Quantitative Recovery of *Listeria monocytogenes* and *Salmonella enterica* from Environmental Sampling Media

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Abstract

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Environmental sampling is a pathogen monitoring technique that has become important in the food industry. Many food processing companies have adopted environmental sampling as a way to verify good manufacturing practices and sanitation plans in their facilities. Environmental sampling is helpful because it gives better information on the source of product contamination than end product sampling. Two specific pathogens of concern to the food industry are *Listeria monocytogenes* and *Salmonella enterica.* Environmental samples are rarely analyzed immediately, but instead may be batched for later analysis or shipped to an off site testing facility. Multiple media on the market today is used for storage and transport of environmental samples. These various media types, differences in holding temperatures and time create variability in test sample conditions. Select time, temperature and media combinations were tested to determine their effect on *Listeria monocytogenes* and *Salmonella enterica* populations during transport and storage of samples. Cocktails of *Listeria monocytogenes* and *Salmonella enterica* were added separately to sample tubes containing D/E Neutralizing Broth, Neutralizing Buffer or Copan SRK Solution. Bacterial counts at 0, 12, 24 and 48 hours post inoculation were compared. Neutralizing Buffer and Copan SRK Solution maintained consistent bacterial populations at all

temperatures. At 10° and 15°C, D/E Broth supported bacterial growth. This study helps validate the use of D/E Neutralizing Broth, Neutralizing Buffer and Copan SRK Solution for environmental sample transport and storage at proper holding temperatures. At temperatures >10°C Neutralizing Buffer or Copan SRK solution should be used if quantifying microbial recovery.

Dedication

I dedicate this work to my mother and father for all of their support throughout my growing up and their commitment to being there for me throughout my education.

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I. Introduction

Environmental sampling has become a very important tool in all aspects of biosecurity. It is a way for investigators to determine the level of contamination in a site that is being investigated, as well as what track the contaminant may be taking in entering the site under investigation.

In the food industry, environmental sampling has become a common method for detection of some pathogens such as *Listeria monocytogenes* and an additional tool for sampling some other organisms such as *Salmonella enterica*. In some cases, this type of sampling minimizes the need for end product sampling, which can reduce costs for the food processors. Also, it can give the investigators a good idea of what might be causing product contamination and where the contaminant may have entered the food production line.

Two major pathogens of concern in the food industry are *Listeria monocyotgenes* and *Salmonella enterica*. These two pathogens are often sampled for in food processing facilities and are major concerns for public health officials. *Listeria monocytogenes* is a foodborne pathogen that is associated with many types of food and has become an increasingly important issue recently. Listeriosis, the disease associated with an infection with *Listeria monocytogenes* has a high mortality rate, especially among pregnant women and the immunocompromised (Brooks et al., 1998). Because of multiple recent outbreaks of listeriosis throughout the country, and the subsequent public outcry, the United States Department of Agriculture's Food Safety Inspection Service (USDA-FSIS) released a directive in 1999 and updated it in 2002 to assess *Listeria monocytogenes* as a pathogen of concern in the food industry that must be considered when food facility

safety procedures and HACCP plans are created (FSIS, 1999). In 2002, this directive was updated to further classify the risks associated with various food processing plants and to announce that all environmental sampling data that was collected by a food processing plant would now have to be shared with the USDA-FSIS (FSIS, 2002).

Salmonella enterica has long been a concern in the food industry because of its pathogenesis in humans. *Salmonella* can cause a variety of diseases from salmonellosis to typhoid fever (Salyers and Whitt, 2002). Many strains of *Salmonella enterica* are associated with human diseases, but the three serotypes used in this experiment represent the strains most readily associated with infection (Typhimurium, Enteritidis) and most dangerous to the patients they infect (Typhi).

