Improving Disease Resistance for Shrimp through Application of Probiotics in Feed

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Abstract

Diseases affecting shrimp contribute to billions of dollars of economic loss yearly to the aquaculture industry. Recently, one of the primary causative agents of disease has been *Vibrio parahaemolyticus*; in 2009, a new strain causing Early Mortality Syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND) in shrimp emerged. Shrimp losses attributed to pathogens can be greatly reduced through probiotic use, which are known to act as natural immune enhancers and promote pathogen resistance. However, research on probiotic treatment against EMS disease is lacking. The overall project goal was to improve intensive shrimp production through direct application of probiotics in aquaculture feeds.

The value of probiotics for the shrimp industry was evaluated by (1) reducing severity or mortality of *V. parahaemolyticus* disease in shrimp, (2) qPCR confirmation of *Bacillus* spore germination in shrimp gut, and (3) probiotic effectiveness evaluation for improving disease resistance. The virulence of several *Vibrio* spp. strains was examined and it was concluded the *V. parahaemolyticus* strain identified as the causative agent of EMS was the most lethal; EMS-infected shrimp exhibited 100% mortality within 36-hours of feed inoculation. The number of bacterial cells added to feed directly correlated with pathogenicity and only cells, not filtrate, was capable of causing death. Probiotic strains were evaluated and it was concluded that probiotic strains A, A/B blend, and B were the best candidates for promoting disease resistance against EMS. This research will provide the shrimp farming industry with information vital to developing a means for reducing economic loss from *Vibrio*-infected shrimp.

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List of Abbreviations

1/10 EMS, 1/10 diluted overnight EMS; 1/100 EMS, 1/100 diluted overnight EMS; 1/1000, 1/1000 diluted overnight EMS; AHPND, Acute Hepatopancreatic Necrosis Disease; AMPs, antimicrobial peptides; ARB, antibiotic-resistant bacteria; BSC, biosafety cabinet; B-cells, blister cells; BSL2, biosafety level 2; CDC, Centers for Disease Control; CFU/mL; colony forming units per milliliter; E-cells, embryonic cells; EMS, Early Mortality Syndrome; F-cells, fibrillar cells; G cells, granular cells; GlcNAc, *N*-acetylglucosamine; H&E, hematoxylin and eosin dye; H cells, hyaline cells; IHHNV, infectious hypodermal and hematopoietic necrosis virus; IMNV, infectious myonecrosis virus; LM5312, LM; LPS, lipopolysaccharide; lpm, liters per minute; MPa, megapascal; ;NTC, non-treated control; OD, optical density; O/N, overnight; PAMPs, pathogen-associated molecular patterns, PBA, prawn blood agar; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PG, peptidoglycan; Pir, *Photorhabdus* insect-related; PMGC, posterior midgut ceca; proPO, prophenoloxidase; ppt, parts per thousand; PRP, pattern recognition proteins; qPCR, quantitative Polymerase Chain Reaction; RAS, recirculating aquaculture system; R-cells, resorptive cells; RER, rough endoplasmic reticulum; RIMD, RIMD2210633; rpm, revolutions per minute; SER, smooth endoplasmic reticulum; SG cells, semi-granular cells; G3SS, type 3 secretion system; T3SS1, type 3 secretion system I; T6SS1, type 6 secretion system I; T6SS2, type 6 secretion system 2; TAN, total ammonical nitrogen; TDH, thermostable direct haemolysin; TRH, thermostable direct haemolysin-related haemolysin; TSA2+, Trypticase Soy Agar with additional 2% NaCl; TSB2+, Trypticase Soy Broth with additional 2% NaCl; TSV, Taura Syndrome Virus; v/w, volume per weight; WSSV, Whitespot Syndrome Virus; YHV, Yellow Head Virus.

Chapter 1:

Introduction

Disease outbreaks in the aquaculture industry contribute to several U.S. billions of dollars of economic loss yearly (Defoirdt et al., 2011). Antibiotics have been frequently and excessively used to treat these disease outbreaks, however, there is now an increased prevalence of antibiotic-resistant bacteria (ARB) and awareness of the negative environmental impacts of antibiotics (Cabello, 2006). The farmed shrimp industry has especially experienced the consequences of antibiotic use when shrimp production in the Philippines dropped by 55% from 1995 to 1997 due to ARB (Kesarcodi-Watson et al., 2008). Residual antibiotics can have a negative impact on the environment because they can modify the composition of the sediment microbiota, disperse to farther marine sites, and be ingested by wild aquatic species (Cabello, 2006). The farmed shrimp industry has never been able to fully recover from this devastating loss; the Philippine shrimp industry once worth US $760 million was worth only US $240 million in 2007 (Kesarcodi-Watson et al., 2008). Alternative prophylactic treatments are warranted in order to decrease the spread of antibiotic resistant diseases and to prevent further harm to the aquatic environment.

In 2009, a new *V. parahaemolyticus* strain causing Early Mortality Syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND) in shrimp emerged (Nunan et al., 2014). EMS first appeared within stocking ponds in southern China and Hainan island causing 100% mortality and has spread to nearby Southeast Asian countries such as Vietnam, Malaysia, and Thailand and was further confirmed in the northern states of Mexico (Lightner et al., 2012; Nunan et al., 2014). Shrimp farms have experienced severe production losses from EMS and, furthermore, this disease has also affected employment, social welfare, and international trade.

Histological examination of EMS-infected shrimp indicate infection is limited to the hepatopancreas (Tran et al., 2013). Initial infection stages show a decrease in fat storage cell vesicles, disappearance of B, F, and R cells, and diminished secretory cell activity (Lightner et al., 2012; Tran et al., 2013). In the later infection stages, basophilic cells and secretory cells deteriorate and separate from the tubule basement membrane and is shed into the tubule lumen

(Lightner et al., 2012). Methods for the detection of the causative bacterium of EMS were developed using PCR and the histology of infected shrimp can be used for confirmation (Nunan et al., 2014). Although the routes of infection, detection, and confirmation of EMS have been studied, data on the prevention methods for this disease are notably lacking. There has not been a study to examine probiotic incorporation into aquaculture feed to enhance disease resistance against the EMS *V. parahaemolyticus* strain.

Probiotics have become a popular method of pathogen control in aquaculture (Wang et al., 2008). Utilization of probiotics is defined as the supplementation of live, naturally-occurring bacteria to the environment in which animals live that is advantageous to the health of a host (Irianto and Austin, 2002). This proactive method can improve overall shrimp gut health, hinder pathogenic bacteria, increase desirable and marketable characteristics in shrimp, and reduce economic losses due to disease in shrimp (Lakshmi et al., 2013; Mohapatra et al., 2013; Wang et al., 2012). Utilizing probiotics as a disease reduction method can greatly minimize shrimp losses in the aquaculture industry and can also provide industry with other marketable advantages.

The long-term goal of this study was to improve the health of shrimp species raised by the domestic aquaculture industry using probiotics. Promoting the use of probiotics is a cost-effective and environmentally sustainable method for the aquaculture business because costs resulting from shrimp stock reduction due to disease and poor water quality can be reduced. In order to be a viable option, probiotic-incorporated aquaculture feeds must be competitively priced and easily accessible. For the work proposed herein, shrimp (*Litopenaues vannamei*) served as a model organism and the *V. parahaemolyticus* EMS strain served as the model disease.

The major objective of the project was to identify specific probiotic strains that can be incorporated into aquaculture feed formulations that colonize within the shrimpgut. Probiotic colonization in the shrimp gut can reduce the presence of pathogenic bacteria by providing competition with *Vibrio* spp. for adhesion sites. Specific objectives included the following: virulence expression of *Vibrio* spp. in shrimp, confirmation of spore germination, utilization of probiotics strains, and confirmation of probiotic ability to decrease mortalities caused by *Vibrio* spp.

Chapter 2:

Literature Review

Aquaculture Industry. Aquaculture is the fastest growing sector of the agriculture industry in the world (Henriksson, 2011). Aquaculture is the practice of raising juvenile fish and invertebrates in hatcheries or collecting wild juveniles and rearing them to a larger or marketable size (Pomeroy et al., 2006). Benefits of the aquaculture practice includes a lessened strain on wild stocks, increased universal production, and species conservation (Tlusty, 2002). There are greater than 220 species of finfish and shellfish that are farmed worldwide ranging from species such as giant clams to salmon (Naylor et al., 2000). Finfish, bivalves, and crustaceans consist of three-quarters of global aquaculture production collectively. Although aquaculture initially offered many benefits such as relief from over-fishing and species conservation, the exploitation of the industry has shed light on unsustainable conditions and food safety concerns.

Between 2000-2012, world aquaculture production expanded at an average annual rate of 6.2% (FAO, 2014). Reliance on farmed fish production has increased with the growing human population while ocean fisheries stock worldwide has decreased (Naylor et al., 2000). The International Food Policy Research Institute predicts that by 2020, the annual increase in seafood consumption will be approximately 1.5 kilograms per person (Diana, 2009). The substantial increase in seafood demand must be met by aquaculture or else depletion of natural fish stocks may occur. The growing human population can also augment the potential for coral reef destruction (Pomeroy et al., 2006). Damaging fishing operations such as the use of dynamite and cyanide for the easy capture of fish in order to meet the population demand are extremely detrimental to coral reefs. If recent trends on fishing practices continue, it is estimated that nearly 80% of coral reefs in Southeast Asia and 60% in the Pacific are at risk for depletion. The growth in the fish market can also be attributed to the increased awareness of the nutritional benefits such as the high protein content of fish (Naylor et al., 2000). Approximately 20% of global animal protein is contributed by fish consumption (Deutsch et al., 2007). As aquaculture has become a major food production sector, large-scale production has intensified leading to disease-sustaining conditions and unsustainable practices (Lakshmi et al., 2013; Naylor et al., 2000).

Current intensive aquaculture practices now rely on the global market for providing production inputs such as fertilizer, commercial feed, antibiotics and pesticides (Deutsch et al., 2007). Antibiotics have been used as a prophylactic treatment in the aquatic environment; however, the unrestrained use has led to increased antibiotic resistance of the bacteria in the environment (Cabello, 2006). Concerns about antibiotic administration and its potential consequences have resulted in pending bans on antibiotic use in animal feeds while simultaneously gaining support for prophylactic alternatives such as the utilization of probiotics (Hong, Cutting, 2005).

Shrimp Culture. Shrimp capture production numbers reached a new peak at 3.4 million tons in 2012 (FAO, 2014). Fisheries located in the Northwest and Western Central Pacific, Indian Ocean, and Western Atlantic regions contribute to more than half of the capture production of shrimp. It is estimated that aquaculture makes up for 50% of the world shrimp supply (Arquitt et al., 2005). Market trends have shown shrimp as the most valuable farmed species and seafood species with the uppermost trade value due to its high protein content (Bondad-Reantaso et al., 2012). *Litopenaues vannamei* shrimp are the most commonly farmed shrimp in the western hemisphere with escalated production from 193,000 tons to 270,000 tons between 1998 and 2004 (FAO, 2014; Saoud et al., 2003). The biggest producers of this species in Asia are mainland China and the Taiwan Province of China (Briggs et al., 2004). *Litopenaues vannamei* are also known as Pacific White shrimp and by their former scientific name *Penaues vannamei* (Li et al., 2012). *Litopenaues vannamei* are normally translucent in color, have maximum sizes of 23 cm, and have weights of 20 grams for males and 28 grams and up for females at 6 to 7 months of age (FAO, 2006-2015). The species thrive in tropical climates where water temperatures are above 20°C and are native to the Eastern Pacific coast from Sonora, Mexico in the North, through Central and South America. *Litopenaues vannamei* can inhabit waters with salinity ranges from 1 to 40 parts per thousand (ppt) (Saoud et al., 2003).

Shrimp Diseases. Diseases affecting shrimp stocks are an important concern because as shrimp production and trade has become widespread, there is a greater threat of biological pollution (Naylor et al., 2000). Foreign pathogens can be introduced with the movement of aquaculture stocks and diseases can also be transferred to wild fish stocks (Naylor et al., 2000). It is

interesting to note that most diseases up to this point that have affected shrimp are mostly viral and not bacterial infections.

There have been significant *L. vannamei* production losses due to viruses (Naylor et al., 2000). Whitespot and Yellowhead virus diseases have been prevalent since the early 1990s and their appearance has been reported in farms in the United States, across Asia, and in numerous countries in Central and South America (Naylor et al., 2000). The Whitespot Syndrome Virus (WSSV) has gained attention in the shrimp industry due to its ability to cause 90-100% mortality within a seven-day period. Prevention of this disease is challenging due its ability to reside and survive in the aquatic environment for up to two years (Yang et al., 2001). Losses from WSSV have been approximately U.S. $3 billion annually (McColl et al., 2004). Similar to WSSV, the Yellow Head Virus (YHV) is another virus that is of great concern to the shrimp industry in Asia due its ability to cause mass mortalities and therefore production losses (Nunan et al., 1998).

The Taura Syndrome Virus (TSV) has also been pandemic in shrimp industries (Bonami et al., 1997). This virus originated in the Western Hemisphere and spread to Ecuador, Peru, the Pacific and Caribbean coasts of Colombia, Honduras, Guatemala, El Salvador, northeast Brazil, Nicaragua, and Belize. Mortalities can range from 50% to 90% among populations with Taura Syndrome infections. Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is another viral infection that has affected shrimp in the Western Hemisphere (Dhar et al., 2000). Two shrimp species are primarily affected by this disease: *Penaues stylirostris and Penaues vannamei.* IHHNV can cause up to 90% mortality in *P. stylirostris* but does not cause mortality in *P. vannamei.* Instead, it causes production losses due to reductions in shrimp growth and physical deformities such as runt deformity syndrome. In 2004, infectious myonecrosis virus (IMNV) was first reported in Brazil (Senapin et al., 2007). This virus was visible in the necrosis of striated muscles mostly in the distal abdominal segments and the discoloration of affected muscles.

Shrimp Immune System. The shrimp immune system relies solely on the innate immune system, which contains hemocytes, numerous plasma components, and multimeric systems (Aguirre-Guzman et al., 2009). Shrimp possess three lines of defense: a physical barrier, humoral immune defense, and cell-mediated immune defense. Although the exact mechanism of

the exoskeleton is unknown, it serves as an important physical barrier through the diverse spread of hemocyanin and catalytic phenol oxidation of the exocuticle and endocuticle of crustaceans. These proposed mechanisms are believed to contribute to the role of cuticle hardening and phenoloxidase. The humoral immune defense is more complex than the physical barrier and involves the use of pattern recognition proteins (PRP), antimicrobial peptides, and lysosomal enzymes.

Another line of defense is the cell-mediated immune defense. The cell-mediated immune defense involves many different mechanisms including: phagocytosis, encapsulation and nodule formation, an antioxidant system, oxyradical scavenging system, melanization, cytokines, and the clotting protein cascade. The cephalothorax, more predominantly in the dorsolateral surface of the foregut, consists of hematopoietic tissue that is a vast network comprised of packed tubules (Aguirre-Guzman et al., 2009; Smith et al., 2010). This tissue is responsible for hemopoiesis, otherwise known as the production of new hemocytes (Smith et al., 2010). There are three categories of hemocytes that vary in size and number of cells within the cytoplasm: hyaline (H), semi-granular (SG), or granular (G) cells (Smith et al., 2010). Hemocytes are involved in various process such as phagocytosis, encapsulation, nodule formation, wound repair, clotting, and prophenoloxidase (proPO) activation (Aguirre-Guzman et al., 2009). Hemocytes distinguish invading diseases by using pathogen-associated molecular patterns (PAMPs) of the intruder and respond using phagocytosis or encapsulation and, later on, discharge antimicrobial peptides (AMPs) such as penaidins (in shrimp) and crustins, lysozymes and lectins (Rowley and Pope, 2012). Hyaline cells are small cells that start the coagulation process while granulocytes phagocytize and encapsulate foreign material through the storage of the enzyme proPO (Aguirre-Guzman et al., 2009; Martin, Hose, 2010). β-1-glucans, peptidoglycans (PG), and lipopolysaccharides (LPS) are able to initiate granulocytes to release enzymes and undergo exocytosis. Semi-granulocytes possess many primary functions including phagocytosis, encapsulation, and clotting.

Shrimp Digestive Tract. There are three basic components of the shrimp alimentary system: the esophagus and foregut, the midgut, and the hindgut (Felgenhauer, 1992). The esophagus is a short, J-shaped structure that leads to a large anterior chamber that opens up to a smaller posterior chamber. Chitinous folds in the esophagus are suspected to inhibit the regurgitation of

ingested material. The anterior chamber, also known as the cardiac chamber, is a sac containing an array of internal structures that aid in the sorting and mastication of consumed food. The following posterior chamber, also known as the pyloric chamber contains an ampulla, which filters the particles to allow only fine particles to enter the hepatopancreas. Larger particles pass directly into the midgut via channels. The hepatopancreas is a large digestive gland that surrounds the foregut. Both the anterior and posterior chambers are made up of chitinous plates that are connected to each other by membranous ligaments that allow movement of the extrinsic muscles to control action on the foregut. The foregut is terminated at the junction of the posterior chamber and midgut and also indicates the end of the first basic component (Dall, 1967; Felgenhauer, 1992). The hepatopancreas has the combined roles of the liver, pancreas, and intestine that is seen in vertebrates as it is involved in a variety of functions such as food absorption, transport, secretion of digestive enzymes, and storage of lipids, glycogen and a number of minerals (Caceci et al., 1988; Felgenhauer, 1992). Four cell types are located in hepatopancreas tubules: E-, F-, R- and B-cells (Felgenhauer, 1992). Embryonic cells (E-cells) are small cells located at the terminal ends of the tubules. E-cells are distinguished by a large nucleus with a distinct nucleolus, rough and smooth endoplasmic reticulum, scarce Golgi profiles, and absence of brush border. Fibrillar cells (F-cells) have a fibrillar appearance and are located at the nucleus base and contain a developed rough endoplasmic reticulum (RER), numerous mitochondria and golgi profiles, and small vesicles spread throughout the cytoplasm. They are involved in protein production and the mineral storage. Resorptive cells (R-cells) are tall, columnar cells that contain a brush border, centrally located nucleus, and numerous storage vesicles (Al-Mohanna, Nott, 1987; Felgenhauer, 1992). They are involved in food absorption and can also gather calcium, magnesium, phosphorus, sulfur and other mineral deposits (Felgenhauer, 1992). R-cells are the most abundant cell type in the hepatopancreas. Blister cells (B-cells) are large cells that contain a smaller brush border and have a prominent vesicle that is surrounded by a cytoplasm filled with RER. B-cells are secretory cells that function as the primary producers of digestive enzymes. For more than a century, there have been investigations on how the four cell types are correlated in the hepatopancreas (Caceci et al., 1988). The most popular scheme was created in 1930 and suggests the E-cell as the initial cellular origin but thereafter, exhibits two independent lines of development: E → F → B and E → R. This scheme assumes that the E-cell has the sole function of creating new cells that are capable of assisting

with digestion and that the B-cell functions as a secretory cell. An alternative sequence that was proposed was created in 1928 suggests the follow sequence: E → R → F → B (Caceci et al., 1988).

The midgut connects the foregut to the subsequent portion of the hepatopancreas into the abdominal somites before connecting to the hindgut. Although the purpose of the midgut is unclear, the following activities are believed to partake in the midgut: osmoregulation, nutrient absorption, and the production of a peritrophic membrane (Felgenhauer, 1992). The midgut is terminated by blind-ended anterior and posterior midgut ceca in various locations and patterns. The posterior midgut ceca (PMGC) is located at the midgut-hindgut juncture and has unique cells that contain myelinlike figures that are typically found within surfactant-producing type II alveolar cells of vertebrate lungs. The PMGC contains many organelles such as mitochondria, RER, smooth endoplasmic reticulum (SER), and Golgi complexes.

The third part of the alimentary canal is the hindgut. The hindgut is similar to the foregut in that it is lined with chitin. A unique feature of the hindgut is that there is a presence of cuticular scales that always position the spines in the direction of the anus and seemingly assist fecal mass route towards the anus.

Shrimp Molting Cycle and *Vibrio spp.* Attachment. Shrimp contain an integumentary system that consists of an exoskeleton comprised of chitin, proteins, minerals, and lipids (Corteel, Nauwynk, 2010). Chitin is a polysaccharide comprised of linear chains of β-(1,4)-linked *N*-acetylglucosamine (GlcNAc) residues that are important for providing cuticles with resistance against tension (Corteel and Nauwynk, 2010; Keyhani and Roseman, 1999). Protein deposits surround chitin strands and in between nanofibrils to create a chitin-protein complex that is impermeable to mechanical compression (Corteel and Nauwynk, 2010).

There are three primary molt cycles: post-molt, inter-molt, and pre-molt (Corteel, Nauwynk, 2010). Molt stages are determined using the appearance of the epidermis and the setae such as pigmentation, setogenesis and presence of the matrix or internal coni in the setal lumen and development of setal organs at the setae base. After the pre-molt stage, shrimp experience a sequence of muscle contractions that release the old exoskeleton. Microorganisms play a crucial role in the chitin recycling process by adhering to molt surfaces and initiating the degradation process (Keyhani and Roseman, 1999). Chitin offers a microbial adhesion surface

for many chemoorganotrophs such as *Vibrio* spp. compared to the marine water, which offers no nutrients such as phosphate, nitrogen, or ammonia for attachment. Marine microorganisms such as *Vibrio* spp. are able to attach to chitin through random collision and utilization of polar flagella for swimming and lateral flagella for movement across solid surfaces. Studies have indicated the ability to revive pathogenic *Vibrio* spp. and *Aeromonas* spp. strains from the exoskeletons of aquatic arthropods, thus implicating zooplankton and phytoplankton as possible locations for pathogenic strains to thrive in. In the marine ecosystem, there is approximately 10^{11} metric tons of chitin produced every year offering numerous attachment locations for pathogenic bacteria and simultaneously, possible routes of infection for many marine inhabitants.

Water Quality. Nitrogenous waste control is important for shrimp farmers to consider during production. Non-optimal levels of nitrate, nitrite, and ammonia can contribute to shrimp health deterioration and/or mortality especially with inland marine shrimp production (Kuhn et al., 2010). Animal waste and decomposing organic solids, such as unconsumed food, can cause increased ammonia and nitrite levels (Maillard et al., 2005). Ammonia levels are measured as total ammoniacal nitrogen (TAN) and TAN levels can be controlled using submerged flow biofilters, bench-scale fluidized bed reactors, and continuous bioreactors using immobilized alginate beads (Shan and Obbard, 2001). TAN levels measure NH_3 and NH_4^+ which are two forms of ammonia that exist in aqueous solutions (Chen et al., 2006; Shan and Obbard, 2001). Although both forms of ammonia can be toxic to shrimp, the unionized form (NH_3) is of bigger concern because it is toxic at lower concentrations (Chen et al., 2006). Biofilters and autotrophic-based bioflocs within intensive shrimp production raceways are often used to remove ammonia using a nitrification process (Chen et al., 2006; Kuhn et al., 2010). Frequently used biofilters in recirculating aquaculture systems (RAS) involve the utilization of oxygen to aid in nitrification which involves the conversion of ammonia to nitrate (Gutierrez-Wing and Malone, 2006). *Nitrosomonas* spp. and *Nitrobacter* spp. are slow growing, autotrophic bacteria that can be used in biofilters (Shan, Obbard, 2001). *Nitrosomonas* spp. and *Nitrobacter* spp. are naturally inherent in marine water but are easily removed during water exchange of the production cycle (Shan and Obbard, 2001). In order to have sufficient nitrification in RAS systems, a high cell density of *Nitrosomonas* spp. and *Nitrobacter* spp. must be maintained in the log phase (Shan and Obbard, 2001). This can prove to be challenging because nitrifying bacteria

may experience insufficient substrate levels, inter-specific inhibition, and inadequate acclimation periods to affect bioremediation (Shan and Obbard, 2001).

The safety level of ammonia in *L. vannamei* juveniles in 25 ppt salinity is 0.15 mg/l NH_3-N (Lin and Chen, 2001). The unionized and ionized forms are inversely related; if there is more unionized ammonia in an aqueous solution than there is less ionized ammonia present and vice versa (Chen et al., 2006). More ionized forms of ammonia are present with lower pH and increased temperature and salinities (Bower and Bidwell, 1978; Chen et al., 2006). Temperature and pH have a stronger effect on the forms of ammonia present while salinity has a lesser effect (Bower and Bidwell, 1978). A balance between unionized ammonia and ionized ammonia must be achieved to provide optimal conditions for aquaculture species. In the first step of the nitrification process, ammonia is oxidized into nitrite (Gutierrez-Wing and Malone, 2006). Similar to increased ammonia levels, increased nitrite levels can also create a harmful environment to *L. vannamei.* Nitrite abundance in the water can contribute to poor water conditions, increase risk of shrimp mortality, and decrease dissolved oxygen concentration and shrimp growth performance (Lin and Chen, 2003). "Safe" nitrite levels for *L. vannamei* juveniles in was calculated to be 15.2 mg/l nitrite-N (Lin and Chen, 2003). The final step of the nitrification process is the conversion of nitrite to nitrate (Gutierrez-Wing and Malone, 2006). Although high nitrate levels are not as big of a concern as nitrite and ammonia levels are, excess nitrate levels exceeding 200ppm nitrate-N at 11.0ppt water can cause gill abnormalities, lesions in the hepatopancreas, and shortened antenna lengths (Kuhn et al., 2010). Water exchange and adequate water treatment can remove nitrate levels that may have been elevated in production systems as they are the end product of the nitrification process (Kuhn et al., 2010).

Optimum temperature levels for stimulating rapid growth in *L. vannamei* are between 29-30°C (Kuhn et al., 2010). *Vibrio parahaemolyticus,* a pathogen that is commonly isolated from raw seafoods and capable of causing mortalities in aquaculture stocks are detected when temperatures are above 15°C (Su and Liu, 2007). It is important to consider temperature in these regards, however it is also important to consider the temperature effects on nitrification. Higher temperatures increase the nitrification rate as biochemical bacterial processes are driven with the increase in temperature in a suspended growth system (Chen et al., 2006). Alternatively, temperature has different effects on nitrification kinetics in fixed film filters; diffusion mass transport is a concern and temperature has a lesser effect on nitrification kinetics when compared

to a suspended growth system (Chen et al., 2006). The dissolved oxygen concentration requirement for shrimp culture is a minimum of 3.0 ppm (Kuhn et al., 2010). The amount of dissolved oxygen present is important for a RAS design because nitrification utilizes oxygen (Chen et al., 2006). Tentative oxygen requirements based on stoichiometric equations are 3.43 mg for oxidation of 1mg NH_3-N and 1.14 mg for oxidation of 1 mg NO_2-N (Chen et al., 2006). The dissolved oxygen concentration range to achieve nitrification is between 0.6 and 3.4 mg/L (Chen et al., 2006). It is important that dissolved oxygen levels are kept high as dissolved oxygen is a limiting substrate for nitrification and is also pertinent for shrimp longevity (Chen et al., 2006; Kuhn et al., 2010).

Vibrio parahaemolyticus. Disease outbreaks in shrimp production such as virus epidemics and vibriosis has become prevalent with the surge in shrimp demand. *Vibrio parahaemolyticus* is a Gram-negative, halophilic, and curved, rod-shaped bacterium that is 0.5 by 1.3-3 micrometers (Percival and Williams, 2014). *Vibrio parahaemolyticus* also possess a lateral, polar flagella that allows migration across semi-solid surfaces by swarming and increased motility in liquid media (Fraser and Hughes, 1999; Percival and Williams, 2014). It is an opportunistic, facultative anaerobe that is commonly found in estuarine and coastal marine waters and in the normal microbiota of marine animals (Hsern Malcolm et al., 2015; Sung et al., 2001; Zhou et al., 2012). They are non-sporulating, catalase-positive, and tend to be oxidase-positive (Percival and Williams, 2014). Growth in seawater is often detected when temperatures reach 15°C or above; *Vibrio parahaemolyticus* densities have been observed to increase to or by 1000cell/100mL in waters when temperatures rise to 25°C (Su and Liu, 2007). During the wintertime, the organism can persist in the seawater sediment until the warmer temperatures of late spring or early summertime, when the organism will then spread to the seawater (Su and Liu, 2007).

Vibrio parahaemolyticus is one of the leading causes of foodborne illness with an estimated 4,500 cases each year in the United States, however, not all strains are pathogenic (Hsern Malcolm et al., 2015). The pathogenicity mechanism of *V. parahaemolyticus* is not entirely understood but it is clear that there are key virulence factors that are associated with this bacteria (Hsern Malcolm et al., 2015). There are over 200 serogroups of *Vibrio spp.* and some strains exhibit the presence of key virulence genes such as cholera enterotoxin (Ace), zonula occludens toxin (Zot) and *Vibrio* cholera toxin (ctx) (Percival and Williams, 2014).

Thermostable direct hemolysin (TDH) and/or TDH-related hemolysin (TRH) is a pathogenicity island that distinguishes pathogenic strains of *V. parahaemolyticus* because they are visible only in pathogenic strains (Makino et al., 2003). TDH-producing isolates are known as Kanagawa-positive and can be identified using β-hemolysis on a Wagatsuma blood agar (Makino et al., 2003).

Vibrio parahaemolyticus can affect a broad range of hosts from humans, finfish, and seafood such as codfish, sardines, mackerel, flounder, clams, octopus, shrimp, crab, lobster, crawfish, scallops, and oysters. In 2012, there have been 75 total illnesses and 6 hospitalizations from the consumption of raw seafood that have been contaminated with *Vibrio* spp. (CDC, 2013a). As *Vibrio* spp. are opportunistic and inherent in the microbiota of various marine animals, they can cause decreases in growth and death of livestock if *Vibrio* spp. numbers reach certain thresholds (Percival and Williams, 2014; Sung et al., 2001). *Vibrio* spp. incidence can be increased if environmental stresses lead to diminished diversity in the pond water community (Sung et al., 2001).

