was found to have lowest in T3, followed by T1. Moreover, T2, T4 and T5 showed survival functions in between 70-80% (Figure 4.2).

The result demonstrated that the probability of survival for T2, T4, T5 and C1 groups was more time extended compared to T1, T3 and C2. That means Cinnamaldehyde might support characteristics for the survival of shrimp and also antagonistic effect against pathogenic *Vibrio parahaemolyticus*.

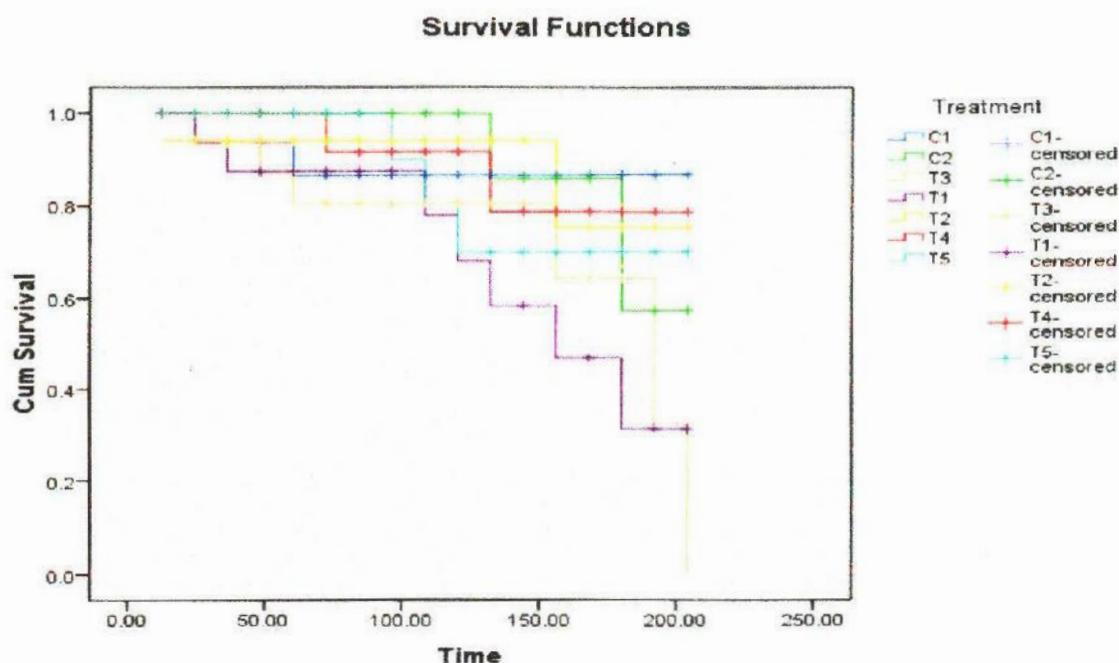


Figure 4.2 Kaplan Meler survival function graph of *P. monodon* over ten days challenge test. Censoring indicate the different horizontal coloured lines.

4.1.3 Hazard function

The probability of cumulative hazard to mortality of shrimp in this experiment was found to be higher in T1 and T3 compared to other treatments. Similarly, it was higher in C2 than that of other groups. C1 was found to have the lowest hazardous might be due to the absence of pathogens as well as free of chemicals. However, lower probability of cumulative hazards in T2, T4 and T5 indicate that Cinnamaldehyde might save the shrimp from being dead or being infected by any pathogenic organisms, like *Vibrio* spp. (Figure 4.3).

