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CONSUMER AND PROCESSOR METHODS TO CONTROL SALMONELLA AND LISTERIA IN SHRIMP

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

In

The Department of Food Science

by
Genevieve Anne Edwards
B.S., Louisiana State University, 2009
May, 2012

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ABSTRACT

A common method used by consumers to determine if shrimp are thoroughly cooked when boiling, is to wait until the shrimp float to the top of the water and are pink in color. The purpose of this study was to determine whether the current floating and color cooking method is adequate to ensure the elimination of *Listeria* and *Salmonella* species. Furthermore, to determine if processing and storage methods reduce the effectiveness of this method. Shrimp samples were submerged into bacterial suspensions for 30 min then allowed to air dry for 1 hr and color parameters were measured using a colorimeter. Shrimp samples were separated into three groups; day 0, 1, or 2, and stored at 4°C. The shrimp samples were then treated by placing into boiling water (100°C) on days 0 (inoculation day), 1 and 2. The shrimp were immediately removed from the boiling water once they started floating and color parameters were measured. Bacterial counts were determined by making serial dilutions, spread plating, incubating plates at 37°C for 24 h and calculating Log CFU/g. Typical storage conditions, the use of the additive sodium tripolyphosphate and freezing methods were then tested for their effect on the control of these pathogens in boiled shrimp. Initial bacterial counts ranged from 3.0 to 5.4 Log CFU/g of shrimp. On day 0, 1, and 2 all bacterial counts were reduced to non-detectable levels for shrimp samples that floated in the boiling water. The bacterial counts remained at non-detectable levels during refrigerated (4°C) storage. The redness (a*), yellowness (b*) and lightness (L*) were significantly higher ($p<0.0001$) in the cooked shrimp compared to uncooked for all days tested.

However, the standard deviation for the redness (a*) in the cooked shrimp was large indicating a wide range of pink coloration for all days tested. The flash freezing method led to development of heat resistance in *Listeria monocytogenes*. Brine freezing was determined to be a better method for microbial reduction. Our results suggest that boiling shrimp until they float will significantly reduce *Listeria* species and *Salmonella* species but color change will not and color variation can occur.

CHAPTER 1 INTRODUCTION

Seafood is considered a nutrient dense addition to a healthful diet and its consumption has been linked to a number of health benefits. Consequently, seafood consumption in the United States has increased over recent decades and reached a new high this decade with average Americans consuming 15.8 pounds per year in 2009 (NOAA, 2009). According to the National Fisheries Institute (NFI), shrimp and catfish were two of the most consumed seafood products in the United States in 2009. Shrimp remains the number one most popular seafood product with consumption at 4.10 pounds per year (NFI, 2010). Unfortunately, with increased consumption also comes increased risk of eating contaminated seafood products.

Fresh seafood is highly perishable and microbiological spoilage is one of the main causes of limited shelf-life and safety. A number of factors can lead to contaminated seafood products. Sources of contamination have included overboard sewage discharge into harvest areas, illegal harvesting from sewage-contaminated waters, and sewage runoff from points inland after heavy rains or flooding (Iwamoto et al., 2010). Furthermore, seafood can become contaminated during handling, processing and preparation. At these stages, causes for contamination may be linked to improper storage and transportation temperatures, contamination by an infected food handler, or cross-contamination through mishandling (Iwamoto et al., 2010). Another source of unsafe seafood is aquacultured seafood and imported seafood. There has been an increase in aquaculture and imported seafood in order to meet demand. Approximately 80% of the seafood consumed in the United States is imported (NOAA, 2010). The FDA is unable to inspect all foreign imports which can result in hazardous seafood reaching the consumer.

Heat treatment, usually in the form of cooking, plays a major role in the safety and sensory acceptance of seafood. Cooking is defined as "the application of heat to a food to modify raw product properties in order to meet sensory expectations of consumers and to reduce its microbial load, which improves its safety and may extend its shelf life" (NACMCF, 2008). According to this definition, it is crucial to determine consumers' acceptance of cooked seafood, in order to ensure their willingness to practice proper cooking methods. A popular method of cooking shrimp is by boiling. Consumers will often boil shrimp until they float to the surface of the water. Additionally, a common method used by consumers to determine doneness when cooking is to observe the color change of the cooked product. For example, when boiling shrimp, the color change of the shrimp from grey to pink is associated with it being thoroughly cooked. This color change is used by consumers for safety evaluation and it is also associated with sensory acceptance. Therefore, scientific research needs to support the claim that this color change correlates to a destruction of pathogens to a level safe for consumption. Currently, improper cooking and storage contributes to a large amount of foodborne illness outbreaks. Therefore, it is necessary to test the reliability of these widely used cooking methods and to provide guidelines for the consumers to follow to ensure the safety of their food. As a result, further research is needed to develop guidelines for proper cooking of shrimp.

Some additives in shrimp have been shown to inhibit the pink color change which can furthermore result in a safety hazard to consumers. The addition of phosphates, particularly sodium tripolyphosphate, is commonly used during shrimp processing in order to avoid excess moisture loss of shrimp during processing, distribution, storage, and preparation which in turn results in maintenance of the quality and consumer acceptance of the shrimp (GonÇalves and Ribeiro, 2008). However, if overused, this ingredient may also result in excess retention of water

weight. This excess water can result in adulteration of products and have negative effects on the organoleptic properties of shrimp and prevent color change of shrimp during cooking. Further research is needed on the effect of phosphate treatment on the pink color change of shrimp during cooking.

Different factors such as thermal resistance of microorganisms and storage conditions can also affect the cooking time and temperature to ensure destruction of pathogens in a product. Freezing has become the most widely used method to preserve the quality of shrimp immediately after harvest. Almost all shrimp sold have been previously frozen. The shrimp is usually frozen onboard the boat soon after harvesting by two common methods, either brine freezing or blast freezing. The shrimp is then stored at frozen storage (-20°C) until sold directly to consumers or distributed for wholesale where it is commonly thawed and stored at chilled temperatures for purchase by consumers. Generally, consumers store shrimp at refrigerator temperatures (2-4°C) for up to 2 days before cooking as recommended by the FDA. At 2-4°C, certain organisms such as *Listeria monocytogenes* can grow and multiply. Growth under cold stress could promote development of heat resistance in some pathogens. Products may also be stored in the refrigerator after cooking by the consumer. Additionally, some distributors sell already cooked, ready to eat seafood products that are stored at refrigeration temperatures. As a result, the risk of growth or contamination during refrigerated storage of previously cooked shrimp products must be determined. All of these considerations need to be evaluated when determining proper processing and storage conditions to control *Listeria* and *Salmonella* species in shrimp and shrimp products.

The Committee on Microbiological Criteria for Foods (NACMCF) was asked to provide scientific guidance to the Food and Drug Administration (FDA) and the National Marine

Fisheries Service (NMFS) on cooking procedures of seafood products for consumers. The committee compiled a report addressing certain questions and discovered that most consumer methods for cooking seafood were based on quality parameters and not on scientific information to ensure destruction of foodborne pathogens. As a result of these conclusions, the aim of this project was to ensure that the common consumer method used in U.S. households of boiling shrimp until floating and pink in color is adequate in reducing *Listeria* and *Salmonella* species to non detectable levels. This was conducted by determining the thermal log reduction of *Listeria a*nd *Salmonella* species on the surface of whole shrimp to non-detectable levels and by determining a correlation between pathogen reduction to non detectable levels and the degree of color change after boiling shrimp until floating. Additionally, the effect of two common freezing methods, brine freezing and flash freezing, followed by typical cold storage conditions was studied to determine the effects on the microbial quality of the shrimp. The goal is to then provide consumers with science-based information in a language easy for them to understand so that it will empower them to make informed decisions on the proper cooking and storage of their shrimp.

CHAPTER 2 LITERATURE REVIEW

2.1 Seafood.

The term seafood refers to molluscs [(e.g., oysters, clams, and mussels)], finfish [(e.g., salmon and tuna)], marine mammals [(e.g. seal and whale)], fish eggs (roe), and crustaceans [(e.g., shrimp, crab and lobster)] (Iwamoto et al., 2010). According to the National Marine Fisheries Service (NMFS, 2011), seafood is a low-fat source of top-quality protein and the health benefits of eating seafood make it one of the best choices for growing children, active adults, and the elderly (NMFS, 2011). Seafood has been accepted as a nutrient-rich component of a healthful diet, and its consumption is associated with potential health benefits, including neurologic development during gestation and infancy and reduced risk of heart disease (Iwamoto et al., 2010). As a result, the U.S. department of Agriculture (USDA, 2011) updated its dietary guidelines in January 2011, recommending that consumers eat at least two servings of seafood each week and that women who are pregnant or breastfeed eat at least 8 to 12 ounces of seafood per week (NMFS, 2011). The United States is one of the top three countries, along with China and Japan, for consuming fish and shellfish (NOAA, 2011). In 2009 Americans consumed a total of 4.833 billion pounds of seafood (NOAA, 2010). Of the total seafood consumption, shrimp has remained the number one choice for the U.S. at 4.1 pounds per person annually, a level unchanged since 2007 (NOAA, 2010).

2.2 Shrimp.

Shrimp are defined as decapod crustaceans and are characterized by jointed appendages and periodically molted exoskeletons (FAO, 2008). Shrimp are low in saturated fat, high in cardio-protective omega-3 fatty acids, and a good source of protein, selenium and vitamin B12.

An increase in knowledge of the nutritional value of shrimp has lead to a strong market demand for shrimp. Shrimp has continuously been one of the most popular seafood items internationally, and the export value of shrimp (U.S$ 16.47 billion) has made it the highest commercial value product in seafood trade (FAO, 2009). According to recent statistical data, shrimp production from both wild harvest and farm culture has reached estimated levels of approximately 6624 million metric tons totaling a value of more than U.S. $23 billion (Norhana et al., 2010). The practice of shrimp aquaculture has increased over the last few years as a way to meet the demand that can no longer be met by wild harvest shrimp. Unfortunately, with an increase in shrimp production and consumption also comes an increased risk of hazards associated with unsafe shrimp consumption.

2.3 Foodborne Outbreaks.

Foodborne illness outbreaks are a serious problem and result in significant deaths, hospitalizations and economic losses each year. The Centers for Disease Control and Prevention estimates that each year approximately one in six Americans (or 48 million people) become ill, 128,000 are hospitalized, and 3, 000 die of causes attributed to foodborne disease (CDC, 2011). These outbreaks have negative effects on food industry components due to decreased consumer confidence and high costs due to lawsuits and loss of earnings.

Seafood has been the cause of a significant number of foodborne diseases and outbreaks in the United States and worldwide. Known bacterial pathogens have been considered one of the main causes of foodborne outbreaks associated with seafood consumption. It has been shown that shrimp and shrimp products, including ready-to-eat (RTE) shrimp can support the survival and/or growth of bacterial foodborne pathogens, and there are reports of foodborne disease

outbreaks where shrimp has been implicated (NACMCF, 2008, Norhana et al., 2010). The potential risk associated with shrimp can often be directly related to the environmental conditions and microbial quality of the water from which it was caught (Feldhusen, 2000). Water qualities such as temperature, salt content, distance between area of catch and polluted waters, and natural occurrence of bacteria in the water can affect the microbial quality of the shrimp (Feldhusen, 2000). Polluted waters have been associated with bacterial contamination of seafood and the sources of pollution have included overboard sewage discharge into harvest areas, illegal harvesting from sewage contaminated waters and sewage runoff from points inland after heavy rains or flooding (Iwamoto et al., 2010). Furthermore, the season during which the shrimp are harvested, the method of catch, chilling conditions and how they are handled, prepared and served can increase the risk of bacterial contamination (Feldhusen, 2000; Iwamoto et al., 2010). The shrimp may also become contaminated as a result of storage or transfer at improper temperatures, contamination by food handlers or through cross-contamination.

The two main pathogens that are commonly associated with shrimp contamination are *Listeria* and *Salmonella* species. These two species were also the main cause of shrimp detention in the U.S. (Norhana et al., 2010). These pathogens are divided into two groups based on their presence in the aquatic environment. One group, known as the indigenous bacteria, is naturally present in the aquatic environment. This group includes *Listeria monocytogenes*. This species is also known to be commonly present in the processing environment. The other group of bacteria is introduced to the aquatic environment by outside sources, such as human or animal faeces and is sometimes referred to as anthropogenic bacteria (NOAA, 2010). *Salmonella* is considered anthropogenic. Both species have been regularly isolated from shrimp culture environments (Norhana et al., 2010). There have been well-documented incidents of seafood and finfish

becoming contaminated with foodborne pathogens such as *Listeria monocytogenes* (Lennon et al., 1984, Mu et al., 1997, and Weagent et al., 1988), and *Salmonella* species (Heinitz et al., 2000).

2.4 Listeria.

Listeria is a bacterial genus containing six species of gram positive bacilli. Of the six species, *Listeria monocytogenes* is most well known due to the fact that it is the only *Listeria* species that is considered a major pathogen and it is the causative agent of the foodborne disease listeriosis. Three main serotypes, 4b, 1/2a and 1/2b, are primarily associated with foodborne outbreaks (Gahan and Collins, 1991). Several different foods including soft cheeses, hot dogs and seafood have been implicated in multiple outbreaks of human listeriosis (Ghandi & Chikindas, 2007). Listeriosis is a relatively rare disease; however, it has an exceptionally high mortality rate [(20-30%) (Todd & Notermans, 2011)] and the highest hospitalization rates (91%) of known foodborne pathogens (Sergelidis, 2009). As a result, it has caused much concern and the FDA has set a zero tolerance limit for this species in all ready-to-eat food products (FDA, 2010). According to the CDC, 1,600 people become seriously ill with listeriosis each year and, of these, 16% die (CDC, 2011). Listeriosis is defined as cases of invasive *Listeria monocytogenes*, which means the organism infects normally sterile parts of the body including the liver, spleen, cerebrospinal fluid and blood (Todd & Notermans, 2011). Once the pathogen enters the host cells through phagocytosis, it is released from the membrane-bound vacuole and begins to multiply (Ghandi & Chikindas, 2007). The pathogen then uses actin polymerization for intracellular movement and cell-to-cell spread allowing it to infect a large range of host tissues (Ghandi & Chikindas, 2007). The disease primarily affects the elderly, pregnant women, newborns and immune compromised adults (CDC, 2011). Symptoms typically include fever and

muscle aches, often followed by diarrhea and other gastrointestinal symptoms (CDC). The symptoms will often vary depending on the person infected. Pregnant women more often experience only mild, flu-like illness, but listeriosis infection during pregnancy can lead to miscarriage, stillbirth, premature delivery or a life-threatening infection to the newborn such as sepsis, pneumonia or meningitis (CDC, 2011; Todd & Notermans, 2011). Infected persons other than pregnant women can often experience headache, stiff neck, confusion, loss of balance and convulsions (CDC, 2011). Infected individuals who have a predisposing condition which has lowered their immunity including people with cancer malignancies, organ transplant, liver disease, HIV/AIDS, and diabetes, can develop sepsis, meningitis, and bacteraemia (Todd & Notermans, 2011). While not as common, healthy adults with no predisposing conditions are still at risk of developing meningitis and bacteraemia (Todd & Notermans, 2011).

Although strict regulations have been placed against *Listeria monocytogenes* in food by agencies such as the FDA, a number of outbreaks have still occurred. As of 2006, the number of sporadic cases of listeriosis increased across Europe (CDC, 2008). Also, a large outbreak occurred in Canada in 2008 resulting in 57 confirmed cases of listeriosis and 23 deaths (PHAC, 2010). The most recent outbreak occurred in the United States and has been linked to consumption of whole cantaloupes from Jensen farms (Holly, CO). According to the CDC, as of October 6 2011, there have been 109 confirmed cases of listeriosis, 21 deaths and one miscarriage resulting from this outbreak (CDC, 2011).

Listeria monocytogenes is prevalent in nature and can be isolated from soil, foliage and faeces of animals and humans (Feldhusen, 2000). This species is indigenous to the marine and estuarine environments, so its association with shrimp should be expected (Norhana et al., 2010). It has also been known to establish itself as an in-house bacterium within a processing facility. It

can create a biofilm on stainless steel surfaces and can be isolated from equipment, cold stores and floors enabling it to recontaminate products in the production environment (Feldhusen, 2000). In house reservoirs of *Listeria monocytogenes* have been reported from fish-processing establishments (Feldhusen, 2000), and the bacterium has been isolated from domestic and imported, fresh, frozen and processed seafood products, including crustaceans, molluscan shellfish and finfish (Elliott et al., 2000). It has also been isolated from ready-to-eat products such as cooked crabmeat, shrimp, and finfish, and from hot-smoked and cold-smoked mussels, salmon and other finfish (Jinneman et al., 1999). It has been revealed that the presence of *Listeria monocytogenes* in fresh and frozen raw shrimp at retailers, wholesalers and importers is common, and occurrence can vary from low to almost 50% (Norhana et al., 2010).

2.5 Salmonella.

Salmonella is a facultatively anaerobic, non spore forming, gram negative bacilli belonging to the family Enterobacteriaceae (Amagliani et al., 2011). Most strains are motile by flagella. There have been approximately 2,500 serovars of *Salmonella* identified (Iwamoto et al., 2011). The serovars are considered to be potential animal and human pathogens and are widely distributed in nature. They are commonly intestinal tract inhabitants of a wide range of animals including birds and humans (Norhana et al., 2010).

Salmonella is considered one of the leading causes of foodborne illness worldwide (Feldhusen, 2000). In 2007, *Salmonella* was identified as the most common bacterial infection reported according to a surveillance report from the Food Diseases Active Surveillance (FoodNet). In that same year it was estimated that the cost associated with *Salmonella* was US$ three billion [(Economic Research Services (ERS), 2009)]. Based on a global report, the non-

typhoid *Salmonella* serotypes most often responsible for human infections are Enteriditis followed by Typhimurium (Grieg & Ravel, 2009). Salmonellosis is the infection caused by *Salmonella* bacteria. Salmonellosis usually causes acute gastroenteritis and persons infected will experience diarrhea, fever, and abdominal cramps 12 to 72 hours after infection (CDC, 2011). The illness commonly lasts 4 to 7 days and does not result in hospitalization or death; however, the elderly, infants, and those with impaired immune systems can experience severe illness and consequences (CDC, 2011). The CDC estimates that there are approximately 40,000 cases of salmonellosis each year resulting in 400 deaths, and children are the most likely to acquire the infection (CDC, 2011).

Most cases of salmonellosis are caused by ingestion of contaminated food (Iwamoto et al., 2011). Outbreaks of *Salmonella* associated with seafood have been caused by shrimp, fish, oysters and clams (Iwamoto et al., 2011). From 1998-2004, *Salmonella* was the leading cause of foodborne illness outbreaks associated with seafood (CDC). The FDA has revealed the presence of *Salmonella* in a variety of fish and shellfish products including RTE seafood (Iwamoto et al., 2011). *Salmonella* contaminates shrimp usually through cross-contamination or by entering the marine environment. The organism enters the aquatic environment through wild animals, domestic stock, poor sanitation and inappropriate disposal of human and animal wastes (Amagliani et al., 2011). As a result of its prevalence in aquatic environments, *Salmonella* is considered a natural flora of the shrimp culture environment (Norhana et al., 2010). Shrimp can also become contaminated with this pathogen during storage and processing (Iwamoto et al., 2010). The presence of *Salmonella* is used by the FDA as an indicator of poor sanitation during production, harvest or processing, and its occurrence is unacceptable (Miget, 2010).

2.6 Additional Concerns Facing the Shrimp Industry.

2.6.1 Persistence of *Listeria monocytogenes* to Adverse Conditions.

A large cause of concern in the food industry is the fact that *Listeria monocytogenes* is a very persistent organism and is able to grow under stressful conditions such as: refrigeration temperatures, in high salt foods, in acidic foods, and within the human immune system (Hill et al., 2002). This pathogen is able to grow and multiply at temperatures from 0 to 45° C, at pH values between 4.6-9.5 and at high salt concentration or water activity as low as 0.92 (Carpentier & Cerf, 2011; Todd & Notermans, 2011). According to the FDA Bad Bug Book, it resists the deleterious effects of freezing, drying and heat remarkably well for a non-spore forming organism (FDA Bad Bug Book, 2009).

