THE MICROBIOLOGICAL QUALITY OF PACKAGED ICE IN GEORGIA AND THE STRUCTURAL DAMAGE OF *SALMONELLA* SPP. INDUCED BY FREEZE/THAW TREATMENTS

by

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(Under the Direction of Mark A. Harrison)

ABSTRACT

Ice receives little to no attention as a possible source of illness, despite evidence that freezing temperatures do not kill all microorganisms. This study determined the microbiological quality of ice produced and bagged on-premises in retail establishments and in self-service vending machines in the state of Georgia and compared the results with that from ice produced by manufacturing companies that are monitored by the International Packaged Ice Association (IPIA). Assays were used to detect indicator organisms present and membrane filtration technique was used for detection of pathogenic organisms. The presence of microorganisms at unsatisfactory levels in packaged ice indicates the need for improved sanitary practices during the manufacturing and packaging of ice. Further studies determined the injury rate of *Salmonella* spp. induced after freezing inoculated water using selective and nonselective media. The structural damage induced by freezing was observed under the scanning electron microscope.

INDEX WORDS: Water, Ice, Freezing, Injury, *Salmonella*, Scanning electron microscope

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CHAPTER 1

INTRODUCTION

The benefit of various food and water processing treatments is the resulting decrease in the level of spoilage microorganisms and pathogens that are potentially present that can threaten the safety of consumers. Freezing is a well-known treatment for food preservation and a crucial step in food processing, yet the freezing process is not effective for inducing cell death and does not destroy all pathogens present in the food and water (1, 2, 4-8). While their numbers decline, some microorganisms survive freezing and although cells may be injured, the remaining microorganisms may have the ability to recover their viability when warmed (1, 3, 4, 8). Past studies have looked at the survival of microorganisms in ice caused by various routes of contamination, like from unhygienic handling of commercial ice, and the possibility for resulting in illness (1, 2). With the growing popularity of purchasing packaged ice from retail establishments and self-service vending machines, the likelihood of finding contaminated ice is a possibility.

This thesis is separated into 2 objectives, both focusing on the survival of microorganisms in ice:

1. To survey the microbiological and chemical quality of ice produced and bagged on-premises in retail establishments and in self-service vending machines in the state of Georgia and compare the results with that from ice produced by manufacturing companies that are monitored by the International Packaged Ice Association (IPIA);

2. To determine the injury rate of *Salmonella* spp. induced after freezing inoculated water for different time intervals and to observe structural damage of the frozen *Salmonella* by scanning electron microscopy (SEM).

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CHAPTER TWO

LITERATURE REVIEW

Drinking Water Quality

Water is the main chemical component in the human body, making up approximately 55-78% of the body weight (26). It functions as a means to flush toxins out of vital organs, to carry nutrients to cells and provides a moist environment for ear, nose and throat tissues (26). However, the body loses a great deal of water every day through breathing, perspiration, urination, and bowel movements. This water must be replaced through beverages and foods in order to avoid dehydration, which could impair the body from carrying out normal functions. According to The Institute of Medicine, the adequate intake (AI) for men is around 13 cups of total beverages a day, and for women, it is about 9 cups of beverages a day (26). Generally, the 'at least eight 8-ounce glasses of water a day' is the rule of thumb followed by most individuals and is recommended by dietitians. According to the American Water Works Association, approximately 3.9 trillion gallons of water are consumed in the United States per month (26).

All of the distribution and consumption of water originating from public water systems in the U.S. is overseen and regulated by the Environmental Protection Agency (EPA) whose mission is to protect human health and the environment. In 1974, a federal law, the Safe Drinking Water Act (SDWA), was passed by Congress to protect the consumers by regulating the nation's public drinking water supply and to ensure its safety and quality (7). Under the SDWA, the EPA oversees that states, counties, and suppliers implement the required standards

for drinking water quality (7). There are a number of threats that could contaminate the public's drinking water, such as improperly disposed chemicals and animal and human wastes, as well as inadequate water treatment and improperly maintained distribution system (6, 7). The SDWA works to ensure the quality of drinking water by protecting it from source to consumption. The EPA has set national standards for drinking water based on science and research, establishing the National Primary Drinking Water Regulations which sets mandatory water quality standards with maximum contamination levels (MCLs) for particular contaminants allowable in drinking water (6). Their purpose is to protect the public against the consumption of water contaminates that put the consumer at risk for illness. These MCLs include various limits on several microorganisms, disinfectants, and chemicals. Additionally, the National Secondary Drinking Water Regulations were established for non-mandatory water quality standards for fifteen other contaminants, listing them as secondary maximum contaminant levels (SMCLs) (6). These were put in place as guidelines to assist public water systems in managing their drinking water for subjective quality, such as taste, color, and odor, and are not considered to present any risk to consumers.

The National Primary and Secondary Drinking Water Regulations are legally enforced by the EPA and they have set several quality standards for the public water systems. These standards exist to protect public health by limiting the levels of contaminants in drinking water. Tables 2.1 and 2.2 exhibit a few standards that touch on the microbial and chemical quality that are required by the EPA. It is expected that no coliforms or fecal coliforms, such as *E. coli*, as well as pathogenic bacteria are present in the water system that could cause illness to consumers (6). As mentioned above, it is important to test for these criterions frequently to guarantee the continuing safety of our drinking water. Table 2.2 shows the criteria for some key

physicochemical qualities that were set by the EPA. These factors do not necessarily threaten the safety of the water but do affect the aesthetic qualities of the water. For an example, a pH that is too high will give the water a metallic taste and strong odors, which could be a sign of contamination, and would cause the water to be undesirable (6).

The World Health Organization states that drinking water should be suitable for human consumption and all purposes, including personal hygiene (76). It should be safe for use for consumers in all different stages of life, from infant to elders to immune-compromised individuals. Water should be free from solids, bacteria, flavors and odors and minerals should be dissolved at the lowest possible level (76). No pathogenic bacteria, such as *E. coli* O157:H7 or *Salmonella*, should be present that could remain viable during the freezing process and storage of water and ice (24, 76). Water must also be examined regularly and frequently because pollution and contamination is often intermittent and may not be detected if not done on a regular basis with a sufficient sample volume (22, 76). Testing should examine the water quality based on simple tests for fecal indicator bacteria, which will be described in a later section, rather than specific tests for pathogenic bacteria

Ice as Food

Ice is water frozen into a solid state when temperatures drop below zero degrees Celsius. Despite its simplicity, ice functionality ranges from food perseveration to cooling a beverage that is to be consumed. Centuries ago, before ice was recognized as a simple method to keeping food from spoiling, food was preserved through salting, spicing, pickling, or smoking, all which are time consuming and alters the quality and taste of the product. Meat was slaughtered only for the day's trade. Dairy products, fresh fruits and vegetables were subject to spoilage if not sold in the local markets within an appropriate time period, and milk was often pulled to the city

markets at night when the temperatures were cooler (62). All of these methods were eventually deemed impractical with the growing population and demand in the food supply, so harvesting of ice became the solution. Before the invention of artificial refrigeration, where possible, ice was harvested every winter from ice storms and was stored in large ice houses. The ice was sold to shippers of fresh fish, meat, and produce for train deliveries to the city (62). However, by 1868, the demand for ice was high and with the variable supply and availability. The Louisiana Ice Manufacturing Company appeared as the first operation to produce and sell 'artificial' ice (62). By 1878, the company expanded into multiple facilities, quickly expanding the ice industry. By 1925, factory-made ice had entered the realm of industry, and natural ice had become a thing of the past (62). As ice became plentiful and shortage was no longer a concern, the culinary world took advantage of its cooling qualities with the discovery of chilled and frozen desserts. As an example, in 1914, L.H. Larned published one of the first cookbooks that was devoted to the new ice-centered genre, entitled *One Hundred Cold Desserts*, where her directions included putting the dessert "on ice" (62). In 1904, at the World's Fair in St. Louis, MO, a tea plantation owner R. Blechynden, wanted to give away samples of his hot tea, but the weather was hot and with few interested visitors, he poured ice into his tea to cool the beverage and iced tea became a hit (23). As time continued, there have been more and more uses for ice which has stimulated the expansion of the ice industry and its availability to the public. Today, it has become easy and popular to buy packaged ice in bulk for everyday needs, such as keeping a cooler cold or to cool beverages at a party. Packaged ice is commonly bought directly from retail stores, such as grocery stores and gas stations, and convenient self-service vending machines that are found on the side of road or in the parking lot of a shopping center.

According to the International Packaged Ice Association (IPIA), it is estimated that a total of 2 billion bags of ice are sold each year from retail, wholesale, and vending producers and from that number, 200 million bags are sold from vending machines (33). The U.S. Food and Drug Administration (FDA) estimated that each American buys approximately 4 bags of packaged ice every year, with about 80% of the bags purchased during the summer months (33).

Regulations

Ice is defined as a food by the FDA and the marketing of packaged ice is regulated by the FDA for interstate commerce, just like any other food (1, 25). However, the same regulations for ice that is produced and distributed by manufacturing companies do not cover packaged ice produced and bagged on-premises of retail establishments nor self-service vending machines that are only intrastate sales.

There are a little over 700 commercial ice-making companies in the United States. Approximately 500 of these companies are represented by the International Packaged Ice Association (IPIA) (33). The IPIA is an internationally-known organization whose mission is to provide retailers and consumers with guaranteed safe, high-quality ice and gives accreditation to their members who meet the mandatory safe packaged ice standards (33). All manufacturing companies are required to follow the Good Manufacturing Practices (GMPs) established by the Association of Food and Drug Officials (AFDO) (1, 33). Accredited members of the IPIA go beyond the minimal safety requirements for packaged ice. Nevertheless, for both members and non-members of the IPIA, packaged ice labels must meet FDA food labeling requirements. The labels must list the name and place of business of the manufacturer or distributor of the ice and the net weight of the product must be viewable to the purchaser (33). Since ice is a single ingredient food, packaged ice does not need listing of ingredients nor does it require a nutrition

facts label, unless the package has a nutrient content claim (such as low in sodium). If ice is labeled as being from a specific source, such as spring water or artesian well water, it must be truthfully labeled and not misleading (33). The source water must meet all the requirements for such types of source water, as described in FDA regulations.

All ice manufacturing companies are required to follow the GMPs published by the AFDO because it is a food. Therefore, ice is subjected to the GMPs Regulations for Foods contained in the Code of Federal Regulations, Title 21, Chapter 1, Part 110 (25). The purpose of these regulations is to provide guidelines to uniformly apply GMPs to packaged ice manufacturing and handling operations. These regulations address the facilities where ice is manufactured, the quality of source water, and the sanitary practices of employees during ice production (1, 25). It is stated that ice manufacturers must produce, hold, and transport ice in a clean and sanitary condition, monitor the cleanliness, and hygiene of employees, use properly cleaned and maintained equipment, and use water that is safe and from a reliable source (1, 25). The ice should be tested periodically for the presence of bacteria (every 90 to 120 days) but should be done more frequently when internal conditions do not conform to GMPs guidelines (25). States are granted the option of regulating ice manufacture plants further within their jurisdiction, but most state laws are based on individual state agencies which regulate the food and reaffirm the federal GMP regulations (25, 32). Inspections conducted vary from state-to-state in which manufacturing and handling processes of packaged ice is evaluated for compliance with the GMP regulations and guidelines (25).

The IPIA has adopted its own health and safety standards to control the manufacturing and distribution of packaged ice since there is minimal day-to-day state or federal oversight. These standards are entitled the Packaged Ice Quality Control Standards (PIQCS), which is

based on both GMPs and IPIA's Sanitary Standards and adhering to the PIQCS is a required condition for membership in the IPIA (32, 34). To be accredited by the IPIA, there are specific terms and conditions that are required by each manufacturing company. For new members, the basic PIQCS program includes following the criteria specified by the FDA and EPA for drinking water quality, having a HACCP-based Preventive Food Safety Plan, implementing microbial testing of the finished ice product based on stated frequencies and limits, developing a recall plan, and completing mandatory plant audits by qualified third party auditors (32, 34). The PIQCS-Plus, provides a higher level of safety and quality principles for the other members. To reach this level, the company needs to meet the requirements under the basic PIQCS plan as well as additional standards beyond what is normally expected (32, 34). The extras include the implementation of a sanitation standard operating procedures (SSOP) plan, following the EPA National Primary Drinking Water Regulations, the implementation of a thorough chemical analysis of the finished product, and implementation of a customized and effective HACCP program (32, 34).

The Hazard Analysis Critical Control Point (HACCP) system is a science based tool that identifies and assesses specific hazards and the control systems that focuses on the prevention of these hazards rather than relying on end-product testing (3). The HACCP program enhances food safety by identifying all physical, biological, and physical hazards that are likely to occur during a step of the food production and distribution process (3). This prevention system is usually applied through the food chain and is guided by the scientific evidence that risks to human health are present (3). It requires a multidisciplinary approach that may include expertise in production, microbiology, public health, food technology, environmental health, and quality management (3).

As stated in previous sections, implementing a HACCP plan is not required for non-members but is for the members of the IPIA in order to gain accreditation (32, 34). The PIQCS manual provides guidelines for developing and implementing a plan and provides the essential framework of a HACCP plan (32, 34). It would be beneficial for companies to have a plan in place and to identify the critical control points during the manufacturing of ice. A critical control point is a control step during processing that is essential to prevent or eliminate a food safety hazard or reduce it to an acceptable level (3). Figure 2.1 illustrates a basic, generic flow chart of the processing steps that occurs during the production of ice and the most common points in which critical limits would be set for any physical, biological, or chemical hazard that may occur. For an example, the sanitizing step is a critical control point within the process because if certain precautions or control steps are not established and followed, there is a risk for chemical and biological contamination of the water used.

More likely than not, the ice purchased by consumers in a retail establishment was produced, bagged, and handled on-premises of that location. These establishments include convenient stores, gas stations, food service sites, and liquor stores. The FDA does not inspect these small packaged ice producers that make and package ice directly for the consumer nor do they inspect food service establishments that make ice for direct use (e.g., for drinks or cooling food) (1, 47). However, retail food stores and foodservice establishments are subject to regulation by state and local authorities (1, 47). The FDA Food Code is a model code and reference document for state, city, and county agencies that cover restaurants, retail food stores, vending and foodservice operations (25). The code establishes practical, science-based guidance and enforceable requirements for controlling risk factors known to cause foodborne illnesses (25). The regulation of on-site production of ice and the self-service vending machines differ

from state-to-state and so do sanitary standards. Each individual state has an agency that oversees all retail establishments and production and distribution of ice in those establishments (5). In the state of Georgia, the Department of Agriculture is responsible for enforcing state laws, rules, and regulations by conducting sanitation inspections of retail food stores, vending machines, food storage warehouses, etc. (5). These inspections evaluate the manufacturing and handling processes for ice for compliance with the GMP regulations, the same guidelines that are required for larger manufacturing plants (1).

