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Determination of minimum safe cooking temperatures for shrimp to destroy foodborne pathogens

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**DETERMINATION OF MINIMUM SAFE COOKING TEMPERATURES
FOR SHRIMP TO DESTROY FOODBORNE PATHOGENS**

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
Sailaja Chintagari
B.Sc. (Hons), Osmania University, 2005
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ABSTRACT

Shrimp is one of the most common seafoods and a favorite among consumers. Like any other food there are safety concerns about shrimp. *Listeria* species, *Salmonella* species, *Clostridium* species and *Vibrio* species are among the pathogens of prime importance. Most of these pathogens can be eliminated by cooking. However, the extent of cooking and temperatures greatly influence the safety of these foods. The current study is focused on the determination of minimum cooking temperatures for shrimp to eliminate *Listeria* species, *Salmonella* species and *Vibrio* species. Shrimp were surface inoculated with the three different species mentioned above to about 5.00 log CFU/g of shrimp and then incubated for two days. Shrimp samples were treated at five different temperatures on days 0 (day of inoculation), 1 and 2 by boiling. The effects of heat treatments by boiling on bacterial counts were determined by plating and calculating the log CFU/g reduction for each temperature. The experiment was repeated with different temperatures for each bacterium until the bacterial load in the shrimp was at non-detectable levels. The internal temperature of 85°C was the lowest temperature that was needed to kill all the bacteria tested. *Vibrio* species were less resistant to heat with bacterial counts reaching non-detectable levels at 55°C. 75°C was the minimum temperature required to eliminate *Salmonella* species, while *Listeria* species showed highest resistance up to 85°C. This study is mainly intended to design a simple, easy and unbiased consumer guide for cooking shrimp to enhance safety while handling and cooking them at home. This can also serve as a guide for manufacturers of ready-to-eat shrimp products while designing and planning CCP's in HACCP plans during production.

CHAPTER 1
INTRODUCTION

1.1 Introduction

Seafood is a balanced food that is nutritionally rich and delectable. Fresh seafood, in particular shrimp are highly perishable and microbiological spoilage is one of the important reasons that limit the shelf-life and safety. Fresh seafood can be contaminated at any point from rearing or harvesting to processing, transport or cross contamination while handled by the consumer at home.

There are incidents of shrimp contaminated with foodborne pathogens like *Listeria* species, (Lennon and others, 1984; Mu and others, 1997; and Weagant and others, 1988), *Salmonella* species (Heinitz et al., 2000) and *Vibrio* species (Fatma et al., 2005). These foodborne pathogens cause serious health risks if the food is not cooked adequately to kill them. Most of the time consumers rely upon the color of the flesh as a parameter for doneness during cooking which does not ensure the safety.

Heat treatment is an important method to reduce the microbial load in food products. In shrimp, heat treatment has an important role in the safety of the product. The freshness and safety of seafood especially shrimp varies depending on many factors like contamination during farming, harvesting and handling or other post-harvest activities. Consumers can store fresh raw shrimp for 2 days at refrigerated temperatures ranging from 0.5 - 4.5°C (Tim Roberts and Paul Graham, 2001). However, at this temperature some pathogenic bacteria like *L. monocytogenes* could grow. Therefore, in our current study, 3°C has been used for storage to study the effect of refrigerated storage on thermal resistance of three bacterial species. Internationally, there have been several reports of farmed shrimp being contaminated with *Salmonella* due to their culture in poor quality waters (Phan et.al., 2005, Koonse et al., 2005). In many countries the acceptable microbiological level in cooked crustaceans is set at 0 bacteria per 25 grams of sample (Osborne et al., 2003). The three species of *Vibrios* used in this study have been cited as the reasons for

various foodborne outbreaks especially associated with seafood (CDC, 2005). Establishment of safe guidelines for heat processing of seafood is mandatory for the prevention of *V. vulnificus* septicemia. (Kim et al., 1997). Shrimp present a particular risk for bacterial foodborne diseases because although they are generally cooked, they are often consumed only lightly cooked or even raw (Dalsgaard et al., 1995).

Thermal resistance can vary from one bacterium to another and also between species. Thermal resistance can also be affected by other factors such as conditions under which foods contaminated with pathogens are stored. Consumers generally store the shrimp at refrigerated temperatures for 1 or 2 days before using. Therefore, studying the effect of duration of cold storage (3°C) on thermal resistance is also an important factor for designing safe cooking temperature guides for consumers. Our study is aimed at determining the minimum internal temperature of the shrimp required to reduce *Listeria* species, *Salmonella* species, and *Vibrio* species to non-detectable levels by boiling the shrimp samples to reach different internal temperatures.

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CHAPTER 2
LITERATURE REVIEW

2.1 Shrimp

Shrimp is one of the most popular and valuable seafood products in the world with production at about 3.5×10^5 tons. The consumption of seafood among American consumers has steadily increased over the past decade and shrimp has risen to the position of America's number one favorite seafood (NOAA, 2008) leaving behind canned tuna which previously held this position. This could be due to greater awareness of health attributes of seafood. The U.S. Department of Agriculture projects that seafood and fish will increase 26% in per capita consumption between 2000 and 2020 (Sloan, 2005). Increasing consumer awareness of the nutritional value of seafood has stimulated a strong demand for seafood and seafood products (Pigott and Tucker, 1990).

2.2 Foodborne Illnesses; Risks Associated with Shrimp

Consumption of foods contaminated with foodborne pathogenic microorganisms and toxins produced by them cause deaths, illnesses, hospitalization, and economic losses. Foodborne illnesses have major implications for the food industry through lawsuits, lost earnings, and damaged consumer confidence (Dalton and Douglas 1996; O'Brien and others 2002; Dalton and others 2004). Foodborne illnesses cause a high cost to the US economy and results in thousands of deaths each year. The Center for Disease Control and Prevention has stated that foodborne diseases cause approximately 76 million illnesses, 325,000 hospitalizations, and 5000 deaths in the United States each year, in which about 14 million illnesses, 60,000 hospitalizations, and 1800 deaths are caused by known pathogens (Mead et al., 2000). Many of today's concerns for pathogens such as *Campylobacter jejuni*, *Escherichia coli* O157:H7, *L. monocytogenes*, and *Cyclospora cayetanensis* were not recognized as causes of foodborne illnesses about 20 years ago (Mead et al., 2000). There are a series of techniques such

as, washing methods, chlorine sprays, organic acid sprays and steam treatment procedures to reduce the microbial load on food (Kochevar et al., 1997; Reagan et al., 1996; Cutter and Siragusa, 1995; and Prasai et al., 1995). Still, these various methods have not reduced the numbers of recalls and outbreaks.

Contamination by bacterial pathogens e.g. Salmonella, Vibrio, and presence of antibiotics may have potentially hazardous effects on consumers, handlers, and the environment. It is largely accepted that the microbiological quality of the production environment impacts the microbiological quality of the fish and ultimately the processed product. Bacterial pathogens represent a threat to human health when they are consumed raw.

Raw fish and shrimp were linked to an outbreak of *L. monocytogenes* which caused nine deaths in New Zealand (Lennon and others, 1984). Since this outbreak, the seafood industry has been concerned with the ability of *L. monocytogenes* to grow to high levels in shrimp when stored at refrigerated temperatures (Lennon and others, 1984; Mu and others, 1997; and Weagant and others, 1988). Consumers buy raw shrimp from their local grocery store and cook them at home which greatly reduce the risk of outbreaks but the extent of cooking depends upon various factors like the size of the shrimp, and type of cuisine or dish. The shelf life of seafood is greatly influenced by microbial load and moreover these are highly perishable commodities (Mu et al., 1997). Food safety becomes more complicated when foodborne pathogens like *L. monocytogenes*, *V. cholerae* 01 and Salmonella are involved because seafood processing plants are ideal environments for these kinds of organisms to proliferate adding to the ever growing issues of food safety.

For decades the consuming public has believed that “fresh is best.” Unfortunately, fresh is not always best, sometimes it’s old, rancid, or loaded with bacteria. While consumers always

say they prefer fresh rather than frozen, previously frozen or “thawed for convenience,” the fact is most of the shrimp sold in the domestic markets has been frozen.

Bacteria are part of our environment. Where there is food there may be bacteria. Proper food handling and cooking are the best ways to prevent foodborne illness. Consuming raw or undercooked seafood or shellfish may increase the risk of foodborne illness, especially for children, the elderly, pregnant women and those who have chronic illnesses or compromised immune systems.

2.3 Pathogens

2.3.1 *Listeria* Species

In the United States there is zero tolerance policy for *L. monocytogenes* (Cooked crustaceans). *Listeria* is a major concern for the seafood industry as well as the common consumers because its consumption can result in death. A high fatality rate was associated with *Listeriosis* resulting in *L. monocytogenes* being responsible for 27.6% of all deaths due to foodborne pathogens in the United States (Mead et al, 1999). The projected cost relating to *L. monocytogenes* alone was estimated at \$233 million per year in the US (Kanuganti et al., 2002). It is widespread in the environment and can colonize on processing surfaces. It can multiply on foods stored at refrigerated temperatures.

Listeria species are found in almost every medium in the environment, soil, excrement, canal waters, plants, animals, animal feeds and foods. They contaminate food by various means from these sources; because of their ability to grow at +4°C can contaminate many kinds of foods under every kind of storage condition. It is not very hard for them to enter seafood. It is also very important to eliminate pathogens and keep them away.