It is believed that *L. monocytogenes* must have the ability to evaluate and process information about its environment quickly in order to be able to adapt to its changing conditions (Hill et al., 2002). Stress adaptation usually occurs when a bacterium is exposed to sub-lethal stress and as a result may become more resistant to further applications of the same stress or sometimes to a different stress (Hill et al., 2002). The adaptation of *L. monocytogenes* to adverse environmental conditions requires changes in gene transcription which is facilitated by the alternative stress sigma factor, sigmaB (Ghandi & Chikindas, 2011).

The survival and growth of *L. monocytogenes* at refrigeration temperature makes it difficult to control the organism in foods due to the fact that refrigeration is one of the most widely used methods to extend the shelf life of food products (Ghandi & Chikindas, 2011). The adaptation of bacteria to low temperatures includes three phases: initial cold shock, acclimation, and cold adapted status (Chan & Wiedmann, 2011). As the temperature of the environment

decreases, the metabolic rate of bacterial cells also decreases, leading to a change in membrane lipid composition to maintain proper membrane fluidity for enzyme activity (Chan & Wiedmann, 2011; Ghandi & Chikindas, 2011). Cold shock proteins (Csps) are produced by *L. monocytogenes* in response to a temperature down shock, and cold acclimation proteins (Caps) are synthesized during balanced growth at low temperatures (Ghandi & Chikindas, 2011). When the bacteria is subjected to cold shock, a rapid switch from the bacterium's optimum growth temperature to a lower temperature, the synthesis of non-cold-shock proteins is inhibited and the synthesis of cold shock proteins (Csps) is greatly increased (Chan & Wiedmann, 2011). Additionally, it has been shown that *L. monocytogenes* uses the general stress sigma factor, sigmaB, to accumulate the compatible solutes glycine betaine and carnitine which work together as cryoprotectants during refrigerated storage (Ghandi & Chikindas, 2011).

It has been determined that the organism can multiply at refrigerator temperatures (2-4°C), and after refrigeration it is possible for food to contain significant levels of *L. monocytogenes*, even though it has been stored at FDA recommended temperatures (Elliott et al., 2000). One study showed that regardless of whether shrimp were raw or cooked, *L. monocytogenes* had a significantly ($p<0.01$) higher ability to grow on them when stored aerobically at 4°C compared to beef or chicken (Shineman & Harrison, 1994; Norhana et al., 2010). In addition, the growth rate of *L. monocytogenes* in cooked shrimp is reported to be greater than in most RTE foods and this may be due to ideal growth conditions, including pH range, water activity and salt content of shrimp (Fsanz, 2002). The occurrence of the organism is considered high in cooked shrimp (1.5-25.0%) and shrimp salad (23.0%) that have undergone commercial cooking processes (Norhana et al., 2010). These facts could mean that even if low levels of *L. monocytogenes* are present in cooked shrimp, high levels could be reached if the

product is stored at refrigerator temperatures commonly used in chilled storage at retail stores. With the increase in consumption of RTE products stored at chilled temperatures that receive no further cooking treatment comes an increased risk of foodborne disease due to psychotrophic pathogens such as *Listeria monocytogenes*. Additionally, the FDA has also revealed the presence of *Salmonella* in a variety of RTE seafood products (Amagliani et al., 2011).

Another major problem in the food industry is the ability of *L. monocytogenes* to create biofilms in food processing facilities. A biofilm exists when the bacterial cells are attached to a surface and enclosed in a matrix primarily made of polysaccharide material (Gandhi & Chikindas, 2011). Biofilms can form on food processing equipment, food processing or handling surfaces, or food storage areas. Biofilms can also establish themselves in harborage sites which are generally hard to reach and even harder to clean (Carpentier & Cerf, 2011). It has been determined that biofilms of *Listeria* are more resistant to disinfectants and sanitizing agents making it hard to eliminate them from food processing facilities (Gandhi & Chikindas, 2011). The main concern in the food industry is that these biofilms can be transferred to food products.

2.6.2 Heat Resistance of Pathogens.

It has been shown that non-chitinolytic *Listeria* and *Salmonella* have been able to attach and colonize shrimp carapace and tissue (Norhana et al., 2009). This attachment and colonization of bacteria to surfaces can increase their resistance to stress, particularly if the surfaces have naturally protective microhabitats like shrimp carapace (Norhana et al., 2010). This adaptation could result in increased resistance to heat treatments. In a recent study, attached and colonized cells of *Listeria* and *Salmonella* on shrimp showed significantly higher ($p<0.05$) resistance to heat (~1.3-2.6 fold increase in *D*-values) than their planktonic counterparts

(Norhana et al., 2010). It has also been determined that some bacteria increase their resistance to heat as a result of exposure, for a short time, to moderately elevated temperatures, normally above their maximum growth temperatures (Sergelidis et al., 2009). As a result, proper cooking must be able to eliminate pathogens in their most heat resistant state in order to ensure safe food for consumers.

2.6.3 Safety Issues with Aquaculture and Imported Shrimp.

Shrimp consumption has steadily increased over the past decade and shrimp and shrimp products are in high demand. This demand can no longer be met by either U.S. production or by wild caught shrimp. As a result, approximately 84 percent of the seafood consumed in the U.S. is imported (NOAA, 2010). China is the leading seafood exporter followed by Thailand and Vietnam (FAO, 2010a). Shrimp has now become the highest valued internationally traded seafood commodity (Gillet, 2008). Unfortunately, an increase in global fish trade leads to an increased risk of spreading food pathogens between countries. Due to the fact that a large amount of imported seafood comes from developing countries and is shipped a long distance, a greater risk has been associated with imported products. Also, there is currently no international agreement on the acceptable levels of *Salmonella* or *L. monocytogenes* on food, and it is estimated that the US Food and Drug Administration is only able to inspect less than 2% of imported seafood (Norhana et al., 2010).

In a recent report, shrimp was responsible for the largest proportion of import items detained during trade in the U.S. (Norhana et al., 2010). The main cause for detention was the presence of pathogenic bacteria and the two main species that caused detention were *Salmonella* (35.6%) and *Listeria* (4.1%) (Norhana et al., 2010). In addition to detentions, recalls of shrimp

have also been made due to contamination with these two pathogens (Norhana et al., 2010). Furthermore, in a nine year study performed by the FDA, the incidence of *Salmonella* was identified in 7.2% of 11,312 imported samples compared to 1.3% of 768 domestic samples (Heinitz et al., 2000). The presence of *Salmonella* and *Listeria* on the international shrimp trade is estimated to have a substantial impact due to financial losses through detentions, recalls and re-inspections.

Of the imported shrimp in the U.S., farm cultured shrimp accounts for approximately half (NOAA, 2010). Aquaculture production has greatly increased outside of the U.S., particularly in the Asia-Pacific region, and now supplies 46% of the world's seafood supply (FAO, 2010a). This trend could lead to increased risk of safety hazards related to shrimp consumption due to biological and chemical contamination in farm culture waters compared to natural seawater as a result of the proximity of culture farms to urban areas (Feldhusen, 2000). A number of factors have been shown to influence the safety of aquaculture products including location, farmed species, husbandry practices, postharvest processing, and cultural habits of food preparation and consumption (Amagliani et al., 2011). Also, *Salmonella* contamination in cultured shrimp products is a problem, and contamination of culture environments can occur through the following routes: run off of organic water into ponds during rainfall; animal waste; introduced directly (bird droppings or frogs) or indirectly (runoff); fertilization of ponds with non-composted manures; integrated farming systems with animals housed in proximity to ponds; toilets discharging into ponds; contaminated source water; unsanitary ice, water, containers, and poor hygienic handling practices; and contaminated feed (FAO, 2010a; Amagliani et al., 2011). A study by the FDA showed that aquacultured seafood was more likely to contain *Salmonella* than wild caught seafood (Koonse et al., 2005). Additionally, several reports have been made on

the prevalence of *Salmonella* in shrimp culture environments (Amagliani et al., 2011). It is considered "reasonably likely" (a HACCP term) that *Salmonella* will be present on, or in the intestinal tract of farmed shrimp (Miget, 2010).

Another major issue facing the aquaculture industry is the fact that extensive use of antibiotics in agricultural animal production can result in the development of antibiotic resistant pathogens and that these pathogens can infect and transfer resistance to humans (Holmström et al., 2003). The development of resistant pathogens in aquaculture environments has been well documented and the transfer of resistance encoding plasmids between aquaculture environments and humans has been reported (Holström et al., 2003). The term resistance refers to the microorganism's ability to adapt and survive antimicrobials (Amagliani et al., 2011). When an organism is exposed to a sub-lethal level of an antimicrobial agent, it can lead to adaptation and development of resistance to higher levels of the antimicrobial or cross-resistance to other agents (Ghandi & Chikindas, 2007). The consequences of resistance to public health include failure of treatment, increased severity and duration of infections, hospitalization and mortality (Newell et al., 2010). Antibiotics and other substances that are authorized for use in aquaculture include oxytetracycline, florfenicol, chorionic gonadotropin, formalin solutions, tricaine methanesulfate, sulfadimethoxine/ormetropin, and hydrogen peroxide (FDA center for Veterinary Medicine, www.fda.gov/cvm).

The concern of antibiotic resistant pathogens has been spotlighted by an increased prevalence of antimicrobial resistant *Salmonella* in shrimp and other claims that RTE shrimp is an international vehicle of antibiotic resistant bacteria (Norhana et al., 2010). Both the European Food Safety Authority (EFSA) and National Antimicrobial Resistance Monitoring System (NARMS) have reported on resistant and multiresistant *Salmonella* isolates (Amagliani et al.,

2011; EFSA, 2007; FDA, 2006). A *Salmonella* strain resistant to ampicillin, ceftriaxone, gentamicin, streptomycin and trimethoprim from farm cultured shrimp imported into the U.S. has been reported (Boinapally and Jiang, 2007). Also, a *Salmonella* strain resistant to extended spectrum betalactamalase has been recognized worldwide (Newell et al., 2010). One study showed that of 4072 imported foods into the U.S., fifteen percent of the *Salmonella* isolates recovered from the foods were resistant to at least one antimicrobial and five percent were resistant to three or more antimicrobials, and the resistant isolates were predominantly found in seafood (Zhao et al., 2003). Moreover, a study conducted on the antibiotic use in shrimp farming in Thailand revealed that the use of antibiotics among farmers in that area could result in a severe risk of development of antibiotic resistant bacterial strains (Holmström et al., 2003). Of particular concern in this study was the prophylactic use of antibiotics at sub therapeutic levels. The study claimed that 74% of the farmers used antibiotics in farm management and a minimum of thirteen different antibiotics were used. Additionally, the farmers either used higher doses or what they considered more potent antibiotics for treatment rather than prevention. It was also revealed that many of the farmers studied did not have enough information on efficient or proper use of the antibiotics. A main cause of concern was the widespread use of fluoroquinolones among the farmers in the study which are important due to their treatment of a broad range of human pathogens (WHO, 1998; Holmström, 2003).

Furthermore, antimicrobial resistance in *Listeria* has also come to light in recent years. A study by Walsh et al. (2001), revealed that 10.9% of 1001 *Listeria* isolates from retail foods were resistant to one or more antibiotics. Most resistance was to penicillin or tetracycline and neither of these are currently used for treatment of listeriosis (Walsh et al., 2001). Likewise, a study conducted by Prazak et al. (2002) concluded that 95% of twenty-one isolates of *L.*

monocytogenes were resistant to two or more antibiotics. Of the isolates 85% were resistant to penicillin and one of the strains was also resistant to gentamycin (Prazak et al., 2002). These results are critical because they reveal multidrug resistant *L. monocytogenes* strains found in food and the environment and the occurrence of resistance to gentamycin which is sometimes used to treat listeriosis. Therefore, it is extremely important to eliminate *Salmonella* and *Listeria* during cooking to prevent persons from becoming infected with strains that cannot not be treated with antibiotics.

2.6.4 The Use of Sodium Tripolyphosphate.

Color is one of the most important characteristics evaluated by consumers due to its relation to product acceptability (Fernandez-Lopez et al., 2003). Often times color is used by consumers to determine doneness of a product when cooking which in turn relates to sensory acceptance. If the color is not right, the consumer usually considers the product to be improperly processed, spoiled or of low quality (Latscha, 1989).

In shrimp carotenoids are the primary compounds responsible for the coloration (pigmentation) and are present in the sub epidermal chromatophores and/or in the principal layers of the exoskeleton (Latscha, 1989). The most prevalent carotenoid is astaxanthin, representing 65-98% of the total carotenoids present in the integumental tissues (Latscha, 1989). The astaxanthin is commonly in carotenoid-protein complexes termed carotenoproteins or chromoproteins which results in the pigment-protein bond exhibiting colors ranging from green to purple which are very different from the color of the pigment itself (red) (Latscha, 1989). As the shrimp cooks, the protein becomes denatured resulting in unbinding of the carotenoid-protein

complex, and the astaxanthin becomes free to absorb light and produce the red color commonly associated with cooked shrimp.

Lately, there have been concerns associated with the additive sodium tripolyphosphate altering the red color change of cooked shrimp. Sodium tripolyphosphate is commonly added as a way to retain moisture in the shrimp and to therefore enhance the end product quality. Water is the main component of shrimp comprising 70-80% and, as a result, it has a major influence on the sensorial characteristics and quality (Gonçalves & Ribiero, 2008). A large amount of water can be lost during capture, processing, storage, and distribution. Therefore, there is an emphasis on retaining water to ensure quality of the final product and for economic reasons due to the fact that seafood is sold by weight (Gonçalves & Ribiero, 2008). As a result, phosphates have been widely accepted as additives in seafood to increase water retention in fresh products (Rattanasatheirn et al., 2008).

The alkaline phosphates are salts of the purified phosphoric acid (Hourant, 2004). Their name will change depending on the number of P atoms they have (Hourant, 2004). For example, tripolyphosphates have three P atoms. The two main characteristics of the phosphates are their chain length and their pH. These two characteristics define their four properties: buffering agent, sequestering power, dispersing power and water holding capacity (Hourant, 2004). Sodium tripolyphosphates have a high pH which increases their ability for water retention (Schilling et al., 2010) by increasing the muscular proteins' water holding capacity (Gonçalves & Ribiero, 2008). However, if the shrimp have too much moisture it can lead to adulteration and economic fraud (Gonçalves & Ribiero, 2008).

Sodium tripolyphosphate is commonly applied by exposure to prepared solutions by spray, dips, soaks, soaks with or without mechanical tumbling, or by direct packaging with the product prior to freezing (Otwell, 1993). Shrimp may be soaked after catch, onboard where the phosphate is mixed with the water and added to the slush ice with the shrimp (southereasternfish.org). In this case, the phosphate is commonly prepared as a 2 to 3 percent solution (w/v) of food grade sodium tripolyphosphate and held at 41°F or colder for about 2 hours (Miget, 2010). The concentration of the phosphate solution in the shrimp after soaking can range according to concentration of the phosphate solution and soak time. There are currently no formally approved regulations set by the FDA for the use of sodium tripolyphosphate. The additive has FDA GRAS (Generally Recognized as Safe) status and the guidelines by the FDA state that it should be "used according to good manufacturing practices" (FDA, 2011). It is then left up to the processor to decide what "good manufacturing processes" are, and this is where the problem of adulteration and economic fraud can occur. Additionally, it can become a safety hazard for the consumer due to the fact that misuse of sodium tripolyphosphate can result in excess moisture content and a translucent appearance of the shrimp even after it has been cooked. This could prevent the consumer from being able to use the red color change during cooking as a doneness indicator. As a result, this factor must be taken into account when developing safe cooking guidelines for shrimp products.

2.7 Shrimp Safety.

Handling of raw shrimp between capture and distribution to the consumer has a major impact on the bacteriological quality and safety of shrimp. The time between harvesting and processing periods is crucial, and shrimp must be properly handled and stored in order to prevent microbial degradation (Hossain et al., 2010). After death the flesh of shrimp is still active and

biochemically alive and enzymes and microbes immediately begin the process of organic decomposition (Boonsmrej et al., 2007). Frozen storage of shrimp is imperative to extend its shelf life by limiting microbial and enzymatic activity which causes deterioration (Tsironi et al., 2009). Once shrimp comes onboard the shrimp boat, there is a race against time and temperature to maintain quality (Miget & Haby, 2003). Low temperatures reduce the growth of bacteria, which is why it is crucial to chill and freeze shrimp as soon as possible (Falgout & Bell, 2008). The highest quality shrimp can be obtained in shrimp immediately frozen after harvesting (Boonsumrej et al., 2007). As a result of its extended shelf life, frozen shrimp is a product of high commercial value and increasing demand (Tsironi et al., 2009). Moreover, the freezer boat method has an additional economic advantage over the traditional ice boat method due to the fact that it can stay out in the water longer because it does not have to deal with ice melting and product decomposing (Nagle et al., 1980). The freezer boats can shrimp until they reach full capacity and do not have to waste fuel by making trips back to port to replenish ice (Nagle et al., 1980).

Two methods are commonly used to freeze shrimp after harvest, brine freezing and flash freezing which is also commonly referred to as blast freezing. Both of these methods result in quick freezing of the shrimp which prevents the formation of large ice crystals allowing the growth of only small ice crystals. The small ice crystals will not puncture as many cell walls resulting in only minimal water loss and a higher quality product when thawed (Nagle et al., 1980).

Brine freezing is an effective and economical freezing method that results in premium quality shrimp. Due to the fact that extremely low temperatures are not required to rapidly freeze the shrimp in a brine solution, it is also an energy efficient freezing method (Bankston et al.,

1996). Brine freezing is based on the fact that a brine solution, concentrated salt water, is able to lower the freezing point of water. Furthermore, cold circulating water removes heat from shrimp ten times faster than cold, circulating air (Miget & Haby, 2003). In order to achieve an effective brine system, salt should be added to reach a concentration of 23.31% which will lower the freezing point of water to -6° F. The actual working temperature of the brine system is usually between 5° F and 0°F which allows the shrimp to completely freeze within 20 minutes (Miget & Haby, 2003). It is important that the shrimp freeze within 20 minutes in order to prevent the uptake of salt by the shrimp and loss of water from the shrimp (Falgout & Bell, 2008).

In order to have a successful brine freezing system, several steps must be taken. The first step is to measure the gallon fill capacity of the brine tank. Then, the proper amount of salt must be added to achieve a concentration of 23%. This is usually achieved by adding 2.53 pounds of salt per gallon (Falgout & Bell, 2008). The brine mixture must then be cooled to the lowest temperature possible which is usually a working temperature of 5° F and 0° F. The shrimp may then be added to the circulating brine tank and should freeze within twenty minutes. Shrimp are either frozen in mesh bags that can hold up to 30 lbs of shrimp or perforated boxes that can hold up to 20 lbs of shrimp (Falgout & Bell, 2008). There should never be more than 15 pounds of shrimp per 100 gallons of water in a brine tank because it will "heat shock" the brine solution and prevent complete freezing within 20 minutes (Miget & Haby, 2003). Additionally, because some of the salt will remain on the shrimp and be removed from the tank after freezing, the brine solution must be recharged by adding salt to maintain the proper concentration. Otherwise, the operating temperature will increase and it will take longer to freeze the shrimp. The brine tank should be recharged after freezing approximately 1,000 pounds of shrimp (Miget & Haby, 2003). Moreover, in order to achieve minimal freezing times, shrimp are pre-chilled in an ice bath

consisting of ice and water in a 50/50 ratio in order to get the shrimp close to 32°F prior to freezing (Miget & Haby, 2003).

Blast freezing or flash freezing involves subjecting the shrimp to cryogenic temperatures in order to freeze them in a short time period. After harvest the shrimp are first pre chilled in an ice bath to lower their temperature. The shrimp are then sorted and placed into plastic bags and packed into 5 pound boxes. The boxes are then placed inside a -40° C freezer until completely frozen, usually within 1 hour, and then stored at -18 to -20° C (Tsironi et al., 2009). This method is very straight forward and mistakes are less common than with the brine freezing method. Also, because the shrimp are frozen in their natural state and do not have a brine solution coating them, they are easy to thaw and separate and then peel, making them very acceptable to the consumer. However, the disadvantage is that the blast freezers are more expensive and take up more space on the shrimp boats.

Freezing has become a very effective preservation for shrimp, however, some deterioration in shrimp quality can still occur during storage (Boonsumrej et al., 2007). It is possible for some microorganisms to remain viable to different extents in frozen foods during storage, and this can be dependent on the rate of cooling or thawing and the storage temperatures (Hatha et al., 1998). It is possible for shrimp to be subjected to temperature fluctuations during transportation to processors and distribution sites as well as during storage. Shrimp might also undergo thawing and refreezing cycles before it reaches the consumer (Tsironi et al., 2009). During this time bacteria might have the opportunity to grow and multiply in the shrimp making it a potential hazard to the consumer.