Microbial and Chemical Quality of Packaged Ice

It is stated by the World Health Organization (WHO) that ice to be consumed or come into direct contact with food that is to be consumed is expected to be at the same quality and safety level as drinking water (76). Commercial packaged ice should be safe to consume because it is ingested directly when added to beverages or indirectly when used to refrigerate foods, such as fish and produce. However, several outbreaks have raised awareness on the prevalence of contaminated ice and there are several published reports that have demonstrated the association between contaminated ice and enteric infections.

The Centers for Disease Control and Prevention (CDC) defines an "outbreak" as the occurrence of more cases of disease than normally expected within a specific place or group of people over a given period of time. They have reported several outbreaks over the past several decades where the cause of the illness was contaminated ice. One of the most significant outbreaks was in 1987, which occurred in the states of Delaware and Pennsylvania. Nearly five thousand people who attended four different events between the two states experienced symptoms of nausea, vomiting, cramps, and diarrhea (2). It was determined that the source of illness was caused by contaminated ice that was distributed from a manufacturing company

whose wells and septic tanks were flooded by water from a nearby creek after a heavy rainfall (2). The water was not chlorinated before production resumed. Elimination of this essential control step resulted in water that used to produce the ice with fecal coliform levels above acceptable limits (2). It was determined that the ice was the vehicle of infection. This turned out to be a crucial event that turned the public health's attention to the problems associated with ice manufacturing, distribution, and consumption. However, in 1992, there was a spread of the Norwalk virus infection that affected over a hundred individuals on a cruise ship touring the Hawaiian Islands. The infection was traced back to several ice machines that serviced the dining room that did not have appropriate air-gap devices to prevent sewage backup, causing contamination (16, 42). In 1999, over three hundred people were infected with *Salmonella* Muenchen, with one death, after drinking either bottled or bulk unpasteurized orange juice from restaurants, hotels, and other food establishments in fifteen states. It this particular outbreak, it was found that the imports of orange juice were transported from Mexico to Arizona in a truck that was cooled with ice contaminated with *Salmonella* Muenchen and three other *Salmonella* strains (2, 68). At the time, this was the largest *Salmonella* outbreak associated with unpasteurized orange juice.

There have been several studies conducted on the microbial and chemical quality of packaged ice and the survival of bacterial pathogens that poses a risk to consumer health. Dickens et al. (18) focused on the survival of four common enteropathogens that were frozen in ice and then exposed to various popular drinks. They found that even after being frozen for 24 h and allowed to melt in the beverages, none of the organisms were completely eliminated indicating that ice in drinks should not be disregarded as potential source of infection.

Falcão et al. (24) collected commercial ice, ice used in fish markets, and ice used in street markets to refrigerate fish and seafood in Brazil. It was found that the majority of the ice samples had undesirable hygienic conditions due to the presence of fecal and heterotrophic indicator microorganisms and *Salmonella* Enteritidis was isolated from one of the samples.

Moyer et al. (51) evaluated packaged ice that was sold around Iowa, investigating the microbial and chemical quality of bags from both ice manufacturing plants and also retail establishments and convenience stores. They found that the quality of the packaged ice sold in Iowa reflected the quality of the water source used and the sanitary conditions during the manufacturing of the ice. Ice produced in convenience stores was consistently poorer in terms of microbiological quality than ice produced in by major commercial producers. Schmidt et al. (66) did a similar study in Florida, concentrating on the difference between microbial, physical, and chemical quality of packaged ice that was either made on-premises of retail establishments or off-premises at ice manufacturing plants. They found there was a difference in the quality of ice between the two kinds of bagged ice and concluded that new regulations are needed for the improvement in quality.

These studies demonstrated the importance of manufacturing and handling of ice under strict hygienic conditions as it can be an unsuspected source of nonpathogenic and pathogenic bacteria and may present a public health risk to consumers.

Indicator Organisms

Pathogens are not readily detected in water and ice samples, so nonpathogenic indicator organisms are a fundamental monitoring tool used to evaluate the hygienic condition and quality of water and the possible presence of target pathogens (24, 73). Testing for all pathogens would be too expensive and time consuming and the technology needed to test each pathogen is not

readily available for all labs. Typically, indicator organisms used are coliforms, *E. coli*, enterococci, and aerobic heterotrophic organisms (9, 73). The presence of these organisms reflects the general hygienic status of commercial ice and contamination that may be attributed to contaminated water sources, contact with storage bins and equipment, and staff handling practices (9, 73).

Heterotrophic bacteria, which provide the number of aerobic organotrophic bacteria present, are naturally occurring in water and the use of the heterotrophic plate count (HPC) test, also known as standard plate count, is a typical indicator test for drinking water cleanliness and quality (63). It provides information about the total number of aerobic organotrophic bacteria present and is an indication of the total organic composition of the water (9). The HPC is a useful tool for monitoring the efficiency of the water treatment process, including disinfection, measuring bacterial regrowth or after growth potential in treated drinking-water, and monitoring bacterial population changes following treatment modifications, such as a change in the type of disinfectant used (60, 63, 76). The results of the HPC reflect the general hygiene during ice production and handling (4, 43, 52, 63). The presence of heterotrophic bacteria does not necessarily signify a risk for illness because low numbers are normally found in treated water, but it does give a good indication of sanitary conditions during storage, handling and the efficiency of water treatments from the water source and manufacturing plant (60, 63, 76). According to the WHO, the HPC value is a good indication of effective coagulation, filtration, and disinfection steps during water treatment and is generally used in validation and verification of the pathogen removal during normal treatment plant procedures; however, it has no health significance itself (62, 76). There is no evidence, either from epidemiological studies or from

correlation with occurrence of waterborne pathogens that HPC values alone directly relate to health risk (63, 76).

The standard HPC pour plate is the conventional method used to test for the bacteria and is approved by the EPA (9). However, an enzyme-based assay, called SimPlate®, was developed by Idexx Laboratories, Inc., to determine the Most Probable Number (MPN) of the microorganisms in food and water, and is approved by the Association of Official Analytical Chemists (AOAC) (11, 35). It uses the Multiple Enzyme Technology™ (MET) to detect the HPC in water by using a reagent that contains multiple unique enzyme-substrates, each targeting a different bacterial enzyme (11, 35). All these enzyme-substrates are hydrolyzed by microbial enzymes to release 4-methylumbelliferone, which fluoresces blue under ultraviolet (UV) light after incubation (11, 35).

Several studies have been completed to support that the SimPlate® for HPC testing is strongly equivalent to the standard HPC pour plate method and is a suitable tool for monitoring changes in bacterial water quality over time (11, 30, 35). Studies support that SimPlate® is simple to use, with little preparation, is easy and reliable to read and there is no need for dilutions or duplicate plates, making it ideal to use over standard HPC pour plates (11, 30, 35). The disadvantages to SimPlate®, as pointed out by Beuchat et al. (11) is that it is essentially a MPN technique, so actual numbers of colony-forming units cannot be determined. It is also impossible to distinguish any specific microorganisms that may be predominant in the sample or to isolate a pure colony for identification (11).

Coliforms belong to the family *Enterobacteriaceae* and they are facultative anaerobic, gram-negative, non-sporeforming, rod-shaped bacteria that ferment lactose with gas and acid formation within 48 h at 35°C (9, 21). Some coliforms are natural inhabitants of soil or

vegetation and are seen as generally harmless, whereas fecal coliforms, such as *E. coli*, are bacteria that are found in intestines of warm-blooded animals and are useful as an indicator for fecal contamination (15). Many bacteria in the genera of *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Serratia* meet the criteria for coliforms and are widely found in the environment; however, they are not of fecal origin and do not imply a health risk (22). A high concentration of coliforms in drinking water does not necessarily mean there is fecal contamination, but there is a possibility for the presence of pathogenic enteric bacteria (38). For coliforms, drinking water is not a natural environment and their presence is seen as a possible threat or indicative of water quality deterioration (64). Positive total coliform samples in treated water may suggest ineffective water treatment, loss of disinfectant, intrusion of contaminated water into the potable water supply, or regrowth problems in the distribution system (64, 48). The presence in consumable ice could suggest unsanitary production conditions and improper handling during the production and distribution of ice with poor hygienic practices by workers (51, 52, 64, 66).

Testing for *E. coli* is seen as the optimal choice because it is a good indicator for fecal contamination, and the methods for detecting the organisms are inexpensive, cost effective, simple, sensitive, and specific (22). The most conventional method of detection of coliforms is by most probable number, or the MPN method. The standard methods to detect coliforms in water samples uses multiple-tube fermentation techniques, or membrane filter technique (9, 64). However, it has been found that these methods have limitations, such as long durations of incubation, antagonistic organism interference, lack of specificity, uncertainty in reading results and poor detection of slow-growing or viable but non-culturable microorganisms (VBNC) (71). Over the past decade, enzyme-based methods that can detect both total coliforms and *E. coli* have gained popularity and are widely accepted as the standard for the analysis of water, mainly

due to rapid detection and easy-to-read results (37, 53). These tests are based on the detection of the enzyme β-D-galactosidase and β-D-glucuronidase, which is produced by coliforms and *E. coli*, respectively, within 48 hours when incubated at 35°C (9, 37, 53). An example of the common, EPA-approved, enzyme-based method, is the Colisure® assay (Idexx Laboratories, Inc., Westbrook, ME), which is used to detect the presence of total coliforms as well as *E. coli*. Colisure® uses CPRG (chlorophenol red β-D-galactopyranoside) and MUG (4-methyl-umbelliferyl-β-D-glucuronide) as nutrient indicators that are cleaved by the enzymes β-D-galactosidase and β-D-glucuronidase, respectively (15, 53). The cleaving causes the water sample to change from yellow to a red or magenta color in the presence of coliforms. Presence of *E. coli* causes the sample to fluoresce blue when exposed under an UV light (15, 53). The resulting fluorescing color is due to *E. coli* metabolizing the MUG indicator. This method is also known as the Defined Substrate Technology® (DST) (15, 53).

Several studies have compared the conventional coliform and *E. coli* detection methods to these enzyme-based assays to determine consistencies and validation. The advantages to using assays, such as the Colisure® assay, includes the ability to detect total coliforms and *E. coli,* directly from drinking water and other from varied sources of water. Therefore, it is faster, less expensive, and said to have an improved accuracy (22, 64). McFeters et al. (49) determined that these tests also provide a more realistic estimate of the actual population of indicator bacteria in the water supply. It has also been concluded that there is no significant difference in the recovery for the enumeration of *E. coli* between the Colisure® assay and the membrane filtration technique, while the total coliform recovery was greater using Colisure® assay (64). However, it has also been recommended that one should not be dependent on the results of these assays. It was found that Colisure® is sensitive to background bacteria, such as heterotrophs.

High levels of *Aeromonas* species and *Pseudomonas*, noncoliform bacteria, may give a false positive because they also produce low levels of β-D-galactosidase (53). However, it is argued that these bacteria are usually suppressed and generally will not produce a positive response unless there is more than 10^4 cfu/ml present (9). Heterotrophs were also looked at as suppressing coliform growth and giving a false negative response (22). Overall, it is recommended by many that precise species identification be done when total coliforms or *E. coli* are found to determine the potential health risks posed by the water supply (9, 22, 64).

The *Enterococcus* group of organisms is a subgroup of the fecal streptococci, which includes *S. faecalis* and *S. faecium* (21). These facultative anaerobic organisms are gram positive cocci and are common commensal organisms found in the intestines of humans. This subgroup is a valuable indicator for determining the extent of fecal contamination in water and is thought to be the most efficient bacterial indicator of water quality (9). Enterococci are approximately 100 to 1000-fold less numerous than *E. coli* but have about the same lifespan as *E. coli*. There are a number of methods that are both sensitive and specific for enterococci, including the MPN and membrane filter techniques (9). There is also the enzyme-based assay that uses Defined Substrate Technology® (DST), Enteroelert® by Idexx Laboratories, Inc. This method is essentially the same as the rapid detection test for total coliforms and *E. coli,* with using a nutrient-indicator that fluoresces when metabolized by the enterococci (53). The enterococci produce the enzyme β-glucosidase, which metabolizes MUG, the same indicator used in the detection of *E. coli.* A blue fluorescence indicates a positive result (53).

EPA studies have indicated that both enterococci and *E. coli* are both effective indicators for predicting the presence of gastrointestinal illness causing pathogens in drinking water. However, testing for enterococci should be in addition to testing for *E. coli* and not a

replacement (22, 73). This is because *E. coli* is a better indication of the quality of drinking water whereas enterococci, which survives better in high salt levels, is better for marine water. The disadvantage of including this additional test is the increased cost required (22, 73).

Pathogenic Bacteria in Water

Salmonella spp. are gram-negative, rod-shaped, facultative anaerobes that belong to the *Enterobacteriaceae* family (21). These pathogenic bacteria can cause infections if ingested, resulting in symptoms of diarrhea, fever, vomiting, and abdominal cramps, lasting 4 to 7 days (21). Most cases pass by without treatment; however, the CDC estimates that approximately 400 people die each year in the U.S. from *Salmonella* infections. They are the leading cause for hospitalizations in regards to foodborne illnesses, with about 10% of these infections being waterborne (2). *Salmonella* contamination in the food industry, from processing plants to retail establishments, may be due to their natural presence in the environment and in raw ingredients and water. This pathogen has the ability to survive in various environments and is known to survive in water and ice for weeks. However, the purpose of drinking water and wastewater treatment is to reduce the numbers of viable organisms to acceptable levels and to remove or inactivate all pathogens (9). Water-contamination and transmission of *Salmonella* spp. can be the result of malfunctioning sanitary process systems during water treatment, cross-contamination during food processing, or unhygienic practices (22). The isolation of *S.* Enteritidis from commercial ice in a survey published by Falãco et al. (24) demonstrates the ability of *Salmonella* to be transferred through water from source contaminant and survive the extreme freezing temperatures.