Researchers have studied the thermal resistance of *Listeria innocua*, *Listeria seeligeri*, *Listeria welshimeri*, and *L. monocytogenes* (Bradshaw et al., 1991). They found that the heat

resistance of *L. monocytogenes* appeared somewhat greater than other *Listeria* species in milk products.

L. monocytogenes has been shown to grow at refrigeration temperatures by several studies (Harrison et al., 2000). Investigators have studied the growth of *L. monocytogenes* under modified atmospheres of 5% O₂: 10% CO₂: 85% N₂ and at 4 and 8°C (Harrison et al., 2000). The general relationship between the maximum and minimum specific growth temperature (-2 to 45°C) was studied for *L. monocytogenes* by Bajard et al., (1995). These investigators showed the existence of growth between 10 and 15°C, furthermore below these temperatures *L. monocytogenes* grew faster than would be expected (Bajard et al., 1995).

In general it has been reported that *L. monocytogenes* can survive for 10-30 days in tap water at 28-30°C and for 7-110 days at 5-10°C. In pond water, *L. monocytogenes* has been reported to survive for more than 8 weeks (air temperature – 26 to 9°C), (Bremer et al., 2003). *L. monocytogenes* has been reported to survive for at least 3 weeks and probably significantly longer in seawater. Survival in seawater was dependent on seawater temperature and *L. monocytogenes* strain (Bremer et al., 2003).

L. monocytogenes has many adaptive physiological traits that enable it to survive under a wide range of environmental conditions. It can overcome various types of stress, including the cold stress associated with the low temperatures of food production environments (Jemmy et al., 2006). Researchers have proposed that this cold tolerance phenomenon in these microorganisms is a function of multiple genetic and physiological factors that sense the cold stress threat and efficiently induce appropriate cellular responses. These mechanisms render the current use of low temperatures and refrigeration, which control most foodborne pathogens in food environments (Wouters et al., 2000)

Slabyj et al., (2008) reported that *L. monocytogenes* could survive for five-minutes in boiling water inside shrimp tails. It is important to note that the pathogen was not detected immediately after the boiling, but was detected when the shrimp tails were analyzed after three days of refrigeration. The delayed recovery of *L. monocytogenes* from shrimp tails is important to note because it implies that the few surviving cells that had sustained sub-lethal damage during processing could (given three days to recover) repair the damage that was sustained during the boiling step at refrigerated temperature. The recovery of the pathogen is not unexpected since shrimp is a nutritious product, which will support bacteria growth.

2.3.2 *Salmonella* Species

Acute gastroenteritis caused by *Salmonella species* continues to be a worldwide public health concern (Obana et al., 1996). In humans, Salmonellosis is usually due to the consumption of contaminated food or water. The fecal wastes from infected animals and humans are important sources of bacterial contamination of the environment and the food chain (Thong et al., 2002). During a 9-year study (1990–1998), the FDA noted an overall incidence of *Salmonella* in 7.2% of 11,312 samples from imported and 1.3% of 768 samples from domestic USA seafood (Heinitz et al., 2000). *Salmonella* has also been detected in US market oysters (Brands et al., 2005) and in other imported seafood from different countries (Khan et al., 2006). The incidence of *Salmonella* in seafood is highest in the central Pacific and African countries and lowest in Europe including Russia, and North America (12% versus 1.6%)(Heinitz et al., 2000). The presence of *Salmonella* species in seafood has been reported in Vietnam (Phan et al., 2005), India (Varma et al., 1985; Iyer and Shrivastava, 1989; Kumar et al., 2003), Sri Lanka (Fonseka, 1990), Thailand (Rattagol et al., 1990), Taiwan (Chio and Chen, 1981) and Japan (Saheki et al., 1989) which make significant exports of shrimp to US markets.

USFDA has conducted studies which showed that aquacultured seafood was more likely to contain Salmonella than wild-caught seafood (Koonse et al., 2005). Many researchers also have evaluated the presence of Salmonella, fecal coliforms and *E. coli* in shrimp aquaculture ponds (Bhaskar et al., 1998; Iyer and Varma, 1990; Reilly and Twiddy, 1992; Dalsgaard et al., 1995). The relationship between the occurrence of Salmonella in shrimp from aquaculture operations and the concentration of fecal bacteria in the source and grow-out pond water has been recently described by Koonse et al (2005). These could be the possible routes of contamination of Shrimp and other seafood with the pathogen.

2.3.3 *Vibrio* Species

Scientific studies conducted in countries within warm climate zones demonstrated that shellfish can be contaminated by a variety of pathogenic organisms (Colakoglu et al, 2005). Some members of Vibrionaceae have a specific emphasis among these pathogenic organisms. The members of Vibrionaceae such as *Vibrios*, *Aeromonas*, and *Plesiomonas* are natural contaminants of aquatic habitats which are also inhabited by shellfish (Huss, 1997; Oliver, 1989). Fatma et al., (2005) have isolated *Vibrio* species from raw shrimps in Turkey. During the last several decades researchers have continuously emphasized foodborne infection cases in humans which were caused by consuming contaminated fresh-raw shellfish (Colakoglu et al., 2006). Occasionally, *Vibrio* species has been identified as the most significant cause of foodborne hospitalizations; even as a cause of death (Diesterweg, 1992; Kunz, 1988). The magnitude of the risk increases when food preparation and consumption trends change towards raw or undercooked seafood (Stolle, 2002).

V. cholerae 01 due to its higher resistance compared to the other microorganisms, could lead to safety hazards in seafood products (Rippen & Hackney, 1992). Shrimp are cooked at home by the consumer, or in commercial/institutional settings. This reduces the number of

microorganisms in shrimp (Erdogdu et al 2001), but the extent of cooking greatly plays a role in destruction of this bacteria.

V. cholerae O1 is recognized as an autochthonous bacterium in the marine environment (Colwell et al., 1981). Marine foods have been identified as vehicles for the transmission of cholera (Centers for Disease Control, 1986a, b, 1991a, and b). The factors and mechanisms that affect the survival in the aquatic environment are not completely understood. But some research groups have stated that this pathogen is able to attach to abiotic surfaces, to zooplankton and phytoplankton, and to the carapaces of crustaceans such as shrimp and crab (Castro-Rosas and Escartín 2002; Hood and Winter, 1997; Huq et al., 1983, 1984, 1986, 1990; Tamplin et al., 1990). Vibrios are generally considered to be heat sensitive (Hackney and Dicharry, 1988) but there are some reports which show that the *V. cholerae* O1 can show some resistance in hot foods (Makukutu and Guthrie, 1986).

2.4 VBNC - Viable but Non Culturable State

Viable but Non-Culturable state (VBNC) trends have been observed in bacteria such as *Listeria*, *Salmonella* and *V. cholerae*. Both O1 and O139 strains of *vibrio cholerae*, may also be singled out because it has been suggested that the VBNC state accounts for the seasonal nature of cholera outbreaks due to survival for long periods e.g. in river sediments (Oliver, 2005).

2.5 Foodborne Outbreaks

2.5.1 Occurrence of Foodborne Disease Outbreaks

Of the places identified, the frequency of foodborne disease outbreaks in the United States is listed as follows by The Idaho Food Safety and Sanitation Manual

- Restaurants, cafeterias, delicatessens and other commercial food establishments (57%)
- Homes (29%)

- Schools (6%)
- Church functions (3%)
- Picnics (3%)
- Camping (2%)

Because many foodborne disease outbreaks are not recognized or just considered "a bug that's going around," many foodborne disease outbreaks go unreported. It is estimated that the actual number of outbreaks is 10 to 100 times more than reported.

2.5.2 Causes of Outbreaks

- Investigations of foodborne disease outbreaks have revealed the following as the most important contributing factors. (Idaho food safety and sanitation manual)
- Improper holding temperatures (34%)
- Poor personal hygiene (18%)
- Inadequate cooking (15%)
- Contaminated equipment (14%)
- Food from unsafe source (9%)
- Other (10%)

2.6 Food Safety- Cooking

In the past century, advances in making the food supply safe for consumers have occurred with the use of irradiation and other thermal treatments as new technologies, however we are still having outbreaks related to seafood. These technologies have largely not been accepted by the consumer. One of the main reasons is the lack of non-biased information to the consumer (Skovgaard, 2007). A lot of foodborne outbreaks occur due to improper cooking of food by consumers. There is a need for consumer friendly guidelines in order to ensure maximum possible food safety.

Cooking processes are not usually designed to eliminate spores of pathogens (FDA, 2001; Rippen 1998). When discussing cooking, we must be aware that the core of the food particles heats significantly slower than the surrounding liquid. Storing the cooked shrimp under refrigeration for a day results in very little chance of bacterial infection but it is not guaranteed.

The thermo tolerance of *L. monocytogenes* is estimated to be one of the highest among nonperformers. Inadequate cooking was cited as a contributing factor in 67% of the *Salmonella* related outbreaks and has also been investigated as a source of Listeriosis (Bean and Griffin, 1990; D'Sa et al., 2000).