A common illness of *V. parahaemolyticus* in humans is gastroenteritis; an illness with symptoms including diarrhea (sometimes bloody and watery) with abdominal cramps, nausea, vomiting, headache, chills, and low-grade fever (CDC, 2013a). Human illness can occur through the consumption of raw or undercooked shellfish, especially oysters (CDC, 2013). There are also rare cases in which *V. parahaemolyticus* infection can occur through an open wound exposure to warm seawater (CDC, 2013). *Vibrio parahaemolyticus* infection can be diagnosed through the isolation of cultures of stool, wound, or blood (CDC, 2013). Thiosulfate, citrate, bile salts, and sucrose (TCBS) agar is commonly used as a selective medium for *Vibrio* spp. (CDC, 2013). Treatment of *V. parahaemolyticus* infections in humans does not warrant antibiotics unless the infection is severe or prolonged. If antibiotics are used, tetracycline or ciprofloxacin are commonly used. Patients are recommended to drink plenty of fluids throughout the treatment period. Prevention of infection is through proper cooking of shellfish products to inactivate the bacteria. Organisms are sensitive to heat at 49-50°C and cold at -18°C or -24°C for 15-28 weeks. High hydrostatic pressure (HPP) at 450 megapascal (MPa) for 6 minutes, 350MPa for 6 minutes, or 450MPa for 4 minutes can also be used to destroy bacterial cells in food without affecting the nature of the food (Mootian et al., 2013). Cobalt-60 gamma radiation

at 0.75 kilogray is another method used to reduce bacteria to undetectable levels (<10 CFU/g) however, consumers are reluctant to consume irradiated foods (Su and Liu, 2007).

EMS. Early Mortality Syndrome (EMS) also known as Acute Hepatopancreatic Necrosis Disease (AHPND) is caused by a *V. parahaemolyticus* strain that is thought to have initially emerged in 2009 and has gained much attention due its high effectiveness in causing mortality in shrimp upon exposure (FAO, 2014). Not much research has been conducted on EMS as it is a recently emerged pathogen. PCR analysis of the 63 clinical and 66 environmental *Vibrio parahaemolyticus* isolates obtained between 2008-2014 was conducted using AP2 primers that were specific to DNA sequences from the EMS plasmid and AP3 primers that were unique to the toxin gene of EMS (Kongrueng et al., 2014). Results from the PCR analysis confirmed that the EMS strain was newly emerged because all isolates that were collected were negative for the AP2 and AP3 primers. Virulence genes of the EMS strain detected using PCR techniques indicate a Type 6 secretion system I (T6SS1) and a Type 3 secretion system I (T3SS1) in EMS isolates. The existence of T6SS1 and T3SS1 suggests the EMS ability to adhere to host cells and prevent other bacteria from growing in the environment.

The presence of a 69-kb plasmid from *V. parahaemolyticus* strains causing EMS is believed to be responsible for injecting *Photorhabdus* insect-related (Pir) toxins, specifically *pirA*- and *pirB*- like toxins, into shrimp (Han et al., 2015). Experiments conducted using the injections of *pirA*- and *pirB*- like toxins and knockout mutants have demonstrated that these toxins are etiological factors for EMS. Histological examinations and laboratory bioassays have been previously used to diagnose EMS, however molecular diagnostic methods based on conventional PCR that target *pirA*- and *pirB*- like genes can be used for identification of EMS causing *V. parahaemolyticus* strains. Utilization of qPCR methods allows for the optimal detection of EMS compared to previous methods as it is has the advantages of speed, sensitivity, and specificity.

Mortality due to EMS can be seen in Thailand (FAO, 2014). Thailand, a major shrimp-producing country, reached a maximum shrimp production of 1.4 million tons in 2009 but experienced a decrease in shrimp production from 2010-2012 due to EMS. EMS has also impacted *Penaeus vannamei* shrimp culture in Southeast Asian countries such as China, Vietnam, and Malaysia and has recently spread to northern states of Mexico such as Nayarit,

Sinaloa, and Sonora (Nunan et al., 2014). Northern states of Mexico reported a 65% drop in tons of shrimp produced in 2013 compared to tons of shrimp produced in 2011. The Global Aquaculture Alliance estimates $1 billion have been lost in production due to EMS (FAO, 2014). As shrimp capture production numbers continually increase, there is a much needed focus on sustainable shrimp aquaculture (Bondad-Reantaso et al., 2012). The mortality-causing effectiveness of the EMS strain is of great concern and much attention is warranted if efficient shrimp production is to continue under intensive aquaculture systems.

EMS disease is limited to the shrimp hepatopancreas and causes the hepatopancreas to shrink and discolor (Nunan et al., 2014). Histopathology of EMS infected shrimp have revealed degradation of the hepatopancreas due to sloughing of central tubule epithelial cells and lack of B, F, and R cells (Leaño, Mohan, 2012). Infiltration of hemocytes was also visible in the shrimp hepatopancreas which was indicative of immune system dysfunction (Tran et al., 2013). Hemocytes are involved in numerous cellular responses such as encapsulation, phagocytosis, melanization, cytotoxicity, cell-to-cell communication, clotting, and proPO system activation (Witteveldt et al., 2004). The EMS restriction to the hepatopancreas imply a toxin-mediated etiology (Tran et al., 2013).

Previous studies have shown immersion, reverse gavage, and inoculated feed challenge tests can be used to introduce EMS to the shrimp (Nunan et al., 2014; Tran et al., 2013). Results from Lightner's studies showed that bacteria-free filtrate of the EMS grown for 18-hours overnight was capable of causing mortalities when challenged with reverse gavage to shrimp indicating that an extracellular bacterial toxin may be associated with EMS (Tran et al., 2013). Although the histopathology of EMS has been reviewed extensively, research on the incorporation of probiotics in the shrimp diet to promote disease resistance against EMS has not been studied.

There are currently no disease prevention methods for EMS infection that have been tested in shrimp. The specific *V. parahaemolyticus* strains that are associated with EMS are ambiguous, therefore the optimal prevention method is to utilize approaches that reduce the presence or activity of *Vibrio* spp. in general (De Schryver et al., 2014). Complete disinfection of the pond bottom and water to eliminate possible EMS sources is not recommended because the elevation in nutrient availability and decrease in microbial community promotes the growth of fast-growing bacteria. Sensitivity of the *V. parahaemolyticus* strain causing EMS to

antibiotics was tested using the disc diffusion method of the following most commonly used antibiotics in aquaculture: ampicillin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), sulphamethoxazole/trimethoprim (25 μg), gentamycin (10 μg), erythromycin (15 μg), and norfloxacin (10 μg) (Kongrueng et al., 2014). Results from the antibiotic sensitivity tests showed that EMS isolates were resistant to all seven antibiotics tested, which was not congruent with the antibiotic susceptibility patterns of control isolates. This indicates that the EMS strain is vastly different than other *V. parahaemolyticus* strains that already occur in the aquatic environment. Microbial management that involves the incorporation of mature microbiota or the colonization of slow-growing innocuous bacteria in the environment should be used instead. These microbial management techniques can provide competition for nutrients with *Vibrio* spp. and hinder pathogenic bacterial growth and decrease animal mortality in the marine environment.

Methods of Shrimp Disease Detection. "Classical" diagnostic methods are commonly used to diagnose and detect shrimp disease such as: review of disease history, clinical sign observation (visible lesions, behavior, abnormal growth), direct microscopy, histopathology, electron microscopy, culture and biochemical identification, enhancement, bioassay, serological methods, hematology and clinical chemistry, toxicology, DNA probes, polymerase chain reaction (PCR) and tissue culture (Lightner and Redman, 1998). Among these, the observation of gross and clinical signs along with laboratory test application using the light microscope and classical microbiological methods of isolation and culturing of the agent, regular histology and histochemistry are prominent in diagnosing shrimp diseases. PCR methods have now become a common technique in identifying viral, bacterial, and parasitic shrimp diseases (Nunan et al., 2014).

Non-infectious diseases due to environmental stresses (temperature, salinity, and pH), nutritional imbalances and deficiencies, and toxicants (shrimps own metabolites or industrial and agricultural) can cause shrimp mortalities and loss of shrimp stock (Lightner and Redman, 1998). Non-infectious diseases can be differentiated from infectious diseases because various diseases show distinctive signs and lesions. In most cases, the particular shrimp disease can be confirmed through initial observation of the histopathology and lesions but appropriate diagnostic methods,

case history, and/or analytical results are necessary in order to provide further confirmation for definitive diagnosis of the shrimp disease.

Antibiotics. Antibiotics are defined as "a compound produced by a microorganism which inhibits the growth of another organism" (Kümmerer, 2009). The use of antibiotics is a common strategy used to combat disease and promote the growth of food-producing animals including aquaculture species (CDC, 2013b). Approximately 13.3 million kilograms of antimicrobial drugs were approved for use in food-producing animals in 2010 and between 2009 to 2012, the total amount of antimicrobials sold for use in food-producing animals increased by 16% (FDA, 2014b; c). Drugs that are currently approved for aquaculture use by the FDA are: chloramine-T, chlorionic gonadotropin, florfenicol, formalin, hydrogen peroxide, oxytetracycline dehydrate, oxytetracycline hydrochloride, sulfadimethoxine/ormetoprim, tricaine methanesulfonate (FDA, 2014c). Although antibiotic utilization is widespread, there are increasing concerns about common adverse side effects. A direct correlation between improper antibiotic use and increased resistant bacteria has become a public concern. Improper antibiotic use can be characterized as antibiotic dosage miscalculations, which are not sufficient to eliminate the target bacteria completely, and applying antibiotics when it is unwarranted. Inadequate antibiotic application can cause a selective pressure for bacteria that are impervious to antibiotics and the adaptive evolution of the bacteria to survive the antibiotic use making it difficult to resume antibiotic treatment (Andersson, 2003; Kümmerer, 2009). In some cases, adaptive evolution has been so effective that antibiotics cannot be used against the bacterial infection (Andersson, 2003). In a study conducted in 2000, 76 interviews conducted of shrimp farmers in Thailand along the eastern Gulf Coast, the Andaman Coast, and the southern Gulf Coast showed that commonly used antibiotics were norfloxacin, oxytetracycline, enrofloxacin, and different sulphonamides (Holmström et al., 2003). Eighty-six percent of shrimp farmers in the study used antibiotics as a prophylactic treatment by feeding antibiotics daily to shrimp and administering higher doses of antibiotics as amounts they considered to be "potent". Twenty-seven percent of the farmers were unaware of the proper uses of antibiotics to treat diseases.

The selective pressure for resistant bacteria is not only unfavorable for animal species but humans as well. Antibiotic use in food-producing animals can allow resistant bacteria from the animals to enter into the human food supply and cause adverse human health consequences

(CDC, 2013b). Health consequences can include selection for antibiotic-resistant bacteria and the modification of the normal microbiota that increases vulnerability to bacterial infections (Cabello, 2006). In 2013, the CDC conservatively estimated that more than two million people were ill every year with antibiotic-resistant infections and a minimum of 23,000 died as a result in the United States (CDC, 2013b). The treatment of antibiotic-resistant infections is a costly process compared to infections that are easily remedied with antibiotics due to prolonged and/or expensive therapy, extensive hospital stays, multiple doctor visits and healthcare use, and increased risk for disability and death. Problems of allergies to antibiotics and toxicity can arise from unprotected workers in the aquaculture industry through absorption of large quantities of antibiotics via skin, intestinal and bronchial tracts that are dispensed to animals (Cabello, 2006). These problems are often difficult to diagnose because there is insufficient information on antibiotic absorption in humans. Although it is challenging to evaluate and compare the quantity of drugs used in food animals and the quantity used in humans, there is indication that there is more use of antibiotics in food animals (CDC, 2013b). Due to the numerous health hazards that antibiotics present to both animal and human species, the Centers for Disease Control and Prevention (CDC) promotes efforts to reduce antibiotic misuse in humans and animals. A 2014 guidance from the U.S. Food and Drug Administration has begun to phase out antibiotic use to promote growth in food-producing animals and requires veterinary supervision of antibiotic application (FDA, 2014a).

Environmental contamination through antibiotic use is also a major public concern. Antibiotics can migrate from unconsumed food and fish faeces containing antibiotics into the sediment where aquatic animals live (Cabello, 2006). Antibiotic residue on the sediment floor is detrimental to aquaculture because wild fish and crustaceans can become exposed to the antibiotics through ingestion while on the sediment floor. Antibiotic use in aquaculture also serves as a problem because drugs can linger in the aquatic environment and flow out of the aquaculture farms and into exposed waterways or sewage systems (Benbrook, 2002). Remaining antibiotics can also cause selective pressure by changing the microbiota of the sediment and antibiotic-resistant bacteria to flourish (Cabello, 2006). Resistance genes from the emerged antibiotic-resistant bacteria can be transmitted to bacteria of the terrestrial environment through horizontal gene transfer via conjugation and conjugative transposition. The first case of antimicrobial resistance in South America in 1994 showed that after implementation of

antibiotics to control non-cholerae *Vibrio* infections in shrimp hatching ponds may have exerted environmental pressure leading to antibacterial resistance against *V. cholera* O1 (Weber et al., 1994). The presence of antimicrobial resistance implicate that resistance may have been transferred to *V. cholerae* O1 through a plasmid from other bacteria.

Delivery of antibiotics can be stressful for the animals and labor demanding especially if antibiotics are delivered via injection (Heppell and Davis, 2000). Antibiotic injections also may not be practical for marine animals as small and numerous as shrimp due to the difficulty of netting and holding individual shrimp long enough to administer injection. Alternatively, antibiotics can be delivered via oral and immersion routes, however oral antibiotics are less effective than antibiotics delivered through injections and immersion. Immersion antibiotics can be less labor intensive however there is a higher injection dosage required for the animals. Unwarranted use of antibiotics in aquaculture can negatively impact human and animal health and cause economical loss globally. Antibiotic use presents many environmental concerns, health hazards, and time and labor demands the shrimp industry must be wary of. Although the U.S. government does not permit the import of seafood with high levels of antibiotics, there has been little enforcement of the rule (Benbrook, 2002). The administration of antibiotics has caused many health and environmental concerns for the past few decades and warrants the need for alternative treatments.

Probiotics. The term "probiotics" has been redefined over the years and can be summarized as a viable microorganism that upon ingestion can provide beneficial health effects; typically through biochemical and physiological effects of the probiotic microbe (Aly et al., 2008; Farzanfar, 2006). Probiotic use can also aid in reducing economic losses due to disease (Hong and Cutting, 2005). Some probiotic microbes are able to reduce pathogenic bacterium colonization by competitive adhesion that hinder attachment of the gastrointestinal tract by a pathogen, and through the production of antimicrobial substances such as bacteriocins, bacteriocin-like inhibitory substances, and antibiotics (Casula and Cutting, 2002; Hong and Cutting, 2005). The growing popularity of shrimp production necessitates research on probiotics as a viable solution to counter antibiotic resistance. Probiotics as a method of disease control is beneficial for shrimp production because they can improve overall gut health, hinder pathogenic bacteria from

colonizing and increase desirable and marketable shrimp characteristics (Lakshmi et al., 2013; Mohapatra et al., 2013; Wang et al., 2012).

Bacillus spp. bacteria are commonly used probiotic bacteria for aquaculture due to its common occurrence in marine sediments and natural digestion by shrimp who feed in or around the marine residues (Moriarty, 1999). *Bacillus subtilis* spores are frequently used as probiotics for both humans and animals (Casula and Cutting, 2002). Strains of *Bacillus subtilis* used as probiotics have especially grown in the agricultural industry with growing public understanding of the benefits of probiotics as an alternative to antibiotics. Previous studies have shown that *B. subtilis* contains antitumor and immunomodulatory activities (Aly et al., 2008). There have also been indications from other studies that *B. subtilis* can stimulate the establishment of lactic acid bacteria in the intestinal tract of humans and certain animals.

Some probiotics are commonly incorporated into the diet as spores (Hong and Cutting, 2005). Once the spore is ingested, the spore goes through the process of germination, reproduction, and resporulation once nutrients are depleted. Results from fecal sampling show that *Bacillus* spore-forming species are frequently found in the gut of animals and insects. Facultative anaerobic strains of *B. subtilis* are able to proliferate anaerobically if they are able to use nitrate or nitrite as an electron acceptor or through fermentation in the lack of an electron acceptor. Consumed spores are robust and able to survive the intestinal enzymes and acidic environment of the stomach. Spores can be induced out of their dormant state because the small intestine provides an environment that is rich in nutrients. The *Bacillus* spp. spores are then able to germinate and proliferate and resporulate in the gastrointestinal tract. The gastrointestinal tract is an optimal growth environment for many bacteria because it is nutrient-rich, however, probiotic colonization in the tract can be affected by a variety of host-related factors and microbe-related factors (Balcázar et al., 2006; Wang et al., 2008). Host-related factors can include parameters such as body temperature, redox potential levels, enzymes, and genetic resistance and microbe-related factors can include the effects of antagonistic microorganisms, proteases, and bacteriocins (Balcázar et al., 2006).

Probiotic supplementation of feeds has been shown to stimulate nutrient utilization while enhancing the immune system (Balcázar et al., 2006). Probiotic bacteria can initiate the production of digestive enzymes such as protease, amylase and lipase. The proliferation of these digestive enzymes lead to increased metabolism and efficient nutrient absorption that results in

greater final weight and daily weight gains (Wang et al., 2012). Inosinic acid, a desirable, umami compound found in poultry, livestock, fish and shrimp, was also produced in greater concentrations in shrimp that were fed probiotics compared to shrimp that were not fed probiotics. The increased concentration of inosinic acid and digestive enzymes provide an additional marketable aspect to consumers. Studies have also shown that *Bacillus* spp. can improve water quality and survival and growth rates of shrimp species (Wang et al., 2008). Gram-positive bacteria are more efficient in the conversion of organic matter to CO_2 than Gram-negative bacteria (Balcázar et al., 2006). The presence of elevated levels of Gram-positive bacteria can decrease the accumulation of dissolved and particular organic carbon. Oxygen demand can be greatly reduced with the addition of *Bacillus* spp. near the proximity of pond aerators (Hong, Cutting, 2005). Therefore, the incorporation of probiotics in aquaculture feeds may be simultaneously a cost effective and profitable endeavor to aquaculture production.

The preparation of probiotic application requires that the probiotic have elevated levels of viable microorganisms for increased efficacy (Wang et al., 2008). Probiotics can be sensitive and can lose viability during preparation and storage of the strain. The bacterial strain itself and the manufacturer of the strain may also affect the retention of viability in probiotics. It is also important for industry to consider that the majority of liquid/frozen probiotic cultures may require refrigerated storage and distribution so there may be an added cost and inconvenience in order for probiotics to be applied throughout the aquaculture industry. *Bacillus* spp. spores are robust compared to other probiotic spores because of their physical and biological nature allowing them to be resilient to the aquaculture environment and have extended shelf life. Factors such as water activity, temperature, pH, osmotic pressure, mechanical friction and oxygen also have an impact on the probiotic stability. Another factor to consider for probiotic use is the environmental conditions in which the probiotic will be used (Karunasagar et al., 2010). The intended probiotic product must be adapted to the range of conditions in which it will be applied or else it will be unable to be used to its best capacity. In order for probiotics to become a viable antagonist to inhibit pathogenic organism colonization in the gut, probiotic numbers must be significantly greater than the pathogen, however this is variable on the specific probiotic strain and its target pathogen (Vaseeharan and Ramasamy, 2003). The probiotic must either be continuously introduced to test subjects regularly or must have the ability to reproduce in the host.

Conclusions

The need for prophylactic methods to reduce pathogenic *Vibrio* spp. is evident from the severe production losses *Vibrio* spp. strains have caused in the aquaculture industry. EMS is caused by a *V. parahaemolyticus* strain that has rapidly spread from Southeast Asian countries such as China, Vietnam, and Malaysia and to the northern states of Mexico. This particular strain has gained much attention in the shrimp industry due to its high lethality rate. Results from previous studies identify the hepatopancreas, a large digestive gland that surrounds the foregut, as the only affected organ from EMS disease. Further investigation into probiotics as a method of disease control against EMS is warranted. Probiotic mechanisms include hindering pathogenic bacteria by providing competition with *Vibrio* spp. for adhesion sites in the gut. In addition, probiotics as a method of disease control is beneficial for shrimp production because they can improve overall gut health and increase desirable and marketable shrimp characteristics, which reduces economic losses due to disease. The growing popularity of shrimp production and spread of EMS disease necessitates research on probiotics as a viable solution to counter disease incidence. There are currently no disease reduction methods for EMS that have been tested for shrimp. The specific *V. parahaemolyticus* strains that are associated with EMS are ambiguous, therefore optimal prevention methods are to utilize approaches that reduce the presence or activity of vibrios in general or to completely disinfect ponds. These options may not be economically or environmentally sustainable options to the shrimp industry.

This study aimed to identify specific probiotic strains that can be incorporated into aquaculture feed formulations for gut colonization in shrimp species (*Litopenaues vannamei*). Specific objectives included the following: confirmation of spore germination, virulence expression of *Vibrio* spp. in shrimp species, identification of best probiotic strain candidates as a supplement to shrimp aquaculture feeds, utilization of probiotic strains, and confirmation of probiotic ability to decrease mortalities caused by *Vibrio* spp.

Chapter 3:

Virulence Expression of *Vibrio* spp. in Shrimp Species

Introduction

Vibrio parahaemolyticus can cause disease in a broad range of hosts from humans, finfish, and seafood such as codfish, sardines, mackerel, flounder, clams, octopus, shrimp, crab, lobster, crawfish, scallops, and oysters (Makino et al., 2003). *V. parahaemolyticus* is one of several causative agents of shrimp disease that has become prevalent with the surge in shrimp demand. It is a frequent cause of foodborne illness in humans with an estimated 4,500 cases each year in the United States, however not all strains are pathogenic (Hsern Malcolm et al., 2015). As *Vibrio* spp. are opportunistic and inherent in the microbiota of various marine animals, they can cause decreases in growth and death of livestock if *Vibrio* spp. numbers reach certain thresholds in the animal (Percival, Williams, 2014; Sung et al., 2001).

The study focused on the ability of certain *V. parahaemolyticus* strains to result in chronic virulence using a shrimp model. In this study, acute virulence was defined as virulence that caused 50% survival or less within 24 hours as opposed to chronic virulence, which was defined as virulence that caused 50% survival or less more than 24 hours later. In order to ascertain the *V. parahaemolyticus* strain that would cause chronic virulence, a *V. parahaemolyticus* strain that is pathogenic to shrimp species must be sought out first. Three different *V. parahaemolyticus* strains (RIMD2210633, LM5312, and Early Mortality Syndrome 13-028/A3) were grown using uniform parameters and exposed to shrimp via feed inoculation. RIMD2210633 (RIMD) was a human clinical isolate that was obtained in 1996 from a patient with travellers' diarrhea at the Kansai International Airport (Makino et al., 2003). LM5312 (LM) was an environmental strain that was obtained from Bangladesh in the early 1980s (Jensen et al., 2013). Early Mortality Strain 13-028/A3 (EMS) originated from Vietnam and is also known as acute hepatopancreatic necrosis disease (AHPND). It is a known shrimp pathogen that recently emerged in 2009 and gained much attention due to its high effectiveness in causing shrimp mortality upon exposure (FAO, 2014; Han et. al, 2014). A virulence expression method in shrimp species was needed to properly evaluate the pathogenicity of *V. parahaemolyticus* strains to shrimp species, as there is currently no standard method to do so.

The *objectives* of this study were to evaluate mortality rates of shrimp inoculated with three different *V. parahaemolyticus* strains and to establish the quantity of each of the pathogenic *V. parahaemolyticus* strains to cause chronic virulence in *L. vannamei.*

It was hypothesized that the EMS strain will result in significantly lower survival rates than the control (no *V. parahaemolyticus* strain inoculation) survival rate. It was also hypothesized that different EMS dilutions will result in significantly different survival rates.

Materials and Methods

General Methods: *Starter freezer cultures for EMS 13-028/A3 (EMS), RIMD2210633 (RIMD), and LM5312 (LM)*

Glycerol stocks of EMS cultures were preserved by placing 1 mL of EMS strain grown to an optical density measured at wavelength 600 nm (OD_{600}) to 0.5 and temperature 30°C in Trypticase Soy Broth with additional 2% NaCl (TSB2+) into glycerol. Glycerol stocks were then frozen at −80°C. Trypticase Soy Broth was supplemented with an extra 2% NaCl, which made the overall salinity 25 parts per thousand (ppt) to match the salinity of the water in the experimental beakers used throughout this study. RIMD and LM strains were obtained from Dr. Ann Stevens from the Department of Biological Science in Virginia Tech, Blacksburg, VA and the EMS strain was obtained from Dr. Donald Lightner from the Department of Veterinary Science and Microbiology in the University of Arizona, Tucson, AZ. Similarly, starter freezer RIMD and LM cultures were preserved by placing 1 mL of RIMD or LM strain grown to an OD_{600} = 0.5 in TSB2+ and temperature 30°C into glycerol and frozen at −80°C.

General Methods: *Overnight (O/N) EMS growth*

At the start of each trial, a starter EMS culture (OD_{600} = 0.5) was placed into 30 mL of TSB2+ making the starting OD_{600}= ~0.02. The starter EMS culture with TSB2+ was placed into a shaking incubator for 18 hours. The incubator was set to agitate at 250 revolutions per minute (rpm) at 30°C. EMS was enumerated by a 10-fold serial dilution and spread plating onto Trypticase Soy Agar (Difco) with additional 2& NaCl (TSA2+). Similar to TSB2+, Trypticase Soy Agar was supplemented with an extra 2% NaCl, which made the overall salinity 25 ppt to match the salinity of the water in the experimental beakers used throughout the study.

General Methods: *Spread plate Method*

The spread plate medium used throughout the experiments was TSA2+. All spread plates were made in duplicate and used methods adapted from Buck and Cleverdon's study (1960). TSA2+ medium was tempered at 45°C and approximately 15 mL of the agar was poured into petri plate. Dilutions of bacterial strains were made so that ejected 0.1 mL on the agar surface resulted in the

proper dilution. A 5 mm glass rod was then used to spread dilutions evenly over the surface of the agar using a circular motion. The glass rod was flame sterilized in between dilutions.

Spread plates were left at room temperature at 20°C overnight and counted 24 hours after inoculation. An incubator was not used to store spread plates because incubation at 30°C in preliminary trials resulted in swarming on the EMS spread plates, making it difficult to quantify CFU/mL.

General Methods: *Biosafety Cabinet Housing Apparatus Setup in a Biosafety Level 2 (BSL2) lab*

All trials using shrimp models were conducted in a biosafety cabinet (BSC) to reduce risk to lab personnel. Two polypropylene vessels were constructed to each contain and stabilize 12 one-liter polypropylene beakers in a 3x4 grid, and form-fitted to the dimensions of the BSC. Prior to each trial, beakers were filled with 500 mL of 25 ppt with synthetic sea salt (Crystal Sea Marine Mix, Marine Enterprises International, Baltimore, MD, USA) constituted with distilled water. Evaporated water was replaced daily using distilled water. Continuous aeration was provided to each beaker through a submerged glass pipette coupled to a diaphragm pump positioned external to the BSC. Airflow was manually adjusted at a multivalve manifold to achieve balanced and moderate bubbling between all beakers. Perforated polycarbonate lids were built for each beaker to contain shrimp within the beaker, to prevent cross-splash, and to permit atmospheric exchange of dissolved gasses.

General Methods: *Weighing of Shrimp*

Shrimp were weighed prior to each experiment to determine the approximate amount of feed to inoculate. A negative method was utilized in order to minimize weighing errors due to water on the surface of the shrimp. Shrimp were placed into a plastic container with a paper towel lining the interior and an additional paper towel placed on top of the container in order to prevent shrimp from escaping. The plastic container and shrimp were then placed onto the scale and tared. The shrimp was then placed into the holding tank while making sure the paper towels were kept from coming into contact with any surfaces. Afterwards, the container and paper towels were placed backed onto the scale to record the negative weight.

General Methods: *Feeding Regime*

Prior to the start of the trials, shrimp were not fed for 48-hours to ensure that shrimp would consume all *V. parahaemolyticus* inoculated feed. This was later confirmed during time checks after the start of *V. parahaemolyticus* exposure. *V. parahemolyticus* strains were introduced to each test group via feed inoculation. Feed was premeasured to be 3% of the total average shrimp weight in grams. Strains were applied at a 1:1 ratio of grown *V. parahaemolyticus* strains in milliliters to weight of feed in grams. All shrimp were fed pelleted feed (Shrimp Hyperintensive Feed, Ziegler Bros. Inc., Gardners, Pennsylvania, US). One spoon was allocated to each beaker and each of the treatments was directly applied to feed on a spoon.

After *V. parahaemolyticus* strains were applied to the feed, a 3-minute absorptive period followed to ensure the treatments were completely absorbed by the feed. To guarantee that all of the strains were delivered to each shrimp, the entire spoon was dipped into the beaker along with the strain applied so that unabsorbed EMS application was still introduced to the shrimp. Shrimp were fed inoculated feed once at the start of the 96-hour trial.