Listeria belongs to the *Clostridium* family and *L. monocytogenes* is one six species in the genus (21). It is a gram-positive, non-sporeforming, facultative anaerobic bacterium that is

capable of surviving in the presence of oxygen, at low water activity levels, cold temperatures, and a high salt environment (21). *Listeria* has been isolated from surface waters (rivers and lakes), soil, feces, plant material and vegetables as well as feed and sewage (13, 65). It has also gained attention because of its persistence in food processing environments and the cross-contamination risk when processing ready-to-eat foods (21). *L. monocytogenes* has emerged to be a major foodborne pathogen with outbreaks traced back to ready-to-eat foods (especially deli meats), unpasteurized milk and other dairy products (21). Budzinska et al. (13) showed water can play an important role in the transmission of *Listeria* because of its ability to survive in a water environment at low temperatures. The adaption of *Listeria* at low temperatures, even below 0°C, results in the slowing of all their vital processes (65). This results in the cells showing more effective resistance to harsh environments, allowing them to survive longer in water. This could result in the contamination of drinking water in treatment plants and food establishments if proper sanitation and disinfection is not used. However, at present, there is no epidemiological data suggesting the occurrence of human infection from water contaminated with *L. monocytogenes* (65). It is seen as unlikely that this pathogen will reach numbers in contaminated water that will directly cause infection, but could potentially cause indirect infection from fish or seafood from contaminated waters, such as lakes and streams (65).

When testing water for pathogens, it is generally recommended to test for indicator organisms first or simultaneously because the presence of pathogens is sporadic and the survival times in the environment are variable (9). Although no single indicator provides assurance that water is free of pathogens, the monitoring of the indicators assumes the relationship of the indicators to the pathogens (9). For example, the presence of total and fecal coliforms and *E.*

coli correlate with bacterial enteric pathogens, and it is rare to find these pathogens in the absence of fecal contamination (9).

There is no one best standard method for *Salmonella* and *L. monocytogenes* detection in water. The occurrence of these pathogens in water is highly variable and usually in low numbers, so there are several limitations in the sensitivity and selectivity in the accepted procedures (9). When using only standard selective plate count for detection, the method loses some of its value for cells may not be present in the small sample pipetted onto the plate. A common detection method that is recommended for water sampling is the membrane filter (MF) technique, which is approved by the EPA. The filtration is used to concentrate and retain all the bacteria cells contained in any quantity of water before culturing for the specific pathogen (17, 65). Water is filtered through a membrane with 0.45-µm pores that traps the cells in the suspension. As recommended by *The Standard Methods for the Examination of Water and Wastewater* (9) as well as Watkins et al. (24) filtration is combined with enrichment and then sub-cultured onto selective media, as this will give better recovery of the pathogens present. Other common methods include the most probable number (MPN) and also the presence/absence test with selective media. Some advantages of using the MF technique includes that it is less labor intensive, requires less culture media and glassware, and better accuracy with the ability to use a higher sample volume, which is the key benefit over using spread plates (76). The disadvantages of using the MF technique over the MPN method includes that it is a less sensitive method, it is not applicable for turbid water samples, and it can be costly for some regions and laboratories (76). It was also found by Ryser and Marth that the recovery of injured cells is limited, especially with high levels of competing cells in the sample (65).

Cell Injury and Recovery After Freeze Damage

Several studies and past outbreaks have demonstrated that pathogens have the ability to survive in ice for extended periods and cause infections in humans. However, it is possible for bacteria to sustain damage within the cell, causing injury and temporarily inhibiting its ability to cause infection.

Bacteria injury can be defined as a sub-lethal injury to a microorganism that implies structural damage and the temporary or permanent loss of function (30, 28, 74). Injury could be caused by chemical or physical treatment on an organism and occurs during food processing as means to control microorganisms in foods. Stresses that could cause injury to cells include intrinsic factors such as water activity, pH, and competitive cultures, as well as extrinsic factors such as exposure to acid, heat, cold, starvation, and oxidants (8, 50, 74). Structural injury is defined as the inability for the cell to proliferate or survive on selective media, which is usually due to damaged cell wall or membrane permeability (12, 74). Many studies have documented morphological changes in cellular shape of injured *Salmonella*, *E. coli*, and *Listeria* (45, 46, 48, 74). Metabolic injury due to low or freezing temperatures results in damage to various functional components of the cell, such as the synthesis of essential cellular proteins (70). These metabolic injured cells are described by their inability to grow on minimal media without any selective agent that could inhibit its growth (13, 28, 74)

Sensitivity of bacteria to low and freezing temperatures vary greatly from organism to organism and is based on population density, cooling rate, and environmental medium (water presents a more stressful environment than broth and food matrices) (44, 55, 74). Looking more closely, freeze injury results from continued exposure to concentrated solutes and physical damage caused by ice crystal formation; however, it is generally accepted that freezing is

ineffective in microbial inactivation (44, 74). It has been determined that severely injured, yet metabolically active, foodborne bacteria can enter a viable-but-nonculturable (VBNC) state and still maintain their pathogenicity (54, 56, 69, 74).

Under the appropriate and ideal environment, such as the availability of nutrients or exposure to optimal temperature or relative humidity, many injured cells will repair and regain the characteristics of normal cells and in addition, exhibit increased stress tolerance as a result of stress adaption (15, 58, 59, 74, 77). Many bacteria, including, *S*. Enteritidis, respond to abrupt changes in temperature by synthesizing the protective cold shock proteins (CSPs) which facilitate in maintaining various cellular and physiological functions in the cell and promotes cell repair in response to the stress (30, 74).

To assess the microbial cell damage due to freezing, the differential plating method is often used and most preferred. This method uses a selective medium containing a substance that inhibits growth of injured cells and only allows healthy cell growth and a nonselective medium with no inhibition for stressed cells that enumerates the entire viable population. The difference in plate counts between selective and nonselective media is used to quantify sublethal injury as a result from a stress-causing treatment (10, 59, 60, 66, 70, 74, 77).

Ray et al. (70) studied repair of injury induced by freezing *S.* Anatum using xylose-lysine-peptone-agar (XLP) and XLP with sodium deoxycholate added (XLDP) as the nonselective and selective media, respectively. After treatment, the number of healthy cells was determined by the growth on the XLDP plates and the number of injured cells was determined by calculating the difference in the number of colonies between the XLP and XLDP plates. This method in detecting injured cells is important because failure to detect stressed but viable cells, whether they are pathogenic or indicator organisms, in natural waters is a problem because as

many as 90% of microbial cells present may not be detected on a selective medium, such as the XLDP medium used by Ray et al. (70). This could prevent accurate detection of microorganisms in the water samples and could be a potential threat to public health.

The presence of injured microorganisms in food and water poses significant public health concerns because they can go undetected during routine quality control checks during processing (74). When an injured bacterial cell is provided with an ideal, nutritional environment, it can undergo cellular repair, eventually allowing for growth and potential spoilage and production of toxins and other virulence factors (37, 74). It has been shown that both injured indicator organisms and pathogens, when ingested through water or food, can survive in the stomach and the small intestines, and can resuscitate, multiply, and become virulent, causing illness (59).

Scanning Electron Microscope

The scanning electron microscope (SEM) was first commercially introduced in the mid-1960s and is considered a significant advancement to the field of microscopy because it has become a useful tool to investigate surface and intracellular details of plant and animal tissues as well as microorganisms (27, 41). Since then, there has been a rapid progression in both the methodology of sample preparation and in the instrument design. This has improved the use of this microscope and allowed for a wider application in biology and biomedical areas.

The SEM produces micrographic images by a beam of electrons being scanned over the surface of a pattern and images are constructed on a point-by-point basis on the face of the cathode ray tube (28). The electrons in a SEM are emitted by the heating of a tungsten filament (the cathode) that is within the gun in the upper portion of the microscope. As a result, this electron gun produces an electron source (41). The electromagnetic condenser lenses, which operate in a vacuum, accelerate the electrons and helps create a small, focused electron probe on

the specimen (41). This electron beam can typically interact with the specimen to a depth of approximately 1 μm. In order to produce images, the electron beam is focused into a fine probe, which is then scanned across the surface of the specimen with the help of scan coils (41). Every point on the specimen that is struck by the beam of electrons emits a signal in the form of electromagnetic radiation (41). Selected portions of this radiation are collected by a detector and the resulting signal is amplified and displayed on a viewing monitor (41).

The scanning electron microscope is widely used in environmental microbiology because of its ability to characterize surface structure, to measure cell attachment, and changes in morphology of bacteria due to adaption or stress (40). The SEM can reveal topographical details of a surface with clarity and great detail that cannot be seen with any other type of microscope, making it ideal to use with identifying microorganisms and injured cells (29). It can also detect surface potential distributions, subsurface conductivity, surface luminescence, surface composition, and crystallography (29). The SEM has several characteristics that make it ideal for use over other common microscopes, such as light microscopes (LM) and transmission electron microscope (TEM). Unlike the 3-D images that can be provided by SEM, LM and TEM can only produce 2-D images as they can only focus on one plane so the depth of field is limited (41). With the LM, only shapes of the cells can be identified at low magnifications. Other advantages that SEM may have over TEM and LM include the capability of viewing a large size specimen without limitation to specimen thickness, a broader magnification range, finer resolution, the ease of varying magnification without changing focal length so that the depth of field remains constant, and more information can be obtained from the specimen with the images produced (40, 41). A disadvantage to using SEM in investigating microorganisms is its inability to provide necessary genetic information about the cell (40). Other disadvantages include the

time consuming preparation methods, multiple preparation steps with several materials needed, and the potential cost of labor and the actual microscope itself (40, 41).

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Table 2.1. Common microbial quality standards for drinking water as set by EPA (6)

Standard	Acceptable Limits
Heterotrophic Plate Count	<500 cfu/ml
Total Coliforms	<1.0 MPN/100ml
E. coli	<1.0 MPN/100ml
Pathogenic Bacteria	None Present

Table 2.2. Common physicochemical quality standards for drinking water as set by EPA (6)

Standard	Acceptable Limits
pH	6.5-8.5
Turbidity	<1 NTU
Nitrate	<10 mg/L
Odor	3 threshold odor number
Color	Colorless

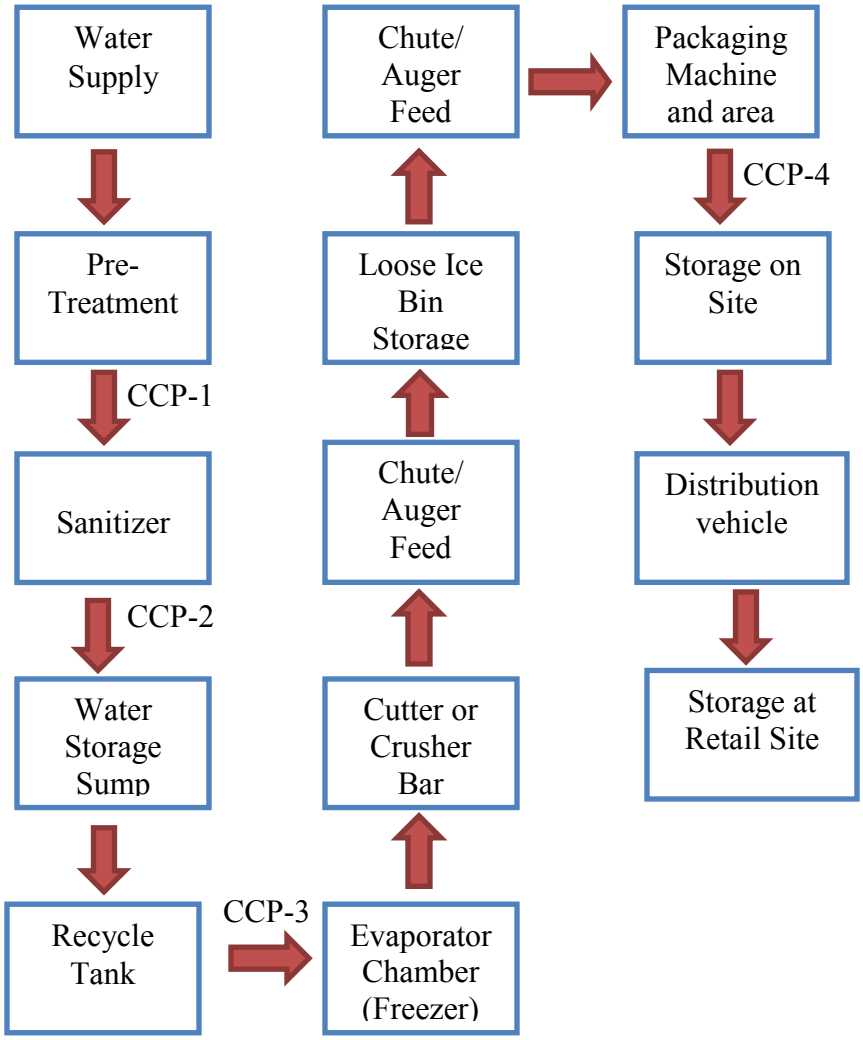

Figure 2.1. Example of an ice manufacturing flow chart (32)

CHAPTER THREE

MICROBIOLOGICAL QUALITY OF ICE MADE AND BAGGED ON-PREMISES IN RETAIL STORES AND IN SELF-SERVICE VENDING MACHINES IN COMPARISON TO MANUFACTURED PRODUCED ICE IN GEORGIA[1]

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ABSTRACT

Ice receives little to no attention as a possible source of illness, despite evidence that freezing temperatures do not kill all microorganisms that can potentially cause harm to consumers. This study determined the microbiological and chemical quality of ice produced and bagged on-premises in retail establishments and in self-service vending machines in the state of Georgia and compared the results with that from ice produced by manufacturing companies monitored by the International Packaged Ice Association (IPIA). Two hundred and fifty bags of packaged ice samples were obtained from retail locations and self-service vending machines, along with 25 bags of packaged manufactured ice. Ice samples were melted within 24 h of collection and HPC SimPlates® were used to detect heterotrophic bacteria present. Colisure® and Enterolert® assays were used to enumerate coliforms, non-pathogenic *E. coli* and enterococci. Membrane filtration coupled with enrichment was used to detect of *Salmonella* and *Listeria monocytogenes*. Confirmation tests were done for presumptive positive pathogens. Six percent of the ice samples bagged at retail sites and from vending machines contained unsatisfactory levels of heterotrophs compared to the limits set by the IPIA (>500 MPN/100ml). Twenty-six percent of all samples contained an unsatisfactory level of coliforms (>2.2 MPN/ml), 1% contained nonpathogenic *E. coli* and 13% contained enterococci (>1.0 MPN/ml). One sample tested positive for the presence of *Salmonella* and another positive for *Enterobacter agglomerans*. The presence of microorganisms at unsatisfactory levels in packaged ice indicates the need for improved sanitary practices during the manufacturing and packaging of ice at on-premises retail locations and vending machines.