Recently, there have been increased food borne outbreaks associated with restaurants and private residences, and the two main causes have been improper food handling and inadequate cooking. Pathogenic bacteria, such as *Salmonella* and *Listeria monocytogenes*, are one of the main causative agents for the food borne outbreaks associated with shrimp. Shrimp becomes contaminated with these pathogens due largely to fecal pollution of the aquatic environment, pathogens naturally present in the aquatic environment, and through industrial, retail, restaurant or home processing and preparation (Norhana et al., 2010). Also, imported and aquacultured shrimp present contamination problems. However, most of the pathogenic bacteria are present in low levels in shrimp and do not pose a great threat when the shrimp is adequately cooked. Therefore, improper cooking contributes to a large number of food borne outbreaks caused by the consumption of shrimp.

Cooking, which refers to the application of heat, plays a major role in the safety and sensory acceptance of shrimp. Cooking is achieved by exposing the shrimp to moist heat, dry heat, microwave energy, radiant heat or their combinations (NACMCF, 2008). There are a wide variety of cooking recommendations available, however, The National Committee on Microbiological Criteria for Foods stated that most recommendations for cooking seafood are based on subjective measures for determining whether a fishery product is adequately cooked (NACMCF, 2008). When cooking shrimp, cookbooks generally claim that the shrimp is adequately cooked when the flesh is opaque and the shell has turned to a red-orange color. Likewise, the FDA recommends that shrimp should be cooked until the flesh is pearly-opaque in color (FDA, 2011). Consequently, "doneness" refers to the visual appearance that leads consumers to believe that the shrimp is adequately cooked. Moreover, when boiling shrimp they

are believed to be done once they have floated to the surface of the water and are red-orange in color. These guidelines are not science based and do not ensure proper elimination of pathogens.

The inactivation of pathogens in seafood depends on the type of seafood, the cooking method, and the type of pathogen. As a result, the thermal inactivation kinetics should be determined for the most prevalent and heat resistant pathogen of concern in shrimp (NACMCF, 2008). Currently, there is not adequate information on the thermal inactivation data for relevant pathogens in seafood, and one reason for this is the wide variety of methods for cooking applied to shrimp and seafood products (NACMCF, 2008). Also, there is currently no single temperature that will ensure the safety of all cooked fishery products while providing an acceptable product in terms of palatability and appearance (NACMCF, 2008). Another factor that must be taken into account is that shrimp vary in size and thickness and will therefore require different cooking times and temperature. Additionally, the FDA recommends that shrimp may be stored in the refrigerator for up to two days. However, refrigerator temperatures may vary and allow for the growth of bacteria to high initial levels prior to cooking or allow the growth of bacteria in stored leftovers. Therefore, it is crucial to develop science based cooking methods for shrimp to ensure safety for the consumers. It is equally important to test the reliability of current widely accepted methods, such as the previously described method for boiling shrimp until floating and red in color, to ensure the destruction of pathogens in shrimp. This science based information should then reach consumers in a way that is easy for them to understand in order to enable them to make informed decisions when cooking shrimp.

CHAPTER 3 MATERIALS AND METHODS

3.1 Determining the Thermal Log Reduction of *Listeria* and *Salmonella* Species in Shrimp After Boiling Until Floating.

3.1.1 Culture Preparation.

Salmonella enteriditis (13076), *Salmonella infantis* (CDC, Atlanta), *Salmonella typhimurium* (ATCC 14028), *Listeria innocua* (*Lm* F4248, CDC, Atlanta), *Listeria monocytogenes* (1/2a) (Lm F4263, CDC, Atlanta) and *Listeria welshimeri* (ATCC, 35897) were obtained from the Louisiana State University, Department of Food Science frozen culture collection. Frozen cultures were thawed and a loop full (~ 10µl) of each *Salmonella* serotype was suspended into 9 mL of Brain Heart Infusion (BHI) (Acumedia Manufacturers, Inc., Lansing, MI) broth and a loop full of each *Listeria* serotype was suspended into 9 mL of Tryptic Soy Broth [(TSB) (Becton, Dickinson & Co., Sparks, MD)]. All were incubated at 37° C for 16 hours in order to achieve stock cultures that were approximately 10^7-10^9 cell density. After 16 hours, a loop full of each *Salmonella* serotype was then transferred from the broth and streaked onto BHI agar (Acumedia Manufacturers, Inc., Lansing, MI) slants and a loop full of each *Listeria* serotype was transferred from the broth and streaked onto tryptic soy agar (Becton, Dickinson & Co., Sparks, MD). slants. The slants were incubated for 16 hours at 37° C and then maintained at room temperature (23°C) for future use. Prior to using the cultures for inoculation, *Salmonella* and *Listeria* species were subcultured by suspending a loop full of cells from the agar slants into 9 mL of BHI broth for *Salmonella* and 9 mL of TSB broth for *Listeria* and incubated for 16 hours at 37° C. This resulted in an approximately 10^7 cell density working culture.

3.1.2 Sample Preparation.

Shrimp samples were purchased from a local seafood market (Maxwell's Market, Baton Rouge, LA). The samples were large (21-30 count per pound) Gulf coast shrimp with the shells and heads on. Individual shrimp weighed approximately 16 ± 2 grams. On the day of inoculation, the samples were washed thoroughly with tap water followed by sterile distilled water immediately before inoculation. Fifty mL of respective 16 hour culture was added to a sterile container holding 500 mL of sterile 0.1% Phosphate Buffered Saline (PBS) solution which consisted of 2.4 g sodium phosphate monobasic (Sigma-Aldrich, Inc., St. Louis, MO), 2.84 g sodium phosphate dibasic (Sigma-Aldrich, Inc., St. Louis, MO), and 8.5 g NaCl (VWR International LLC, West Chester, PA) in 1 L of dH_2O. This gave an approximate 10^6 cell density bacterial suspension. The shrimp samples were soaked in the culture solution for 30 minutes and then allowed to air dry for one hour. This procedure was followed for each of the six bacterial species used in this study. The final concentration of the bacteria was 3.5 to 5.5 $\log_{10}$ CFU/g. After inoculation, the shrimp samples were randomly picked and separated into three different groups for sampling; days 0, 1 or 2. Day 1 and 2 sample groups were stored at 4° C before heat treatments.

3.1.3 Thermal Destruction Procedures.

The day of inoculation was assigned day 0 and immediately after surface inoculation and drying, individual shrimp samples were placed into a boiling water bath (100°C) using an immersible 6 quart multi-cooker (National Presto Industries, Inc., Eau Claire, WI) containing 1 L of tap water and were removed immediately once they floated to the surface of the water and were pink in color. The time it took for the shrimp to float to the surface of the water was

measured and recorded. A traceable thermometer with a digital probe (Control Company, Friendswood, TX) was used for monitoring the internal temperatures. The thermometer probe was inserted at the cold spot of the shrimp and monitored individually. The cold spot of the shrimp is its thickest part which is between the first and second abdominal segment.

FIGURE 1: Cold Spot of the Shrimp.

The numbers 1 and 2 represent the 1st and 2nd abdominal segments and the circle indicates where the thermometer probe was inserted to take a temperature reading.

Additionally, surface temperature was measured using a ThermoTrace Model 15036 infrared laser thermometer (Delta Trak, Pleasanton, CA). After recording the temperature measurements, the heated samples were immediately transferred to whirl pak filter sample bags (Nasco, Salida, CA, USA) using sterile tongs. The bags were then submerged into an ice cold water bath in order to stop further cooking of the sample.

3.1.4 Enumeration of Bacteria.

The samples were weighed and the weight was recorded. Equal (wt/vol) amounts of PBS were added to the samples in the whirl pak bag and the samples were homogenized by placing in a Lab-Blender 400 Stomacher (Tekmar Co., Cincinnati, OH) at normal speed for one minute. Under aseptic conditions, decimal dilutions of each sample were prepared and the dilutions were plated in triplicate on Xylose Lysine Deoxycholate [(XLD) (Remel – Thermo Fisher Scientific, Lenexa, KS)] plates for *Salmonella* species and Oxford Listeria agar (Acumedia Manufacturers, Inc., Lansing, MI) plates that had been modified by adding Listeria selective enrichment supplement (Acumedia Manufacturers, Inc., Lansing, MI) for *Listeria* species. Plates were incubated overnight at 37°C. After incubation, colonies were counted and Log CFU/g was calculated for each plate.

The same procedure was followed for all the days tested. On days 1 and 2, the samples designated day 1 or 2 were taken out of refrigerated storage and the above procedures for heat treatment and enumeration were followed. All experiments were repeated three times.

3.2 Time Needed for Pathogens to Reach Non-Detectable Levels in Shrimp.

This study was performed in order to determine the time point during boiling until floating when the pathogenic bacterium is killed. The average time to floating for individual shrimp was determined from the previous study to be 1:20-1:30 minutes depending on the size of the shrimp. In order to determine the bacterial destruction on the shrimp during boiling, five time points were measured; time 0, 25 seconds, 50 seconds, 1:15 seconds and 1:30 seconds. For this study, the previously described methods for sample preparation were followed except using only *S. typhimurium* and *L. monocytogenes*. The individual shrimp were then placed in the boiling

water bath and removed at the different time points. Enumeration of bacteria was then performed for each shrimp at each different time point. The study was performed in triplicate.

3.3 Growth of Pathogens on the Surface of Shrimp at 4°C.

In order to determine the growth of pathogenic bacteria on the surface of shrimp during refrigerated (4° C) storage, an inoculum study was performed. For this study the shrimp were inoculated on Day zero following the same procedures as previously described but only *S. typhimurium* and *L. monocytogenes* were used. Only these two pathogens were studied because they are the most resistant to environmental conditions such as low temperatures. Enumeration of bacteria on shrimp was performed on day zero. Shrimp were then placed in refrigerated storage (4° C) for six days. Enumeration of bacteria was performed on samples each day from day zero to day six to observe the growth of the bacteria during refrigerated storage. The number of days was decided as six in order to mimic typical cold chain conditions of shrimp. The FDA recommends that consumers may store shrimp for up to two days at refrigerator temperatures (FDA, 2011), however, shrimp have most likely already been stored at refrigerator temperatures for a number of days during transportation and chilled display at the retail outlet before it reaches the consumer. After six days of refrigerated storage the shrimp were then boiled until floating and enumeration of bacteria was performed in order to test whether the surviving bacteria had become more heat resistant.

3.4 Cooking Portion Study.

Since it would be highly unlikely for a consumer to boil and eat just one individual shrimp at a time, a cooking portion study was performed. In this study a more common cooking size of shrimp was used which was one pound of large (21-30) shell on, head on shrimp

(Maxwell's Market, Baton Rouge, LA). For this study, the same procedures for sample preparation as the original study were followed, except only the most pathogenic and resistant strains, *S. typhimurium* and *L. monocytogenes*, were studied. Typical cookbooks recommend boiling a pound of shrimp in 4 cups of water so 4 cups of water was used to boil the shrimp in this study. The water was brought to a boil (100°C) in the water bath and then the pound of shrimp was added. The time to floating for the shrimp was measured and recorded. The samples were then transferred to sample bags and the steps for enumeration of bacteria were then followed. The study was performed in triplicate.

3.5 Control of Pathogens in Shrimp Boiled in Salt Water.

Due to the fact that it is common for shrimp to be boiled using certain shrimp boil seasoning mixes which contain salt, it was necessary to determine the effect of salt on the boiling temperature, time and bacterial quality of the shrimp. For this study, sample preparation was performed following the same steps as the original study except using only *S. typhimurium* and *L. monocytogenes*. In order to determine the appropriate salt content of the water, the salt content of two widely used shrimp boil mixes, Zatarain's Crawfish, Shrimp and Crab Boil (Zatarain's, New Orleans, LA) and Louisiana Crawfish, Crab and Shrimp Boil (Louisiana Fish Fry Products, Baton Rouge, LA) were averaged and the average value was then used. The average salt content was calculated to equal 17.5 grams or ~1.7% salt for 1 pound of shrimp boiled in 4 cups of water. Food grade table salt (Wal-Mart, Springdale, AR) was added to 4 cups of water and brought to a boil. Individual shrimp were added to the boiling water and removed immediately once they floated to the surface of the water. The time to floating, and the internal and external temperature were measured and recorded. Also, the salt content of the shrimp before and after boiling with salt was measured and recorded using a handheld YSI model 30 salinity,

conductivity, and temperature instrument (YSI Inc., Yellow Springs, OH). The steps for enumeration of bacteria were then followed. Additionally, a pound of shrimp was boiled until floating under the same conditions to observe if there was any affect on the time to floating for a pound of shrimp boiled in water with salt added.

3.6 Determining the Possibility of Bacterial Growth During Refrigerated Storage of Cooked Shrimp.

3.6.1 Bacterial Counts of Inoculated Shrimp at 4°C.

Shrimp samples were inoculated as previously described and separated into days 0, 1 and 2. On day 0 all three test groups were then given the same heat treatment as the original study. For day 0, the procedure for enumeration of bacteria was followed after heat treatment. The cooked Day 1 and Day 2 samples were stored at 4°C. The same methods for the enumeration of bacteria as described earlier were then performed for the previously cooked shrimp samples after 1 and 2 days of refrigerated storage.

3.7 Determining a Correlation Between Pathogen Reduction to Non Detectable Levels and the Degree of Color Change After Boiling Shrimp Until Floating.

3.7.1 Color Analysis of Cooked Shrimp.

The color of the cooked and uncooked shrimp shell was measured by a Minolta spectrophotometer (CM-508d series, Osaka, Japan). The color of cooked and uncooked shrimp was evaluated after the heat treatment was performed. Before taking color measurements, the device was calibrated by first taking a reading of the ambient surroundings and then by using a white color standard (L=97.21, a=0.55, and b=2.12). Color readings were taken on the same spot for each shrimp which was again at the thickest part, between the first and second abdominal

segments. The spectrophotometer was used to take 3 color readings. Values for “L” (luminance), “a” (red to green) and “b” (yellow to blue) were obtained using the Hunter Lab System which was programmed into the instrument.

3.7.2 Determining the Effect of Sodium Tripolyphosphate on the Color Change of Shrimp During Cooking.

The methods used for this study were the same as the original study and the color analysis was the same as previously described. The difference was that the shrimp was purchased directly from a local Louisiana fisherman (AnnaMarie Seafood, Houma, LA) who had not applied any sodium tripolyphosphate to the shrimp.

3.8 Statistical Analysis.

Differences in survival of all species after the heat treatment of boiling until floating were analyzed for significance using Student’s t-test following one-way analysis of variance (ANOVA) JMP-IN (version 9.0, SAS Institute Inc., Cary, NC). The statistical difference was at $p<0.05$. Data analysis for color evaluation was done by taking the mean of the color readings for each temperature treatment per replicate. Data was analyzed using one-way ANOVA.

3.9 Effect of Different Freezing Methods and Subsequent Storage on the Microbial Quality of Shrimp.

3.9.1 Brine Freezing.

This study was performed in order to determine the effect of brine freezing on the microbial quality of shrimp immediately after freezing and during subsequent cold chain storage. The methods for brine freezing used in the lab closely mimic the methods used onboard shrimp vessels. Likewise, the cold chain storage closely mimics that of a retail operation.

Large (21-30 count), head on, shell on shrimp were purchased from a local Louisiana fisherman (Four Winds Seafood, New Orleans, LA). The shrimp had not been previously frozen onboard the vessel nor had the shrimp been subjected to a salt box or sodium metabisulfite. The shrimp were first inoculated with *S. typhimurium* and *L. monocytogenes*. These pathogens were used because they are the most prevalent and most pathogenic strains associated with shrimp. For this study, a high inoculum (~10^6 cell density) was used for each bacterial strain. Prior to inoculation the shrimp were first washed with tap and distilled water. For the inoculum, the bacterial suspension was prepared by adding 50 mL of respective 16 hour culture to a sterile container holding 500 mL of sterile 0.1% Phosphate Buffered Saline (PBS) solution. Twenty-eight shrimp samples were used for each bacterial strain.

Following inoculation, a total of eight shrimp, 4 *S. typhimurium* and 4 *L. monocytogenes*, were set aside and used as a control. Enumeration of bacteria was performed on the control samples following the previously described procedures in order to determine the bacterial load on the shrimp prior to brine freezing. Additionally, the salinity of the homogenized control samples was measured using a handheld YSI model 30 salinity, conductivity, and temperature instrument (YSI Inc., Yellow Springs, OH) in order to determine the salinity of the shrimp prior to freezing in the brine solution. The remaining shrimp were then separated into labeled mesh bags according to the bacterial strain and the inoculum level.

The shrimp were then immediately placed into an ice bath consisting of a 50/50 ratio of ice and water in order to lower the internal temperature of the shrimp to about 33° F prior to freezing.

FIGURE 2. Image of Shrimp Inside Labeled Mesh Bags Used for Brine Freezing.

A 7.58 L (2 gallon) brine solution was prepared in a sterile container for freezing the shrimp. For each gallon (3.79 L) 2.53 pounds (1,301.308 grams) of table grade salt (Wal-mart, Springdale, AR) was added to a sterile container holding 3.79 L of dH_2O water and dissolved in order to achieve a final concentration of 23% salt solution determined by using a handheld YSI model 30 salinity, conductivity, and temperature instrument (YSI Inc., Yellow Springs, OH). The solution was then placed in the 0° F freezer and monitored using a thermometer with a digital probe (Control Company, Friendswood, TX) until it reached a working temperature of ~0° F.

Prior to freezing, shrimp were removed from the chilled ice bath and the temperature of the shrimp was measured and recorded using a thermometer with a digital probe. The shrimp were then immediately placed into the container with the brine freezing solution for 20 minutes or until completely frozen (internal temp ~ -20° C).

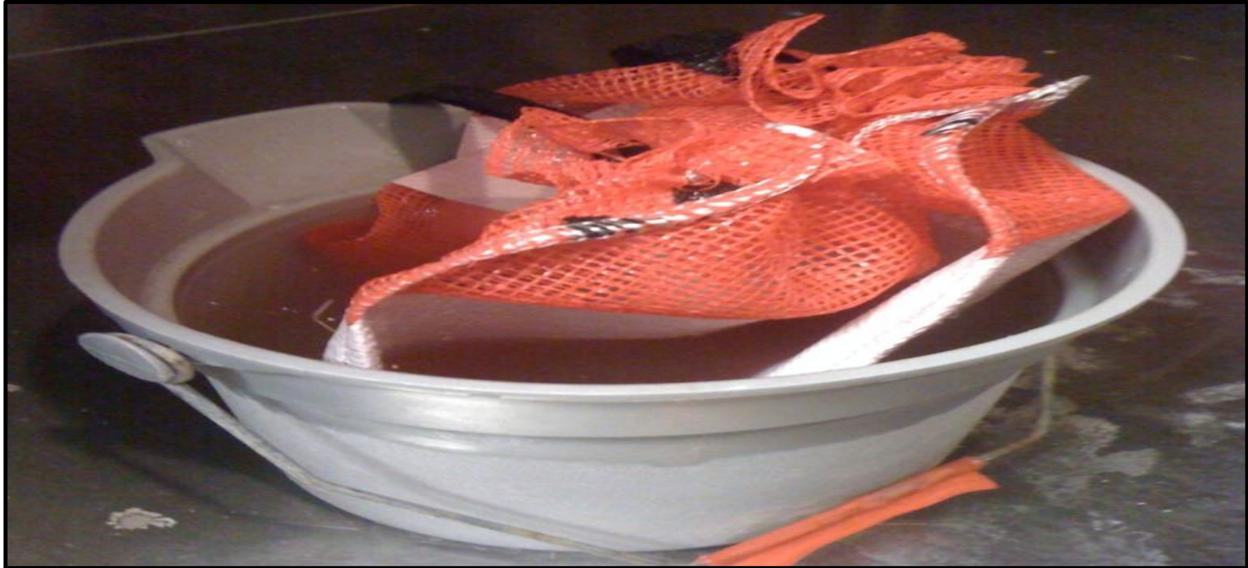


FIGURE 3. Shrimp in Mesh Bags Placed in ~0°F Brine Solution Inside 0°F Freezer.

The temperature after freezing was measured and recorded. Also, the salinity of the shrimp was measured using the handheld YSI 30 instrument and recorded after brine freezing in order to determine salt uptake by the shrimp from the brine during freezing.

