# THE USE OF PROBIOTICS IN PACIFIC WHITE SHRIMP (*LITOPENAEUS VANNAMEI)* AQUACULTURE TO INCREASE GROWTH, SURVIVAL AND TO REDUCE GENE EXPRESSION OF HEAT SHOCK PROTEIN 70 WHILE SUBJECTED TO ACUTE THERMAL STRESS

by

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**Abstract**

Shrimp farmers face numerous challenges associated with the vulnerability of shrimp to disease and environmental stress. Pacific white shrimp (*Litopenaeus vannamei*) is one of the most commonly produced and high yielding cultured shrimp species. Growth and survival are two factors that dictate the overall production; probiotics which are 'beneficial bacteria,' can help improve both. Moreover, extreme temperatures are also a concern for shrimp production and can negatively impact growth and survival. Elevated temperatures can trigger the expression of Heat Shock Proteins (HSPs) in shrimp, which aid in repairing denatured proteins.

To assess the impact of probiotics on shrimp, two experiments were conducted. The first experiment, which consisted of three trials, analyzed the effect of probiotics on growth and survival of post-larvae (PL) shrimp. Initially, only one trial was intended, however, because there were setbacks, two additional trials were conducted. After multiple weeks, the remaining PL shrimp from each group were counted to ascertain survival, and group weighed to calculate growth. For trial one, the Media Control (MC) appeared to produce the fastest growth followed by ISO 11 and ISO 12. PL in the MC also appeared to have the highest survival at harvest. However, a mortality event was experienced during the first trial due to various confounding factors including excessive temperatures, feeding rates, and stocking density, which prompted the initiation of trial two. For trial two, ISO 5 seemed to have the fastest growth followed by the MC and ISO 11. Survival for the Negative Control (NC), along with ISO 11 and ISO 12 were

higher at the time of harvest compared to the MC and ISO 5. However, system design and sample size, in addition to other limiting factors, which influenced shrimp performance undermine the validity of the findings from trial two, so a third trial was planned. During the third trial, probiotics did not affect growth. At the time of harvest, it was found that nearly all containers contained the same number of shrimp, as nearly half of the shrimp from all experimental containers died during the first few days of the study. Due to the errors and issues experienced during all three trials, a definitive conclusion cannot be made regarding the true effectiveness of the applied probiotics on shrimp performance.

In the second experiment, the objective was to determine if any of the selected probiotics effect gene expression of HSPs in juvenile shrimp. Shrimp were divided into groups and assigned to one of 12 ((3 probiotics + 1 control) x 3 temperatures)) treatments. Shrimp were pre-exposed to assigned probiotics for six days prior to the planned temperature stress event. Shrimp were sampled in two phases, exposure and recovery, to measure HSP expression with reverse transcriptase PCR. Results were compared across treatments to determine which probiotic had the greatest effect on shrimp. During this study, the intention was to collect the hepatopancreas, because it is this organ which highly expresses and regulates gene function in shrimp. However, due to a mistake in organ identification, the foregut was extracted instead. In addition to the foregut, a section of the abdominal muscle was also collected. Also, due to improper handling, and contamination of tissue samples, there was a statistically significant difference found in the quality of RNA between the NC and ISO 11 treatments at different temperatures across all sampling periods. Similarly, there was a statistically significant difference found in the expression of the HSP 70 between the NC and ISO 11 treatment at different temperatures across all sampling periods, as a possible result of thermal stress and high level of contamination. Due

to the high level of contamination that was experienced during this study, a definitive conclusion cannot be made regarding the true effectiveness of the applied probiotics on mitigating shrimp stress.

Key Words: Pacific White Shrimp, Probiotics, Heat Shock Proteins, Growth, Survival

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# CHAPTER 1: INTRODUCTION

Shrimp production is a major sector of the aquaculture industry worldwide. The demand for shrimp has increased over time, which has led to the growth of the shrimp aquaculture industry. This is an important source of income for farmers throughout the world (Primavera 1997). Two commonly farmed species of shrimp are the Pacific white shrimp (*Litopenaeus vannamei*), and the giant tiger shrimp (*Penaeus monodon*) (Ronnback 2001). Historically, the Pacific white shrimp, also known as the white leg shrimp, was the primary shrimp species produced in the western hemisphere (Ronnback 2001). In addition, the adoption of Pacific white shrimp for commercial aquaculture production worldwide is attributed largely to the fact that they are not as vulnerable to diseases as are giant tiger shrimp (Ronnback 2001).

Shrimp farming faces numerous challenges, many of which are associated with shrimp's vulnerability to diseases and environmental stressors. Before the introduction of probiotics, farmers resorted to using a wide range of chemical therapeutics. Some of these chemicals led to unforeseen complications ranging from ecological problems, such as poor water quality, to disease outbreaks (Lakshmi *et al.* 2013). Such problems can result in limited growth and high mortality (Lakshmi *et al.* 2013). Commercial shrimp aquaculture began in the 1970s in Southeast Asia (Boyd and Clay 1998), though shrimp aquaculture techniques were developed in Japan, with the culture of Kuruma or Japanese tiger shrimp (*Marsupenaeus japonicus*) in the 1930s (Benzie 2009). These beginnings allowed for the growth of the shrimp industry in response to market demand for shrimp, which are relatively inexpensive, and a good source of protein (Lakshmi *et al.* 2013). Increasing demand for shrimp calls for a more efficient means of production, and a greater demand to keep them healthy.

Despite the economic importance of the shrimp farming industry, it is important to point out that this industry has had a storied history with respect to its environmental impact (Páez-Osuna 2001), which have resulted in development of more sustainable practices. One major shrimp farming practice that makes it unsustainable is the use of antimicrobials to prevent shrimp losses in order to maximize yields (Xue *et al.* 2015; Decamp *et al.* 2008; Gomez-Gill *et al.* 2000). Chemicals that were used by some farmers, including nitrofurans and chloramphenicol, have been prohibited due to their potential harmfulness to humans and the animals such as shrimp that were being cultured (Hassan *et al.* 2013). The use of these chemicals can have long-lasting impacts on the environment, and human health. Antibiotics can lead to disease resistance and the accumulation of excess antibiotic residues in shrimp (Bermúdez-Almada and Espinosa-Plascencia 2012). Because not all pathogens are killed off by exposure to antibiotics, it increases the likelihood of some pathogens surviving and developing antibiotic resistance (Moriarty 1999). The accumulation of excess antibiotics in shrimp tissues could alter human intestinal flora when consumed, which can cause foodborne illnesses and release toxins that cause shellfish allergens (Bermúdez-Almada and Espinosa-Plascencia 2012).

The overuse of antibiotics has resulted in increased governmental regulations, resulting in a need for aquaculture farmers to adopt more sustainable practices as the use of probiotics (Nayak 2010). The term '*probiotic*' refers to live microorganisms, which are believed to improve the health of humans and animals when consumed (Lakshmi *et al*. 2013). Therefore, probiotics can be introduced into shrimp production systems to aid in the regulation of disease management and outcompete pathogenic bacteria. In addition, probiotics could help to promote the growth of cultured organisms, as well as induce tolerance to environmental stressors, such as thermal stress (Farzanfar 2006).

All terrestrial and aquatic species exhibit some level of thermal tolerance (Gao *et al.* 2014; Portner 2010). In response to extreme environmental conditions, heat shock proteins (HSPs) are produced to help mitigate tissue damage. The denaturing or malfunction of a protein can occur due to heat stress or other stressors such as exposure to heavy metals or microbial contaminants (Schlesinger 1990). Therefore, the primary function of HSPs is to aid in the repair of denatured proteins (Feder and Hoffmann 1999). HSPs adhere to the malfunctioning protein and hydrolyze it by releasing nucleotides that help in protein synthesis, folding, and transportation of the affected protein back to its appropriate cellular compartment (Feder and Hoffmann 1999). The initial discovery and description of HSPs were made by Ritossa (1962) from *Drosophila busckii* (Rungrassameea *et al.* 2010). HSPs have been studied in various organisms ranging from bacteria to higher plant and animal species (Gao *et al.* 2014; Hoffmann *et al.* 2003; Feder and Hofmann 1999; Sorensen 2010).

When aquatic animals, such as shrimp, are exposed to extreme temperatures and other stressful conditions like hypoxia, toxins, or disease, HSP functions are enhanced (Gao *et al.* 2014; Feder and Hofmann 1999; Sorensen 2010; Sorensen *et al.* 2003). As with other animals, Pacific white shrimp express HSPs in response to environmental stress. Expression profiles of HSP genes in shrimp can be categorized by their molecular weight into six common families, HSP60, HSP70, HSP80, HSP90, HSP100, and HSP110 (Qian *et al.* 2012). Qian *et al.* (2012) tested the reaction of Pacific white shrimp to acute environmental stressors such as temperature, pH, and heavy metals. They concluded that HSP60 and HSP70 were the most commonly expressed HSPs, meaning they can serve as potential biomarkers to measure pH and thermal stress.

The current study consisted of two separate experiments to ascertain the effect of various probiotics on shrimp. The objective for the first study was to determine if selected probiotic strains could increase the growth and survival of post-larval shrimp. The objective for the second study was to determine if selected probiotic strains could mitigate stress while shrimp were subjected to acute thermal stress as measured by expression of HSP 70.

## CHAPTER 2: REVIEW OF LITERATURE

As the human population and demand for seafood increases, Pacific white shrimp has become one of the major farmed raised shrimp species with an annual production of 300 million metric tons (Li *et al.* 2018). This is partly due to the fact that Pacific white shrimp can be cultured under a wide range of environmental conditions such as low salinity levels (Li *et al.* 2017), and temperatures. The optimal temperature for this shrimp species ranges between 23 - 30 °C (Cheng *et al*. 2005; Wyban *et al.*1995). Also, because Pacific white shrimp do not require a high protein diet as compared to other shrimp species, they can adapt to diets containing different protein percentages (Correia *et al*. 2014; Hu *et al*. 2008), which results in them being cost-efficient. They also serve as good candidates for selective breeding due to their ability to spawn in captivity (Sui *et al*. 2016), which allows for relatively easy selection of desirable traits such as disease resistance. In addition, when cultured under optimal conditions, they can be reared in high stocking densities in recirculating systems as well as ponds. Although, several factors need to be considered when culturing this species. These factors includes making sure that larvae are pathogen free, shrimp are fed proper diets, and ensuring that aeration systems and water quality are maintained (Rengpipat *et al.* 2003).

As seafood production becomes more aquaculture dependent, there is a need to maximize yields while maintaining healthy animals. Antibiotics have been used for years to mitigate disease. However, the misuse, and overuse, of antibiotics can have a negative impact on the animals (Lui et al. 2017). According to Santos and Ramos (2016), one problem that arises from the over use of antibiotics is that susceptible pathogens can become antibiotic resistant. Due to such issues caused by the misuse and overuse of antibiotics, probiotics have been used as an alternative (Sequeiros et *al*. 2015). For a bacterium to be classified as a probiotic it must have

certain beneficial characteristics and meet certain criteria. A bacterium must be able to be ingested by the host and be able to colonize the digestive tract of the host (Hai 2015). Likewise, a bacterium must be able to survive and work efficiently within the host (Tan *et al*. 2016). In addition, bacteria should not be pathogenic or toxic to the host (Pérez-Sánchez *et al*. 2014). Probiotics have proven helpful when producing marine animals (Soundarapandian and Sankar 2008; Sissons 1989). Not only do probiotics enhance growth, improve water quality and immune system function, they can also prevent disease, aid in reproduction, help reduce stress, enhance the functioning of the digestive tract, and increase survival rates (Cruz *et al.* 2012). Probiotics have a high multiplying rate, which allows them to colonize and adhere to the GI tract for long periods where they can outcompete harmful bacteria (Cruz *et al.* 2012). This can contribute to increase survival and disease prevention. In addition, probiotics has the ability to synthesize extracellular enzymes such as protease and lipids and provide an extra source of vitamins and fatty acids to the host (Cruz *et al.* 2012). This allows nutrients to be absorbed more efficiently, which contributes to the effectiveness of the digestive tract. Applies probiotics can lower cortisol, lactate and plasma glucose levels, which becomes elevated under stressful conditions (Cruz *et al.* 2012). Furthermore, growth and survival are two major factors that dictate the overall shrimp production (Immanuel *et al.* 2007), and probiotics can be used to help improve both (Wang *et al.* 2008).