INTRODUCTION

Ice is water frozen into the solid state when temperatures drop below 0°C. It has many purposes dating back centuries ago when people used ice for food preservation. Ice is often consumed on its own or mixed with beverages and allowed to melt to cool drinks for refreshment. According to the International Packaged Ice Association (IPIA), it is estimated that a total of 2 billion bags of ice are sold from all retail, wholesale, and vending producers and from those sales, 800 million bags are attributed to retail producers and 200 million bags are from vending machine sales (15). The U.S. Food and Drug Administration (FDA) estimated that each American buys approximately 4 bags of packaged ice every year, with about 80% of the bags purchased during the summer months (6). Overall, the ice industry has increased the production in the past century. There are close to 700 commercial ice-making companies in the United States with approximately 500 of these represented by the IPIA (3, 6).

Ice is defined as a food by the FDA, which regulates packaged ice for interstate commerce (1, 6). The Association of Food and Drug Officials (AFDO) have published Good Manufacturing Practices (GMPs) in order to regulate the sanitary manufacturing of packaged ice (1). The GMP regulations state that ice manufacturers must produce, hold, and transport ice in a clean and sanitary condition, monitor the cleanliness, and hygiene of employees, use properly cleaned and maintained equipment, and use water that is safe and from a reliable source (1, 6). All ice manufacturing companies are required to follow these basic GMPs, but are enforced differently from state to state. There are little to no specific packaged ice processing regulations at the state and federal level (19). The IPIA has published the Packaged Ice Quality Control Standards (PIQCS) manual which is based on GMPs but is tailored specifically to packaged ice (14). The establishment of the PIQCS/PIQCS-Plus Program within the manufacturing company

is required in order to gain membership in the IPIA (14). Although the development and implementation of a Hazard Analysis and Critical Control Point (HACCP) plan is not required by the FDA, the implementation of a HACCP and HACCP pre-requisites are required by IPIA (14).

The World Health Organization (WHO) has stated that ice to be consumed or come into direct contact with food that is to be consumed is expected to be at the same quality and safety level as drinking water (25). The Centers for Disease Control and Prevention (CDC) has reported foodborne illness outbreaks over the past several decades where the cause of the illness was contaminated ice (2). The CDC reports there are over 50,000 cases per year of reported foodborne illnesses where the origin is unknown, but ice is not one of the first food products investigated, if at all, as the source of the illness, even though it is a widely used commodity in retail establishments (2).

Freezing is a well-known method for food preservation and a crucial step in food processing, yet the freezing process does not destroy all pathogens present in the food (4, 11, 17). While their numbers decline, some microorganisms survive freezing and although cells may be injured, the remaining microorganisms have the ability to recover their viability when ice melts (4, 17). Past studies have indicated that the microorganism's capability to recover after frozen storage makes ice an ideal vehicle for transmission of pathogenic bacteria and viruses to food and beverages (4, 17). Kim and Harrison demonstrated that *E. coli* O157:H7 can transfer to lettuce from melted ice made with contaminated water (16). They concluded that ice is a possible route for *E. coli* O157:H7 to lettuce either by direct contact with water from melted contaminated ice or from contaminated lettuce to uncontaminated lettuce with melted ice being the vehicle of transmission (16). The packaging and transportation of food products that are

typically kept on ice, such as produce and fish, are shown to be at risk for contamination if the recommended precautions are not observed.

Another misconception is if pathogenic bacteria are present in ice, they can be killed when the ice is added to beverages of high alcoholic content, with high acidity, and with carbonation, such as soda (7, 10). Dickens et al. tested the survival of several bacterial enteropathogens in ice of popular drinks, including cola, scotch, and tequila (9). Although multiple factors could vary the outcome, such as the number and type of organisms present in the initial water and the length of time frozen, Dickens concluded that none of the organisms were completely eliminated in the test drinks when contaminated ice was added (9). There are concerns about the safety of drinking water and the ice used in beverages, especially when traveling to foreign locations with a questionable water supply.

Poor quality water or lack of hygiene during the production and handling of water to produce ice can contribute to the presence of harmful microorganisms (4, 6, 9, 10, 13, 17). In addition, ice may be sold commercially that may not be closely or consistently monitored for proper sanitation and hygienic conditions. This survey determined the microbiological and chemical quality of ice produced and bagged on-premises in retail establishments and in self-service vending machines in the state of Georgia and compared the results with that from ice produced by manufacturing companies that are monitored by the IPIA.

MATERIALS AND METHODS

Sample Collection - A total of 250 bags of packaged ice were collected in the state of Georgia between mid-August and October 2012 from retail establishments that produced ice on-premise and self-service vending machines. The number of retail samples and vending machine samples

selected was based on the population density of communities throughout the state (Table 3.1). One hundred and forty-nine samples were collected from retail and convenient stores, and 101 samples were collected from vending machines, with one bag collected per location. Samples were collected from retail establishments that were known to produce and package ice on the location's premises. The type of establishments in which these samples were bought ranged from gas stations, foodservice franchises, and liquor stores. The ice was collected in the bags provided at the locations and closed using their method, such as with a twist tie, metal clip, or knot. The original bags were doubled bagged with large, sterile, 5 kg bags (Whirlpak®, Nasco, Fort Atkinson, WI) in case there were holes in the first packaging to prevent potential cross-contamination. Both bags were numbered with a permanent marker which corresponded with a number on a datasheet containing information and key identifying points for that particular sample, including name and type of retail establishment in which the sample was collected, the address, the type of closure of the bag, and retail labeling on the bag. Noticeable defects of the sample or packaging were recorded, and the samples were kept in the cooler until they were ready to be tested.

Twenty-five bags of packaged ice were collected from 2 different ice manufacturing companies near Atlanta, Georgia during January 2013. Both companies are IPIA members. Thirteen samples were collected from plant A and 12 samples from plant B. The sample bags were numbered and the location was recorded on the datasheet. The samples were kept in the cooler until they were ready to be tested. Table 3.2 shows the distribution of all 275 samples of packaged ice and the area of Georgia in which it was collected.

Sample Preparation **-** The ice was kept in the coolers in a 4°C refrigerator following collection and sample preparation was completed within 24 h of collecting the sample. Ice samples were removed from their original bags and separated into separate bags and containers with corresponding numbers. Approximately 1 L (or 1,000 g) of ice was separated into a large, sterile, 2.6 kg bag (Whirlpak®, Nasco) for microbial analysis, and approximately 1 L (or 1,000 g) was separated into sterilized plastic bottles for chemical analysis. Excess sample was discarded. The original bags were kept for recordkeeping. The ice was allowed to melt completely at room temperature (24°C) before testing.

Microbiological Examination **-** The heterotrophic plate count (HPC) was enumerated using SimPlate® for HPC Multi Dose (Idexx Laboratories Inc., Westbrook, ME). One ml of the melted ice sample was slowly pipetted onto the center of the SimPlate® and 9 ml of the manufacturer-provided media that was hydrated with 100 ml of sterilized deionized water in its original bottle, was pipetted onto the center of the plate, on top of the 1 ml sample. The plate was covered with its lid and gently swirled to mix and distribute the sample into all the wells. The plate was tilted forward to drain the excess liquid into the absorbent pad and inverted before it was incubated at 35°C for 48 h. After incubation, the plates were observed under a 365 nm ultraviolet (UV) light and wells that fluoresced were counted. The total number of positive wells counted and the most probable number (MPN) table that was specific to the Simplate® was used to determine the MPN of HPC bacteria present in the sample.

Total coliforms and *E. coli* were enumerated using the Quanti-Tray* and the Colisure® Assay test kit (Idexx Laboratories Inc.). One hundred ml of the melted ice sample was pipetted into a sterile media vessel. The contents from the provided Colisure® reagent packet were added and 3-4 drops of an antifoam solution (Idexx Laboratories Inc.) was added to the vessel. Each

solution was shaken until the large media particles were dispersed. The sample/reagent mixture was poured into a Quanti-Tray* tray and sealed with the Quanti-Tray* Sealer (Idexx Laboratories Inc.). The sealed tray was incubated with the wells lying facing down, at 35°C for 24 h. Results were read based on the color of the well and if the well fluoresced under a 365 nm UV light. If the well was yellow/gold, it was negative for both total coliforms and *E. coli*. If the well was red/magenta, it was positive for total coliforms and if it was red/magenta and fluoresced, it was positive for *E. coli*. The number of positive wells was then referenced to the MPN table specific to the Quanti-Tray* to determine the MPN of total coliforms and/or *E. coli* in the sample.

Enterococci (*Enterococcus faecalis*) were enumerated using the Quanti-Tray* and the Enteroelert® Assay test kit (Idexx Laboratories Inc.). One hundred ml of the melted ice sample was pipetted into a sterile media vessel. The contents from the provided Enteroelert® reagent packet were added and 3-4 drops of an antifoam solution were added to the vessel. Each solution was shaken until the large media particles were dispersed. The sample/reagent mixture was poured into a Quanti-Tray* tray and sealed with the Quanti-Tray* Sealer. The sealed tray was incubated with the wells lying face down at 41°C for 24-48 h. The presence of enterococci in the wells was detected by the presence of blue fluorescence under a 365 nm UV light. The number of positive wells was referenced to the MPN table specific for the Quanti-Tray* to determine the MPN of enterococci in the sample.

The presence or absence of *Salmonella* and *L. monocytogenes* was determined using membrane filtration and enrichment methods. One hundred ml of the melted ice sample was filtered through a 0.45µ MicroFunnel™ Filter Funnel (Pall Life Sciences, Ann Arbor, MI). Using sterilized tweezers, the filter was separated from the funnel, placed into a stomacher bag

with 100 ml of universal preenrichment broth (Becton Dickinson and Company, Sparks, MD), stomached for 1 min, and incubated at 35°C for 24 h.

For the enrichment of *Salmonella*, 0.1 ml was transferred from the UPB sample into a tube of Rappaport Vassiliadis (RV) broth (Becton Dickinson), 1.0 ml was transferred into a tube of tetrathionate (TT) broth (Becton Dickinson), and the tubes were incubated for 24 h at 42°C and 35°C, respectively. After incubation, portions of each broth were streaked using a sterilized loop onto separate plates of bismuth sulfite agar (BSA), xylose-lysine-desoxycholate (XLD) agar and Hektoen-Enteric (HE) agar (Becton Dickinson). The plates were incubated at 35°C for 24 h. Presumptive positive colonies were subcultured to triple sugar iron (TSI) and lysine-iron-agar (LIA) slants (Becton Dickinson) for additional characterization. For TSI and LIA slants that had positive reactions typical for *Salmonella*, an Enterobacteriaceae Micro-ID® (Thermo Fisher Scientific, Lenexa, KS) was used to confirm the identity of the *Salmonella* present.

For *L. monocytogenes*, a portion of UPB was streaked onto the selective modified Oxford agar (Becton Dickinson) using a sterilized loop and was incubated for 24 h at 35°C. Presumptive positive colonies were subcultured to a chromagar plate (Becton Dickinson) for selective enrichment, and if the plate was positive with typical *L. monocytogenes* colonies, a Micro-ID® *Listeria* (Thermo Fisher Scientific) was used to confirm the *L. monocytogenes* identification.

Chemical Analysis – All tests completed for chemical analysis was done in a separate lab and only on samples collected from the retail establishments and vending machines. The Hach HQ 440d Benchtop Dual Input, Multi-Parameter Meter (Hach Company, Loveland, CO) was used to determine conductivity, pH and the level of nitrate, using the appropriate probes, CDC40101, PHC28101, and ISENO318101, respectively. The manufacturer's instructions were followed, and the instrument was calibrated for each probe before each sample period. Turbidity was

determined using the LaMotte 2020 We Turbidity meter (LaMotte Company, Chestertown, MD). The manufacturer's instructions were followed, and the instrument was calibrated before each sample period. The alkalinity of the water samples was measured using the titration method instructions published in section 2320 of *The Standard Methods for the Examination of Water and Wastewater* (8).

Statistical Analysis **-** Statistical analysis was completed on the results from the microbiological and chemical testing of the packaged ice. The significance and independence of variables were determined by using common statistical tests. The analysis of variance (ANOVA) was used to determine if the relationship between 2 independent variables (i.e., HPC levels and bag closures) was statistically significant as the Fisher exact tests and Chi-square tests were both used determined if these variables were independent of one another. The likelihood ratio test and the logistic regression analysis were used to express how many times more likely the data under one variable will occur than another variable.

RESULTS AND DISCUSSION

In this study, packaged ice from multiple locations in Georgia was tested for total heterotrophic bacteria and indicator organisms, as well as *Salmonella* and *Listeria monocytogenes*. Heterotrophs and indicator organisms are used to evaluate the sanitation and hygienic conditions of the production areas, the contamination of foods, including ice, and for the possible presence of pathogens (10, 21). These organisms all reflect the sanitary quality of the ice, the ice machine and scoop, the quality of the water the ice is made from, cross-contamination from food contact surfaces, and the hygiene of the staff handling the ice (10, 19,

21). The presence of *E. coli* and enterococci, such as *E. faecalis*, indicate possible fecal contamination (10).

The International Packaged Ice Association (IPIA) established limits for these indicator tests as a quality control measure to keep ice safe for consumers. These limits state that the heterotrophic plate count of water should not exceed 500 MPN/ml of water, the total coliform count should not be greater than 2.2 organisms/100 ml of water using MPN, and fecal coliforms, *E. coli,* and enterococci organisms should not be present in any packaged ice sample. No pathogenic bacteria, such as *Salmonella* and *Listeria monocytogenes*, should be present.

Heterotrophic Plate Count

Heterotrophic bacteria are naturally occurring in water and the contamination level is a common indicator of the cleanliness and quality of drinking water. It reflects the general hygiene of the ice production and handling process (4, 13, 21). The presence of heterotrophic bacteria does not necessarily signify a risk for illness because low numbers are normally found in treated water, but it does give a good indication of sanitary conditions during storage and handling and the efficiency of water treatments from the water source and manufacturing plant (5, 7, 23). According to the WHO, the HPC value is a good indication of effective coagulation, filtration, and disinfection steps during the water treatment process (23). In this survey, 178 samples (71%) of all retail and vending machine produced ice contained some level of heterotrophs, with 16 samples (6.4%) exceeding IPIA's recommended limits of less than 500 MPN/ml of water (Table 3.3). The majority of samples that exceeded the limits were bagged ice from retail establishments and were primarily gas stations. Chi-square analysis revealed the HPC values were dependent on the type of ice, whether it is from retail outlets or vending machines ($p < 0.0001$). There is also a 3.5 times greater chance the ice purchased from a retail

store will have a higher HPC value than ice from vending machines, which could be attributed to the increase handling of ice from workers. The high levels of heterotrophs may indicate improper personnel hygienic practices of the workers at the retail establishment, cross-contamination, and poor water source. The ice that was collected from the manufacturing plants did not have any growth that was above the recommended limit, with only 2 samples showing little growth present in the ice (Table 3.3). This indicates possibly better hygienic control in an ice manufacturing facility that follows the more specific guidelines (i.e., GMPs, PIQCS, etc.). Ice produced in these facilities is done so with little hands-on exposure from workers and with less chance for cross-contamination from food-contact surfaces. The study on the quality of packaged ice collected in Iowa completed by Moyer et al. (20) included members and nonmembers of the IPIA in their survey and no samples collected from IPIA accredited companies exceeded the HPC limit of 500 MPN/ml, supporting the claim of higher-quality ice.

