Use of Direct-Fed Microbes to Enhance Shrimp Resistance to a *Vibrio parahaemolyticus* strain Causing Early Morality Syndrome

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Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
in
Life Science
in
Food Science and Technology

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May 10th, 2019
Blacksburg, VA

Keywords: *Litopenaeus vannamei,* Pacific white shrimp, probiotics, direct fed microbes, Early Mortality Syndrome (EMS), *Vibrio parahaemolyticus*

USE OF DIRECT-FED MICROBES TO ENHANCE SHRIMP RESISTANCE TO A *VIBRIO PARAHAEMOLYTICUS* STRAIN CAUSING EARLY MORALITY SYNDROME

ZACHARY WILLIAM TAYLOR

Academic Abstract

Early Mortality Syndrome (EMS) is a widespread bacterial infection of shrimp, attributed to pathogenic *Vibrio parahaemolyticus* strains (VP-EMS). This disease threatens aquaculture production and global food security. A valuable and alternative approach to using antibiotics for pathogen control, is the practice of incorporating direct-fed microbes (DFM) or probiotics. In order to evaluate the hypothesis that probiotics (specific strains of *Bacillus subtilis* spores) are able to provide shrimp, *Litopenaeus vannamei,* protection to the EMS disease, a pathogen growth model, disease challenge model, and probiotic feed coating methodologies were developed and refined, allowing independent shrimp probiotic trials to be piloted.

A single probiotic strain of *Bacillus subtilis*: O14VRQ and a blend of *Bacillus subtilis* strains: Plus10, were evaluated as feed additives or as water additions, for their efficacy. Accordingly, two independent trials were conducted in which shrimp were fed daily with a probiotic-coated feed for seven days, before a challenge with VP-EMS. Each trial consisted of a negative control (no VP-EMS exposure, no probiotic) and positive control (VP-EMS exposure, no probiotic), with five additional probiotic treatment groups, which were fed and exposed to VP-EMS in the same manner as the positive control. Shrimp were observed for clinical signs of disease after the initial exposure and were continuously exposed every 24 hours until 50% of the population remained in the positive control treatment. Both probiotics studied were shown to

significantly ($p < 0.05$) improve shrimp survival. Overall the data presented in this work demonstrates that probiotic prophylaxis is reliant upon probiotic dose, regardless of application.

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General Audience Abstract

Aquaculture is one of the fastest growing agricultural sectors in the world allowing it to greatly contribute to global food security. Seafood products are known for their excellent health benefits, providing good sources of protein, fatty acids, and vitamins. However, the animals raised in this industry, like in many facets of animal agriculture, are susceptible to disease. Diseases can be costly to treat and if no treatment exists, can be detrimental to farms, especially to highly valued species such as shrimp. Traditionally, many diseases have been treated with antibiotics, however this can promote the growth of antibiotic resistant bacteria, which is a public health concern especially when involving animals fit for human consumption.

An alternative to this approach is administering probiotics or beneficial bacteria to these animals. When incorporated with feed or applied to water, these beneficial bacteria can prevent diseases and help promote the growth of healthy animals. Two novel probiotics were fed to shrimp, before exposing them to the bacteria, *Vibrio parahaemolyticus*, which causes Early Mortality Syndrome, and is responsible for annual shrimp losses of more than $1 billion USD. Signs of this disease and survival were observed to assess if this probiotic could provide protection against this bacterium. Results from these studies show that these probiotics were capable of offering protection to shrimp when they were fed or introduced into tank water in high concentrations. Such probiotic applications could have beneficial effects on intensive shrimp aquaculture and help prevent this disease.

Dedication

“If I have seen further, it is by standing upon the shoulders of giants. “

– Sir Isaac Newton

I would like to dedicate this work to those who have laid the foundation before me and to those who will follow. I have had many who have supported me along the way and have helped me to see further in life. Many thanks to you all! Specifically, I would like to thank my wife Rebekah, who’s love, encouragement, and support in life helped make this possible.

Acknowledgements

Funding for this study was provided in part by Novozymes Biologicals (Salem, VA, USA). Special thanks to Dr. David Drahos, Meaghan Thompson, and Uri Levine for their support and collaboration. Additionally, thanks to Dan Taylor and Moonyoung Choi while in affiliation with Virginia Tech for their contributions to this work.

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List of Abbreviations

Acute Hepatopancreatic Necrosis Disease (AHPND); American Public Health Association (APHA); antibiotic resistant bacteria (ARBs); direct-fed microbes (DFM); feed conversion ratios (FCRs); dissolved oxygen (DO); Early Mortality Syndrome (EMS); EMS strain of *V. parahaemolyticus* (VP-EMS); Food and Drug Administration (FDA); gastrointestinal tract (GIT); Generally Recognized As Safe (GRAS); genetically modified organisms (GMOs); Good Aquaculture Practice for Fish Farming (GAP-FF); gut passage times (GPTs); lipopolysaccharide (LPS); nitrate (NO_3-); nitrite (NO_2); parts per million (ppm); parts per thousand (ppt); peptidoglycan (PG); pattern recognition receptors (PRR); photorhabdus insect-related (Pir); post larvae (PL); reactive oxygen species (ROS); recirculating aquaculture systems (RAS); TDH-related hemolysin (*trh*); thermostable direct hemolysin (*tdh*); thiosulphate citrate bile salts sucrose (TCBS); Toll-like receptors (TLRs); total ammonia nitrogen (TAN); β-1,4-linked N-acetylglucosamine (GlcNAc)

CHAPTER 1: Introduction

Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Disease (AHPND) is an epizootic bacterial infection of shrimp which has become a major threat to global shrimp production causing billions of dollars in economic loss. According to the Global Aquaculture Alliance, the shrimp aquaculture production by world regions indicated an annual production rate loss of 5.2% between 2006 and 2011, with a decrease in production of 6% between 2011 and 2013 (Anderson, Valderrama, & Jory, 2016). This decrease and slow rebound in production is correlated and attributed to pathogenic strains of *Vibrio parahaemolyticus*, which is the causative agent of EMS. There have been many endeavors to control pathogenic bacteria, such as the use of chemical additives and antibiotics which have been applied to aquacultural practices. However, when used improperly these measures fail to treat the bacterial root of the disease. Moreover, such practices can have negative implications such as increasing pathogen virulence by promoting antibiotic resistance. To prevent these diseases, aquaculturists must first control the environment by implementing good aquaculture practices such as basic water quality and sanitation practices. These measures assist in reducing environmental stressors placed upon the animals. However as aquaculture practices continue to intensify, animals are encountering increasing physical, chemical, and biological stresses (Mohapatra, Chakraborty, Kumar, DeBoeck, & Mohanta, 2013). Therefore, special attention must be also placed on biosecurity which prevents the introduction of potential pathogens, as stressed animals are more vulnerable to disease. Outside of these measures, the host may also be supported by nutrients and feed supplements, as functional foods have been shown to improve animal health and enhance disease resistance (Farzanfar, 2006).

There are many intervention strategies including bioremediation, development of vaccines and immunostimulants (Mohapatra et al., 2013). However, this is not always a uniform solution. Another strategy to combat diseases is the practice of applying direct-fed microbes (DFM) or probiotics to aquacultural systems. Selected bacterial strains are thought to shift microbial flora, reducing pathogen presence and persistence within hatchery tanks and aquaculture ponds through competitive exclusion. DFM treatment of penaeid shrimp and other aquatic species can also provide heightened or augmented immunological protection against pathogens, with a greater likelihood of success and fewer negative biological complications (Rengpipat, Phianphak, Piyatiratitivorakul, & Menasveta, 1998; Tseng et al., 2009). Furthermore, DFM treatment may stabilize water quality parameters (decrease toxic forms of NH_3 and NO_2), decrease feed conversion ratios (FCRs) and improve survival rates (Verschuere, Rombaut, Sorgeloos, & Verstraete, 2000). While this emerging field is promising, it is also important to quantify the effectiveness of DFM application, outside of subject mortality as the specific mechanisms of probiotic effectiveness remains unknown. Some parameters to consider DFM effectiveness include, pathogen antagonism, intestinal colonization, and heightened resistance to host pathogens (Gatesoupe, 1999). Furthermore, little is known regarding the persistence and environmental dispersion of DFM treatment, let alone pathogenic bacterial species in aquaculture systems. Bacterial dispersion in recirculating systems remains largely unknown, as current studies have only focused on the oral transmission and colonization of bacteria in the shrimp gastrointestinal tract, when their surrounding aquatic environment is of upmost importance. The aim of this research was to explore these interactions between antagonistic and pathogenic bacteria, as well as their role in shrimp (*Litopenaeus vannamei*), with special emphasis on the EMS strain of *V. parahaemolyticus* (VP-EMS). Therefore, the following overall objective and specific aims were proposed:

Overall Objective:

Optimize the protection of Pacific white shrimp (*Litopenaeus* vannamei) against the water-borne pathogen *Vibrio parahaemolyticus* using mono- and mixed-cultures of DFMs.

> Central Hypothesis: Direct-fed microbes (DFMs) or probiotics can provide and enhance shrimp protection to the EMS/AHPNS disease via natural microbial exclusion of pathogenic *Vibrio parahaemolyticus* strains.

Aim 1: Model and establish consistent growth of the *Vibrio parahaemolyticus* strain causing EMS

Aim 2: Determine lethal dosages of *Vibrio parahaemolyticus* EMS *in vivo* using lethal dosage methodology

Aim 3: Incorporate probiotic spore products onto commercially available feeds via spray top coating and confirm appropriate dosages to be applied

Aim 4: Compare two DFM spore products: *Bacillus subtilis* O14VRQ vs. a probiotic blend, Plus10, using feed-borne and tank delivery applications

CHAPTER 2: Literature Review

Aquaculture Industry

Aquaculture refers to the broad practice of farming in water, where aquatic organisms including fish, shellfish, bivalves, and plants, are reared in various environmental systems to produce food for human consumption, enhance wild harvest stocks, restore ecosystems, and reintegrate threatened and endangered species (NOAA, 2019). While aquaculture serves many purposes, this practice most greatly contributes to the agricultural production of seafood, or fish and shellfish products used for human consumption. According to the Food and Agriculture Organization of the United Nations (FAO), aquaculture is one of the fastest growing food-producing industries accounting for greater than 50 percent of all fish produced globally for human consumption (FAO, 2018). To put this into perspective, aquaculture was responsible for approximately 3 million tons of fish produced in the 1970s and as of 2015 world aquaculture production reached 76.6 million tons of fish which is valued at 157.9 billion US dollars (FAO, 2018). This increase in production can be directly correlated to aquaculture's growing popularity and more importantly its need worldwide.

According to the United Nations Department of Economic and Social affairs, the world population is expected to reach 8.5 billion by 2030 (UN DESA 2019). This growth presents a complex problem, as current and traditional agricultural practices and domestic food production cannot provide food security for the projected population. Aquaculture, therefore, serves as an alternative agricultural outlet to meet global food demands, while improving food security and nutrition in many parts of the world. In 2013, fish provided greater than 3.1 billion people with approximately 20 percent of their average per capita of animal protein (FAO, 2016). This alone accounted for 17 percent of the global population's intake of animal protein. However, this may not be as apparent domestically, as Americans typically consume 15.3 pounds of fish and shellfish per person per year. In comparison to countries such as the Philippines, farmed fish accounts for at least 12 percent of their animal protein intake (FAO, 2019). The United States is currently ranked as the third largest consumer of fish and shellfish, following China and Japan (NOAA, 2019).

Aquaculture is, moreover, practical in many ways in which conventional meat-protein agricultural practices are not. For instance, aquaculture is resource efficient, as fish, shellfish, and other aquatic organisms can convert more of the food they consume into edible body mass. In comparison to traditional land farmed animals, such as cattle or poultry, fish have lower feed conversion ratios or FCRs. It takes approximately 8.7 pounds of feed to produce 1 pound of beef, 5.9 pounds of feed to produce 1 pound of pork, and 1.9 pounds of feed to produce 1 pound of chicken. Strikingly, it takes only 1.2 pounds of feed to produce 1 pound of salmon (NOAA, 2019).

Products of aquaculture are also beneficial from a nutritional perspective. Fish are an excellent source of low-fat protein, which may provide many health benefits. For instance, omega-3 fatty acids, which are found in fillets, have been shown to reduce risk of stroke and heart disease by reducing cholesterol levels. Other benefits such as increased cognitive function and development are thought to arise from these fatty acids (Burger & Gochfeld, 2009).

Shrimp Culture

Shrimp comprise a large percentage of global aquaculture production as they are considered a high-value species and are exported to many industrialized countries (Subasinghe, Soto, & Jia, 2009). According to FAO, "*The global production of farmed shrimp in 2017 was estimated between 2.9–3.5 million tons, with 75 to 80 percent of the production originating in Asia-*

Pacific" (FAO, 2018). While most shrimp aquaculture occurs in China, many south-east Asian countries such as Thailand, Vietnam, Indonesia, Bangladesh, Malaysia, Philippines, Myanmar and Taiwan contribute significantly to global production and exportation. Shrimp and shellfish in general, remain one of the most popular seafood items in many countries (Otwell, W. S. & G. J. Flick, JR, 1995). Furthermore, they are high in protein and essential amino acid content, as well as, other vitamins and minerals, which offer a variety of health benefits (Venugopal & Gopakumar, 2017). In the United States, 2010 consumption of shrimp was 4.0 pounds per capita, which is significantly higher than the consumption of canned tuna and fish fillets of any other species at 2.7 pounds (Asche, Bennear, Oglend, & Smith, 2012). Penaeid shrimp are largely responsible for the increase in production and consumption trends observed. Among all shrimp species, *Litopenaeus vannamei*, is the most commonly farmed shrimp in the Western hemisphere, in Central and South American countries and in Asia and South-east Asia (Srinivas, Venkatrayulu, & Swapna, 2016).