General Methods: *Davidson's fixative*

Davidson's fixative was prepared under a chemical fume hood and contained the following ingredients: 95% ethyl alcohol, 100% formalin (saturated aqueous solution of formaldehyde gas, 37-39% solution), glacial acetic acid, and distilled water (Andrade et al., 2008). Davidson's fixative was stored at room temperature. Utilization of the fixative preserved tissues and stopped autolysis of the highly metabolic activities of the hepatopancreas. After sectioning, the hematoxylin and eosin stain (H&E) dye was used to stain tissue samples previously preserved in Davidson's fixative (Fischer et al., 2008).

General Methods: *Statistics*

The Kaplan-Meier estimate was used for survival analysis to measure the portion of subjects living for a certain amount of time after feed inoculation (Goel et al., 2010). The estimate calculated the probability of occurrence of an event at a specific time point and multiplying successive probabilities by previously calculated probabilities to get a final estimate. Any

shrimp that jumped out of the beaker during the 96-hour trials were labeled as right-censored observations. Right-censored shrimp provided partial survival information because it was known that death occurred or will occur sometime after the incident causing the subject to drop out of the study. The log-rank test was used as a post-hoc test to differentiate whether survival times between two groups were significantly different ($p<0.05$). The log-rank test assumed that the hazard ratio is constant over time. The hazard ratio was the fraction of the risk of hazard occurring at a specific time point in one treatment compared to another treatment at the same time point.

1. Bacterial growth curve of *V. parahaemolyticus* strains. 24-hour bacterial growth curves of EMS, RIMD, and LM strains were obtained using a multimode microplate reader (Infinite 200, Tecan Group AG, Switzerland). Non-inoculated TSB2+ was run alongside these strains as a negative control to ensure that no other contamination occurred.

Starter cultures of EMS, RIMD, and LM were thawed and placed into 30 mL of TSB2+ in three individual flasks. 200 µL of the resulting solution of each of the respective strains with three replicates were aliquoted into wells on a Corning 96-Round bottom Transparent Polystyrol plate (Sigma-Aldrich, St. Louis, MO, USA). The multimode microplate reader was set to incubate the strains at 30°C for 144 kinetic cycles or 24 hours and each measurement was taken every 10 minutes with shaking for 5 seconds prior to each measurement. Absorbance measurements were obtained at wavelength 600 nm and at shaking amplitude 6 mm. Growth rate and lag phase of each strain were calculated. Absorbance was averaged and plotted against time.

2. Virulence evaluation. In Trial 2.1, three different *V. parahaemolyticus* strains were evaluated by dividing the system into four test groups (n=6 shrimp; average shrimp weight=3.3±0.58 grams): TSB2+ (non-inoculated control), EMS, RIMD, and LM. All three strains were grown using identical parameters: 18-hour growth time, 30 mL TSB2+ growth medium, and incubator conditions (temperature at 30°C and agitation at 250 rpm). Feed was inoculated immediately after the 18-hour growth period using an automatic pipette to apply strains directly onto the feed and the log CFU/g of feed was determined through serial dilutions of the strains and spreading 0.1 mL onto TSB2+ agar. Spread plates were created for each

treatment group using TSA2+ to measure colony-forming units per mL (CFU/mL) or viable cells.

In Trial 2.2, the EMS, RIMD, and LM strains were evaluated by dividing the system into four test groups (n=6 shrimp; average shrimp weight=3.21±0.91 grams): TSB2+ (control), EMS grown to an OD_{600}=0.5 (concentration 2.54×10^8 CFU/mL), RIMD grown to an OD_{600}=0.5 (concentration 6.1×10^8 CFU/mL), and LM grown to an OD_{600}=0.5 (concentration 2.00×10^8 CFU/mL). All three strains were grown using identical parameters: 18-hour growth time, 30 mL TSB2+ growth medium, and incubator conditions (temperature at 30°C and agitation at 250 rpm). Optical density was measured using a Spectronic 20+ (Spectronic Instruments, United Kingdom) every 30 minutes by placing 100 µL of each strain into a cuvette. 100 µL of TSB2+ was used to blank the Spectronic 20+ for each measurement. All strains were incubated simultaneously and used to inoculate feed when the strains reached OD_{600}=0.5. Spread plates were created for each treatment group using TSA2+ to measure CFU/mL.

In Trial 2.3, the RIMD and EMS strains with different parameters were evaluated by dividing the system into five test groups (n=5 shrimp; average shrimp weight=2.33±0.57grams): TSB2+ (control), RIMD grown to an OD_{600}=6.0 (concentration 5.3×10^9 CFU/mL), EMS grown to an OD_{600}=6.0 (concentration 3.8×10^9 CFU/mL), 12-hour grown RIMD (concentration 1.1×10^5 CFU/mL), and 12-hour grown EMS (concentration 2.3×10^{10} CFU/mL). All treatments were grown using the same incubator settings: temperature at 30°C and agitation at 250 rpm. Optical density was monitored using a Spectronic 20+ every 30 minutes by placing 100 µL of each strain into a cuvette. 100 µL of TSB2+ was used to blank the Spectronic 20+ for each measurement. Once the optical density of the strains reached 1.0, optical density was measured using 1/10 diluted sample of strains. All strains were incubated simultaneously and used to inoculate feed when the strains reached OD_{600}=6.0 or when the 12-hour growth period elapsed. Spread plates were created for each treatment group using TSA2+ to measure CFU/mL.

3. Effects of different EMS components (filtrate only, cells only, overnight EMS (filtrate and cells)). The pathogenicity of different EMS components was compared by dividing the system into four test groups (n=6 shrimp): TSB2+ (control), overnight (O/N) EMS, filtrate only

of EMS, and washed cells only of EMS. Overnight EMS was defined as EMS grown for 18-hours in 30 mL TSB2+ and incubator conditions set to temperature at 30°C and agitation at 250 rpm. Each experiment was conducted for a 96-hour duration and two replicate trials were performed. The mean weight of shrimp used was 3.5±0.65 grams for Trial 3.1 and 3.6±0.80 grams for Trial 3.2.

1 mL of the O/N of EMS was spun at 5000 rpm for 5 minutes and filter sterilized using a 0.2 μL filter to obtain the filtrate. This filtrate was used to inoculate the filtrate only treatment group. Washed cells were obtained by spinning 1 mL of O/N EMS culture at 5000 rpm for 5 minutes. The supernatant was then removed and 1 mL TSB2+ was then applied to the pellet. TSB2+ and pellet were spun again at 5000 rpm for 5 minutes. The supernatant was removed again and 1 mL of TSB2+ was used to reconstitute the pellet.

Histology samples were preserved during the first trial by collecting shrimp within 2 hours of mortality or when they were moribound. Moribound shrimp were classified as shrimp that were not responding to external stimuli but still exhibited gill movement. Histology was also performed on three of the control shrimp that were sacrificed at the end of the trial to be used as a negative control for histological examination. Histology was performed on shrimp using methods presented in Bell and Lightner (1988). Shrimp were cut between the carapace and abdominal segment I and immersed in Davidson's fixative for a minimum of 48 hours prior to sectioning, slide preparation, and microscopy.

4. Dilution series. A dilution series of O/N EMS was conducted in order to achieve a chronic virulence as opposed to an acute virulence. In this study, chronic virulence was defined as virulence that resulted in 50% survival more than 24 hours after inoculation versus acute virulence, which was defined as virulence that resulted in 50% survival in less than 24 hours. Methods used to conduct LD_{50} (number of bacteria necessary to kill 50% of the sample) assays were adapted in this study (Zorrilla et al., 2003). Five test groups were used (n=5 shrimp): TSB2+ (negative control), undiluted O/N EMS (positive control), 1/10 diluted O/N EMS (1/10 EMS), 1/100 diluted O/N EMS (1/100 EMS) and 1/1000 diluted O/N EMS (1/1000 EMS). TSB2+ was used as dilution blanks for the series. It was estimated that EMS bacterial culture

doses would range from 10^6 to 10^9 CFU/mL as the average spread plate counts of previously grown O/N EMS was ~10^9 CFU/mL. Each experiment was conducted for a 96-hour duration and replicated twice. The mean weight of shrimp used was 3.3±0.58 grams for Trial 4.1 and 4.05±0.12 grams for Trial 4.2.

Results & Discussion

1. Bacterial growth curve of *V. parahaemolyticus strains*. Bacterial growth curves of three different *V. parahaemolyticus* strains demonstrated that EMS had a delayed exponential growth phase compared to the RIMD and LM strains (Figure 3.1). RIMD and LM strains exhibited a shorter lag phase of approximately 0.5 hours followed by an immediate exponential growth phase. The EMS Phase I lag phase was much longer and lasted for approximately 2.5 hours before the exponential growth phase began. Although the strains hit the exponential phase at different rates, it was interesting to observe that RIMD exhibited the largest growth rate (μ) during the exponential phase, followed by EMS, and then LM (Table 3.1). The RIMD strain was the first to show the stationary phase around 4.7 hours into the cycle, which was followed by the LM strain with stationary phase at around 8 hours. Although the EMS strain hit stationary phase at around 8.5 hours, the strain displayed diauxic growth initiating at 12 hours. Therefore the EMS Phase I stationary phase was simultaneously the lag phase for EMS Phase II, which lasted approximately 3.5 hours. Diauxic growth was represented by two peaks in the EMS growth curve, which was not observed in the RIMD and LM growth curves.

Diauxic growth is distinguished by the appearance of two exponential growth phases separated by a lag phase, which is also known as diauxic lag (Narang and Pilyugin, 2007). It is a phenomenon in which microbial cells sequentially consume two carbon sources to proliferate. The first carbon source consumed is the one that will promote a faster growth rate for the organism (Kompala et al., 1986). Diauxic lag allows time for bacteria to build up peripheral enzymes to consume the second substrate (Narang, Pilyugin, 2007). For example, diauxic growth has been exhibited in *Escherichia coli,* which can chronologically consume glucose and lactose sources, and *Vibrio cholera,* which initially consumes glucose and then galactose or fructose as the second substrate (Bag, 1974; Narang and Pilyugin, 2007).

The diauxic growth present in the EMS curve provides possible explanations to why this strain is so successful in surviving in the marine environment. The ability of EMS to utilize multiple different carbon sources provides further evidence that this *V. parahaemolyticus* strain has some unique adaptations. It also provides another reason why the shrimp industry is having difficulty when trying to find EMS disease prevention and disinfection methods. Further

investigation is currently underway to identify the carbon sources that EMS utilizes for growth and this information will help to provide further investigation on the EMS growth mechanism.

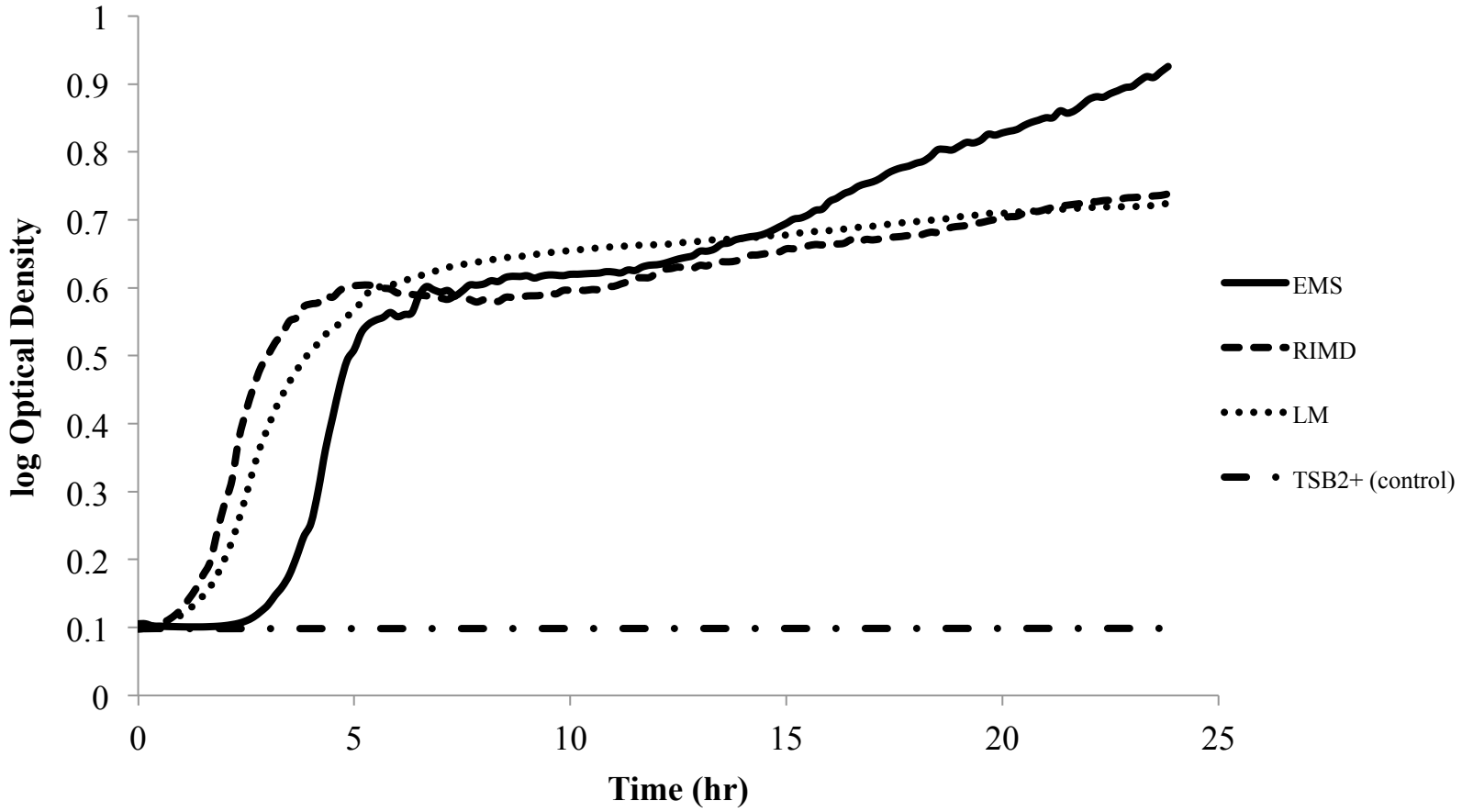

Figure 3.1. *Comparison of bacterial strain growth of EMS, RIMD, and LM. Absorbance values are mean values of n=3 replicate analysis.*

Table 3.1. *Growth rate (μ), Mean Generation Time (MGT) (1/ μ), and lag time (hr) of the exponential phase of EMS, RIMD, and LM strains relating the log optical density vs. time (hr) of incubation.*

Strain	μ	MGT (1/μ)	Lag Time (hr)
EMS Phase I	0.243	4.11	2.5
EMS Phase II	0.0250	40.0	3.5
RIMD	0.262	3.82	0.5
LM	0.193	5.17	0.5

2. Virulence evaluation. Spread plate results of 18-hour cultures of EMS, RIMD, and LM broth strains used to inoculate feed in the study revealed that the EMS strain was more efficient at growing for 18-hours than the RIMD and LM strains. The EMS strain was able to achieve plate counts up to $3.7x10^9$ CFU/mL, which made the actual feed inoculation concentration $3.7x10^8$ CFU/g. RIMD and LM strains exhibited much lower plate counts; the RIMD culture was only able to achieve up to $5.3x10^3$ CFU/mL growth and the LM culture with an even lower growth of $1.59x10^3$ CFU/mL. Actual feed inoculation concentrations for RIMD and LM respectively were $5.3x10^2$ CFU/g and $1.59x10^2$ CFU/g. Possible limiting factors for RIMD and LM strain viability can be attributed to the depletion of nutrients, buildup of toxic metabolic products, and shifts in the ion equilibrium (Monod, 2012). Although plate counts of strains seem to conflict with the optical density curve obtained previously, it is important to remember that optical density measures turbidity while plate counts measure only viable cells or colony forming units (Sutton, 2011).

The diauxic growth shown in the EMS bacterial growth curve obtained previously using the Infinite 200 (Tecan Group AG, Switzerland) provides a possible explanation why the EMS strain was so successful in proliferating throughout the 18-hour incubation while RIMD and LM strains could not. The EMS strain is able to consume two different substrates through the buildup of peripheral enzymes to consume the second substrate while LM and RIMD can only consume one substrate as demonstrated in the bacterial growth curve (Narang, Pilyugin, 2007). The EMS proliferation and viability after 18-hours provides a possible explanation for the difficulty shrimp industries face when trying to find EMS disease prevention and disinfection methods.

A limitation of the bacterial growth curve obtained using the Infinite 200 in the previous experiment was that the bacterial strains were not grown at identical parameters compared to strains grown for the virulence evaluation. Although the temperature setting, media used, and inoculation methods were identical, there were discrepancies in both protocols. Strains in the Infinite 200 were not constantly agitating at 250 rpm as they were in the shaking incubator; instead, strains were shaken for 5 seconds prior to each measurement that was taken at 10-minute intervals.

In Trial 2.1, the EMS strain was the most lethal in the virulence evaluation; the EMS-infected shrimp exhibited 100% mortality within 24 hours of feed-inoculation (Figure 3.2).

These results were consistent with previous feed inoculation studies which showed feed-inoculation with EMS resulted in 100% mortality within 72 hours (Nunan et al., 2014). Feed inoculation with the EMS strain showed significant differences ($p<0.05$) in survival rates when compared to the survival rates of the control. At the end of the trial, feed inoculation with the RIMD strain exhibited 67% survival and feed inoculation with the LM strain exhibited 100% survival, however these results were not significantly different from the control, which exhibited 83% survival.

In addition to difference of the concentration of viable cells due to the strains, the differences in survival rates among the strains may be due to the difference in genes that are present in each strain. Quantitative polymerase chain reaction (qPCR) analysis results of a previous study conducted by Kongrueng et al. (2014) show that EMS strains contain one type 3 secretion system (T3SS), T3SS1, and two type 6 secretion system systems, T6SS1 and T6SS2 (Yang et al., 2001). EMS lacks a T3SS2 that is present in RIMD and LM strains (Makino et al., 2003; Yang et al., 2001). A thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) are also absent in the EMS strain (Kongrueng et al., 2014). TDH, TRH, and the T3SS2 are present in only *V. parahaemolyticus* isolates that are human pathogens. RIMD supports this concept because as it is a known human pathogen, it contains TDH, TRH, and T3SS2 along with T3SS1, T6SS1, and T6SS2 (Makino et al., 2003). LM is an environmental strain that when compared to RIMD, it lacks the TRH gene (Jensen et al., 2013). The inability for RIMD and LM strains grown for 18-hours to cause significant differences in survival rates compared to the control, suggests that neither strain possess the genes necessary to cause shrimp mortality. However, the 18-hour grown EMS strain displayed cell viability that was almost 6 logs CFU/mL greater than 18-hour grown RIMD and LM strains. The EMS cell viability can be attributed to the fact that the EMS strain is in the diauxic growth phase or second exponential phase while the RIMD and LM strains are in the stationary phase at 18 hours, which could be seen in the bacterial growth curve obtained previously. A trial in which a more uniform strain concentration of EMS, RIMD, and LM is warranted to test whether the viable cells of each strain are capable of causing mortality.

The ability of the EMS strain to replicate for 18 hours while the other strains entered stationary phase at 4.7 hours and 8 hours respectively indicates that the diauxic growth in the EMS strain contributed to increased cell viability. In Trial 2.2, shrimp were exposed to EMS,

RIMD, and LM strains grown to an OD_{600}=0.5. The optical density and the duration of the strain were chosen to standardize the strain dose concentration to be uniform for all shrimp.

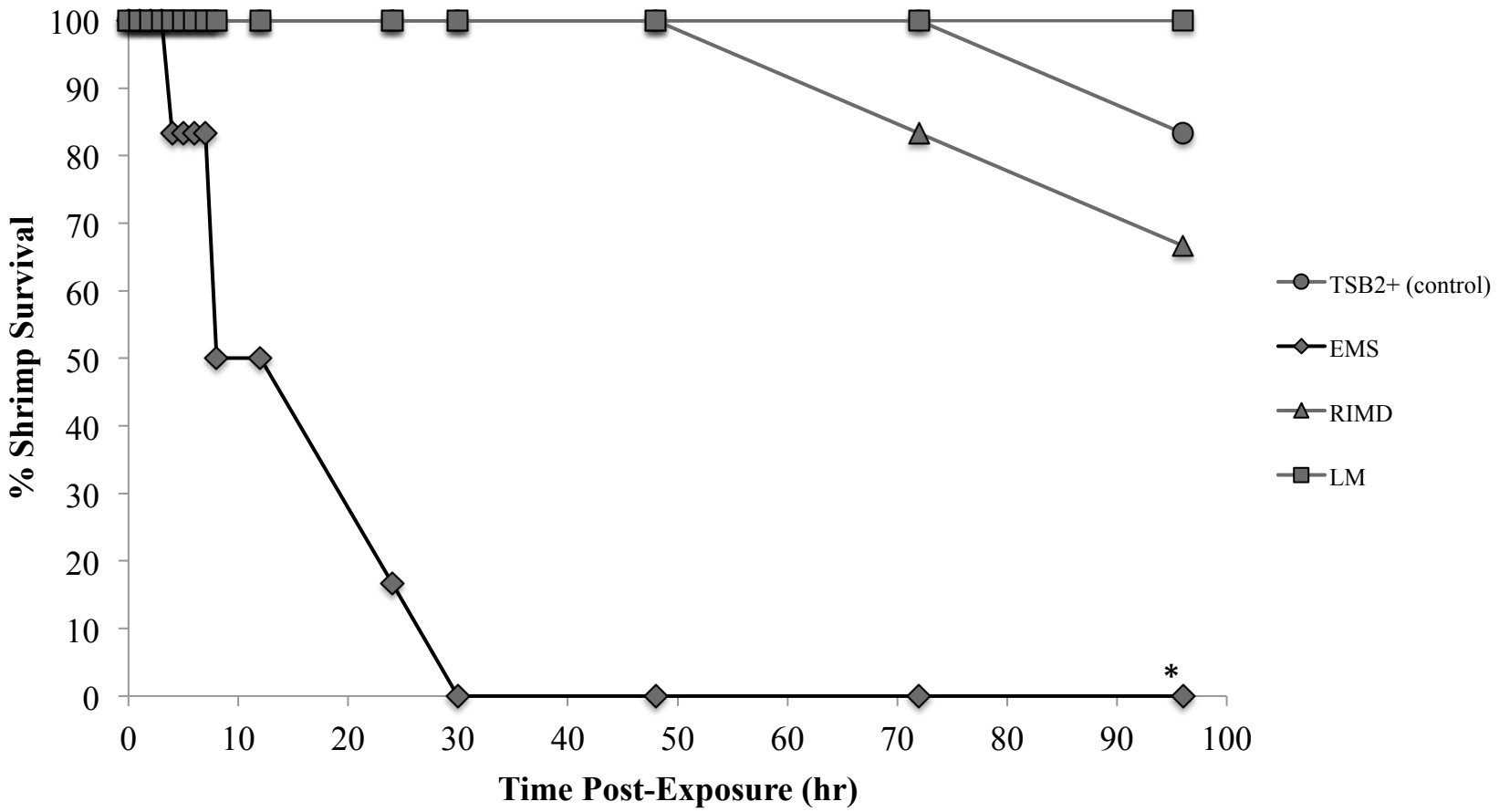

Figure 3.2. *Shrimp survival rate of Litopenaues vannamei species exposed to 18-hour grown EMS, RIMD, and LM strains in Trial 2.1. Values are % of n=6 replicate analysis. Survival curves* with asterisks are significantly different ($p<0.05$) from the control. P-values for significantly different treatments are EMS with 0.0006.*

In Trial 2.2, EMS, RIMD, and LM strains were grown to an OD_{600}=0.5 and respectively exposed to shrimp. Spread plate counts of RIMD, EMS, LM broth culture used to inoculate feed showed that the EMS culture had colony counts of $2.54x10^8$ CFU/mL, the RIMD culture had colony counts of $6.1x10^8$ CFU/mL, and the LM culture had $2.00x10^8$ CFU/mL. These results showed that there was a more standard concentration for comparison as all of the concentrations were within the same log. Feed inoculation concentrations of EMS, RIMD, and LM respectively were $2.54x10^7$ CFU/g, $6.1x10^7$ CFU/g, and $2.00x10^7$ CFU/g.

At 72 hours, one shrimp mortality was observed in both EMS and LM treatments during the 96-hour trial making the survival rates for EMS and LM equal to 83.33% (Figure 3.3). At the end of the trial, TSB2+ and RIMD treatments showed 100% survival compared to the EMS and LM treatment groups, which showed 83.33% survival. In this trial, EMS, RIMD and LM strains were captured at OD_{600}=0.5 as it was the optical density in which the strains were slightly after the mid-exponential phase. Capturing the strains at this stage was important as it has been shown that in other *Vibrio* spp., there was hemolysin production during the mid-exponential phase that decreased in the early stationary phase (Kim et al., 2003). Alternatively as hemolysin production decreases, protease production is initiated. Hemolysins and protease are significant virulence factors that are believed to play important roles in pathogenicity (Iyer et al., 2000). There were no significant differences ($p<0.05$) in shrimp survival rates between EMS, RIMD, and LM treatments when compared to the control survival rate suggesting that the RIMD strain was not virulent at 6.1×10^8 CFU/mL and the LM strains was not virulent at 2.00×10^8 CFU/mL. Further research is warranted using a hemolysin or protease assay to conclude whether there was hemolysin or protease production at the specified points of the strains.

Results from Trial 2.1 and 2.2 suggest that the RIMD or LM does not have an infectious dose necessary to cause mortality in shrimp at either OD_{600}=0.5 or after an 18-hour growth period. Although one mortality was observed for the LM strain grown to OD_{600}=0.5 and one mortality was observed for the RIMD strain grown for 18 hours, the survival rate was not significant compared to the survival rate of the control. Results in the study conducted by Kongrueung et al. (2014) indicate the EMS strain isolates exhibited haemolytic activity on prawn blood agar (PBA) while environmental *V. parahaemolyticus* strains could not. It can be proposed from that study, Trial 2.1, and Trial 2.2. results that EMS may contain haemolysin genes that are capable of causing significant mortality in shrimp subjects when grown for 18-

hours that are not expressed by EMS at OD_{600}=0.5, RIMD at OD_{600}=0.5 or 18-hour grown RIMD, and LM OD_{600}=0.5 or 18-hour grown LM strains.

The comparison of EMS, RIMD, and LM spread plates at both 18-hour incubation and at OD_{600}=0.5 indicate EMS and RIMD strains exhibited swarming on spread plates while the LM strain did not at both parameters (Figure 3.4). Quorum sensing is a cell-to-cell communication process that bacteria use to determine cell population density using a low-molecular-mass signal molecule (Daniels et al., 2004; Henke and Bassler, 2004). Swarming is indicative of bacterial quorum sensing and is the occurrence of bacteria moving across the agar (Daniels, et al., 2004). Thus it can be proposed that EMS and RIMD strains have the quorum sensing ability. Quorum sensing allows bacterial swarming in order to permit optimal spread of bacterial cells when the population has outgrown its single given niche. There is some evidence that shows that certain swarmer cell states can be correlated with the expression of specific virulence factors. Further research is warranted to see what virulence factors are expressed in the EMS and RIMD strains that caused the swarming phenomenon.