Foodborne pathogens can have different responses or reactions after exposure to stresses or sub-lethal treatments. One of responses is the sigma factor RpoS, or the stationary phase sigma factor, which has been identified in a number of Gram-negative bacteria and similar systems operate in other bacteria (Skovgaard, 2007). The RpoS response confers resistance to a range of stresses, and exposure to one factor such as low pH or high osmotic pressure, can confer increased resistance to other stresses such as heat. Some research groups have also noted that the resistance can be contagious (Skovgaard, 2007). The implication of this for food safety is considerable because not only do they suggest that stresses micro-organisms can encounter during food processing might increase resistance to other stresses, but they could also increase the virulence of any pathogen present (Adam and Moss, 2000).

A study by Guzewich and Ross's (1999) that reviewed 81 foodborne outbreaks from 1975 to 1998 found that 89% of these outbreaks were associated with the transmission of pathogens to foods by workers' hands. Ha and others (2003) suggested that drinking water, employees' hands, refrigerators, and aprons obtained from school foodservices could also be factors related to foodborne illness. This emphasizes that contamination of the food can also

take place at and during cooking and handling. The immediate and important measure to overcome this problem is cooking which can eliminate the pathogens.

Risk factors identified as causes for foodborne illness are foods from unsafe sources, inadequate cooking, improper holding temperature, contaminated equipment, and poor personal hygiene (USDHHS-FDA-CFSAN, 2000). Apart from this while cooking, cutting boards may cause cross-contamination after they have been sanitized in the dishwasher (Sneed and others 2004; Staskel and others 2007).

Inadequate cooking and storage of food is considered to be the main cause of foodborne infection, (Ryan et al., 1996) it has also been suggested that domestic household conditions and inadequate heating account for 11% and inappropriate storage for up to 50% of outbreaks. This leaves room for extensive research in food safety for developing guidelines at the consumer or domestic house hold level that will aid in reducing the number of outbreaks.

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CHAPTER 3

SAFE COOKING TEMPERATURES TO DESTROY *VIBRIO SPP*, *LISTERIA SPP* AND *SALMONELLA SPP* ON SHRIMP

3.1. Methods and Materials

3.1.1 Culture Preparation

Vibrio cholerae 01, *Vibrio parahaemolyticus* (ATCC 33847) and *Vibrio vulnificus* (ATCC 1007), *Salmonella enteritidis*(13076), *Salmonella infantis* (CDC, Atlanta) and *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 culture collection. Frozen pure cultures were thawed and a loop full was streaked on nutrient agar for *V. cholerae* 01 and Nutrient agar with 2% salt for *V. vulnificus* and nutrient agar with 3% NaCl slants for *V. parahaemolyticus*. *Salmonella enteritidis*, *Salmonella infantis* and *Salmonella typhimurium* were streaked on BHI agar slants and *Listeria innocua*, *Listeria monocytogenes* (1/2a) and *Listeria welshimeri* were streaked on TSB agar slants and incubated for 16 hrs at 37°C after that these slants were maintained at room temperature for future use. *Vibrios* were sub cultured by suspending a loop full of cells from nutrient agar slants to nutrient broth with NaCl supplemented according to the requirement of each species. *Listeria* species and *Salmonella* species were sub cultured into TSB and BHI broths respectively and incubated at 37°C and this process was repeated before using the cultures for inoculation studies.

3.1.2 Sample Preparation

Shrimp samples were purchased from a local seafood market. The samples were sorted for uniform weight and only samples weighing 72 ± 3 grams were selected as larger shrimp take more time to reach the internal temperature. Samples were tested for the presence of *Vibrio* species following the protocols explained in the FDA/BAM rules of food testing. All the samples used in this study were free of *Vibrio* species. These samples were stored at -20°C until

the next day. On the day of inoculation the samples were thawed at room temperature and were first washed thoroughly with tap water and then with sterile distilled water. Fifty ml of respective 16 hour culture was added to a sterile container holding 1,000 ml of sterile 0.1 % Peptone water. The shrimp samples were soaked in culture solution for 30 minutes and then allowed to air dry for about 1 hour. This process was followed for all the 9 bacteria used in this experiment. The final concentration of the bacteria was about 5.00 to 6.00 log CFU/g.

After inoculation the shrimp samples were randomly picked and separated into three different portions and were assigned the following Day 0, 1 or 2. The samples were packed in double layered freezer bags and were stored at 3°C in an incubator until treated.

3.1.3 Heat Treatment – Boiling

The day of inoculation was designated day 0 and immediately after surface inoculation and drying, shrimp samples were placed in a boiling water bath and were removed at different internal temperatures. Thermometer probes with digital display were used for monitoring the temperatures. The thermometer probe was inserted at the cold spot of the whole shrimp which was between the third and fourth abdominal segment. Two samples were inserted with the thermometer probes and temperatures were monitored individually. Shrimp samples were subjected to internal temperatures ranging from 30°C to 50°C for *Vibrio* species, 50°C to 75°C for *Salmonella* species and 65°C to 85°C for *Listeria* species. When the internal temperature of the shrimp samples attained the predetermined temperature the samples were quickly removed from the water bath and immediately transferred aseptically to pre-labeled Whirl-Pak bags using sterile stainless steel tongs. These bags containing the heated shrimp samples were immediately plunged into an ice water bath in order to stop further cooking of the sample and were left in the ice bath for 1 minute and then weighed.

3.1.4 Enumeration of Bacteria

The weight of the samples was recorded on the exterior of the bags. Equal (wt/vol) amounts of PBS were added to the samples and then they were stomached to homogenize. Homogenized samples were transferred into a sterile filter bag. From here the filtered sample was obtained for subsequent decimal dilutions. Then each dilution was plated in triplicate on Thiosulphate Citrus Bile Salts (TCBS) plates (Acumedia, Baltimore, MD, U.S.A.) for *Vibrios* (Ali et al., 2005), Xylose lysine deoxycholate agar (XLD agar) plates (Acumedia, Baltimore, MD, U.S.A.) for *Salmonella* species (Nye et al., 2001) and Modified Oxford Agar plates (Acumedia, Baltimore, MD, U.S.A.) for *Listeria* species (Beverly et al., 2006). The same procedure was followed for all the temperature treatments.

Day 1 and Day 2 —the samples designated as day 1 or day 2 were taken out of the incubator and the above said procedure for heat treatment and enumeration was followed. The experiment was repeated three times for each of the species used and the values were analyzed.

3.1.5 Verification Studies

On Day 0, Day 1 and Day 2 for each bacterial pathogen used two shrimp samples were cooked to the assigned highest internal temperature. The cooked samples were then incubated at 37°C for 24 hours. After incubation the samples were stomached by adding equal (wt/vol) amount of sterile PBS, filtered in a sterile filter bag and the filtrate used for subsequent dilutions which were plated in triplicates on respective enumeration media for each bacteria.

3.1.6 Statistical Analysis

Differences in survival of *Vibrio* species, *Salmonella* species and *Listeria* species were analyzed at different internal temperature treatments for significance using Student's t test following one-way analysis of variance (ANOVA) JMP-IN (version 5.0, SAS Inst. Inc., Cary,

North Carolina, USA). The statistical difference was set at $p < 0.05$. All experiments were done in triplicate.

3.2 Results

V. vulnificus, *V. parahaemolyticus* and *V. cholerae* 01 inoculated shrimp samples showed different responses to cold storage at 3°C and also to the thermal treatment by boiling to different internal temperatures. The thermal resistance patterns varied both by the species and also by the day of storage.

On day 0, the initial counts of all the three *Vibrio* species inoculated onto the surface of shrimp samples were about 5.00 log CFU/g. When shrimp samples were subjected to heat treatment by boiling to different internal temperatures, all three *Vibrio* species showed significant reduction in bacterial counts. The counts of *V. cholerae* 01 and *V. parahaemolyticus* at 35°C internal temperature were about 2.35 and 2.12 log CFU/g, and *V. vulnificus* was the most sensitive with 1.98 log CFU/g. When the shrimp samples were treated to reach the internal temperature of 40°C all the three *Vibrio* species were reduced to non-detectable levels (Fig.1a).