Shrimp Husbandry and Production

L. vannamei, is native to Central and South America, i.e. Mexico and extending to Peru, where tropical marine environments and habitats are found. This species flourishes there due to temperate waters which remain above 20˚C throughout the year (FAO, 2006). This is vitally important for all shrimp production, as shrimp growth and feed conversion ratios (FCRs) or the amount or ratio of feed required to produce a desired biomass, is largely influenced by temperature. The optimum temperature of *L. vannamei* varies and is dependent upon their life cycle stage. Wyban et. al. found that growth is directly related to temperature but varies inversely with size and life stage (Wyban, Walsh, & Godin, 1995). Furthermore, *L. vannamei* may also be sensitive to subtle changes in temperature (Wyban et al., 1995). Shrimp smaller than 5 g, generally thrive at temperatures greater than 30˚C, but a decrease in growth feeding rates are observed in larger shrimp, in which the optimum temperature is 27˚C (Wyban et al., 1995). There is research which indicates *that L. vannamei* is tolerant to temperatures as low as 15˚C, allowing them to be cultured in cold seasons (Briggs & Nations, 2005). The native habitat of *L. vannamei* may also be responsible for its tolerance to a wide range of salinity. Adult and juvenile shrimp may exhibit hyper–hypo-osmoregulatory patterns in response to changing salinities ranging from freshwater to 50 parts per thousand (ppt). Optimally, the iso-osmotic salinity for shrimp is approximately 25 ppt (Chong-Robles et al., 2014). Growth rate and survival of Penaeid species is not directly dependent upon this range, especially in post-larvae and young shrimp (Zein-Eldin, 1963).

Shrimp have six main life stages, including several larvae stages: nauplii, zoea, mysis, and further maturation stages: post-larvae, juvenile and adult (FAO, 2006). Commercially, broodstock are encouraged to spawn and the first stage, nauplii, are hatched. During hatchery production, nauplii are separated and stocked into environmentally controlled tanks, where they are allowed to develop for approximately 2 days. Nauplii are semi-motile and survival only on egg yolk reserve (FAO, 2006). Zoea are considered the second larval stage and the first true feeding stage. Zoea diet is generally comprised of algae and artemia. This stage occurs for 3 to 5 days until eyes are developed. The third stage of development is known as mysis and last about 3 to 5 days. Here shrimp develop pleopods, or swimming appendages. With this addition, mysis are able to actively seek out food as they vigorously swim. After completion of the 3 larval stages of development, shrimp are fully developed and are classified as post-larvae or PLs. At this stage, farming operations usually obtain post-larvae at 10 to 12 days old, denoted PL10-12, and introduce or stock them directly into concrete tanks or earth ponds for grow out (FAO, 2006). The shrimp life cycle and corresponding metamorphosis are regulated by the molt cycle or molting, in which the exoskeleton or cuticle is periodically shed to facilitate growth. Since many crucial metabolic and endocrinological functions are dependent upon this phenomenon, it is a critical event in shrimp maturation (Corteel et al., 2012). Post-larval shrimp are able to achieve maturation and a marketable size at the age of 6-7 months, with male weights of approximately 20 grams and females weighing upwards of 28 grams (FAO, 2006).

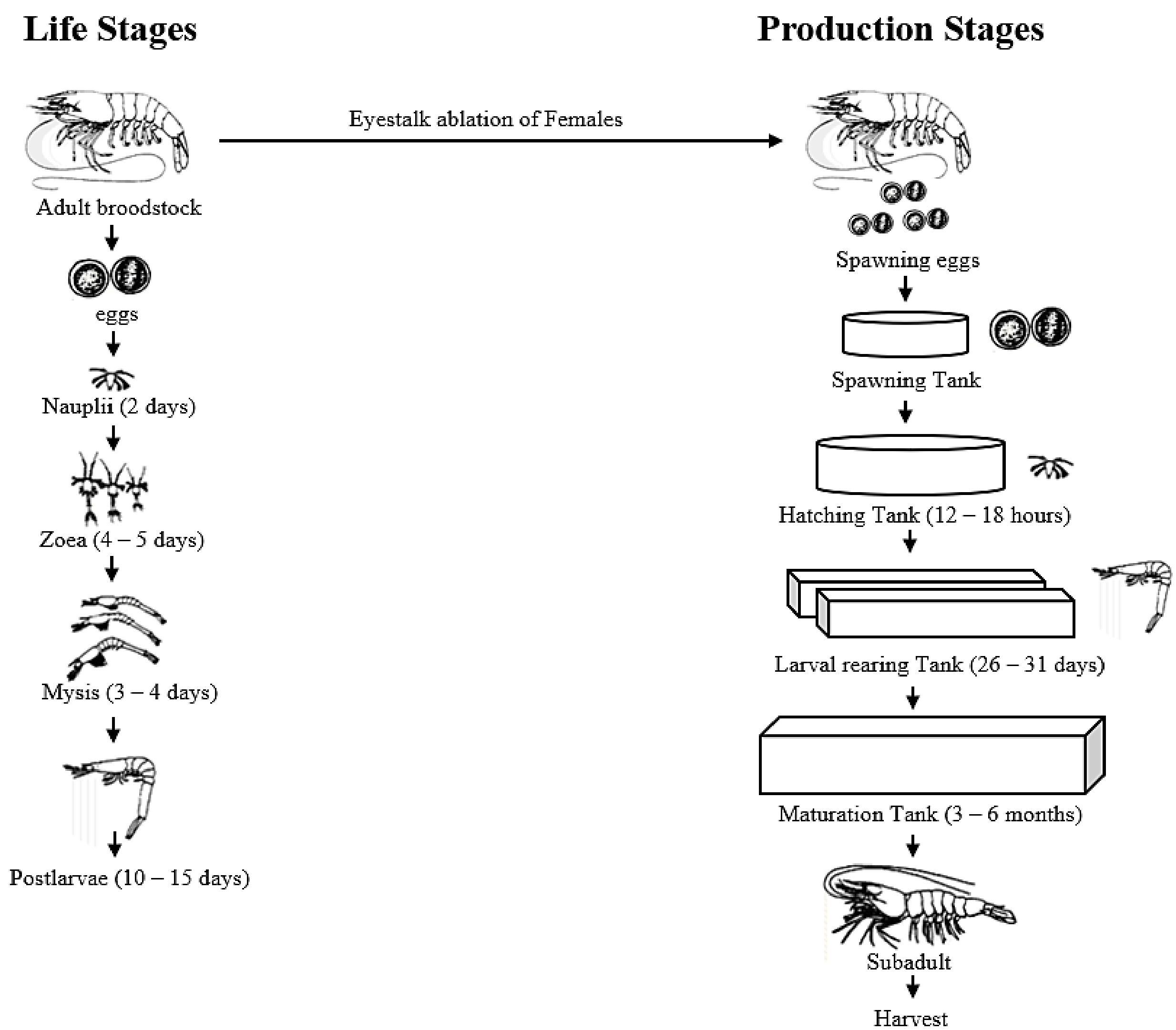

Figure 1. *General production cycle of farmed Penaeid shrimp adapted from FAO, 2006.*

Shrimp Anatomy

The external anatomy of *L. vannamei* is characterized by the segmented chitin comprised exoskeleton, containing a dorsal carapace and individual tergums (Carpenter & FAO, 2002). This external shell has accompanying jointed appendages located ventrally. Shrimp can be divided into two main segments: the anterior cephalothorax, containing a fused head and thorax with a characteristic rostrum, and the posterior abdomen, which is divided into six segments, as shown in Figure 2 (Alday-Sanz, 2010). Shrimp have a pair of compound eyes found at the end of a stalk; along with antennules and antennae; they are responsible for sensory functions and detecting external stimuli in the shrimp's environment. Attached to the cephalothorax are paired appendages known as maxillipeds and pereopods, each which have a distinct anatomical function. Maxillipeds are known as feeding appendages and are located near the mouth, while pereopods are considered ambulatory appendages used for walking. However, some pereopods contain specialized chela used for grasping, cutting, or crushing items (Alday-Sanz, 2010). Internal organs, such as gills, heart, hepatopancreas (combining the function of a liver and pancreas), and stomach are located within the cephalothorax. Posteriorly, shrimp are comprised of mainly muscle and contain five pairs of pleopods located on abdominal segments one through five. These appendages are used in swimming and responsible for rhythmical propulsion (Alday-Sanz, 2010). Located on the sixth abdominal section are a pair of uropods, or "tail fin", which enable shrimp to escape predators by rapidly propelling the shrimp in a backwards direction (Alday-Sanz, 2010).

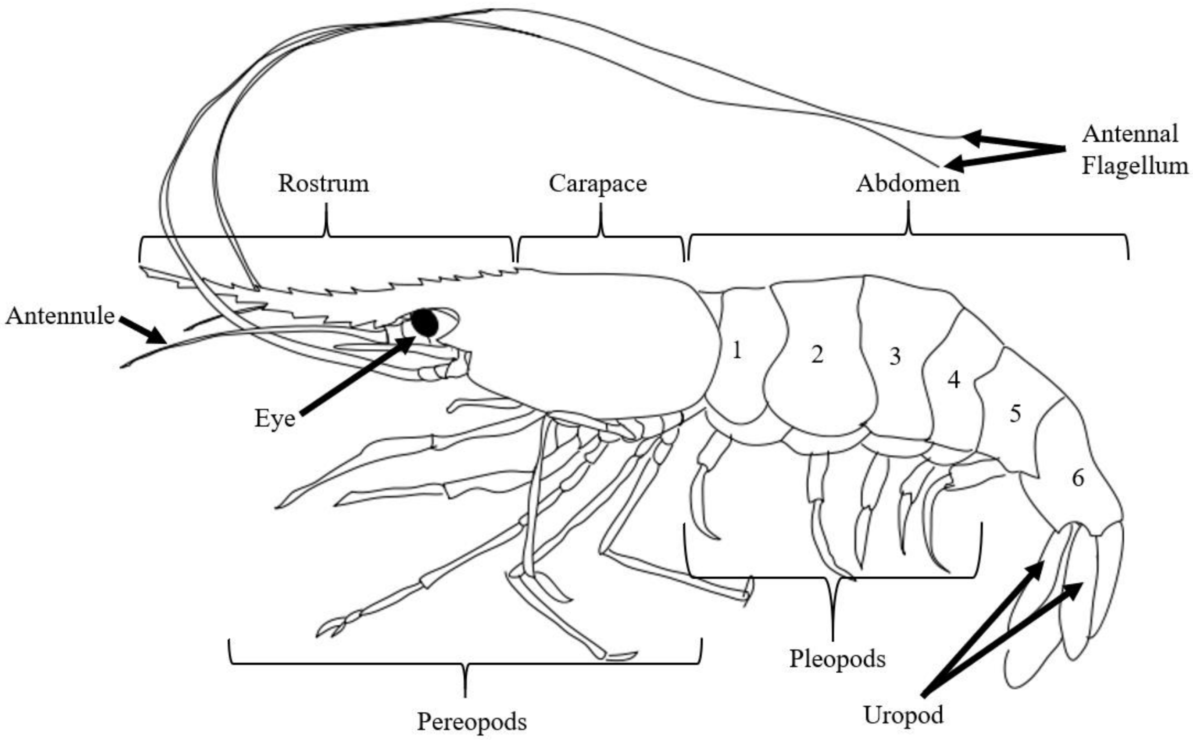

Figure 2. *General external anatomy of Litopenaeus vannamei adapted from Carpenter & FAO, 2002.*

Shrimp Immune System and Defenses

Immunity and defense against pathogens are critical, considering that shrimp and many other marine organisms, are in immediate contact with their surrounding aquatic environment, which often carry and harbor bacteria and viruses. While the chitinous exoskeleton provides a physical and chemical barrier, it is not invulnerable. Damage to this shell can create an entry point for opportunistic microorganisms. This barrier cannot provide consistent protection, as it is shed during the molting process to allow growth and development (Söderhäll & Cerenius, 1992). There is therefore a great need for an efficient immune system. However, unlike many other aquatic organisms which have an adaptive immune system, shrimp only rely upon the molecular defenses provided by an innate immune system to protect them against pathogenic microorganisms. Innate immunity or non-specific immunity includes both cellular and humoral defenses (Jiravanichpaisal et al., 2006), which are triggered by pathogens. They are recognized via highly conserved pathogen-associated molecular patterns, or PAMPs. Examples of these surface proteins or structures include lipopolysaccharide (LPS), peptidoglycan (PG), bacterial flagellin proteins, and nucleic acids (Alday-Sanz, 2010). This recognition is made possible via free or membrane-bound receptor molecules, which have similar functionality as pattern recognition receptors (PRR), such as Toll-like receptors (TLRs) (Alday-Sanz, 2010). These specialized pattern recognition receptors stimulate a series of immune responses leading to activation of either cellular, i.e. phagocytosis and nodule formation and encapsulation, or humoral, i.e. phenol oxidase, reactive oxygen species (ROS), antimicrobial peptides, and anticoagulant proteins, host-defense systems (Ji et al., 2009). Additionally, these pattern recognition receptors can recognize substances and structures similar to β-1,3-glucan, indicative of some fungi, as well as, double stranded RNA characteristic of viral pathogens (Ji et al., 2009).

Shrimp Digestive System

The shrimp digestive system is responsible for a variety of nutritional functions including ingestion, digestion, absorption and storage of nutrients, followed by packaging and passing of excrement. The digestive system of shrimp is comprised of three main segments, the foregut, midgut and hindgut (Ceccaldi, 1989). The foregut begins with an external portion of specialized mouth parts such as the mandibles, responsible for mastication and leads to the stomach comprised of a muscular esophagus and proventriculus (Alday-Sanz, 2010). This portion of the digestive tract consequently serves as a primary route of infection. However, shrimp are able to deter and eliminate many viruses and bacteria, as this portion has a chitinous membrane with an arsenal of host defense acids and enzymes (Jiravanichpaisal et al., 2006). Food enters the mouth and is passed through the anterior and posterior chamber of the proventriculus, where a complex series of movements coordinated with teeth reduces particle sizes. The posterior chamber or pyloric chamber allows ingested material to pass along to the midgut (Alday-Sanz, 2010).