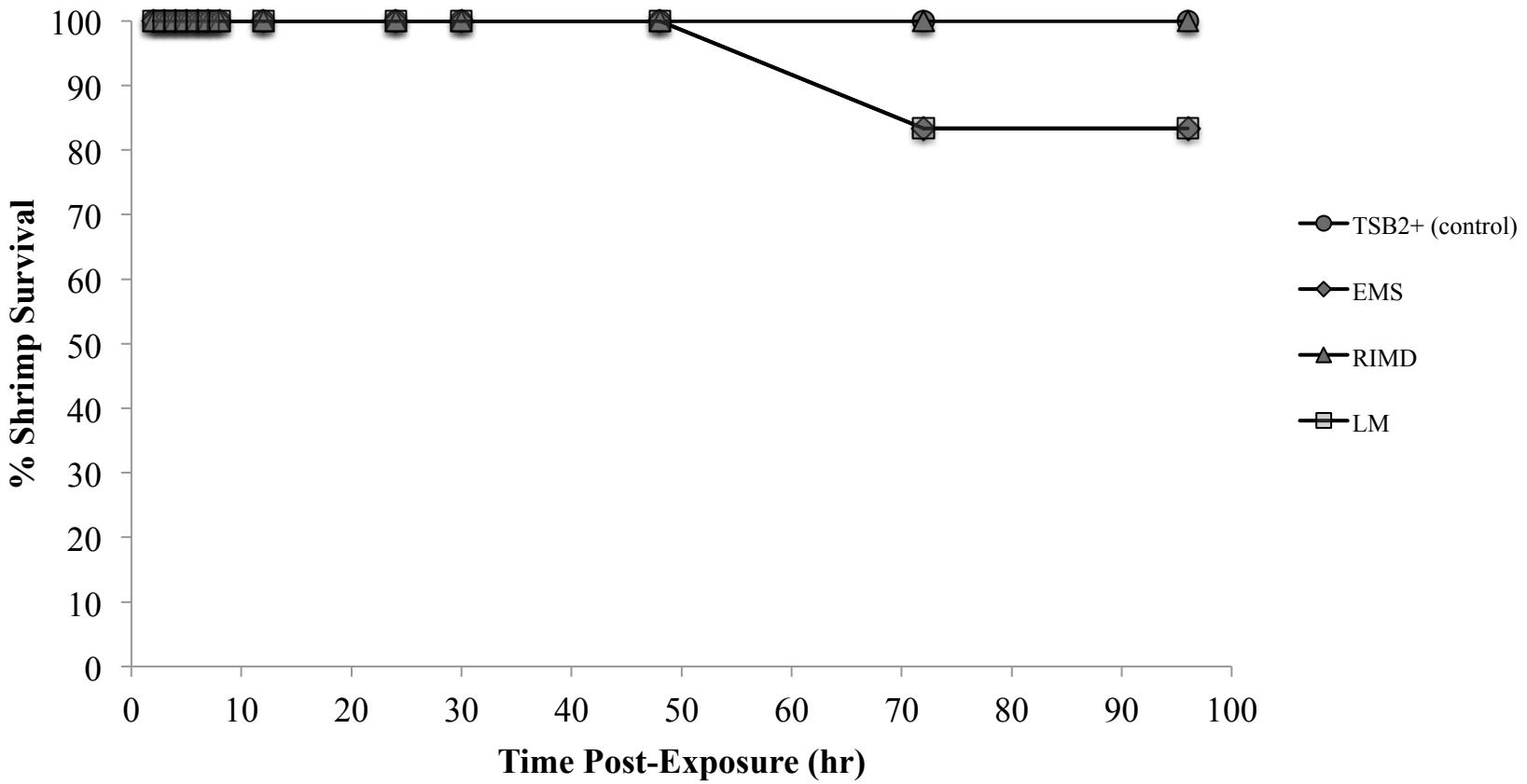

Figure 3.3. *Shrimp survival rate of Litopenaues vannamei species exposed to EMS, RIMD and LM at OD_{600}=0.5 in Trial 2.2. Concentrations of EMS, RIMD, and LM were $2.54x10^8$ CFU/mL, $6.10x10^8$ CFU/mL, and $2.00x10^8$ CFU/mL respectively. Values are % of n=6 replicate analysis. There were no significant differences in this trial.*

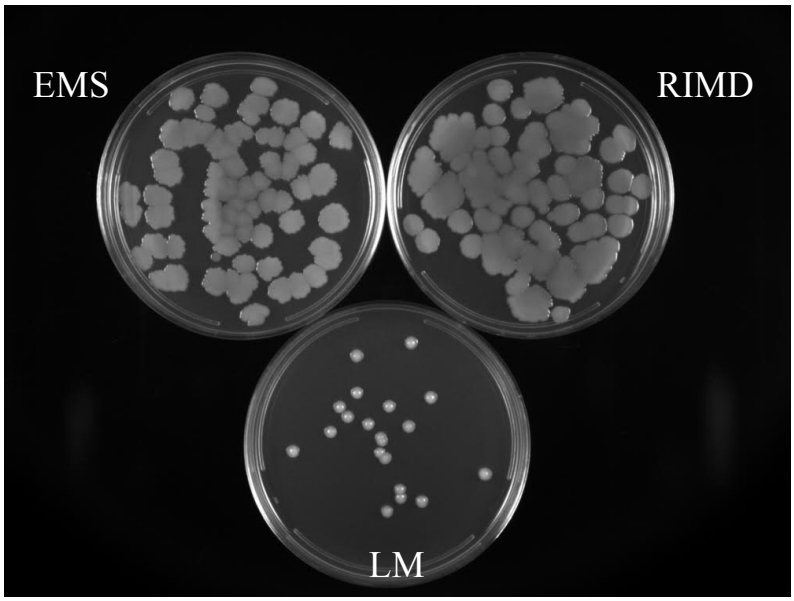

Figure 3.4. *Comparison of EMS, RIMD, and LM spread plates after 24-hour incubation at 20°C. EMS and RIMD spread plates consistently showed swarming on spread plates while LM spread plates did not.*

In Trial 2.3, RIMD and EMS strains were both grown and respectively exposed to shrimp when grown to an OD_{600}=6.0 and grown for 12-hours. LM was not used in this trial as it was an environmental strain. The human clinical isolate, RIMD, and EMS, which was a newly emerged pathogen known for causing mortality in shrimp was of higher interest in this trial. At OD_{600}=6.0, the RIMD culture had colony counts of $5.3x10^9$ CFU/mL and the EMS culture had colony counts of $3.8x10^9$ CFU/mL making it a standard concentration for comparison. Feed inoculation concentrations at OD_{600}=6.0 for RIMD and EMS were $5.3x10^8$ CFU/g and $3.8x10^8$CFU/g respectively.

EMS and RIMD were captured at OD_{600}=6.0 as they were at a higher concentration by one log compared to when they were grown to an OD_{600}=0.5 in Trial 2.2. Both strains had not yet entered the death phase as can be seen by the viability of both strains. Results for the 12-hour growth of RIMD and EMS were similar to the concentrations of the previously grown 18-hour RIMD and EMS strains in Trial 2.1. The EMS culture was more effective at growing during the 12-hour duration, achieving growth of $2.3x10^{10}$ CFU/mL compared to the RIMD culture, which was only able to achieve a growth of $1.1x10^5$ CFU/mL. Feed inoculation concentrations of EMS and RIMD respectively were $2.3x10^9$ CFU/g and $1.1x10^4$ CFU/g.

The EMS strain grown to an OD_{600}=6.0 and 12-hour duration was the most lethal in the virulence evaluation study; the 12-hour EMS-infected shrimp exhibited 100% mortality within 24 hours of feed-inoculation and EMS at OD_{600}=6.0 exhibited 100% mortality at the end of the 96-hour trial (Figure 3.5). RIMD grown to an OD_{600}=6.0 and 12-hour duration exhibited 0% mortality during the 96-hour trial. RIMD and EMS were captured at OD_{600}=6.0 as it was the optical density in which there was a higher concentration of viable cells compared to previously grown strains captured at OD_{600}=0.5. The concentrations of RIMD and EMS captured at OD_{600}=6.0 were also within the same log, which was not the case for the RIMD and EMS strains grown for 18 hours. Comparing the strains using the parameters of RIMD and EMS strains at OD_{600}=6.0 and 12-hour duration was important to see if there was an increase or absence of hemolytic production that was not seen in RIMD and EMS strains at OD_{600}=0.5. As mentioned previously, it has been shown in other studies that in other *Vibrio* spp. there was hemolysin production during the mid-exponential phase that decreased in the early stationary phase (Kim et al., 2003). Alternatively as hemolysin production decreases, protease production is initiated. Hemolysins and protease are significant virulence factors that are believed to play important

roles in pathogenicity (Iyer et al., 2000). As EMS and RIMD strains were captured at OD_{600}=6.0, the strains had just approached the lag phase. It was important to see whether possible haemolysin production or protease production had a role in causing shrimp mortality.

The RIMD strain was unable to cause mortality in shrimp at both OD_{600}=6.0 and 12-hour growth, suggesting that this strain may not be pathogenic regardless of possible hemolysin or protease production in shrimp despite the phase in which the strain was captured at. The EMS strains at OD_{600}=6.0 and 12-hour grown EMS were able to cause 0% survival suggesting that hemolysin or protease production may not be present at OD_{600}=0.5 but was present at OD_{600}=6.0 and 12-hour grown EMS. Further research is warranted using a hemolysin or protease assay to conclude whether there was hemolysin or protease production at the specified points of the strains.

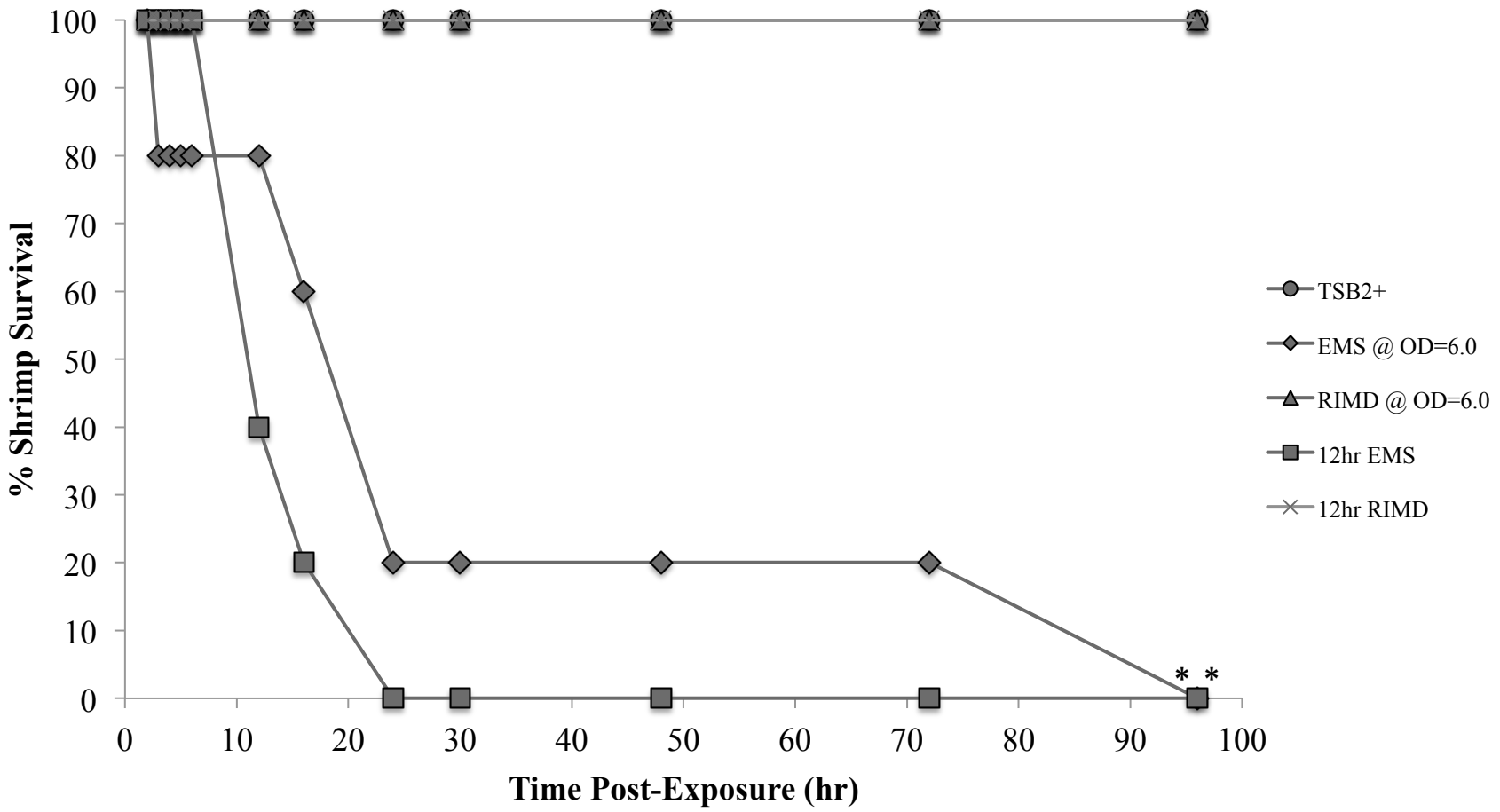

Figure 3.5. *Shrimp survival rate of Litopenaues vannamei species exposed to EMS and RIMD at different parameters (OD_{600}=6.0 vs. 12-hour growth). Values are % of n=5 replicate analysis. Survival curves* with asterisks are significantly different ($p<0.05$) from the control. P-values for significantly different treatments: 12hr EMS – 0.0015 and EMS @ OD=6.0 – 0.0021.*

3. Effects of different EMS components (filtrate only, cells only, O/N EMS (filtrate and cells)). Two replicate trials were conducted to determine the effects of EMS components on survival rates. Survival rates of shrimp subjected to various components of EMS were different. In Trial 3.1, the O/N EMS culture and cells only treatments in shrimp exhibited 0% survival within 24 hours of feed inoculation (Figure 3.6). The control shrimp group showed 83% survival and the filtrate treatment showed 100% survival at 96 hours. There were significant differences ($p<0.05$) in survival rates of shrimp subjected to O/N and cells only treatments when compared to the survival rate of the control. The filtrate only treatment did not have a significant difference in shrimp survival rate when compared to the control survival rate. Results from this trial indicated that only cells and not filtrate was capable of causing shrimp mortality, which indicated that there was no extracellular toxin and that the infectious dose correlated to the EMS virulence. Spread plate counts of the cells only treatment culture was $1.8x10^{10}$ CFU/mL Estimated Standard Plate Count (ESPC) and O/N culture was $3.0x10^{9}$ CFU/mL ESPC. There were no growth on the TSA2+ spread plates for the negative control and filtrate only treatments. Feed inoculation concentrations of cells only treatment and O/N cultures were respectively $1.8x10^{9}$CFU/g and $3.0x10^{8}$CFU/g. Overdilution of the sample during the spread plating procedure showed CFU/mL below the countable range of 25-250 CFU/mL. Therefore, ESPC procedures were used to quantify concentration. In Trial 3.2, standard plate counts (SPC) were used because the proper dilution factor for spread plating was utilized.

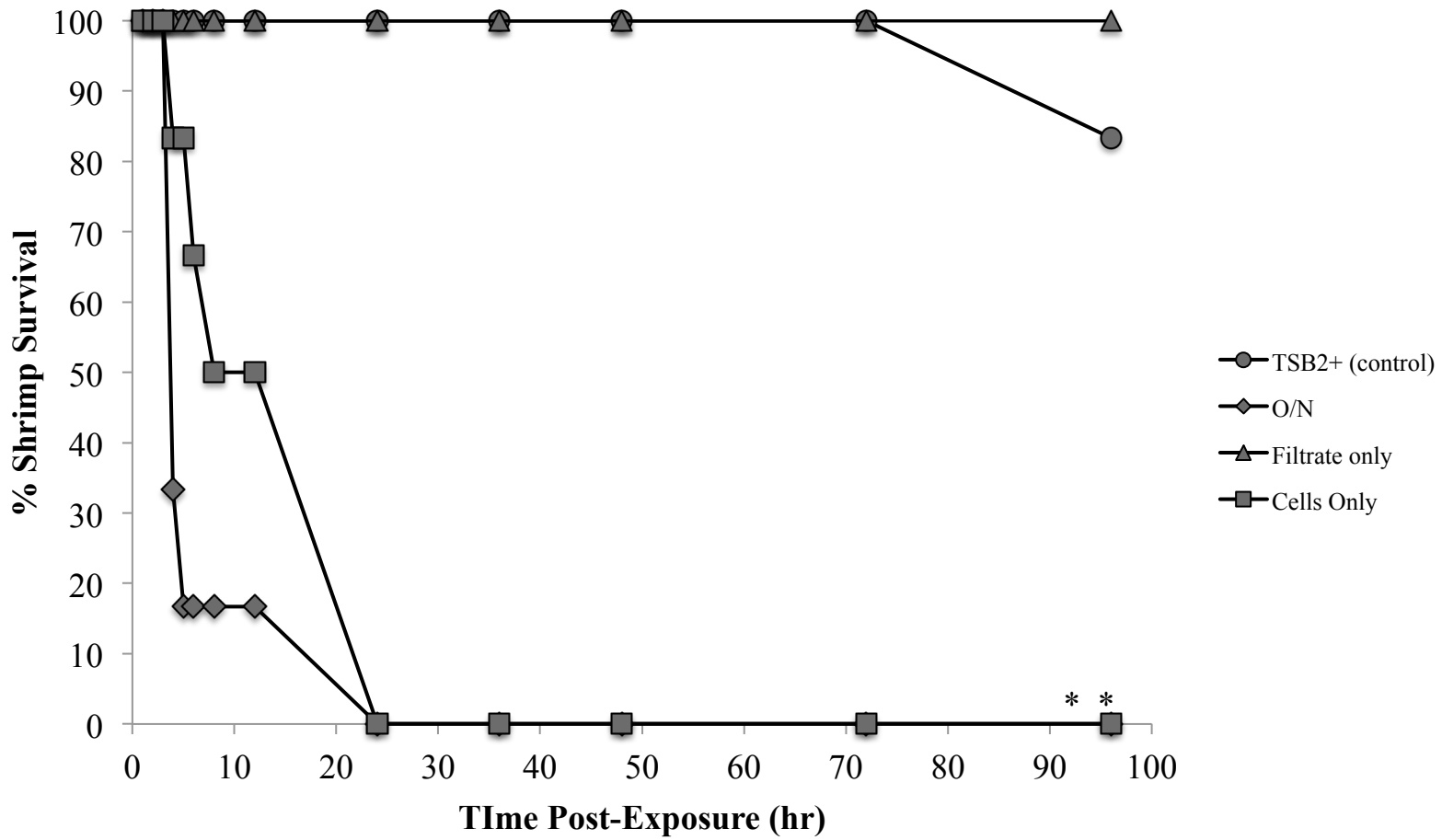

Figure 3.6. *Shrimp survival rate of Litopenaues vannamei species after exposure to overnight grown EMS, filtrate only, and cells only in Trial 3.1. Values are % of n=6 replicate analyses. Survival curves* with asterisks are significantly different ($p<0.05$) from the control. P-values for significantly different treatments are as follows: cells only – 0.0011 & O/N – 0.0004.*

In Trial 3.2, the O/N and cells only treatment exhibited 0% survival within 24 hours of feed inoculation (Figure 3.7). The filtrate only and control groups both showed 66.7% survival. There were significant differences ($p<0.05$) in survival rate of the O/N and cells only treatments when compared to the survival rate of the control. There was no significant difference in survival rate of the filtrate only and survival rate of the control group. Spread plate counts of the cells only broth culture was $4.8x10^9$ CFU/mL and O/N EMS broth culture exhibited growth of $3.6x10^9$ CFU/mL, while there was no growth on the TSA2+ spread plates for the negative control and filtrate only treatments. Feed inoculation concentration of cells only was $4.8x10^8$ CFU/g and O/N EMS was $3.6x10^8$ CFU/g.

Trial 3.1 and 3.2 survival rate results for O/N EMS were consistent with results from Trial 2.1 and Nunan et al.'s (2014) feed inoculation studies. In all previously mentioned trials, feed-inoculation with 18-hour grown EMS resulted in 100% mortality within 72-hours (Nunan et al., 2014). However survival rates for the filtrate only group in Trials 3.1 and 3.2 did not correlate with previous inoculation studies using reverse gavage treatment of the EMS filtrate. Shrimp that were reverse gavaged with filtrate exhibited 100% mortality after 72-hours in previous studies (Tran et al., 2013). The Tran et al.'s (2013) study concludes that the lethality of the EMS filtrate indicate the possibility of a filterable toxin as there were characteristic tubule lesions in the shrimp hepatopancreas upon histological examination. Discrepancies between Trials 3.1 and 3.2 results and Tran et. al's (2013) study may be attributed to the significance of filter sterilization. While the filtrate in Trials 3.1 and 3.2 were filter sterilized, it was unclear as to whether the filtrate in Tran et. al's (2013) study was. Results from Trials 3.1 and 3.2 indicate that only cells and not filtrate was capable of causing shrimp mortality.

Histology was performed on shrimp that were sampled to confirm the presence of EMS disease in the shrimp hepatopancreas in Trial 3.2 (Figure 3.8). Shrimp from the control were sacrificed at the end of the trial and submitted for histological examination. Histology slides from Trial A were congruent with histological findings from the Nunan et al.'s (2014) study. Tubule degeneration indicated by the sloughing of epithelial cells into the lumen and hemocytic infiltration was visible in both histological examinations in Nunan et al.'s (2013) study and Trial 3.2. Hemocytic infiltration increased due to the presence of EMS as hemocytes are able to detect invading diseases using pathogen-associated molecular patterns (PAMPs) (Rowley and Pope, 2012). Hemocytes respond to diseases by using phagocytosis and later on discharging

antimicrobial peptides (AMPs) such as penaidins and crustins, lysozymes and lectins. The disappearance of B cells was also apparent in histology of shrimp exposed to EMS. This indicates that there was most likely a disruption in digestion as B-cells are primary producers of digestive enzymes (Felgenhauer, 1992).

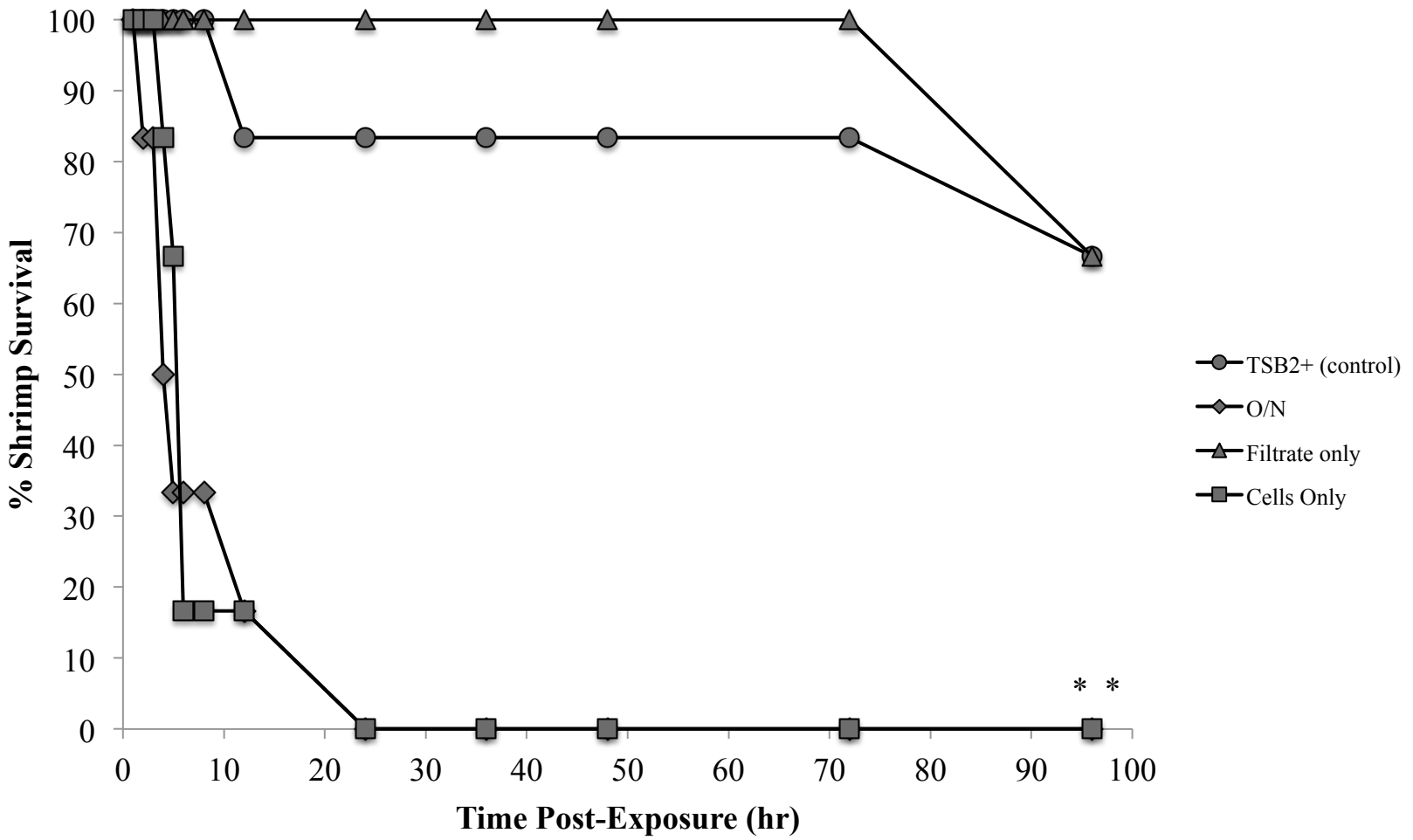

Figure 3.7. *Shrimp survival rate of Litopenaues vannamei species after exposure to overnight grown EMS, filtrate only, and cells only in Trial 3.2. Values are % of n=6 replicate analyses. Survival curves* with asterisks are significantly different (p<0.05) from the control. P-values for significantly different treatments are as follows: cells only – 0.0021 & O/N – 0.0022.*

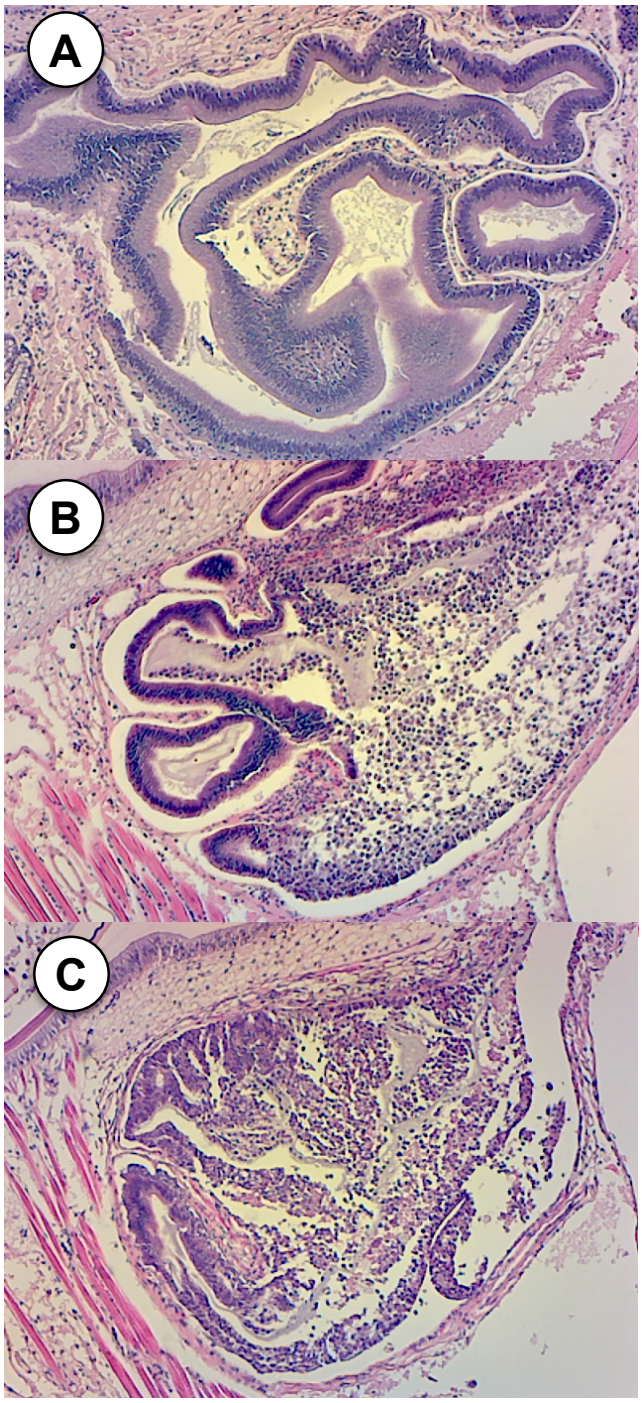

Figure 3.8. *Representative histological results from the feed inoculation bioassay revealed pathogenicity of EMS obtained from the midgut ceca. Litopenaues vannamei shrimp were used. (A) Normal appearance of the hepatopancreas. Control shrimp was sacrificed for histological comparison. (B) Early Mortality Syndrome apparent in shrimp fed feed inoculated with overnight EMS (containing cells and filtrate). (C) Early Mortality Syndrome apparent in shrimp fed EMS cells only inoculated feed.*

4. Dilution Series. In order to develop a disease challenge model for future studies, a dilution series of the O/N EMS culture was created to find the dilution that would cause chronic virulence. Two trials were conducted using feed inoculations of O/N EMS (10^9 CFU/mL), 1/10 EMS (10^8 CFU/mL), 1/100 EMS (10^7 CFU/mL), and 1/1000 EMS (10^6 CFU/mL).

In Trial 4.1, spread plate counts of the undiluted EMS broth culture exhibited growth of $4.6x10^9$ CFU/mL. Bacterial doses of EMS broth dilution series therefore ranged from 10^6 to 10^9 CFU/mL and after the broth application onto feed, feed concentrations ranged from 10^5 to 10^8 CFU/g. Shrimp that were fed feed inoculations with 10^9 CFU/mL exhibited 0% survival, 10^8 CFU/mL exhibited 20% survival, 10^7 CFU/mL exhibited 60% survival and 10^6 CFU/mL exhibited 80% survival (Figure 3.9). The control group exhibited 100% survival. Significant differences ($p<0.05$) in survival rate were seen in shrimp that were fed feed inoculations using 10^9 CFU/mL and 10^8 CFU/mL when compared to the survival rate of the control group. There were no significant differences in survival rates in shrimp that were fed feed inoculated with 10^7 CFU/mL and 10^6 CFU/mL when compared to the survival rate of the control group. It can be concluded from these results that a broth culture concentration of 10^8 CFU/mL was necessary in order to achieve chronic virulence that exhibited significantly different survival compared to the control.