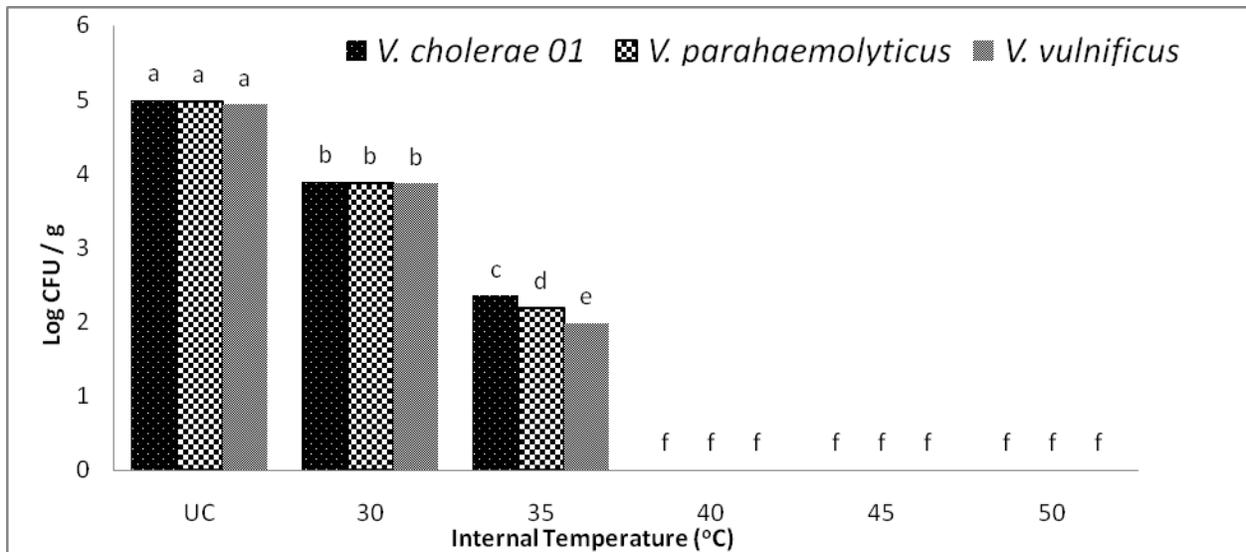


Fig.1a. Thermal resistance of *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* 01 at different internal temperatures in shrimp when subjected to boiling on day 0. UC = Uncooked shrimp sample. Data presented in the bar diagram is the mean of three different experiments and the bars with same data letters are not significantly different from each other. ($p < 0.05$)

Storing the shrimp samples inoculated with *Vibrio* species at 3°C for one day resulted in 0.78, 0.82 and 0.54 log CFU/g reduction of *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* respectively (Fig.1b).

An internal temperature of 45°C was lethal enough to reduce *V. vulnificus* to non-detectable levels and reduce *V. cholerae* and *V. parahaemolyticus* to 1.6 and 0.8 log CFU/g, respectively for sample stored first day prior to boiling. The highest internal temperature that was necessary to reduce all the three *Vibrio* species to non-detectable levels on day 1 was 50°C.

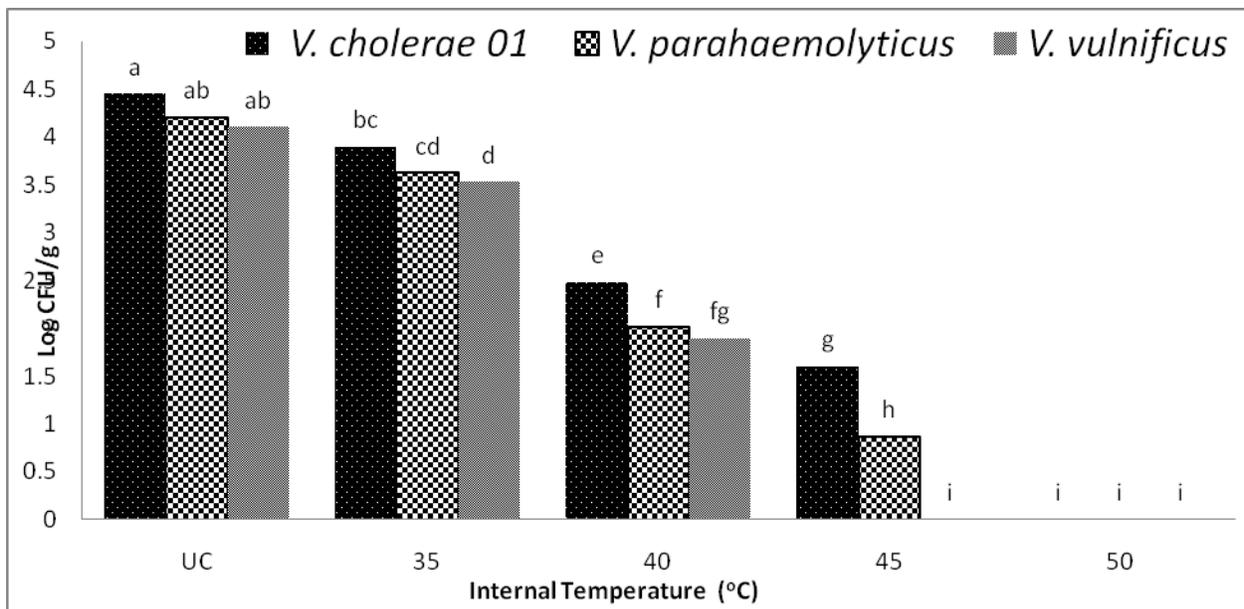


Fig.1b. Thermal resistance of *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* 01 at different internal temperatures in shrimp when subjected to boiling after storing at 3°C for 1 day. UC = Uncooked shrimp sample. Data presented in the bar diagram is the mean of three different experiments and the bars with same data letters are not significantly different from each other. (p<0.05)

On day 2, *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* 01 counts inoculated onto the surface of shrimp samples and stored at 3°C were significantly reduced by 1.16, 1.11 and 0.8 log CFU/g (Fig.1c) after 48 hours of storage.

Shrimp samples stored for 2 days at 3°C showed a different pattern of thermal resistance at 40°C for *V. cholera* 01, *V. parahaemolyticus* and *V. vulnificus* with counts of 2.42, 2.13 and

2.01 Log CFU/g. 50°C was the highest internal temperature required to reduce all the three *Vibrio* species to non-detectable levels on Day 2 (Fig.1c).

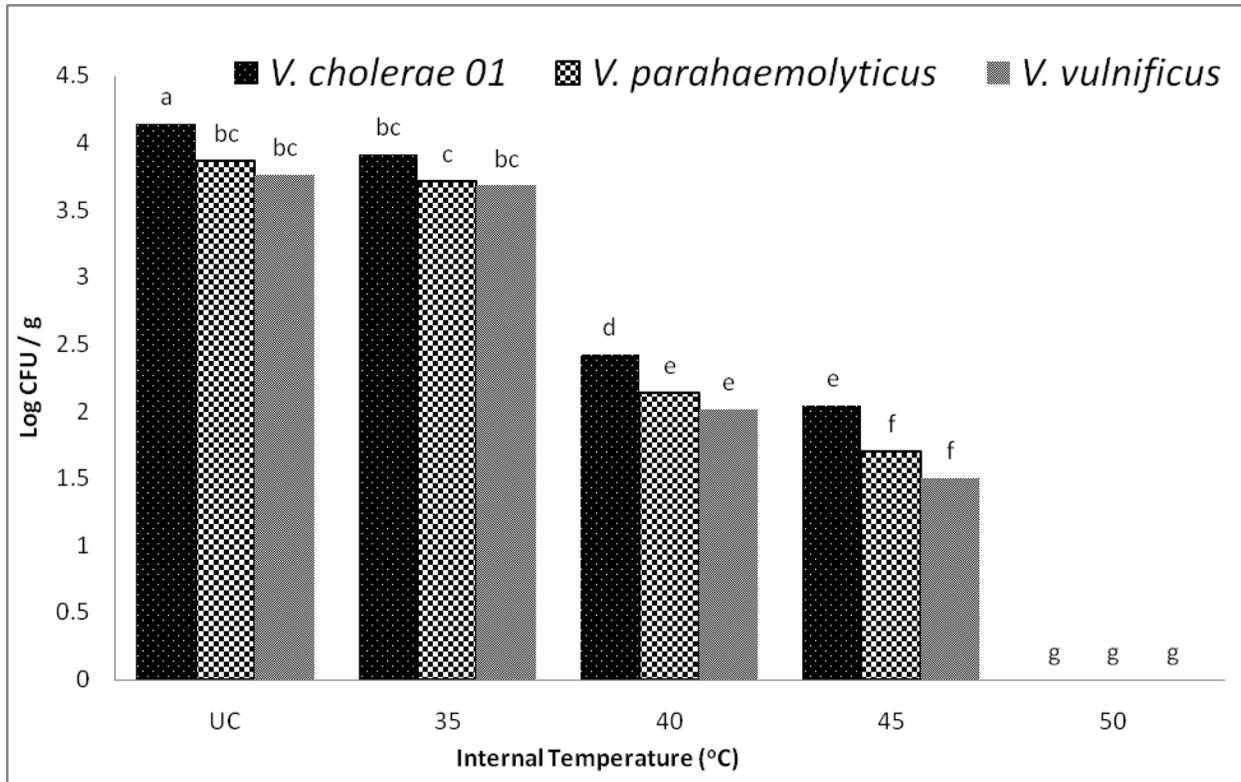


Fig.1c. Thermal resistance of *V. vulnificus*, *V. parahaemolyticus* and *V. cholerae* 01 at different internal temperatures when subjected to boiling after storing at 3°C for 2 days. UC = Uncooked shrimp sample. Data presented in the bar diagram is the mean of three different experiments and the bars with same data letters are not significantly different from each other. ($p < 0.05$)

On day 0 the plate counts for *S. enteritidis*, *S. infantis* and *S. typhimurium* inoculated shrimp stored at 3°C were 5.87, 5.86 and 5.58 Log CFU/g.

Shrimp samples boiled to an internal temperature of 65°C had 1.68, 0.22 and 0.99 Log CFU/g for *S. enteritidis*, *S. infantis* and *S. typhimurium*, respectively. At 70°C *S. infantis* and *S. typhimurium* were reduced to non-detectable levels whereas an internal temperature of 75°C was required to reduce all the three serovars to non-detectable levels (Fig.2a).