Total Coliforms and *E. coli*

Coliforms are indicator organisms and are used to evaluate the hygienic conditions, the possible fecal contamination, and potential presence of pathogens (4, 10). A total of 64 samples (25.6%) from retail establishment locations and vending machines exceeded the recommended limits of total coliforms (less than 2.2MPN/100mL), with the majority from gas stations (Table 3.4). Two samples bought from gas stations also had nonpathogenic *E. coli* present. The logistic regression analysis showed the odds of a retail bag of ice having an unacceptable level of total coliforms are 1.18 times more than those of a bag from a vending machine. In comparison to past studies done by Schmidt et al., who tested packaged ice from Florida, Gerokomou et al. who tested packaged ice from Greece, and Moyer et al. the percentage of unacceptable level of total coliforms and *E. coli* were slightly higher in this study (13, 20, 24). Although the distribution of

sample collection varied some among these studies, the results are significant enough to indicate sanitation problems. These unacceptable samples could indicate a contaminated water source, un-sanitized scoops or utensils, unsanitary packaging process or unhygienic staff handling (13, 19, 20). Unless self-service vending machines are maintained properly and the presence of insects and animals controlled, there could be a greater chance for contamination. No coliforms or *E. coli* were detected in the ice from manufacturing plants (Table 3.4), which indicates good sanitary, hygienic practices may have been in place. The IPIA members from the study done by Moyer et al. (20) also showed no positive results during their survey (20).

Enterococci

Enterococci bacteria, commonly *Enterococcus faecalis*, can typically be found in human and animal intestines and can also be an indicator of poor sanitary and hygienic conditions during the production of ice (18). Just as with the total coliforms and nonpathogenic *E. coli*, the presence of enterococci does not necessarily signify that illness will occur; however, it may indicate the presence of fecal pathogens that could cause nausea, vomiting, abdominal pain, and diarrhea (18). In this survey, 32 samples (12.8%) from retail establishments and vending machines contained unacceptable levels of enterococci, exceeding the limit of 1.0 MPN/100 mL. Positive samples were found in both self-service vending machines and retail locations, in particular gas stations (Table 3.5). Based on the likelihood ratio test, the odds of a retail bag of ice having an unacceptable level of enterococci are 3.3 times more likely than the samples from the vending machines. No sample from the ice manufacturing plants tested positive for enterococci. These results could be contributed to the process involved in the production of the different types of ice. Ice from the vending machines and manufacturing plants are less likely to be handled by employees and come into contact with contaminants. Again, these results indicate

that manufactured ice may be produced under more sanitary and controlled conditions than the packaged ice sold at retail establishments and self-service vending machines.

Salmonella and Other Organisms

Regardless of source, no samples tested positive for *L. monocytogenes*. *Salmonella* was not detected in the manufactured ice, but one sample from a retail establishment, a foodservice franchise, tested positive for the presence of *Salmonella.* Since only the presence of this organism was confirmed and the number of cells is unknown, it was not possible to determine whether or not the consumption of this contaminated ice would have caused illness. However, the mere presence still raises concern about the conditions of the location where this sample was bought. The presence of *Salmonella* demonstrates a more serious level of contamination and the need for attention and intensive cleaning.

Enterobacter agglomerans was also detected in a sample collected from a self-service vending machine. It is a common *Enterobacter* species with an unknown infectious dose and is found in the stool of healthy humans (12). This microorganism can cause acute gastroenteritis with symptoms including vomiting, nausea, abdominal pain, and diarrhea (12). The frequency of these microorganisms is relatively unknown among the CDC because the symptoms are sometimes mild and go without complaint (12). The presence of *E. agglomerans* is a little more significant than the presence of the indicator organisms, such as coliforms or heterotrophic bacteria, because it has been linked to foodborne illness.

Sample Distribution of Retail-Produced and Self-Service Vending Machine Bagged Ice

Table 3.6 shows the distribution of unacceptable samples collected in Georgia while Figure 3.1 shows the map of Georgia to assist in visualizing the areas where the majority of unacceptable samples were obtained. The area with the greatest likelihood of poor quality of ice

was the southern region of the state of Georgia. More than half the samples from the southeast region contained an unacceptable level of coliforms and enterococci. Results from the likelihood ratio test indicate that there are higher odds that towns in the southeast portion of the state are more likely to have a higher unacceptable level of HPC, total coliforms, and enterococci than the rest of the state. The unacceptable levels of total coliforms are dependent on the location in which the samples were obtained ($p < 0.0001$) but the level of HPC and enterococci are not.

The Influence of Type of Bag Closure on the Microbial Quality

All samples that were obtained from manufacturing companies were closed using metal clips, and as stated in the previous sections, there was little to no microbial growth present. Therefore, the statistical analysis was only done on on-site- and vending machine-produced ice. Examples of bag closures that were found to secure packaged ice from retail outlets and vending machines are shown in Figure 3.2. The majority of the samples from vending machines were closed with a twist tie, mainly because all ice samples from the vending machines had twist ties available at each location, with the exception of five machines/samples. More than a quarter of these sample closed with twist ties had unacceptable levels of coliforms (Table 3.7). Two bags were closed with tape and both were unacceptable for coliforms and one for enterococci. Although the Fisher Exact test determined that there was no significant relationship between all microbial variables and bag closures, the results suggest there is a need for more secure, sanitary methods of closing these bags, such as mechanically sealing. The sample where the bag was automatically sealed from the vending machine had no positive growth for any of the organisms. A more sanitary, automated method of closure for packaged ice could result in a better quality bagged ice product.

Chemical Analysis

Included in the IPIA PIQCS program are safety standards for the chemical quality of the water used to produce packaged ice. In order to meet the terms for the PIQCS accreditation, manufacturers need to follow the current standards set by the Environmental Protection Agency (EPA) and their National Primary and Secondary Drinking Water Regulations (7, 14). According to the EPA secondary standards, the recommended acceptable pH level for drinking water is 6.5-8.5, less than 1.0 nephelolometric unit (NTU) for turbidity, a nitrate level less than 10 mg/L, and an alkalinity level less than 500 mg/L $CaCO_3$ (7). There are no specific limits set for the conductivity levels of drinking water. The results of the chemical analysis done on the packaged ice collected from retail establishments and self-service vending machines are shown in Tables 3.8 and 3.9. Of the 250 samples, 95 (38%) samples of the packaged ice fell out of the acceptable range for pH, with 37 (39%) samples from vending samples and 58 (61%) samples from retail being unacceptable. According to the Fischer's Exact test, there is a distinct relationship between the pH value and the type of ice, for ice produced at a retail establishment has a higher probability of unacceptable low pH while vending ice tends to have a higher probability of unacceptable high pH (p-value < 0.0001). More than half (50.6%) of the samples from gas stations and from the food service establishments (57.1%) were outside of the acceptable range of 6.5 to 8.5. Water with a pH that is too high or too low does not mean it is unsafe, but does have aesthetic effects on taste and odor. When a pH is too low, the water may have a bitter or metallic taste, which could be due to the acid leaching metals from the pipes that it is traveling through due to the corrosive action, such as lead and copper (7). The level of metal in the water could also be a potential problem. A pH that is too high can produce a 'slippery' feel to the water and a soda taste to the consumer due to the high levels of alkalinity minerals

present (7). The alkalinity level is the measure of minerals and the concentration of earth metals present, and is used to determine the efficiency of water treatments. No sample exceeded the limit set by the EPA, with the highest value measured at 127.68 mg/L $CaCO_3$ from a foodservice outlet in northern Atlanta. Only 3 samples had turbidity levels that exceeded the recommended level of 1 NTU, 1 sample being from a vending machine and 2 samples collected from gas stations. Turbidity is the measure of the cloudiness of water and is used to show the quality of water and filtration effectiveness (7). A higher turbidity level is associated with higher levels of microorganisms present in the water and there is a higher risk for potential illness if consumed (7). The sample with high turbidity collected from the vending machine was also shown to have an unacceptable level of total coliforms. However, the two samples with high turbidity levels did not have significant growth of bacteria showing little correlation between turbidity level and bacterial growth in this survey. No samples were measured to have unacceptable levels of nitrates in the water. These problems can usually be fixed through proper filtration and consistent testing, which is required by the IPIA to be PIQCS accredited (7, 14).

CONCLUSION

Overall, this study indicates a need for more sanitary practices in the packaging of ice on the premises of retail locations and for cleaner vending machines. The manufacturing companies are required to follow the GMPs that were set up by the AFDO (1). Additionally, members of organizations, like the IPIA, are required to follow not only these GMPs but also the PIQCS guidelines that incorporate a HACCP program for these companies (14). Congress directed the FDA to work to educate manufacturers regarding safe production of ice (19). The issuance of a Food Facts sheet informing the public about existing FDA regulations that apply to ice

manufacturers could also be beneficial if applied to locations who insist on making and selling their own ice. It is significantly important to train and educate workers at these locations about appropriate hygienic practices, the importance of regular cleaning and sanitizing, the risks of transferring contaminated water and ice, and prevention techniques they can take to avoid causing any foodborne illness. Consumers who purchase ice should also be educated about the risk they take by purchasing this product and ways they can also prevent cross-contamination in their own homes.

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Table 3.1. Types of ice samples collected from manufacturing companies, retail establishments, and self-service vending machines.

	# of Samples	% of Total Samples	% of Total Retail
Total Samples	275	100	-
Manufactured	25	9.8	-
Vending	101	36.7	-
Retail	149	54.2	-
Gas Stations	81	29.5	54.4
Liquor Stores	19	6.9	12.8
Food Service	49	17.8	32.8

Table 3.2. Sample distribution of packaged ice purchased from retail establishments with on-site production of ice, self-service vending machines, and ice produced in a manufacturing plant.

Regions (cities)	# of Retail (%)	# of Vending (%)	# of Manufactured (%)	Total (%)
Northeast (Athens Commerce Gainesville)	19 (12.8)	22 (21.8)	0 (0)	41 (14.9)
East (Augusta)	7 (4.7)	12 (11.9)	0 (0)	19 (6.9)
Northwest (Atlanta Marietta Alpharetta Griffin)	33 (22.0)	15 (14.8)	25 (100)	73 (26.6)
South (Valdosta Albany Macon)	32 (21.5)	30 (29.7)	0 (0)	62 (22.5)
Southeast (Savannah)	29 (19.5)	12 (11.9)	0 (0)	41 (14.9)
West (Columbus LaGrange)	29 (19.5)	10 (9.9)	0 (0)	39 (14.2)
Total	149	101	25	275

Table 3.3. Frequency of acceptable and unacceptable[a] levels of heterotrophic bacteria in retail establishments and self-service vending machine produced ice based on the different retail sources.

	# of Total Samples	# of Samples within Acceptable Limits (%)	# of Samples within Unacceptable Limits (%)
Manufactured Ice	25	25 (100.0)	0 (0.0)
Total On-site Samples	250	234 (93.6)	16 (6.4)
Vending	101	97 (96.0)	4 (4.0)
Retail	149	137 (91.9)	12 (8.1)
Gas stations	81	72 (88.8)	9 (11.1)
Liquor Stores	19	18 (94.7)	1 (5.3)
Food Service	49	47 (95.9)	2 (4.1)

[a] Unacceptable level was based on the IPIA level of >500 MPN/ml of water

Table 3.4. Acceptable and unacceptable[a] levels of total coliforms for retail establishments and self-service vending machine produced ice based on of the different retail locations.

	# of Total Samples	# of Samples within Acceptable Limits (%)	# of Samples within Unacceptable Limits (%)
Manufactured Ice	25	25 (100.0)	0 (0.0)
Total On-site Samples	250	186 (74.4)	64 (25.6)
Vending	101	78 (77.2)	23 (22.8)
Retail	149	108 (72.5)	41 (27.5)
Gas stations	81	55 (67.9)	26 (32.1)
Liquor Stores	19	16 (84.2)	3 (15.8)
Food Service	49	37 (75.5)	12 (24.5)

[a] Unacceptable level was based on the IPIA level of >2.2MPN/100 ml of water

Table 3.5. Acceptable and unacceptable[a] levels of enterococci for retail establishments and self-service vending machine produced ice based on different retail locations.

	# of Total Samples	# of Samples within Acceptable Limits (%)	# of Samples within Unacceptable Limits (%)
Manufactured Ice	25	25 (100.0)	0 (0.0)
Total On-site Samples	250	218 (87.2)	32 (12.8)
Vending	101	95 (94.1)	6 (5.9)
Retail	149	123 (82.5)	26 (17.5)
Gas stations	81	65 (80.2)	16 (19.8)
Liquor Stores	19	15 (78.9)	4 (21.1)
Food Service	49	43 (87.7)	6 (12.3)

[a] Unacceptable level was based on the IPIA level of >1.0MPN/100 ml of water

Table 3.6. Number of samples exceeding acceptable[a] limits based on distribution regions for packaged ice collected from retail establishments and self-service vending machines for heterotrophic plate counts (HPC), total coliforms, and enterococci.

Regions	# of Total Samples	# with unacceptable levels of HPC (%)	# with unacceptable levels of Coliforms (%)	# with unacceptable levels of Enterococci (%)
Northeast	41	2 (12.5)	5 (7.8)	2 (6.3)
East	19	0 (0.0)	1 (1.6)	1 (3.1)
Northwest	73	3 (18.8)	3 (4.7)	7 (21.9)
South	62	0 (0.0)	21 (32.8)	10 (31.2)
Southeast	41	9 (56.2)	26 (40.6)	5 (15.6)
West	39	2 (12.5)	8 (12.5)	7 (21.9)
Total	275	16 (100.0)	64 (100.0)	32 (100.0)

[a] Unacceptable level was based on the IPIA level for HPC (>500 MPN/100 ml of water), total coliforms (>2.2 MPN/100 ml of water), and enterococci (>1.0 MPN/100 ml of water).