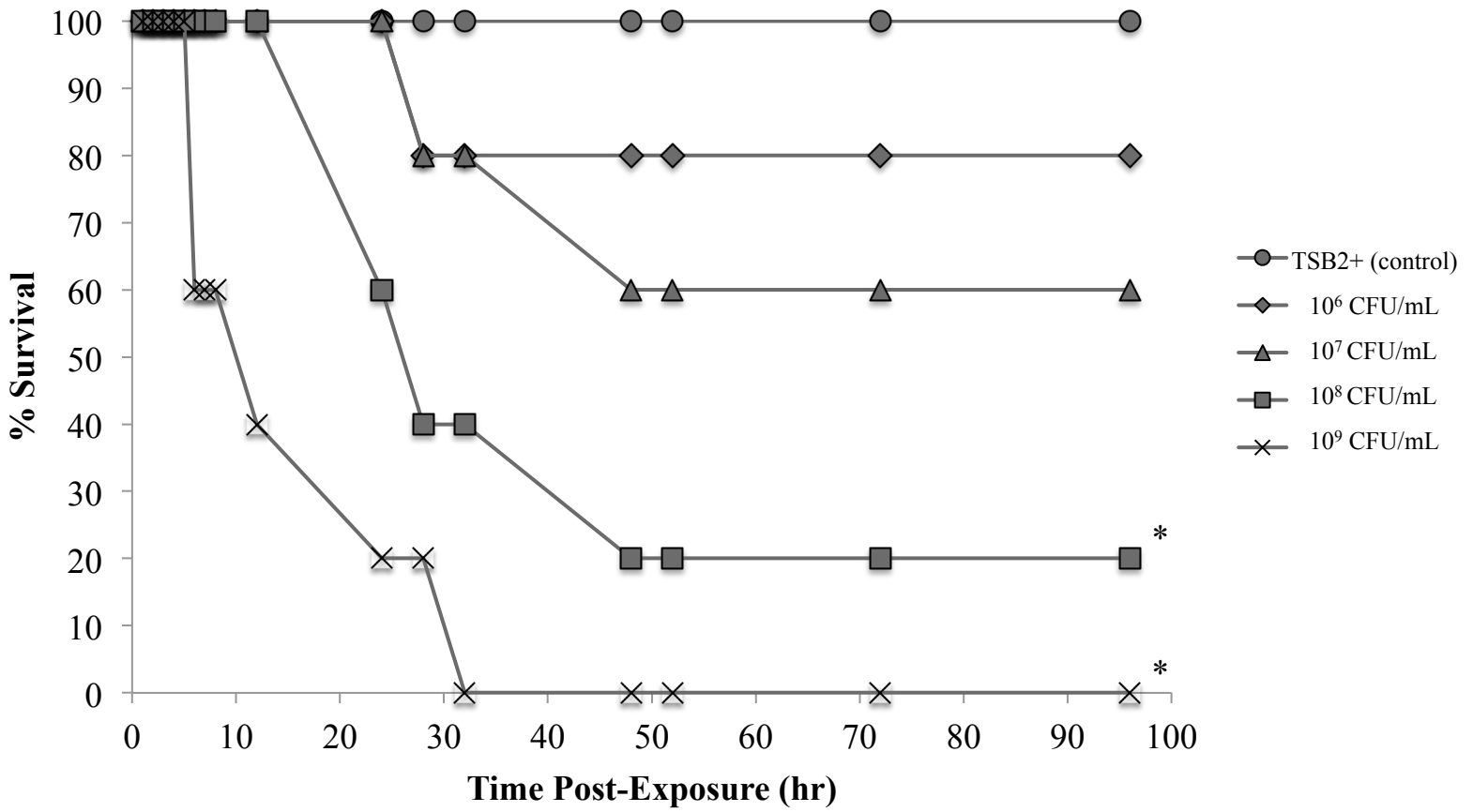

Figure 3.9. *Infectious dose evaluation of 18-hour EMS (10^6, 10^7, 10^8, 10^9 CFU/mL) in Trial 4.1. Values are % of n=5 replicate analyses. Survival curves* with asterisks are significantly different ($p<0.05$) from the control. P-values for significantly different treatments are as follows: 10^9 CFU/mL – 0.0017 & 10^8 CFU/mL – 0.0128.*

In Trial 4.2, spread plate counts of the undiluted EMS broth culture exhibited growth of $3.46x10^9$ CFU/mL. Bacterial doses of EMS broth cultures therefore ranged from 10^6 to 10^9 CFU/mL and after the broth application onto feed, feed concentrations ranged from 10^5 to 10^8 CFU/g. Shrimp that were fed feed inoculations with 10^9 CFU/mL exhibited 0% survival, 10^8 CFU/mL exhibited 20% survival, and 10^7 CFU/mL exhibited 70% survival (Figure 3.10). The 10^6 CFU/mL and control group exhibited 100% survival. Significant differences ($p<0.05$) in survival rate were seen in shrimp that were fed feed inoculations using 10^9 CFU/mL and 10^8 CFU/mL groups when compared to the survival rate of the control group. It can be concluded from these results that a broth culture concentration of 10^8CFU/mL was necessary in order to achieve chronic virulence that exhibited significantly different survival compared to the control.

Results from Trial 4.1 and 4.2 showed that the more concentrated EMS cultures correlated with increased survival rates in both trials. The number of bacterial cells was able to cause mortality. The data shows that a later LD_{50} was experienced in shrimp that were fed feed with broth culture concentration 10^8 CFU/mL compared to 10^9 CFU/mL.

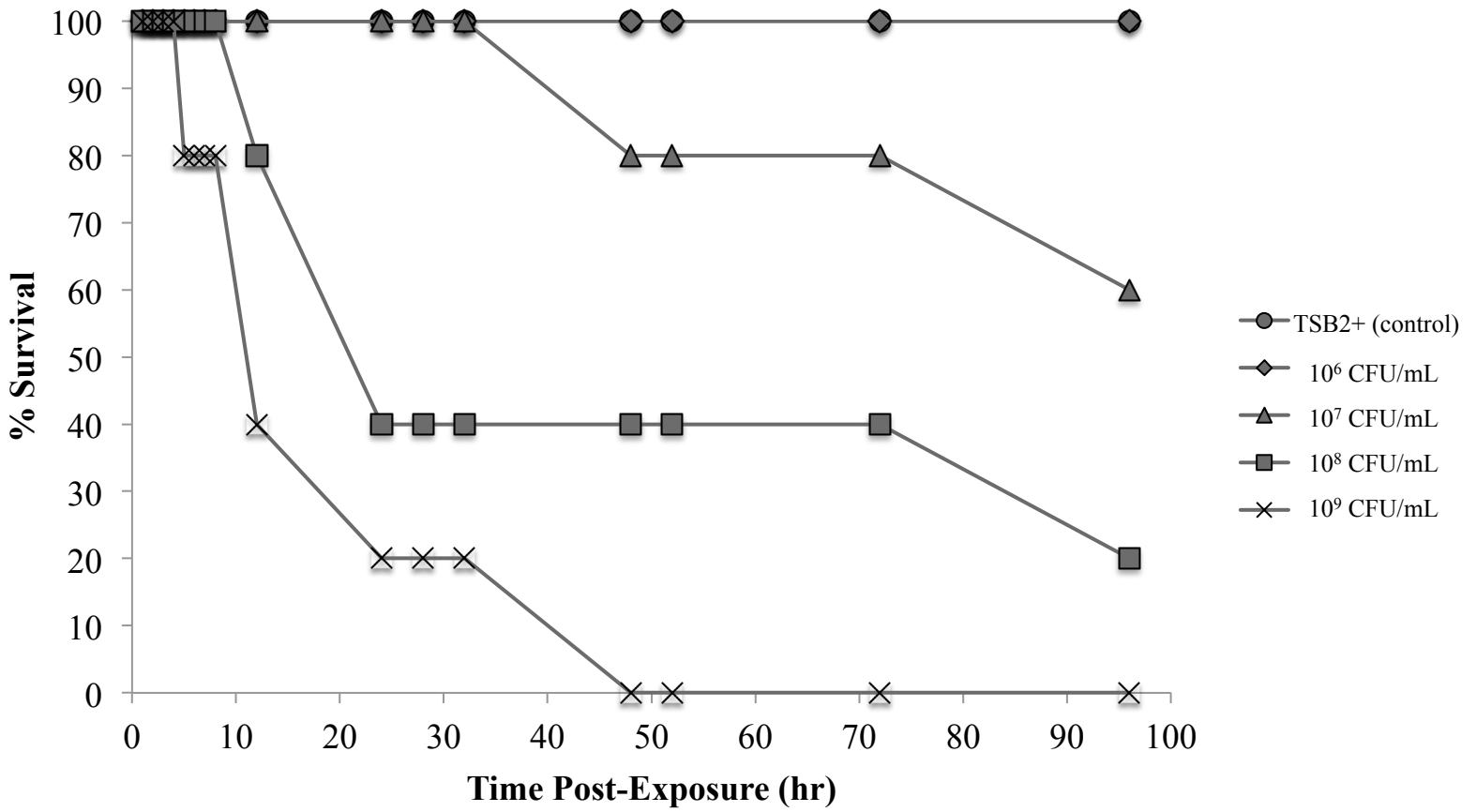

Figure 3.10. *Infectious dose evaluation of 18-hour EMS (10^6, 10^7, 10^8, 10^9 CFU/mL) in Trial 4.2. Values are % of n=5 replicate analyses. Survival curves* with asterisks are significantly different ($p<0.05$) from the control. P-values for significantly different treatments are as follows: 10^9 CFU/mL – 0.0018 & 10^8 CFU/mL – 0.0135.*

Conclusions

The objective of this study was to evaluate three different *V. parahaemolyticus* strains and their ability to cause significantly different mortality in shrimp species through a virulence evaluation. The feed inoculation method employed in the study was successful in causing mortalities in shrimp subjects thus making it an appropriate method to be used for the challenge studies. The EMS at OD_{600}=6.0, 12-hour grown EMS, and 18-hour grown EMS were capable of causing significantly different survival than the control survival rate. Survival rates of EMS, RIMD, and LM strains at OD_{600}=0.5, RIMD at OD_{600}=6.0, 12-hour grown RIMD, and 18-hour grown RIMD and LM strains were not significantly different from the control survival rate. Although previous studies have used the EMS filtrate to cause death in shrimp subjects, the filtrate in this study was not capable of causing death. The number of bacterial cells, not filtrate, was able to cause mortality. Light microscopy of samples collected of shrimp killed by EMS revealed that there was a lack of central hepatopancreatic B-cells and massive sloughing of central tubule epithelial cells compared to shrimp not exposed to EMS. These results strongly suggest a degenerative pathology. The dilution series of the 18-hour EMS strain was evaluated and demonstrated there were significant differences in survival rate of shrimp that were fed feed inoculated with 10^8 CFU/mL and 10^9 CFU/mL EMS strains compared to the survival rate of the control. The broth culture concentration 10^8 CFU/mL use to inoculate feed was capable of causing chronic virulence as defined in this study. Therefore, the two objectives of this chapter were successfully achieved.

Further research is warranted to determine the carbon sources that EMS utilizes that contribute to its diauxic growth and therefore its ability to proliferate for a longer duration compared to the RIMD and LM strains.

Chapter 4:

Probiotic Studies

Introduction

Probiotics as a prophylactic method has gained popularity, as it is an alternative method for antibiotic use and has demonstrated many rewarding benefits when utilized. The term probiotics has been redefined over the years and can be summarized as a viable microbial feed supplement that upon ingestion, can provide beneficial health effects by improving the intestinal microbiota through biochemical and physiological effects (Aly et al., 2008; Farzanfar, 2006). Probiotic use can also aid in reducing economic losses due to disease (Hong and Cutting, 2005). Estimated use of antibiotics in aquaculture species in the mid 1990s fell within a range of 204,438 and 433,397 pounds (Benbrook, 2002). Shifting from antibiotic use to probiotic use can save the aquaculture industry on funds that can be allocated elsewhere. Probiotics are able to enhance the immune system through the reduction of pathogenic bacterium colonization by competitive adhesion that hinder colonization of the gastrointestinal tract by a pathogen and the production of antimicrobial substances such as bacteriocins, bacteriocin-like inhibitory substances, and antibiotics (Casula, Cutting, 2002; Hong, Cutting, 2005). The growing expansion of shrimp production necessitates research on probiotics as a viable solution to counter disease resistance. Probiotics as a method of disease control is beneficial for shrimp production because they can improve overall gut health, hinder pathogenic bacteria from colonizing and increase desirable and marketable shrimp characteristics (Lakshmi et al., 2013; Mohapatra et al., 2013; Wang et al., 2012).

Early Mortality Syndrome (EMS) also known as acute hepatopancreatic necrosis disease (AHPND) is caused by a *V. parahaemolyticus* strain that is thought to have initially emerged in 2009 and has gained much attention due its high effectiveness in causing mortality in shrimp upon exposure (FAO, 2014). EMS has impacted shrimp culture in Southeast Asian countries such as Thailand, China, Vietnam, and Malaysia and has recently spread to northern states of Mexico such as Nayarit, Sinaloa, and Sonora (Nunan et al., 2014). The Global Aquaculture Alliance estimates $1 billion have been lost in production due to EMS (FAO, 2014). As shrimp capture production numbers continually increase, there is a much needed focus on sustainable shrimp aquaculture (Bondad-Reantaso et al., 2012). The mortality-causing infectivity of the EMS strain is of great concern and much attention is warranted if efficient shrimp production is to continue under intensive aquaculture systems.

Although probiotic use has increased in popularity, there are no studies that have investigated these claims in regards to promoting disease resistance against Early Mortality Syndrome (EMS). Few studies have also sought to evaluate the lethality of the EMS strain. In this study, a shrimp model was used in which shrimp were fed probiotic coated pelleted feed for one week prior to experimentation and shrimp that were fed non-treated probiotic feed. Survival rates of probiotic-fed shrimp were compared to survival rates of the negative control (non-probiotic treated feed and no EMS-exposure) and positive control (non-probiotic treated feed only).

The *objective* of this study was to determine if probiotic diets reduce EMS colonization in the shrimp hepatopancreas and increase shrimp survival rate.

It was hypothesized that daily feeding of probiotic diets will significantly increase survival rate of probiotic-fed shrimp compared to non probiotic-fed shrimp after exposure to the EMS causing *V. parahaemolyticus* strain.

Materials and Methods

General Methods: *Tank setup*

Eighteen 80-liter tanks were filled with 40-liters of 25 parts per thousand (ppt) synthetic sea salt (Crystal Sea Marine Mix, Marine Enterprises International, Baltimore, MD, USA) constituted with distilled water. After the addition of synthetic sea salt, 1-liter airlift biofilters stocked with nitrifier-established media (KMT K1) were stocked into each of the tanks. Each tank had its own filtration system. Pressurized air supply was filtered using a 1μm disc filter and equally distributed to all tank airlift filters for simultaneous provision of atmospheric oxygen, carbon dioxide purging, and biofiltration of nitrogenous wastes. Propylene lids were constructed to form fit tanks, with holes to provide positive and negative pressure air supplies. Nalgene tubing was run between the air supply manifold and airlift filters, as well as between the tank and 0.45μm filtered vacuum source. This air-evacuation system provided an extra layer of safety from aerosolization and maintenance of water quality by exchanging atmospheric air. All tanks were set up in a biosafety level 2 (BSL2) room. Evaporated tank water was replaced daily.

General Methods: *Weighing of Shrimp*

Shrimp were weighed prior to each experiment to determine the approximate amount of feed to inoculate. A negative method was utilized in order to minimize weighing errors due to water on the surface of the shrimp. Shrimp were placed into a plastic container with a paper towel lining the interior and an additional paper towel placed on top of the container in order to prevent shrimp from escaping. The plastic container and shrimp were then placed onto the scale and tared. The shrimp was then placed into the holding tanks while making sure the paper towels were kept from coming into contact with any surfaces. Afterwards, the container and paper towels were placed backed onto the scale to record the negative weight.

General Methods: *Growth of V. parahaemolyticus causing EMS strain*

Starter freezer EMS cultures were preserved by placing 1 mL of EMS strain grown to an OD_{600} = 0.5 into glycerol and frozen at −80°C. At the start of each trial, a starter EMS culture (OD_{600} = 0.5) was placed into 30mL of Trypticase Soy Broth supplemented with an additional 2% NaCl (TSB2+) making the starting OD_{600} ~0.02. The 2% NaCl addition to Trypticase Soy Broth made

the overall salinity 25 parts per thousand (ppt), which matched the salinity of the water in the experimental tanks used throughout this study. The starter EMS culture with TSB2+ was placed into a shaking incubator for 18 hours. The incubator was set to agitate at 250 rpm at 30°C. EMS strain concentrations grown throughout the treatment were measured using spread plate counts.

General Methods: *Spread plate method*

The plating medium used was Trypticase Soy Agar (Difco) with additional 2% NaCl (TSA2+). Similar to TSB2+, Trypticase Soy Agar was supplemented with an extra 2% NaCl, which made the overall salinity 25 ppt to match the salinity of the water in the experimental beakers used throughout the study. All spread plates were made in duplicate and used methods adapted from Buck and Cleverdon's study (1960). TSA2+ medium was tempered at 45°C and approximately 15 mL of the agar was poured into petri plates. Dilutions of EMS strains were made so that ejected 0.1 mL onto the agar surface resulted in the proper dilution. A 5 mm glass rod was then used to spread dilutions evenly over the surface of the agar using a circular motion. The glass rod was flame sterilized in between dilutions.

Spread plates were left at room temperature at 20°C overnight and counted 24 hours after inoculation. An incubator was not used to store spread plates because incubation at 30°C in preliminary trials resulted in swarming on the EMS spread plates, making it difficult to quantify CFU/mL.

General Methods: *Probiotic Feed Application*

Eleven different *Bacillus subtilis* strains of probiotic spores were supplied by Novozymes Biological Inc. (Salem, VA). Due to confidentiality agreements, probiotic strains were named under a different alias in this chapter. Spores were applied to dry feed pellets by low pressure air spraying. Feed was weighed and placed in a stainless steel countertop stand mixer bowl and assembled to the mixer with a flat beater attached (KitchenAid, Benton Harbor, MI, USA). Spores were mixed with sterile Butterfield's phosphate buffered water (PBW) at a ratio of 1:1 volume per weight (v/w) of spore solution to feed to achieve $1x10^7$ spores per gram of dry feed. The feed was mixed continuously at the lowest setting concurrently with continuous direct spraying of the spore solution at 25 liters per minute (lpm) with a siphon-fed airbrush (Paasche

H3, Paasche Airbush, Chicago, IL, USA). Following complete expenditure of the spore solution, 10 mL of non-inoculated phosphate-buffered saline (PBS) was sprayed onto the feed to flush any residual spores from the airbrush. Feed was then placed into a food-safe high-density polyethylene container and spread to a layer no deeper than 5 cm. This container was then stored unsealed in a refrigerator at 4°C for 24 hours.

General Methods: *Feeding Regime during Trial 1, 2, and 3*

Treatments were introduced to each test group via feed inoculation. Feed was premeasured to be 3% of the total average shrimp weight in grams. Treatments were applied at a 1:1 ratio of inoculation in milliliters to weight of feed in grams. Non-probiotic fed shrimp were fed non-treated probiotic inoculated feed while probiotic fed shrimp were fed probiotic inoculated feed (Ziegler Bros. Inc., Gardners, Pennsylvania, US). One spoon was allocated to each tank and each of the treatments was directly applied to feed on a spoon.

After treatments were applied to the feed, a 3-minute absorption period followed to ensure the treatments were completely absorbed by the feed. To guarantee that all of the treatments were delivered to each shrimp, the entire spoon was dipped into the tanks along with the treatment applied so that unabsorbed EMS application was still introduced to the shrimp. Shrimp were fed inoculated feed every 24 hours using this feeding regime during the 96-hour exposure trial. Prior to each feeding, solid waste was suctioned out of the water to reduce nitrogenous waste that may contribute to poor water conditions. Treatments were randomly assigned to each tank.

General Methods: *Davidson's fixative*

Davidson's fixative was prepared under a chemical fume hood and contained the following ingredients: 95% ethyl alcohol, 100% formalin (saturated aqueous solution of formaldehyde gas, 37-39% solution), glacial acetic acid, and distilled water. Davidson's fixative was stored at room temperature. Utilization of the fixative preserved tissues and stopped autolysis of the highly metabolic activities of the hepatopancreas. The hematoxylin and eosin (H&E) stain was used to stain tissue samples previously preserved in Davidson's fixative (Fischer et al., 2008).

General Methods: *Statistics*

The Kaplan-Meier estimate was used for survival analysis to measure the portion of subjects living for a certain amount of time after feed inoculation (Goel et al., 2010). The estimate calculated the probability of occurrence of an event at a specific time point and multiplying successive probabilities by previously calculated probabilities to get a final estimate. Any shrimp that jumped out of the beaker during the 96-hour trials were labeled as right-censored observations. Right-censored shrimp provided partial survival information because it was known that death occurred or will occur sometime after the incident causing the subject to drop out of the study. The log-rank test was used as a post-hoc test to differentiate whether survival times between two groups were significantly different ($p<0.05$). The log-rank test assumed that the hazard ratio was constant over time. The hazard ratio was the fraction of the risk of hazard occurring at a specific time point in one treatment compared to another treatment at the same time point.

Germination Trial. Two treatment groups (n=35 shrimp/tank; shrimp mean weight=12.5 grams were used: feed with no *B. subtilis* probiotic spores and feed with *B. subtilis* probiotic spores. Each treatment group provided three replicate tanks and shrimp were fed 6.5 grams once a day. A composite sample consisted of a sample of guts from 10 shrimp. Two composite gut samples were collected prior to the experiment and three composite gut samples were collected from each treatment on Day 7 and 14 of the experiment. Prior to the germination evaluation, shrimp were fed a standard shrimp diet.

At Day 7 and 14, shrimp guts were extracted and placed into sterile containers and frozen in a standard freezer at −80°C. Samples were then sent to Novozymes (Salem, VA) where composite samples were weighed out and placed into a 15 mL falcon tube. A total volume of 1.5 mL using phosphate buffer was placed into each tube taking into consideration the weight of the guts.

Gut samples with phosphate buffer were homogenized using a Qiagen TissueRuptor system (approximately 0.20 to 0.35 grams of guts per composite sample). DNA was recovered using both the MoBio PowerLyzer system (MoBio Laboratories, Inc., Carlsbad, CA, USA) to quantify

spores and vegetative cells and the MoBio PowerSoil system (MoBio Laboratories, Inc., Carlsbad, CA, USA) to quantify vegetative cells only. Quantitative polymerase chain reaction (qPCR) was performed using a unique-strain primer/probe method for SB3086.

Duplicates of each composite sample were run and qPCR for 16s rRNA was run on the extractants. Operating conditions for the qPCR run were as follows: 1 cycle of preincubation, 36 cycles of amplification, and 1 cycle of cooling. The annealing temperature was set at 60°C and the denaturing temperature was set at 95°C.

SB3086 primer probe sets used for analysis on the Roche LightCycler 480 were as follows:

- SB3086 Probe (P2)
  - 5' [FAM] AAGGTCGAAGTTGAGGCAAA[BHQ1a~6FAM] 3'
- SB3086 Reverse Primer (R2)
  - 5' GCTAACTCTGCAGGTACCCC 3'
- SB3086 Forward Primer (L2)
  - 5' CTGTTCTCATGAACTGGGGC 3'

Trial 1. Six treatment groups (n=5 shrimp/tank; average shrimp weight = 2.69±.08 grams) were used: non-probiotic treated control (NTC), strain A, strain B, strain C, strain I, and strain J. Probiotic strains in this treatment were strains A, B, C, I, and J. Tanks were administered graduated doses of EMS applied to feed. For each treatment, the first tank was inoculated with undiluted EMS, the second tank was inoculated with 1/10 diluted EMS (1/10 EMS), and the third tank was inoculated with 1/100 diluted EMS culture (1/100 EMS). Dilutions were made with TSB2+ media. The trial was carried out for 96 hours.

Trial 2. Nine treatment groups (n=10 shrimp/tank; average shrimp weight = 1.19±0.16 grams) were used: non-probiotic treated control (NTC), strain A, strain B, strain C, strain D, strain E, strain F, strain G, and strain H. Each treatment group provided two replicate tanks. Tanks were administered undiluted EMS through feed inoculation. The trial was carried out for 96 hours.

Histology samples were preserved during this trial by obtaining shrimp within two hours of mortality or when they were moribound. Moribound shrimp were classified as shrimp that did not respond to external stimuli but still exhibited gill movement. Histology was also performed

on probiotic-fed shrimp that were not inoculated with EMS to determine the effects of probiotics on the gut tract. Histology was performed on shrimp using methods presented in Bell and Lightner (1988). Shrimp were cut between the carapace and abdominal segment I and immersed in Davidson's fixative for a minimum of 48 hours prior to sectioning, slide preparation, and microscopy.

Histology slides were viewed using a light microscope. Microscopy slides obtained were longitudinal as they were sectioned from one-half of a longitudinally cut thorax, which was orientated so that the inside of the shrimp would be sectioned along the long axis of the thorax. A scoring system to rate the histology using a 3-point scale was developed (1= normal cells, 2= moderately diseased cells, 3= severely diseased cells). Grading was based on the appearance of sloughing of central tubules and hemocytic infiltration. These methods were adapted from Bell and Lightner (1988).

Trial 3. Six treatment groups (n=9 shrimp/tank; average shrimp weight = 2.97±0.20 grams) were used: non-probiotic treated control (NTC) with no EMS exposure (negative control), NTC (positive control), strain A, strain B, strain C, and strain A/B blend together. Each treatment group provided three replicate tanks. The A/B blend was constituted with a 50/50 blend of probiotic strain A and B mixed together. All tanks were administered undiluted EMS except for the non-treated probiotic control that served as negative control. The negative control served as a measurement to ensure that shrimp were in good health throughout the trial and water quality was not an issue. The trial was carried out for 96 hours.

Results and Discussion

Germination Trial. The qPCR results of the composite shrimpgut samples of shrimp that were fed diet with probiotic spores collected on Day 7 provided $7.32x10^4$ CFU/g vegetative cells, $2.68x10^4$ CFU/g spores, making the total cell count (total = spore + vegetative cells) $1x10^5$ CFU/g (Table 4.1, Figure 4.1). On Day 14, qPCR results of the diet with probiotic spores provided $3.66x10^4$ CFU/g vegetative cells, $3.66x10^4$ CFU/g spores, making the total cell count $7.32x10^4$ CFU/g in composite samples collected on Day 14. In summary, the diet with probiotic spores contained 73.2% vegetative cells in composite samples on Day 7 while there were 50% vegetative cells in composite samples compared to total counts at that specific time point. There were no probiotic spores or vegetative cell counts in the composite samples of the control diet and in the prefeed samples.

The qPCR results demonstrated that probiotic spores were able to go through the process of germination once they were ingested by shrimp. Once a spore is ingested, the spore can go through the process of germination, reproduction, and resporulation once nutrients are depleted (Hong and Cutting, 2005). Consumed spores are robust and able to survive the intestinal enzymes and acidic environment of the stomach. Spores can be induced out of their dormant state because the small intestine provides an environment that is rich in nutrients. The *Bacillus* spp. spores are then able to germinate and proliferate and re-sporulate in the gastrointestinal tract. The gastrointestinal tract is an optimal growth environment for many bacteria because it is nutrient-rich, however, probiotic colonization in the tract can be affected by a variety of host-related factors and microbe-related factors (Balcázar et al., 2006; Wang et al., 2008). Host-related factors can include parameters such as body temperature, redox potential levels, enzymes, and genetic resistance and microbe-related factors can include the effects of antagonistic microorganisms, proteases, and bacteriocins (Balcázar et al., 2006).

In the preliminary study, feeding shrimp for seven days was sufficient for spore germination in the gut. The comparison of Day 7 and 14 composite samples indicate that feeding shrimp for 7 days actually provided slightly higher vegetative cell counts than for 14 days. Thus for the following trials (Trial 1, 2, and 3), shrimp were fed for seven days prior to experimentation.

Table 4.1. *Vegetative and total concentrations of shrimp composite samples collected on Day 0, 7, and 14 using qPCR analysis.*

Diet	Day	Concentration (CFU/g)
Vegetative Concentrations		
Prefeed	0	0
Control	7	0
	14	0
Diet w/ Spores	7	7.32E+04
	14	3.66E+04
Total Concentrations		
Prefeed	0	0
Control	7	0
	14	0
Diet w/ Spores	7	1.10E+05
	14	7.32E+04

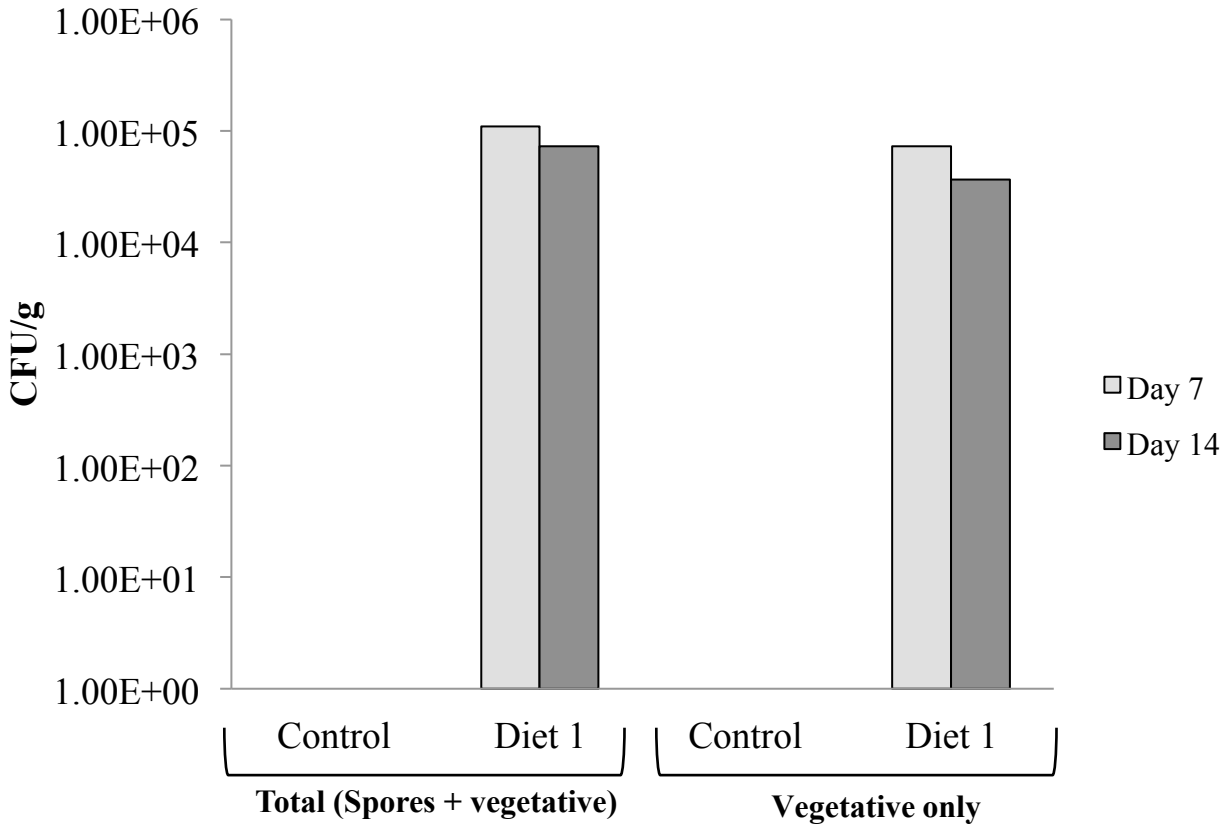

Figure 4.1. *Comparison of total count (spores + vegetative) vs. vegetative cells only in Day 7 and 14 shrimp samples from germination trial. Concentration values are based on the standard curve derived from concentration vs. amplicon values. n=3 replicate analyses from the preliminary trial.*

Trial 1. Five different *B. subtilis* probiotic strains using an EMS dilution series (undiluted EMS, 1/10 EMS, and 1/100 EMS) were evaluated. Spread plates results of undiluted EMS concentrations used to inoculate feed daily were $1.88x10^9$ CFU/mL (Table 4.2). Bacterial populations of diluted EMS broth cultures therefore ranged from 10^6 to 10^9 CFU/mL and after the broth application onto feed, feed concentrations ranged from 10^5 to 10^8 CFU/g.