The midgut houses a specialized organ, called the hepatopancreas, which combines the function of a liver and pancreas. In general, the shape and color of the organ is indicative of shrimp health, as it is sensitive to environmental or microbial toxins. This multifunctional organ also carries out many metabolic functions, such as synthesis and secretion of digestive enzymes, nutritional absorption, lipid and carbohydrate metabolism, and distribution of stored nutrients during the molting cycle (Ceccaldi, 1989). This organ is segmented or lobed as it surrounds the stomach on both sides inside the cephalothorax. Composed of a series of tract ducts or channels, the hepatopancreas releases digestive enzymes to the stomach and also allows fine particles to enter (Ceccaldi, 1989).

Non-digestible materials are rejected into the hindgut. Located dorsally, the hindgut is a chitin-line chute which begins at the rectal gland and continues posteriorly through abdomen, rectum and ending at the anus (Ceccaldi, 1989). The lining of the hindgut contains a folded epithelium which forms a hook structure and points posteriorly. This is thought to aid in transit and evacuation of feces (Alday-Sanz, 2010). The time it takes for feed to pass completely through the gut and exit the anus or gut passage times (GPTs) is variable and has not shown to be dependent upon the composition of feeds, i.e. fiber, protein, or lipid content, nor the size of shrimp (Beseres et al., 2006). However, this evacuation may be influenced if shrimp are starved or if they have continuous access to foods.

Water Quality

While aquaculture is attractive when compared to conventional terrestrial practices, it faces its own unique set of challenges. Factors such as temperature and salinity have historically been considered as the most important physical factors which influence aquaculturally raised animals, however biological factors add to this complexity (Ponce-Palafox et al., 1997). One particular challenge is the aquatic medium in which animals are reared. Unlike traditional farmed animals, fish and shellfish must combat waste products of their own metabolism, i.e. ammonia and other nitrogenous species including nitrite and nitrate.

Generally, removal of these toxic by-products is managed in recirculating aquaculture systems (RAS) via biofiltration, in which biological filters containing autotrophic species of nitrifying bacteria reduce harmful ammonia species to nitrate through the process of nitrification (Chen, Ling, & Blancheton, 2006). However, many shellfish practices are incorporating 'biofloc' technologies, in which suspended microbial floc growth is encouraged. Here nitrogenous nutrient waste is recycled and used in microbial biomass production, which is later consumed by the animals or can be further processed and incorporated into feeds (Bossier & Ekasari, 2017). Biofloc applications may reduce the quantity of water used to maintain traditional water exchange RAS systems, while also mitigating accumulation of toxic ammonia, and decreasing FCR and feed costs (Bossier & Ekasari, 2017; Rode, 2014).

In nitrification, ammonia is oxidized into nitrite (NO_2-) by bacterial species such as *Nitrosomonas spp*. The less toxic nitrogen species, nitrate (NO_3-) is converted by the oxidation of nitrite by important bacterial species such as *Nitrobacter spp.* (Chen et al., 2006). Nitrification requires an adequate supply of oxygen and inorganic carbon such as bicarbonate (alkalinity) for the successive bacterial species to produce cell biomass and complete the cycle (Chen et al., 2006). Nitrogenous species can easily accumulate in RAS due to poor design, management, or simply intensity of shrimp culture. Total ammonia nitrogen (TAN) can rapidly accumulate simply due to high metabolic excretion found in high stocking density ponds and systems (Obbard & Shan, 2001). Optimum conditions for shrimp require a concentration of less than 0.1 parts per million (ppm) unionized ammonia at pH 8.0 and temperature of 28–30 °C (Obbard & Shan, 2001).

Following a progressive pattern, as ammonia decreases, subsequent nitrogenous species such as the intermediate product, nitrite, increase. Much like ammonia, accumulation of nitrite can reduce overall water quality, inhibit growth, increase mortality, reduce dissolved oxygen concentrations, and increase susceptibility to pathogens (Lin & Chen, 2003). While levels of ammonia in culture ponds have been reported to be as high as 4.6mg/L, the lethal concentration depends on stage of development (i.e. juvenile or adult) (Lin & Chen, 2003).

Simple evaporative losses in RAS systems can also lead to toxic nitrate levels. While nitrate is generally considered less toxic than other nitrogen species, at concentrated levels due to less water volume, it can be problematic (Kuhn et al., 2010). Nitrate at levels greater than 200 ppm at a salinity of 11.0 ppt can prevent growth and biomass accumulation, as well as have adverse effects on shrimp physiology including damages to the hepatopancreas and gills (Kuhn et al., 2010).

Specific water quality requirements will also depend largely upon species and stage of development. For instance, adult *Litopenaeus vannamei*, spawn in salt water with salinities of at least 28 ppt, while early larval stages can tolerate a wider range, since their natural estuarian habitat can experience extreme fluctuations (Crawford, 1999). A general list of water quality parameters for Penaeid shrimp culture can be found in Table 1, however the environment and animal stage of development should be considered to maintain optimum conditions, reduce stress, and encourage growth.

Table 1. *Water quality parameters for shrimp culture adapted from Crawford, 1999.*

Water Quality Parameter	Range
Temperature	28 – 32°C
Dissolved Oxygen	5.0 – 9.0 ppm
pH	7.0 – 8.3
Salinity	20 – 35 ppt
Calcium Hardness ($CaCO_3$)	≥ 100 ppm
Total Alkalinity ($CaCO_3$)	≥ 100 ppm
Unionized Ammonia (NH_3)	≤ 0.03 ppm
Nitrite (NO_2)	≤ 1 ppm
Nitrate (NO_3)	≤ 60 ppm
Chlorine	≤ 10 ppb

Special attention must be placed upon the water quality of systems which house aquacultured animals. While various approaches are taken to ensure water quality is optimum, conservation of resources and steps to reduce environmental impacts must also be considered. Recirculating aquaculture systems, which limit water use and exchange, are commonly used, however much of commercially produced aquaculture products are farmed in open waters.

Diseases

Perhaps the biggest challenge facing agricultural practices, including aquaculture, today is disease. Diseases in the aquaculture sector alone, result in losses upwards of several billion US dollars per year, globally (Defoirdt, Sorgeloos, & Bossier, 2011). Disease in aquaculture can furthermore be amplified in animals, simply by the water surrounding them which acts as a medium for viral, fungal, bacterial, and parasitic infections. This aquatic environment supports many pathogens independently of their host, whereas terrestrial or land-based agricultural environments cannot reach these high pathogen densities and directly interact with the host animal outside of ingestion or injury (Defoirdt et al., 2011).

Antibiotics

Met with the challenge of keeping animals healthy while promoting growth, aquaculture much like traditional agricultural practices, has advanced the expanding use of antibiotics. Antibiotics can be defined as any natural or synthetically derived compound which has some capacity to kill or inhibit growth of microorganisms, while not harming the host (Hernández Serrano, 2005). Feeding of antibiotics to animals has historically been shown to improve growth via increased absorption of intestinal nutrients, decreasing host energy investments and subsequently improving the efficiency of how these nutrients are used (Hernández Serrano, 2005). Prophylactic use of antibiotics is illegal in the United States and has been largely consumer driven as preferences have forced many companies to reduce their use in animals (Boeckel et al., 2017). Many countries are implementing regulations to limit use in animal production, yet legislative loopholes may still allow antibiotics to be routinely administered, with no apparent evidence of disease or the presence of pathogens (Defoirdt et al., 2011). It is estimated that eighty percent of antibiotics within the United States are not used in human healthcare, but are directly applied to agriculture, including animal agricultural practices, such as aquaculture (Hollis & Ahmed, 2013). This number is striking considering that globally more than 131,000 tons of antibiotics were used in food animals in 2013 and this use is projected to be more than 200,000 tons by the year 2030 (Boeckel et al., 2017). Antibiotic and antimicrobial use in animals is largely targeted and intended to increase growth rates and serve as a prophylaxis, which is a stark contrast considering clinal use of antibiotics in humans are used as a treatment strategy post-infection (Boeckel et al., 2017).

Over use of antibiotics has negative implications such as creating antibiotic resistant bacteria (ARBs), which in animals farmed as food, raises concern for public health, as these pathogens may not only infect the cultured animal, but perhaps even the humans consuming them (Holmström et al., 2003). Antibiotics used in food-producing animals are often administered through feed. Considering the biomass of animals raised for food exceeds that of humans, food animals serve as a reservoir in which a greater source of resistance genes is likely to be found (Boeckel et al., 2017). Development of antibiotic resistant genes can not only be found in the intestinal bacteria of food animals but also in their environment. Specifically considering aquaculture, ARBs are likely to be found in the host and in the surrounding aquatic environment. Since bacteria have the capability of sharing resistance genes via horizontal gene transfer, the possibility exists that this exchange may reach terrestrial environments including human and animal pathogens (Cabello, 2006). This can be further amplified via the contamination of water sources with agricultural waste or untreated sewage, as unconsumed food and animal feces often contains these antibiotics, which can then reach sediments or be easily transported via this aquatic vehicle (Cabello, 2006). A further concern is that many countries producing and exporting large aquacultural commodities, do not monitor the type or quantity of antibiotics used in the rearing of these animals (Defoirdt et al., 2011). For instance, in a study conducted in 2000 which interviewed shrimp farmers along the Thai coast, 74% used antibiotics in their practice and at least thirteen different antibiotics were used (Holmström et al., 2003). This concern has led many government entities and aquaculture certification programs, such as Good Aquaculture Practice for Fish Farming (GAP-FF) and Best Aquaculture Practices Certification, to limit and restrict the use of certain unapproved antibiotics, like chloramphenicol (a broad-spectrum antibiotic used clinically in humans to treat infection). Reducing and preventing the use and reliance on such antibiotics is therefore crucial to maintain healthy and otherwise safe food production (Rengpipat et al., 1998; Tseng et al., 2009; Verschuere et al., 2000).

The use of antibiotics or any type of antimicrobial agent, such as chemical disinfectants, has an associated risk of increasing the frequency of bacterial resistance to the agent used. Not only is this of importance to maintain food quality and safety, but antibiotic use and overuse may soon play a role on economics with such great importation and exportation of seafood. Unfortunately, once the decision is made to use an antibiotic or anti-microbial, the risk of creating antibiotic resistance cannot be avoided (Alday-Sanz, 2010).

Probiotics

Many chemical disinfectants and biological treatments such as vaccinations are used in aquaculture to control parasitic and microbial infections and contribute to decreasing the use of antibiotics. However, they can often be ineffective, labor intensive, difficult to regulate and approve, and inappropriate for species such as shellfish (Costello et al., 2001). While many finfish operations apply these measures, most shellfish and shrimp hatcheries and operations use antibiotics, as shrimp do not possess the ability to create a secondary immune response, as is seen in fish and mammals by the conventional antigen-antibody based vaccine (Alday-Sanz, 2010). This factor is considered a major hinderance in development of consistent and quality shrimp aquaculture production (Ninawe & Selvin, 2009). Considering this limitation, the approach is quickly shifting to finding natural immune enhancing substances which can be administered via water immersion or ingested directly as oral supplements (Alday-Sanz, 2010). Additionally, the fear of antibiotic overuse and onset of disease outbreaks has led to global support of prophylactic alternatives, such as probiotics, which have been shown to provide heightened or augmented immunological protection against pathogens, while minimizing antibiotic resistant bacteria. The increasing demand and public pressure upon the shrimp industry, continues to drive application of more natural alternatives.

The term probiotics originated from a Greek word which translates to "for life" (Farzanfar, 2006). Historically probiotics have been defined as bacteria which provide some benefit to humans and animals, with much of this technology and definition curtailed to terrestrial animals, which do not encounter some of the same challenges as aquacultured animals. Beneficial effects of probiotics, such as increasing growth, improving overall health, and increasing disease resistance, have been well documented in pig, cattle and poultry, however this is a relatively new avenue for aquaculture. (Farzanfar, 2006) Robert Fuller defines probiotics as, '*A live microbial feed supplement which beneficially affects the host animal by improving intestinal microbial balance*' (Fuller, 1989). Authors such as Moriarty have extended this definition and application to further include aquatic animals and their surroundings, as '*the addition of live bacteria to tanks and ponds in which the animals live…*(and) *modify the bacterial composition of the water and sediment; the health of animals is thus improved by the elimination of pathogens or at least minimizing the effect of pathogens…*' (Moriarty, 1998). This is an important consideration, moving forward with probiotic development, as bacteria are present in and on every surface of the earth, including the aquatic environment. Therefore, from the first stage of development, an aquatic organism's microbiota is established based upon its surrounding environment (Farzanfar, 2006). Probiotics, can therefore serve to develop this microbial barrier against pathogens, which may also be encountered in the same medium (Farzanfar, 2006).

There is a wide range of probiotics which have been evaluated for aquacultural use, including Gram-negative and Gram-positive bacteria, bacteriophages, and yeast (Irianto & Austin, 2002). Of these options, bacteria are most commonly used. Lactic acid bacteria and *Bacillus* spp. are often used agriculturally; though, it is often specific strains of these bacteria which of are interest, due to the plethora of positive effects demonstrated (Farzanfar, 2006). *Bacillus* spp. bacteria are being exploited more commonly due to their ability to sporulate, which provides increased survival in the gastrointestinal tract, as well as, stability when processed and stored (Elshaghabee, Rokana, Gulhane, Sharma, & Panwar, 2017). Furthermore, *Bacillus* spp. can produce many different antibiotic compounds, secretory proteins and enzymes which can degrade biofilms (Elshaghabee et al., 2017). In addition, *Bacillus* spp. have been shown to inhibit other bacteria via competition for nutrients in the environment and the gut (Moriarty, 1998). Isolated *Bacillus* strains have a proven track record of providing improvements in the survival and growth of shrimp species, in addition to, providing disease resistance to several pathogenic *Vibrio* species (Gomez-Gil, Roque, & Turnbull, 2000; Moriarty, 1998; Rengpipat et al., 1998; Wang, Xu, & Xia, 2005).