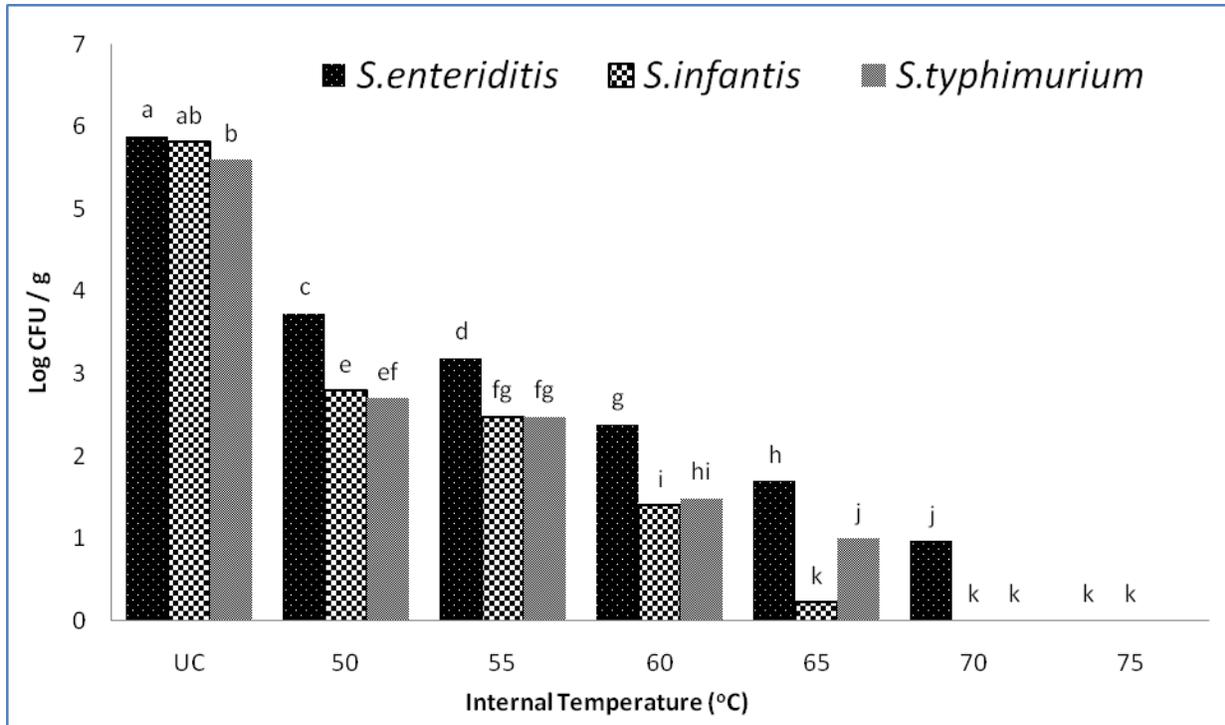


Fig. 2a. Thermal resistance of *S. enteritidis*, *S. infantis* and *S. typhimurium* at different internal temperatures in shrimp when subjected to boiling on Day 0. UC= Uncooked. Data presented in the bar diagram is the mean of three different experiments and the bars with same data letters are not significantly different from each other. ($p < 0.05$)

The inoculated shrimp samples stored at 3°C for one day had counts of 5.55, 5.53 and 5.37 Log CFU/g for *S. enteritidis*, *S. infantis* and *S. typhimurium* respectively. On Day 1 Shrimp with an internal temperature of 60°C had significant reduction of all three serovars with 1.11, 0.12 and 1.12 Log CFU/g for *S. enteritidis*, *S. infantis* and *S. typhimurium*, respectively. An internal temperature of 65°C was lethal enough to reduce *S. infantis* to non-detectable levels and at 70°C all the three species were reduced to non-detectable levels on Day 1(Fig.2b).

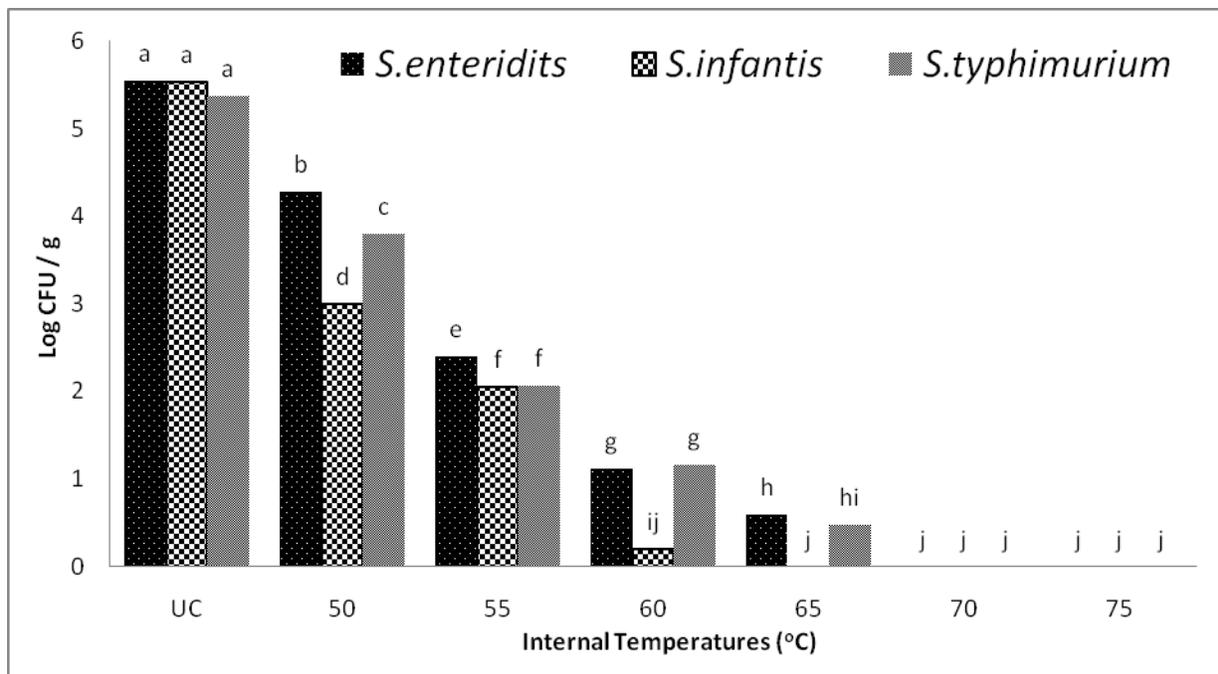


Fig. 2b. Thermal resistance of *S. enteritidis*, *S. infantis* and *S. typhimurium* at different internal temperatures in shrimp when subjected to boiling after storing at 3°C for 1 day. UC= Uncooked. Data presented in the bar diagram is the mean of three different experiments and the bars with same data letters are not significantly different from each other. ($p < 0.05$)

There was reduction of 0.61, 0.72 and 0.39 Log CFU/g for *S. enteritidis*, *S. infantis* and *S. typhimurium* respectively after 2 days of storage at 3°C (Fig.2c).

On Day 2 the inoculated shrimp samples had significant reductions in bacterial counts at internal temperatures of 50°C to 60°C but at 65°C there were only 0.29 Log CFU/g of *S. enteritidis* while *S. infantis* and *S. typhimurium* had reached to non-detectable levels and at 70°C all the serovars were at non-detectable levels.

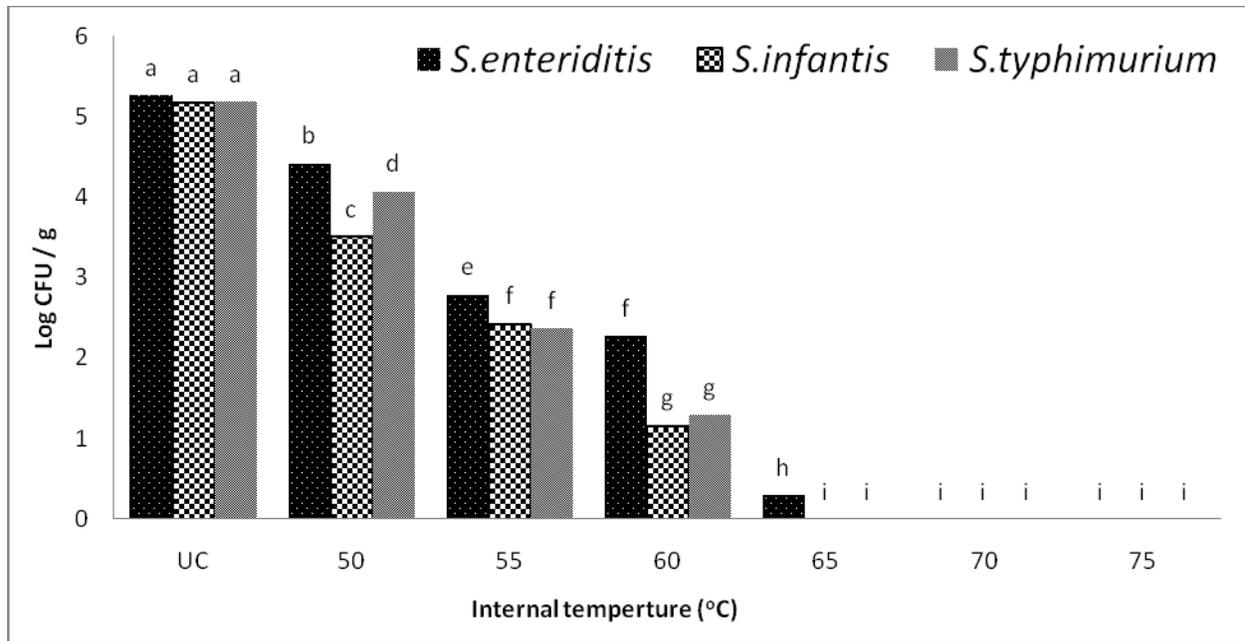


Fig. 2c. Thermal resistance of *S. enteritidis*, *S. infantis* and *S. typhimurium* at different internal temperatures in shrimp when subjected to boiling after storing at 3°C for 2 days. UC= Uncooked. Data presented in the bar diagram is the mean of three different experiments and the bars with same data letters are not significantly different from each other. ($p < 0.05$)

The three *Listeria* species used in this study showed divergent responses when subjected to different cooking temperatures. *L. monocytogenes*, *L. innocua*, *L. welshmeri* showed distinct patterns of survival when inoculated on the surface of the shrimp.