The following comparisons were for shrimp exposed to 10^9 CFU/mL EMS after 96-hours. Shrimp that were fed the probiotic strain B diet had the highest shrimp survival rate of 80% (Figure 4.2). Shrimp fed the probiotic strain A diet exhibited the second highest survival rate of 40% followed by shrimp fed probiotic strain I diet with 20% survival. Shrimp fed NTC, probiotic strain C, and probiotic strain J diets exhibited 0% survival. A significant difference ($p<0.05$) in survival rate was seen in shrimp fed probiotic strain A ($p=0.0889$) and B diets ($p=0.0203$) when compared to survival rate of shrimp fed the NTC diet after 96-hours. Shrimp survival rates improved by 80% in shrimp fed probiotic diet B and 40% in shrimp fed probiotic diet A compared to shrimp fed the NTC diet. Shrimp fed probiotic strains C, I, and J diets did not show significant differences in survival rate when compared to the NTC survival rate.

When shrimp were exposed to 10^8 CFU/mL EMS, mortalities were first observed for shrimp fed the following probiotic diets: strain A at 24 hours; NTC, strain C, and strain J at 48 hours; and strain B at 72 hours (Figure 4.3). The following survival rates were observed for shrimp exposed to 10^8 CFU/mL EMS at the end of the 96-hour period: shrimp fed the probiotic strain I diet showed 100% survival, followed by shrimp fed the probiotic strain B diet with 80% survival, shrimp fed probiotic strain J and NTC diets with 60% survival, and lastly shrimp fed probiotic diets strains A and C with 40% survival.

Although there was some mortality when shrimp were exposed to 10^8 CFU/mL EMS, there were overall no significant differences ($p<0.05$) in survival rate among the different probiotic strains (Table 4.3). The inability for the 10^8 CFU/mL inoculation to cause significant differences among probiotic strains rendered it an inadequate infectious dose for the purposes of the study. No mortalities were seen when shrimp were exposed to 10^7 CFU/mL thus making the 10^7 CFU/mL inoculation an ineffective infectious dose as the purpose of this study was to evaluate the reduction of mortality from disease (Figure 4.4). Thus, onward probiotic studies in this chapter will focus using on using the infectious dose of 10^9 CFU/mL EMS (undiluted EMS) for feed inoculations as it causes significant differences among treatments.

Table 4.2. *Mean CFU/g of EMS in feed inoculations used in Trial 1.*

Time (hr)	Broth Concentration (CFU/mL)	Feed Concentration (CFU/g)
0	$1.56x10^9$	$1.56x10^8$
24	$2.17x10^9$	$2.17x10^8$
48	$1.50x10^9$	$1.50x10^8$
72	$2.27x10^9$	$2.27x10^8$
Average	$1.88x10^9$	$1.88x10^8$

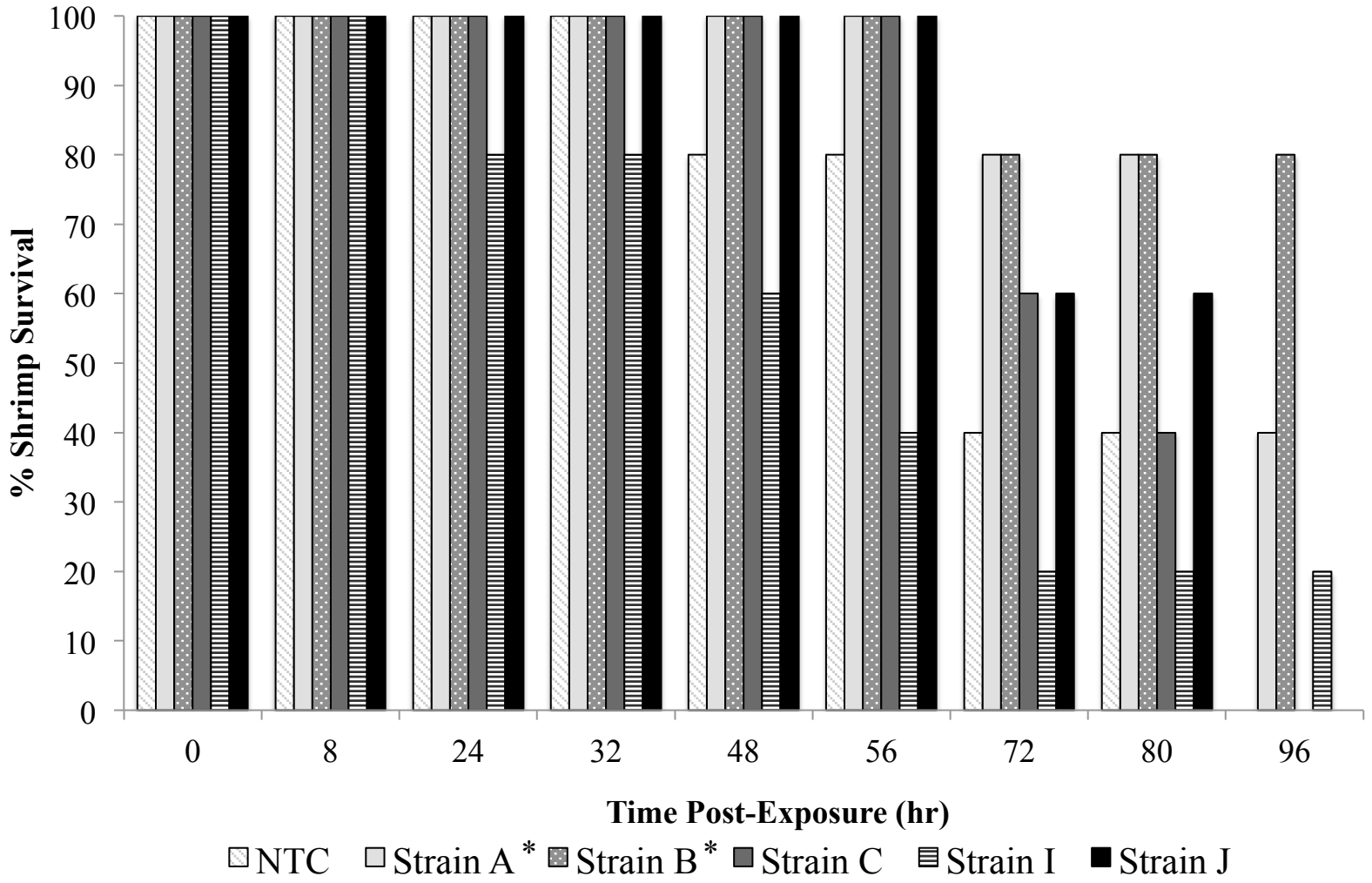

Figure 4.2. *Survival trend evaluation of probiotic strains A, B, C, I, and J upon exposure to 10^9 CFU/mL EMS in Trial 1. Values are % of n=5 replicate analysis. NTC = non-treated control (no probiotic application). Treatments* in legend with asterisks are significantly different ($p<0.05$) from NTC. P-values for significantly different treatments are as follows: probiotic strain A – 0.0889 & Strain B – 0.0203.*

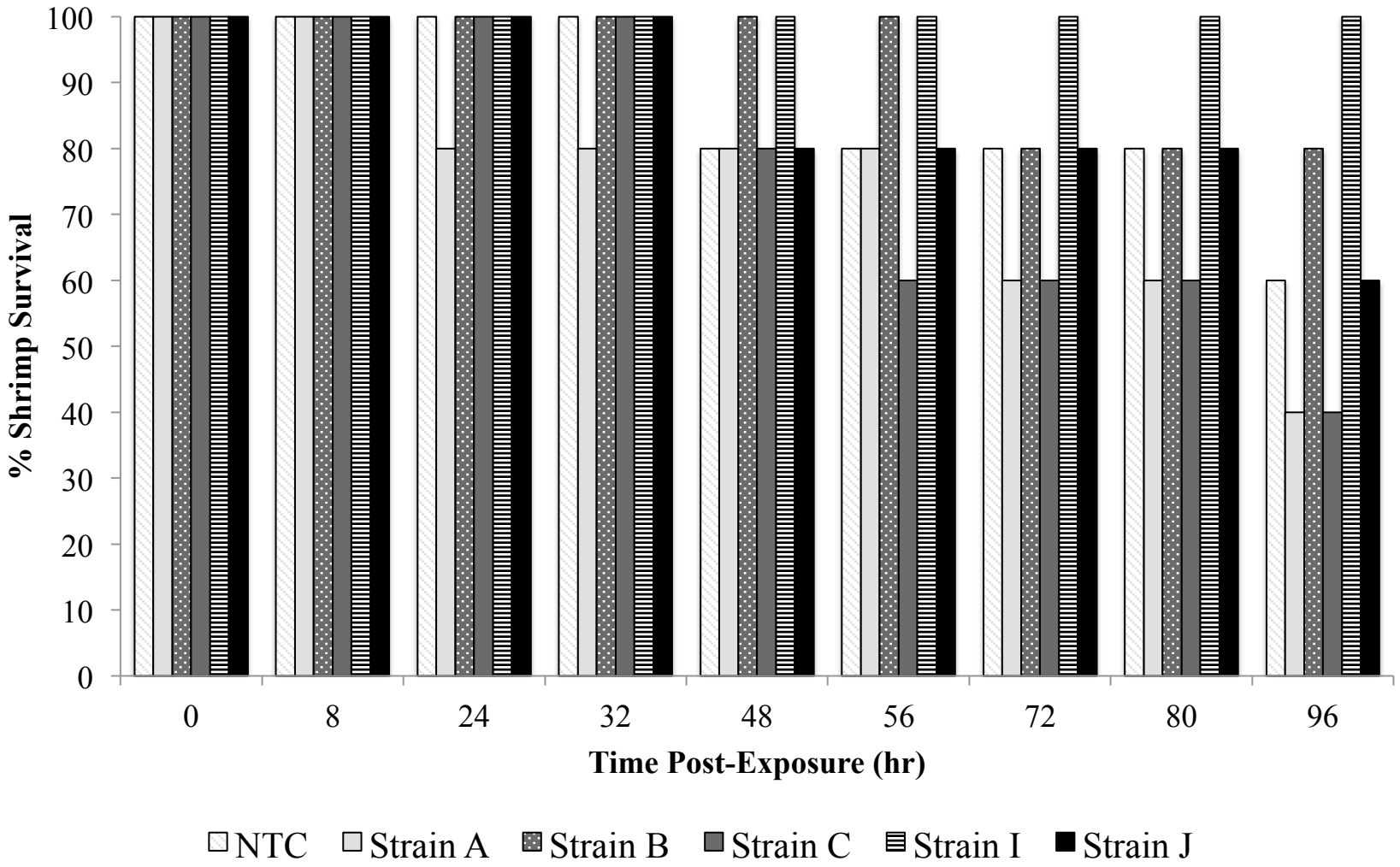

Figure 4.3. *Survival trend evaluation of probiotic strains A, B, C, I, and J upon exposure to 10^8 CFU/mL in Trial 1. Values are % of n=5 replicate analysis. NTC = non-treated control (no probiotic application). There were no significant differences in treatments compared to the NTC.*

Table 4.3. *p-value outputs for different EMS concentrations in Trial 1.*

EMS Concentration (CFU/mL)	p-value
10^7	1.0
10^8	0.3461
10^9	0.0378

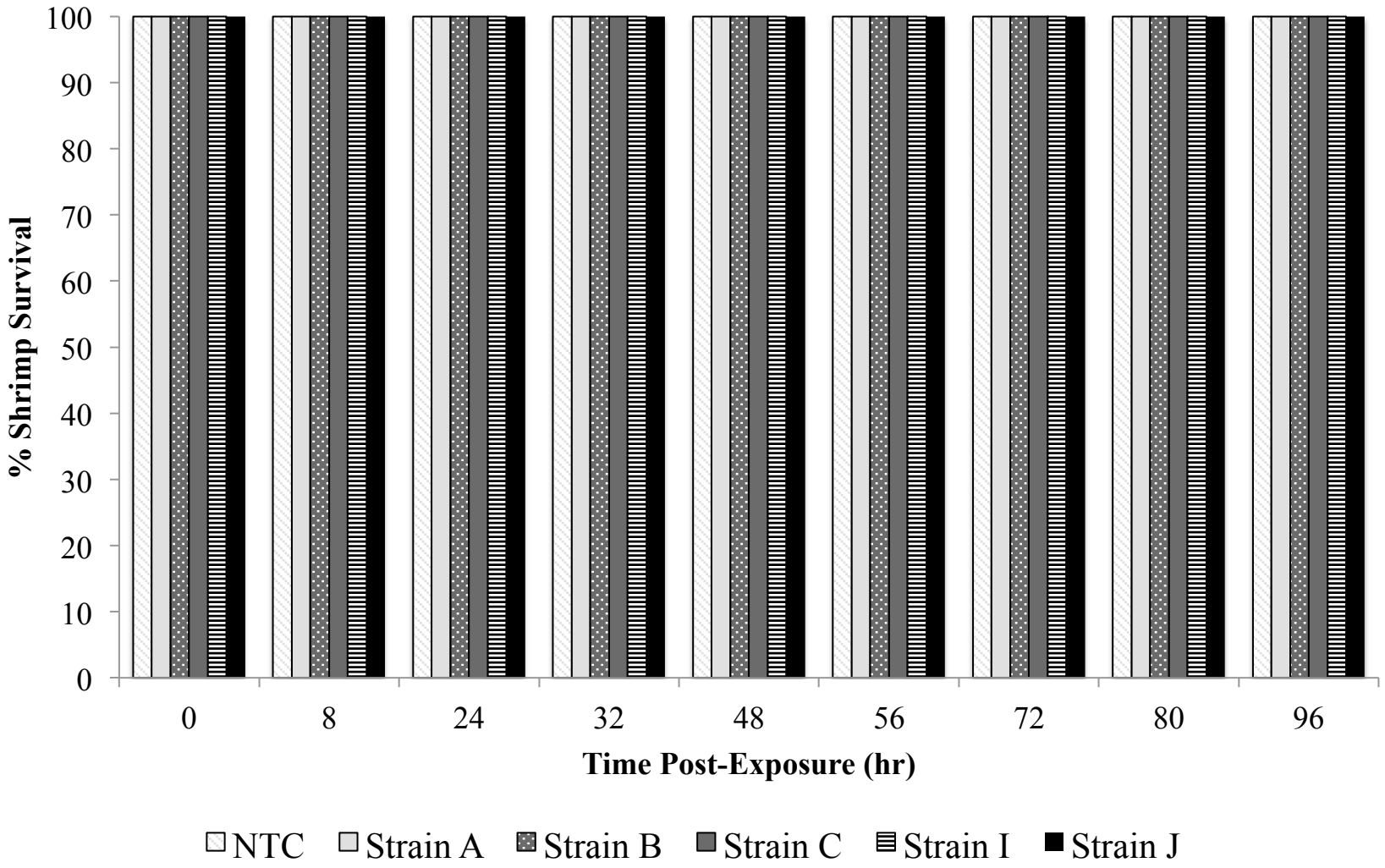

Figure 4.4. *Survival trend evaluation of probiotic strains A, B, C, I, and J upon exposure to 10^7 CFU/mL EMS in Trial 1. Values are % of n=5 replicate analysis. NTC = non-treated control (no probiotic application). There were no significant differences compared to the NTC.*

Trial 2. Eight different *B. subtilis* probiotic strains were evaluated using the infectious dose 10^9 CFU/mL EMS feed inoculations. The two best performing probiotics from Trial 1, strain A and strain B, were used again in this Trial 2. Strain C was used again as a negative control because the probiotic bacteria contained in this strain was known to have neither positive nor negative effects and to provide evidence that foreign bacteria supplementation in the shrimp gut was benign to shrimp. Average spread plate counts of the overnight EMS strains used to inoculate feed daily was $3.35x10^9$ CFU/mL consistently (Table 4.4). Shrimp fed probiotic strains A and B diets exhibited the highest survival rate of 40% after EMS exposure (Figure 4.5). Shrimp fed probiotic strains D and E diets exhibited the second highest survival rate of 20%. Following these strains were shrimp fed the probiotic strain F diet with 15% survival and shrimp fed probiotic strain G diet with 10% survival. Shrimp fed probiotic strains C and H and NTC diets had the lowest survival rate of 5%. Significant differences ($p<0.05$) in survival rate were seen in shrimp fed probiotic strains A ($p=0.0015$) and B ($p=0.0231$) diets when compared to the survival rate of shrimp fed the NTC diet. There were no significant differences in survival rate in shrimp that were fed probiotic strains C-H diets. Results from Trial 2 were consistent with results from Trial 1 in that shrimp that were fed probiotic strains A and B diets were able to achieve higher survival rates compared to shrimp fed other probiotic strain diets.

Histology samples were collected and graded using representative samples of grades 1=normal cells, 2=moderately diseased cells, and 3=severely diseased cells (Figure 4.6). Histological grading during this trial indicated shrimp fed probiotics Strain A and Strain B were achieved a grade of 2 showing moderately diseased cells (Table 4.5). Shrimp fed the NTC and Strains C-H diets achieved grades of 3 showing severely diseased cells. Grades assigned to histology slides from different treatments roughly correlated with survival rate. Groups that scored lower grades had lesser diseased-appearance cells had higher survival rates.

Examination of histology samples indicated that EMS progressed from the interior to apical surface (Figure 4.7). The gradient that was exhibited in EMS-exposed shrimp also indicates that the etiology of the strain is through the feed and not through the gills from the ingestion of environmental water. In previously performed EMS immersion studies by Tran et al. (2013) however, an immersion bath method was able to cause mortality in shrimp. The method used in the Tran et. al study (2013) was to immerse shrimp for 15 minutes using 150 mL prepared TSB2+ broth culture of EMS at $2x10^8$ cells/mL and then placing the TSB2+ EMS

culture along with the shrimp in tank water afterwards making the final dilution $2x10^6$ cells/mL. The average spread plate count of EMS used to inoculate feed daily in this study was $3.35x10^9$ CFU/mL. Therefore once the feed was placed into 40 liters of tank water, the EMS dilution would have been reduced to $8.38x10^8$ CFU/mL. This dilution was similar to the concentration used in Tran et al.'s (2013) study. The immersion of shrimp for 15 minutes into $2x10^8$ CFU/mL may be crucial in attaining mortality through the water. Shrimp used in Tran et al.'s (2013) study were also between 0.5 grams to 2 grams, which was much smaller than the average shrimp used in Trial 2. Further studies are warranted to see if there is a correlation between shrimp size and EMS concentration to see if smaller sized shrimp are prone to EMS exposure through the water than bigger shrimp.

Light microscopy of histology slides of all EMS-exposed shrimp were consistent with findings of Nunan et al (2014). Histological slide analysis revealed gradual degradation of epithelial cells and an increase of hemocyctic activity where the epithelial layer should be in the midgut cecum (Nunan et al., 2014). Sloughing of tubule epithelial cells was also apparent in EMS-exposed shrimp. Histology of the hepatopancreas indicates disappearance of B cells and increase in hemocytic activity. Hemocytic infiltration increased due to the presence of EMS as hemocytes are able to detect invading diseases using PAMPs (Rowley and Pope, 2012). Hemocytes respond to diseases by using phagocytosis and later on discharging AMPs such as penaidins and crustins, lysozymes and lectins. The disappearance of B-cells indicates that there was most likely a disruption in digestion as B-cells are primary producers of digestive enzymes (Felgenhauer, 1992).

Table 4.4. *Mean CFU/g of EMS in feed inoculations used in Trial 2.*

Time (hr)	Broth Concentration (CFU/mL)	Feed Concentration (CFU/g)
0	$4.4x10^9$	$4.4x10^8$
24	$4.7x10^9$	$4.7x10^8$
48	$1.4x10^9$	$1.4x10^8$
72	$2.9x10^9$	$2.9x10^8$
Average	$3.35x10^9$	$3.35x10^8$

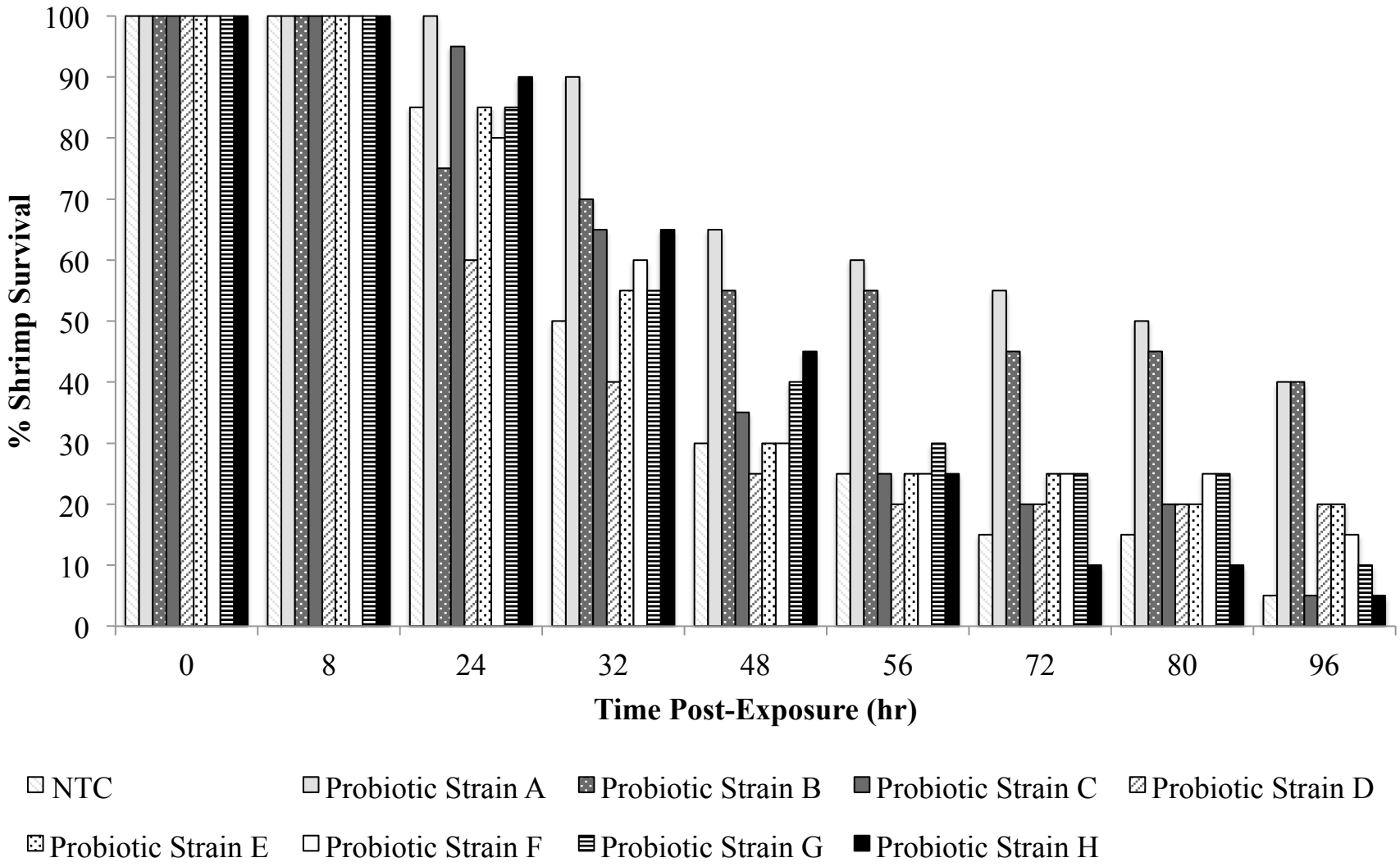

Figure 4.5. *Survival trend evaluation of probiotic strains A, B, C, D, E, F, G, and H upon EMS exposure in Trial 2. Values are % of n=10 replicate analysis. Treatments* in legend with asterisks are significantly different ($p<0.05$) from NTC. P-values for significantly different treatments are as follows: probiotic strain A – 0.0015 & Strain B – 0.0231.*

Table 4.5. *Results from histology grading for Trial 2 shrimp that remained after the 96-hour period. All remaining shrimp were placed in an ice bath before being histology grading. (1=normal cells, 2=moderately diseased cells, 3=severely diseased cells). The average grade was rounded to the closest whole number.*

Strain	Individual Grade for each Shrimp	Average Grade
NTC	3	3.0
A	2, 2, 2, 2, 3, 3	2.3
B	2, 2, 2, 2, 3, 3	2.3
C	3, 3	3.0
D	3, 3	3.0
E	3, 3, 3, 3	3.0
F	2, 2, 3, 3, 3, 3	2.7
G	3, 3	3.0
H	2, 3	2.5

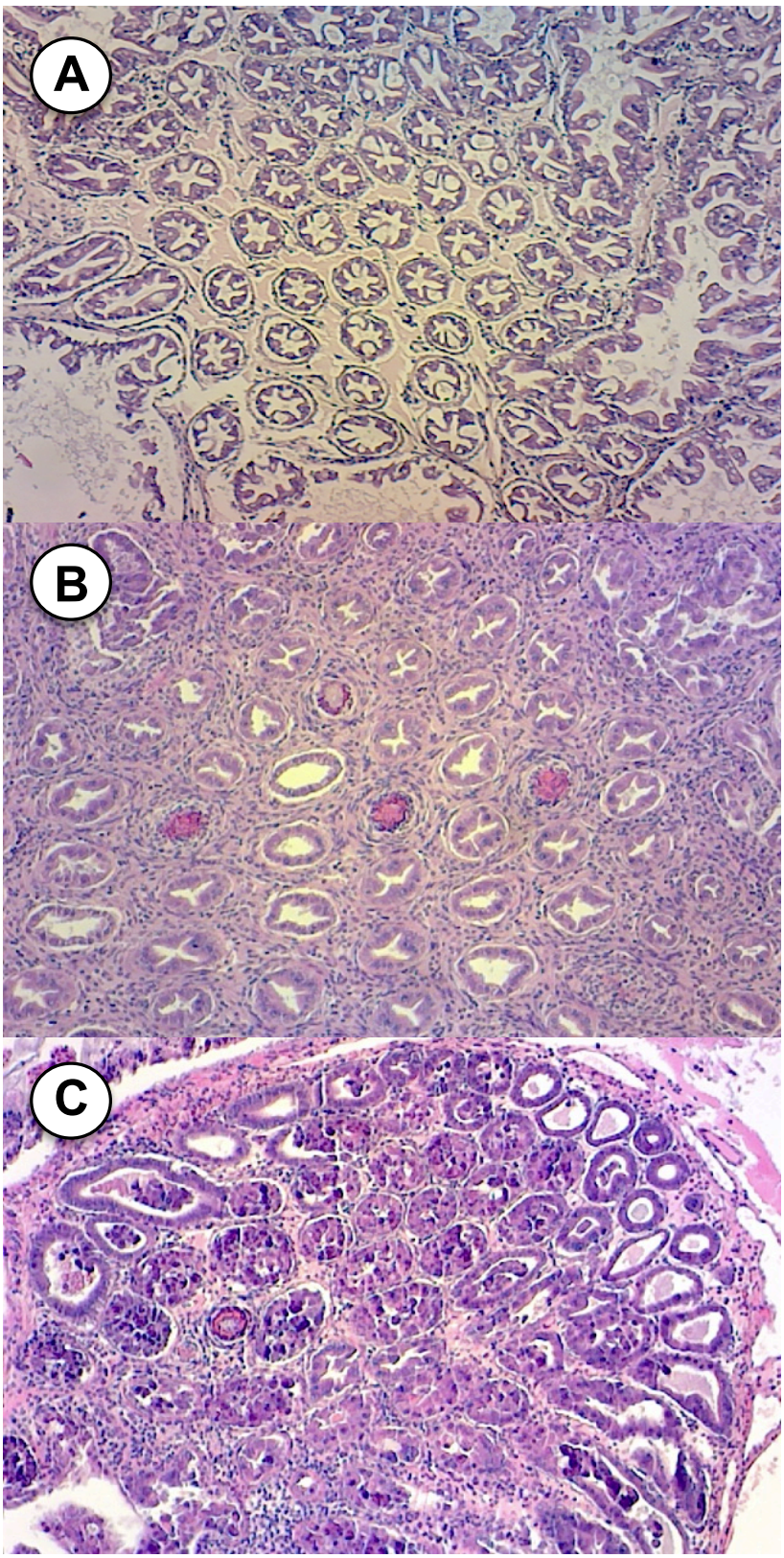

Figure 4.6. *Representative images of the following scores: (A) 1= normal cells, (B) 2 = moderately diseased cells, and (C) 3= severely diseased cells*

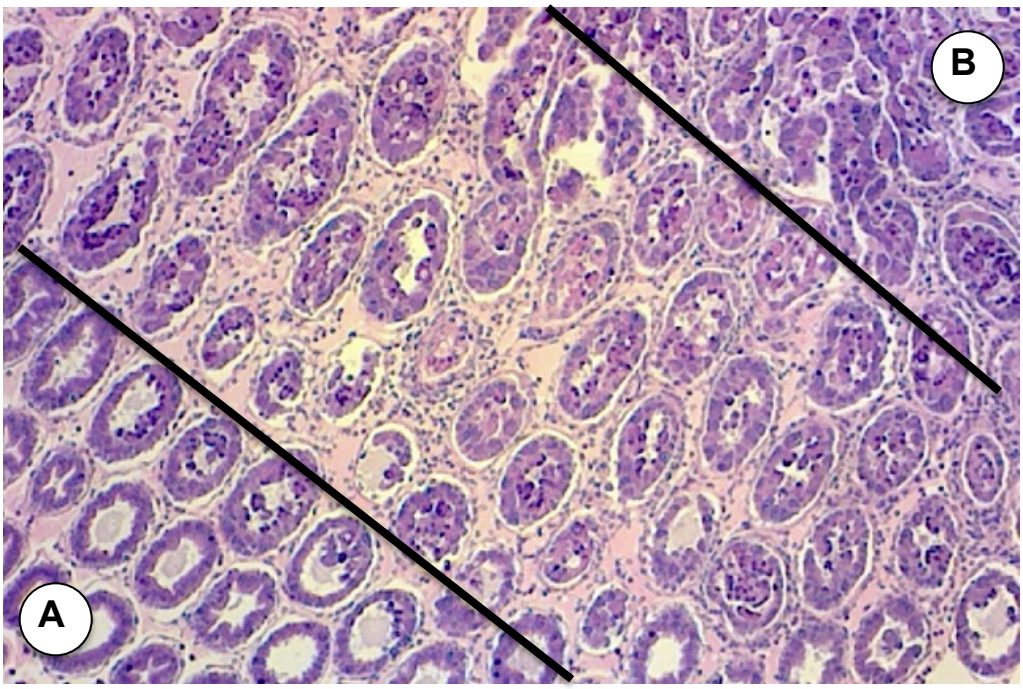

Figure 4.7. *Light microscopy analysis of histology slide prepared from remaining shrimp fed the Strain D diet in Trial 2. Two extreme regions are depicted: (A) apical surface shows no EMS degradation and (B) interior surface shows extreme with EMS degradation depicted by central tubule sloughing.*

Trial 3. Probiotic strains A and B were blended together and applied to feed to see if the combined strains of bacteria could achieve higher survival rates in shrimp after feeding the probiotic A/B diet. Average spread plate counts of the overnight EMS strains used to inoculate feed daily were at 4.30×10^9 CFU/mL (Table 4.6). In Trial 3, four different probiotic strains were examined: probiotic strain A, strain B, strain C, and a blend of strains A and B. A negative treatment group (no probiotic diet and no EMS exposure) was created to ensure that water quality parameters were not causing any unexpected mortalities. Shrimp that were fed the NTC diet and not exposed to EMS (negative treatment) exhibited 100% survival during the trial (Figure 4.8). Following the negative treatment, shrimp that were fed probiotic strains A, B, and A/B blend diets exhibited the highest rate of 15% survival after EMS exposure. Following these diets, shrimp that were fed the NTC diet showed 4% survival. Shrimp that were fed probiotic strain C diet had the lowest survival rate of 0%. A significant difference ($p<0.05$) in survival rate was seen in shrimp that were fed probiotic strain A ($p=0.0051$), strain B ($p=0.0424$), and A/B blend ($p=0.0378$) diets when compared to survival rate of shrimp that were fed the NTC diet.