Another reason for the selection of these two organisms for this investigation was their different cell envelope structure. *Listeria monocytogenes* is a gram positive organism with a thick outer peptidoglycan layer covering its cell membrane in its cell envelope. *Salmonella enterica* is gram negative and has an outer and inner membrane encapsulating a much thinner peptidoglycan layer in its envelope. (White, 2000) Also, *Salmonella enterica* and most gram negative organisms have a convoluted outer cell wall which gives it a much higher surface area/volume ratio than a gram positive cell wall. (Madigan et al., 2000)

One of the issues of concern with environmental sampling is its reliability. There have been several research reports optimizing sampling techniques and procedures. One aspect of the process that has not been widely studied is the storage and transport of environmental samples between the times they are taken (e.g. swabbed) and the time they are processed (e.g. plated onto microbiological media). In many cases, environmental

samples are shipped to off site labs and may be in the transport and storage chain for up to 48 hours. This delay is critical to the reliability of the tests.

There are many types of transport media that are often used for environmental sampling. Two of the most commonly used media were used in this research, as well as a new medium that is just being introduced. The two common transport broths tested were Dey / Engley Neutralizing Broth (D/E Broth), and Neutralizing Buffer. The third medium tested was a Copan Swab Rinse Kit transport solution.

The time between when environmental samples are taken and when they are processed in a lab can vary depending on the facility being tested and the distance between the facility and the lab where it will be tested. In some cases, processing facilities have labs on site for immediate sample analysis; however there may be a delay due to the need for batching samples before testing. In some cases, a plant may not have a lab on site and will need to ship these samples to an off-site test lab. This can take up to two days.

The temperature at which samples are held during storage and transport may vary as well. In most cases, samples in transit are refrigerated, but in some cases refrigeration temperature (0 to 4°C) may not be maintained throughout the duration. In other cases, the samples may be kept at freezer temperatures (-10 to -4°C).

These variables are important to investigate with regards to environmental sampling. It is important to develop an optimum time/temperature/medium combination for the recovery of various microorganisms of interest. It is also important to determine what combinations will best maintain cell populations, in the event that a quantitative analysis of contamination in a processing facility is desired.

The purpose of this research was to improve environmental sampling protocols by determining optimal sample transport and storage variables. The pathogens of concern that were used for the research are often the target of environmental sampling and offer a public health risk that is important to the food processors. The two different organisms tested offer a variance in cell envelope physiology and therefore are representative of two different classes of bacteria.

II. Review of Literature

A. *Listeria monocytogenes*

1. Characteristics and nature of disease

The *Listeria* species of bacteria are non-spore forming, gram positive bacilli (FDA, 2004a). They are ubiquitous in the environment and present in the intestinal tract of many animal species (Blackburn and McClure, 2002). *Listeria* are facultatively anaerobic organisms. They are also psychrotrophic which is an important competitive advantage for growth at low temperatures compared to most food contaminant species.

There are many species of *Listeria* but only certain serotypes of *Listeria monocytogenes* species are pathogenic to humans (Blackburn and McClure, 2002). *Listeria monocytogenes* has been isolated from such various sources as soil, dust, animal feed, water, sewage and many animals (Cliver, 1990). It has been found in 37 species of mammals and 17 species of birds. Some fish and shellfish are also possible carriers of the organism. It has been suggested that 1-10% of humans are intestinal carriers of the organism as well (FDA, 2004a).

The major vector for human infection from *Listeria monocytogenes* is food. Because of it's prevalence throughout the environment, *Listeria monocytogenes* can come in contact with food products in just about any raw processing procedures (3). Food processors must take this into consideration when developing food safety plans for their facilities. *Listeria monocytogenes* is a psychrotrophic organism. Because of this, *Listeria monocytogenes* is also at a competitive advantage against other microorganisms in food processing facilities that tend to operate under cool temperatures. This adaptive feature also helps the organism survive and grow during refrigeration of food products.

Listeriosis is the disease caused by infection by the organism *Listeria monocytogenes*. Listeriosis is a systemic infection that can cause meningitis, bacteremia, sepsis and focal infections. (Cliver, 1990) It is also commonly associated with spontaneous abortions in infected pregnant women. Listeriosis annually accounts for nearly 1600 illnesses and 415 deaths (FDA, 2004a). This high mortality rate of infected individuals (close to 20%) is of major concern to the public health and food industry. In addition, the fact that it has such disturbing effects on pregnant women and children makes *Listeria monocytogenes* an increasingly prevalent topic in the press.