By achieving a balance between beneficial and harmful bacteria within the body of the target species, probiotics can help regulate overall immune health (Sekhon and Jariath 2010). It has been found that *Bacteroides spp.* and *Clostridium spp.* make significant contributions to the overall nutrition of the host by supplying vitamins and fatty acids (Rolfes *et al.* 2014). Other probiotic bacteria may also enhance digestion through the production of extracellular enzymes,

including proteases and lipases, which can make digestion faster and easier for the target species (Nayak 2010). Therefore, it is necessary to ensure that the correct probiotics are selected for use in aquatic environments because certain strains of probiotics have different effects (Kesarcodi-Watson *et al.* 2008). Probiotics can serve as biological agents that can aid in the promotion of optimal water quality and/or enhance shrimp performance (Angahar 2016). For example, *Bacillus spp*., a gram-positive bacterium, acts as an efficient converter of organic matter by consuming food waste. This bacterium has been widely studied as a probiotic and is shown to contribute positive benefits, such as increasing overall growth and survival rates, to treated organisms (Xue *et al.* 2015).

While there are many different bacteria species that have been used as probiotics in shrimp aquaculture, there are a few species that has proven more effective than others. *Lactobacillus plantarum* is used to enhance growth (Nguyen *et al*. 2018), survival (Zheng *et al*. 2018), and improve immune health (Dash *et al*. 2015). According to Vieria *et al.* (2016), when used as a dietary supplement, *L. plantarum* has increase survival, improved feed efficiency, and altered the intestinal microbiota in shrimp. Also, another study conducted by Bolivar-Ramirez *et al.* (2017) concluded that *L. plantarum* could increase survival and disease resistance against *Vibrio alginolyticus*. *Bacillus spp.* (Sadat et al. 2018; Hai 2015; Hong et al. 2005) and *Shewanella spp.* (Interaminense *et al.* 2018; Jiang et al. 2013) are two additional bacteria strains that have also been widely used as probiotics in shrimp. More specifically, *B subtillis* has been used in shrimp to enhance growth (Abdollahi-Arpanahi *et al.* 2018; Keysami *et al.* 2012), survival (Jamali *et al.* 2015), and to improve immune health (Zokaeifar *et al.* 2012; Shen *et al.* 2010). Similarly, the use of *S. algae* as a probiotic has increased in shrimp aquaculture (Ariole and Eddo 2015; Suantika *et al.* 2013; Shakibazadeh *et al.* 2008). This gram-negative bacterium

can serve as a probiotic due to its ability to improve disease resistance (Jiang *et al.* 2013) and increase survival (Interaminense *et al.* 2018).

In a marine environment, aquatic species are in direct contact with their environment, making them particularly vulnerable to environmental alterations. Water temperature changes are one of the most common abiotic stressors experienced by aquatic organisms (Huang *et al*. 2017; Long *et al*. 2013), whose body temperatures change to adapt to the surrounding environment (Feder and Hofmann 1999). Temperature stress is a concern for many aquatic species including shrimp. Temperature, along with salinity, can influence shrimp growth and survival (Kumlu *et al* 2010).

Exposure to elevated or low temperatures can trigger expression of HSPs in both aquatic animals and plants (Rungrassameea *et al*. 2010; Anderson *et al*. 1994; Sabehat *et al*. 1998). HSPs are produced in the hepatopancreas of shrimp as well as other tissues such as muscle tissue, and gills (Ceasar and Yang 2007). They can either be present continuously (constitutive expression) or be induced by stress (stress-inducible) through transcription and translation mechanisms (Whitley *et al*. 1999). A major role of HSPs is aiding in the repair of proteins that have been denatured from stress (Tomanek 2010), as such, they are an essential component of maintaining homeostasis within an organism (Qian *et al*. 2012). Because of this, HSPs are also classified as "molecular chaperones" (Wang *et al.* 2004), given that HSPs help ensure that protein folding is accurate and efficient. They help to ensure that the quantity of unravelled proteins are kept to a minimum (Finka *et al.* 2016). Although they are named "heat shock proteins," they have the capability to respond to other stressors as well, including extreme ion concentrations (Shi *et al*. 2016) and the depletion of cellular energy (Stracuh and Haslbeck 2016).

HSPs are grouped into functional families based on their molecular weight (Zhang et al. 2015). HSP 70 is the most commonly studied HSP as a physiological stress indicator (Hupal *et al.* 2018; Kültz 2003), because HSP 70 can be activated and respond to various environmental stress factors (Hupal *et al.* 2018; Lee and Vierling 2000). Numerous studies have shown that high expression of HSP 70 indicates that an organism has a high sensitivity to thermal stress (Hupal *et al.* 2018; Cottin *et al.* 2015; Morris *et al.* 2013; Tomanek and Somero 2000). Junprung *et al*. (2016) concluded that HSP 70 can defend the immune system of shrimp when shrimp are subjected to acute thermal stress and are exposed to *V. parahaemolyticus*. Sung *et al.* (2018) proved that the build-up and expression of HSP 70 in the caudal tissue of the shrimp can serve as a stress reducer when shrimp are exposed to various stressors such as heat, heavy metals, and ammonia. Moreover, exposing shrimp to probiotics may help regulate stress and therefore mitigate the expression of HSPs. Vaseeharan and Ramasamy (2003) pretreated shrimp with *B. subtills* for six days before subjecting them to *V. harveyi.* This pretreatment phase allowed for the probiotic to colonize within the shrimp so that the probiotic effective against *V. harveyi*, resulting in higher shrimp survival, and lower *V. harveyi* counts in treated shrimp than in control shrimp.

## CHAPTER 3: MATERIALS AND METHODS

Post larval (PL) Pacific white shrimp (ave wt. 0.0098105 g) were sourced from Shrimp Improvement Systems breeding and hatchery facility (Islamorada, FL) to conduct research studies at Delaware State University Aquaculture Research and Demonstration Facility (ARDF) in Dover, Delaware. Upon arrival, PL (n = 24,000) were acclimated to water conditions at ARDF before being stocked into four 2,850-L tanks (parent population). Water for use in the experiments was obtained from Indian River Inlet and stored in fiberglass holding tanks at the ARDF. Raw, full strength seawater (31.0 ± 2 ppt) was disinfected by chlorinating with 10 mg/L Cl, aerating for 24 hours, and then removing residual chlorine with sodium thiosulfate. This water was diluted to the working salinity with well water and used to fill experimental containers for both experiments.

Probiotics used during both studies were obtained from an ongoing study aimed at identifying probiotic strains from a euryhaline fish (*Fundulus heteroclitus*) and testing their application in other aquatic species. From this study, 230 isolates were obtained. Of these, ISO 5 and ISO 11 (*Bacillus spp.* strains), and ISO 12 (a *Shewanella spp.* strain) were the only three shown to have probiotic activity as evidenced by their ability to inhibit pathogen growth in disk diffusion assays. In addition to ISO 5, ISO 11 and ISO 12, a commercially available probiotic strain (CSX), a media control (MC); which is the glycerol media that the probiotic strains were cultured in, and a negative control (NC) were used. These probiotic strains were used for the current studies to determine if they could increase growth and survival of post-larval (PL) shrimp, and mitigate stress while shrimp were subjected to acute thermal stress.

### 3.1. The Effect of Probiotics on Growth and Survival of Pacific White Shrimp

Three growth and survival trials were conducted to analyze the effect of selected strains of probiotics on the growth and survival of Pacific white shrimp. Initially only one trial was planned. However, two additional trials were undertaken. This is because of husbandry issues, resulting in massive mortality that occurred during the first trial, which compromised the quality of the results. Table 1 outlines the specific conditions in each of the three trials.

Table 1. Experimental details of each of the three trials conducted to determine if specific probiotic strains have a beneficial effect on the growth and survival of post-larval shrimp when the probiotics are added to the culture water.

| **Experimental Details** | **Trial 1** | **Trial 2** | **Trial 3** |
|---|---|---|---|
| Duration (days) | 22 | 28 | 28 |
| Density (PL/L) | 25 | 1 | 8 |
| Average Stocking Size (g) | 0.010 | 1.13 | 0.20 |
| Feed Rate (% biomass/day) | 25 | 5 | 10 |
| # Feedings/Day | 4 | 3 | 3 |
| Salinity (ppt) | 10.0 ± 2 | 10.0 ± 2 | 31.0 ± 2 |
| Water Exchange (%/day) | 25 | 75 | 90 |
| Water Quality Testing | Every other day | Every other day | Daily |
| Water Quality Parameters Measured | pH, Ammonia, and Nitrite | pH, Ammonia, and Nitrite | pH and Ammonia |
| Treatments Used | MC, NC, ISO 5, ISO 11, and ISO 12 | MC, NC, ISO 5, ISO 11, and ISO 12 | NC, ISO 5, ISO 11, ISO 12, and CSX |
| Water Bath Used | No | No | Yes |
| Mortality Recorded | End of trial | End of trial | Daily |

Fifteen 2-L polycarbonate containers were used for each of the three trials, with individual experimental containers separated into five treatment groups. Each treatment group consisted of three containers, which were used as biological replicates. Given that the experiment consisted of four independent variables or treatment groups plus a negative control, a complete randomized design with multiple treatment groups was used during all three trials. Although, the randomization of the distribution of replicates for each probiotic treatment was an issue. For trials one and two, randomization was based on treatment groups where experimental containers were placed on a styrofoam surface and replicates of each treatment were grouped together (Fig. 1-A). This is was corrected during trial three, where both treatment groups and replicates were randomly distributed (Fig. 1-B). All experimental containers for trials one and two were filled with 1-L of prepared 10.0 ± 2 ppt seawater. For trial three, all experimental containers were filled with 1-L of sterilized seawater at 31.0 ± 2 ppt.

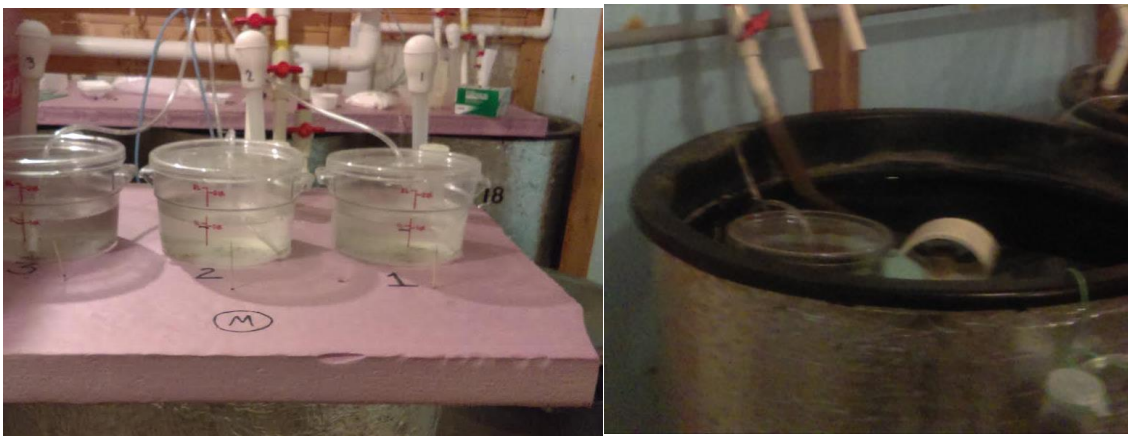


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Figure 1. A schematic showing the layout of the 2-L experimental containers used during the growth and survival trials.