Table 4.6. *Mean CFU/g of EMS in feed inoculations used in Trial 3.*

Time (hr)	Broth Concentration (CFU/mL)	Feed Concentration (CFU/g)
0	$1.91x10^9$	$1.91x10^8$
24	$2.18x10^9$	$2.18x10^8$
48	$4.45x10^9$	$4.45x10^8$
72	$8.67x10^9$	$8.67x10^8$
Average	$4.30X10^9$	$4.30X10^8$

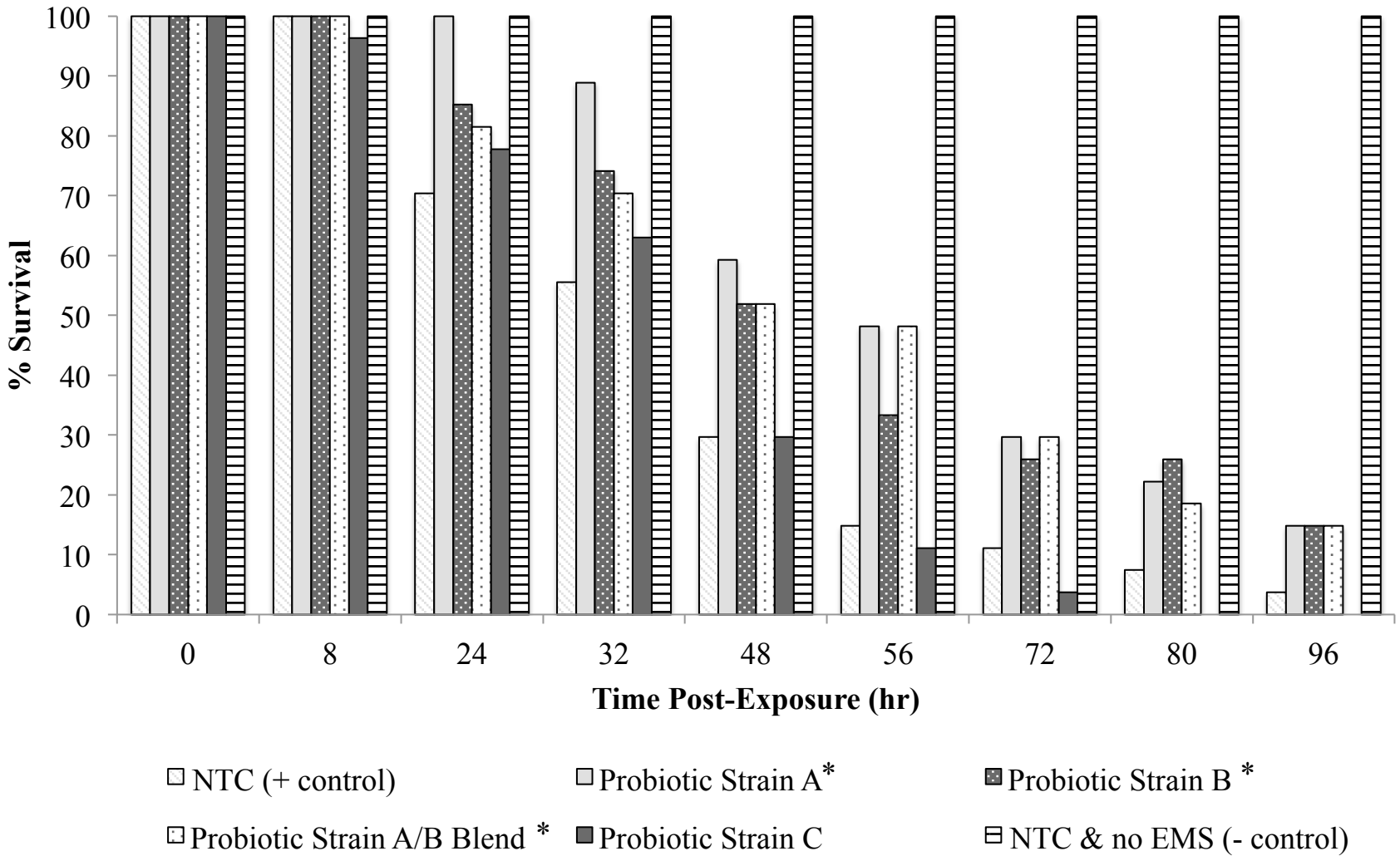

Figure 4.8. *Comparison of probiotic strains A, B, A/B blend, and C in Trial 3. Values are % of n=9 replicate analysis. Treatments with asterisks are significantly different ($p<0.05$) from the non-probiotic diet. Treatments* in legend with asterisks are significantly different ($p<0.05$) from NTC. P-values for significant different treatments are: probiotic strain A – 0.0051, strain A/B blend – 0.0378, & strain B – 0.0424.*

Results from Trial 3 suggest that water quality did not have an effect on mortalities that were observed during the 96-hour trial. Probiotic strain A, B, and A/B were the top probiotic strains that were able to inhibit EMS colonization in the shrimp hepatopancreas and may be of use as a proactive method for disease reduction.

Overall Probiotic Strain Evaluation. Shrimp that were fed probiotic strains A and B consistently showed significant differences in survival compared to shrimp that were fed the NTC diet in Trial 1, 2, and 3. When a mixture of probiotic strain A/B blend was used as a treatment in Trial 3, there was a significant difference in survival when shrimp were fed A/B blend when compared to shrimp that were fed the control diet. Previous studies have shown the beneficial health effects of probiotics by improving the intestinal microbiota, providing competition for adhesion sites in the gastrointestinal tract, and producing antimicrobial substances. However, there was no analysis performed during this study to confirm the production of antimicrobial substances and further research is warranted. Although there have been several studies in the literature examining probiotics' effects on reducing *Vibrio* spp. in general, there is insufficient information on the reduction method targeted specifically for the recently emerged EMS causing *V. parahaemolyticus* strain. Reduction methods targeted for the EMS strain is warranted due to its high lethality and rapid spread of the disease.

The ability of certain probiotic diets to achieve higher survival rates upon EMS exposure may be attributed to the strains ability to survive or colonize in the shrimp gut (Farzanfar, 2006). Previous studies using nonpathogenic *Bacillus* probiotics to reduce *V. harveyi* colonization in the shrimp gut have indicated that the probiotics are able to enhance survival through competitive exclusion (Rengpipat et al., 1998). Probiotics with higher survival rates from Trials 1, 2, and 3 may have colonized better in the shrimp gut, which provided competition against bacterial pathogens (Ninawe and Selvin, 2009). The synthesis of antimicrobials such as bacteriocins, antibiotics, and bacteriocin-like inhibitory substances is another mechanism in which probiotics can utilize to minimize pathogenic bacterial colonization in the gastrointestinal tract (Hong and Cutting, 2005). Previous research on certain *B. subtilis* probiotic isolates has demonstrated the probiotics' ability to produce a protease-resistant isocouamrin antibiotic, aminocoumacin A. The production of antimicrobial substances along with noncompetitive exclusion may have increased

survival rate of shrimp that were fed probiotics compared to the survival rate of shrimp on the control diet.

Probiotics can be sensitive and therefore lose viability during the preparation and storage of strains (Wang et al., 2008). The bacterial strain itself and the manufacturer of the strain may also affect the retention of viability in probiotics. Data was collected to see the actual concentration of probiotics applied to used in Trials 1, 2, and 3 were collected (Table 4.7). This data was characterized as probiotic retention data. Although the target for probiotic concentrations was 10^7 CFU/mL, there were varying concentrations among the different strains. *Bacillus* spp. spores are robust compared to other probiotic spores because of their physical and biological nature. Despite this nature, the spore application to feed and the storage conditions in which probiotic feeds may have affected probiotic concentrations.

Table 4.7. *Probiotic strain retention data for all probiotic strains used in Trial 1, 2, and 3.*

Probiotic Strain	CFU/mL
A	2.40×10^6
B	1.73×10^6
A/B	2.10×10^6
C	6.20×10^6
D	5.48×10^6
E	2.67×10^5
F	7.08×10^6
G	2.03×10^6
H	5.33×10^5
I	2.32×10^6
J	5.77×10^6

Conclusion

The present study aimed to determine whether probiotic diets can reduce EMS colonization in the shrimp hepatopancreas and therefore, increase shrimp survival rates against EMS. Probiotic strains A, A/B blend, and B significantly increased survival in shrimp after feed inoculation with EMS compared to survival in shrimp that were the fed the non-probiotic diet. The histology grading of shrimp from Trial 2 correlated with these results as there was less cell degeneration and hemocyte infiltration in shrimp tissues that were fed strain A and B diets compared to shrimp tissue from other strain diets used in the experiment. Strains A and B were better at minimizing EMS colonization in the hepatopancreas compared to other strains. Light microscopy of EMS-exposed shrimp suggests the EMS etiology to be primarily through the feed and not through the gills because there was a gradual degradation of cells from the interior to apical surface.

Additional studies of prolonged feeding of diluted EMS *in vivo* are warranted to evaluate probiotics' effects on longer EMS exposure and the ability of EMS to proliferate in the environment. It would also be of interest to conduct zone inhibition studies using each probiotic study on EMS on TSB2+ plates and evaluate whether higher survival rates correlate with larger zones of inhibition. The qPCR analysis for the EMS pathogen in shrimp tissues should also be assessed to see if there is a reduction of EMS in the gut and or hepatopancreas in probiotic-fed shrimp compared to nonprobiotic-fed shrimp. Research on methods for optimal probiotic application methods in order for probiotics to retain viability during preparation and storage of the strain are also warranted.

Chapter 5:

Conclusions and Future Work

The objectives of this study were completed through virulence expression of *Vibrio* spp. in shrimp species, confirmation of spore germination in the shrimpgut, and the identification of the best probiotic strain candidates as a supplement to shrimp aquaculture feeds to decrease mortalities caused by *Vibrio* spp. Survival was compared to survival of control shrimp to evaluate the virulence of three different *V. parahaemolyticus* strains and to test the EMS dilution necessary to cause chronic virulence. Eleven probiotic strains were tested to determine whether the probiotic diets increased shrimp survival rate. Survival of shrimp that were fed probiotic diets were statistically analyzed and compared to survival of shrimp that were fed the non-probiotic diet. Probiotic strains A and B demonstrated the greatest survival compared to survival of shrimp fed the non-treated control diet post-EMS exposure.

Results from this study suggest several future directions. First, a long-term probiotic study using various EMS dilutions for exposure is necessary to evaluate the proliferation of EMS in the marine environment. Second, it would be interesting to see whether *in vitro* studies of zone of inhibition studies using probiotic strains used in Chapter 4 correlate with higher survival rates in *in vivo* studies. Third, qPCR analysis for the EMS pathogen in shrimp tissues should also be assessed to see if there is a reduction of EMS in the gut and or hepatopancreas in probiotic-fed shrimp compared to nonprobiotic-fed shrimp. Some research has already been conducted in this area and has exhibited promising results. Last but not least, a more efficient probiotic application in order to achieve higher retention during application of probiotics to feed and during storage of probiotic strains should be sought out and evaluated.

References

Aguirre-Guzman, G., Sanchez-Martinez, J.G., Campa-Cordova, A.I., Luna-González, A., Ascencio, F., 2009. Penaeid shrimp immune system. Thai J. Vet. Med. 39, 205-215.

Al-Mohanna, S.Y., Nott, J.A., 1987. R-cells and the digestive cycle in Penaeus semisulcatus (Crustacea: Decapoda). Mar. Biol. 95, 129-137.

Aly, S.M., Abdel-Galil Ahmed, Y., Abdel-Aziz Ghareeb, A., Mohamed, M.F., 2008. Studies on Bacillus subtilis and Lactobacillus acidophilus, as potential probiotics, on the immune response and resistance of *Tilapia nilotica (Oreochromis niloticus)* to challenge infections. Fish & Shellfish Immunology. 25, 128-136.

Andersson, D.I., 2003. Persistence of antibiotic resistant bacteria. Current Opinion in Microbiology. 6, 452-456.

Andrade, T.P.D., Redman, R.M., Lightner, D.V., 2008. Evaluation of the preservation of shrimp samples with Davidson's AFA fixative for infectious myonecrosis virus (IMNV) in situ hybridization. Aquaculture. 278, 179-183.

Arquitt, S., Honggang, X., Johnstone, R., 2005. A system dynamics analysis of boom and bust in the shrimp aquaculture industry. System Dynamics Review. 21, 305-324.

Bag, J., 1974. Glucose inhibition of the transport and phosphoenolpyruvate-dependent phosphorylation of galactose and fructose in *Vibrio cholerae*. Journal of bacteriology. 118, 764-767.

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

Bell, T.A., Lightner, D.V., 1988. A handbook of normal penaeid shrimp histology.

Benbrook, C.M., 2002. Antibiotic drug use in US aquaculture. Institute for Agriculture and Trade Policy Report. 2.

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

Bondad-Reantaso, M.G., Subasinghe, R.P., Josupeit, H., Cai, J., Zhou, X., 2012. The role of crustacean fisheries and aquaculture in global food security: Past, present and future. Journal of Invertebrate Pathology. 110, 158-165.

Bower, C.E., Bidwell, J.P., 1978. Ionization of Ammonia in Seawater: Effects of Temperature, pH, and salinity. Journal of the Fisheries Research Board of Canada. 35, 1012-1016.

Briggs, M., Funge-Smith, S., Subasinghe, R., Phillips, M., 2004. Introductions and movement of *Penaeus vannamei* and *Penaeus stylirostris* in Asia and the Pacific. RAP publication. 10, 92.

Buck, J.D., Cleverdon, R.C., 1960. The Spread Plate as a Method for the Enumeration of Marine Bacteria Limnology and Oceanography. 5, 78-80.

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

Caceci, T., Neck, K.F., Lewis, D.D.H., Sis, R.F., 1988. Ultrastructure of the hepatopancreas of the Pacific white shrimp, *Penaeus vannamei* (Crustacea: Decapoda). Journal of the Marine Biological Association of the United Kingdom. 68, 323-337.

Casula, G., Cutting, S.M., 2002. Bacillus probiotics: spore germination in the gastrointestinal tract. Applied and environmental microbiology. 68, 2344-2352.

CDC, 2013a. Vibrio Illness (Vibriosis). in: Prevention, C.f.D.C.a. (Ed.).

CDC, 2013b. Antibiotic Resistance Threats in the United States, 2013. in: Services, U.S.D.o.H.a.H. (Ed.), CDC.
Centers for Disease Control and Prevention, Foodborne Outbreak Online Database (FOOD). in: Prevention, C.f.D.C.a. (Ed.).
Chen, S., Ling, J., Blancheton, J.-P., 2006. Nitrification kinetics of biofilm as affected by water quality factors. Aquacultural Engineering. 34, 179-197.
Corteel, M., Nauwynk, H.J., 2010. The Integument of Shrimp: Cuticle and its Moult Cycle. in: Alday-Sanz, V. (Ed.), The Shrimp Book. Nottingham University Press.
Dall, W., 1967. The functional anatomy of the digestive tract of a shrimp *Metapenaeus bennettae* Racek & Dall (Crustacea : Decapoda : Penaeidae). Australian Journal of Zoology. 15, 699-714.
Daniels, R., Vanderleyden, J., Michiels, J., 2004. Quorum sensing and swarming migration in bacteria.
De Schryver, P., Defoirdt, T., Sorgeloos, P., 2014. Early Mortality Syndrome Outbreaks: A Microbial Management Issue in Shrimp Farming? PLoS Pathogens. 10, e1003919.
Defoirdt, T., Sorgeloos, P., Bossier, P., 2011. Alternatives to antibiotics for the control of bacterial disease in aquaculture. Current Opinion in Microbiology. 14, 251-258.
Deutsch, L., Gräslund, S., Folke, C., Troell, M., Huitric, M., Kautsky, N., Lebel, L., 2007. Feeding aquaculture growth through globalization: Exploitation of marine ecosystems for fishmeal. Global Environmental Change. 17, 238-249.
Dhar, A.K., Roux, M.M., Klimpel, K.R., 2001. Detection and quantification of infectious hypodermal and hematopoietic necrosis virus and white spot virus in shrimp using real-time quantitative PCR and SYBR Green chemistry. Journal of clinical microbiology. 39, 2835-2845.
Diana, J.S., 2009. Aquaculture Production and Biodiversity Conservation. BioScience. 59, 27-38.
FAO 2006-2015. Cultured Aquatic Species Infomration Programme. Penaues vannamei. Cultured Species Information Programme. Text by Briggs, M. In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 7 April 2006. [Cited 2 March 2015]. http://www.fao.org/fishery/culturedspecies/Litopenaues_vannamei/en
Farzanfar, A., 2006. The use of probiotics in shrimp aquaculture. FEMS Immunology & Medical Microbiology. 48, 149-158.
FDA, 2014a. FDA's Strategy on Antimicrobial Resistance - Questions and Answers. in: Administration, U.S.F.a.D. (Ed.), Animal & Veterinary.
FDA, 2014b. 2012 Summary Report on Antimicrobials Solds or Distributed for Use in Food-producing Animals. in: Services, D.o.H.a.H. (Ed.).
FDA, 2014c. Approved Drugs. in: Administration, U.S.F.a.D. (Ed.), Animal & Veterinary. U.S. Department of Health and Human Services.
Felgenhauer, B.E., 1992. Internal anatomy of the Decapoda: an overview. Microscopic anatomy of invertebrates. 10, 45-75.
Fischer, A.H., Jacobson, K.A., Rose, J., Zeller, R., 2008. Hematoxylin and eosin staining of tissue and cell sections. Cold Spring Harbor Protocols. 2008, pdb. prot4986.
FAO, 2014. The state of world fisheries and aquaculture. FAO Rome, Italy.
Fraser, G.M., Hughes, C., 1999. Swarming motility. Current Opinion in Microbiology. 2, 630-635.

Goel, M.K., Khanna, P., Kishore, J., 2010. Understanding survival analysis: Kaplan-Meier estimate. International journal of Ayurveda research. 1, 274.

Gutierrez-Wing, M.T., Malone, R.F., 2006. Biological filters in aquaculture: Trends and research directions for freshwater and marine applications. Aquacultural Engineering. 34, 163-171.

Han, J.E., Tang, K.F., Pantoja, C.R., White, B.L., Lightner, D.V., 2015. qPCR assay for detecting and quantifying a virulence plasmid in acute hepatopancreatic necrosis disease (AHPND) due to pathogenic *Vibrio parahaemolyticus*. Aquaculture. 442, 12-15.

Henke, J.M., Bassler, B.L., 2004. Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. Journal of Bacteriology. 186, 3794-3805.

Henriksson, P.J., Guinée, J.B., Kleijn, R., de Snoo, G.R., 2012. Life cycle assessment of aquaculture systems—a review of methodologies. The International Journal of Life Cycle Assessment. 17, 304-313.

Heppell, J., Davis, H.L., 2000. Application of DNA vaccine technology to aquaculture. Advanced Drug Delivery Reviews. 43, 29-43.

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

Hong, H.A., Cutting, S.M., 2005. The use of bacterial spore formers as probiotics. FEMS microbiology reviews. 29, 813-835.

Hsern Malcolm, T.T., Cheah, Y.K., Radzi, C.W.J.W.M., Kasim, F.A., Kantilal, H.K., Huat John, T.Y., Martinez-Urtaza, J., Nakaguchi, Y., Nishibuchi, M., Son, R., 2015. Detection and quantification of pathogenic *Vibrio parahaemolyticus* in shellfish by using multiplex PCR and loop-mediated isothermal amplification assay. Food Control. 47, 664-671.

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

Iyer, L., Vadivelu, J., Puthucheary, S., 2000. Detection of virulence associated genes, haemolysin and protease amongst *Vibrio cholerae* isolated in Malaysia. Epidemiology and infection. 125, 27-34.

Jensen, R.V., DePasquale, S.M., Harbolick, E.A., Hong, T., Kernell, A.L., Kruchko, D.H., Modise, T., Smith, C.E., McCarter, L.L., Stevens, A.M., 2013. Complete genome sequence of prepandemic *Vibrio parahaemolyticu*s BB22OP. Genome announcements. 1, e00002-00012.

Karunasagar, I., Karunasagar, I., Alday-Sanz, V., 2010. Immunostimulants, probiotics, and phage therapy: Alternatives to Antibiotics, The Shrimp Book. Nottingham University Press.

Keer, J.T., Birch, L., 2003. Molecular methods for the assessment of bacterial viability. Journal of Microbiological Methods. 53, 175-183.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.J., Gibson, L., 2008. Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. Aquaculture. 274, 1-14.

Keyhani, N.O., Roseman, S., 1999. Physiological aspects of chitin catabolism in marine bacteria. Biochimica et Biophysica Acta (BBA) - General Subjects. 1473, 108-122.

Kim, S.Y., Lee, S.E., Kim, Y.R., Kim, C.M., Ryu, P.Y., Choy, H.E., Chung, S.S., Rhee, J.H., 2003. Regulation of *Vibrio vulnificus* virulence by the LuxS quorum-sensing system. Molecular Microbiology. 48, 1647-1664.

Kompala, D.S., Ramkrishna, D., Jansen, N.B., Tsao, G.T., 1986. Investigation of bacterial growth on mixed substrates: Experimental evaluation of cybernetic models. Biotechnology and Bioengineering. 28, 1044-1055.

Kongrueng, J., Yingkajorn, M., Bunpa, S., Sermwittayawong, N., Singkhamanan, K., Vuddhakul, V., 2014. Characterization of *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease in southern Thailand. Journal of fish diseases.

Kuhn, D.D., Smith, S.A., Boardman, G.D., Angier, M.W., Marsh, L., Flick, G.J., 2010. Chronic toxicity of nitrate to Pacific white shrimp, *Litopenaeus vannamei*: impacts on survival, growth, antennae length, and pathology. Aquaculture. 309, 109-114.

Kümmerer, K., 2009. Antibiotics in the aquatic environment – A review – Part I. Chemosphere. 75, 417-434.

Lakshmi, B., Viswanath, B., Sai Gopal, D.V., 2013. Probiotics as antiviral agents in shrimp aquaculture. J Pathog. 2013, 424123.

Leaño, E.M., Mohan, C., 2012. Early mortality syndrome threatens Asia's shrimp farms. Global Aquaculture Advocate, 38-39.

Li, C., Weng, S., Chen, Y., Yu, X., Lü, L., Zhang, H., He, J., Xu, X., 2012. Analysis of *Litopenaeus vannamei* Transcriptome Using the Next-Generation DNA Sequencing Technique. PLoS ONE. 7, e47442.

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

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

Lin, Y.-C., Chen, J.-C., 2001. Acute toxicity of ammonia on *Litopenaeus vannamei* Boone juveniles at different salinity levels. Journal of Experimental Marine Biology and Ecology. 259, 109-119.

Lin, Y.-C., Chen, J.-C., 2003. Acute toxicity of nitrite on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. Aquaculture. 224, 193-201.

Maillard, V.M., Boardman, G.D., Nyland, J.E., Kuhn, D.D., 2005. Water quality and sludge characterization at raceway-system trout farms. Aquacultural Engineering. 33, 271-284.

Makino, K., Oshima, K., Kurokawa, K., Yokoyama, K., Uda, T., Tagomori, K., Iijima, Y., Najima, M., Nakano, M., Yamashita, A., Kubota, Y., Kimura, S., Yasunaga, T., Honda, T., Shinagawa, H., Hattori, M., Iida, T., 2003. Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*. The Lancet. 361, 743-749.

Martin, G.G., Hose, J.E., 2010. Functional Anatomy of Penaeid Shrimp. in: Alday-Sanz, V. (Ed.), The Shrimp Book. Nottingham University Press.

McColl, K.A., Slater, J., Jeyasekaran, G., Hyatt, A.D., Crane, M.S., 2004. Detection of White Spot Syndrome virus and Yellowhead virus in prawns imported into Australia. Australian Veterinary Journal. 82, 69-74.

Mohapatra, S., Chakraborty, T., Kumar, V., Deboeck, G., Mohanta, K., 2013. Aquaculture and stress management: a review of probiotic intervention. Journal of animal physiology and animal nutrition. 97, 405-430.

Monod, J., 2012. The growth of bacterial cultures. in: Ullman, A. (Ed.), Selected Papers in Molecular Biology by Jacques Monod, Pasteur Institute, Paris, France, pp. 139.