Specific strains of *Bacillus subtilis* are of interest to commercial aquaculture for the above reasons and more. Not only is *B. subtilis* economically feasible to produce, growing on a number of low-cost carbon and nitrogen sources, but it has great probiotic capacity, via spore production, fast growth rate and versatility, enzymatic and antimicrobial production, and development in both aerobic and anaerobic conditions (Olmos & J, 2014). Additionally, this multifunctional probiotic is Generally Recognized As Safe (GRAS) by the Food and Drug Administration (FDA) (Olmos & J, 2014).

Vibrio species and Vibrio parahaemolyticus

Vibrio spp. are responsible for an estimated 80,000 cases of foodborne illness and approximately 100 deaths in the United States each year (CDC, 2018). Vibriosis is most commonly associated with the consumption of raw or undercooked seafood or exposure of an open wound to seawater (CDC, 2018). While most infections are not life threatening, symptoms of ingesting *Vibrio* spp. leading to an illness known as gastroenteritis, can include severe diarrhea, abdominal cramps, nausea and vomiting, fever, and headache (Hsern Malcolm et al., 2015). *Vibrio* spp. are Gram-negative, halophilic, and naturally found in warm coastal marine waters (Hsern Malcolm et al., 2015). While *Vibrio* spp. have a broad infection range including human, finfish, and shellfish such as shrimp, not all species are pathogenic. In fact, many *Vibrio* spp. are opportunistic pathogens. There are approximately 100 different species of aquatic bacteria which have been identified, with only a few that are associated with human illness, most notably: *Vibrio cholerae*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus* (Jones, 2014). Prevention of foodborne illness associated with *Vibrio* spp. requires proper cooking of seafood to inactivate the bacteria, as *Vibrio* are very susceptible to heat (Jones, 2014).

Vibrio parahaemolyticus is a bacterium with a single polar flagellum which grows optimally in salt concentrations of 20-25 parts per thousand (ppt) and at temperatures between 30-35˚C (Jones, 2014). It is one of the most common *Vibrio* spp. isolated from seafood. This phenomena may be due to *Vibrio* interactions and association with chitin, a polysaccharide which is composed of β-1,4-linked N-acetylglucosamine (GlcNAc) residues, which is most commonly associated with the shell or exoskeleton of aquatic arthropods and crustaceans such as shrimp, lobster, and crab (Pruzzo, Vezzulli, & Colwell, 2008). These organisms produce approximately 100 billion tons of chitin each year, as they grow and develop (Yan & Chen, 2015). Adhesion and binding to chitin is a complex process, however this bacterial-substrate interaction rewards capable species with advantages and nutrients (Pruzzo et al., 2008). Hydrolysis of chitin via chitinases, a characteristic enzyme of *Vibrio* spp. which cleaves glyosidic bonds, not only provides nutrients to bacterial species, but also aids in the biogeochemical recycling of carbon and nitrogen in marine environments (Pruzzo et al., 2008). *Vibrio parahaemolyticus* has been shown to cause infections in humans, as it contains two hemolysin virulence factors: a thermostable direct hemolysin (*tdh*) and a TDH-related hemolysin (*trh*), as well as, type III and type VI secretion systems, further contributing to infectivity (Letchumanan, Chan, & Lee, 2014).

EMS/AHPNS Strains

Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS) is a bacterial disease of penaeid shrimp, which emerged around 2009 (Lee et al., 2015). Typically impacting post-larval shrimp within 20-30 days post-stocking, this disease can cause up to 100% mortality and is responsible for losses estimated at $1 billion USD (Schryver, Defoirdt, & Sorgeloos, 2014). Species effected are *Litopenaeus vannamei* (Pacific white shrimp) and *Litopenaeus monodon* (black tiger shrimp) (Tran et al., 2013). The causative agent is known to be the waterborne bacterial pathogen *Vibrio parahaemolyticus*. The otherwise opportunistic pathogen is strain specific, as these strains contain a ~70 kb plasmid which encodes for insect-related (Pir) toxin-like genes (Han, Tang, Tran, & Lightner, 2015; Lee et al., 2015). Further virulence factors, which may contribute to this disease, have yet to be characterized, however the presence of hemolytic activity and type III and type VI bacterial secretion systems are likely to play a role (Kongrueng et al., 2015).

EMS/AHPNS strains of *Vibrio parahaemolyticus* are transmitted orally and localize in the gastrointestinal tract (GIT), where the bacteria begins to produce toxins that cause atrophy and bacterial necrosis of the hepatopancreas, an organ which functions as both a pancreas and liver (Tran et al., 2013). Infected shrimp have distinct clinical signs. Effected shrimp display lethargy, slow response to stimuli, decreased growth, and empty stomach midgut, as well as, the most distinguishing sign: a pale to white atrophied hepatopancreas (Kongrueng et al., 2015). The most common detection methods, are isolation of *V. parahaemolyticus* from the intestinal tract, stomach, or hepatopancreas using selective media such as thiosulphate citrate bile salts sucrose (TCBS) or CHROAMagar *Vibrio* agars (Kongrueng et al., 2015; Letchumanan et al., 2014). However, molecular methods such as PCR or PFGE exist to distinguish between pandemic strain isolates (Letchumanan et al., 2014).

Currently, no disease treatments exist for EMS/AHPNS. There is interest in breeding resistance to the disease, through generational breeding and selection of shrimp. Howbeit, this is laborious, time intensive, and met with the opposition of limiting genetically modified organisms (GMOs) in the food supply. Preventive vaccination as a control strategy is not applicable due to immunological limitations. Antibiotics are not an option, as there is a push to decrease use, and in this case, EMS isolates have been shown to be resistant to most antibiotics tested, including ampicillin, tetracycline, chloramphenicol, sulphamethoxazole/trimethoprim, gentamycin, erythromycin, and norfloxacin (Kongrueng et al., 2015). Since *V. parahaemolyticus* is commonly found in coastal waters, where shrimp are raised, prevention methods have primarily focused on controlling the activity of the environmental bacteria. Activities such as total disinfection of pond sediment and water to eliminate EMS/AHPNS strains, may further contribute to this disease, as the microbial ecosystem is disturbed and diversity is highly reduced (Schryver et al., 2014). What is left is a nutrient rich environment, favoring the fastest growing species, i.e. vibrios with little natural microbial antagonism or competition (Schryver et al., 2014). Further complicating prevention strategies is the fact that the virulent 69 kb plasmid, pVPA3-1 (Jee Eun Han, Tang, Tran, & Lightner, 2015; Hernández Serrano, 2005; Kongrueng et al., 2015) is obtained or acquired via horizontal gene transfer and subsequent transposition or homologous recombination, allowing seemingly harmless species to become pathogenic (Lee et al., 2015). Microbial management of waters and stocking ponds has been presented as the best prophylactic measure as proper densities and diversities of colonized bacteria can provide natural exclusion of pathogenic bacterial growth and decrease animal mortalities (Schryver et al., 2014). Nonetheless, this strategy cannot act as a curative treatment for shrimp once infected (Schryver et al., 2014).

Conclusions

Given aquaculture's potential for growth and unique set of challenges to be faced, there are many opportunities for improvement and development. Bacterial diseases threaten this industry and further expansion is impeded by incorporating hazardous practices such as applying antibiotics, which can in turn amplify diseases by selecting for resistant species. The presence of EMS-causing strains of *Vibrio parahaemolyticus* is a current example of this and warrants the need for novel and natural alternatives. With no viable or economically feasible options, probiotics stand to fill this void and offer excellent benefits, such as mitigation of toxic waste by-products, improvement of health, and at the molecular level: resource competition and exclusion against pathogens. Many of these ecological processes are yet to be understood, but by manipulating these microbial parameters in the environment and in the animal, research has been shown to drastically affect bacterial species growth and mortality rates, leading to major shifts in microbial dominance. Applied to the EMS endemic, probiotics could provide competition with *Vibrio* and shift the flora in favor of beneficial bacterial which hinder pathogen gut adherence and persistence, while improving and promoting gut health (i.e. digestibility and absorption of nutrients, etc.) However, the probiotic that is added must be appropriately selected for specific functions, added in the appropriate concentration, and at the right environmental condition, to achieve desirable outcomes. (Moriarty, 1998) Therefore, this study aimed to incorporate select probiotic strains onto aquaculture feeds, to provide or enhance shrimp (*Litopenaeus vannamei*) resistance to pathogenic *Vibrio* species responsible for EMS.

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CHAPTER 3: *Vibrio parahaemolyticus* EMS pathogenesis in *Litopenaeus vannamei*

1. Introduction

The widely distributed marine bacterium, *Vibrio parahaemolyticus,* is commonly associated with seafood, particularly shellfish. It has gained attention as a human pathogen as the causative agent of gastroenteritis or vibriosis, a foodborne illness which occurs after consumption of raw or undercooked seafood (Su & Liu, 2007). This illness appears to have an increasing prevalence, as global warming contributes to warmer marine waters and the consumption of shellfish continues to rise (Oliver & Jones, 2015). However, *V. parahaemolyticus* can also cause disease in a broad range of hosts. In fact, bacteria which belong to the family *Vibrionaceae*, are considered one of the most prominent causes of mortality in cultured Penaeid shrimp (Ananda Raja et al., 2017). Diseases caused by *Vibrio parahaemolyticus*, such as Acute Hepatopancreatic Necrosis Disease (AHPND) or also known as, Early Mortality Syndrome (EMS) are responsible for high mortality rates (up to 100%) and have resulted in losses upwards of $1 billion USD annually in the aquaculture industry (Ananda Raja et al., 2017; Defoirdt, 2016).

Considering the destructive impacts of *V. parahaemolyticus* to this industry, it is logical to model not only the bacteria, but the pathogenicity profile in the shrimp host. Previous studies have developed LD_{50} models based upon intramuscular injection or reverse gavage (Ananda Raja et al., 2017; Lee et al., 2015). Such research is important clinically; however, it does not effectively model natural infectivity and may be unrealistic to develop mitigation strategies in intensive shrimp farming. Therefore, it is warranted to ascertain the growth of *V. parahaemolyticus* strains causing AHPND/EMS, so that a shrimp model utilizing a more natural, chronic disease challenge may be further refined. It was hypothesized that different concentrations of EMS culture would result in significantly different survival rates. The overall objective of this study was to confirm and improve upon our current working model in order to establish the optimum dose of *V. parahaemolyticus* to achieve consistency for infecting shrimp.

2. Materials and Methods

The following protocols were conducted in accordance with the Institutional Biosafety Committee at Virginia Tech (VT IBS #17-057).

2.1 General Methods: *Vibrio parahaemolyticus* Growth

Preparation of starter cultures

EMS strains of *Vibrio parahaemolyticus* were obtained from Novozymes Biologicals (Salem, VA). VP-EMS glycerol stocks ("starter cultures') were used in subsequent modeling of VP-EMS growth and for shrimp exposure. Glycerol freezer stocks of VP-EMS cultures were prepared by growing the obtained culture in 50 mL of TSB2+ (BBL Trypticase Soy Broth - Becton Dickinson and Company, Sparks MD USA), supplemented with 2% NaCl (Carolina Biological Supply Company, Burlington NC, USA) at 30˚C shaking (250 RPM) until an optical density (OD_{600}) of 0.5 was reached, which correlated to logarithmic phase growth. One mL of this culture was added to a final concentration of ~20% glycerol, vortexed to ensure homogeneity and subsequently frozen at -80˚C. Trypticase Soy Broth was supplemented with an extra 2% NaCl, which made the overall salinity 2.5% NaCl (25 parts per thousand, ppt) to match the salinity of the water in which shrimp would be later maintained.

Modeling *Vibrio parahaemolyticus* EMS growth

Vibrio parahaemolyticus EMS strain culture growth was confirmed via spectrophotometry and aerobic plate counts, measuring the culture and plating in triplicate, approximately every hour for 18 hours. The plating medium used was Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) (Becton Dickinson and Company, MD, USA) which is selective and differential for *Vibrio* spp. Dilutions of VP-EMS culture were prepared using phosphate buffer saline (PBS), and 0.1 mL was plated over the agar surface using a spiral plater (Whitley WASP Touch Automated Spiral Plater, Don Whitley Scientific Limited, West Yorkshire, UK). Spread plates were incubated at room temperature (20˚C) overnight and counted approximately 24 hours after plating. Aerobic plate counts were analyzed using ProtoCOL SR/HR Software 1.47.0. (ProtoCOL SR, Synbiosis, Cambridge, UK).

Manipulation of *Vibrio parahaemolyticus* EMS culture for Shrimp Exposure

Prior to the first feed inoculation, one freezer vial was thawed, added to 50 mL of TSB2+ and grown for 18 hours at 30˚C and shaking (250 RPM). After this time, dilutions of an 18-hour culture of VP-EMS were made using TSB2+ and applied to all feed treatments except the negative control, at approximately a volume/weight ratio of 1 mL working solution/1 g feed. Negative control feed treatments were inoculated only with sterile TSB2+ in the same manner. After inoculation, a 5-minute adsorption period followed to ensure complete saturation of feed with VP-EMS culture. Further exposure and feeding regime are outline in section 2.2.