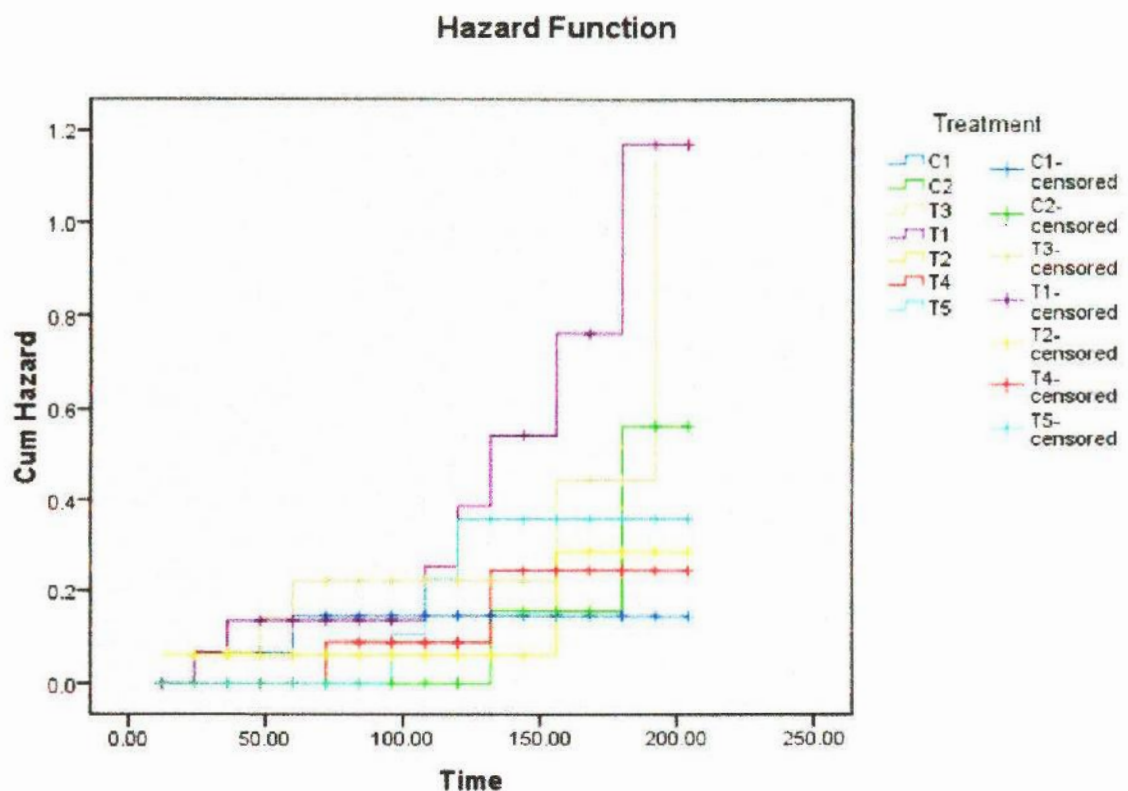


Figure 4.3 Kaplan Meier hazard function graph of *P. monodon* over ten days challenge test. Censoring is indicated by different horizontal coloured lines.

((1.34±1.03)×10⁸ CFU/g), C2 ((2.01±1.21)×10⁷ CFU/g), T3 ((3.13±1.61)×10⁷ CFU/g), T1 ((5.42±3.19)×10⁷ CFU/g), T2 ((4.11±3.07)×10⁷ CFU/g)), and T5 ((3.28±2.43)×10⁷ CFU/g) (Figure 4.4).

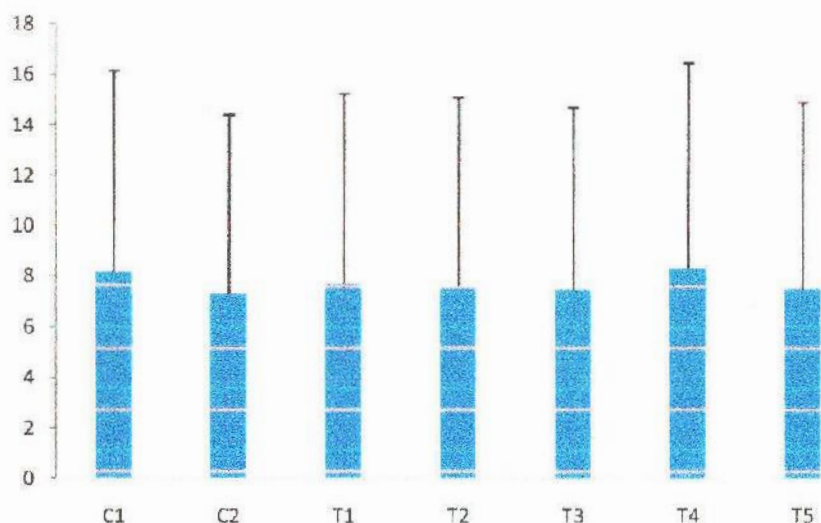


Figure 4.4 Total bacterial load in the gut and hepatopancreas of *P. monodon* after a 10 days challenge test using *Vibrioparahaemolyticus*, indole, and cinnamaldehyde. The error bars represent the standard deviations of three replicates.