After brine freezing, 8 shrimp, 4 *S. typhimurium* and 4 *L. monocytogenes*, were set aside in order to perform enumeration of bacteria. This was done to determine the bacterial load of the shrimp after brine freezing. The remaining shrimp were separated into previously labeled whirl pak sample bags according to the bacterial strain. The shrimp were then stored in the -20° C freezer.

After 1 day of frozen storage, the shrimp samples were removed, thawed and enumeration of bacteria was performed. Remaining shrimp samples were then placed back into the -20° C freezer and this step was repeated after 2 days of frozen storage. This was performed in order to mimic frozen storage and freeze thaw cycles that shrimp would commonly undergo during distribution from shrimp vessels to processing facilities. After 2 days of frozen storage,

the shrimp was stored on ice and at refrigerator temperatures (4° C). Enumeration of bacteria was performed after 1 and 2 days of refrigerated storage. This was done due to the fact that once shrimp reaches the retail outlet, it is placed on ice in a chilled storage cabinet where it is thawed and stored prior to being purchased by the consumer. After 2 days of storage on ice at 4° C the shrimp samples were boiled until floating and enumeration of bacteria was performed.

3.9.2 Flash Freezing.

This study was performed in order to determine the effect of flash freezing on the microbial quality of shrimp immediately after freezing and during subsequent cold chain storage. The methods for flash freezing and cold chain storage used in the lab closely mimic the methods used onboard shrimp vessels and retail operations. However, most shrimp vessels use an air blast freezer set at -40° C which was unavailable in this lab. Instead, the shrimp were subjected to cryogenic temperatures by being frozen in a -80° C freezer.

Large (21-30 count), head on, shell on shrimp were purchased from a local Louisiana fisherman (Four Winds Seafood, New Orleans, LA). The shrimp had not been previously frozen onboard his vessel nor had he subjected them to a salt box or sodium metabisulfite. The shrimp were first inoculated with *S. typhimurium* and *L. monocytogenes*. Prior to inoculation the shrimp were first washed with tap and distilled water. For the inoculum, the bacterial suspension was prepared by adding 50 mL of respective 16 hour culture to a sterile container holding 500 mL of sterile 0.1% Phosphate Buffered Saline (PBS) solution. Twenty-eight shrimp samples were used for each pathogenic strain.

Following inoculation, a total of eight shrimp, 4 *S. typhimurium* and 4 *L. monocytogenes*, were set aside and used as a control. Enumeration of bacteria was performed on the control

samples following the previously described procedures in order to determine the bacterial load on the shrimp prior to flash freezing. Additionally, the salinity of the homogenized control samples was measured using a handheld YSI model 30 salinity, conductivity, and temperature instrument (YSI Inc., Yellow Springs, OH) in order to determine the salinity of the shrimp prior to cryogenic freezing.

The remaining shrimp were placed into previously labeled whirl pak sample bags according to the bacterial strain and inoculum level. The shrimp were then immediately placed into an ice bath consisting of a 50/50 ratio of ice and water in order to lower the internal temperature of the shrimp to about 33° F prior to freezing. Temperature of the shrimp was monitored using a thermometer with a digital probe and once the shrimp reached an internal temperature of ~ 32-33°F, they were ready to be frozen. The sample bags containing shrimp were placed into the -80° C freezer (Revco – Kendro Laboratory Products, Asheville, NC) for 1.5 hours or until completely frozen (internal temp @ -20° C).

The temperature after freezing was measured and recorded. Also, the salinity of the shrimp was measured using the handheld YSI 30 instrument and recorded after freezing in order to determine effect on salinity of shrimp by cryogenic freezing.

After flash freezing, 8 shrimp, 4 *S. typhimurium* and 4 *L. monocytogenes*, were set aside in order to perform enumeration of bacteria. This was done to determine the bacterial load of the shrimp after flash freezing. The remaining shrimp were then separated into previously labeled whirl pak sample bags according to the bacterial strain. The shrimp were then stored in the -20° C freezer.

FIGURE 4. Image of Revco -80° C Freezer Used for Cryogenic Freezing.

FIGURE 5. Image of Shrimp Samples Being Frozen in Revco -80° C Freezer.

After 1 day of frozen storage, the shrimp samples were removed, thawed and enumeration of bacteria was performed. Remaining shrimp samples were then placed back into the -20° C freezer and this step was repeated after 2 days of frozen storage. This was performed in order to mimic frozen storage and freeze thaw cycles that shrimp would commonly undergo during distribution from shrimp vessels to processing facilities. After 2 days of frozen storage, the shrimp was stored on ice and at refrigerator temperatures (4° C). Enumeration of bacteria was performed after 1 and 2 days of refrigerated storage. This was done due to the fact that once shrimp reaches the retail outlet, it is placed on ice in a chilled storage cabinet where it is thawed and stored prior to being purchased by the consumer. After 2 days of storage on ice at 4° C the shrimp samples were boiled until floating and enumeration of bacteria was performed.

3.10 Comparison of Freezing Methods.

Enumeration of bacteria was performed and Log CFU/g was calculated at each step during the procedures for both freezing methods. The results were then compared in order to observe the effect of each method on the destruction or control of the two pathogens, *S. typhimurium* and *L. monocytogenes*, on the shrimp. This information was then used to determine which method was more successful at controlling or eliminating harmful pathogens during processing.

CHAPTER 4 RESULTS AND DISCUSSION

4.1 Thermal Log Reduction of *Listeria* and *Salmonella* Species to Non Detectable Levels in Shrimp After Boiling Until Floating.

The results of the original boiling until floating experiment revealed that after heat treatment the bacterial load was reduced to non-detectable levels (<1 CFU/25 g) for all species on all days tested. Figures 6a-c show the thermal reduction of *Salmonella* species after boiling until floating. Original bacteria counts for *Salmonella* were on average 5.3 Log CFU/g and post cooking were reduced to non-detectable levels.

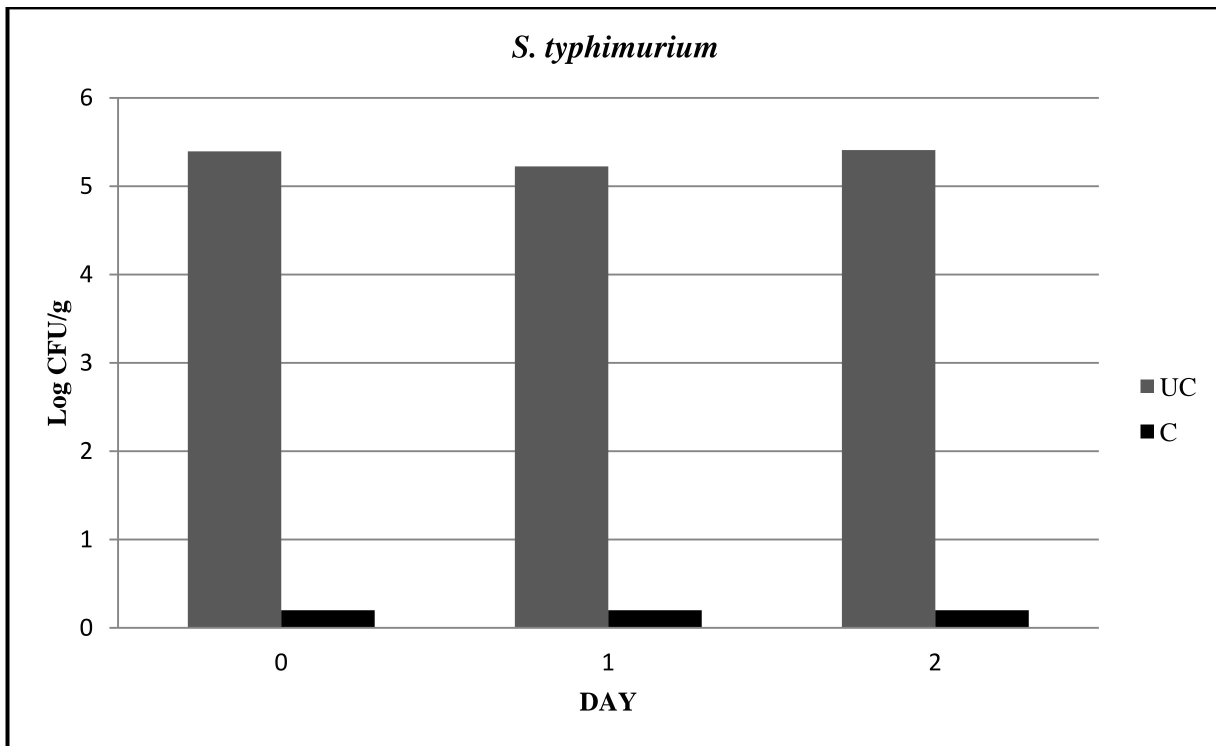


FIGURE 6a. **Thermal reduction of *Salmonella typhimurium* species after boiling until floating**.

The original bacterial load was 5.37 Log CFU/g and was reduced to non detectable levels after heat treatment on all days tested.

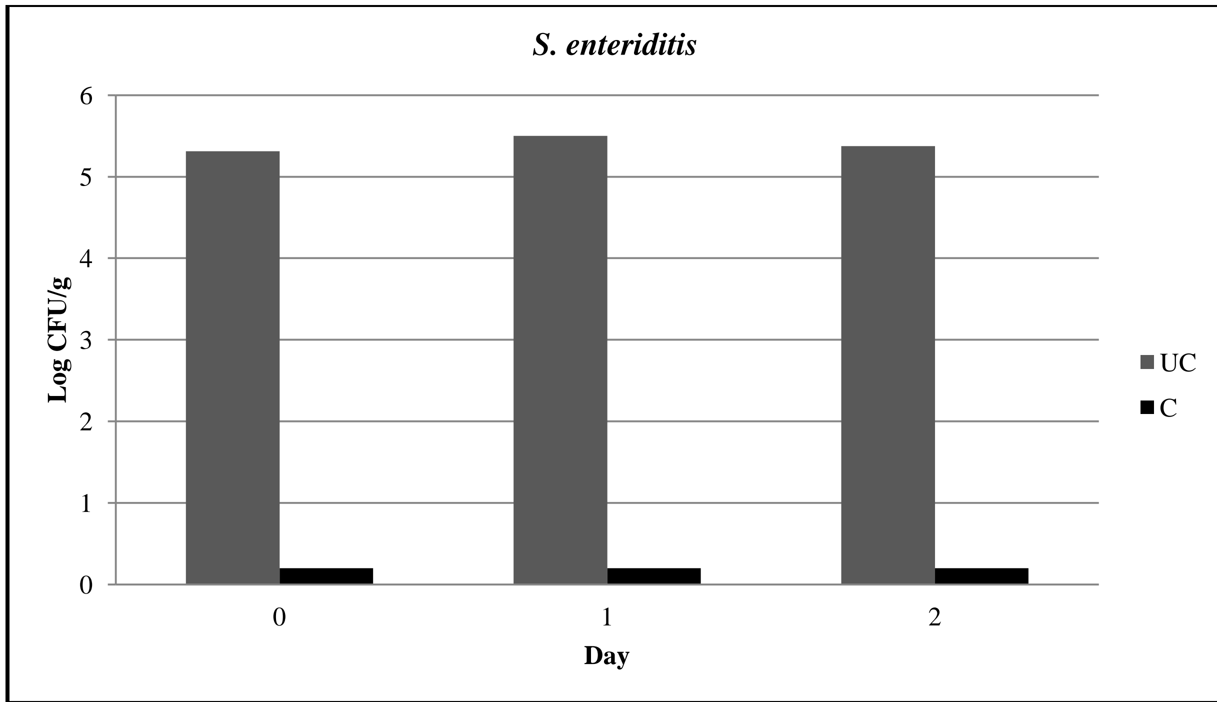


FIGURE 6b. Thermal reduction of *Salmonella enteriditis* species after boiling until floating.

The original bacterial load was 5.29 Log CFU/g and was reduced to non detectable levels after heat treatment on all days tested.

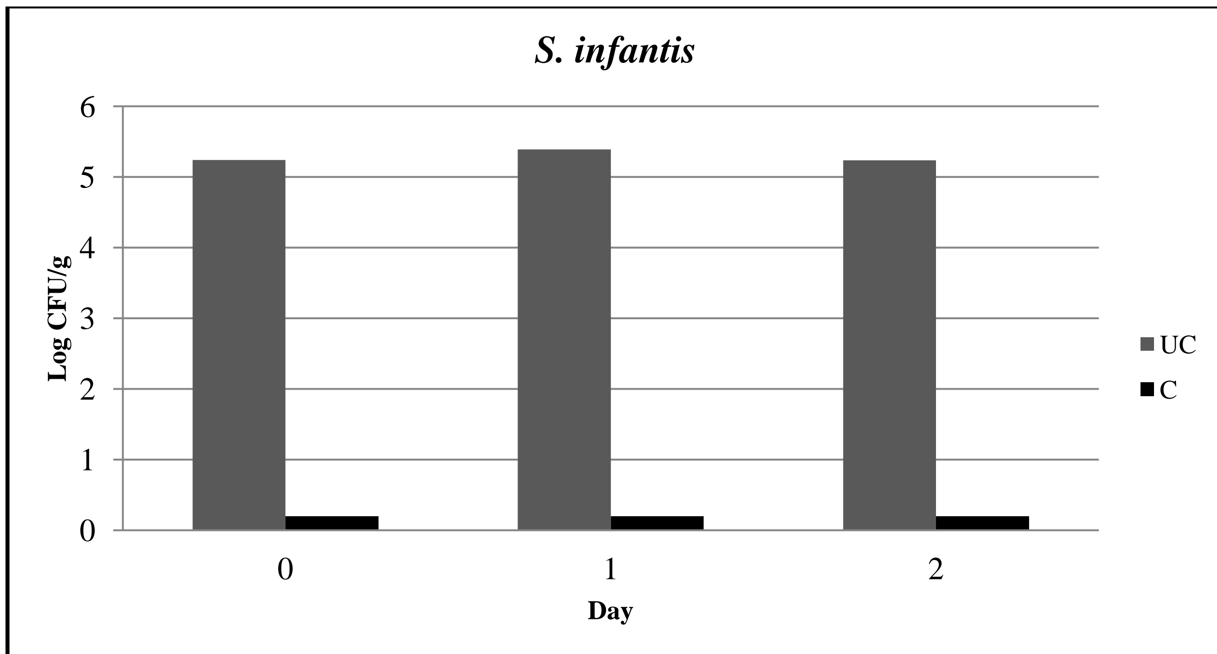


FIGURE 6c. Thermal reduction of *Salmonella infantis* species after boiling until floating.

The original bacterial load was 5.28 Log CFU/g and was reduced to non detectable levels after heat treatment on all days tested.

Figures 7a-c reveal the thermal reduction of *Listeria* species after boiling until floating. Original bacteria counts for *Listeria* ranged from 3.5-4.5 Log CFU/g and post cooking were reduced to non detectable levels.

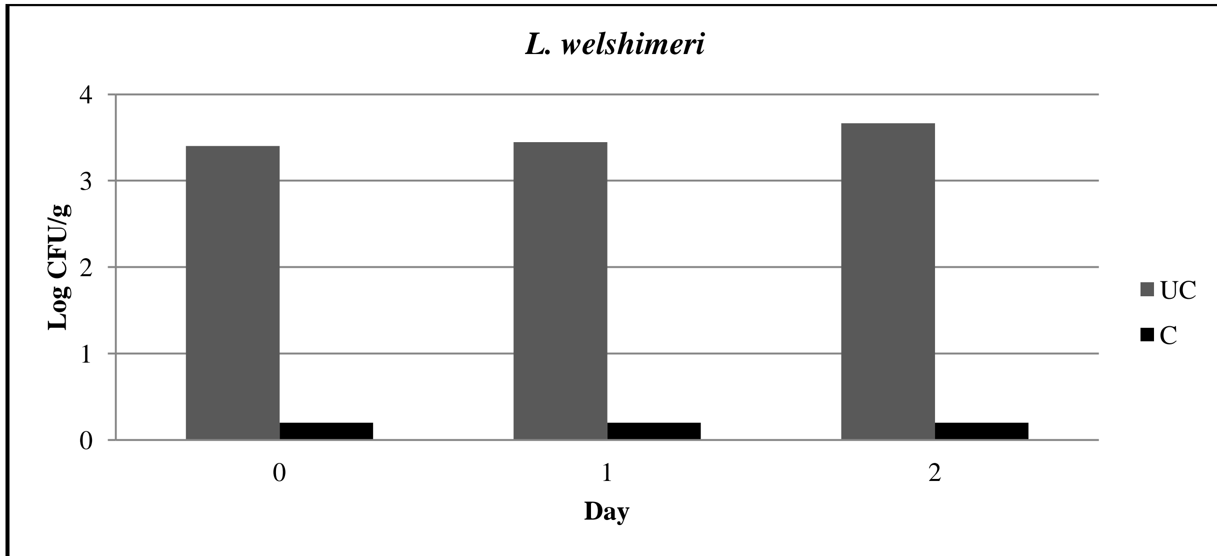


FIGURE 7a. Thermal reduction of *Listeria welshimeri* species after boiling until floating.

The original bacterial load was 3.6 Log CFU/g and was reduced to non detectable levels after heat treatment on all days tested.

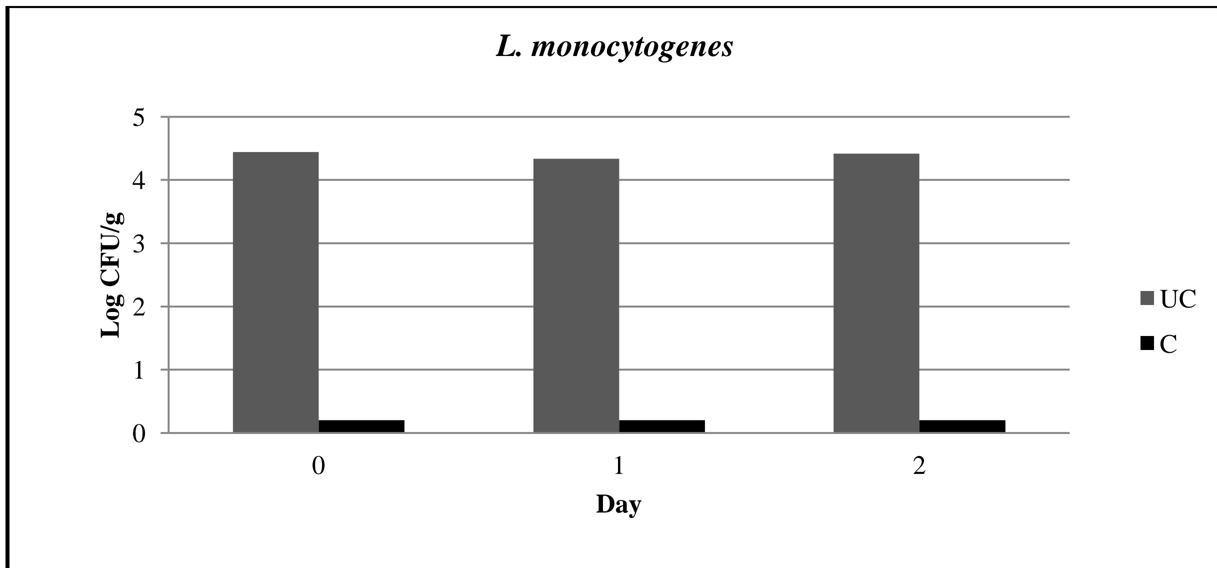


FIGURE 7b. Thermal reduction of *Listeria monocytogenes* species after boiling until floating.