On day zero there was no significant difference in the initial number of each bacteria that were inoculated onto the surface of shrimp samples with 5.65, 5.69 and 5.70 log CFU/g of *L. monocytogenes*, *L. innocua* and *L. welshmeri*. When the shrimp samples were subjected to different internal temperatures, there were no notable differences observed regarding the thermal resistances showed by the three species of *Listeria* at internal temperatures of 65°C and 70°C. But, at 75°C there was a significant decrease in the level of *L. welshimeri* on the surface of the shrimp that was reduced to non-detectable levels where as *L. innocua* and *L. monocytogenes* had counts of 1.12 and 2.23 Log CFU/g respectively. All the three *Listeria* species were reduced to non-detectable levels when the shrimp samples were cooked to an internal temperature of 80°C

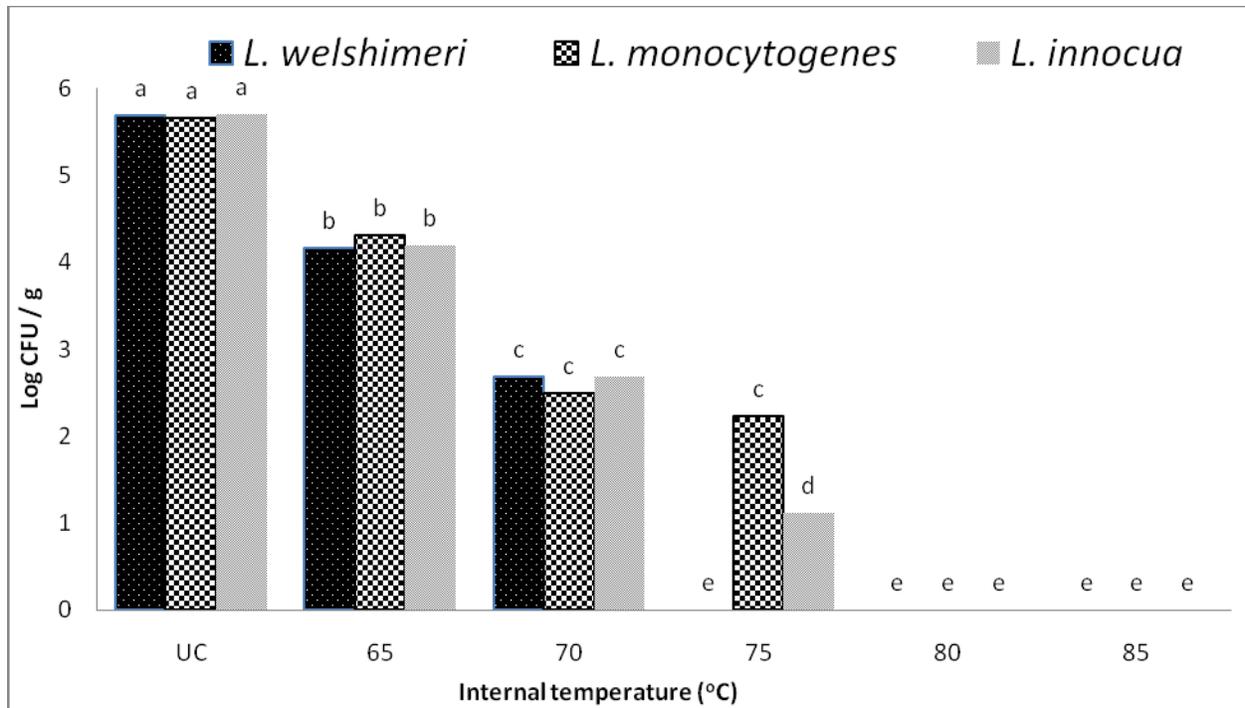


Fig.3a. Thermal Resistance of *L. monocytogenes*, *L. innocua* and *L. welshimeri* at different internal temperatures in shrimp when subjected to boiling on Day 0. UC = Uncooked. . Data presented in the bar diagram is the mean of three different experiments and the bars with same data letters are not significantly different from each other. ($p < 0.05$)

Shrimp samples stored at 3°C for one day had counts for *L. welshimeri*, *L. monocytogenes* and *L. innocua* of with about 5.64, 5.61 and 5.68 log CFU/g, respectively (Fig 2).

On day 1 when the shrimp samples were boiled to achieve an internal temperature of 65°C *L. monocytogenes*, *L. innocua* and *L. welshimeri* showed almost similar pattern of thermal resistance with a reduction of 1.34, 1.51 and 1.52 log CFU/g respectively. At 75°C *L. welshimeri* was reduced to non-detectable levels and in shrimp with an internal temperature of 80°C *L. monocytogenes* had counts of 1.8 log CFU/g while the other two species were reduced to non-detectable levels. All the three species were reduced to non-detectable levels when the internal temperature of the shrimp samples was 85°C.

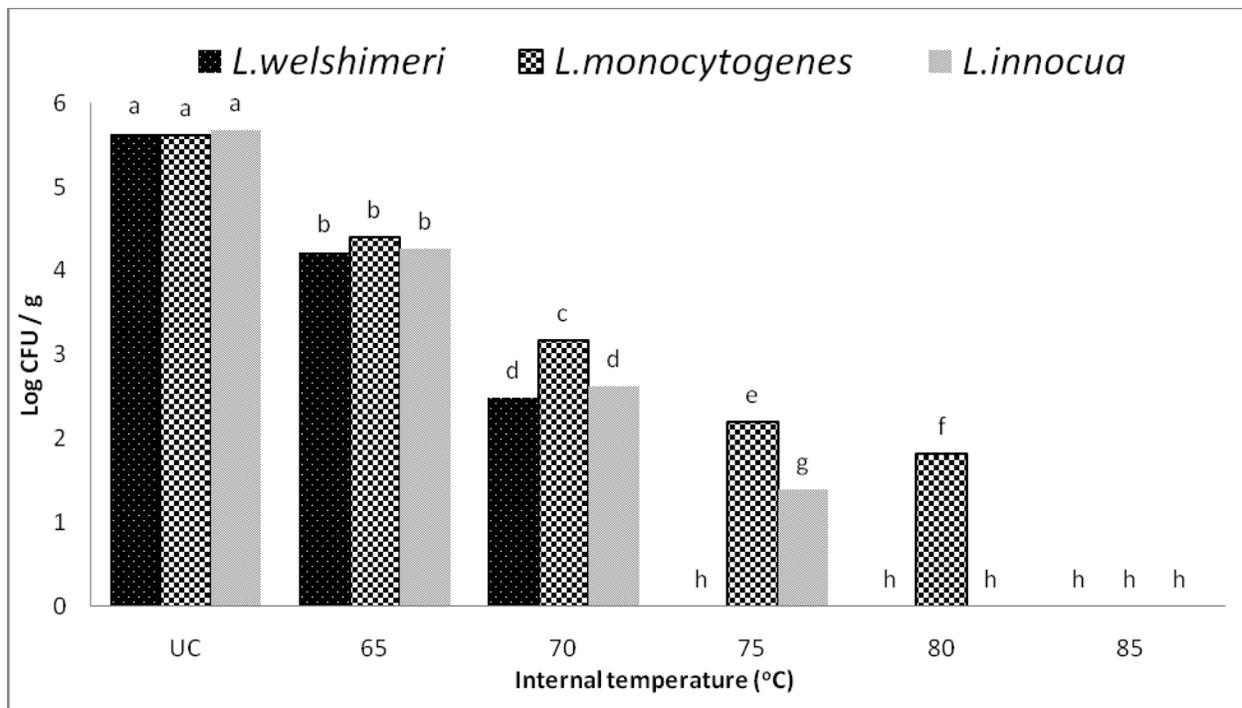


Fig.3b. Thermal Resistance of *L. monocytogenes*, *L. innocua* and *L. welshimeri* at different internal temperatures in shrimp when subjected to boiling after storing at 3°C for 1 day. UC= Uncooked shrimp sample. Data presented in the bar diagram is the mean of three different experiments and the bars with same data letters are not significantly different from each other. (p<0.05)

The bacterial counts of *L. monocytogenes*, *L. innocua* and *L. welshimeri* on day 2 when stored at a temperature of 3°C were 5.61, 5.63 and 5.64 log CFU/g respectively.