4.2.2 Total *Vibrio* spp. load in *P. monodon* juvenile

In contrast to total bacterial load, total *Vibrio* spp. count in juvenile after 10 days challenge test was found to have higher in T4 (1.31±1.29)×10⁶CFU/g and T3 ((5.20±3.68)×10⁵CFU/g) compared to positive control C2 ((2.23±1.45)×10⁵CFU/g), T1 ((1.27±0.68)×10⁵CFU/g), negative control C1 (1.23×10⁵CFU/g), and T2 ((9.21±8.98)×10⁴CFU/g) (Figure 4.5). Unfortunately, due to sampling error the samples of T5 were lost before starting *Vibrio* spp. enumeration. Therefore, we could not be able to present the *Vibrio* spp. data here for T5.



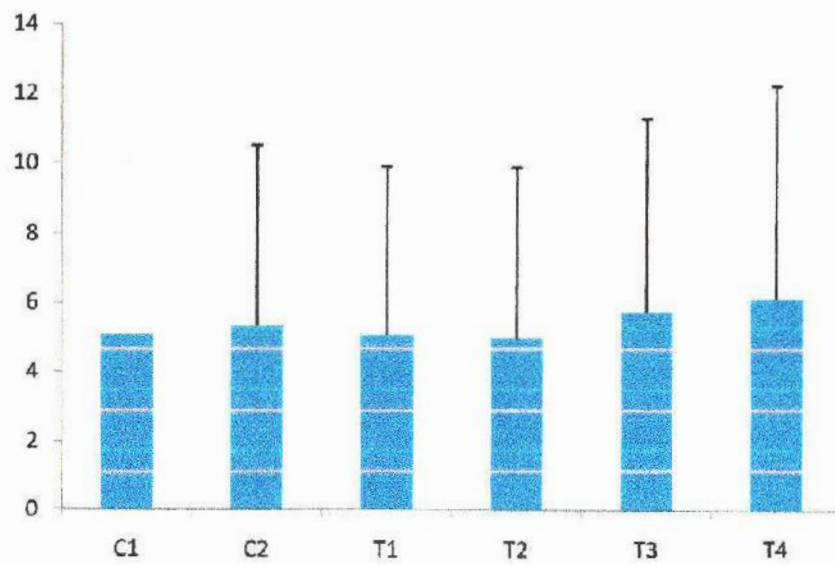


Figure 4.5 Total *Vibrio* spp. load in shrimp juvenile after a 10 days challenge test. The error bars represent the standard deviations of three replicates.

Chapter Five

DISCUSSION

5 Discussion

In this study, the virulence of *Vibrio parahaemolyticus* to shrimp juvenile was investigated. In addition, two well-known biodegradable chemicals was used to confirm their anti-virulence activity against the bacterium and finding a solution of antibiotic application in shrimp aquaculture.

The results of this experiment demonstrated that *Vibrio parahaemolyticus* is pathogenic to shrimp juvenile (Figure 4.1). Positive control group showed lower survival compared to other treatments, which means this bacterium has virulent characteristics to the target animal. In addition, treatment T1 and T2 with 100 μ M of indole and cinnamaldehyde respectively represented a minor toxicity to the animals as the survival were close to the control group C1. Indole (T3) as well as cinnamaldehyde (T4) were found to have protective capability of shrimp juvenile against *Vibrio parahaemolyticus* (C2). On the other hand, combined effect of indole and cinnamaldehyde could not protect the larvae shrimp where survival was 26%(Figure 4.1). It has been reported that indole is influencing to *Vibrio* spp. virulent towards shrimp juvenile (Gatesoupe, 1999). Cinnamaldehyde is an effective inhibitor of the growth of yeasts, bacteria, and molds as well as toxin production by microorganisms. On the otherhand, indole is an aromatic heterocyclic organic compound and can be produced by a variety of bacteria. Recently, it was showed that cinnamaldehyde and indole have quorum sensing inhibitory and stimulatory activities, respectively (Natrah *et al.*, 2011). However, both compounds significantly increased the survival of challenged juvenile, although the survival was lower in the treatments receiving *Vibrio parahaemolyticus* when compared to those receiving indole and cinnamaldehyde (Natrah *et al.*, 2012).