Temperature in the experimental containers for trials one and two was controlled by adjusting ambient room temperature. Temperature of the individual experimental containers was not checked throughout the duration of the trials because they were found to be consistently 2 °C lower than the measured room temperature prior to the beginning of the study. For trials one and two, target water temperature was 28 °C. A glass thermometer was used to display room temperature during the first trial. A digital room thermometer was used during the second trial. In the third trial, experimental containers were randomly placed at the surface of individual 56-L water bath tanks conditioned at 28 °C to minimize temperature fluctuations within individual containers and across treatments (Fig. 1-B). The water bath tanks were part of a recirculating system, with its temperature controlled by an electric immersion heater. To support the experimental containers and limit their movement in the water bath tank, a Styrofoam ring was placed around each experimental. Aeration for all three trials was supplied to experimental containers by a common regenerative blower connected to an air distribution manifold. Each experimental container had one 1.23 cm x 4 cm ceramic air stone (AS1, Pentair Aquatic Eco-Systems, Apopka, FL).

Shrimp used in these trials were obtained from the parent population. A live group weight was taken from a sub-sample of 375 shrimp for the first trial, 15 shrimp during the second trial, and 120 shrimp for the third trial (Table 1). This was done to ascertain the average stocking size, and to determine the initial feed ratio. Individual shrimp in each experimental container were used as technical replicates. For trial one, 25 shrimp were placed into each experimental container in a random order. For trial two, one shrimp was placed into each experimental container in a random order. For trial three, eight shrimp were placed into each experimental container in a random order. During trial three, once shrimp were stocked, a one-week

acclimation period ensued. During this acclimation period, any mortalities were replaced with shrimp from the parent population. No acclimation period was granted for trials one and two.

For all three trials, shrimp in the experimental containers were fed a PL Raceway Plus (Shrimp Post Larvae Feed with VpakTM, 50% crude protein, 15% crude lipid; Zeigler Brothers, Inc., Gardners, PA). However, feed size and amount fed varied based on the size and number of animals in each trial. For trial one, shrimp were fed a #1 crumble at a rate of 17-25% of their total biomass four times a day. For trial two, shrimp were fed a #3 crumble diet at a rate of 10% of their total biomass. For trial three, shrimp were fed a #2 crumble diet at a rate of 5% of their total biomass. During trials two and three, shrimp were fed three times a day. Feed rates were adjusted weekly based on projected shrimp weights, which were calculated based on expected survival, and weighed samples from the parent shrimp population (Table 2). During trial one, the mortality for experimental containers was estimated based on an assumption made from the estimated weekly survival averages from the parent population. Survival estimates were not made during trial two, given that only one shrimp were stocked in each experimental container. For trial three, mortality in the experimental containers was recorded daily. This allowed for feed rates during trial three to be adjusted more accurately based on projected shrimp weights from the parent population and weekly survival averages from experimental containers.

Table 2. Weekly feed rates for *L. vannamei* based on projected weekly growth for three trials.

| | Feed Type | Sample Wt. (g) | Feed Rate (%) | Feed/Day (g) | # Feedings/day |
|---|---|---|---|---|---|
| **Week** | | | **Trial 1** | | |
| 0 | PL 1 | 0.0098105 | 25 | 0.0153 | 4 |
| 1 | PL 1 | 0.0288478 | 22 | 0.0389 | 4 |
| 2 | 50/50 | 0.0600675 | 20 | 0.0721 | 4 |
| 3 | PL2 | 0.1444038 | 20 | 0.1697 | 4 |
| **Week** | | | **Trial 2** | | |
| 0 | PL 3 | 1.0977 | 10 | 0.0024 | 3 |
| 1 | PL 3 | 2.1650 | 10 | 0.0048 | 3 |
| 2 | PL 3 | 2.5222 | 10 | 0.0056 | 3 |
| 3 | PL 3 | 2.9887 | 10 | 0.0066 | 3 |
| 4 | PL 3 | 3.0235 | 10 | 0.0067 | 3 |
| **Week** | | | **Trial 3** | | |
| 0 | PL 2 | 0.2033 | 5 | 0.0152 | 3 |
| 1 | PL 2 | 0.4519 | 5 | 0.0212 | 3 |
| 2 | PL 2 | 0.6334 | 5 | 0.0178 | 3 |
| 3 | PL 2 | 0.3257 | 5 | 0.0092 | 3 |
| 4 | PL 2 | 1.3856 | 5 | 0.0390 | 3 |

Water quality in the experimental containers was monitored regularly. Ammonia, nitrite, nitrate, hardness, alkalinity, and pH were measured every other day, during trials one and two; only ammonia and pH were measured during trial three. Ammonia was measured using LaMotte Insta- TEST® Strips (3023-G, LaMotte, Chestertown, MD). LaMotte 5-way Insta-TEST® Strips (3038-G, LaMotte, Chestertown, MD) were used to measure nitrite, nitrate, hardness, alkalinity, and pH during trials one and two. During trial three, pH was measured using LaMotte Insta-TEST® Wide Range pH Test Strips (2974, LaMotte, Chestertown, MD). In all three trials, water quality monitoring was conducted before daily water exchanges took placed.

Daily water exchanges were conducted on all experimental containers to minimize ammonia buildup and remove solid waste (excess feed, fecal material and molts). Following the water quality monitoring, water was siphoned from each experimental container, and replaced with fresh make-up water at the correct salinity (Table 1). For trial one, experimental containers

underwent a 25% daily water exchange; for trial two, experimental containers underwent a 50% daily water exchange; and for trial three, experimental containers underwent a 90% daily water exchange. For each trial, make up water of the correct salinity was held in a 121-L fiberglass holding tank in the study area and adjusted to ~28 °C with an aquarium heater (T11202, Hydor USA, Inc Sacramento, CA). Temperature in the holding tank was spot checked during trials one and two. Both holding tank temperature and the water bath temperature were checked daily in trial three. Temperatures in the holding tank and water bath were checked using an YSI 556 multi-probe meter (YSI Inc, Yellow Springs, OH).

Probiotics were added to the experimental containers to achieve target concentration of $10^6$ CFU/mL. In trials one and two, probiotic additions began one day after stocking. During trial three probiotics were not added until after the seven day acclimation period to ensure that all shrimp in this trial were exposure to the probiotics for the same length of time. For all three trials, an additional 0.1 ml (or 100 µl) aliquots of probiotics were added to the respective containers every other day after the daily water exchanges were completed to maintain the target concentration of $10^6$ CFU/mL.

Trial one was intended to run for eight weeks. However, shrimp were harvested at the end of three weeks due to a mass mortality event stemming from extreme temperatures, and excessive feed rates. As a result of the unexpected mortality, a decision was made to reduce the two succeeding trials from eight weeks to four weeks. Trial two used larger animals, and a reduced stocking density leading to issues with inadequate sample sizes given the experimental design. Due to the issues experienced during trial two, trial three used PL's similar in size to those used during trial one. Trial three also used a reduced stocking density. However, the

stocking density during trial three was lower than trial one and higher than trial three to eliminate issues of inadequate samples sizes.

The effect of the tested probiotics was determined by analyzing shrimp growth and survival rates. For each trial, all surviving shrimp in each experimental container were counted and group weighed at the time of harvest. After harvest, surviving shrimp from the first trial were euthanized, while surviving shrimp from trials two and three were transferred to a holding tank. Raw data (shrimp weights, survival, water quality, feeding, etc.) was entered into MS Excel (Microsoft Corp. Microsoft Excel for Windows 10. Redmond, WA) for archiving, data verification, calculating specific growth rates (SGR), and feed conversion ratios (FCR). SGR and FCR were calculated separately for each experimental container using the following equations:

$$SGR = \frac{(\ln(Harvest\ Weight\ (g)) - \ln(Stocking\ Weight\ (g)))}{Production\ Period * 100}$$

Where the harvest weight is the average weight of shrimp at the end of each study, and stocking weight was the average weight of shrimp at the beginning of the trial. The production period is the length in days of the duration of the trial.

$$FCR = \frac{TFI\ (g)}{ADG(g)}$$

Where TFI (Total Feed Intake) is the total weight of feed offered over the course of the trial, and ADG (Average Daily Gain) is calculated by subtracting the initial shrimp weight (g) from the average final weight (g) and dividing that value by the number of days the trial lasted.

All treatment averages were used to obtain overall descriptive data for each respective variable. Data were analyzed using SPSS (IBM Corp. IBM SPSS Statistics for Windows, Version 24. Armonk, NY). A one-way ANOVA was used to test for differences among treatments for FCR, SGR, and final size in all three trials, along with survival for trial one. Due

to the mass mortality event at the end of trial one, the NC and ISO 5 treatments each lost one of the three replicates. During trial two, two NC replicates along with one ISO 5 replicate had shrimp that escaped at different time points, so these replicates were excluded from statistical analysis. Also, during trial two, two MC replicates died a few hours prior to terminating this trial. Because they were not decomposed, they were still weighed to obtain an average final size for those treatment replicates and included in the statistical analysis. During the last week of trial three a CSX replicate was lost due to the shrimp escaping from the container. Therefore, there was no data collected for that container for analysis during that week. To combat the issue of missing data from trial one and three, the mean substitution method (Bennett 2001) was used to fill in the blanks for missing size, water quality parameters (ammonia and pH), and FCR data points. A Chi-square test was used to test for differences among treatments in survival for trial two. For trial three, survival was analyzed by using the Kaplan-Meier survivor analysis test. In addition, the log-rank (Mantel-Cox) test was used to determine if there were any significant differences for survival throughout the trial. Homogeneity of the variance was tested for all three trials by using a Leven Test with a p-value of 0.05. Normality for all three trials was checked using a Shapiro-Wilk test with a significance value of 0.05. A generalized linear model was used to test for differences in water quality parameters such as pH, ammonia, and nitrite.

### 3.2. The Effect of Probiotics on the Expression of Heat Shock Protein 70 in Pacific White Shrimp Subjected to Acute Environmental Stress

An 18-hour trial was conducted to determine if select strains of probiotics could alter the expression of HSPs in shrimp under acute thermal stress. To assess this, expression of HSP 70 was measured over time: before, during, and after a period of extreme temperature stress. Thirty-six 19-L experimental containers were used for this experiment, with each container representing one combination of temperature (n = 3) and probiotic (n = 4) with triplicates of each combination. Probiotic treatments that were used were ISO 11, ISO 12, and a commercially available probiotic strain (CSX), plus a negative control (NC), and temperature treatments were 28, 32, and 36 °C.

Four hundred juvenile shrimp (mean 5.00 ± 0.356 g), were obtained from the parent population, and divided among four 88-L probiotic inoculation tanks filled with prepared seawater at 10.0 ± 2 ppt. Shrimp were kept in the inoculation tanks for six days to undergo a pretreatment acclimation phase. The pretreatment acclimation phase had to be repeated. During the initial attempt tanks experienced elevated temperatures (>30.0 °C), and deteriorating water quality. These issues were controlled and maintained during the second attempt. During the pretreatment inoculation period, ammonia and pH were checked daily to ensure these parameters were within a tolerable range for the shrimp. Inoculation tanks underwent a 90% daily water exchange to help maintain water quality. After draining, tanks were then refilled with filtered 10.0 ± 2 ppt seawater obtained from the parent population holding system. Once tanks were refilled with the seawater, respective tanks were dosed with 8.8 ml (880 µl) aliquots of probiotics every other day to maintain the target concentration of $10^6$ CFU/mL. Temperature in the inoculation tanks was maintained at 28 °C with electric aquarium heaters (T11202, Hydor USA, Inc Sacramento, CA). While in the inoculation tanks, shrimp were fed a maintenance diet (PL

Raceway Plus, Zeigler Brothers, Inc., Gardners, PA) at 5% biomass per day offered in two feedings.