The original bacterial load was 4.5 Log CFU/g and was reduced to non detectable levels after heat treatment on all days tested

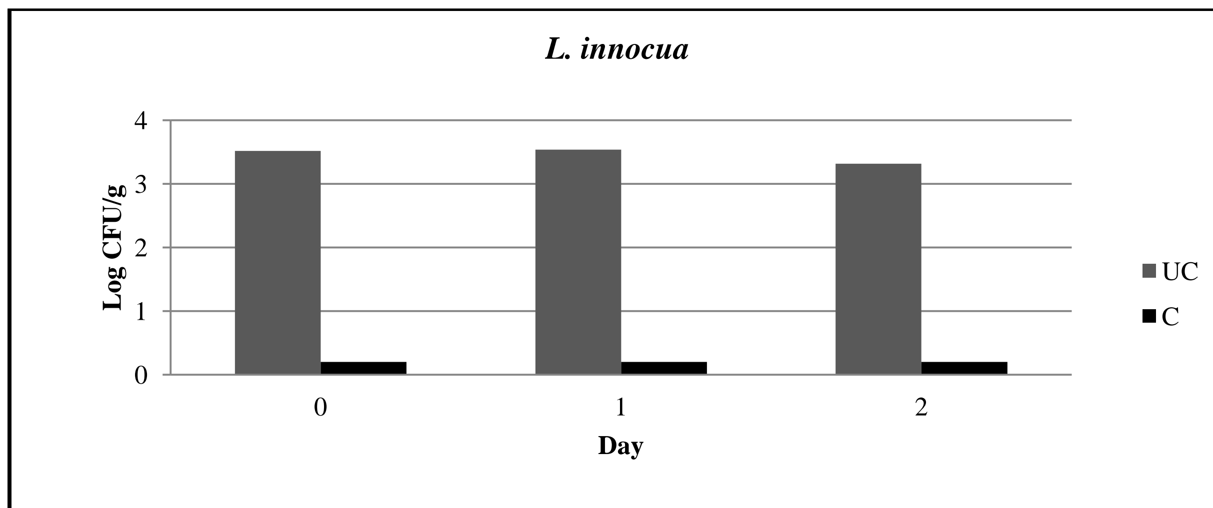


FIGURE 7c. Thermal reduction of *Listeria innocua* species after boiling until floating. The original bacterial load was 3.5 Log CFU/g and was reduced to non detectable levels after heat treatment on all days tested.

After boiling until floating the average internal temperature was 68.61 ± 2.29°C with the minimum internal temperature recorded at 65.2° C. The average surface temperature was 76.67 ± 4.9° C. The variation in both the internal and surface temperatures was due to the variation in the size of the shrimp. The average size of the shrimp was 16.09 ± 2 grams. The temperature had a direct relationship with the size and larger shrimp reached a higher internal temperature. Consequently, larger shrimp also took longer to float to the surface of the water. The average time it took the shrimp to float to the surface of the water when boiled was 1:36 ± 0:08 seconds.

The FDA and USDA both recommend that seafood should be cooked until it reaches an internal temperature of 62.7° C (145° F) (FDA, 2011; USDA,2011). This temperature is recommended in order to eliminate *L. monocytogenes*, the target pathogen due to the fact that it is the most heat resistant pathogen that does not form spores associated with food (FDA, 2010). The results of this study revealed that after boiling until floating, the minimum internal temperature attained was still higher than the FDA's recommended temperature which explains

why all of the bacteria were reduced to a level safe for consumption on all days tested. Furthermore, because the surface temperature results had a large standard deviation compared to the standard deviation for the internal temperature results it was considered a less reliable source for determining doneness when cooking shrimp.

Additionally, a concern for those who enjoy eating shrimp is the fact that it is a delicate product and may be overcooked easily resulting in an undesirable firm and rubbery texture and the loss of most of its flavor. One visual way to determine if shrimp has been overcooked is to look at the shape of the tail. When doing this, two things must be remembered: “C” means cooked and when shrimp is fully cooked it will curl up and form the letter C and “O” means overcooked and when shrimp is overcooked it will curl up into a complete circle and form the letter O (www.rubiconresources.com, & various authors). After boiling until floating, the shrimp in this study formed the letter “C” indicating that it had been fully cooked but had not been overcooked. Consequently, this would be an acceptable method of cooking for consumers. Moreover, this affirms that chefs in restaurants or cooks in private residences do not have to sacrifice quality for safety when applying this method for cooking shrimp.

4.2 Time Needed for Pathogens to Reach Non-Detectable Levels in Shrimp.

The results of the time point study showed that both *Listeria monocytogenes* and *Salmonella typhimurium* were both eliminated by boiling in water at 100° C at 1 minute 15 seconds. At this time point the internal temperature reached an average of 68.25 ± 3°C. This internal temperature is very similar to what was obtained in the previous results for boiling until floating which was 68.61 ± 2° C. Furthermore, the results of the time point study revealed that at 1:30 seconds the average internal temperature was 69.95 ± 1° C. There was not a significant

FIGURE 8. Image showing the difference between a fully cooked, boiled until floating shrimp versus a shrimp that has been overcooked.

change in temperature between 1:15 and 1:30. This further explains how in the original study even with a 0:08 second standard deviation in the mean 1:30 cooking time, all of the bacteria was eliminated. Figures 9a-b show the bacterial reduction in *S. typhimurium* and *L. monocytogenes* at the different time points during boiling.

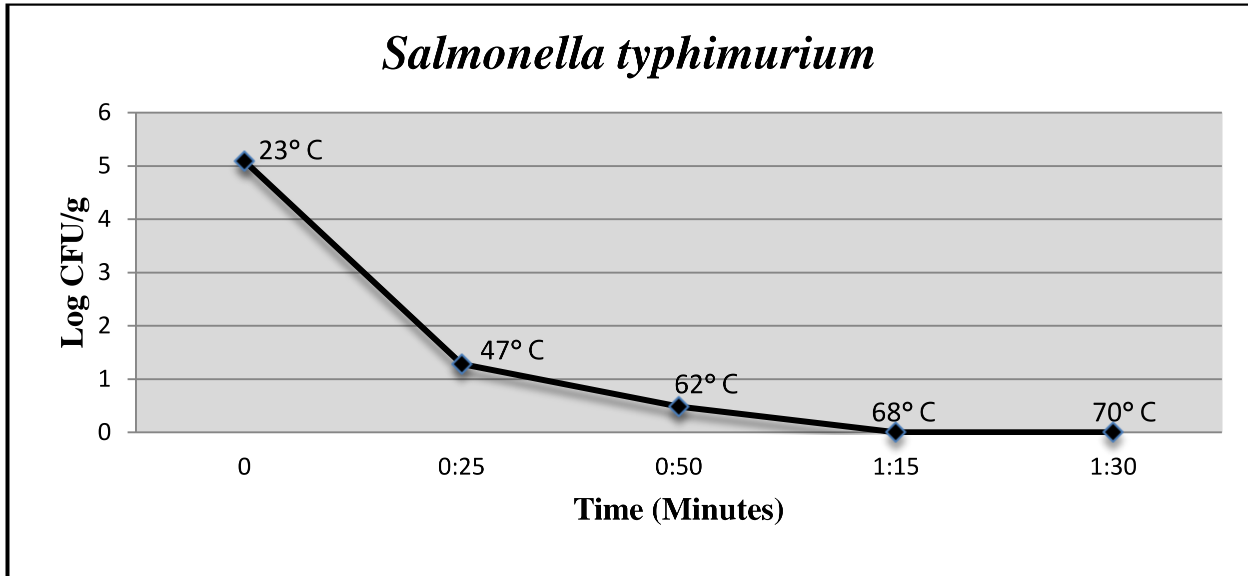


FIGURE 9a. Results of *Salmonella* destruction at different time points.

This graph illustrates that at 50 seconds and an internal temperature of 61° C the bacteria was not completely eliminated. At 1:15 with an internal temperature of 68° C, the bacteria were eliminated from the shrimp.

At time 0, the bacterial load was 5.09 Log CFU/g. After heat treatment for 0:25 seconds the bacterial load was 1.28 Log CFU/g and the internal temperature of the shrimp was 47° C. A 3.81 Log reduction occurred from time 0 to the first time point. At 0:50 seconds the bacterial load of the shrimp was .48 Log CFU/g with an internal temperature of 62° C. A .8 Log reduction occurred between time point 2 and time point 3. At 1:15 seconds the bacterial load was reduced to non detectable levels and the internal temperature reached 68° C. A .48 Log reduction occurred between time point 3 and time point 4.

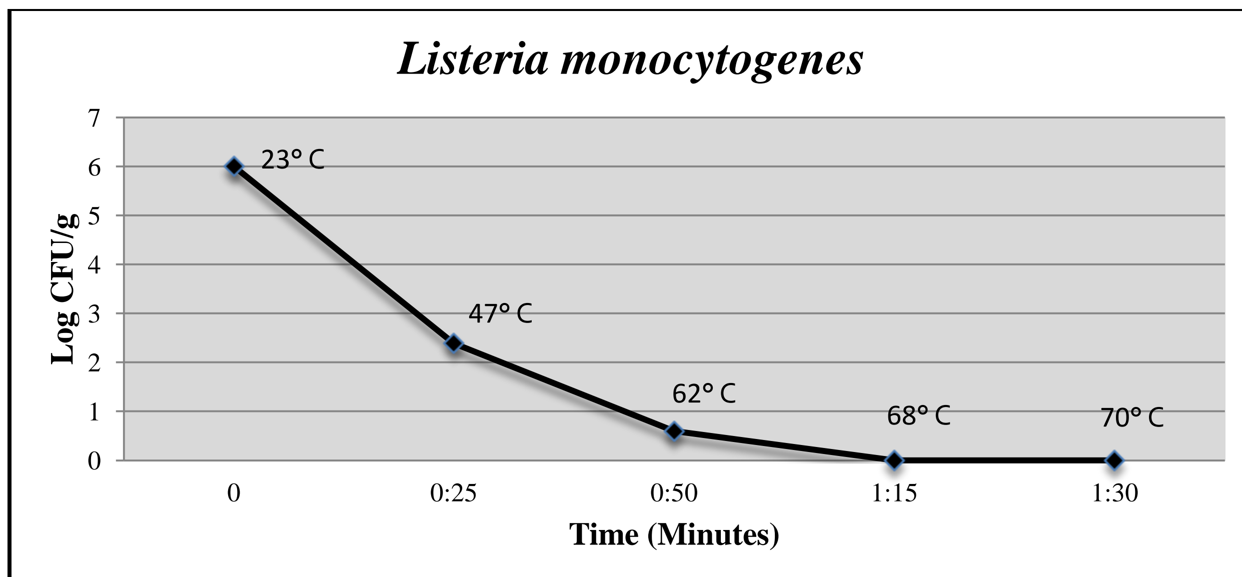


FIGURE 9b. Results of *Listeria* destruction at different time points.

This graph illustrates that at 50 seconds and an internal temperature of 62° C the bacteria was not completely eliminated. At 1:15 with an internal temperature of 68° C, the bacteria were eliminated from the shrimp.

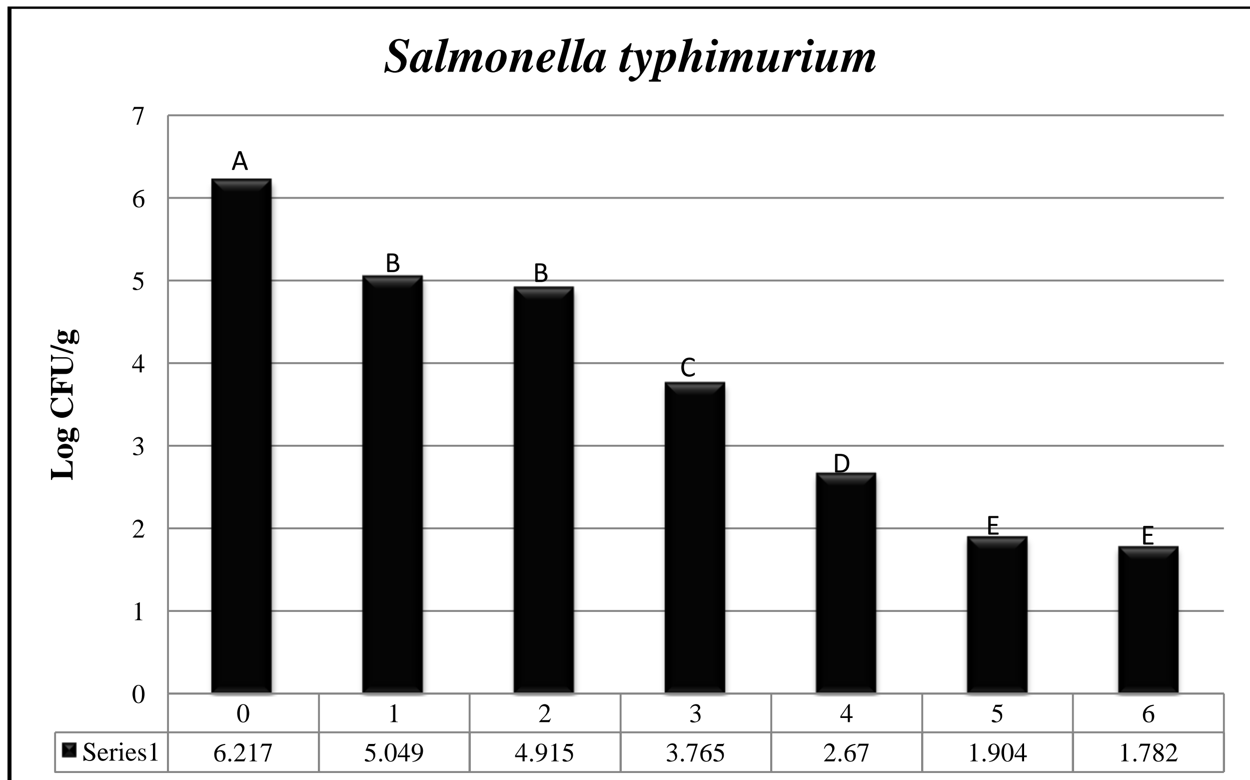
At time 0, the bacterial load was 6 Log CFU/g. After heat treatment for 0:25 seconds the bacterial load was 2.39 Log CFU/g and the internal temperature of the shrimp was 47° C. A 3.61 Log reduction occurred from time 0 to the first time point. At 0:50 seconds the bacterial load of the shrimp was .6 Log CFU/g with an internal temperature of 62° C. A 1.79 Log reduction occurred between time point 2 and time point 3. At 1:15 seconds the bacterial load was reduced to non detectable levels and the internal temperature reached 68° C. A .6 Log reduction occurred between time point 3 and time point 4.

The results present that at 0:50 seconds of boiling at 100° C the shrimp reached an internal temperature of approximately 62° C. This is very close to the minimum safe internal temperature of 62.7° C recommended by the FDA when cooking seafood. However, at 62° C the pathogenic bacteria had not been completely eliminated from the shrimp. It can therefore be

concluded from these results that shrimp should be boiled to a minimum internal temperature of 68° C to ensure the elimination of pathogens and, consequently, safety for the consumer.

4.3 Growth of Pathogens on the Surface of Shrimp at 4°C.

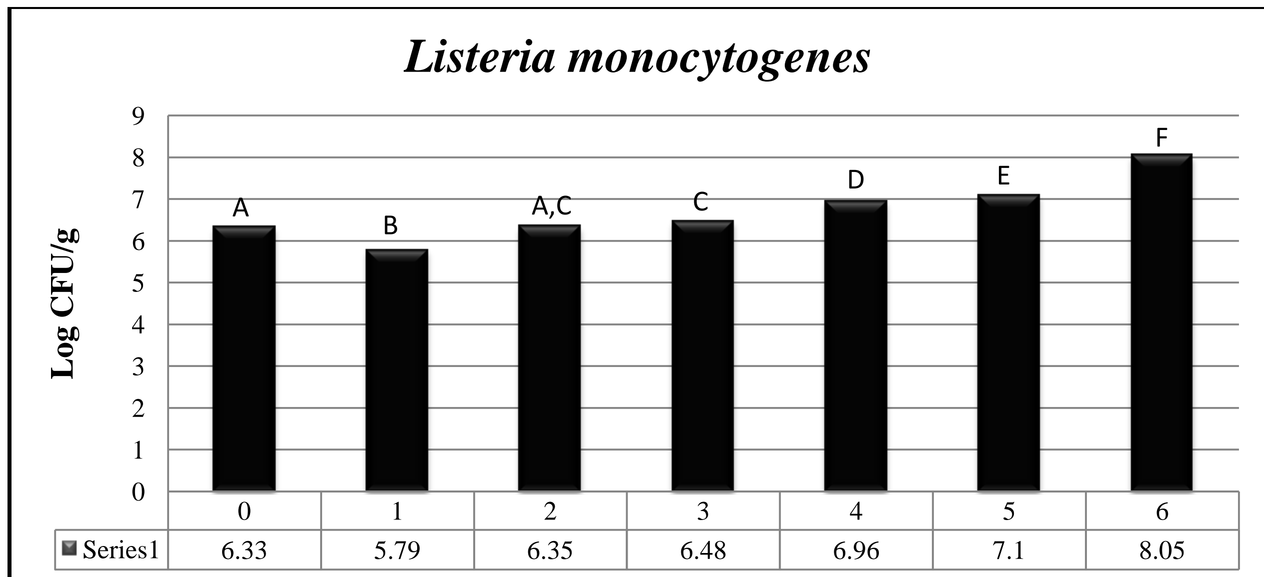
The results of the inoculum study revealed that *Salmonella typhimurium* continually decreased in Log CFU/g with each day over a 6 day period of refrigerated (4° C) storage. Oppositely, *Listeria monocytogenes* decreased after the first day of refrigerated study and then continually increased in Log CFU/g over the remaining days of storage. Figures 10a-b present the results of the inoculum study.



	0	1	2	3	4	5	6
Series1	6.217	5.049	4.915	3.765	2.67	1.904	1.782

***Bars not connected by the same letter are significantly different (p<.0001, α=.05)**

FIGURE 10a. ***Salmonella typhimurium*** **bacterial counts during 6 days of refrigerated storage.**



***Bars not connected by the same letter are significantly different (p<.0001, α=.05)**

FIGURE 10b. ***Listeria monocytogenes*** **bacterial counts during 6 days of refrigerated storage.**

The results for *S. typhimurium* were to be expected because while this organism has been shown to survive at refrigerator and even freezer temperatures (Norhana et al., 2010), it is not known to be able to multiply at low temperatures. Overall, a 4.44 Log reduction occurred in the *Salmonella* bacterial load on the shrimp between day 0 and day 6 of refrigerated storage. Likewise, the results for *L. monocytogenes* were also to be expected because this organism is known to grow and multiply at refrigerator temperatures. Initially, a significant .54 Log reduction occurred in the *L. monocytogenes* number on the shrimp after 1 day of refrigerated storage. This could be attributed to the acclimation phase of cold shock adaptation during which non cold shock protein synthesis is inhibited and cold shock protein synthesis increases greatly (Chan & Wiedmann, 2011). During the first day of refrigerated storage the bacteria was most likely in the adaptation phase and had not fully acquired its ability to adjust to the cold environment. After its acclimation phase, the bacteria were able to adapt to its environment

enabling it to be able to grow during subsequent storage at a low temperature. A 1.72 Log increase in *Listeria monocytogenes* was observed between day 0 and day 6 of refrigerated storage.

4.4 Cooking Portion Study.

It was concluded that boiling a common serving size of 1 pound of shrimp until floating to the surface of the water was adequate in eliminating the bacteria on the shrimp. Additionally, the time to floating for 1 pound was 1:45 ± 0:02 seconds as opposed to 1:36 ± 0:08 seconds for an individual shrimp.

4.5 Control of Pathogens in Shrimp Boiled in Salt Water.

Preliminary results revealed that the shrimp boiled in water with salt added took slightly less time to float to the surface of the water. The average time to floating for individual shrimp was 1:15 seconds and the average internal temperature was 67° C. The average time to floating for a 1 pound serving size in water with salt added was 1:40 seconds. All of the bacteria were reduced to non detectable levels. The addition of 2% salt did not affect the boiling point of the water (100° C). This is consistent with another study stating that a 2% salt solution has a boiling point of 100° C and, increasing salt concentrations result in increasing boiling point temperatures (Niamnuy et al., 2007). The salinity of the shrimp before boiling was 5.5 parts per trillion (ppt) and after boiling it was still 5.5 ppt so, no uptake of salt by the shrimp occurred.

4.6 Bacterial Growth During Refrigerated Storage.

The results of the refrigerated storage study showed that on Day 0 bacterial counts were reduced to non detectable levels for all species in all shrimp tested after boiling until floating.

Furthermore, no growth was observed for any of the bacterial species in whole shrimp after days 1 or 2 of refrigerated storage at 4° C.

Figures 11a-c reveal the thermal reduction of *Salmonella* species in shrimp after boiling until floating on day 0 and show that no growth was detected after day 1 or day 2 of refrigerated storage. Original bacterial counts for *Salmonella* ranged from 5.28 – 5.39 Log CFU/g and were reduced to non detectable levels post cooking.