Table 3.7. Number of samples exceeding the acceptable[a] limits based on the different type of bag closures provided by retail establishments and self-service vending machines for heterotrophic plate count (HPC), total coliforms and enterococci.

Types of Bag Closures	# of Total Samples	# with unacceptable levels of HPC (%)	# with unacceptable levels of Coliforms (%)	# with unacceptable levels of Enterococci (%)
Knotted	17	1 (6.3)	3 (4.8)	2 (6.5)
Metal Clip	23	3 (18.7)	3 (4.8)	4 (13.0)
Nothing Provided	5	0 (0.0)	0 (0.0)	0 (0.0)
Thread	33	1 (6.3)	6 (9.7)	6 (19.3)
Twist Ties	169	11 (68.7)	50 (80.7)	19 (61.2)
Total	247[b]	16 (100.0)	62 (100.0)	31 (100.0)

[a] Unacceptable level was based on the IPIA level for HPC (>500 MPN/100 ml of water), total coliforms (>2.2 MPN/100 ml of water), and enterococci (>1.0 MPN/100 ml of water).
[b] Three samples were not included in analysis due to insignificant numbers for that type of bag closure.

Table 3.8. Summary of the pH, turbidity, conductivity, alkalinity and nitrate concentrations for ice samples from retail establishments and vending machines.

Variable	N	Mean	Std Dev	Minimum	Maximum
pH	250	7.24	1.01	4.65	9.83
Turbidity	250	0.21	0.33	-0.07	4.40
Conductivity	250	70.39	75.32	2.33	574.50
Alkalinity	250	16.94	21.65	1.64	127.68
Nitrate	250	1.15	0.63	0.02	3.69

Table 3.9. Chemical analysis of ice collected from retail establishments and self-service vending machines based on limits set by the Environmental Protection Agency.[a]

	pH		Turbidity		Nitrate Concentration	
	# Acceptable (%)	# Unacceptable (%)	# Acceptable (%)	# Unacceptable (%)	# Acceptable (%)	# Unacceptable (%)
Total	155 (62.0)	95 (38.0)	247 (98.8)	3 (1.2)	250 (100.0)	0 (0.0)
Vending	64 (63.4)	37 (36.6)	100 (99.0)	1 (1.0)	101 (100.0)	0 (0.0)
Retail	91 (61.1)	58 (38.9)	147 (98.7)	2 (1.3)	149 (100.0)	0 (0.0)
Gas Stations	40 (49.4)	41 (50.6)	79 (97.5)	2 (2.5)	81 (100.0)	0 (0.0)
Liquor Stores	17 (89.5)	2 (10.5)	18 (100.0)	0 (0.0)	19 (100.0)	0 (0.0)
Food Service	21 (42.8)	28 (57.2)	49 (100.0)	0 (0.0)	49 (100.0)	0 (0.0)

[a]Limits set by the EPA for pH (6.5-8.5), turbidity (<1.0 NTU), and nitrate concentrations (<10mg/L).

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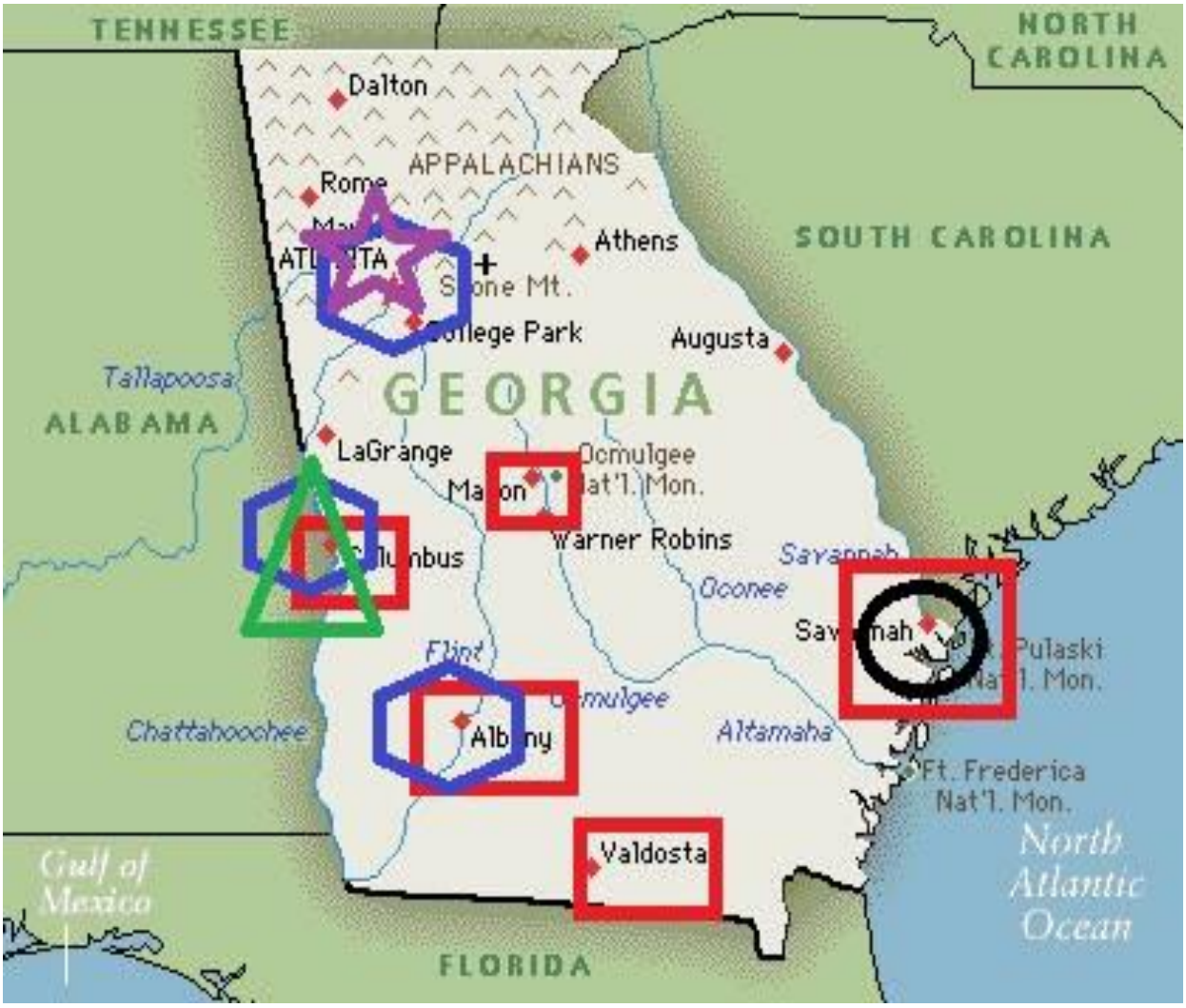

Figure 3.1. Map of Georgia highlighting the locations with the highest number of samples that exceed the acceptable limit of microbial growth.

Circle: Heterotrophic Counts
Square: Total Coliforms
Triangle: *E. coli*
Hexagon: Enterococci
Star: *Salmonella*

A. B. C. D.

Figure 3.2. Example of bag closures that are commonly used to secure packaged ice from retail outlets and vending machines. From left to right: A. Twist Ties B. Metal Clip C. Drawstring D. Knotted

CHAPTER FOUR

STRUCTURAL DAMAGE OF *SALMONELLA* SPP. INDUCED BY FREEZE/THAW TREATMENTS[1]

[1] S.L. Mako and M.A. Harrison. To be submitted to the Journal of Food Protection

ABSTRACT

Freezing of food or water is generally seen as an ineffective method for inducing cell death but may be injurious to bacterial cells, affecting cell viability and membrane integrity. This study determined the injury rate of *Salmonella* spp. induced after freezing inoculated water for different time intervals and observed the structural damage of *Salmonella* by scanning electron microscope. Five different *Salmonella* spp. serotypes were inoculated into tap water, which was frozen and held for increments of 0, 24, 72, and 120 h. After each interval, the ice was melted, surviving *Salmonella* were enumerated onto selective (XLD) and nonselective (TSA) media for recovery, and the percentage of cells killed and injured were determined. After 24 h of freezing, there was 74% lethality. Of the 26.0% viable survivors, 26.9% remained uninjured and 73.1% of the cells were injured. After 120 h, there was a slight increase in the percentage of cell death (74.0% to 76.5%) and an increase in the number of injured cells (73.1% to 88.6%). Scanning electron microscope analysis showed examples of cell membrane damage induced by the freeze/thaw treatment of each serotype of *Salmonella*. Microbial injury is important to food safety for injured cells may not be detected at the time of post-processing sampling and may undergo repair if given an appropriate environment, resulting in a food safety risk to consumers.

INTRODUCTION

One purpose of food and water processing treatments is to decrease the level spoilage microorganisms and pathogens that are potentially present. Lethality of microbial cells can be induced by various treatments, including physical and chemical stresses. Examples of physical stresses include subjecting the cells to freezing, heating, drying, and radiation, while chemical stresses include exposure to organic acids, sanitizers, and preservatives (9, 17, 22). There are various levels of stresses ranging in severity, resulting in different responses from the cell. Treatments that induce minor or low levels of stress may only cause a temporary adaptation response to the changed environment with minor physiological changes and increased stress tolerance while not affecting the growth rate (3, 9, 11, 22). On the other end, lethal stress causes cell death. Cell death results from the permanent loss of essential cell functions, such as inactivation of enzymes and cell membrane components (3, 9, 11, 22). However, a moderate stress may result in microbial injury that maybe temporary and repairable or severe and permanent, depending on the harshness of the stress and the characteristics of the individual cells effected (3, 9, 11, 22). In regards to food processing, this kind of stress is seen as insufficient because it results in sublethally injured microbes that still are a threat to the safety and shelf stability of the food.

Bacterial injury is influenced by time, temperature, concentration of the injurious stressor, and strain of the target microbe (6). The type of injury a cell suffers can be described as either metabolic or structural. Metabolic injury is defined as the impaired capability to synthesize essential cellular components and the loss of intracellular nutrients and RNA (21). This type of injury is commonly induced by freezing, heating, chemical exposure, radiation and other treatment stresses. Structural damage is the cell's inability to proliferate or survive on

media containing selective agents with no obvious inhibitory effects on unstressed cells (21). This type of injury is characterized by the disruption of the cell membrane's permeability, causing cell leakage of essential components (17, 21, 22). Stress can cause the destabilization of weak bonds, such as hydrogen bonds, of some macromolecules, and this can change the normal conformation of the cell, causing the collapse of the wall's integrity (17, 21, 22). Structural injury is commonly due to damage caused by freezing, heating, and drying.

Studies have looked at the subject of bacterial injury, such as how it is induced, microbial resuscitation, and its impact on the food industry. Initially, researchers tried to emphasize the importance and the need for microbiological evaluations of injured pathogens in food processing and semi-preserved food, but this failed to gain interest of most (6, 7). It was not until 1959 that Straka and Stokes (21) looked at the metabolic injury of *Escherichia coli* and *Pseudomonas* spp. that were subjected to freeze/thaw stress that notable interest was sparked in the research of bacterial injury. They found that injury due to low temperatures impaired the ability of the bacteria to synthesize essential cellular components, which prevented the cells from growing on minimal media but not on the nutrient-rich media that supported the repair (21). More studies followed that focused on injury induced by freezing and heating, later on physical and chemical treatments, and continued on to determine the sites of cellular damage and mechanisms of cellular repair through plating media supplemented with specific nutrients (17). Ray et al. (16) studied the rate of injury and repair of freeze-damaged *Salmonella* Anatum cells after being exposed to various environments and found injured cells were able to repair themselves after being introduced to an environment rich in phosphate, citrate and $MgSO_4$ as well as in the presence of complex organic nutrients. This raised awareness of the potential of the presence of

injured bacteria in certain foods that would support the repair of the cell, allowing the bacteria to become viable once again.

Freezing of food or water is generally seen as an ineffective method for inducing cell death but is proven to be injurious to bacterial cells (16, 21, 22). Freeze/thaw damage can result from continued exposure to concentrated solutes from food particles and physical damage caused by ice crystal formation. Although injurious, pathogens are known to survive in water and through the freezing process, putting consumers at risk when ingesting contaminated ice, as seen in past outbreaks (1). This study determined the injury rate of *Salmonella* spp. induced after freezing inoculated water for different time intervals and observed structural damage of the *Salmonella* by scanning electron microscopy (SEM).

MATERIALS AND METHODS

Preparation of Inoculum **-** *Salmonella enterica* serovars Anatum, Enteritidis, Typhimurium, Montevideo, and Poona were obtained from the culture collection in the Department of Food Science and Technology, University of Georgia, Athens. These cultures were kept on beads (Microbank, Pro-Lab Diagnostics, Austin, TX) at -80°C in frozen storage and were reactivated by 3 consecutive 1 mL transfers into 9 mL of tryptic soy broth (Becton Dickinson and Company, Sparks, MD), incubated at 35°C at 24 h intervals and incubated at 35°C. On the day of the experiment, prior to the inoculation of the tap water and freezing, 10 mL of each serovar were centrifuged (Forma Scientific, Inc., Marietta, OH) separately at a relative centrifugal force (RCF) of 3300 x g for 10 min and the supernatant was removed. The pellets were resuspended in 10 mL of 0.1% peptone water (Becton Dickinson) by vortexing and centrifuged again for 10 min at 3300 x g. The supernatant was removed and the pellets were resuspended in 10 mL of 0.1%

peptone water by vortexing. The serovars were centrifuged once more for 10 min at 3300 x g and the supernatant was removed and the pellets were resuspended in 5mL of 0.1% peptone water for final use. Each serovar was diluted with additional 0.1% peptone water to be of equivalent turbidity to McFarland Latex 0.5 (Hardy Diagnostics, Santa Maria, CA). Each culture contained approximately 10^8 cfu/mL.

Inoculation and Freezing of Water - Four mL of each serovar were pooled together and vortexed to create a 20 mL cocktail. Tap water that was kept at room temperature (24°C) until use was measured in a sterilized graduated cylinder to 200 mL and was poured into a sterilized 500 mL glass bottle for inoculation. The 20 mL cocktail was transferred to the bottle of water for a 1/10 dilution and the sample was shaken by hand for 1 min. Ten mL of the inoculated tap water was pipetted into 6 different wells of 3 separate plastic ice trays (Kroger Market Place, Athens, GA) totaling to 60 mL of sample per tray. The trays were covered with aluminum foil and placed into a conventional freezer (KendroLab Products, Asheville, NC) set -10°C. After 2 h in the freezer to allow sufficient time for the water to completely freeze, the clock started to begin the 24, 72, 120 h time interval.