Thirty six 19-L experimental containers were filled with 15-L of conditioned 10.0 ± 2 ppt seawater, and 12 were assigned to each of three temperature treatments groups (28, 32, or 36 °C), and placed into one of three 1,514-L water baths to maintain treatment temperature (Figure 2). Each water bath was fitted with a 208-volt immersion heater (ESA-1811-P1, Process Technology, Mentor, OH). Air was supplied to each experimental container by one ceramic 7.6 x 3.8 cm air stone (ASW885, Pentair Aquatic Eco-Systems, Apopka, FL) connected to a central air system.

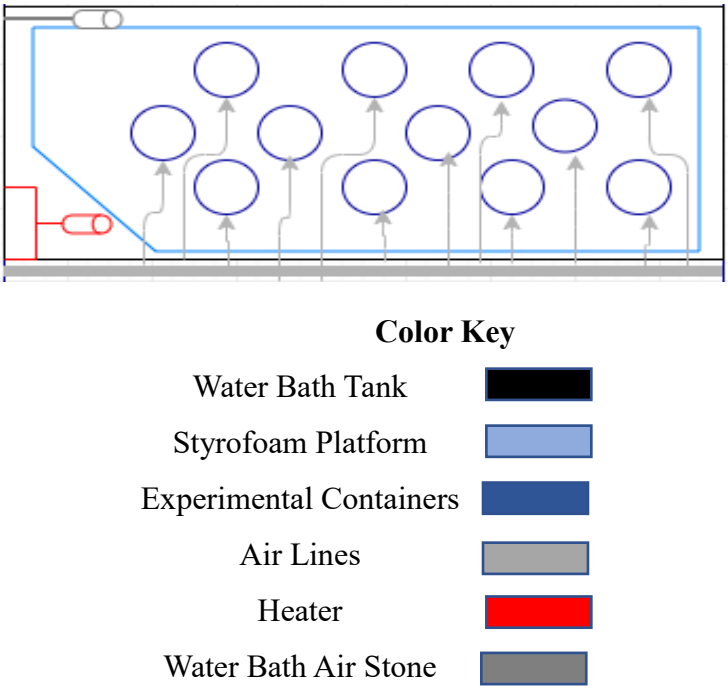


Figure 2. A schematic showing the layout of the 19-L experimental containers in one of the 1,514-L water bath tanks.

After experimental containers were acclimated to the respective treatment temperatures, they were randomly assigned to one of four probiotic treatment groups (ISO 5, ISO 11, ISO 12, and *CSX*), using a completely randomized block design (3 temperatures x 4 probiotics). There were three containers per probiotic treatment in each water bath to serve as biological replicates. Experimental containers were then dosed with 1.5 mL of prepared probiotic stock to achieve a final concentration of $10^6$ CFU/mL. Given the short trial period, shrimp in the experimental containers were not fed, and water exchanges were not conducted. Following the six-day probiotic inoculation period, experimental containers were at their target temperatures, and were dosed with probiotics, shrimp were stocked. Shrimp were transferred from each of the four inoculation tanks and divided into 3 groups of 12, where 10 shrimp were placed in the respective experimental containers (4 probiotic treatments x 3 treatment temperatures x 3 replicates = 36 experimental containers in total) (Figure 2). The 10 shrimp in each experimental container were used as technical replicates.

Expression of HSP 70 in shrimp was sampled during two phases: exposure and recovery. During the exposure period, one shrimp from each experimental container was sampled at four time points (0, 2, 4, and 6 hours of exposure to acute thermal stress), allowing expression of HSP 70 to be assessed over time. For the 0 hour time point, three shrimp were sampled directly from each of the 88-L probiotic inoculation tanks. Similarly, to assess recovery post- thermal stress exposure, shrimp were sampled at six and 12 hours after being returned to pre-stress holding temperatures. This was accomplished by transferring all of the 19-L experimental containers from the treatment water baths to a common 5,700-L water bath. The temperature for the recovery phase was intended to be the same as the control temperature of 28 °C, however, the heater for the recovery water bath was not turned on in enough time to sufficiently raise the

temperature. As such, the temperatures of recovery water bath tanks were between 24 °C and 26 °C throughout the recovery sampling period. The amount of time needed for the water in the experimental containers to adjust to the water bath temperatures was measured beforehand to ensure that the temperature of the experimental containers acclimated to the desired temperatures within the allotted sampling time frame. The water adjustment period was recorded to be 1.5 hours.

At each sampling point (0, 2,4, 6,1 2, and 18 hours after exposure), collected shrimp were individually weighed, and then euthanized by being injected with 0.5 mL of RNAlater solution (Ambion, Inc., Austin, Texas) into the muscle tissue to stop and preserve the expression of the HSP 70. Individual shrimp were then placed into individual plastic bags and held on ice at 4 °C until further processing. At each given sampling point, after all of the shrimp were euthanized, individual shrimp were dissected with a sterilized scalpel to obtain tissue samples. Our intention was to collect the hepatopancreas, because it is this organ which highly expresses and regulates gene function in shrimp. However, due to a mistake in organ identification, the foregut was extracted instead. In addition to the foregut, a section of the abdominal muscle was also collected. Collected tissue samples were placed into individual 2-mL vials filled with RNAlater. The vials containing the RNAlater solution and tissue sample were then stored at -20°C until they could be analyzed.

Total RNA was extracted from preserved shrimp tissues using the Trizol reagent method as described by Chomczynski and Mackey (1995) using the manufacturer's protocol. The quality of the extracted RNA was analyzed by placing 1 µL of the extracted RNA onto a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The Nanodrop reveals the purity of the extracted RNA samples, which indicates if the RNA samples qualifies to

be used for PCR analysis. The quantity of extracted RNA was determined by mixing 2 µL of the RNA with 3 µL of loading dye and loading the mixture onto a 1% agarose gel block (60 ml of 1 X TAE, 0.6 g of Agarose) to perform electrophoresis. DNase (3 µL of RNA template, 30 µL of TURBO DNase buffer, 1 µL of DNase) was used as a first step to remove excess DNA that may be left in the RNA samples. Following the DNase step, cDNA synthesis was then performed on RNA samples using the manufacturer's protocol (Proto-Script II First Strand cDNA Synthesis Kit, New England Bio-Labs Inc., Ipswich, MA). To confirm that all RNA samples were rid of genomic DNA, negative controls (RNA samples without a reverse transcriptase enzyme) and a positive control (genomic DNA) were used, and analyzed by PCR using β actin primers. Extracted RNA was stored at -80 °C and cDNA samples were stored at -20 °C for later use.

To measure the expression of HSP 70, reverse transcriptase PCR or RT-PCR was conducted on preserved RNA samples. A 330-amplicon primer for HSP 70 (GenBank accession no. EF495128.1) and a 260-amplicon primer for β- actin (GenBank accession no. AF300705.2) as the housekeeping gene (Wu et al. 2008) were used to run the analysis (Table 3). The β-actin was used to further determine the quality of the isolated RNA. The expression of β-actin reveals that the extracted RNA is well compose and is of good quality, whereas, the lack of gene expression indicates RNA degradation. An RT-PCR analysis was prepared using a 25 µL reaction procedure using the manufacture's protocol. The reactions were carried out using a 96 well sequence Thermal Cycler (Applied Biosystems, Foster City, CA). Thermal Cycler conditions used were as followed: 95 °C for 3 mins, followed by 34 cycles of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 1 min.

Once RT-PCR products were analyzed on an agarose gel, RNA band amplification was scored using the following symbols: '+' for amplification, and '-'for no amplification, and

assigned to numbers: '1' for amplification, '0' for no amplification which were used for statistically analysis. Raw data was archived in MS Excel (Microsoft Corp. Microsoft Excel for Windows 10. Redmond, WA). All data sets were analyzed with SPSS (IBM Corp. IBM SPSS Statistics for Windows, Version 24. Armonk, NY). A repeated-measures ANOVA (rANOVA) was used to determine if there were any statistically significant differences between the four probiotic strains and the covariates (temperature). Scores from band amplification and band quality were used to conduct this analysis. However, given the aim of this study, using a rANOVA is not an appropriate statistical analysis to use; which is explained in further detail in chapter five. Homogeneity of variance was tested for the generated scores by using a Leven Test ($\alpha = 0.05$), and normality was checked using a Shapiro-Wilk test ($\alpha = 0.05$).

Table 3. Primer sequences used in reverse transcriptase PCR to amplify HSP 70 and β - actin genes from *L. vannamei* tissues after exposure to extreme temperatures reared with and without the presence of probiotics.

| **Primer** | **Sequence 5'-3'** | **Amplicon size (bp)** |
|---|---|---|
| HSP 70 Froward | CCT GCC TAC TTC AAC GAT TCT | 330 |
| HSP 70 Reverse | GTT CTC ACT TGG GTC CTT CTT | |
| β - actin Forward | TCC ACG AGA CCA CCT ACA A | 260 |
| β - actin Reverse | CTG CTT GCT GAT CCA CAT CT | |

Accession no. EF495128.1 (HSP 70), and AF300705.2 ($\beta$ – actin) (Wu et al. 2008).

# CHAPTER 4: RESULTS

## 4.1. The Effect of Probiotics on Growth and Survival of Pacific White Shrimp

Shrimp performance metrics for all three trials are displayed in Table 4. During trial one, it appeared as if the MC had the highest growth with an average final size of 3.2 g, followed by ISO 11 and ISO 12 with an average final size of 1.2 and 1.5 g, respectively. A one-way ANOVA performed on the final shrimp size data for trial one showed that there was a statistically significant difference among treatments ($p = 0.011$) (Table 5). Final size of shrimp in MC was statistically higher/lower than the NC ($p = 0.009$) and ISO 5 ($p = 0.021$). There were no statistically significant differences found among treatments for SGR ($p = 0.165$) or FCR ($p = 0.143$) for trial one. Similarly, at the time of harvest, it appeared as though the MC had the highest survival (76%). The four remaining treatments had survival less than 50%, in which the NC had the least amount of surviving shrimp resulting in 16% survival ($p = 0.251$). A one-way ANOVA was performed on the final survival data and showed that there were no statistically significant differences among treatments ($p = 0.097$). During trial one, the overall survival average was 37%.