Mootian, G.K., Flimlin, G.E., Karwe, M.V., Schaffner, D.W., 2013. Inactivation of *Vibrio parahaemolyticus* in Hard Clams (Mercanaria mercanaria) by High Hydrostatic Pressure

(HHP) and the Effect of HHP on the Physical Characteristics of Hard Clam Meat. Journal of Food Science. 78, E251-E257.
Moriarty, D.J., 1999. Disease control in shrimp aquaculture with probiotic bacteria, Proceedings of the 8th International Symposium on Microbial Ecology, pp. 237-243.
Narang, A., Pilyugin, S.S., 2007. Bacterial gene regulation in diauxic and non-diauxic growth. Journal of Theoretical Biology. 244, 326-348.
Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C., Clay, J., Folke, C., Lubchenco, J., Mooney, H., Troell, M., 2000. Effect of aquaculture on world fish supplies. Nature. 405, 1017-1024.
Ninawe, A., Selvin, J., 2009. Probiotics in shrimp aquaculture: avenues and challenges. Critical reviews in microbiology. 35, 43-66.
Nunan, L., Lightner, D., Pantoja, C., Gomez-Jimenez, S., 2014. Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. Diseases of Aquatic Organisms.
Nunan, L.M., Poulos, B.T., Lightner, D.V., 1998. The detection of White Spot Syndrome Virus (WSSV) and Yellow Head Virus (YHV) in imported commodity shrimp. Aquaculture. 160, 19-30.
Percival, S.L., Williams, D.W., 2014. Chapter Twelve - Vibrio. in: Gray, S.L.P.V.Y.W.W.M.C.F. (Ed.), Microbiology of Waterborne Diseases (Second Edition). Academic Press, London, pp. 237-248.
Pomeroy, R.S., Parks, J.E., Balboa, C.M., 2006. Farming the reef: is aquaculture a solution for reducing fishing pressure on coral reefs? Marine Policy. 30, 111-130.
Pope, E.C., Powell, A., Roberts, E.C., Shields, R.J., Wardle, R., Rowley, A.F., 2011. Enhanced cellular immunity in shrimp (*Litopenaeus vannamei*) after 'vaccination'. Plos one. 6, e20960.
Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S., Menasveta, P., 1998. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture. 167, 301-313.
Rowley, A.F., Pope, E.C., 2012. Vaccines and crustacean aquaculture—A mechanistic exploration. Aquaculture. 334, 1-11.
Saoud, I.P., Davis, D.A., Rouse, D.B., 2003. Suitability studies of inland well waters for *Litopenaeus vannamei* culture. Aquaculture. 217, 373-383.
Senapin, S., Phewsaiya, K., Briggs, M., Flegel, T.W., 2007. Outbreaks of infectious myonecrosis virus (IMNV) in Indonesia confirmed by genome sequencing and use of an alternative RT-PCR detection method. Aquaculture. 266, 32-38.
Shan, H., Obbard, J., 2001. Ammonia removal from prawn aquaculture water using immobilized nitrifying bacteria. Applied Microbiology and Biotechnology. 57, 791-798.
Smith, V.J., Roulston, C., Dyrnda, E.A., 2010. The Shrimp Immune System. in: Alday-Sanz, V. (Ed.), The Shrimp Book. Nottingham University Press.
Su, Y.-C., Liu, C., 2007. *Vibrio parahaemolyticus:* a concern of seafood safety. Food microbiology. 24, 549-558.
Sung, H.-H., Hsu, S.-F., Chen, C.-K., Ting, Y.-Y., Chao, W.-L., 2001. Relationships between disease outbreak in cultured tiger shrimp (*Penaeus monodon*) and the composition of *Vibrio* communities in pond water and shrimp hepatopancreas during cultivation. Aquaculture. 192, 101-110.
Sutton, S., 2011. Measurement of microbial cells by optical density. J. Validation Techn. 17, 47-49.

Tlusty, M., 2002. The benefits and risks of aquacultural production for the aquarium trade. Aquaculture. 205, 203-219.

Tran, L., Nunan, L., Redman, R.M., Mohney, L.L., Pantoja, C.R., Fitzsimmons, K., Lightner, D.V., 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis. Aquat. Organ. 105, 45-55.

Vaseeharan, B., Ramasamy, P., 2003. Control of pathogenic Vibrio spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Letters in applied microbiology. 36, 83-87.

Wang, Y., Fu, L., Lin, J., 2012. Probiotic (*Bacillus coagulans*) Cells in the Diet Benefit the White Shrimp *Litopenaeus vannamei*. Journal of Shellfish Research. 31, 855-860.

Wang, Y.-B., Li, J.-R., Lin, J., 2008. Probiotics in aquaculture: Challenges and outlook. Aquaculture. 281, 1-4.

Weber, J., Mintz, E., Canizares, R., Semiglia, A., Gomez, I., Sempertegui, R., Davila, A., Greene, K., Puhr, N., Cameron, D., 1994. Epidemic cholera in Ecuador: multidrug–resistance and transmission by water and seafood. Epidemiology and infection. 112, 1-11.

Witteveldt, J., Vlak, J.M., van Hulten, M.C.W., 2004. Protection of *Penaeus monodon* against white spot syndrome virus using a WSSV subunit vaccine. Fish & Shellfish Immunology. 16, 571-579.

Yang, F., He, J., Lin, X., Li, Q., Pan, D., Zhang, X., Xu, X., 2001. Complete genome sequence of the shrimp white spot bacilliform virus. Journal of virology. 75, 11811-11820.

Zhou, J., Fang, W., Yang, X., Zhou, S., Hu, L., Li, X., Qi, X., Su, H., Xie, L., 2012. A Nonluminescent and Highly Virulent *Vibrio harveyi* Strain Is Associated with "Bacterial White Tail Disease" of *Litopenaeus vannamei* Shrimp. PLoS ONE. 7, e29961.

Zorrilla, I., Arijo, S., Chabrillon, M., Diaz, P., Martinez‐Manzanares, E., Balebona, M., Morinigo, M., 2003. *Vibrio* species isolated from diseased farmed sole, Solea senegalensis (Kaup), and evaluation of the potential virulence role of their extracellular products. Journal of Fish Diseases. 26, 103-108.

Appendices

Information included in this section is from trials that were not included in the thesis. These trials provided valuable insight and areas of improvement for the trials that were incorporated into the manuscript.

Appendix A: Probiotic Studies Trial A.1 using Biosecurity Cabinet (BSC) Apparatus

Scope:

Goal is to establish a method that is consistent in exhibiting virulence in *L. vannamei* with *V. parahaemolyticus* and to establish a preventative method using probiotics

Set-up:

- Experiment will be conducted at the Virginia Tech Life Science I Building (Blacksburg, VA)
- EMS frozen culture (13-028/A3) was obtained from Arizona University (Tucson, AZ) and introduced to the shrimp via feed
- *L. vannamei* will be kept within bounds of optimal growth conditions: Salinity (25ppt) and temperature (28°C)
- 5 different test groups were used: TSB2+ (control), EMS (undiluted), 1/10 EMS, EMS(und)+Pro, 1/10 EMS + Pro
- Probiotic strain J will be used
- 5 shrimp per treatment, 1 shrimp per beaker
- 25 beakers were set up with 500 mL of 25ppt DI water
- 100mL of salinity 25ppt DI water was placed into the beaker daily to account for evaporation
- Water quality was measured in a separate preliminary experiment to ensure that ammonia and O_2 levels would not be a confounding factor
- 4-day toxicity exposure: looking at survival rates
- Pathology of shrimp will be examined during this period: shrimp will be preserved in Davidson's fixative upon mortality

Weighing Procedure:

- Shrimp were weighed in order to determine the approximate amount of feed to inoculate
- A negative weight method was utilized
    - Shrimp were placed into a plastic container with a towel lining the interior
    - An additional paper towel was placed on top of the container in order to prevent shrimp from escaping
    - Plastic container and shrimp were placed onto the scale and tared
    - The shrimp was then placed in the holding tank. Paper towels were kept from coming into contact with any surfaces especially water.

- The container and paper towels were then placed back on the scale to record "negative weight"

- The negative weight method was formed on the following sampling schemes:
  - 15 shrimp were selected randomly and weighed individually
  - The remainder were weighed on a group basis (how many you net and feel comfortable weighing at a time)
  - If the shrimp group weight was similar size to the individual weighed shrimp (i.e. within 95%), the feed weight (function of the mean weight) will be valid

Testing:

11/11/14 @ 9:00AM: 500mL of 25ppt DI water was transferred into beakers and shrimp were transferred into the holding tank

Shrimp were weighed and averaged (ex. 3.5 ± 0.65 grams) and fed at 3% body weight per shrimp (ex. 105mg)

11/11/14 @ 3:30 PM: Starter EMS culture (OD_{600}= 0.5) was placed into 30mL of TSB2+ making the starting OD_{600} ~ 0.02.

Starter freezer EMS culture was preserved by placing 1 mL of EMS strain grown to an OD_{600}= 0.5 into glycerol and frozen.

11/12/14 @ 9:30 AM:

- 1mL TSB2+
- 2mL EMS (undiluted)
- 2mL 1/10 EMS

- Overnight EMS culture was grown for 18 hours while aerating at 30°C
- 105µL of each treatment was added to the food at a 1:1 ratio
- Probiotic shrimp were fed probiotic feed inoculated with EMS while nonprobiotic (TSB2+, EMS (undiluted), and 1/10 EMS) shrimp were fed non-probiotic feed inoculated with EMS

11/12/14 @ 10:00AM: First inoculation

Table A.1. *Average shrimp weights and measured feed (3% body weight of average shrimp weight) used in Trial A.1.*

	Non-Probiotic (TSB2+, 1:10EMS, 1:100EMS)	**Probiotic**
Avg. Shrimp Wt. (g)	3.88	1.96
Feed Wt. (mg)	116	58.8

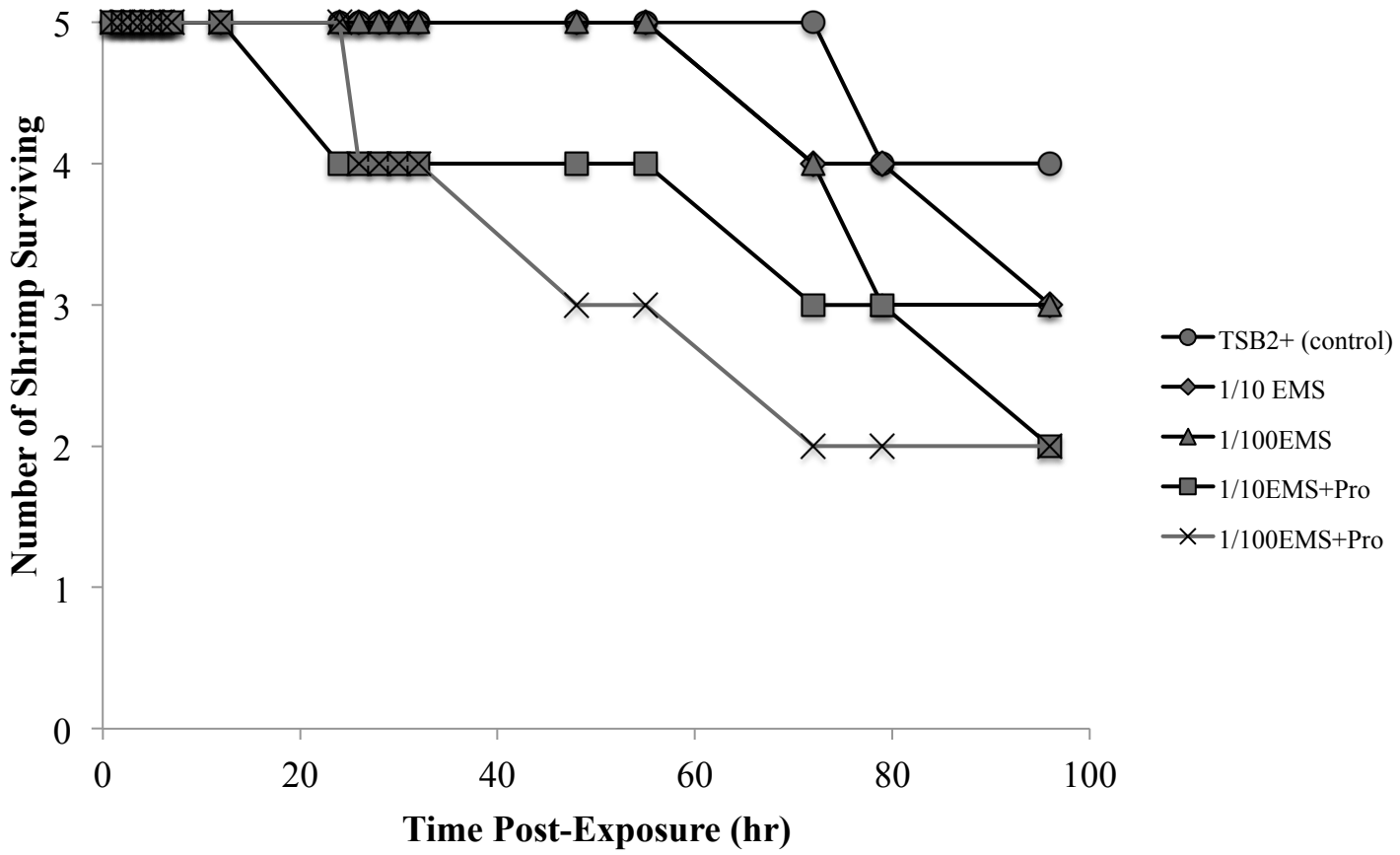

Figure A.1. *Survival rate comparison of non-probiotic fed and probiotic-fed shrimp exposed to 1/10 diluted EMS and 1/100 diluted EMS exposure in Trial A.1. Values are % of n=5 replicate analyses.*

Appendix B: Probiotic Studies Trial A.2 using BSC Apparatus

Scope:

Goal is to establish a method that is consistent in exhibiting virulence in *L. vannamei* with *V. parahaemolyticus* and to establish a preventative method using probiotics

Set-up:

- Experiment will be conducted at the Virginia Tech Life Science I Building (Blacksburg, VA)
- EMS frozen culture (13-028/A3) was obtained from Arizona University (Tucson, AZ) and introduced to the shrimp via feed
- *L. vannamei* will be kept within bounds of optimal growth conditions: Salinity (25ppt) and temperature (28°C)
- 5 different test groups were used: TSB2+ (control), EMS (undiluted), 1/10 EMS, EMS(und)+Pro, 1/10 EMS + Pro
- Probiotic strain J will be used
- 5 shrimp per treatment, 1 shrimp per beaker
- 25 beakers were set up with 500 mL of 25ppt DI water
- 100mL of salinity 25ppt DI water was placed into the beaker daily to account for evaporation
- Water quality was measured in a separate preliminary experiment to ensure that ammonia and O_2 levels would not be a confounding factor
- 4-day toxicity exposure: looking at survival rates
- Pathology of shrimp will be examined during this period: shrimp will be preserved in Davidson's fixative upon mortality

Weighing Procedure:

- Shrimp were weighed in order to determine the approximate amount of feed to inoculate
- A negative weight method was utilized
    - Shrimp were placed into a plastic container with a towel lining the interior
    - An additional paper towel was placed on top of the container in order to prevent shrimp from escaping
    - Plastic container and shrimp were placed onto the scale and tared
    - The shrimp was then placed in the holding tank. Paper towels were kept from coming into contact with any surfaces especially water.

- The container and paper towels were then placed back on the scale to record "negative weight"

- The negative weight method was formed on the following sampling schemes:
  - 15 shrimp were selected randomly and weighed individually
  - The remainder were weighed on a group basis (how many you net and feel comfortable weighing at a time)
  - If the shrimp group weight was similar size to the individual weighed shrimp (i.e. within 95%), the feed weight (function of the mean weight) will be valid

Testing:

11/11/14 @ 9:00AM: 500mL of 25ppt DI water was transferred into beakers and shrimp were transferred into the holding tank

Shrimp were weighed and averaged (ex. 3.5 ± 0.65 grams) and fed at 3% body weight per shrimp (ex. 105mg)

11/11/14 @ 3:30 PM: Starter EMS culture (OD_{600}= 0.5) was placed into 30mL of TSB2+ making the starting OD_{600} ~ 0.02.

Starter freezer EMS culture was preserved by placing 1 mL of EMS strain grown to an OD_{600}= 0.5 into glycerol and frozen.

11/12/14 @ 9:30 AM:

- 1mL TSB2+
- 2mL EMS (undiluted)
- 2mL 1/10 EMS

- Overnight EMS culture was grown for 18 hours while aerating at 30°C
- 105µL of each treatment was added to the food at a 1:1 ratio
- Probiotic shrimp were fed probiotic feed inoculated with EMS while nonprobiotic (TSB2+, EMS (undiluted), and 1/10 EMS) shrimp were fed non-probiotic feed inoculated with EMS

11/12/14 @ 10:00AM: First inoculation

**Initial problem with differing shrimp weights so shrimp were split into 3 groups to minimize shrimp weight differences

Table B.1. *Average shrimp weights and measured feed (3% body weight of average shrimp weight) used in Trial A.2.*

	Control	**Probiotic**	**Non-Probiotic**
Avg. Shrimp Wt. (g)	1.25	2.45	3.00
Feed Wt. (mg)	37.4	73.4	90

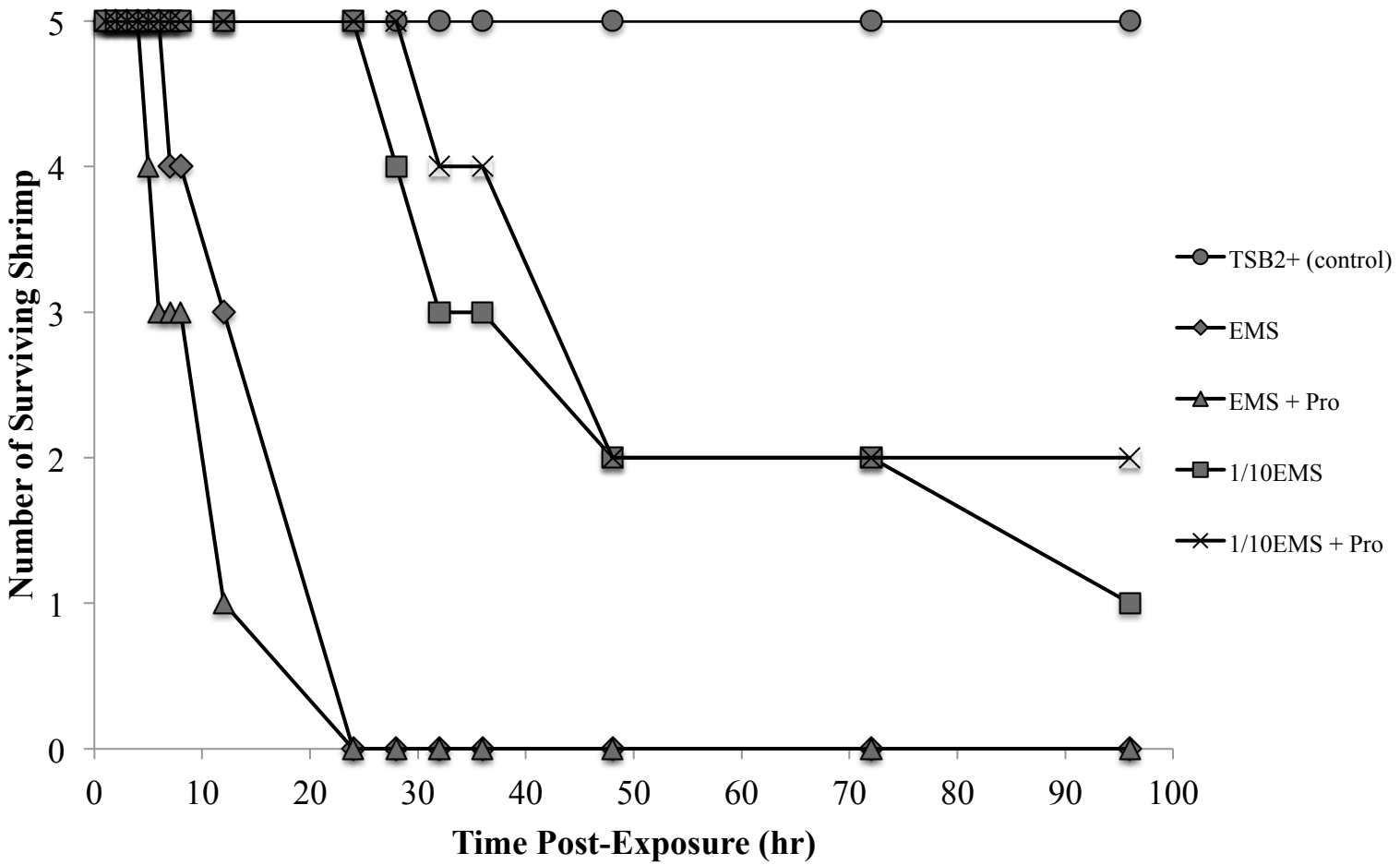

Figure B.1. *Survival rate comparison of non-probiotic fed and probiotic-fed shrimp exposed to undiluted and 1/10 diluted EMS in Trial A.2. Values are % of n=5 replicate analyses.*

Appendix C: Probiotic Studies Trial A.3 using BSC Apparatus

Scope:

Goal is to establish a method that is consistent in exhibiting virulence in *L. vannamei* with *V. parahaemolyticus* and to establish a preventative method using probiotics

Set-up:

- Experiment will be conducted at the Virginia Tech Life Science I Building (Blacksburg, VA)
- EMS frozen culture (13-028/A3) was obtained from Arizona University (Tucson, AZ) and introduced to the shrimp via feed
- *L. vannamei* will be kept within bounds of optimal growth conditions: Salinity (25ppt) and temperature (28°C)
- 5 different test groups were used: TSB2+ (control), 1/10 EMS, 1/100 EMS, 1/10 EMS + Pro, 1/100 EMS + Pro
- Probiotic strain K will be used
- 5 shrimp per treatment, 1 shrimp per beaker
- 1 additional beaker will serve as a positive control; shrimp will be fed feed inoculated with undiluted EMS
- 26 beakers were set up with 500 mL of 25ppt DI water
- 100mL of salinity 25ppt DI water was placed into the beaker daily to account for evaporation
- Water quality was measured in a separate preliminary experiment to ensure that ammonia and O_2 levels would not be a confounding factor
- 4-day toxicity exposure: looking at survival rates
- Pathology of shrimp will be examined during this period: shrimp will be preserved in Davidson's fixative upon mortality

Weighing Procedure:

- Shrimp were weighed in order to determine the approximate amount of feed to inoculate
- A negative weight method was utilized
    - Shrimp were placed into a plastic container with a towel lining the interior
    - An additional paper towel was placed on top of the container in order to prevent shrimp from escaping
    - Plastic container and shrimp were placed onto the scale and tared

- The shrimp was then placed in the holding tank. Paper towels were kept from coming into contact with any surfaces especially water.
- The container and paper towels were then placed back on the scale to record "negative weight"

- The negative weight method was formed on the following sampling schemes:
  - 15 shrimp were selected randomly and weighed individually
  - The remainder were weighed on a group basis (how many you net and feel comfortable weighing at a time)
  - If the shrimp group weight was similar size to the individual weighed shrimp (i.e. within 95%), the feed weight (function of the mean weight) will be valid

Testing:

11/17/14 @ 9:00AM: 500mL of 25ppt DI water was transferred into beakers and shrimp were transferred into the holding tank

Shrimp were weighed and averaged (ex. 3.5 $\pm$ 0.65 grams) and fed at 3% body weight per shrimp (ex. 105mg)

11/17/14 @ 3:30 PM: Starter EMS culture (OD_{600}= 0.5) was placed into 30mL of TSB2+ making the starting OD_{600} ~ 0.02.

Starter freezer EMS culture was preserved by placing 1 mL of EMS strain grown to an OD_{600}= 0.5 into glycerol and frozen.

11/18/14 @ 9:30 AM:

- 1mL TSB2+
- 1mL EMS (undiluted)
- 2mL 1/10 EMS
- 2mL 1/100 EMS

- Overnight EMS culture was grown for 18 hours while aerating at 30°C
- 105µL of each treatment was added to the food at a 1:1 ratio
- Probiotic shrimp were fed probiotic feed inoculated with EMS while nonprobiotic (TSB2+, EMS (undiluted), 1/10 EMS and 1/100 EMS) shrimp were fed non-probiotic feed inoculated with EMS

11/12/14 @ 10:00AM: First inoculation

Table C.1. *Average shrimp weights and measured feed (3% body weight of average shrimp weight) used in Trial A.3.*

	Non-Probiotic	**Probiotic**
Avg. Shrimp Wt. (g)	2.72	3.02
Feed Wt. (mg)	81.6	90.5

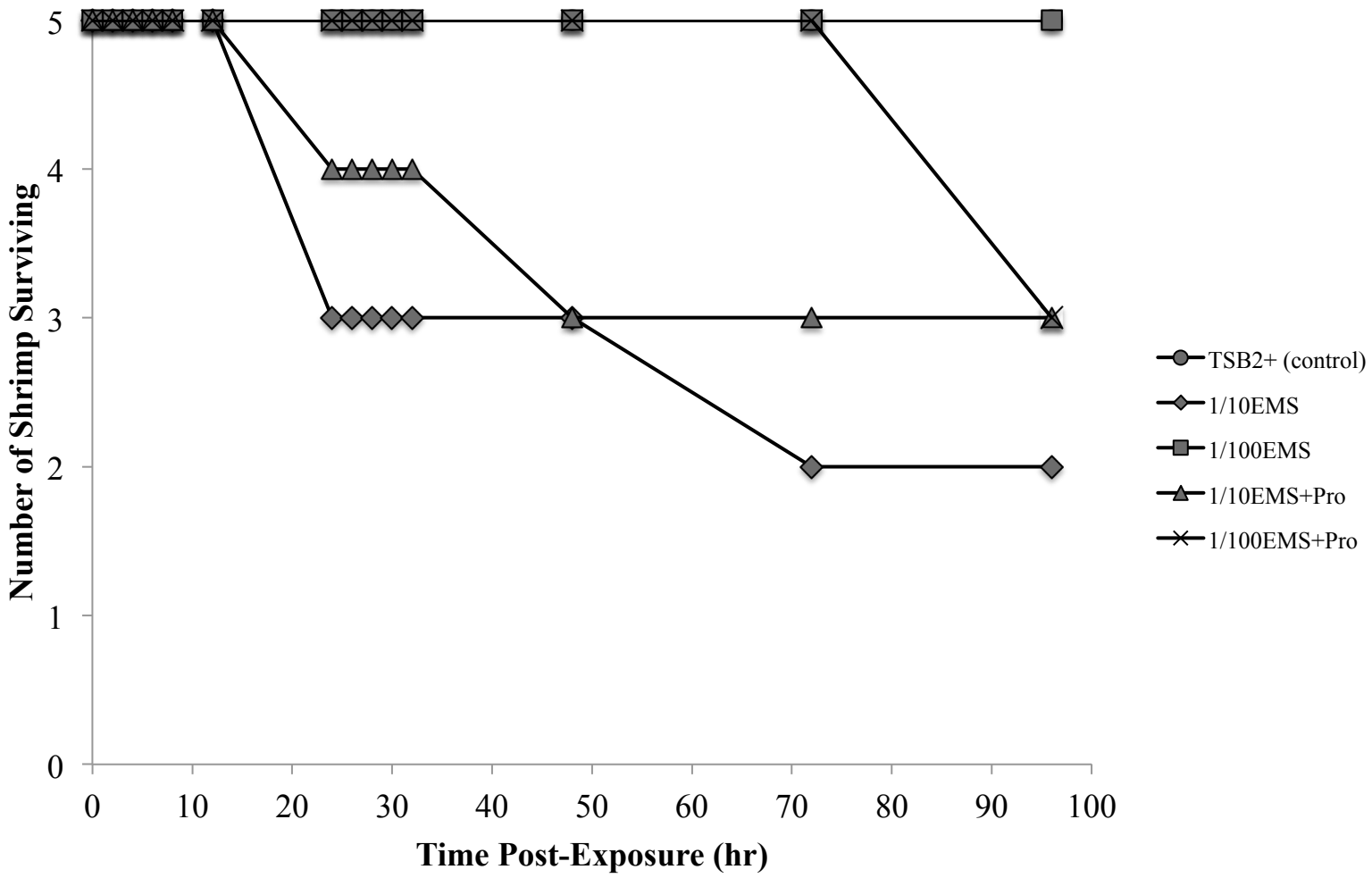

Figure C.1. *Survival rate comparison of non-probiotic fed and probiotic-fed shrimp exposed to 1/10 and 1/100 diluted EMS in Trial A.3. Values are % of n=5 replicate analyses.*

In Trials A.1, A.2, A.3, probiotic diets were used to determine if there was an effect on promoting disease resistance against EMS using a shrimp model. However, there was a problem with varying shrimp sizes (probiotic vs. non-probiotic) that were used especially in Trial A.1. Non-probiotic fed shrimp were almost twice the size of probiotic-fed shrimp causing a standardization issue. Another problem was trying to determine an EMS dilution factor that would be sufficient to use during the probiotic studies. In Trial A.1, the 1/10 diluted and 1/100 diluted EMS were used, however the problem with this trial was whether the undiluted EMS would have caused acute toxicity in addition to problem of the varying shrimp sizes used. In Trial A.2, undiluted EMS was used, however, this dilution factor was too strong as results have shown in previous trials that this dilution factor caused acute toxicity making it difficult to be used for probiotic evaluation. At least one beaker with shrimp exposed to EMS was necessary for trials to ensure the undiluted EMS would have caused mortality. As a solution to this problem, a "canary" beaker was used in Trial A.3. The shrimp in the "canary" beaker was the only shrimp fed undiluted EMS. If the "canary" shrimp exhibited mortality within 30 hours, it could be determine that the undiluted EMS used to inoculate feed was virulent.

Another problem in Trials A.1, A.2, and A.3 was that shrimp were fed only once in the beginning of the trial. Probiotic studies have shown that probiotics must be fed to animals as a daily routine or the probiotics will be secreted eventually. However, the biosecurity (BSC) apparatus does not have an adequate filtration system to support daily feeding of shrimp. Another issue with these trials was that a small volume of water was used with a high concentration of EMS at one time point that was known to cause acute toxicity in shrimp. It is unrealistic that shrimp would be exposed to EMS at such a high concentration at one time point. In order to have a more realistic approach, EMS should be exposed to shrimp on a daily basis but again, the same problem is experienced where the BSC apparatus does not have a sufficient filtration system to support this concept.

In order to counter these problems, there was a switch from using the BSC apparatus setup to a much larger setup using tanks. Using the larger scale method instead of the BSC apparatus allowed more shrimp to be evaluated with constant feeding as the tanks had an efficient filtration system as well as more area for shrimp to thrive in.