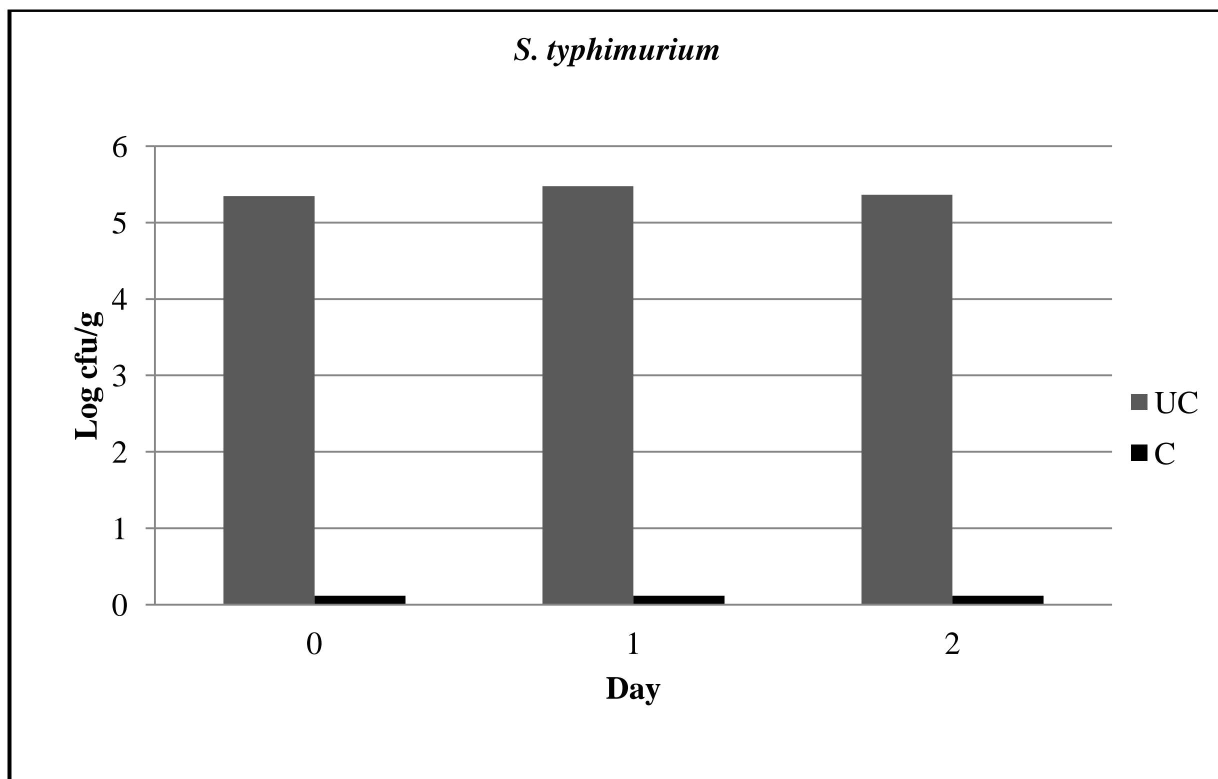


FIGURE 11a. Thermal reduction of *Salmonella typhimurium* species after boiling until floating and subsequent growth during refrigerated storage.

The original bacterial load was 5.39 Log CFU/g and was reduced to non detectable levels after heat treatment. No growth was detected after day 1 or day 2 of refrigerated storage.

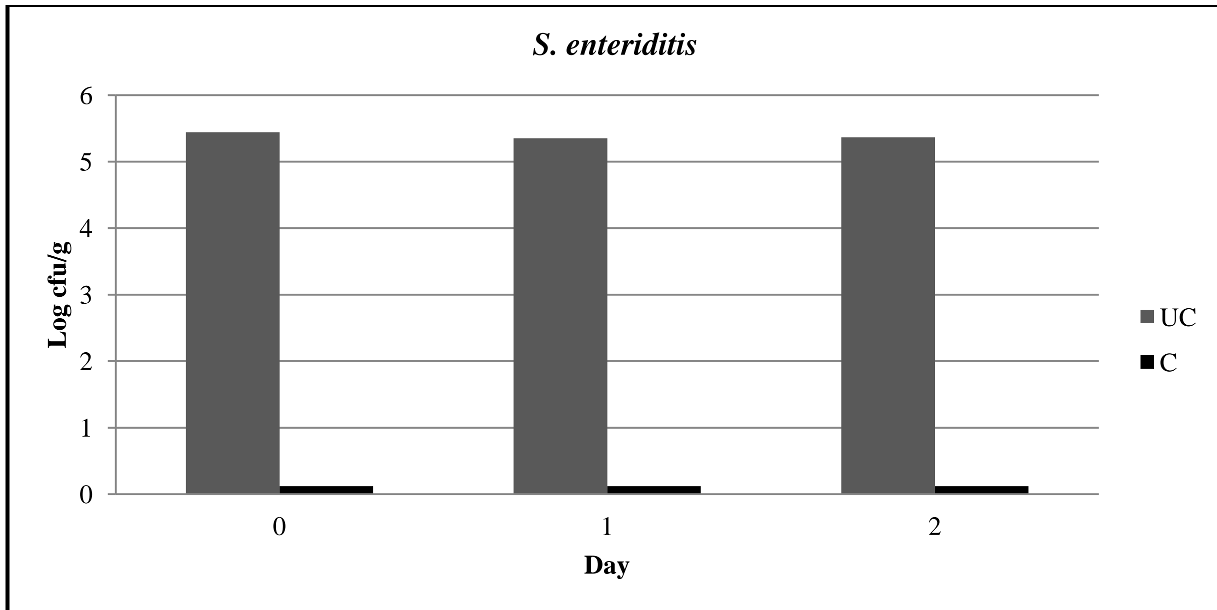


FIGURE 11b. Thermal reduction of *Salmonella enteriditis* species after boiling until floating and subsequent growth during refrigerated storage.

The original bacterial load was 5.38 Log CFU/g and was reduced to non detectable levels after heat treatment. No growth was detected after day 1 or day 2 of refrigerated storage.

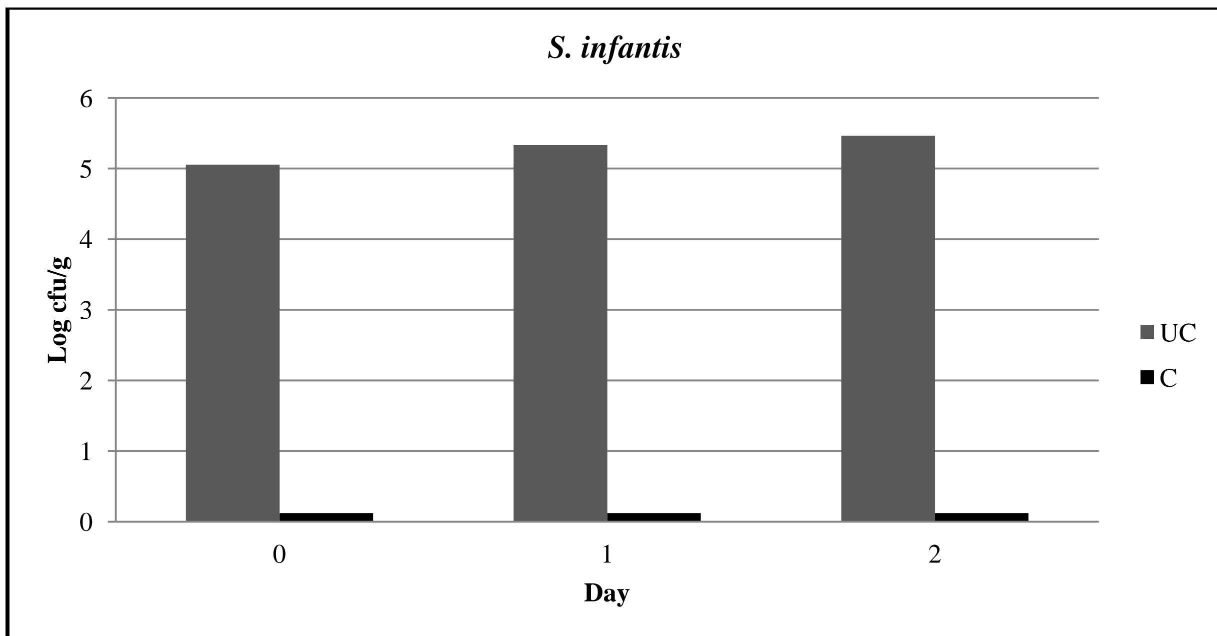


FIGURE 11c. Thermal reduction of *Salmonella infantis* species after boiling until floating and subsequent growth during refrigerated storage.

The original bacterial load was 5.28 Log CFU/g and was reduced to non detectable levels after heat treatment. No growth was detected after day 1 or day 2 of refrigerated storage.

Figures 12a-c reveal the thermal reduction of *Listeria* species in shrimp after boiling until floating on day 0 and show that no growth was detected after day 1 or day 2 of refrigerated storage. Original bacterial counts for *Listeria* ranged from 4.29-5.48 Log CFU/g and were reduced to non detectable levels post cooking.

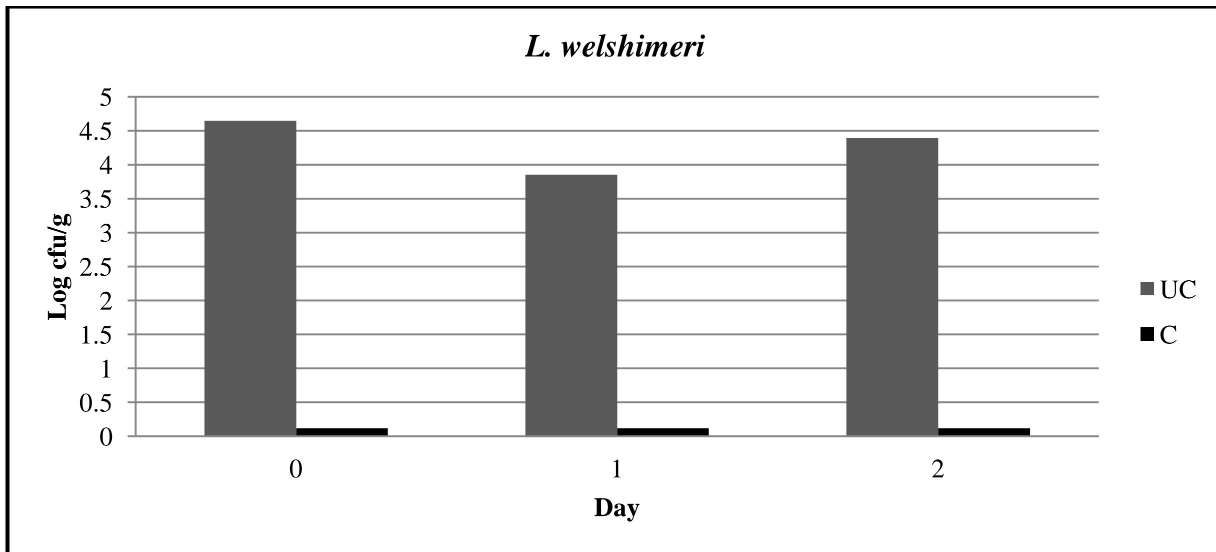


FIGURE 12a. Thermal reduction of *Listeria welshimeri* species after boiling until floating and subsequent growth during refrigerated storage.

The original bacterial load was 4.29 Log CFU/g and was reduced to non detectable levels after heat treatment. No growth was detected after day 1 or day 2 of refrigerated storage.

Previous studies have described the ability of *L. monocytogenes* to grow on cooked shrimp stored at 4° C (Norhana et al., 2011). This has serious implications for the food industry because if this pathogen is present in cooked shrimp, even at a low level, a dangerous level could be reached if the shrimp is stored at temperatures commonly used in chilled display cabinets at retail outlets (Norhana et al., 2011). The findings of this study reveal that boiling until floating eliminates *L. monocytogenes* to a low enough level in cooked shrimp to where it is unable to grow or be detected even after refrigerated storage.

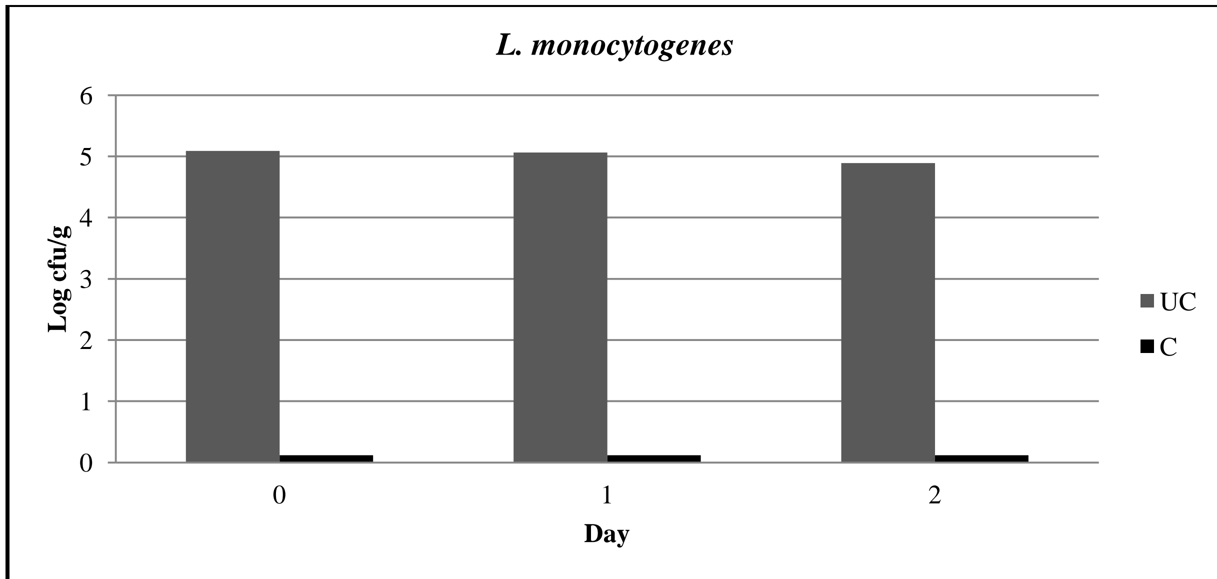


FIGURE 12b. Thermal reduction of *Listeria monocytogenes* species after boiling until floating and subsequent growth during refrigerated storage.

The original bacterial load was 5.0 Log CFU/g and was reduced to non detectable levels after heat treatment. No growth was detected after day 1 or day 2 of refrigerated storage.

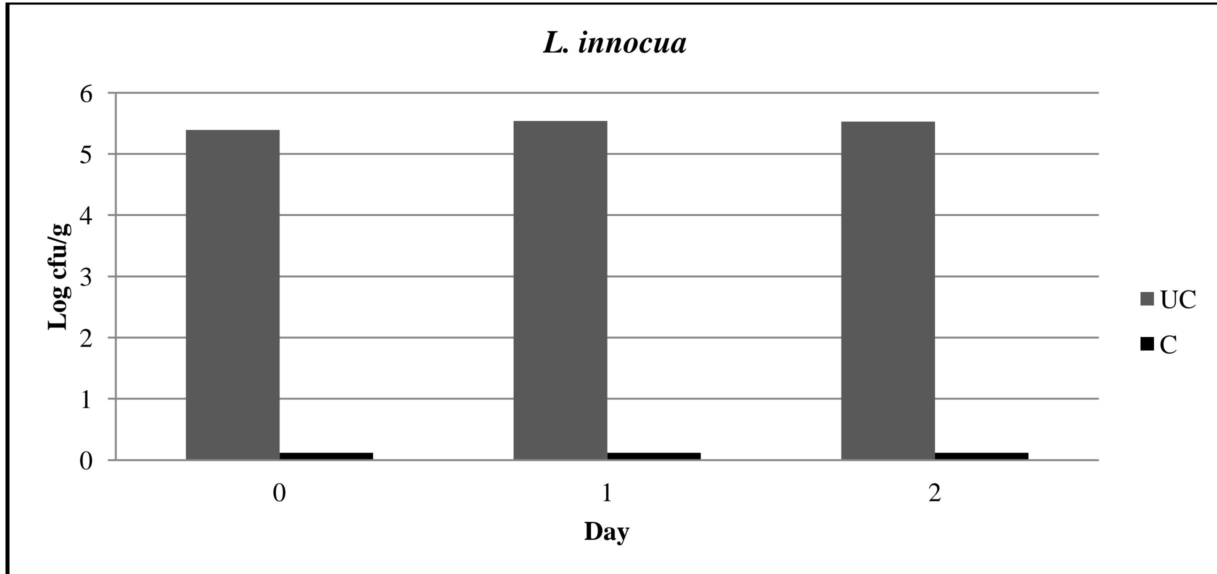


FIGURE 12c. Thermal reduction of *Listeria innocua* species after boiling until floating and subsequent growth during refrigerated storage.

The original bacterial load was 5.48 Log CFU/g and was reduced to non detectable levels after heat treatment. No growth was detected after day 1 or day 2 of refrigerated storage.

4.7 Determining a Correlation Between Pathogen Reduction to Non-detectable Levels and the Degree of Color Change After Boiling Shrimp Until Floating.

Color readings were taken before and after cooking each shrimp and were analyzed after cooking. The results of the color analysis (Figure 13 & Table 1) reveal that the mean redness (a*), yellowness (b*), and lightness (L*) were significantly higher ($p<0.0001$) in the cooked shrimp versus uncooked for all days tested. Additionally, the standard deviation of the redness (a*) values of the cooked shrimp versus the uncooked was large indicating a wide variation in pink coloration for all days tested.

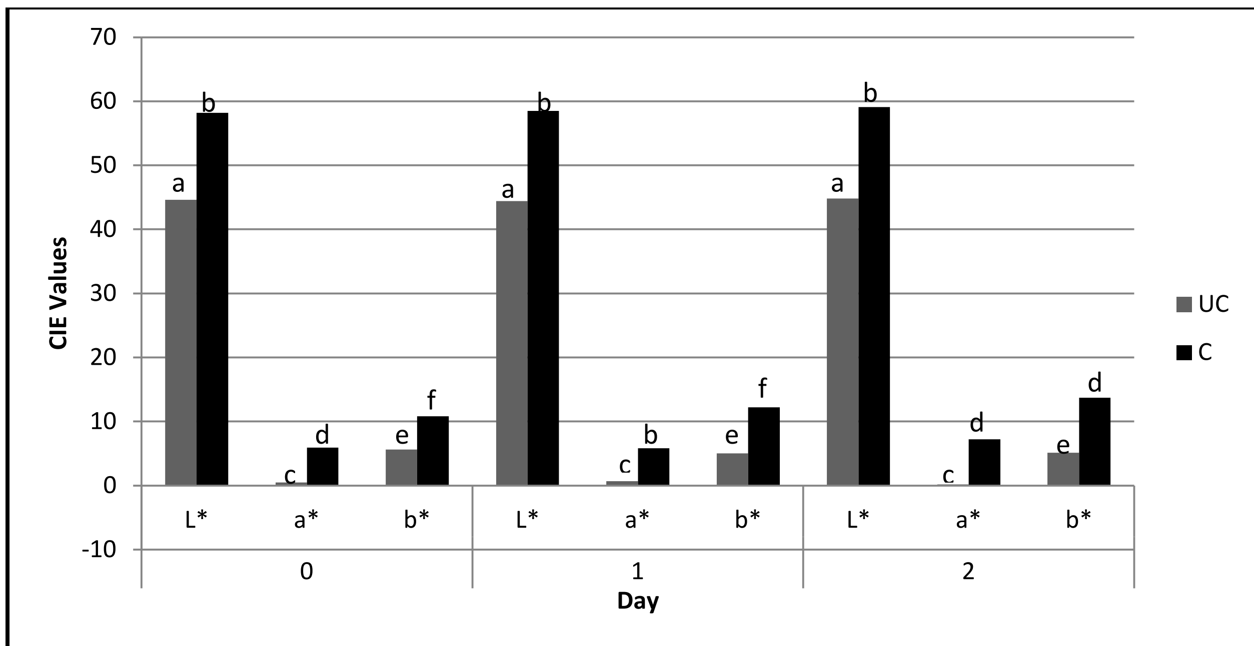


FIGURE 13. Comparison of mean L*, a*, and b* values between cooked and uncooked shrimp on day 0, 1, and 2.

TABLE 1. Mean and standard deviation of a* values for cooked and uncooked shrimp.