Table 4. Overall means ± standard errors of the mean of performance metrics for three trials after several weeks for *L. vannamei* reared with and without probiotic strains added to the water.

| Trials | Metrics | Treatments | | | | | |
|---|---|---|---|---|---|---|---|
| | | Media Control | Negative Control | ISO 5 | ISO 11 | ISO 12 | CSX |
| 1 | Size (g) | 3.22 ± 0.32[a] | 0.45 ± 0.004[b] | 0.79 ± 0.40[b] | 1.25 ± 0.27 [a] | 1.48 ± 0.81 [a] | -- |
| | SGR (%) | 0.12 ± 0.01 [a] | 0.03 ± 0.004 [a] | 0.03 ± 0.04 [a] | 0.07 ± 0.01 [a] | 0.072 ± 0.04 [a] | -- |
| | FCR (%) | 1.29 ± 0.14 [a] | 18.16 ± 0.31 [a] | - 5.68 ± 10.66 [a] | 4.33 ± 1.24 [a] | - 9.993 ± 12.34 [a] | -- |
| | Survival(%) | 76.0 ± 1.53 [a] | 16.0 ± 2.19 [a] | 20.0 ± 5.18 [a] | 36.0 ± 2.08 [a] | 36.0 ± 4.91 [a] | -- |
| 2 | Size (g) | 2.96 ± 1.00 [a] | 2.25 ± 0.00 [a] | 3.12 ± 0.37 [a] | 3.01 ± 0.27 [a] | 2.22 ± 0.28 [a] | -- |
| | SGR (%) | 0.04 ± 0.00 [a] | 0.06 ± 0.00 [a] | 0.03 ± 0.00 [a] | 0.03 ± 0.00 [a] | 0.038 ± 0.01[a] | -- |
| | FCR (%) | 1.77 ± 0.46 [a] | 1.56 ± 0.00 [a] | 1.77 ± 0.20 [a] | 1.73 ± 0.19 [a] | 2.423 ± 0.70 [a] | -- |
| | Survival(%) | 30.0 ± 0.33 [a] | 100.0 ± 0.00 [a] | 100.0 ± 0.00 [a] | 100.0 ± 0.00[a] | 100.0 ± 0.00[a] | -- |
| 3 | Size (g) | -- | 0.31 ± 0.11 [a] | 0.28 ± 0.09 [a] | 0.21 ± 0.05[a] | 0.24 ± 0.10 [a] | 0.43 ± 0.05 [a] |
| | SGR (%) | -- | - 0.06 ± 0.01 [a] | - 0.07 ± 0.02 [a] | - 0.07 ± 0.01 [a] | - 0.08 ± 0.02 [a] | - 0.05 ± 0.00 [a] |
| | FCR (%) | -- | 2.145 ± 0.66 [a] | 2.90 ± 1.54 [a] | 2.92 ± 0.73 [a] | 4.86 ± 3.25 [a] | 1.21± 0.16 [a] |
| | Survival(%) | -- | 37.50 ± 0.58 [a] | 37.50 ± 0.00 [a] | 25.0 ± 0.88 [a] | 25.0 ± 0.58[a] | 12.50 ± 0.88 [a] |

Note: Different superscripts within a row indicate statistically significant differences among treatments.

Table 5. Statistical analysis results from a one-way ANOVA performed on final performance metrics measured over the course of three weeks during trial one of *L. vannamei* reared with and without probiotic strains added to the water.

| Measurement Factor | Source of Variance | Sum of Squares | df | Mean Squares | F | Sig. |
|---|---|---|---|---|---|---|
| Final Size (g) | Between Groups | 13.772 | 4 | 3.443 | 5.839 | 0.011 |
| | With-in Groups | 5.897 | 10 | 0.590 | | |
| | Total | 19.669 | 14 | | | |
| SGR (%) | Between Groups | 0.016 | 4 | 0.004 | 2.034 | 0.165 |
| | With-in Groups | 0.019 | 10 | 0.002 | | |
| | Total | 0.035 | 14 | | | |
| FCR (%) | Between Groups | 395.073 | 4 | 98.768 | 0.748 | 0.143 |
| | With-in Groups | 1319.759 | 10 | 131.976 | | |
| | Total | 1714.832 | 14 | | | |
| Survival (%) | Between Groups | 395.733 | 4 | 98.933 | | 0.097 |
| | With-in Groups | 374.000 | 10 | 37.400 | | |
| | Total | 769.733 | 14 | | | |

In trial two, shrimp that were inoculated with ISO 5 and ISO 11 generated an average final size of 3.1 and 3.0 g, respectively. A one-way ANOVA, however, showed that there were no statistically significant differences among treatments ($p = 0.857$) for size (Table 6). Similarly, there were no statistically significant differences found among treatments for SGR ($p = 0.153$) or FCR ($p = 0.783$) for trial two. Given that only one shrimp was stocked per container during trial two, container survival was either 0 or 100%. However, at the time of harvest, overall survival was 83%. A chi-square analysis showed that there were no statistically significant differences found among treatments with respect to survival for trial two ($p = 0.126$).

Table 6. Statistical analysis results from a one-way ANOVA performed on final performance metrics measured over the course of four weeks during trial two of *L. vannamei* reared with and without probiotic strains added to the water.

| Measurement Factor | Source of Variance | Sum of Squares | df | Mean Squares | F | Sig. |
|---|---|---|---|---|---|---|
| Final Size (g) | Between Groups | 0.354 | 4 | 0.089 | 0.290 | 0.493 |
| | With-in Groups | 2.134 | 7 | 0.305 | | |
| | Total | 2.488 | 11 | | | |
| SGR (%) | Between Groups | 0.001 | 4 | 0.000 | 2.344 | 0.153 |
| | With-in Groups | 0.001 | 7 | 0.000 | | |
| | Total | 0.002 | 11 | | | |
| FCR (%) | Between Groups | 1.098 | 4 | 0.275 | 0.431 | 0.783 |
| | With-in Groups | 4.463 | 7 | 0.638 | | |
| | Total | 1.000 | 4 | 0.250 | 2.625 | 0.125 |
| Survival (%) | Between Groups | 0.667 | 7 | 0.095 | | |
| | With-in Groups | 1.667 | 11 | | | |
| | Total | 1.000 | 4 | 0.250 | 2.625 | 0.125 |

During trial three, shrimp containers treated with probiotics ISO 5 and ISO 12 exhibited similar growth patterns to that of the NC, in which, shrimp from all experimental containers displayed minimal to no growth at the time of harvest. A one-way ANOVA performed on final shrimp size showed that there were no statistically significant differences found among treatments ($p = 0.436$) (Table 7), resulting in a mean final size/growth of 0.271 g. There also were no statistically significant differences found among treatments for SGR ($p = 0.562$) or FCR ($p = 0.640$) for trial three. A Kaplan-Meier survival analysis conducted for trial three showed that were no statistically significant differences found among treatments, resulting in a cumulative survival below 30%.

Table 7. Statistical analysis results from a one-way ANOVA performed on final performance metrics measured over the course of four weeks during trial three of *L. vannamei* reared with and without probiotic strains added to the water.

| Measurement Factor | Source of Variance | Sum of Squares | df | Mean Squares | F | Sig. |
|---|---|---|---|---|---|---|
| Final Size (g) | Between Groups | .086 | 4 | 0.022 | 1.035 | 0.436 |
| | With-in Groups | .208 | 10 | 0.021 | | |
| | Total | .294 | 14 | | | |
| SGR (%) | Between Groups | .002 | 4 | 0.000 | 0.783 | 0.562 |
| | With-in Groups | .005 | 10 | 0.000 | | |
| | Total | .007 | 14 | | | |
| FCR (%) | Between Groups | 21.679 | 4 | 5.420 | 0.649 | 0.640 |
| | With-in Groups | 83.511 | 10 | 8.351 | | |
| | Total | 105.190 | 14 | | | |

The pH for trial one ranged between 7.3 and 8.0. The overall means for pH for trial 1 are displayed in Table 8. A generalized linear model analysis showed that there were no statistically significant differences in pH among treatments ($p = 0.874$) (Table 9). Ammonia levels for all experimental containers during trial one began to rise towards the end of the first week, resulting in high ammonia levels for the duration of trial one, ranging from 4.0 to 6.0 mg/L TAN.

Table 8. Overall means ± standard errors of the mean of water quality parameters for three trials after several weeks for *L. vannamei* reared with and without probiotic strains added to the water.

| | | Treatments | | | | | |
|---|---|---|---|---|---|---|---|
| | Metrics | MC | NC | ISO 5 | ISO 11 | ISO 12 | CS*X* |
| Trial 1 | pH | 8.000 ± 0.000 [a] | 8.000 ± 0.000 [a] | 7.939 ± 0.036 [a] | 7.894 ± 0.052 [a] | 7.788 ± 0.102 [a] | -- |
| | Ammonia | 5.227 ± 0.305 [a] | 5.227 ± 0.305 [a] | 5.136 ± 0.311 [a] | 5.227 ± 0.305 [a] | 5.136 ± 0.311 [a] | -- |
| | Nitrite | 0.000 ± 0.0.000[a] | 0.030 ± 0.030 [a] | 1.550 ± 0.632 [a] | 1.640 ± 0.632 [a] | 1.820 ± 0.682 [a] | -- |
| Trial 2 | pH | 7.026 ± 0.066[a] | 7.346 ± 0.087[b] | 7.154 ± 0.091[a] | 7.218 ± 0.071[a] | 7.359 ± 0.048[a] | -- |
| | Ammonia | 3.385 ± 0.315[a] | 5.538± 0.313[b] | 5.192 ± 0.342[b] | 5.487 ± 0.201[b] | 5.308 ± 0.011[b] | -- |
| | Nitrite | 1.771 ± 0.460[a] | 1.563 ± 0.000[b] | 1.771 ± 0.197[b] | 1.734 ± 0.188[b] | 2.423 ± 0.250[b] | -- |
| Trial 3 | pH | -- | 8.274 ± 0.029 [a] | 8.268 ± 0.031 [a] | 8.262 ± 0.031 [a] | 8.268 ± 0.032 [a] | 8.301 ± 0.031 [a] |
| | Ammonia | -- | 2.393 ± 0.184 [a] | 2.399 ± 0.165 [a] | 2.381 ± 0.166 [a] | 2.220 ± 0.168 [a] | 2.205 ± 0.199 [a] |
| | Nitrite | -- | -- | -- | -- | -- | -- |

Note: Different superscripts within a row indicate statistically significant differences among treatments.

Table 9. Statistical analysis results from a generalized linear model performed on final water quality parameters measured over the course of several weeks during three trials of *L. vannamei* reared with and without probiotic strains added to the water.

| | | **Test of Model Effects** | | | |
|---|---|---|---|---|---|
| | Measurement Factor | Source | GLM | df | Sig. |
| Trial 1 | pH | Date | 2.401 | 10 | 0.992 |
| | | Treatment | 1.244 | 4 | 0.874 |
| | Ammonia | Date | 0.000 | 10 | 1.000 |
| | | Treatment | 0.000 | 4 | 1.000 |
| | Nitrite | Date | 10.400 | 10 | 0.406 |
| | | Treatment | 6.813 | 4 | 0.146 |
| Trial 2 | pH | Date | 57.901 | 12 | < 0.001 |
| | | Treatment | 26.154 | 4 | < 0.001 |
| | Ammonia | Date | 43.061 | 12 | < 0.001 |
| | | Treatment | 41.418 | 4 | < 0.001 |
| | Nitrite | Date | 93.527 | 12 | < 0.001 |
| | | Treatment | 48.799 | 4 | < 0.001 |
| Trial 3 | pH | Date | 62.842 | 27 | < 0.001 |
| | | Treatment | 0.786 | 4 | 0.940 |
| | Ammonia | Date | 310.998 | 27 | 0.000 |
| | | Treatment | 7.797 | 4 | 0.099 |

There were no statistically significant differences in ammonia among treatments ($p = 1.000$), as reported by a generalized linear model analysis. Nitrite levels during trial one ranged from 0.0 to 10.0 mg/L. Nitrite levels were too low to be detected for the MC and NC during trial one. According to a generalized linear model analysis, it was concluded that there were no statistically differences between treatments ($p = 0.146$) for nitrite.

For trial two, overall pH levels were on the low end of the spectrum for this shrimp species, ranging from 6.3 to 7.5. A generalized linear model analysis performed on the final pH data revealed that there was a statistically significant difference among treatments ($p < 0.001$) (Table 9). The parameters estimate showed that all treatments were statistically significantly different from the NC. Based on a generalized linear model analysis that was performed on the final ammonia data, it was determined that there was a statistically significant difference among treatments ($p < 0.001$) (Table 9). The parameters estimate showed that the MC was statistically significant from all the other four treatments. The MC during trial two appeared to have the highest nitrite levels, ranging from 2.0 mg/L to 6.0 mg/L ($p < 0.001$) (Table 9). The parameters estimate showed that the MC was statistically significantly lower from all other treatments.