All the three *Listeria* species behaved similarly when cooked to an internal temperature of 65°C (Fig.3). At 70°C *L. welshimeri* and *L. innocua* had plate counts of 2.65 and 2.7 log CFU/g but the level of *L. monocytogenes* was significantly higher with about 3.2 log CFU/g. On day 2 *L. welshimeri* and *L. innocua* were reduced to non-detectable levels at 80°C but *L. monocytogenes* had 1.06 log CFU/g and at 85°C all the three *Listeria* species were reduced to non-detectable levels.

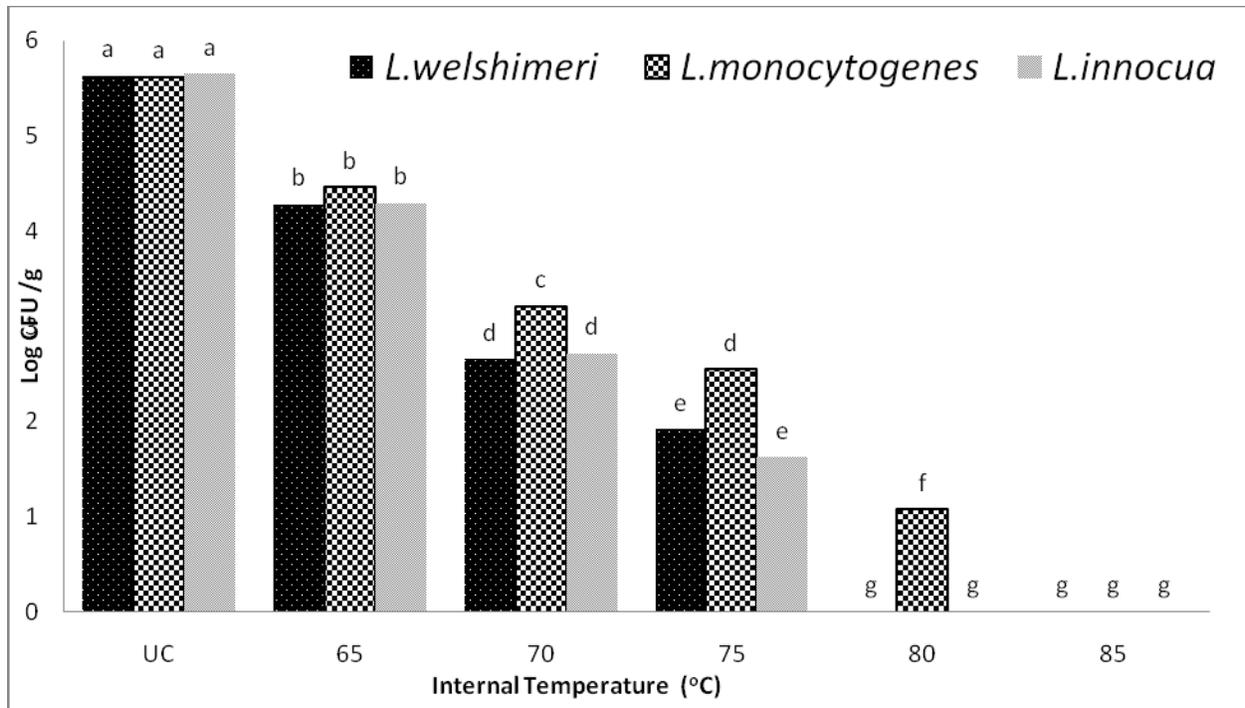


Fig.3c. Thermal resistance of *L. monocytogenes*, *L. innocua* and *L. welshimeri* at different internal temperatures when subjected to boiling in shrimp after storing at 3°C for 2 days. UC = Uncooked shrimp sample. Data presented in the bar diagram is the mean of three different experiments and the bars with same data letters are not significantly different from each other. (p<0.05)

The results from our verification studies showed that none of the samples were positive for the *Listeria species* when boiled to reach an internal temperature of 85°C and similarly none of the samples were positive for *Salmonella species* when boiled to reach an internal temperature of 75°C. But our results showed that 1 out of three replications were positive for *V. cholerae* 01 when boiled to reach an internal temperature of 50°C. However, there were no positive samples at 55°C.

3.3 Discussion

Although the infectious dose for the classical strain of *V. cholerae* has been reported to be 8–9 log CFU/g in water, prior neutralization of gastric acid within the stomach may reduce this to 3–6 log CFU/g (Hornick et al., 1971; WHO Scientific Working Group, 1980). In the current study the initial number of bacteria inoculated on to the surface of shrimp was about 5

log CFU /g. Our results found that bacterial levels were reduced by more than 1 log after two days at 3°C. The data suggests that the pathogen can withstand cold storage temperatures. Comparatively high resistance of *V. cholerae* O1 has been linked to its ability to form colonies on the shell of shrimps. Several studies have demonstrated that once *V. cholerae* O1 has attached to chitin particles or crustacean external surfaces, the microorganism is able to initiate a process of colonization (Castro- Rosas and Escartín, 2002; Huq et al., 1984; Nalin et al., 1979). This process can be associated with an increased resistance to various stresses like temperature but also to those caused by chemical disinfectants (McCarthy and Miller, 1994), low temperatures (Amako et al., 1987), and low pH levels (Nalin et al., 1979). In a research study conducted by Castro- Rosas and Escartín (2005) *V. cholerae* O1 suspended in Isotonic sterile saline solution were inactivated at 70°C for 1 minute, whereas the colonizing cells on shrimp carapaces survived for 1 min at that temperature. Our study found that all the three species were reduced to non-detectable levels when cooked to reach an internal temperature of 50°C. Shultz et al. (1984) also conducted a similar kind of experiment with cockles and concluded that cockles should be cooked until the slowest heating cockles reach 71°C, for 1 minute, the difference in temperature requirements for cockles and shrimp may be due to the differences in sizes and composition.

The optimum temperature for growth of *V. parahaemolyticus* is 35–37°C, while the maximum and the minimal temperature are reported to be 42–44°C and 5°C, respectively, (Beuchat, 1982). Furthermore, this pathogen is reported to be extremely sensitive to thermal inactivation when heated in either laboratory media or shrimp substrates (Beuchat, 1973 ,Covert 1972, Vanderzant,1972). Vanderzant (1972) found a 1-2 log reduction of *V. parahaemolyticus* in shrimp homogenate when stored at 3°C for 1 day and were destroyed after heating at 100°C for one minute. In our study the results showed that *V. parahaemolyticus* inoculated onto shrimp was reduced by 1.2 log CFU/g after storing at 3°C for 2 days.

V. vulnificus was the most susceptible to boiling temperatures in our experiment which reduced it to non-detectable levels at an internal temperature of 45°C on day 0 and day 1 but the temperature requirement to reduce it to non-detectable levels increased to 50°C on day 2. There is little information on the effect of boiling shrimp inoculated with *V. vulnificus*. However, Hesselman et al. (1999) found that *V. vulnificus* levels in oysters (*Crassostrea virginica*) subjected to a commercial heat-shock process, which was 1 to 4 minutes at internal oyster meat temperatures exceeding 50°C, were significantly reduced. The current data also suggests that the *V. vulnificus* have been reduced 1.15 log CFU /g after two days of storage which is not ideal for controlling the pathogen. Similarly, Quevedo and others (2005) also showed that the use of ice immersion as a post-harvest method is not effective in reducing *V. vulnificus* because of the relatively small declines in bacterial load (Ren and Su, 2006).

George et al. (1984) reported that every 12/15 breaded shrimp samples tested in their study had surviving *S. typhimurium* even when fried for 3 min to an internal temperature of 82°C. Our results do not match their findings, which could be due to the difference in the product the breaded shrimp versus whole shrimp and also the medium of cooking which was water in our study. The layer of breading could act as a protective covering. Additionally it has been documented by many studies that heat treatment is the primary method for preserving foods. Several factors, such as growth medium (Annous and Kozempel, 1998; Casadei et al., 1998), growth temperature (Rowan and Anderson, 1998; Martínez et al., 2003), growth phase (Rees et al., 1995; Martínez et al., 2003), water activity (Fernández et al., 2007) and pH (Annous and Kozempel, 1998), may influence the heat resistance of microorganisms. Other mechanisms such as the synthesis of Heat Shock Proteins (HSP) in bacteria could be responsible for this increase in the bacterial heat resistance (Avelino et al., 2008) Therefore, the thermal resistance of bacteria can vary from one type of food or food product to another.

From our results the minimum temperature requirement to kill *S. typhimurium* on day 0 and day 1 was 70°C (Fig 2a&2b). However the requirement decreased to 65°C on day 2. A similar study by Murphy et.al (2001), found chicken patties inoculated with a mixed culture of *Salmonella* serovars then cooked in a convectional oven to an internal temperature of 65°C had no surviving bacterial cells.