An *in vivo* challenge experiments revealed that the survival of challenged *Penaeus monodon* juvenile significantly increased when indole and cinnamaldehyde were added to the culture water at 1 μ M, offering a complete protection (Pande *et al.*, 2014). Importantly, the quorum sensing-disrupting compounds showed no negative impact on the growth of surviving shrimp juvenile. This might indicate that the vibrios are more vulnerable to quorum sensing disruption when during infection of shrimp juvenile. However, we cannot exclude the possibility that in addition to quorum sensing disruption, the compounds have other (yet unknown) activities towards either the juvenile or the bacteria (Pande *et al.*, 2014).

The challenge test results also demonstrated that at the end of the experiment the probability of cumulative survival was significantly higher in C1 compared to other treatments T1, T3 and C2 (Figure 4.1). In contrast, T1, T3 and C2 were found more hazardous than that of other groups, which indicated that as indole is a product of *V. parahaemolyticus*, this bacterium together with indole and cinnamaldehyde concentrations might be hazardous (Figure 4.2). However, indole and cinnamaldehyde concentrations used in this experiment might have impact on higher survival function in shrimp juvenile.

Zhang *et al.* (2017) revealed that total cell densities for all meat, shell, and shrimp samples were always within 10^6 – 10^7 CFU/ml, while there was no *V. parahaemolyticus* detected in natural *Vibrio* species in shrimp. For cinnamaldehyde treatment, *V. parahaemolyticus* decreased from 10^5 CFU/ml to 10^3 CFU/ml for meat samples at day 15 and then gradually increased to 10^4 CFU/ml at day 22 and dropped to minimum level of detection at day 25. For both shell and total shrimp samples, *V. parahaemolyticus* gradually decreased from 10^5 CFU/ml to minimum level of detection at day 4 (Zhang *et al.*, 2017). In contrast, in this study we did not find any significant difference in any of the treatment whether it was treated with indole or cinnamaldehyde, or even combination of both. Similarly, although not significant, but cinnamaldehyde concentration 100 μ M could not be able to reduce the *Vibrio* spp. load during this experiment. This might be due to the indole producing characteristics of *Vibrio* spp., which may demolish the effect of cinnamaldehyde at this concentration.

*C*hapter *S*ix

**CONCLUSION and
RECOMMENDATIONS**

6 Conclusions and recommendations

From this study the following conclusions can be drawn-

1. *Vibrio parahaemolyticus* is virulent to shrimp juvenile.
2. Indole and Cinnamaldehyde might regulates the virulence of *Vibrio parahaemolyticus* to shrimp juvenile.
3. Combination of indole and cinnamaldehyde might be toxic to *P. monodon*.

Recommendation:

1. Further studies should be conducted to uncover the disease protective mechanisms of cinnamaldehyde against *Vibrio parahaemolyticus*.
2. Molecular mechanism of quorum sensing and its regulatory mechanisms in controlling virulence factors of *Vibrio parahaemolyticus* should be investigated for understanding the disease mechanism in shrimp juvenile.

Chapter Seven

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4.2 Microbial analysis

4.2.1 Total bacteria count in *P. monodon* juvenile

The result of this study demonstrated that although not significant, but total heterotrophic bacterial count was found higher $((2.07 \pm 1.31) \times 10^8 \text{ CFU/g})$ in group T4 where shrimps treated with feed, *Vibrio parahaemolyticus*, and cinnamaldehyde compared to control group C1

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