During trial three, pH levels began to fluctuate after the first two weeks of the trial. Although pH levels fluctuated, a generalized linear model analysis showed that there were no statically significant differences found among treatments ($p = 0.940$) (Table 9). Ammonia levels were high during the first week of trial three (2.5 to 6.0 mg/L TAN), however, ammonia levels began to decrease halfway through the second week and remained under 3.0 mg/L TAN for the duration of the trial. According to a generalized linear model analysis, it was concluded that there were no statically significant differences found among treatments for final ammonia ($p = 0.099$) (Table 9). Nitrite was not tested during trial three.

### 4.2. The Effect of Probiotics on the Expression of Heat Shock Protein 70 in Pacific White Shrimp Subjected to Acute Environmental Stress

As mentioned above, due to a mistake in organ identification, the foregut along with abdominal muscle tissue, was extracted instead of the hepatopancreas. The HSP 70 gene is not highly expressed in the foregut compared to other organs such as the hepatopancreas, and abdominal muscle tissue (Rungrassameea *et al.* 2010). As a result, only the abdominal muscle tissue was processed for further analysis to determine the expression of HSP 70.

RNA was extracted from 214 shrimp abdominal muscle tissue samples. A Nanodrop was used as an initial baseline to determine the overall quality of the RNA extracted from muscle tissue for all four treatments (Table 10). Based off the data retrieved from the Nanodrop, 15 of the samples had RNA concentrations under 100 ng/µl (Table 10), indicating poor quality of RNA due to phenol chloroform contamination obtained during the RNA extraction phase. From those 15 samples, eight of the samples had low RNA 260/280 ratios under ~1.8 nm/µl, and of the same 15 samples, 12 of the samples also had 260/230 ratios under ~1.8 nm/µl as well. As a result, to assess the gene expression of HSP 70 from shrimp abdominal muscle tissue, cDNA synthesis and RT- PCR analysis were performed only on the RNA that was extracted from the NC and ISO 11 treatment samples, given that these two treatments had the least amount of samples with contamination.

Table 10. Quality of RNA concentrations and ratios based on temperature and probiotic treatment combinations obtained from muscle tissue of *L. vannamei* sampled at six different incubation periods after being subjected to acute thermal stress.

| | | | 0 hr | | | 2 hrs | | | 4 hrs | | | 6 hrs | | | 12 hrs (Recovery) | | | 18 hrs (Recovery) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Temperature** | **Container** | **Probiotic** | **RNA Con. ng\µl** | **260/ 280 nm\µl** | **260/ 230 nm\µl** | **RNA Con. ng\µl** | **260/ 280 nm\µl** | **260/ 230 nm\µl** | **RNA Con. ng\µl** | **260/ 280 nm\µl** | **260/ 230 nm\µl** | **RNA Con. ng\µl** | **260/ 280 nm\µl** | **260/ 230 nm\µl** | **RNA Con. ng\µl** | **260/ 280 nm\µl** | **260/ 230 nm\µl** | **RNA Con. ng\µl** | **260/ 280 nm\µl** | **260/ 230 nm\µl** |
| pre-exposure | R1 | Neg. Control | 313.9 | 1.92 | 1.41 | | | | | | | | | | | | | | | |
| pre-exposure | R2 | Neg. Control | 1333.8 | 1.93 | 2.07 | | | | | | | | | | | | | | | |
| pre-exposure | R3 | Neg. Control | 4161.7 | 2.05 | 2.07 | | | | | | | | | | | | | | | |
| 28 °C | 1 | Neg. Control | | | | 1314.40 | 1.95 | 2.26 | 610.70 | 1.88 | 1.35 | 664.90 | 1.95 | 2.13 | 1229.20 | 2.03 | 2.04 | 659.60 | 1.99 | 1.63 |
| 28 °C | 4 | Neg. Control | | | | 1952.40 | 2.07 | 2.11 | 1851.20 | 2.02 | 2.10 | 1733.30 | 2.02 | 2.16 | 1061.10 | 1.55 | 1.50 | 505.00 | 1.44 | 1.45 |
| 28 °C | 10 | Neg. Control | | | | 105.9 | 1.85 | 1.08 | 6755.2 | 1.95 | 2.09 | 1121.7 | 2.06 | 2.18 | 1676.9 | 1.8 | 1.45 | 427.2 | 1.59 | 1.68 |
| 32 °C | 2 | Neg. Control | | | | 35.9 | 1.85 | 0.89 | 51.4 | 1.82 | 1.74 | 163.2 | 1.87 | 1.76 | 651.6 | 1.93 | 1.98 | 781 | 1.95 | 2.36 |
| 32 °C | 7 | Neg. Control | | | | 1261 | 1.89 | 1.07 | 1293.5 | 1.79 | 1.21 | 1392.7 | 2.02 | 2.01 | 1744.7 | 1.68 | 1.16 | 3184.8 | 2.03 | 2.06 |
| 32 °C | 10 | Neg. Control | | | | 2710.4 | 2.03 | 1.91 | 4836.5 | 2.05 | 1.99 | 291.8 | 1.87 | 1.38 | 751.1 | 1.99 | 1.42 | 12430.3 | 2.08 | 2.17 |
| 36 °C | 2 | Neg. Control | | | | 180.4 | 1.94 | 1.27 | 1846.7 | 2.04 | 1.99 | 314.2 | 1.9 | 1.04 | NA | NA | NA | 3949.7 | 2.06 | 1.95 |
| 36 °C | 11 | Neg. Control | | | | 98.7 | 1.88 | 1.17 | 16 | 1.1 | 0.28 | 424.4 | 1.96 | 1,65 | 55.7 | 1.44 | 0.48 | 3711.9 | 2.08 | 2 |
| 36 °C | 12 | Neg. Control | | | | 5947.9 | 2.07 | 2.09 | 6021.5 | 2.04 | 1.94 | 2626.4 | 2.07 | 2 | 2919.9 | 2.08 | 2.1 | 58.8 | 1.79 | 1.09 |
| pre-exposure | R1 | *CSX* | 741 | 1.87 | 1.86 | | | | | | | | | | | | | | | |
| pre-exposure | R2 | *CSX* | 306.2 | 2.13 | 1.15 | | | | | | | | | | | | | | | |
| pre-exposure | R3 | *CSX* | 1001 | 1.98 | 2.1 | | | | | | | | | | | | | | | |
| 28 °C | 5 | *CSX* | | | | 417.90 | 1.88 | 1.21 | 729.10 | 1.94 | 1.59 | 253.40 | 1.68 | 0.61 | 1375.80 | 1.85 | 1.59 | 954.00 | 1.98 | 1.62 |
| 28 °C | 6 | *CSX* | | | | 257.1 | 1.91 | 2 | 367.2 | 1.99 | 2.09 | 390.4 | 1.98 | 1.27 | 381.1 | 1.92 | 2.12 | 304 | 1.79 | 1.29 |
| 28 °C | 8 | *CSX* | | | | 209.7 | 1.66 | 0.8 | 141.4 | 1.68 | 1.13 | 388.8 | 1.9 | 1.51 | 50.6 | 1.95 | 1.38 | 285 | 1.75 | 0.84 |
| 32 °C | 6 | *CSX* | | | | 916.8 | 1.99 | 2.1 | 4900.8 | 2.14 | 1.98 | 1306.2 | 2.08 | 2.09 | 418 | 2.1 | 1.85 | 903.8 | 2.12 | 2.1 |
| 32 °C | 8 | *CSX* | | | | 1278.9 | 1.99 | 2.03 | 1378.4 | 1.68 | 1.78 | 1763 | 2 | 2.17 | 3846.8 | 2.06 | 2.02 | 11.7 | 1.65 | 1.32 |
| 32 °C | 11 | *CSX* | | | | 252.6 | 1.97 | 1.93 | 1543.9 | 1.98 | 1.57 | 3503.9 | 2.08 | 2.12 | 1200 | 1.94 | 2.07 | 5637 | 2.02 | 2 |
| 36 °C | 4 | *CSX* | | | | 3007 | 2.07 | 1.84 | 219.3 | 1.87 | 0.93 | 84900 | 2.06 | 2.09 | 718.3 | 1.99 | 1.5 | 5349.9 | 2.04 | 1.96 |
| 36 °C | 7 | *CSX* | | | | 260.8 | 1.88 | 1.43 | 249.6 | 1.87 | 1.13 | 6023.8 | 2.04 | 2.09 | 197.3 | 1.89 | 1.4 | | | |
| 36 °C | 9 | *CSX* | | | | 225.9 | 1.85 | 2.34 | 1511 | 1.99 | 1.04 | 1705.1 | 2.03 | 1.59 | 1090.8 | 1.97 | 1.05 | 18.7 | 1.65 | 1.55 |
| pre-exposure | R1 | ISO 11 | 8443.7 | 2.04 | 2.03 | | | | | | | | | | | | | | | |
| pre-exposure | R2 | ISO 11 | 1985.9 | 1.85 | 1.77 | | | | | | | | | | | | | | | |
| pre-exposure | R3 | ISO 11 | 2233.3 | 1.8 | 1.5 | | | | | | | | | | | | | | | |
| 28 °C | 2 | ISO 11 | | | | 540.40 | 2.11 | 2.16 | 408.50 | 1.99 | 0.95 | 1999.10 | 2.01 | 2.16 | 1108.80 | 2.02 | 2.02 | 1053.30 | 1.99 | 2.23 |
| 28 °C | 9 | ISO 11 | | | | 140.1 | 1.91 | 1.18 | 1070.4 | 1.66 | 0.97 | 496.5 | 1.87 | 0.82 | 184.3 | 1.93 | 1.48 | 690.3 | 1.7 | 0.65 |
| 28 °C | 11 | ISO 11 | | | | 914.2 | 2.04 | 1.91 | 379.7 | 1.93 | 2.15 | 598.9 | 2 | 2.08 | 624.6 | 1.99 | 2.06 | 717.2 | 1.69 | 1.82 |
| 32 °C | 4 | ISO 11 | | | | 879.1 | 1.99 | 1.72 | 689 | 1.91 | 1.16 | 650.8 | 2.28 | 1.29 | 3179.4 | 2.07 | 2.11 | 450.4 | 1.66 | 1.55 |
| 32 °C | 5 | ISO 11 | | | | 165,1 | 2.05 | 1.79 | 798.1 | 2.07 | 2.04 | 1748.4 | 2.07 | 2.16 | 316.3 | 2.11 | 1.8 | 1838.7 | 2.09 | 2.03 |
| 32 °C | 9 | ISO 11 | | | | 15666.8 | 1.34 | 0.72 | 1511 | 1.99 | 1.39 | 3930.1 | 1.95 | 1.75 | 815.8 | 2 | 1.85 | 5205.5 | 1.85 | 1.81 |
| 36 °C | 1 | ISO 11 | | | | 3119.8 | 1.98 | 1.78 | 265.7 | 1.88 | 1.23 | 298.6 | 1.95 | 1.48 | 3914.8 | 2 | 1.82 | 644.9 | 1.95 | 1.33 |
| 36 °C | 3 | ISO 11 | | | | 643.2 | 1.95 | 1.33 | 164.4 | 1.9 | 1 | 596.9 | 1.98 | 1.41 | 2494 | 1.86 | 0.86 | 68331 | 2.04 | 2.04 |
| 36 °C | 5 | ISO 11 | | | | 5751.6 | 2.05 | 1.97 | 9696.9 | 2.04 | 1.99 | 6220.1 | 2.01 | 1.85 | 4463.7 | 2.03 | 1.86 | 387.2 | 1.94 | 1.41 |