2.2. General Methods: Lethal Dosing Experiment

Shrimp

Post-larval 10 to 15-day old (PL-10 to PL-15) shrimp were obtained from Miami Aqua-Culture, Inc (Boynton Beach, FL, USA). Shrimp were housed in a 450 L recirculating aquaculture systems (RAS) containing 260 L of 25 parts per thousand (ppt) synthetic sea salt (Crystal Sea Marine Mix, Marine Enterprises International, Baltimore, MD, USA) reconstituted with carbon filtered municipal water. Recirculating tanks with mechanical and bio-filtration, as well as, aeration systems were each outfitted with two heaters (EcoPlus Aqua Heat Titanium Reservoir and Aquarium Heather, Sunlight Supply INC, TN, USA) which maintained water temperature between 25-30˚C. Kaldness type media (a type of plastic which provides increased surface area for the growth of nitrifying bacteria) was housed in a TetraPond ClearChoice BioFilter PF-1 (Blacksburg, VA, USA). Water quality parameters such as, temperature, dissolved oxygen (DO) and salinity were monitored daily. Total ammonia nitrogen (TAN), nitrite, nitrate, pH, and alkalinity, were measured three times per week and were adjusted to suitable ranges for shrimp health as necessary (Van Wyk, Davis-Hodgkins, Laramore, Mountain, & Scarpa, 1999). Water quality parameters were analyzed using methods adapted from the American Public Health Association (APHA). (Eaton, Clesceri, Rice, & Greenberg, 2012)

Post-larval shrimp were raised on an intermediate diet of powdered feed (Zeigler Bros. Inc. Raceway Plus premium Dry Post-Larval Diet, Gardners, Pennsylvania, USA), until larger pelleted feed (2.4mm) with 35% crude protein and 7% lipid, was appropriate. Juvenile shrimp were fed this commercial diet hereafter until the average shrimp size was greater than 1 gram. Shrimp were weighed prior to each experiment to determine the amount of feed required for each tank and respective treatment. A negative tare method was utilized using an analytical scale (Mettler Toledo model: AG104) when weighing shrimp to negate a water weight on the surface of the shrimp. Individual shrimp were relocated into a plastic container with lid, lined with paper towels. This container and lid with paper towels were placed onto the scale and tared. Shrimp were removed and placed into a separate container filled with 25 ppt saltwater. The empty plastic container, lid and paper towels were then placed back onto the scale and the negative weight, indicative of the shrimp's weight, was recorded.

Experimental Systems

Five, ten-gallon glass tanks (Aqueon, Franklin, WI, USA), housed in a negative pressure, 30˚C heated, BSL2 room, were filled with 20 L of 25 parts per thousand (ppt) synthetic sea salt (Crystal Sea Marine Mix, Marine Enterprises International, Baltimore, MD, USA) reconstituted in de-ionized water. Each tank was outfitted with custom constructed propylene lid, with two holes allowing positive and negative pressure air supply. Negative pressure Nalgene tubing lines were connected to a central vacuum containing a 0.45 µm cellulose paper filter, preventing spread and potential aerosolization of *Vibrio parahaemolyticus.* Tanks were supplied with pressurized air distributed via Nalgene tubing connected to an airstone to supply adequate oxygen to each tank. Additionally, Kaldness type media containing nitrifying bacteria, were added to each tank to promote the removal of nitrogenous waste.

Shrimp Challenge Procedure and Survival

Shrimp were exposed to VP-EMS utilizing a feed-borne delivery method as previously described in Section 2.1. Feed was premeasured to be approximately 3% of the total average shrimp weight in grams. Four different dilutions of an 18-hour culture of VP-EMS were made using TSB2+ and applied to all feed treatments, except the negative control. Dilutions and corresponding delivered CFU/tank are shown in Table 2. This method was utilized to establish a repeatable pathogenicity disease challenge. Negative control treatment feeds received uninoculated TSB2+ broth applied in the same manner, as a control. To ensure complete pathogen exposure, plastic cups containing respective feed treatments inoculated with VP-EMS, were completely submerged into tanks. This guaranteed any unabsorbed VP-EMS was still introduced to shrimp. Shrimp were fed inoculated feed every 24 hours until 96 hours elapsed, at which final mortality was recorded. Dead and morbid-bound shrimp were removed from each tank every day.

Table 2. *Treatments used during lethal dosage methodology trials.*

TREATMENT	DILUTION OF 18-HOUR CULTURE	DELIVERABLE CFU/TANK
Very High	1:2	5×10^9
High	1:10	1×10^9
Low	1:20	5×10^8
Very Low	1:50	2×10^8
Control	TSB2+	n/a

2.3 Statistics

The Kaplan-Meier estimate was used as described by Choi et. al. using JMP® Pro 14.0.0 software. (Choi, Stevens, Smith, Taylor, & Kuhn, 2017). This model is commonly used in clinical trials, where starting time is clearly defined by a given point until the occurrence of another defined event (Goel, Khanna, & Kishore, 2010). This analysis measured shrimp survival (respective of treatment groups) over time, after introduction of a bacterial hazard, assuming this hazard was constant over time. This estimate involves the probability of occurrence of an event at a certain time point, and multiplies successive probability by earlier probabilities to achieve a final estimate (Goel et al., 2010). Successive probabilities were calculated using two treatments, the positive control and respective experimental treatments, to determine if there was a statistical difference in survival ($p < 0.05$). The log-rank test, used as a post hoc test, differentiated this statistical significance between treatments and positive control, shown in Figure 4.

3. Results and Discussion

Growth curves for VP-EMS are presented in Figure 3. The EMS strain of *V. parahaemolyticus* was capable of achieving an OD_{600} of above 1, over 18 hours of growth. When enumerated via bacterial plate counts, the mean (n=3) of an 18-hour culture had a concentration of ~10^{10} CFU/mL. Growth of *V. parahaemolyticus* EMS was similar to published findings of Choi et. al. (Choi et al., 2017). This capability to grow to a high concentration, undoubtably contributes to the virulence of this strain as PirAB toxin production and secretion is directly correlated with growth (Jee Eun Han et al., 2015). These toxins have been shown to immediately target and destroy host hepatopancreas cells, however *V. parahaemolyticus* deletion mutants missing the pVA1 plasmid, show no pathogenicity to shrimp (J. E. Han, Tang, Aranguren, & Piamsomboon, 2017; Lee et al., 2015). Therefore, production of these toxins are key to the EMS disease and shrimp mortality. Previous research has explored utilizing cell free supernatant to introduce AHPND/EMS disease, as toxins should be present in this medium (Lee et al., 2015). However, past efforts have demonstrated mixed results using the supernatant alone, which suggests that the cell density of the culture and bacterial cells are more important in causing mortality by direct ingestion (Choi, 2015).

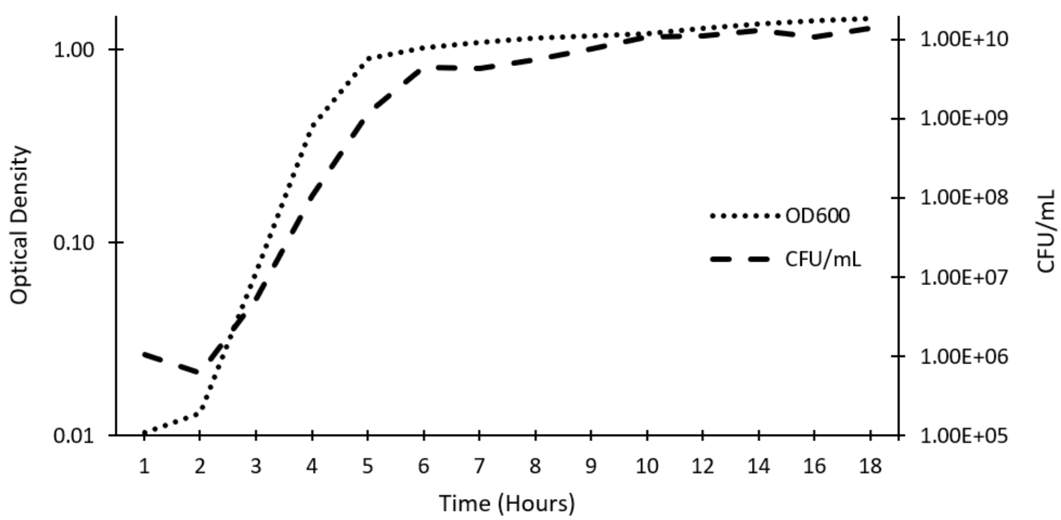

Figure 3. *Growth rate of V. parahaemolyticus EMS strain. Shown are absorbance and enumeration mean values of n = 3 analysis. The left axis displays growth measured at optical density of 600 nm, while the right axis displays bacterial enumerations in CFU/mL, both over the course of 18 hours. The linear slopes of both curves are indicative of exponential growth, while the plateau occurring after this phase indicates the bacteria has reached stationary phase. An uninoculated TSB2+ control (not shown) did not exceed 0.01 absorbance.*

A dilution series of 18-hour VP-EMS cultures was created to further define the concentration that would cause a chronic infection, or infection over a longer period of time. Based on this model, probiotic feed trials could be developed and later piloted, which incorporate a disease challenge to evaluate prophylactic protection. Shown in Figure 4 are the survival rates of Pacific white shrimp (*Litopenaeus vannamei*) after exposure to different dilutions of VP-EMS. This trial involved using feed inoculations of 18-hour VP-EMS cultures (10^{10} CFU/mL) at a 1:2 dilution (5×10^9 CFU/mL), 1:10 dilution (1×10^9 CFU/mL), 1:20 dilution (5×10^8 CFU/mL), and a 1:50 dilution (2×10^8 CFU/mL). Shrimp which were exposed to feed inoculations of the 1:2 dilution exhibited complete mortality within 48 hours. Feed inoculations of 1:10 and 1:20 dilutions had similar mortality trends and were not significantly different from each other but were significantly different ($p<0.05$) when compared to the control TSB2+ feed inoculation. At a 1:50 dilution, no mortalities were observed, with no significant difference from the control. These finding demonstrate correlation between *V. parahaemolyticus* density and pathogenesis, as higher concentrations were more lethal leading to faster mortality rates. Based upon our findings and previous research (Choi et al., 2017), it was concluded that a dilution within the 10^8 CFU/mL range, would be used moving forward to create a long-term exposure model. For future probiotic studies, a 1:20 dilution of the 18-hour culture was used, since there were no significant differences observed between 1:10 and 1:20 dilutions.

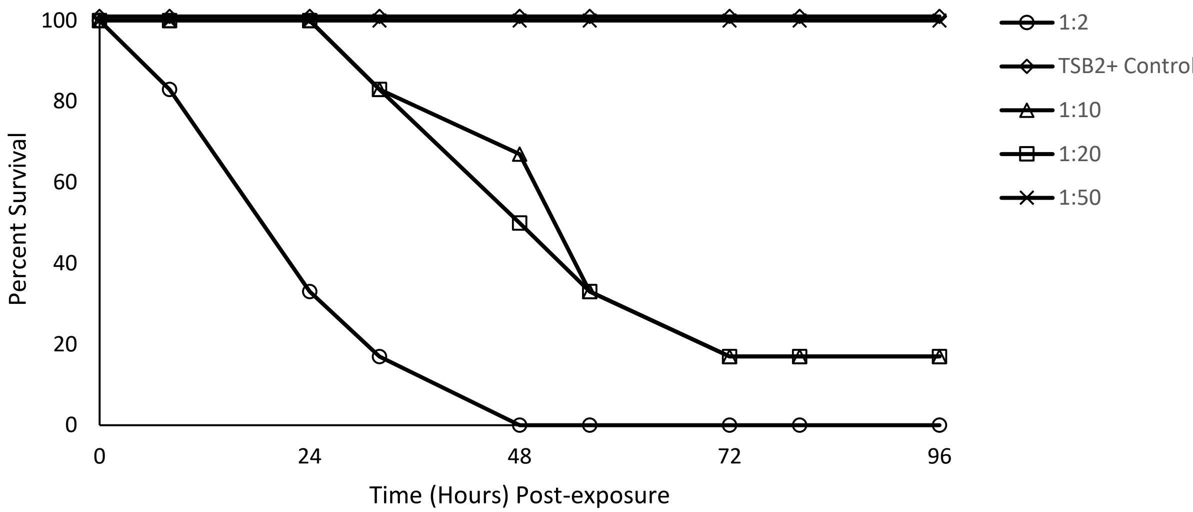

Figure 4. *Lethality evaluation of 18-hour VP-EMS culture dilutions (1:2, 1:10, 1:20, and 1:50). Shown is the percent survival with n=6 replicate analyses over 96 hours. All survival curves are significantly different (p<0.05) when compared to the control, except for the 1:50 dilution (p-value – 1). Significantly different treatments had the following p-values, respectively: 1:2 – <0.0001, 1:10 – 0.0089, 1:20 – 0.006.*

4. Conclusions

The objective of this study was to evaluate various concentrations of *V. parahaemolyticus* EMS strain cultures to determine the optimum dose which would cause a long-term exposure and mimic a natural mortality rate. The feed inoculations and exposure to VP-EMS by direct oral ingestion, was successful in creating the infection and later causing mortality in shrimp, deeming this suitable for future probiotic studies which incorporate a disease challenge. While this model was concurrent with previous research using similar methods, it cannot account for intrinsic factors such as genetic variation in shrimp from batch to batch or control subtle variations in lethality of the pathogen. Therefore, further research is necessary to determine if this method remains consistent and suitable to mimic natural infectivity and pathogenesis among biological differences present in shrimp.