Enumeration of Viable Organisms - The inoculated water was sampled prior to freezing for the initial culture count and then again after 24, 72, and 120 h of freezing. A tray was removed from the freezer and the ice cubes from the 6 wells were taken out and transferred to a stomacher bag to allow the sample to melt completely at room temperature (24°C), which took approximately 2 h. After this, 1 mL was pipetted from the bag, and the appropriate dilutions were made in 0.1% peptone water. At each time interval, dilutions were plated onto the selective medium, xylose-lysine-desoxycholate (XLD) agar (Becton Dickinson), and also onto the nonselective medium,

tryptic soy agar (TSA) (Becton Dickinson). The plates were incubated at 37°C for 24 h before counting the colonies.

Data Analysis **-** This experiment was done 5 different times and the resulting counts on selective and nonselective plates were averaged for each hour interval and the averages were used to determine the percentage of cells that were killed or injured during the freezing process. The calculations used are listed in Table 4.1.

Preparation of Inoculum for SEM Observation **-** On the day prior to cell fixation for SEM, 5 mL of each 10 mL culture suspended in tryptic soy broth was separated and held at 4°C for the following day. The remaining 5 mL were washed and adjusted to the cell density of approximately 10^8 cfu/ml following the steps previously described. Five mL portions of each culture were placed in the freezer at -10°C. After holding for 26 h (2 h allowed for the initial freezing process and 24 h for storage period). The samples were removed from the freezer and thawed at room temperature (24°C), which took approximately 2 h. Additionally, the 5 mL portion of each culture held at 4°C for 24 h were centrifuged separately at 3300 x g for 10 min and the supernatants were removed and the pellet was resuspended in 5 mL of 0.1% peptone water. The samples were centrifuged and washed twice more, like above, before being suspended in 5 mL of 0.1% peptone water for final use. One mL of all samples, healthy and thawed, were transferred to 1.5 µL microfuge tubes and centrifuged (Centrifuge 5415C, Eppendorf AG, Hamburg, Germany) at a RCF of 16000 x g for 5 min. After the pellet was formed, the extra 0.1% peptone water was pipetted out, leaving enough to cover the pellet to keep the cells hydrated.

Preparation of Cells for SEM Observation **-** For the primary fix of the *Salmonella* cells, 1 mL of each 2.0% glutaraldehyde and NaCacodylate buffer were added to the 1.5 µL microfuge tubes,

vortexed, and held for 1 h at 4°C. The cells were washed 3 times in the NaCacodylate buffer, and centrifuged (Eppendorf AG.) at 16000 x g for 10 min. For the secondary fix, the cells were suspended in 1 mL of 1.0% OsO_4 and NaCacodylate buffer, vortexed and allowed to sit for 1 h at 4°C. The cells were washed once with the NaCacodylate buffer and centrifuged at 16000 x g for 10 min and then with 2 additional washes with deionized (DI) water and centrifuged for 10 min. The cells were dehydrated by a consecutive series of ethanol washes using 25, 50, 75, 85, 95, and three increments of 100% ethanol. After each ethanol wash, cells were centrifuged for 10 min at 16000 x g. The samples were filtered through a 0.2 μm membrane filter using a syringe and rinsed with 100% ethanol to collect the cells. The filter was placed on a copper grid with tweezers that was submerged in 100% ethanol. Standard procedures were followed for critical point drying using Samdri Critical Point Dryer (Tousimis Research Corp. Rockville, MD). Steps are listed in Appendix A. After the cells were dried, the filter was stuck to a copper stand using double sided tape. The filter was then coated with gold using SPI Module Sputter Coater (SPI Supplies, Structure Prope Inc., West Chester, PA). Steps are listed in Appendix B. Images of the healthy and damaged cells were obtained using the Zeiss 1450EP variable pressure Scanning Electron Microscope (Carl Zeiss Microimaging, Inc. Thornwood, NY) under high vacuum mode of 2600Pa.

RESULTS AND DISCUSSION

In this study, *Salmonella* spp. were exposed to extreme cold temperatures for a period of time, extending to 5 days, while suspended in frozen tap water. Once thawed and enumerated, the number of cells that survived the treatment, both injured and healthy cells, were determined using the formula listed in Table 4.1. This formula was used by Hartsell who was one of the first

investigators to define injured cells as those capable of forming colonies on nonselective media but not selective media by using frozen *S*. Oranienburg cells (8, 18). This calculation is useful because it can provide information about the relative level of injury rate within a population since bacteria in natural systems are thought to respond to stress individually (8, 18). In this study, XLD was used as the selective media to recover the uninjured cells and TSA was used as the nonselective media to recover all cells that survived, uninjured and injured *Salmonella* cells (Figure 4.1). It was determined that after 24 h of freezing, there was 74.0% lethality of the *Salmonella* cell population while of the 26.0% viable survivors, 26.9% remained uninjured and 73.1% of the cells were injured, as they failed to form colonies on the selective media (Table 4.2). After 120 h, there was a slight increase in the percentage of cell death (74.0% to 76.5%) while there was also an increase in the number of injured cells (73.1% to 88.6%). This corresponds with the decrease in the percentage of uninjured cells after the extended period in freezing temperatures (26.9% to 11.4%). Relating to past investigations, it has been seen that cell injury and death occur more rapidly during initial storage but subsequently slows down as storage time under a stressful environment continues (19, 21). As time progresses, the uninjured cells may lose their ability to resist the damaging effects of the freezing temperatures and cell functionality and stability may eventually become disrupted but may not succumb to total loss of function resulting in death (19, 21).

Studies have looked at the effect of freezing on various *Salmonella* serotypes but under different conditions and substrates (17). The studies that used water as a substrate had percent cell injury ranging from 30 and 40% to 88 and 90%. The differences can be contributed to the different serotypes used within these studies as each serotype may have unique responses and resistance to the freezing temperatures and thawing conditions (16, 18).

The food industry uses freezing as a strategy to halt pathogen growth, while realizing it is not a reliable means for microbial lethality. Different strains of microorganisms differ greatly in sensitivity and resistance to freeze/thaw damage, as freezing could be lethal to some bacteria while causing only moderate stress to others (18, 22). Additionally, various strains of bacteria respond differently to the exposure to an aquatic system, specifically tap water that is treated with a sanitizer, commonly chlorine (18). The highly variable characteristics of different water types result in a number of chemical and physical factors in the drinking water treatment system that is known to cause sublethal physiological and structural changes to bacteria. These stresses may differ to the ones found in food, such as moisture reduction, varying pH, preservatives, etc. (18).

Salmonella spp. are gram-negative facultative anaerobes that belong to the Enterobacteriaceae family and have the ability to cause life-threatening infections in humans and are the leading cause for hospitalizations in regards to foodborne illnesses (6). Approximately 10% of these infections were found to be waterborne related (6). This pathogen has the ability to survive in various environments and known to survive in water and ice for weeks.

Past investigations have determined that along with other various physiological responses from an injured cell, the cytoplasmic membrane is a frequent site of cellular damage (4, 17, 22). Damage caused by freeze injury is caused from the continuous exposure to concentrated solutes and physical damage caused by extracellular ice crystal formation (21, 22). This stress causes structural modifications and morphological changes in cellular shape of *Salmonella* cells (12, 13, 14).

In general, the nature of the bacteria defines its susceptibility to freezing and the lethality after exposure. Gram-negative bacteria, such as *Salmonella* spp. are known to be more subjected

to the damaging effect of freezing than gram-positive bacteria due to the components on the outer membrane and cell wall (4, 6, 15, 22). Gram-positive cell walls are comprised of several layers of peptidoglycan that are connected by cross-linkages and contain teichoic acids and usually have a higher resistance to freeze-damage (4, 6, 19, 22). In contrast, gram-negative cell walls have a thin layer of peptidoglycan and are less sturdy and more prone to damage (4, 6, 19, 22). The differences can also be contributed to the O antigens that are found on the cell surface that consist of the lipopolysaccharide-protein chains and the diverse responses following exposure (18, 22).

Damage to the outer membrane of gram-negative cells causes the release of lipopolysaccharides, lipids, phospholipids, cations necessary for lipopolysaccharide stability, and periplasmic enzymes that may disrupt the membrane permeability (4, 9, 19, 22). Changes in permeability and phase changes in the membrane may induce the formation of small, hydrophilic pores in the outer membrane, which causes intracellular material, such as macromolecules, amino acids, ions, etc. to leak out, to alter nutrient transport across the membrane, and increases ion sensitivity (4, 9, 19, 22).

Figures 4.2-6 are images obtained using the SEM of each *Salmonella* serovar employed in the cocktail during part one of the study. Images on the left of each figure are cells that were refrigerated and suspended in peptone water, without inducing any stress. The cell membranes are intact without noticeable damage that would threaten the cell's function and viability. Images on the right of each figure are cells that were frozen in peptone water overnight and then thawed at room temperature. The arrows indicate examples of injured *Salmonella* cells. The cell membranes are visibly damaged with the loss of structural stability and integrity resulting in a

collapsed cell. This type of damage will result in the change of membrane permeability and temporary or permanent loss in viability and pathogenicity.

The type of organisms that lose their viability in this injured state differ from strain to strain and depend on the type of freezing employed, the type of water, the length of time of freezer storage, and other factors, such as temperature of freezing (4, 11). The bacteria's ability to successfully handle environmental stress, such as freezing and waterborne stress, partially defines its virulence, since the response the stress often includes the expression of various virulence factors (3, 22). Injured cells have the ability to undergo self-repair which requires specific biochemical events that differ based on the type and degree of stress (22). General cellular repair, when exposed to an optimal environment, includes the synthesis of ATP, DNA, RNA, proteins, and the reorganization of macromolecules, and in the case of gram-negative bacteria, of lipopolysaccharides (10, 18, 21, 22). The repair of the cell membrane through lipid synthesis needs to occur quickly so that cells can fully repair from the stress-induced lesions and pores (4, 22). Normal cellular functions may be reestablished by the synthesis of the specific stress proteins, cold shock proteins (CSP). CSPs play a critical role in various cellular functions and assists in the maintenance of the membrane at low temperatures which is necessary for cold adaption and the survival of injured and uninjured *Salmonella* cells (22). For this reason, injured *Salmonella* cells that may go undetected during routine quality control checks during processing because they failed to grow on selective media, poses a significant health risk because of the probability the cells will undergo repair and regain its virulence and result in an increased resistance to treatments.

CONCLUSION

Freezing as a method to reduce or halt the growth of pathogens in food or water has been deemed an insufficient treatment to improve consumer safety. This study showed that the freezing of *Salmonella* cells resulted in a 70-80% kill rate while of the survivors, 70-85% of the cells were injured after an extended storage period. The percentage of healthy and injured cells in a population is based on the pathogen's ability to form viable colonies on selective media versus nonselective media and their response to stress resulting in damaged cell membranes and the changed cell shape or composition. Microbial injury is important to food safety for injured cells may not be detected at the time of post processing sampling and may undergo repair if given an appropriate environment, resulting in a safety or spoilage problem in food and water, posing a health risk to consumers.

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Table 4.1. Calculations implemented by Hartsell et al. to determine the percentage of injured cells induced by freezing temperatures (8).

Percentage (%)	*Equation*
Killed	$\left[1-\left(\frac{CFU\ on\ nonselective\ at\ Hour\ X}{CFU\ on\ nonselective\ at\ Hour\ 0}\right)\right]\times 100\%$
Survivors	$100\% - \%Killed$
Injured	$\left[1-\left(\frac{CFU\ on\ selective\ at\ Hour\ X}{CFU\ on\ nonselective\ at\ Hour\ X}\right)\right]\times 100\%$
Healthy	$100\% - \%Injured$

Table 4.2. The percentage of the *Salmonella* population in ice that was killed or survived the freezing temperatures and of those, the percentage of injured cells after each time interval.

Hours of Storage (h)	% of Initial Population		% of Survivors	
	Dead	Survivors	Uninjured	Injured
24	74.0	26.0	26.9	73.1
72	77.2	22.8	14.5	85.5
120	76.5	23.5	11.4	88.6

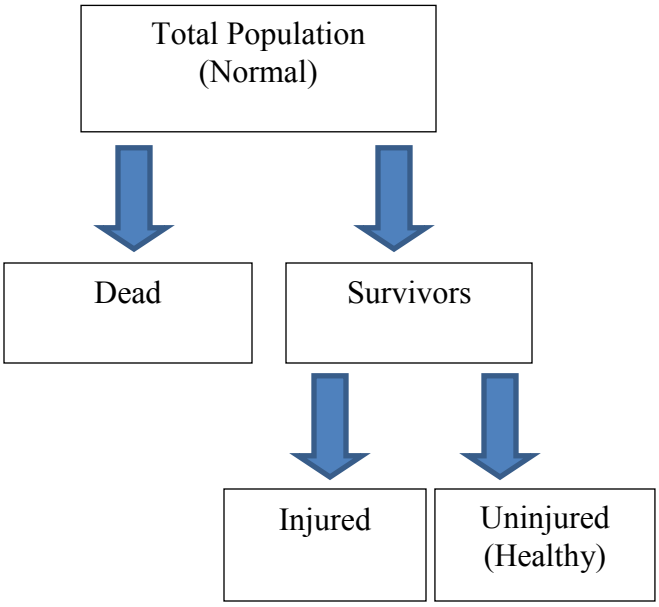

Figure 4.1. The basic overview of the possible fates of *Salmonella* cells induced by the freezing temperatures