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pre-exposure | R1 | ISO 12 | 5469.9 | 2 | 2.21 | | | | | | | | | | | | | | |
| pre-exposure | R2 | ISO 12 | 1563.2 | 2.05 | 2.18 | | | | | | | | | | | | | | |
| pre-exposure | R3 | ISO 12 | 309.1 | 1.94 | 1.68 | | | | | | | | | | | | | | |
| 28 °C | 3 | ISO 12 | | | | 661.70 | 1.93 | 2 | 1116.20 | 2 | 2.14 | 266.00 | 1.93 | 1.75 | 186.50 | 1.97 | 1.30 | 3937.00 | 2.04 | 1.91 |
| 28 °C | 7 | ISO 12 | | | | 2743.9 | 2.01 | 1.72 | 2738.7 | 2.02 | 1.99 | 270.8 | 1.84 | 1.33 | 549 | 1.97 | 1.62 | 549.7 | 1.9 | 2.24 |
| 28 °C | 12 | ISO 12 | | | | 26003.5 | 2.08 | 2.12 | 1364.6 | 1.99 | 1.32 | 1882.4 | 1.99 | 2.07 | 13278.8 | 1.69 | 1.81 | 111.4 | 1.92 | 1.05 |
| 32 °C | 1 | ISO 12 | | | | 140.7 | 2 | 1.44 | 1863.1 | 2.03 | 2.29 | 559.2 | 1.95 | 2.07 | 1673.5 | 2.03 | 2.17 | 98 | 1.79 | 1.82 |
| 32 °C | 3 | ISO 12 | | | | 187.5 | 1.48 | 0.47 | 594.5 | 1.46 | 0.72 | 171,7 | 1.95 | 0.86 | 1094.5 | 1.9 | 2,2 | 186,1 | 1.78 | 0.75 |
| 32 °C | 12 | ISO 12 | | | | 4814.1 | 2.07 | 2.05 | 6780.2 | 2.09 | 2.2 | 596.5 | 2.04 | 2.05 | 255 | 1.92 | 1.95 | 299.5 | 1.95 | 1.54 |
| 36 °C | 6 | ISO 12 | | | | 552.5 | 2.01 | 1.73 | 2039.3 | 1.79 | 1.53 | 3684.6 | 2.03 | 1.77 | 772.9 | 1.95 | 1.14 | 510.7 | 1.96 | 1.32 |
| 36 °C | 8 | ISO 12 | | | | | | | 4771.7 | 2.06 | 1.96 | 227.5 | 1.9 | 0.84 | 202.8 | 1.89 | 1.05 | 458.6 | 1.98 | 1.34 |
| 36 °C | 10 | ISO 12 | | | | 4.4 | 1.18 | 0.92 | 27.6 | 1.66 | 1.94 | NA | NA | NA | 109.6 | 0.94 | 0.44 | 11.9 | 1.5 | 0.95 |

Note: Green shaded cells represent samples with satisfactory RNA concentrations of 100 ng/µl or above and satisfactory RNA ratios of 1.7 ng/µl or above, which measures the absorbance and purity of nuckeic acid content. Blue shaded cells represent samples with low RNA concentrations below 100 ng/µl. Copper shaded cells represent sample with low RNA ratios below 1.7 ng/µl. Black shaded cells represent missing tissue samples.

In respect to the RT- PCR analysis, the β-actin housekeeping gene was also used as an indicator to further determine the quality of the isolated RNA for the NC and ISO 11 samples. Two tissue samples collected at 18 hours (an NC sample subjected to 32 °C, as well as an ISO 11 sample conditioned at 28 °C) contained genomic DNA. These two samples were considered contaminated and therefore are unreliable and excluded from the analysis. It appeared that only half of the NC samples expressed the β-actin gene. For ISO 11, none of the samples extracted at 28 °C during the exposure and recovery phase expressed the β-actin gene. However, two ISO 11 samples that were extracted at time zero expressed the presence of the β-actin gene. Samples extracted from only one ISO 11 replicate container, conditioned at 32 °C amplified, expressing the β-actin gene. Eleven ISO 11 samples, conditioned at 36 °C amplified, expressing the β-actin gene. It appeared that the RNA that was extracted from the NC samples that were processed during the exposure sampling phase appeared to have stronger or brighter bands for the presence of β-actin than samples processed during the recovery sampling phase. Of the samples that did express the β-actin gene from the ISO 11 treatment, which further confirms the purity of the extracted RNA and to become more dominant as temperatures increased (Table 11). As previously mentioned, although it is not a suitable analysis for this specific study, a rANOVA found that there was a statistically significant difference between the NC and ISO 11 treatments at different temperatures across all sampling periods ($p < 0.001$) regarding the quality of the bands produced by β-actin, which was quantified by assigning a band score of 0 or 1.

Table 11. Qualitative band amplification analysis for gene expression of β-actin based on temperature and probiotic treatment combinations obtained from muscle tissue of *L. vannamei* sampled at six different incubation periods.

| | Sampling Periods | | | | | |
|---|---|---|---|---|---|---|
| | | Exposure | | | Recovery | |
| **Treatments** | **0 hr.** | **2 hr.** | **4 hr.** | **6 hr.** | **12 hr.** | **18 hr.** |
| Control - R1 | + | | | | | |
| Control - R2 | - | | | | | |
| Control - R3 | + | | | | | |
| Control - 28.1 | | - | - | - | + | + |
| Control - 28.2 | | + | + | + | - | + |
| Control - 28.3 | | - | - | - | - | - |
| Control - 32.1 | | + | - | - | - | - |
| Control - 32.2 | | - | + | - | - | + |
| Control - 32.3 | | - | + | - | + | + |
| Control - 36.1 | | + | + | - | - | - |
| Control - 36.2 | | + | - | - | - | - |
| Control - 36.3 | | + | + | - | - | + |
| ISO 11 - R1 | + | | | | | |
| ISO 11 - R2 | + | | | | | |
| ISO 11 - R3 | - | | | | | |
| ISO 11 - 28.1 | | - | - | - | - | - |
| ISO 11 - 28.2 | | - | - | - | - | - |
| ISO 11 - 28.3 | | - | - | - | - | - |
| ISO 11 - 32. 1 | | + | + | + | + | - |
| ISO 11 - 32. 2 | | - | - | - | - | - |
| ISO 11 - 32. 3 | | - | - | - | - | - |
| ISO 11 - 36.1 | | - | - | + | + | + |
| ISO 11 - 36.2 | | + | + | - | + | + |
| ISO 11 - 36.3 | | + | + | - | + | + |

Note: ++ indicates strong band amplification. + indicates weak band amplification. – indicates no band amplification.

Again, although it is not a suitable analysis for this particular study, a rANOVA found that there was a statistically significant difference between the NC and ISO 11 treatments at different temperatures across all sampling periods ($p < 0.001$) regarding the quality of the bands produced by HSP 70, which again was quantified by assigning a band score of 0 or 1. The quality, along with the number of bands that amplified for ISO 11 were weakened and decreased across sampling periods. Both the NC and ISO 11 treatments contained samples that expressed

strong bands for the HSP 70 gene during recovery (12 and 18 hours after exposure). Although, a higher number of bands were expressed from the NC samples that were processed during the recovery phase compared to the ISO 11 samples.

Table 12. Qualitative band amplification analysis for gene expression of HSP 70 based on temperature and probiotic treatment combinations obtained from muscle tissue of *L. vannamei* sampled at six different incubation periods.

| | Sampling Periods | | | | | |
|---|---|---|---|---|---|---|
| | | Exposure | | | Recovery | |
| **Treatments** | **0 hr.** | **2 hr.** | **4 hr.** | **6 hr.** | **12 hr.** | **18 hr.** |
| Control - R1 | + | | | | | |
| Control - R2 | - | | | | | |
| Control - R3 | + | | | | | |
| Control - 28.1 | | - | - | - | - | - |
| Control - 28.2 | | - | - | - | - | - |
| Control - 28.3 | | - | - | - | - | - |
| Control - 32.1 | | - | - | - | + | + |
| Control - 32.2 | | - | + | - | + | + |
| Control - 32.3 | | + | + | - | + | + |
| Control - 36.1 | | - | + | + | - | - |
| Control - 36.2 | | + | + | + | + | + |
| Control - 36.3 | | + | + | - | + | + |
| ISO 11 - R1 | - | | | | | |
| ISO 11 - R2 | - | | | | | |
| ISO 11 - R3 | - | | | | | |
| ISO 11 - 28.1 | | - | - | - | - | - |
| ISO 11 - 28.2 | | - | - | - | - | - |
| ISO 11 - 28.3 | | - | - | - | - | + |
| ISO 11 - 32. 1 | | - | - | + | - | + |
| ISO 11 - 32. 2 | | - | - | - | - | - |
| ISO 11 - 32. 3 | | - | - | + | - | - |
| ISO 11 - 36.1 | | + | - | + | + | + |
| ISO 11 - 36.2 | | + | - | - | - | - |
| ISO 11 - 36.3 | | + | - | - | - | - |

Note: ++ indicates strong band amplification. + indicates weak band amplification. – indicates no band amplification.

# CHAPTER 5: DISCUSSION

## 5.1. The Effect of Probiotics on Growth and Survival of Pacific White Shrimp

Shrimp underwent three growth and survival trials to determine the effects that selected probiotics (ISO 5, ISO 11, ISO 12, and CSX) has on shrimp performance. Overall, performance metrics for all three trials were below industry standards. According to industry criteria, shrimp were expected to be 3 to 4 g at the end of four weeks, with 80% survival respectively. Poor shrimp performance may likely be attributed to the various issues that were experienced during each trial, including elevated water temperature and excessive feed rates. Because ammonia levels were also above the tolerable range, it may have caused stress on the shrimp, which may have impacted shrimp performance.

The target temperature for all experimental containers for all three trials was 28 °C. The containers were conditioned by the room temperature during the first two trials. During trial one, the temperature of the study area exceeded 30 °C (maximum on the thermometer). Because of this, water temperatures were also above 30 °C. The tolerable temperature range for this species is between 20 - 30 °C (Cheng *et al*. 2005; Wyban *et al.*1995); temperature levels during my study exceeded these limits. Elevated temperatures could have the potential to hinder shrimp performance by disrupting cellular functions and causing shrimp to become more susceptible to certain diseases.

Similarly, feed rates were also excessive during trial one. Shrimp were fed up to 25% of their total biomass with a low daily water change percent. This likely contributed to the high ammonia levels (> 5.0 mg/L) observed. Excess waste and higher amounts of ammonia can be lethal to shrimp by causing damage to their respiratory system and limiting the productivity of

other vital organs such as the hepatopancreas (Lu *et al*. 2016; Racotta and Hernández-Herrera 2000; Chien 1992). The excessive feed rates with high ammonia levels combined may likely be the cause of the massive mortality that was experienced three weeks into the trial. Due to the massive mortality event, the decision was made to rerun the experiment and combat the issues that were faced.

As previously stated, there were issues that occurred during the succeeding trials, such as pseudo-replication, low sample size, and the loss of replicates. These issues more than likely influenced shrimp performance and the overall validity of the results. Furthermore, during trial three, the majority of shrimp died during the first two weeks of the study. As a result, it was later determined that the chemical residue on my hands left over from the shea butter lotion and hand sanitizer acted as an insecticide, which may have caused a toxic shock, resulting in mortality. The chemicals found in shea butter are toxic and detrimental to shrimp (Essack *et al.* 2014). Shea butter has different chemical ingredients, including titanium dioxide, P-Phenylenediamine, ethanolamine, and resorcinol, which can have a direct and detrimental impact on the biochemical systems of shrimp (DeLorenzo *et al*. 2016). Such chemicals can cause a high degree of toxicity by attacking the shrimp's organ system, including the circulatory and respiratory systems, which can cause mortality (DeLorenzo *et al*. 2016). Therefore, when the chemicals from shea butter are released into an aquatic environment, it can have an immediate impact on the shrimp, resulting in mortality (Essack *et al*. 2014).