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CHAPTER 4: Use of Direct-Fed Microbes to Enhance Shrimp Resistance to a *Vibrio parahaemolyticus* strain causing Early Morality Syndrome

Abstract

Shrimp aquaculture has evolved into a global industry as shrimp are the most highly traded seafood product by value. Penaeid shrimp account for approximately 80% of all shrimp produced and as production increases, this monoculture is becoming increasingly susceptible to disease. Early Mortality Syndrome (EMS) or Acute Hepatopancreatic Necrosis Syndrome (AHPNS) is an epizootic bacterial infection of shrimp. This disease, attributed to pathogenic *Vibrio parahaemolyticus* strains (VP-EMS), threatens shrimp aquaculture production and global food security. A valuable and alternative approach to using antibiotics for pathogen control, is the practice of incorporating direct-fed microbes (DFM) or probiotics. Once established, these beneficial bacteria can improve host survival and overall animal health. Two DFM products, a single strain of *Bacillus subtilis*: O14VRQ and a blend of multiple strains of *Bacillus subtilis*: Plus10, were evaluated at various concentrations as feed additives (applied as a top coat on commercial feed) or as water additions for their ability to provide shrimp, *Litopenaeus vannamei*, protection to the EMS/AHPNS disease. Accordingly, the following assessments involved twenty-one, 20 L systems which allowed exploration of seven treatments performed in triplicate. Each system was comprised of an aquarium outfitted with its own mechanical filter for removing solids, biological filter for nitrification, and aeration. Two trials were conducted in which shrimp were fed daily (~3% bodyweight) with a DFM-coated feed for seven days, before a challenge with VP-EMS. Each trial consisted of a negative control (no VP-EMS exposure, no probiotic) and positive control (VP-EMS exposure, no probiotic), allowing for five additional probiotic treatment groups, which were fed and exposed to VP-EMS in the same manner as the positive control. Inoculation of VP-EMS was performed via a feed-borne delivery method, in accordance with lethal dosing studies. Shrimp were observed for clinical signs and mortalities after the initial exposure and shrimp were continuously exposed every 24 hours until 50% of the population remained in the positive control treatment. After this time period VP-EMS exposure ceased across all treatment groups. While both *Bacillus subtilis* probiotics were shown to significantly ($p < 0.05$) improve shrimp survival, the O14VRQ strain provided the most consistent protection. These results demonstrate that prophylaxis is reliant upon concentration regardless of application. Moreover, this work reveals specific probiotic strains can positively influence survival of shrimp, and in regarding the EMS disease, serve as a promising alternative to traditional pathogen control in shrimp aquaculture.

Keywords: Early Mortality Syndrome (EMS), Acute Hepatopancreatic Necrosis Syndrome (AHPND), probiotic, *Bacillus subtilis*, shrimp, *Litopenaeus vannamei*

1. Introduction

As global shrimp production demand increases, much attention is being placed on emerging *Vibrio parahaemolyticus* strains, which are associated with disease outbreaks and further limit the development of this sector. Such outbreaks are fostered by the supportive aquatic environment, which allow pathogens, independently of their host, to grow to high densities (Defoirdt, Sorgeloos, & Bossier, 2011). Shrimp aquaculture production has seen annual production losses since 2009, when pathogenic strains of *Vibrio parahaemolyticus* attributed to the EMS/AHPND pandemic, were first discovered (Tran et al., 2013). Currently shrimp culture continues to be devastated by this multi-drug resistant bacteria, as it has obtained a plasmid which encodes for photorhabdus insect-related (Pir) toxin-like genes (Jee Eun Han, Tang, Tran,

& Lightner, 2015; Hernández Serrano, 2005; Kongrueng et al., 2015). These toxins target the digestive organ known as a hepatopancreas, which functions as a liver and pancreas (Tran et al., 2013). Infected shrimp have distinct clinical signs, displaying lethargy, slowed response to stimuli, decreased growth, and empty stomach and midgut, as well as, the most distinguishing sign: a pale to white atrophied hepatopancreas (Kongrueng et al., 2015). Farms generally experience outbreaks within the first 30 days of stocking and infected farms can experience losses upwards of 100% of their population (Zorriehzahra, 2015).

Given aquaculture's potential for growth and unique set of challenges faced, there are many opportunities for improvement and development. However, bacterial diseases threaten the shrimp industry and further expansion is impeded by incorporating hazardous practices such as applying antibiotics, which can in turn amplify diseases by selecting for resistant species. The presence of VP-EMS is a current example of this and warrants the need for novel and natural alternatives. For instance, FAO reports that south-east Asians countries, such as Thailand, produce 70 to 75 percent of farmed shrimp globally (FAO, 2018). The Thai Department of Fisheries reported in 2013 that EMS causing strains of *Vibrio parahaemolyticus* were responsible for an approximate 33% reduction in shrimp production (Joshi et al., 2014). China and other south-east Asian countries have experienced similar trends. With no viable or economically feasible options to counter this bacterial disease, probiotics stand to fill this void due to attributes which contribute to pathogen antagonism, such as production of antimicrobial compounds, competition for resources, and rivalry in gastrointestinal tract (GIT) adhesion (Farzanfar, 2006; Gatesoupe, 1999). In addition, they may offer excellent benefits, such as mitigation of toxic waste by-products, improvement of food conversion ratios (FCRs), promotion of gut health (i.e. digestibility and absorption of nutrients, etc.), and naturally stimulating the immune system

(Rengpipat, Phianphak, Piyatiratitivorakul, & Menasveta, 1998; Tseng et al., 2009; Verschuere, Rombaut, Sorgeloos, & Verstraete, 2000). Many of these processes have ecological origins and are not completely understood, but by manipulating these microbial parameters in the rearing environment and in the animal, research has been shown to drastically affect bacterial species growth and mortality rates, leading to major shifts in microbial dominance. Applied to the EMS pandemic, probiotics could shift the microbial flora in favor of beneficial bacterial, while hindering pathogen presence and persistence. However, the probiotic that is added must be appropriately selected for specific functions, added in the appropriate concentration, and at the right environmental condition, to achieve desirable outcomes (Moriarty, 1998).

In previous studies, the EMS strain of *V. parahaemolyticus* was shown to have a higher pathogenicity to shrimp than other strains, perhaps due to its ability to achieve higher concentrations during stationary phase growth (Choi et al., 2017). Additionally, this strain has been shown to have distinctive metabolic capabilities, allowing it to utilize a variety of carbon sources more efficiently than other *V. parahaemolyticus* strains (Williams et al., 2017). Previously, unpublished data suggested that specific strains of *Bacillus subtilis* probiotics could germinate and proliferate in the shrimp gastrointestinal tract after 7 days (Choi, 2015). Additionally, these *B. subtilis* strains demonstrated antagonistic behavior against VP-EMS, suggesting that they could combat the enhanced attributes of VP-EMS. Therefore, it was hypothesized select strains of *Bacillus subtilis* can enhance shrimp protection to the EMS/AHPNS disease via natural microbial exclusion of pathogenic *Vibrio parahaemolyticus* strains. This was accomplished by utilizing various probiotic strains and delivery methods with the goal of improving intensive shrimp production.

2. Materials and Methods

The following protocols were conducted in accordance with the Institutional Biosafety Committee at Virginia Tech (VT IBS #17-057).

2.1 Bacteriology

2.1.1 Vibrio parahaemolyticus EMS preparation

EMS strains of *Vibrio parahaemolyticus* were obtained from Novozymes Biologicals (Salem, VA). VP-EMS glycerol stocks ('starter cultures') were used in subsequent modeling of VP-EMS growth and for shrimp exposure. Glycerol freezer stocks of VP-EMS cultures were prepared by growing the obtained culture in 50 mL of TSB2+ (BBL Trypticase Soy Broth, Becton Dickinson and Company, Sparks MD USA), supplemented with 2% NaCl (Carolina Biological Supply Company, Burlington NC, USA) at 30˚C shaking (250 RPM) until an optical density (OD_{600}) of 0.5 was reached, which correlated to logarithmic phase growth. One mL of this culture was added to glycerol to reach a final concentration of ~20% glycerol, vortexed to ensure homogeneity and subsequently frozen at -80˚C. Trypticase Soy Broth was supplemented with an extra 2% NaCl, which made the overall salinity of 2.5% NaCl or 25 parts per thousand (ppt), to match the salinity of the water in which shrimp would be later maintained.

2.1.2 Vibrio parahaemolyticus EMS growth

Vibrio parahaemolyticus EMS strain culture growth was confirmed via spectrophotometry and aerobic plate counts, measuring the culture and plating in triplicate, approximately every hour for 18 hours. The plating medium used was Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS) (Becton Dickinson and Company, MD, USA) which is selective and differential for *Vibrio* spp. Dilutions of VP-EMS culture were prepared using PBS, and 0.1 mL was plated over the agar surface using a spiral plater (Whitley WASP Touch Automated Spiral

Plater, Don Whitley Scientific Limited, West Yorkshire, UK). Spread plates were incubated at room temperature or 20˚C overnight and counted approximately 24 hours after plating. Aerobic plate counts were analyzed using ProtoCOL SR/HR Software 1.47.0. (ProtoCOL SR, Synbiosis, Cambridge, UK).

2.2 Shrimp

Post-larvae 10-15-day old (PL-10 to PL-15) shrimp were obtained from Miami Aqua-Culture, Inc (Boynton Beach, FL, USA). Shrimp were housed in a 450 L recirculating aquaculture systems (RAS) containing 260 L of 25 ppt synthetic sea salt (Crystal Sea Marine Mix, Marine Enterprises International, Baltimore, MD, USA) reconstituted with carbon filtered municipal water. Recirculating tanks with mechanical and bio-filtration, as well as, aeration systems were each outfitted two heaters (EcoPlus Aqua Heat Titanium Reservoir and Aquarium Heather, Sunlight Supply INC, TN, USA) which maintained water temperature between 25-30˚C. Kaldness type media (a type of plastic which provides increased surface area for the growth of nitrifying bacteria) was housed in a TetraPond ClearChoice BioFilter PF-1 (Blacksburg, VA, USA). Water quality parameters such as, temperature, dissolved oxygen (DO) and salinity were monitored daily. Total ammonia nitrogen (TAN), nitrite, nitrate, pH, and alkalinity, were measured three times per week and were adjusted to suitable ranges for shrimp health as necessary (Van Wyk et al., 1999). Water quality parameters were analyzed using methods adapted from APHA (Eaton, Clesceri, Rice, & Greenberg, 2012).

Post-larval shrimp were raised on an intermediate diet of powdered feed (Zeigler Bros. Inc. Raceway Plus premium Dry Post-Larval Diet, Gardners, Pennsylvania, USA), until larger pelleted feed (2.4 mm) with 35% crude protein and 7% lipid, was appropriate. Juvenile shrimp were fed this commercial diet thereafter until the average shrimp size was greater than 1 gram.

Shrimp were weighed prior to each experiment to determine the amount of feed required for each tank and respective treatment. A negative tare method was utilized using an analytical scale (Mettler Toledo model: AG104) when weighing shrimp to negate a water weight on the surface of the shrimp. Individual shrimp were relocated into a plastic container with lid, lined with paper towels. This container and lid with paper towels were placed onto the scale and tared. Shrimp were removed and placed into a separate container filled with 25 ppt saltwater. The empty plastic container, lid and paper towels were then placed back onto the scale and the negative weight, indicative of the shrimp's weight, was recorded.

2.3 Experimental systems

Twenty-one ten-gallon glass tanks (Aqueon, Franklin, WI, USA), housed in a negative pressure, 30˚C heated, BSL2 room, were filled with 20 L of 25 ppt synthetic sea salt (Crystal Sea Marine Mix, Marine Enterprises International, Baltimore, MD, USA) reconstituted in de-ionized water. Each tank was outfitted with custom constructed propylene lid, with two holes allowing positive and negative pressure air supply. Each tank was outfitted with a corner filter (Lee's Triple-Flow Corner Filter – Medium, Lee's Aquarium and Pet Supplies, San Marcos, CA, USA) filled with 200g of aquarium rock (Topfin Underwater Worlds Premium Aquarium Gravel, PetSmart, Phoenix, AZ, USA), filter pads (MBI Filter Pads, Pentair, Apopka, FL, USA), and Kaldness type media. Tanks were supplied with pressurized air distributed via Nalgene tubing connected to an airstone to supply adequate oxygen to each tank. Filters were installed to reduce tank turbidity, remove solids and promote the removal or biofiltration of nitrogenous wastes. In addition to this mechanical filtration, tanks were inoculated with approximately 10 mL of commercial nitrifying bacterial product (Tetra SafeStart Plus, Spectrum Brands Pet, LLC, VA, USA) to promote nitrification via ammonia species. Negative pressure Nalgene tubing lines were connected to a central vacuum containing a 0.45µm cellulose paper filter, preventing spread and potential aerosolization of *Vibrio.*

2.4 General Methods:

Manipulation of *Vibrio parahaemolyticus* EMS culture for Shrimp Exposure

Prior to the first feed inoculation, one starter culture was thawed, added to 50 mL of TSB2+ and grown for 18 hours at 30˚C and shaking (250RPM). After this time, 1:20 dilutions of an 18-hour culture of VP-EMS were made using TSB2+ and applied to all feed treatments except the negative control, at approximately a volume/weight ratio of 1 mL working solution/1 g feed. Negative control treatment feeds received uninoculated TSB2+ broth applied in the same manner, as a control. After inoculation, a 5-minute adsorption period followed to ensure complete saturation of feed with VP-EMS culture. Further exposure and feeding regiments are outline below.