Analysis of data obtained from the current study shows that 70°C was the highest temperature required to kill *S. enteritidis* on day 1 and 2 (Fig 2b and 2c) but not on the day of inoculation. Thermal inactivation data for *S. enteritidis* in liquid whole eggs was published by Muriana, Hou, and Singh (1996). Their data shows D-values of 16.5 min at 50°C and 0.7 min at 57.5°C. James et.al (2002) proposed that using these values for interpretation indicate that temperatures of over 70°C for less than 1.5 seconds should be capable of reducing *S. enteritidis* populations by as much as 6 log CFU/g. Another study by Bucher et .al (2008) demonstrated that with adequate cooking (71°C), the *Salmonella* serovars including *enterides* and *typhimurium* can be reduced to non-detectable levels in frozen chicken nuggets/strips. There was little information on the thermal resistance studies of *Salmonella infantis*, but the results (Fig 2b) from our study indicate that this is the most susceptible of all the three species used.

A number of studies have previously investigated the growth properties of different *Listeria* species and strains at different incubation temperatures (Rosenow and Marth, 1987; Junttila et al., 1988; Walker et al., 1990; Barbosa et al., 1994). The current study shows that there is no significant difference in counts of *L. welshimeri*, *L. innocua* and *L. monocytogenes* when stored for 2 days at 3°C. Beuchat et al., (1989) and Johnson et al., (1988) made similar observations about *L. monocytogenes* inoculated into minced beef stored at refrigerated temperatures, in which the bacterial counts remained constant throughout a 14 day sampling period. Nufer et al. (2007) found that *L. monocytogenes* strains displayed better cold stress

tolerance. Although factors controlling *L. monocytogenes* growth at low temperatures are not yet clear there are several genetic and molecular factors that are implicated in the cold stress adaptation of these microbes (Liu et al., 2002; Tasara and Stephan, 2006).

Our study found that *L. monocytogenes* was more thermal resistant as compared to *L. innocua* and *L. welshimeri*. The minimum internal temperature of 80°C was required to reduce a 5.6 log CFU/g of *L. monocytogenes* to non-detectable levels on the surface of shrimp at day 0 where as the internal temperature to destroy the pathogen increased to 85°C on day 1 and day 2 when the inoculated shrimp were stored at 3°C. Murphy et al. (2004) found that cooking *L. monocytogenes* inoculated into ground beef/turkey links in an air impingement oven to an internal temperature of 71°C reduced *L. monocytogenes* counts by 7 log CFU/g. In another study researchers found that increasing the product temperature of chicken breast patties from 55 to 80°C reduced *L. monocytogenes* more than 7 logs (Murphy et al., 1999). The difference in our results could be due to the different composition of the foods such as the shell of the whole shrimp which can act as a barrier to the temperature and increase the cooking time. Shrimp can be cooked by different methods like boiling, grilling, frying or baking but the extent of cooking greatly varies with cuisine and style. Doneness of shrimp is measured by the color and hardness of the tissue, in many cases toughness of the tissue is a non-desirable trait which occurs due to over cooking (Mohan et al., 2006). But, it is very important to ensure that the food is cooked safely along while taking care of the sensory characteristics. Studies by different research groups have found that yield loss and hardness of the tissue significantly increased with an increase in the boiling time (Niamnuay, 2008). Therefore, it is very important to have a simple and unbiased method to monitor the safety of the shrimp while balancing the delicacy and the current study shows that this can be achieved by cooking the shrimp to minimum internal temperatures.

L. innocua was the next most heat resistant bacteria and was able to survive at an internal temperature in shrimp up to 75°C on day 0, 1 and 2. Many studies have documented that heat tolerance of *L. innocua* is much similar to *L. monocytogenes* which makes it an ideal surrogate for *L. monocytogenes* in food safety experiments. Freidly et al. (2008) studied the *D*-values of *L. innocua* and *L. monocytogenes* serotypes which ranged from 3.17 to 0.13 min at 62.5 to 70 °C, and the *z*-values of *L. innocua* and *L. monocytogenes* were 7.44 to 7.73 min. The differences in thermal resistance were not very significant in hamburger patty and they concluded that *L. innocua* has the potential to survive as the primary non-pathogenic surrogate with the greatest margin of safety in verifying a new thermal process to destroy *L. monocytogenes*. Murphy et al. (2001) found that *L. innocua* survived in hamburger patties cooked in an air convection oven even after cooking the patties to an internal temperature of 70°C to 80°C with about 3 to 5 log CFU/g. The same group compared their results with their previous experiments in which chicken meat was cooked in a water bath and reduced *L. innocua* to non-detectable levels at 71.1°C. (Murphy, 1999). Our study found *L. monocytogenes* had significantly higher heat resistance when compared to *L. innocua*. From our results the minimum internal temperature of shrimp required to destroy the *Listeria* species tested was 85°C. Furthermore we found that *L. welshimeri* on the surface of the shrimp was less heat resistant when compared to other two species of *Listeria* tested.

Other factors affecting thermal resistance of *L. monocytogenes* are changes in intrinsic factors, including water activity, pH, and protein and/or fat content of the food product (Lihono et al., 2001). Involuntary exposure of microorganisms or food contaminated with microorganisms to conditions that initiate adaptive stress responses may make reduction of the microorganisms in foods more difficult. *L. monocytogenes* has been shown to induce synthesis of about 12 and 32 proteins upon exposure to cold stress (Bayles 1996, Phan-Thanh, 1995).

Additionally, *L. monocytogenes* has been shown to undergo changes in its membrane fatty acid profile upon long-term exposure to reduced temperatures (Annous 1997).

Any temperature above the optimum growth temperature is supposed to have some lethal effect. However, it has been shown that in most microbial species slow heating or heating for short periods of time at temperatures above the optimum temperature for growth induces higher thermo-tolerance (Mackey & Derrick, 1986). This response consists of the synthesis of various proteins, which are also known as heat shock proteins (HSPs), (Lindquist 1986). This could be one of the reasons for the increased heat tolerance of *L. monocytogenes* in our study. Juneja et al., (2003) found that refrigeration prior to heat treatment has increased the heat tolerance of *L. monocytogenes* which is also in agreement with our findings.

The results from our study suggest that cold storage has significant effect on the reduction of the bacterial levels in shrimp but, it is not enough to control the pathogen and maintain the quality of the product and the shrimp should at least be cooked to 50°C of internal temperature to ensure that *Vibrio* species are reduced to non-detectable levels. But, from our verification tests we found that there is a chance of *V. cholerae* 01 surviving at 50°C. Therefore shrimp should be cooked to at least 55°C of internal temperature to control all the three *Vibrio* species tested. In the current study it has also been found that the thermal resistance of *Salmonella* species was decreased when subjected to cold storage for two days prior to thermal treatment. From our current study we also conclude that in order to bring the three *Salmonella* species tested to non-detectable levels, shrimp should be cooked to an internal temperature of 75°C.

Our results showed that the *V. vulnificus* are the most susceptible while *L. monocytogenes* is the most heat resistant of all the nine species tested in this study. The heat

resistance of Salmonella was decreased by cold storage where as it has increased in Vibrios and Listeria.

It can be recommended that consumers should cook raw shrimp to at least 85°C of internal temperature when refrigerated for up to 2 days prior to cooking to destroy all the nine species of bacteria used in this study including *L. monocytogenes*.

3.4 References

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CHAPTER 4
CONCLUSION

4.1 Conclusions

Well acknowledged is the fact that incidence of foodborne infections and intoxications of microbial aetiology are certainly not diminishing (Bean & Griffin, 1990; Rampling, 1993; Cliver, 1997; Motarjemi & Käferstein, 1997; Stephenson, 1997; Slutsker et al., 1998 and Slutsker, L., Altekruze, S. F. and Swerdlow, D. L., 1998). The efforts made to decrease the incidence of such outbreaks is well documented and many previous research studies have focused on improving the food safety and microbiological quality of food at industrial or pilot scales, but there are only a few studies which focused on the development of food safety guidelines at the consumer level.

In our study Vibrios which are known as temperature sensitive microorganisms also showed notable patterns of survival and resistance to cold storage and thermal treatment. It has been observed that *V. cholerae* 01, was the most heat resistant of all Vibrio species tested and when inoculated on to the surface of the shrimp, they were reduced to non-detectable levels when cooked to reach 55°C of internal temperature of shrimp.

Salmonella species tested by inoculating the shrimp surfaces were found to be more temperature resistant than the three Vibrio species tested in our study, they were reduced to non-detectable levels when cooked to a minimum of 75°C of internal temperature of shrimp. It was also found that the thermal resistance of Salmonella species somewhat decreased with the duration of cold storage. *S. enteritidis* was the most heat resistant of all the salmonella tested.

Listeria showed higher thermal resistance than Vibrios and Salmonella. *L. monocytogenes* was the most resistant of all Listeria species and it was observed that the internal temperature required to kill Listeria increased with duration of storage at 3°C. In order to reduce the Listeria species to non-detectable levels on the surface of the shrimp samples, they need to be cooked to reach a minimum internal temperature of 85°C.

From our study it was found that 85°C of minimum internal temperature will kill *Vibrio*, *Salmonella* and *Listeria* species. It has also been found that cold storage does not decrease the bacterial numbers on shrimp samples significantly. There is a need for further study on the response of the foodborne pathogens in different food products and composition and also to study the effect of cold storage and freezing on the thermal resistance of bacteria. This approach of food safety from consumers point will be of immense help in controlling foodborne outbreaks occurring in the US and across the world.

We hope this study will have implications for consumers enhance food safety at consumer level while serving as a easy and handy guide for cooking the shrimp safely at domestic conditions. This can also help the food processors determine CCP's while designing HACCP at industrial level.

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VITA

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