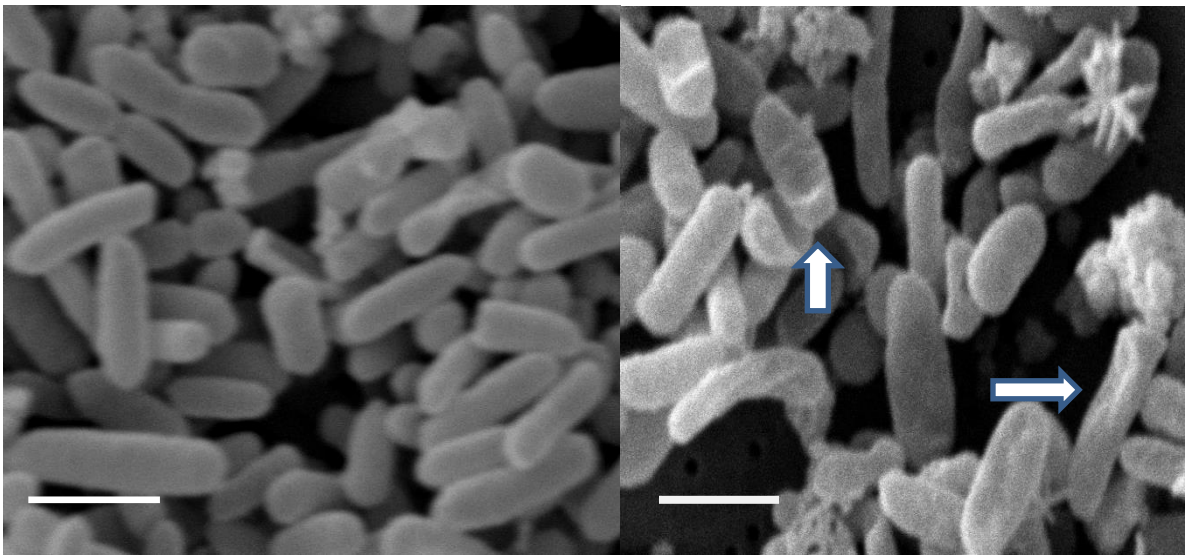

Figure 4.2. Scanning electron image of *Salmonella* Enteritidis cells that were grown under optimal conditions (left) or exposed to freezing temperatures for 24 h (right).
Left to Right: Healthy, fully intact cells at 42k magnification; Collapsed cells with visible damage at 35k magnification. Bar represents 2 µm

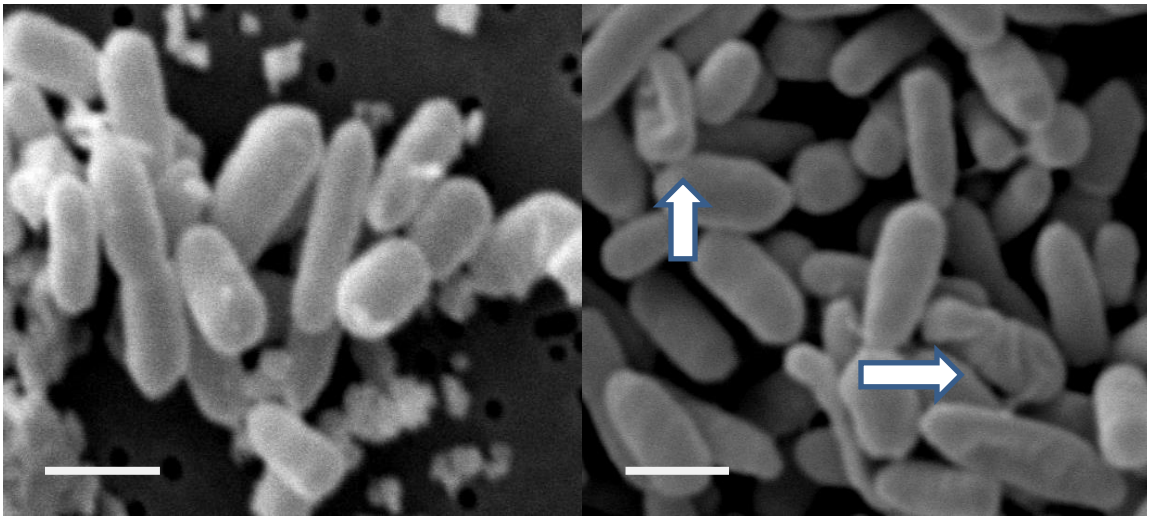

Figure 4.3. Scanning electron image of *Salmonella* Montevideo cells that were grown under optimal conditions (left) or exposed to freezing temperatures for 24 h (right).
Left to Right: Healthy, intact cells at 31k magnification; Damaged, collapsed cells at 37k magnification. Bar represents 2 µm

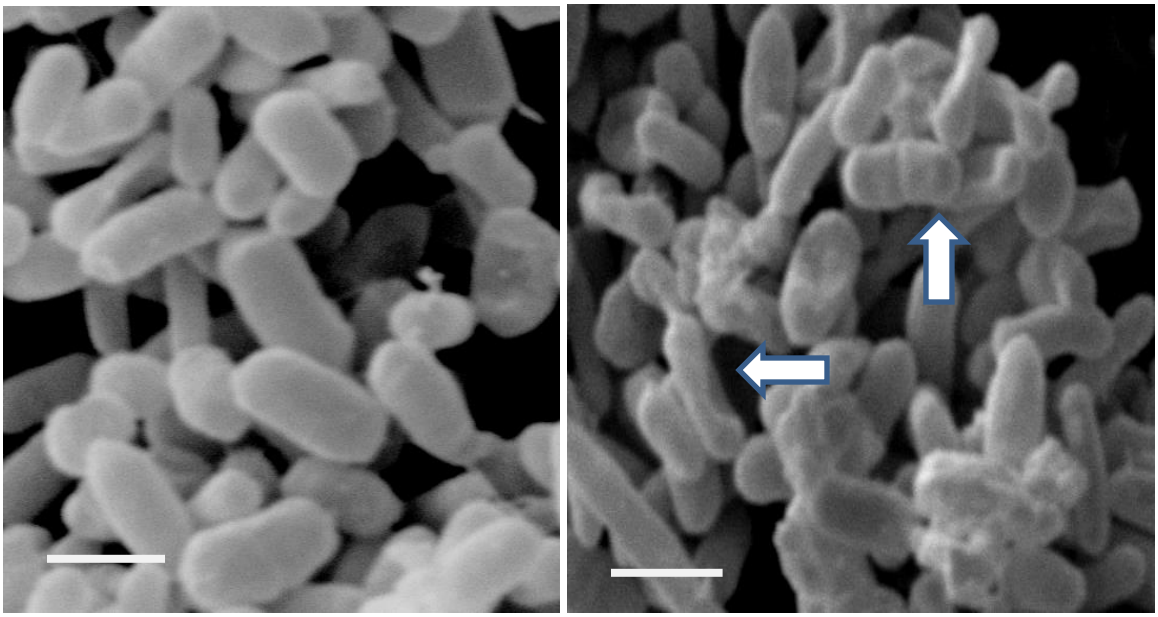

Figure 4.4. Scanning electron image of *Salmonella* Poona cells that were grown under optimal conditions (left) or exposed to freezing temperatures for 24 h (right).
Left to Right: Healthy cells at 30k magnification; Damaged, altered cell conformation at 25k magnification. Bar represents 2 µm

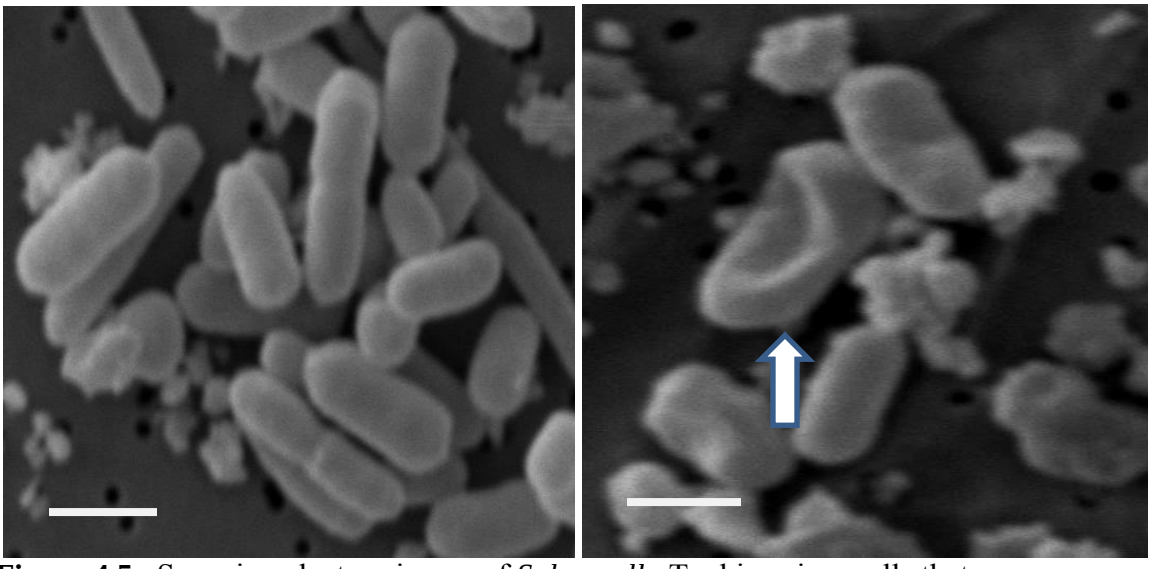

Figure 4.5. Scanning electron image of *Salmonella* Typhimurium cells that were grown under optimal conditions (left) or exposed to freezing temperatures for 24 h (right).
Left to Right: Healthy cells at 30k magnification; Collapsed cell at 35k magnification. Bar represents 2 µm

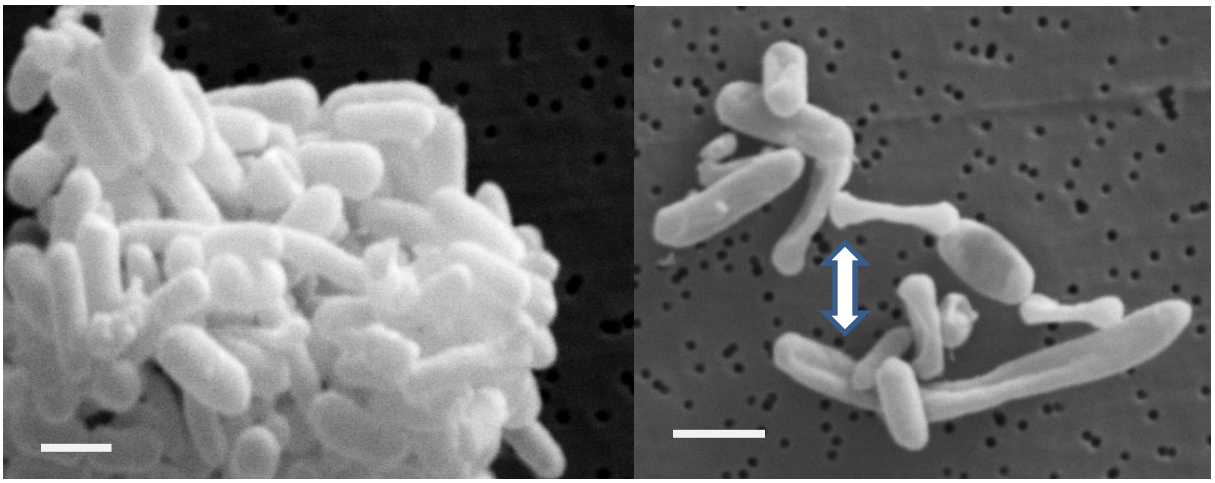

Figure 4.6. Scanning electron image of *Salmonella* Anatum ceiis that were grown under optimal conditions (left) or exposed to freezing temperatures for 24 h (right).
Left to Right: Healthy cells at 16k magnification; Damaged, collapsed cells at 22k magnification. Bar represents 2 μm

CHAPTER FIVE

CONCLUSION

Overall, this thesis supports that freezing as a method to reduce or halt the growth of pathogens in food or water is an insufficient treatment to improve consumer safety. It was seen that the freezing of *Salmonella* cells resulted in only a 70-90% kill rate while of the survivors, 70% of the cells were injured after an extended storage period. Microbial injury is important to food safety for injured cells may not be detected at the time of post processing sampling and may even undergo repair if given an appropriate environment. The presence of unacceptable levels of indicator organisms as well as *Salmonella* in this survey of packaged ice from retail establishments and self-service vending machines from around Georgia indicate the need for improved guidance in proper sanitation techniques. Training and educating workers at these locations about appropriate hygienic practices, the importance of regular cleaning and sanitizing, the risks of transferring contaminated water and ice, and prevention techniques they can take to avoid causing any foodborne illness, should be a significant part of an establishment's training program.

APPENDIX

A. Procedure for Critical Point Dryer

1. Place enough 100%, dry ethanol in the chamber to cover the sample completely.

2. Make sure that O-ring is in place and screw down cover securely.

3. Open main valve on CO_2 tank. Carefully open the cool valve enough to let CO_2 cool the chamber to around 0°C.

4. Once chamber is cooled, close cool valve and carefully open the inlet valve so that a very small stream of liquid CO_2 fills the chamber completely. Once full, open the inlet valve a few more turns.

5. Leaving the inlet valve open, slowly open the purge valve until ethanol can be seen exiting from the Tygon tubing. If temperature of chamber reaches above 10°C, open the cool valve to make it decrease.

6. Once ethanol is completely gone and only CO_2 appears to be spraying out of the tube, close the purge valve.

7. Make sure the chamber is completely full of liquid CO_2 and then close the inlet valve.

8. Make sure all valves are closed, including the main CO_2 tank valve.

9. Turn on both switches. Wait for the temperature to reach 34-38° C and the pressure to reach 1250 psi.

10. Once the temperature and pressure have been reached, let the unit maintain this state for another 2 min.

11. Begin to bleed the gaseous CO_2 by slowly and carefully opening the bleed valve. The pressure should be allowed to escape slowly, around 100 psi/min.

12. Once the unit has reached 250 psi or lower, open both bleed and purge valves to allow the chamber to equilibrate with atmosphere and then carefully open the chamber and remove the specimen.

13. Close all valves and turn both switches to off.

14. Replace cover with or without the O-ring to keep the chamber free of debris.

B. Procedure for SPI-Module Sputter Coater

1. Carefully remove Target Head and place in box provided. Remove and place glass chamber on lint free cloth. Place samples on stage area. Replace glass chamber making sure it is sitting firmly on O-ring; then carefully place target on top to fit snugly.

2. Press on mechanical pump toggle switch and gently but firmly press down on target head to make sure a good seal has been obtained.

3. Press SPI-Module control power switch on. Wait until gauge reads slightly below 2 MBAR.

4. Press SPI-Module sputter coater power switch to on. Briefly press and hold test button and adjust gas leak valve on control unit to where the milliamps read 15 on the plasma current gauge. Turn time-second dial to 60 seconds. Push start. If milliamp needle fluctuates up and down, turn gas leak valve until it remains at the 15 milliamp mark and let coat.

5. Once coating is completed, press both sputter coater and control power switches off. Then press mechanical pump switch off. Slowly turn vent valve and bleed air into chamber. This is done slowly, so fine grained or small particulate samples will not blow off into the chamber. Once vented, make sure vent value is closed and place target head back on box and glass chamber on cloth. Remove samples and replace target head and glass chamber back on coater stage.