Probiotic preparation for experimental diets

Two-hundred grams of commercial (Zeigler Bros. Inc. Shrimp grower (SI-35 2.4 mm), (Gardners, Pennsylvania, USA) pelleted feed were coated with proprietary mono and multi-culture *Bacillus subtilis* strain spore products supplied and prepared by Novozymes Biological Inc (Salem, VA). Spore products were prepared by diluting each to the desired concentration using phosphate buffer saline (PBS). Twenty mL of each respective solution was separately added to the hopper of a high volume, low pressure (HVLP) Spray Gun (ToolForce, Kings Park, NSW, Australia). In line pressure measured in pounds per square inch or PSI was set to 100 PSI (±5 PSI), so that when the trigger was fully engaged stream pressure was maintained at 40 PSI. Commercial feed was weighed and added to a sealed aluminum can with a paddle drill attachment. The spore solution was simultaneously sprayed onto feed as it was mixed in 5 second bursts, until depletion. Coated feeds were removed and spread thinly onto stainless steel trays, approximately 5 cm deep. Feeds were allowed to air dry at ambient room temperature (~20˚C) for 24 hours and then refrigerated at 4˚C in sealed containers. The spraying apparatus was flushed with 50 mL of PBS, followed by 50 mL of 70% EtOH to ensure no residual probiotic remained. Spore counts were verified (n= 3 analysis) by grinding feeds with a sterile mortar and pestle, performing serial dilutions in PBS, and subsequently spread plating onto Tryptic Soy Agar plates (TSA) (Becton Dickinson and Company, MD, USA).

Feed Regiment and Probiotic Introduction

Feed was premeasured to be approximately 3% of the total average shrimp weight in grams. Non-probiotic fed shrimp were fed noncoated commercial feed (Ziegler Bros. Inc., Gardners, Pennsylvania, USA), while probiotic fed shrimp were fed coated commercial feed respective of treatment, as previously described. Probiotics were introduced via feed borne delivery (i.e. coated feeds) or via tank inoculation, once every 24 hours for 7 days to allow germination and colonization of the probiotics in the shrimp gastrointestinal track.

Tank inoculation of probiotics consisted of the same *Bacillus subtilis* strain spore products supplied and prepared by Novozymes Biological Inc (Salem, VA). Each spore product was diluted to predetermined concentrations using phosphate buffer saline (PBS). Each treated tank was then inoculated with a set volume in order to deliver probiotics to shrimp at a desired concentration or dose. Shrimp that were treated with probiotics in this manner followed the same probiotic regiment as coated feed treatments. However, these shrimp were fed noncoated commercial feed, as previously described.

Shrimp Challenge Procedure and Survival

After introduction of probiotics for 7 days, shrimp were exposed to a VP-EMS utilizing a feed borne delivery method as previously described. This method was utilized to establish a repeatable pathogenicity disease challenge. Negative control treatment feeds received uninoculated TSB2+ broth applied in the same manner, as a control. To ensure complete pathogen exposure, plastic cups containing respective feeds inoculated with VP-EMS, were completely submerged into tanks. This guaranteed any unabsorbed VP-EMS was still introduced to shrimp. Shrimp were fed inoculated feed every 24 hours until 50% mortality of shrimp in the positive control group was observed, in accordance with previously established lethality methods. Moving forward using this method, a successful trial was defined as obtaining 50% mortality in the positive control. After this standard was reached, respective treatment feeds were resumed for the remainder of each trial. Tanks were checked three times per day to remove any dead or morbid bound shrimp.

Experimental design Trials 1 and 2

Two different *Bacillus subtilis* probiotic products were evaluated using various delivery applications and in different concentrations using a 1:20 dilution of a ~10^{10} CFU/mL VP-EMS culture as the infectious dose. Seven treatment groups (n=10 shrimp/tank and three replicate tanks; average shrimp weight = 1.85±0.62 grams) were used: non-probiotic treated controls; negative control (no VP-EMS challenge) and positive control (VP-EMS challenge), O14VRQ High feed, O14VRQ Low feed, Plus10 High feed, O14VRQ High In tank, and Plus10 Low In tank. The negative control group served as an indication that shrimp remained healthy and conditions were optimum. Trial 1 utilized the above parameters in 21 tanks and was carried out for 96 hours.

The same parameters and feed applications were duplicated for Trial 2, however in tank inoculations were changed slightly. Seven treatment groups (n=10 shrimp/tank and three replicate tanks; average shrimp weight = 1.74±0.66 grams) were used: non-probiotic treated controls; negative control (no VP-EMS challenge) and positive control (VP-EMS challenge), O14VRQ High feed, O14VRQ Low feed, Plus10 High feed, O14VRQ Low In tank, and Plus10 High In tank. Considering this, a low concentration of O14VRQ was applied at 1.0 x 10^7 CFU/Tank/Day, while Plus10 was applied to achieve a high concentration of 4.0 x 10^7 CFU/Tank/Day. This trial, compared to Trial 1, was carried out for an additional 2 days for a total of 144 hours. Probiotic treatments and respective concentrations for both independent trials are detailed in Table 3.

Table 3. *Treatments used during shrimp probiotic Trials 1 and 2.*

TREATMENT	PROBIOTIC APPLICATION	PROBIOTIC USED	SHRIMP EXPOSED TO VP-EMS	TRIAL 1	TRIAL 2
				Probiotic Concentration in CFU/mL	
Negative Control	Control	No	No	n/a	n/a
Positive Control	Control	No	Yes	n/a	n/a
O14VRQ High	Feed	Yes	Yes	1.3 x 10^7	1.3 x 10^7
O14VRQ Low	Feed	Yes	Yes	2.4 x 10^6	2.4 x 10^6
PLUS10 High	Feed	Yes	Yes	1.6 x 10^7	1.6 x 10^7
O14VRQ	Water	Yes	Yes	4.0 x 10^8	1.0 x 10^7
PLUS10	Water	Yes	Yes	2.0 x 10^6	4.0 x 10^7

Probiotic concentrations are displayed in CFU/mL/g of feed for feed applications and CFU/tank/day for water applications.

2.5 Statistics

The Kaplan-Meier estimate was used as described by Choi et al (Choi et al., 2017) using JMP® Pro 14.0.0 software. This model is commonly used in clinical trials, where starting time is clearly defined by a given point until the occurrence of another defined event (Goel et al., 2010). In these trials, this analysis measured shrimp survival (respective of treatment groups) over time, after introduction of a bacterial hazard, assuming this hazard was constant over time. This estimate involves the probability of occurrence of an event at a certain time point, and multiplies successive probability by earlier probabilities to achieve a final estimate (Goel et al., 2010). Successive probabilities were calculated using two treatments, the positive control and respective experimental treatments, to determine if there was a statistical difference in survivability ($p < 0.05$). The log-rank test, used as a post hoc test, differentiated this statistical significance between treatments and positive control, shown in Table 5.

3. Results

3.1 *Vibrio parahaemolyticus* EMS growth

The growth of VP-EMS was confirmed based upon previous literature and is presented in Figure 5. The EMS strain of *V. parahaemolyticus* was capable of achieving an OD_{600} of above 1, over 18 hours of growth. During shrimp exposure to VP-EMS, 1:20 dilutions of 18-hour cultures were used to inoculate feeds. When enumerated via bacterial plate counts, the mean (n=3) of an 18-hour culture had a concentration of ~10^{10} CFU/mL, with the actual feed inoculation concentration, twentyfold lower.

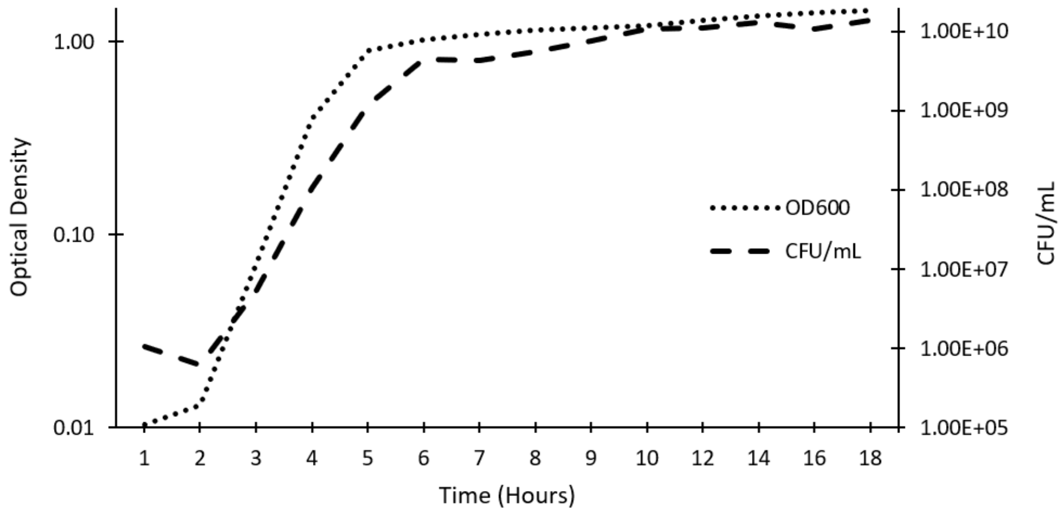

Figure 5. *Growth rate of V. parahaemolyticus EMS strain. Shown are absorbance and enumeration mean values of n = 3 analysis. The left axis displays growth measured at optical density of 600 nm, while the right axis displays bacterial enumerations in CFU/mL, both over the course of 18 hours. The linear slopes of both curves are indicative of exponential growth, while the plateau occurring after this phase indicates the bacteria has reached stationary phase. An uninoculated TSB2+ control (not shown) did not exceed 0.01 absorbance.*

3.2 Probiotic experimental diets

Probiotic coated feeds were enumerated in triplicate via bacterial plate counts onto Tryptic Soy Agar (TSA) and the average CFU/g of feed was recorded and compared to the targeted dose as shown in Figure 6. The high concentration of O14VRQ was found to have 1.3×10^7 CFU/g feed, while the low concentration had 2.4×10^6 CFU/g feed, approximately fivefold lower. The high concentration of Plus10 was enumerated in the same fashion and contained 1.6×10^7 CFU/g feed. The high concentration and low concentration of probiotic products were applied to achieve a similar viable number of cells. In tank inoculum was determined by the starting concentration of spore product and calculated based upon the amount applied, as follows: O14VRQ High [4.0×10^8 CFU/Tank/Day], O14VRQ Low [1.0×10^7 CFU/Tank/Day], Plus10 High [4.0×10^7 CFU/Tank/Day], and Plus10 Low [2.0×10^6 CFU/Tank/Day].

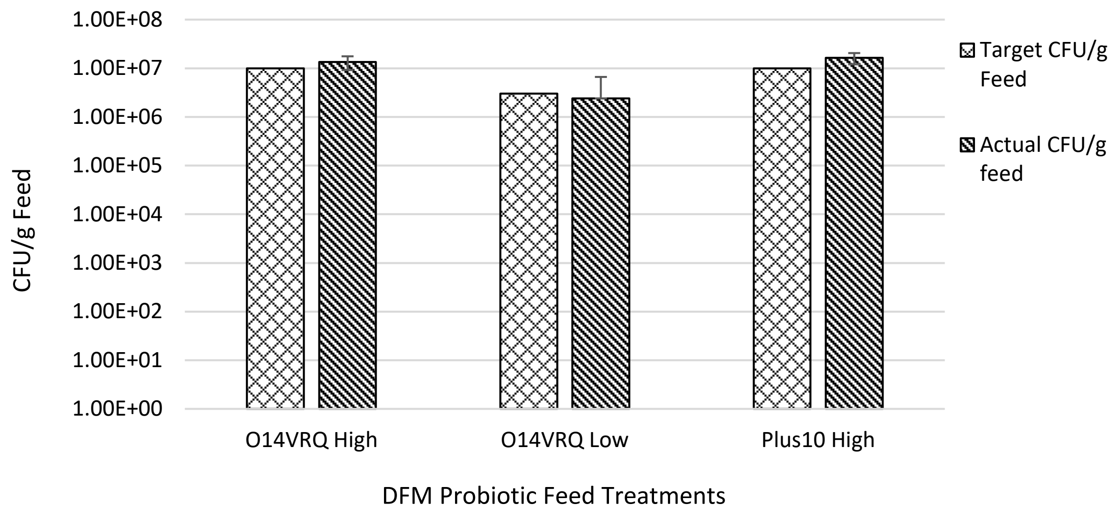

Figure 6. *Enumerations of coated DFM probiotic feeds. Shown are the targeted dose and the mean of actual enumerations, n=3 replicates with standard error, which were administered to shrimp.*

3.3 Shrimp Challenge Studies

Trial 1

Shrimp fed the O14VRQ High, O14VRQ Low, and Plus10 High all exhibited survival greater than 80% with the positive control at only 30% survival. Plus10 provided the best protection at 87% survival, with O14VRQ High at 83% and O14VRQ Low at 80%, as shown in Table 4. Each of these treatments were shown to be significantly different ($p<0.05$) when compared to the positive control. The O14VRQ in tank application was also significantly different than the positive control and had survival of 87%. However, the Plus10 in tank application had the lowest survival rate at 33%, which was not significantly different.

Trial 2

Shrimp fed the O14VRQ High and O14VRQ Low all exhibited survival greater than 60% with the positive control at only 47% survival over a 96-hour period, as shown in Table 4. Plus10 High feed applications showed 23% survival at 96 hours. The best performing in tank application was Plus10 High at 60%, while the O14VRQ showed only 23% survival. When the timeline is extended to 144 hours or 2 days, similar trends are observed, as shown in Figure 7. Of the feed applications O14VRQ High treatment had 53% survival, O14VRQ Low treatment had 30% and the Plus10 High treatment had 10%. In tank applications showed the O14VRQ Low treatment had 10% survival while the Plus10 High showed 37% survival. However, only the O14VRQ High feed treatment group was significantly different ($p<0.05$) when compared to the positive control which exhibited 10% survival. The Plus10 High feed treatment and Plus10 High in tank application did however show potential. While they were not significantly different, the p-value was between 0.05 and 0.10 ($0.05<p<0.10$). This is compared to treatments such as O14VRQ Low feed treatment and O14VRQ Low in tank application which had p-values above 0.10 ($p>0.10$).