In addition, it was noticed that there was a huge size variation within the shrimp population. A huge variation in shrimp size may be an indication of disease, specifically Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV). IHHNV is a highly prevalent virus that is of major concern for penaeid shrimp species (Xia *et al*. 2015). The virus

was first discovered in a culture of blue shrimp (*Litopenaeus stylirostris*) from a shrimp hatchery in Hawaii in 1981 (Lightner *et al*. 1983). Since the virus initial discovery, IHHNV has infected aquaculture shrimp worldwide from the U.S to various Latin and Asian countries (Xia *et al*. 2015). IHHNV is the smallest shrimp virus containing only 4.1 kb in length (Dewangan *et al*. 2015) and a DNA genome that is single-stranded (Ahamed *et al*. 2016). The cell structure of the virus is a non-enveloped icosahedral shape (Escobedo-Bonilla and Rangel 2014) that contains three open reading frames (Xia *et al*. 2015).

Due to the previously described characteristics of IHHNV, IHHNV is classified as a member of the *Parvovridae* family (Chai *et al*. 2016). It has been revealed that although IHHNV is not fatal in white shrimp, the virus is associated with causing "runt and deformity syndrome" (RDS) (Cowley *et al*. 2018; Lightner *et al*. 2012). Shrimp diseases affect the shrimp in different ways that are catastrophic to the species population. RDS causes the suppression of shrimp growth and could cause other deformities in the rostrums, antennal flagella, abdominal segments, and the tail fin (Melena *et al*. 2015). However, the only symptom that was identified during this study was stunted growth. Also, because shrimp were purchased from a commercialized breeding and production facility, the shrimp were certified to be Specific Pathogen Free (SPF). Because of which, shrimp were assumed to be in good health. Hence, the idea of shrimp having a disease prior to beginning the study seemed unlikely and was not taking into consideration until after the termination of trial three.

## 5.2. The Effect of Probiotics on the Expression of Heat Shock Protein 70 in Pacific White Shrimp Subjected to Acute Environmental Stress

Shrimp were exposed to selected probiotic strains (ISO 5, ISO 11, ISO 12, and CSX) to determine the effects these probiotics has on the expression of HSP 70, while shrimp are subjected to elevated temperatures. RNA was extracted from abdominal muscle tissue to elevate the expression of HSP 70. To have acceptable and pure quality RNA, RNA concentrations need to be at a minimum of 100 ng/µl. In addition, both 260/230 and 260/280 RNA ratios need to be at a minimum of ~1.8 nm/µl (Eldh *et al.* 2012). As previously mentioned, a high percentage of extracted RNA samples were contaminated with phenol, which decreased the quality of the RNA samples. The cause for the poor RNA quality may be attributed to the excess traces of trizol and phenyl chloroform left in the samples from inadequate chemical phase separation during the RNA extraction process while RNA samples were suspended in the centrifuge.

During the RNA extraction process, the interphase which contains unwanted DNA and the organic phase which contains proteins and lipids may have become disturbed while extracting the aqueous phase which contains the pure RNA. The RNA could have easily become assorted with the excess DNA, proteins, and lipids, decreasing the quality of the extracted RNA (Tan and Yiap 2009). An RNA Ethanol Precipitation procedure using sodium acetate was conducted to help purify the RNA of some of the samples by removing the excess contaminants. More specifically, the use of sodium acetate was intended to aid in the denaturation and removal of excess proteins along with sodium ions that may be present in the RNA sample to increase the quality of the extracted RNA (Tan and Yiap 2009). However, the procedure did not work as intended. After samples underwent the precipitation procedure, it was found that there were still traces of phenyl in the RNA sample. This indicates that initially there was an improper proportion of phenol chloroform to sample that disallowed the sodium acetate to adequately

precipitate out the contaminants that were in the sample. This corresponds to and was confirmed by the Nanodrop results, which shown RNA ratios that were below ~1.8. This indicates that there was RNA degradation, resulting in poor quality.

In respect to RNA isolation and band quality, a total of 59 out of 96 (61%) of samples did not amplify, indicating that ultimately no RNA was present in those samples, which is possibly due to the high level of contamination obtained during the extraction process. As a result, only 20 of the 48 NC samples expressed the amplification for the β-actin gene. Of the 20 amplified NC samples, 13 of those amplified during the exposure phase. This indicates that the RNA from the NC samples that were extracted from the first half of the study were less contaminated from the extraction phase and were of better quality compared to the RNA that was extracted during the recovery phase. This could possibly be an indirect result due to handling and sample processing. In respect to ISO 11 RNA, only 17 out of 48 samples expressed the amplification of the β-actin gene. Of those 17 samples, 11 of those samples amplified at 36 °C during various sampling periods. This indicates that those 11 samples were less contaminated from the extraction phase and were of better quality compared to the RNA that was extracted from ISO 11 samples at 28 °C and 32 °C across all sampling periods during the recovery phase, which again is possible as an indirect result due to handling and sample processing.

Given the few NC RNA samples that were of good quality, it was found that for the samples that expressed the HSP 70 gene, bands became stronger as temperatures increased. This would suggest that shrimp became more stressed with elevated temperatures. Regarding ISO 11 samples, with the 10 samples that expressed the HSP 70 gene, they produced weak bands. This would be an indication that shrimp were less stressed due to the addition of this probiotic. However, due to the high level of contamination obtained during the RNA extraction process, the

data is insufficient to draw a definitive conclusion regarding whether the applied probiotics ultimately played a role in the cause of the decline of stress in shrimp while subjected to elevated temperatures. Also, the expression level for HSP 70 while shrimp were cultured under 'optimal' temperature conditions (i.e. 28 °C) cannot be confirmed due to the high level of contamination. Moreover, water temperatures of 32 °C and above could cause thermal shock in shrimp. This shock could lead to an increase in stress and could disrupt cellular functions (Van Wyk and Scarpa 1999). This corresponds to the current study where as temperatures increased the expression of HSP 70 increased as well. However, the contamination obtained during the RNA extraction process decreased the accuracy of the results.

As previously stated, the target organ that was initially going to be used to evaluate expression of HSP 70 was the hepatopancreas. However, due to a mistake in organ identification, the abdominal muscle tissue was harvested and processed instead. It would be preferred to analyze gene expression in the hepatopancreas compared to the abdominal muscle tissue because the hepatopancreas serves as the primary acting site for gene regulation, and expression (Wang *et al*. 2014; Vogt 1994). This is because the hepatpancreas consist of pancreatic polypeptides known as F-cells that are responsible for protein synthesis, gene regulation, and aids in storing minerals needed for nutrient digestion (Wang *et al*. 2014; Vogt 1994). F-cells are not present in abdominal muscle tissue, resulting in less gene expression than the hepatopancreas. Furthermore, given the high expression of HPS 70 in the hepatopancrease of a shrimp compared to other organs indicates the main reason it is preferred to that of the abdominal muscle for the analysis of gene expression.

The data during this experiment was analyzed using a rANOVA. This statistical test ultimately was not an appropriate choice to use to analyze the data. It was used, however, because gene expression was interpreted to be a continual measure over time, given the various sampling periods. Furthermore, a rANOVA was used because it was believed that the analysis involve related groups instead of independent variables in addition to a reliant variable, which is the dependent quantitative variable upon which each victim is measured. It is now understood that a rANOVA examines the same variable that is obtained from the same study subject or individual over the course of multiple time periods (Kherad-Pajouh and Renaud 2015). Given that gene expression of HSP 70 was not measured from the same individual shrimp during each sampling period, a rANOVA was not a suitable analysis to use. Given the objective of the current study, a chi-square would be a more appropriate statistical analysis.

A chi-square analysis is a non-parametric test that allows for robust nominal data to be analyzed (McHugh 2013). This means that the data can be analyzed even if the data does not have a normal distribution or homogeneity of the variance. A chi- square analysis can further determine if there is a correlation between two dependent variables by evaluating actual observation against expected outcomes or even the presences or absence of an expected event (McHugh 2013). During the current study, because the ultimate goal was to determine simply if the HSP 70 gene was expressed or not, a chi-square analysis would be suitable. By using a chi-square test, it could be determined if there was a correlation between the combinations of probiotics and temperature treatments and gene expression of HSP 70. Given the circumstances of the poor quality of the extracted RNA samples, preforming a chi-square analysis on erratic data more than likely would have produced a similar outcome to that of the rANOVA. Therefore, the data was not reanalyzed using the chi-square analysis.

# CHAPTER 6: CONCLUSIONS AND FUTURE RECOMMENDATIONS

Many studies have been done and resulted in the successful use of probiotics in shrimp aquaculture (Harpeni *et al.* 2018; Chumpol *et al.* 2017; Shi *et al.* 2016; Yuvaraj and Karthik 2015; Zokaeifar *et al.* 2014; Luis-Villasenor *et al.* 2013; Sliva *et al.* 2012). Utilizing probiotics can have long-lasting positive effects on the host animal and its environment (Cruz *et al.* 2012). However, because of all the combined technical errors that occurred throughout the research, the prevented adequate testing of the probiotics to determine if they were useful to be applied to shrimp aquaculture.

Similar shrimp studies were primarily conducted in larger volumes of water (Pathak *et al*. 2018; Yang *et al*. 2014; Zhang *et al*. 2014; Brito *et al*. 2014). Therefore, for future reference, it may be beneficial that shrimp be stocked in larger containers to obtain a larger water volume. Based on the water quality throughout this study, it may be favorable to conduct two 90% water changes daily, in addition to implementing a carbon filter. However, before adding filtration to these systems, further research is necessary to understand the potential interactions between introduced probiotics and the carbon filter. In addition, plating shrimp samples could determine if the probiotics colonized within the shrimp during the course of the study.

There have been various studies conducted that examine the expression of HSP 70 in shrimp (Sung *et al.* 2018; Gao *et al.* 2017; Junprung *et al.* 2017; Yuan *et al.* 2017; Loc *et al.* 2013; Rungrassame *et al.* 2010; Chuang *et al.* 2007). However, because of all the technical errors made during this study, the true impact that ISO 11, ISO 12 and CSX probiotics had on stress mitigation was not adequately measured. If this experiment were to be repeated, some alterations are suggested to gain a more accurate understanding of the probiotics' full potential as

a stress reducer when shrimp are exposed to acute environmental stress. It is recommended that this study is carried out again using an RNA isolation kit.

Using an RNA isolation kit may help to simply reduce the risk of RNA contamination. A RNA isolation kits contains micro-tube filters that are used to trap contaminants that may be present in the sample, which eliminates the use of chemicals such as phenyl chloroform. Using phenyl to separate and remove contaminants from RNA samples is an intricate process and often times decreases the quality of the extracted sample if not done correctly (Tesena *et al*. 2017). However, phenyl yields a higher quantity of RNA compared to a RNA isolation kit, whereas, a kit produce better quality RNA (Tesena *et al*. 2017). The quality of the samples is primarily important for downstream applications, including PCR and sequencing. An RNA isolation kit ha a limited amount of samples that can be processed. Therefore, a kit was not used during this study given the amount of samples that needed to be processed. Hence, using Trizol and phenyl chloroform was the most cost efficient for the given number of samples.

In essences, prior studies have been conducted that prove probiotics to be an effective resource to enhance performance and mitigate stress in shrimp. The current studies aimed to evaluate similar benefits. However, many issues were encountered over the course of both studies that prevented the ability to produce an accurate outcome. Therefore, further research is needed to adequately assess the true potential of ISO 5, ISO 11, ISO 12, and CSX probiotics strains to have a beneficial effect on growth and survival of shrimp, and aid in mitigating environmental stress.

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