Treatment[a]	Mean[b]	SD[c]
C	6.28403	3.65636
UC	0.39667	1.19442

[a] C, Cooked; UC, Uncooked

[b] Mean of a* values

[c] SD, Standard deviation of a* values

Table 1 shows that the mean and standard deviation for the a* values of the cooked shrimp were large compared to that of the uncooked shrimp. It can be determined from this table that the standard deviation of the a* values for the cooked shrimp was large compared to that of the uncooked due to the larger range of values.

4.8 Effect of Sodium Tripolyphosphate on the Pink Color Change of Cooked Shrimp.

Color readings were taken before and after cooking each shrimp that had not been treated with sodium tripolyphosphate and were analyzed after cooking. The results of the color analysis (Figure 15 & Table 3) reveal that the mean redness (a*), yellowness (b*), and lightness (L*) were significantly higher ($p<0.0001$) in the cooked shrimp versus uncooked for all days tested. Additionally, the standard deviation of the redness (a*) values of the cooked shrimp versus the uncooked was large indicating a wide variation in pink coloration for all days tested.

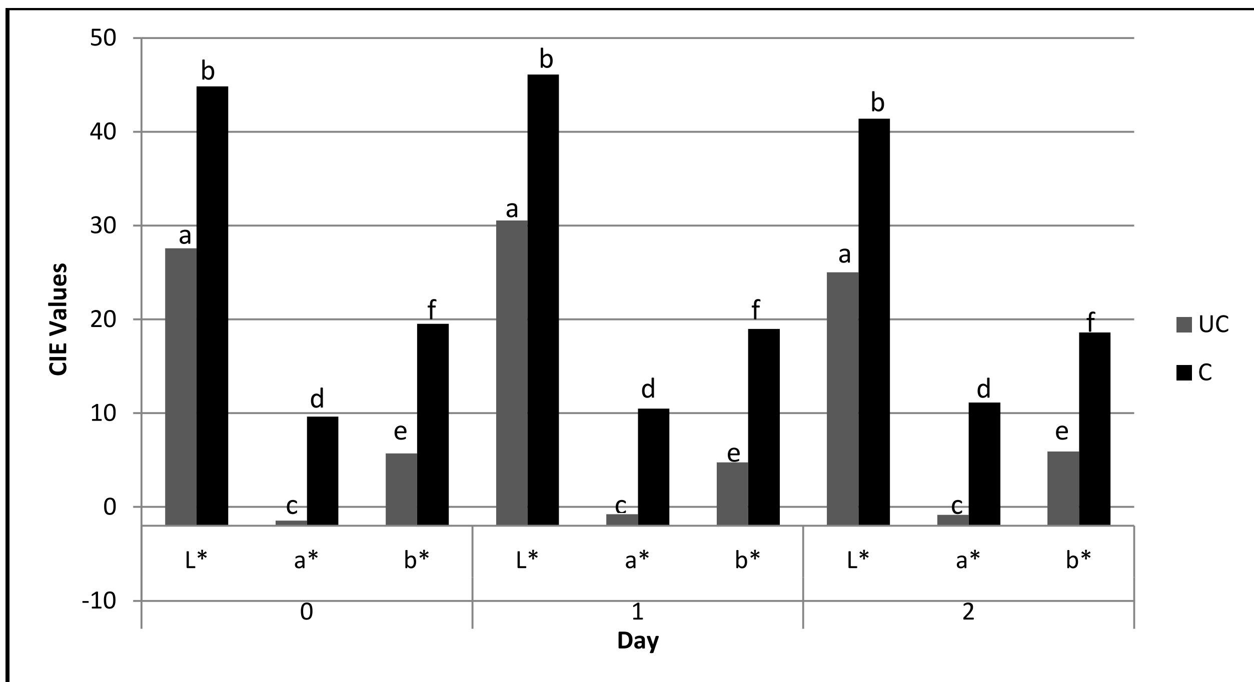


FIGURE 14. Comparison of mean L*, a*, and b* values between cooked and uncooked shrimp not treated with sodium tripolyphosphate on day 0, 1, and 2.

Table 2. Mean and standard deviation of a* values for cooked and uncooked shrimp with no sodium tripolyphosphate.

Treatment[a]	Mean[b]	SD[c]
C	10.3968	2.23771
UC	1.0278	.933174

[a] C, Cooked; UC, Uncooked

[b] Mean of a* values

[c] SD, Standard deviation of a* values

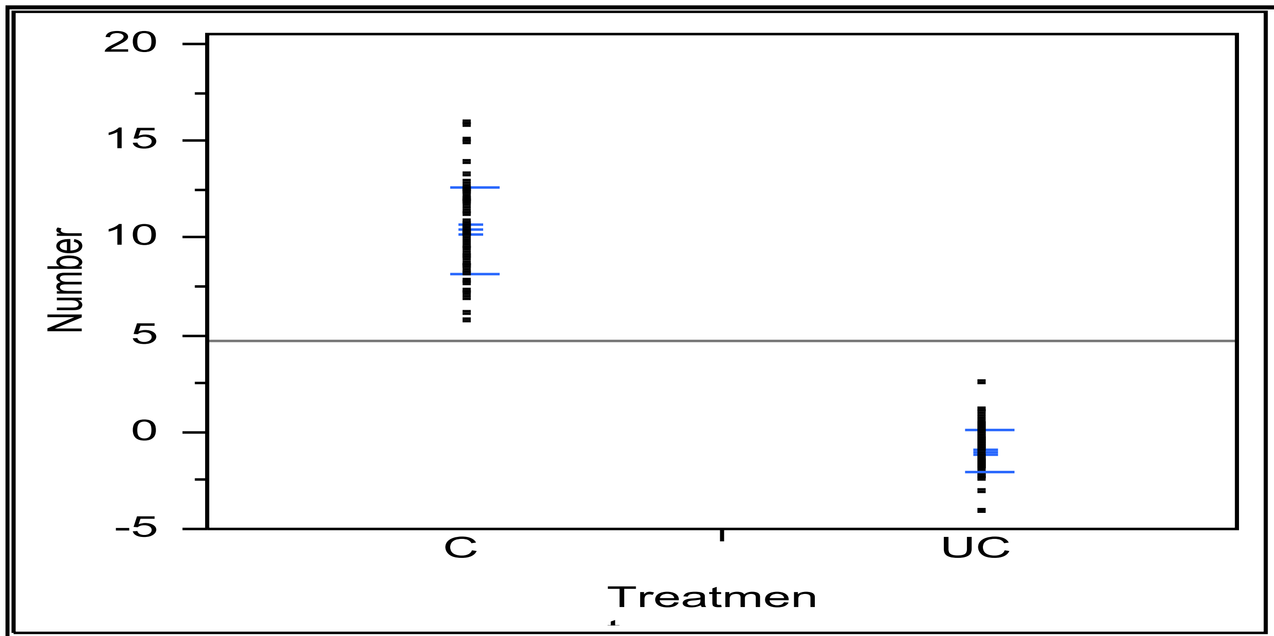


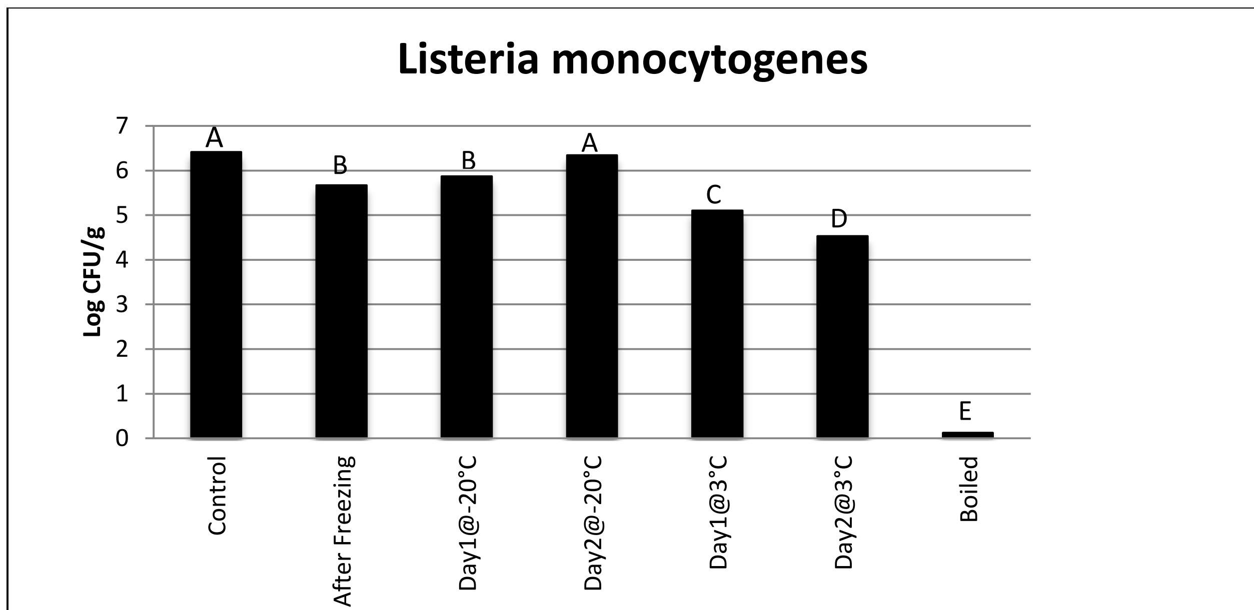
FIGURE 15. One-way analysis of a* values.

Table 4 shows that the mean and standard deviation for the a* values of the cooked shrimp with no sodium tripolyphosphate were large compared to that of the uncooked shrimp. Figure 16 further illustrates this by showing the one-way analysis of a* values of cooked and uncooked shrimp. The large markers in this figure illustrate the standard deviation. It can be determined from this figure that the standard deviation of the a* values for the cooked shrimp was large compared to that of the uncooked due to the larger range of values.

4.9 Effect of Freezing Methods on the Microbial Quality of Shrimp.

4.9.1 Effect of Flash Freezing *Listeria monocytogenes.*

Figure 17 shows that after flash freezing the shrimp there was a significant .74 log reduction in *Listeria monocytogenes.* After one day of frozen storage there was no significant change. After two days of frozen storage, the shrimp significantly increased by .47 logs and reached a Log CFU/g not significantly different from the control. The Log CFU/g then significantly decreased by 1.2 logs followed by .5 logs after one and two days of refrigerated storage. After boiling the shrimp until floating, a significant log reduction occurred, however, the shrimp were not reduced to non detectable levels and .12 Log CFU/g remained on the shrimp. The initial bacterial load on the shrimp was 6.4 Log CFU/g. After flash freezing followed by successive cold chain storage the count was reduced by 1.8 logs overall.



***Bars not connected by the same letter are significantly different (p<.0001, α=.05)**

FIGURE 16. Bacterial reduction of *Listeria monocytogenes* on shrimp after flash freezing followed by subsequent cold storage.

The ability of *Listeria monocytogenes* to grow on the shrimp during storage at freezing temperatures (-20° C) can most likely be attributed to its inherent ability to adapt to stressful environmental conditions. This pathogen is able to adapt to environmental stresses very quickly due to changes in the transcription of genes that is made possible through its general stress sigma factor, σ^B (Gandhi & Chikindas, 2007). *Listeria monocytogenes* is able to resist the destructive effects of freezing (FDA, 2011). It is also able to multiply at low temperatures due its synthesis of cold shock proteins (CSPs) and cold acclimation proteins (CAPs) in response to a temperature downgrade (Chattopadhyay, 2006). CSP production is induced in response to low temperatures but only temporarily (Chan & Wiedmann, 2011). CAP production is also induced in response to low temperatures but its synthesis continues for a longer amount of time (Chan & Wiedmann, 2011). When exposed to stress, the general stress sigma factor, σ^B, mediates changes in gene expression consequently leading to synthesis of various proteins such as CSPs and CAPs (Gandhi & Chikindas, 2007). Additionally, *Listeria* cells that are cold shocked prior to freezing obtain more cryotolerance, therefore, they can resist damage normally caused by freezing temperatures (Phadtare, 2004). In this study, the *L. monocytogenes* was cold shocked prior to freezing when paced in the chilled water bath and consequently could have obtained cryotolerance.

When exposed to low temperatures, *L. monocytogenes* usually encounters a lag phase and slow growth (Chan & Wiedmann, 2011). One study stated that cells abruptly ceased growing and did not acquire a new growth rate until 6 hours after a temperature downshift (Becker et al., 2000). The lag phase could be contributed to a delay in activity of σ^B due to the need to reassemble, repair and and/or resynthesize cold-damaged ribosomes in response to a cold shock (Becker et al., 2000). This is a reasonable explanation for the initial decrease in the *L.*

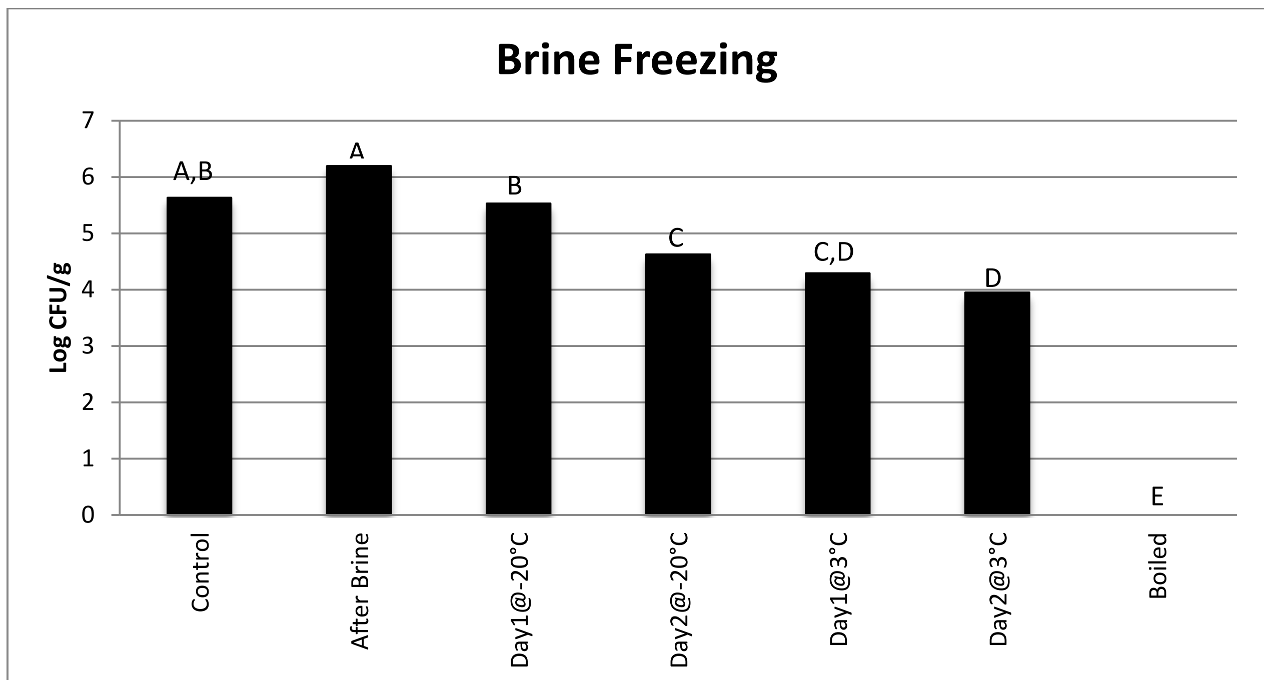
monocytogenes on shrimp immediately following cryogenic freezing observed in this study. Once the σ^B became active it was probably then able to produce CSPs and CAPs in response to the extreme temperature drop. As a result, the cells acquired resistance and adapted to their environment allowing the growth of the pathogen to resume. Also, CAP production was able to continue during storage at low temperatures enabling *L. monocytogenes* to multiply on the shrimp at freezer temperatures.

Furthermore, the survival of the pathogen on shrimp after boiling until floating could most likely be attributed to the development of heat shock proteins (HSPs). HSPs protect the organism from thermal stress. HSPs are also believed to enhance the survival and growth of bacteria at low temperatures (Chattopadyay, 2006). Moreover, HSP synthesis is believed to be an important factor in cold adaption of bacteria and its production is induced by a number of stress conditions other than an increase in temperature (heat shock) (Chattopadhyay. 2006). As a result, HSP production was most likely induced in *L. monocytogenes* in response to the cold stress making it more resistant to heat treatment later.

4.9.2 Effect of Brine Freezing on *Listeria monocytogenes*.

After brine freezing a slight increase occurred in *Listeria monocytogenes* on the shrimp but, the Log CFU/g on shrimp after brine freezing was not significantly different from the control. After one day at -20° C the bacterial load decreased by .66 logs but this reduction was not significantly different from the control. After two days of storage at -20° C the count significantly decreased by .9 Logs. After transferring to refrigerated storage (4° C) the bacterial load was not significantly reduced after day 1 or after day 2 of storage. The initial bacterial load on the shrimp was 5.6 Log CFU/g. Brine freezing followed by successive cold chain storage

reduced the count by 1.7 Logs. Following cold chain storage the shrimp was boiled until floating and *Listeria monocytogenes* was significantly reduced to non detectable levels. No heat resistance was detected. Also, no addition of salt to the shrimp following freezing in the concentrated brine was detected.



***Bars not connected by the same letter are significantly different (p<.0001, α=.05)**

FIGURE 17. Bacterial reduction of *Listeria monocytogenes* on shrimp after brine freezing followed by subsequent cold storage.

The ability of *Listeria monocytogenes* to withstand the brine freezing can be attributed to the fact that it is able to tolerate salt stress. This phenomenon is known as osmoadaptation (Gandhi & Chikindas, 2007). The σ^B factor described earlier also plays a crucial role in this stress adaptation. In order to tolerate salt conditions the σ^B factor mediates gene expression resulting in an increased synthesis of salt shock proteins (Ssps) and salt acclimation proteins

[(Saps) (Gandhi & Chikindas, 2007)]. This mechanism is very similar to the cold adaptation mechanism previously described.

Another mechanism *L. monocytogenes* uses to resist osmotic stress is compatible solutes. In response to increased extracellular osmolarity, compatible solutes can be accumulated at high levels inside the cells, therefore, restoring vigor without affecting cytoplasmic functions (Angelidis et al., 2003). Glycine betaine and carnitine are compatible solutes that act as osmoprotectants in *L. monocytogenes* (Chan and Wiedmann, 2007). Similarly, these solutes also act as cryoprotectants. Glycine betaine and carnitine can be transported into the cell by transporters which are activated by increased osmotic temperature followed by decreased temperature (Angelidis et al., 2003). The compatible solutes behave the same way under osmotic stress and under chill stress. As a result, the *L. monocytogenes* was able to withstand the osmotic and cold shock conditions experienced during the brine freezing.

Additionally, as part of the stress response to high salinity conditions a number of proteins are repressed and some are completely eliminated (Esvan et al., 2000). This could be a likely explanation for the fact that no enhanced resistance of *L. monocytogenes* to freezer or refrigerator temperatures during storage after brine freezing was observed. After brine freezing, the bacterial load continually decreased during both frozen and refrigerated storage and showed no thermal resistance to boiling. Since a number of the pathogens' proteins could have been damaged under the high salinity conditions of the brine, the bacteria was then unable to fully recover and continue growth at low temperatures. It appears as though the salinity of the brine induced an osmoadaptation response in the *L. monocytogenes* instead of a cold shock adaptation. So, in the absence of salt the bacterial cells had no increased resistance and due to destruction of some functional proteins they were unable to adapt to the cold storage.

4.9.3 Comparison of Effects of Brine Freezing and Flash Freezing on *Listeria monocytogenes*.

Figure 19 reveals that brine freezing and flash freezing had significantly different initial effects. Immediately following flash freezing a .74 log reduction in *Listeria monocytogenes* occurred and immediately following brine freezing a .56 log increase occurred. After one day of frozen storage, both methods resulted in similar log reductions. After day 2 at frozen storage, flash freezing resulted in a significantly different log change because it increased in number while brine freezing resulted in a decrease in number. Both methods then had similar log reductions after 1 and 2 days of refrigerated storage.