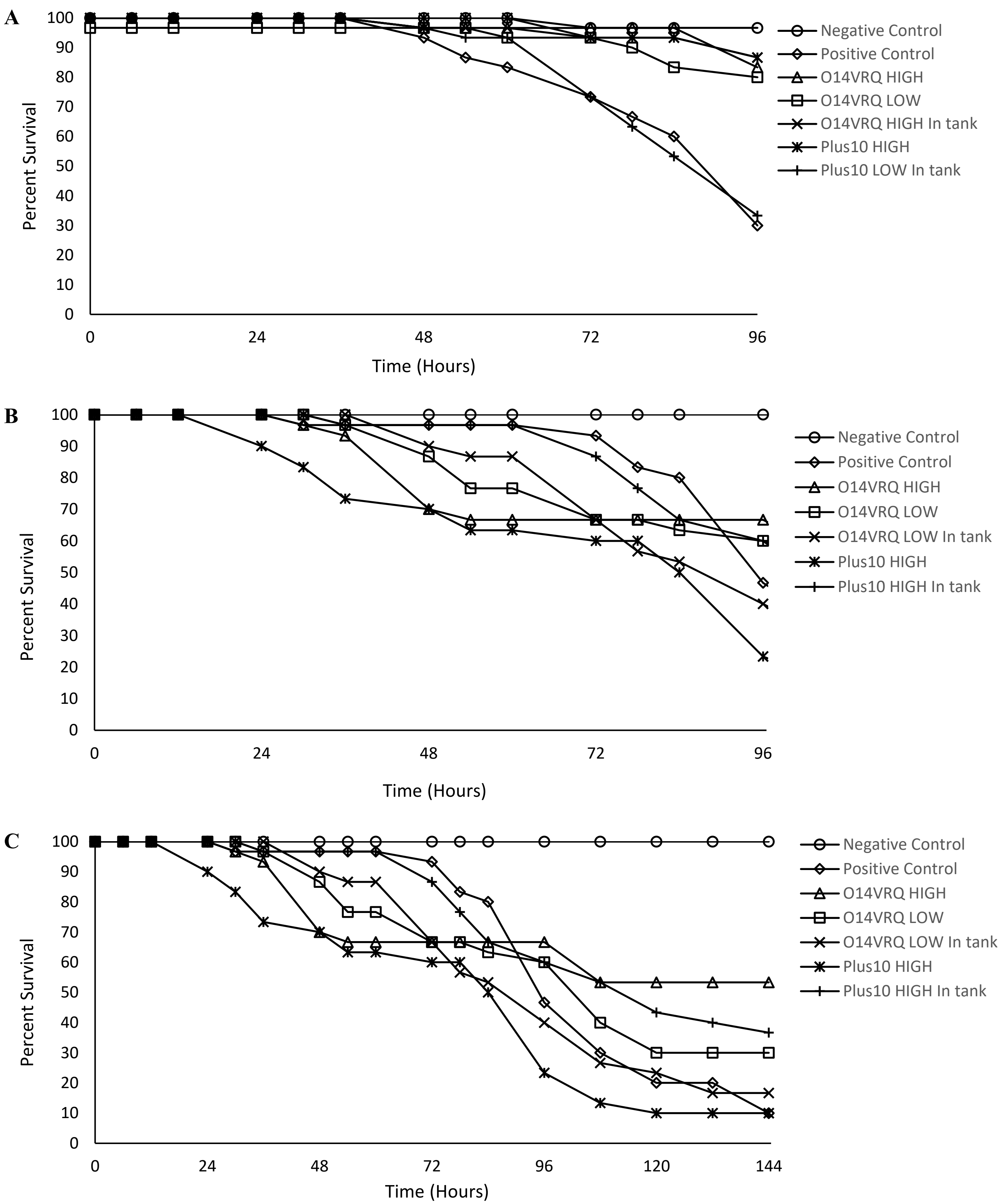

Figure 7. *Survival of shrimp during Trials 1 and 2. Figure A represents Trial 1 and Figure B, Trial 2, respectively, both showing shrimp survival over the course of 96 hours. The third figure C shows shrimp survival of Trial 2 when expanded to 144 hours.*

Table 4. *Survival of probiotic fed shrimp treatments after exposure to V. parahaemolyticus EMS.*

TRIAL	TREATMENT	MEAN SHRIMP SURVIVAL RATE (%) AT RESPECTIVE HOUR							
		0 h	12 h	24 h	48 h	72 h	96 h	120h	144h
1	Negative Control	100	100	100	100	97	97	-	-
	Positive Control	100	100	100	93	73	30	-	-
	O14VRQ High	100	100	100	97	97	83	-	-
	O14VRQ Low	100	97	97	97	93	80	-	-
	Plus10 High	100	100	100	100	93	87	-	-
2	Negative Control	100	100	100	100	100	100	100	100
	Positive Control	100	100	100	97	93	47	20	10
	O14VRQ High	100	100	100	70	67	67	53	53
	O14VRQ Low	100	100	100	87	67	60	30	30
	Plus10 High	100	100	90	70	60	23	10	10

Shown are the results from Trial 1 over a 96-hour period and Trial 2 over a 144-hour period. Statistical differences are shown in Table 5.

Table 5. *Kaplan-Meier estimate statistics for Trial 1 and 2.*

TREATMENT		TRIAL 1	TRIAL 2
Control	Negative Control	<0.0001*	<0.0001*
Feed	O14VRQ High	<0.0001*	0.0149*
	O14VRQ Low	0.0007*	0.2550
	Plus10 High	<0.0001*	0.0962
Water	O14VRQ High	0.0001*	n/a
	O14VRQ Low	n/a	0.7722
	Plus10 High	n/a	0.0726
	Plus10 Low	0.8359	n/a

Each treatment is compared to the Positive control (no probiotic, VP-EMS). A log-rank test was used as a post hoc test to determine significance ($p<0.05$), illustrated by (*).

4. Discussion

Growth of *V. parahaemolyticus* EMS was similar to published findings of Choi et. al. (Choi et al., 2017). This particular strain is able to achieve plate counts of ~10^{10} CFU/mL over the course of 18 hours. This capability to grow to a high concentration, undoubtably contributes to the virulence of this strain as PirAB toxin production and secretion is directly correlated with growth (Jee Eun Han et al., 2015). These toxins have been shown to immediately target and destroy host hepatopancreas cells, however *V. parahaemolyticus* deletion mutants missing the pVA1 plasmid, show no pathogenicity to shrimp (Han et al., 2015). Therefore, production of these toxins are key to the EMS disease and shrimp mortality, as was demonstrated in our assays by the positive control.

Enumeration of probiotic feeds demonstrated the efficiency of the spray top coating methodology used, as targeted dosages were close to actual concentrations administered to shrimp. The stability of this feed, was however, not examined. *Bacillus* spp. generally are advantageous compared to commonly used probiotics such as lactic acid bacteria, largely due to their spore which offers high survival rate, enhanced tolerance to stress, and stability during processing and storage of product (Bader et al., 2012; Elshaghabee et al., 2017). When incorporated onto feeds, metabolically inactive spores do not germinate, which contributes to the extended shelf life (Wang et al., 2008). However, particular strains may lose viability overtime, be sensitive to storage conditions, or preparation pressures, thus, the longevity of our prepared feeds warrants further investigation to ensure efficacy (Bader et al., 2012; Wang et al., 2008).

Overall DFM probiotic evaluation demonstrated that particular strains of *B. subtilis* in specific concentrations were capable of significantly ($p<0.05$) enhancing shrimp survival rates after exposure to VP-EMS. Commercial applications of such direct-fed microbial products, could help to reduce global reliance upon antibiotics and reduce EMS disease outbreaks in intensive shrimp production. Probiotic coated feeds had better efficacy overall, however in tank treatments were limited by their concentration. This trend of certain probiotic diets to achieve higher survival rates may be attributed to several factors and distinct properties retained by the probiotic strain such as advantageous modes of action. Probiotic efficacy is dependent first upon ingestion and secondly, adhesion and colonization of the host gastrointestinal tract (GIT), so naturally it may be anticipated that directly feeding shrimp probiotic cultures would promote survival more so than water inoculations. However, in tank inoculations did provide some level of protection at higher concentrations which advocates the ecological idea that waters may be colonized by the addition of probiotic bacteria, as microbial mature waters eliminate the opportunity for fast growing pathogens (i.e. *Vibrio* spp.) to find a niche and proliferate (Schryver et al., 2014; Verschuere et al., 2000). Regardless of probiotic strain, a clear pattern emerges with regards to in tank applications, as protection was correlated with dose applied. This suggests it may be possible to alter the microbial composition in hatchery tanks or aquaculture ponds, however stoichiometry, may be the biggest factor to consider in commercial application. Taking the volume of water or dilution factor into account, water applications should focus upon modulating the microbiota of larval stages. Penaeid larval stages such as nauplii, are more likely to ingest bacteria through consumption of suspended particles or tank debris, and furthermore their digestive tract and immune system is more readily influenced, as it is still in development (Farzanfar, 2006). *Bacillus* species may be an ideal probiotic candidate for this type of application as they are generally found in marine sediments which juvenile shrimp naturally feed upon (Moriarty, 1999).

Feed applications may have benefited from several plausible probiotic mechanisms. Previously, unpublished data demonstrated that *B. subtilis* spores were able to germinate and proliferate in shrimp GIT within 7 days after ingestion (Choi, 2015). Paired with our current findings, this suggest that *Bacillus* spores are not just transient passengers of the GIT but are ingested and colonize, despite the route of administration. *Bacillus subtilis* has adapted attributes to thrive in conditions found in the GIT, such the ability to sporulate anaerobically and form a biofilm; the low pH of this environment has even been found to assist in the germination process of some *Bacillus s*pecies (Elshaghabee et al., 2017; Tam et al., 2006). Once established, *Bacillus* spp. may promote antagonistic behavior against pathogens, by simple competition for nutrients and space in the gut or in the surrounding aquatic medium (Farzanfar, 2006; Moriarty, 1998; Vaseeharan & Ramasamy, 2003). *Bacillus subtilis* strains used these studies could have simply colonized the shrimp GIT more efficiently and provided shrimp protection through naturally microbial exclusion of VP-EMS. Additionally, our probiotic strains may have produced antimicrobial compounds or stimulated shrimp innate immunity, which had a subsequent antagonistic effect against VP-EMS. Different *Bacillus* strains have been reported produce antimicrobial such as bacteriocins against many pathogens, as well as, immune-modulatory activity (i.e. phagocytic activity and phenoloxidase activity) in shrimp (Elshaghabee et al., 2017; Farzanfar, 2006; Rengpipat et al., 2000; Tseng et al., 2009; Vaseeharan & Ramasamy, 2003).

5. Conclusion

The aim of this study was to determine whether introduction of specific *B. subtilis* strains could provide protection against the EMS disease. Findings demonstrate that when probiotics were properly introduced in the appropriate concentration, *B. subtilis* strains can in fact significantly increase survival rate in shrimp when exposed to VP-EMS. Probiotic strains, O14VRQ and Plus10 successfully provided protection against VP-EMS compared to the survival of shrimp which were only fed a commercial diet. However, application is limited by concentration or dose applied. Additional studies are warranted to confirm and evaluate long term protection provided by these probiotics. Research regarding the minimum inhibitory dosage of these select probiotic strains, should also be explored to maximize commercial application of probiotics.

Acknowledgements

Funding for this study was provided in part by Novozymes Biologicals (Salem, VA, USA). Special thanks to Dan Taylor and Moonyoung Choi while in affiliation with Virginia Tech for their contributions in the development of methods used in this paper.

Conflict of interest

Dr. David Drahos and Mrs. Meaghan Thompson are employees of Novozymes Biologicals Inc. which is the company that provided the probiotics and financial resources to support this study. There are no other potential conflicts to be reported.

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CHAPTER 5: Conclusions and Future Work

The objective of this research was to examine the efficacy of probiotic candidates at varying concentrations *in vivo* for their ability to provide shrimp protection against *Vibrio parahaemolyticus* strains causing Early Mortality Syndrome. Survival of experimental groups (shrimp treated with probiotics) was compared to the survival of control shrimp (no probiotic) to evaluate their effectiveness. Two probiotics, which were previously screened for antagonistic characteristics, were tested at varying concentrations and introduced via feed borne or in tank water inoculation applications. Probiotic coated feeds, specifically, O14VRQ in high concentrations, performed the best as they provided the greatest and consistent survival when compared to the survival of shrimp fed non-probiotic feeds.

This study had promising results which could be directly applied and implemented in intensive shrimp aquaculture. One of the biggest challenges associated with the evaluation of probiotics products is distinguishing between causation and correlation. While a probiotic product may appear to provide disease protection for aquacultural species, the observed effect may be contributed to several compounding factors. Probiotics have been shown to be responsible for modulation of the host gut microbiota and immune response, improving water quality parameters, or decreasing pathogens via natural microbial exclusion. However, without knowing how probiotics specifically provide protection or increase survivability, much of this work could simply be attributed to a random or placebo effects. Therefore, to further the science behind this alternative approach, future directions should focus upon the specific mechanisms or modes of actions concerning probiotic promotion of shrimp survival. With this knowledge, probiotics could be tailored to meet specific needs of the industry, especially when related to prevention of diseases. Such avenues could involve zone of inhibition studies in which a secreted compound could be isolated, or minimum inhibitory concentration (MIC) experiments, demonstrating the concentration at which the probiotic is most effective. Additionally, the quantification of both probiotic and pathogen in the GIT, would provide some insight as to what is occurring *in vivo* with this application.

Long term, several hurdles must be overcome to properly and successfully implement this solution. Goals should include more efficient feed preparation and consideration the practicality of manufacturing such feeds on an industrial scale. Additional research is also warranted to ensure effects observed in a laboratory and controlled settings are mimicked in earthen ponds and traditional shrimp rearing practices, where extrinsic factors may be managed to a lesser extent.

Appendices

All supporting data has been uploaded to the following DOI 10.17605/OSF.IO/87PGW.

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