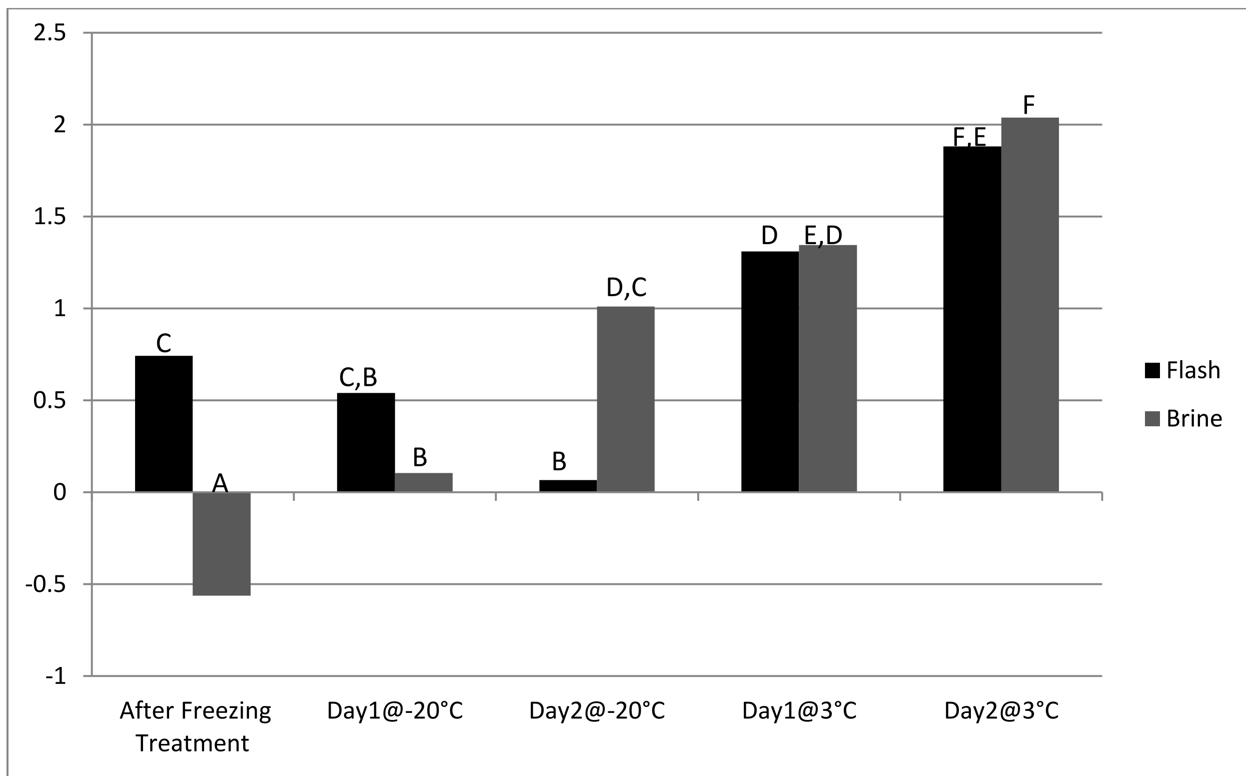


FIGURE 18. Log change in *Listeria monocytogenes* after brine freezing and flash freezing followed by subsequent cold storage.

4.9.4 Effect of Flash Freezing on *Salmonella typhimurium.*

Figure 20 shows that after flash freezing, a significant 1.5 Log reduction in the bacterial load of *Salmonella typhimurium* occurred on the shrimp. The original bacterial count was 5.09 Log CFU/g and this number was reduced to 3.5 Log CFU/g after freezing for 1.5 hours at -80° C. After one day of storage at -20° C the bacterial load was reduced significantly by an additional 2.01 Logs. The bacterial load was again significantly reduced after two days of storage at -20°C. No significant reduction occurred during refrigerated storage. An overall 3.82 Log reduction occurred in the *Salmonella* bacterial load on shrimp after flash freezing and consecutive storage at low temperatures. After cold chain storage the remaining 1.28 Log CFU/g on the shrimp was eliminated by boiling the shrimp until floating. These results were to be expected as *Salmonella* is not known to withstand the deleterious effects of freezing temperatures.

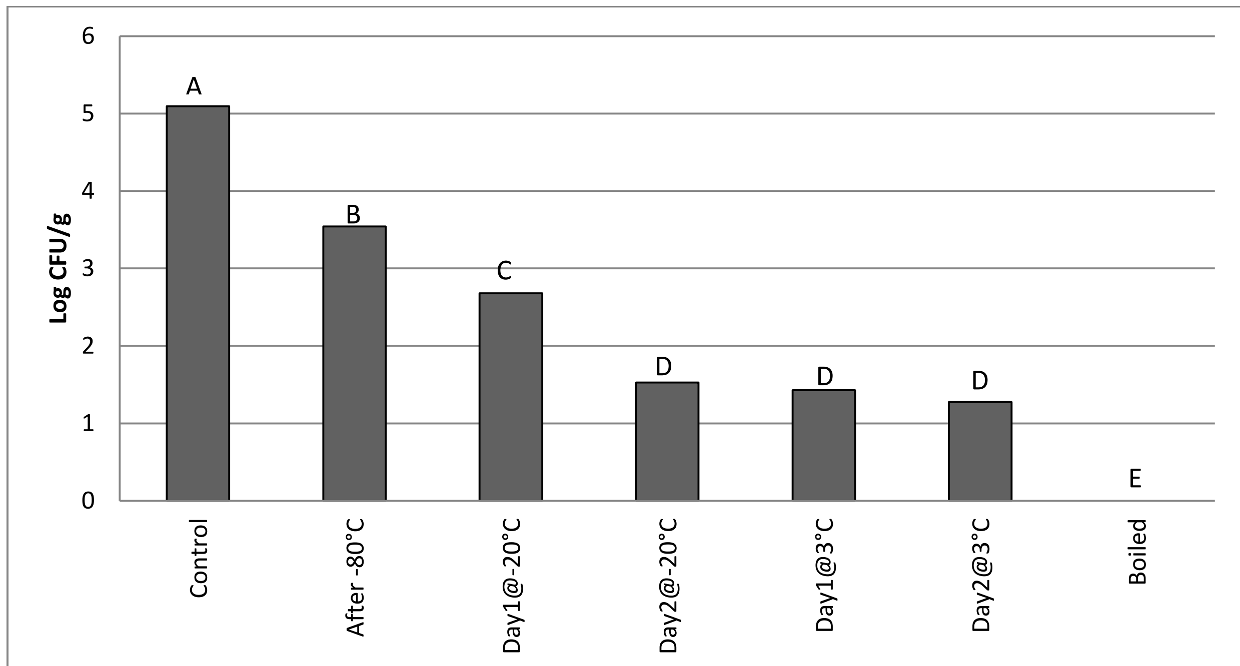


FIGURE 19. Bacterial reduction of *Salmonella typhimurium* on shrimp after flash freezing followed by subsequent cold storage.

4.9.5 Effect of Brine Freezing on *Salmonella typhimurium.*

Figure 21 illustrates that after brine freezing a significant 1.7 Log reduction in the bacterial load of *Salmonella typhimurium* occurred on the shrimp. The original bacterial count was 5.05 Log CFU/g and this number was reduced to 3.3 Log CFU/g after freezing (-20° C) for 20 minutes in a 0°F brine solution. After one day of storage at -20° C the bacterial load was significantly reduced by an additional 1.2 Logs. After two days of frozen storage, the numbers were reduced significantly by .43 Logs. The bacterial load was again significantly reduced after the first day of refrigerated storage but no significant decrease occurred after two days of refrigerated storage. An overall 3.3 Log reduction occurred in the *Salmonella* bacterial load on shrimp after brine freezing and consecutive storage at low temperatures. After cold chain storage the remaining 1.73 Log CFU/g on the shrimp was eliminated by boiling the shrimp until floating. These results were to be expected as *Salmonella* is not known to withstand the deleterious effects of freezing temperatures. The bacterial load reduction was similar to that of the flash freezing. The brine freezing resulted in a slightly higher initial bacterial reduction on the shrimp but a slightly lower overall reduction. Both methods resulted in substantial reduction of initial bacterial counts of *Salmonella* on shrimp and, therefore, both can be considered adequate processing methods.

4.9.6 Comparison of Effects of Brine Freezing and Flash Freezing on *Salmonella typhimurium.*

Figure 22 shows the comparison of both brine and flash freezing methods on the log reduction of *Salmonella typhimurium*. Figure 22 illustrates that there was no significant difference in the log changes that occurred after brine freezing and flash freezing followed by

subsequent cold chain storage. Therefore, both methods are adequate in controlling *Salmonella typhimurium* on shrimp.

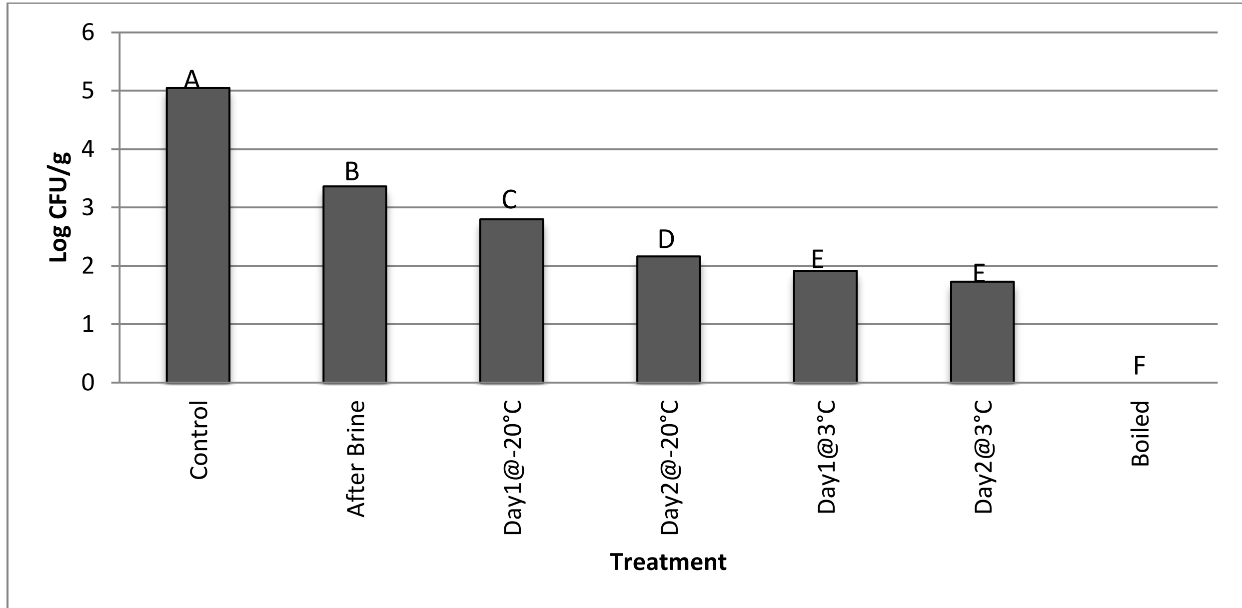


FIGURE 20. Bacterial reduction of *Salmonella typhimurium* on shrimp after brine freezing followed by subsequent cold storage.

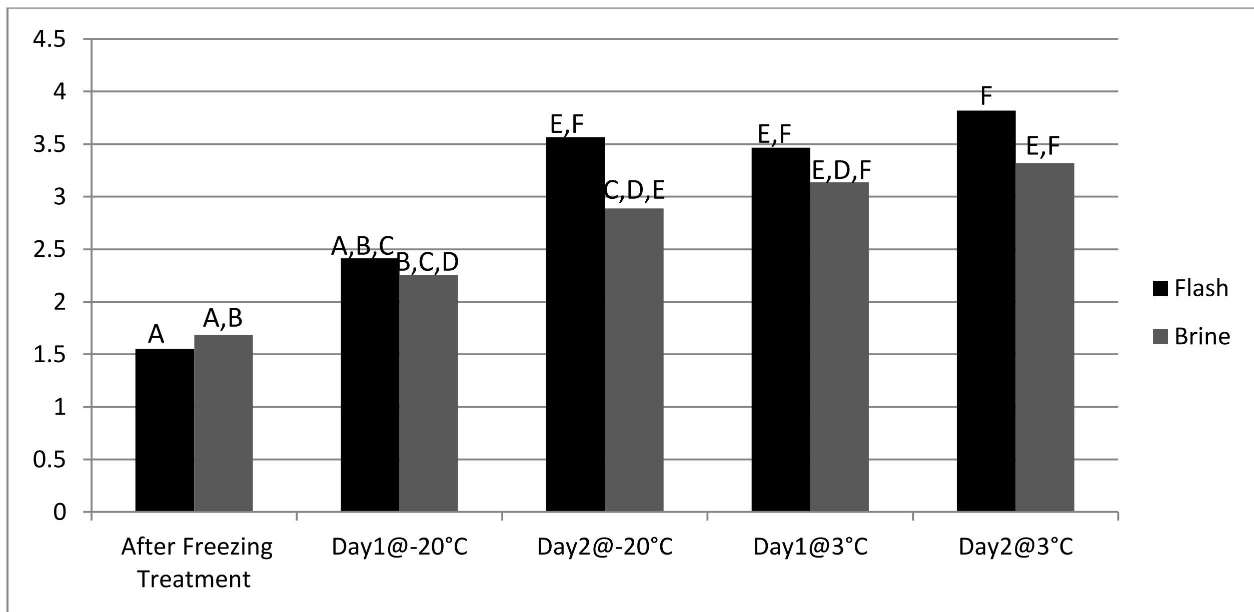


FIGURE 21. Log change in *Salmonella typhimurium* after brine freezing and flash freezing followed by subsequent cold storage.

CHAPTER 5 CONCLUSION

Due to its health benefits and flavorful taste, a high demand has been placed on shrimp in the United States. Unfortunately, an increase in consumption has resulted in increased disease outbreaks associated with shrimp in restaurants and private residences. Shrimp may become contaminated in the environment or postharvest through a number of different routes (NACMCF, 2008). However, most pathogenic bacteria contaminate shrimp in low levels and should not pose a great risk when the shrimp is adequately cooked and stored. Consequently, one of the main causes of the increase in outbreaks is believed to be due to inadequate cooking. Cooking plays a major role in the safety as well as the sensory acceptance of shrimp. The FDA and NMFS have stated that consumers need more guidance regarding temperature and/or time or other indications of "doneness" during cooking to ensure safe seafood (NACMCF, 2008). Color is often a parameter used to indicate doneness when cooking shrimp. Most recipes will recommend cooking shrimp until its shell is "pink" in color. Additionally, there are a wide variety of cooking methods for shrimp including boiling, grilling, steaming, and frying. Boiling is a popular choice and, according to most cookbook recommendations, shrimp is considered "done" when it has floated to the surface of the water and is "pink" in color. There is an absence of scientific information to ensure that this method is adequate in eliminating common pathogens associated with shrimp such as *Salmonella* and *Listeria monocytogenes*. As a result, the purpose of this study was to use scientific methods to determine if this common method would ensure the destruction of primary pathogens associated with shrimp. As well as, to test the effect of different factors, such as processing conditions on the reliability of this method.

From the results of this study, it can be concluded that boiling shrimp until they float is effective in reducing *Listeria* and *Salmonella* species by a significant amount. Initial bacterial

loads ranged from 3.5 to 5.4 Log CFU/g per shrimp. After boiling until floating, all serovars were reduced to non detectable levels, making the shrimp safe for consumption.

Additionally, it has been revealed that the pathogenic strain *Listeria monocytogenes* is psychotrophic and can grow and multiply at refrigerator temperatures of 4° C and below (FDA, 2011) and it is recommended that consumers may store cooked and uncooked shrimp for up to two days at refrigerator temperatures (FDA, 2011). Also, it has been shown that attached cells of *Listeria* and *Salmonella* on shrimp carapace and tissue can have significantly greater resistance to heat (Norhana et al., 2009). There is a concern that if these pathogens can grow and multiply on shrimp during refrigerated storage, then they may also be more resistant to heat due to a higher bacterial load prior to cooking. Furthermore, there has become an increase in demand for ready-to-eat products sold at grocery stores such as chilled, ready-to-eat shrimp that requires little or no further heat treatment. Therefore, the cooking method must ensure full destruction of psychotrophic pathogens to a level low enough that they are unable grow and multiply during refrigerated storage prior to consumption. As a result, this research studied the effect of refrigerated storage for 1 and 2 days prior to cooking and 1 and 2 days after cooking on the growth of bacteria and the development of heat resistance. The results showed that no heat resistance was observed in any of the bacterial strains during boiling until floating and no growth during refrigerated storage after boiling until floating was observed in any of the strains.

Also, the NACMCF stated that "there is no single temperature, with or without specified cooking time, that will ensure the safety of all cooked fishery products", and that there is limited thermal inactivation data currently available for seafood associated pathogens across the different fishery product matrices (NACMCF, 2008). In this study, the internal and surface temperatures of shrimp were measured immediately after boiling until floating. The minimum

internal temperature of shrimp was recorded at 65.2° C. The average internal temperature of shrimp after cooking was 68.61 ± 2.29° C. The average surface temperature of the shrimp after cooking was 76.67 ± 5° C and had an even larger standard deviation of 7.26. The wide degree of variation in internal and surface temperatures of the shrimp was based on the different sizes of the shrimp. The smaller the shrimp is, the faster it will float and vice versa. As a result, boiling the shrimp until it floats is more reliable and easier for the consumer than measuring the temperature of the shrimp when determining if it is done.

In addition, because the "redness" of shrimp is often used as an indicator of "doneness" of cooked shrimp, the redness (a*) was measured using a spectrophotometer and a correlation was made between pathogen reduction to non detectable levels and color change after boiling until floating. The results of this study revealed that the redness (a*) in the cooked shrimp was significantly ($p<0.0001$) higher than uncooked shrimp. However, it was also found that there was a large standard deviation in the a* values of the cooked shrimp indicating a significant variation in the red color of the shrimp for all days tested.

When misused, sodium tripolyphosphate is believed to alter the red color change of cooked shrimp and result in a translucent appearance of the shrimp even after cooking. In order to ensure that the large variation in the red color of shrimp after boiling until floating was not due to the preservative sodium tripolyphosphate, an additional study was performed in the same way but evaluating shrimp that had not been treated with sodium tripolyphosphate. The results of this study showed that the redness (a*) in the cooked shrimp was again significantly ($p<0.0001$) higher than the uncooked for all days tested. Furthermore, the standard deviation of the a* values in the cooked shrimp was again large compared to the uncooked revealing a wide variation in the red color of the shrimp for all days tested even when they had not been treated.

It was determined from the results of this study that boiling shrimp until they float is adequate in reducing *Listeria* and *Salmonella* species to levels safe for consumption, however, color change is not a reliable indication of doneness due to a high degree of variation. These results give scientific evidence that boiling shrimp until they float is a reliable method for cooking in households to ensure safe shrimp for the consumer. Furthermore, this method can be easily understood and used by the consumer in a home setting.

This research also studied the effect of processing parameters on the safety of shrimp. Shrimp begins to spoil due to microbial degradation immediately after harvesting. As a result, freezing the shrimp soon after harvesting is widely used as a way to prevent the growth and negative effects of microbes on shrimp. This study tested the inhibitory effects of the two most widely used freezing methods on board shrimp vessels. It was discovered that brine freezing shrimp had a more desirable effect on the microbial quality. Even though there was an initial increase in the bacterial load no further resistance was detected during consecutive storage at low temperatures or during heat treatment. The growth of *Listeria monocytogenes* at -20° C following flash freezing should be a red flag to the food industry because freezing is widely accepted as a way to prevent or inhibit the growth of pathogens in food. If *Listeria monocytogenes* is able to grow during frozen storage, it could have the potential to multiply to an unsafe level prior to consumption by the consumer. Moreover, the development of heat resistance when subjected to flash freezing is equally alarming. Consumers should be able to eliminate all pathogenic bacteria from their food when it is adequately cooked. In order to keep food products safe it is crucial to inhibit the development of heat resistance and cold adaptation during any point of processing, distribution and storage. The adaptation of *Listeria monocytogenes* to chill stress is a complex process. Future research must focus on the capacity of

this pathogen to grow in refrigerated and even frozen foods and how to prevent this growth. For now, it can be concluded that brine freezing shrimp rather than flash freezing is one way to prevent the development of cold and heat resistance in shrimp.

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VITA

Genevieve Edwards was born in 1987 in Metairie, Louisiana. She received her Bachelor of Science degree in animal science from Louisiana State University in May 2009. She began working for Dr. Marlene Janes in the Louisiana State University food microbiology lab as a student worker during her senior year of undergraduate studies. She then decided to pursue a master's degree in food science with a concentration in food microbiology/safety. She is currently a master's degree candidate and will receive her degree in May 2012.