Listeria monocytogenes method of pathogenesis is relatively simple. It enters the body through consumption of contaminated food. Internalin, a surface protein on the *Listeria monocytogenes* cell interacts with receptors in the intestinal lining and induces phagocytosis of the bacterium (Brooks et al., 1998). Once inside the host cells, the bacterium can use host cell actin to propel them towards the host cells membrane which they push on and create elongated protrusions into neighboring cells (Brooks et al., 1998). The reason the bacteria must use host actin for propulsion is that although *Listeria monocytogenes* have flagella, they are only functional at temperatures below human body temperature (Salyers and Whitt, 2002). In this way, the bacteria are able to spread throughout the cells while avoiding contact with antibodies, complement or polymorphonuclear cells. Other bacteria such as *Shigella flexneri* and *rickettsiae* use similar methods of infection (Brooks et al., 1998).

A gastroenteritis associated with the consumption of *Listeria monocytogenes* has also recently been recognized. This gastroenteritis affects healthy individuals and usually presents symptoms more than 12 hours after the consumption of the contaminated food.

The symptoms are typical of febrile gastroenteritis (IFT, 2004). One reason why this emergence of gastroenteritis as a disease symptom of *Listeria monocytogenes* infection is that it, in some cases, can be an early indicator of systemic listeriosis. The onset of acute gastroenteritis has been shown to be only one of the early symptoms that can indicate more serious disease (IFT, 2004).

2. Presence in food and food processing environment

Listeria monocytogenes has become a major problem in the food industry for a couple of reasons. One major reason that *Listeria monocytogenes* is prevalent in the food industry is its ability to survive and grow at refrigeration temperatures. It can grow at temperatures from 2°C to 45°C (FDA, 2004a). This means that it can survive on ready to eat meats and cheeses and can readily grow when these food products are under refrigeration. *Listeria monocytogenes* can also grow at a relatively broad pH range, from 4.8 to 9.6 (FDA, 2004a). It has been shown to survive for long periods of time at a pH of 5 in such products as cheddar cheese (Ryser and Marth, 1987).

Typical methods for the treatment of wastewater may favor the growth for *Listeria monocyotgenes*. Specifically, bacterial oxidation may help select for the survival of *Listeria monocytogenes* (IFT, 2004). The production of sludge and sewage plant effluents are also conducive to the survival of *Listeria monocytogenes* and therefore the use of these waters for irrigation of crops or any contact with edible foods may be dangerous and controlled (IFT, 2004).

Listeria monocytogenes has been isolated from a number of food sources and environmental sources. The most common foods associated with *Listeria monocytogenes*

include deli meats and soft cheeses. Raw milk has also been associated with *Listeria monocytogenes*, especially when further processed into soft cheeses (IFT, 2004).

Because of this prevalence in nature, it is not uncommon for this organism to be found throughout the food processing facility. It also has very good attachment capabilities and can therefore attach to many food contact surfaces made of various materials. In addition, *Listeria monocytogenes* has been shown to be able to form biofilms on solid food contact surfaces (IFT, 2004).

In a report by the IFT Expert Panel on Food Safety and Nutrition, biofilm production and it's affect on food safety was discussed. In this report, the authors sited several studies that showed *Listeria monocytogenes* as a common producer of biofilms on all types of food contact surfaces (IFT, 2004). This biofilm production has also led to an increased amount of resistance for *Listeria monocytogenes* against common sanitation procedures. These factors heighten the risk of *Listeria monocytogenes* contaminating food products in a food processing facility.

In late 2000 and early 2001, an outbreak of listeriosis occurred in North Carolina. This outbreak was associated with Mexican style soft cheeses. Twelve cases were identified, 11 women and one immunocompromised man. Ten of the women were pregnant and the infection caused five stillbirths, three premature births and two newborns that were infected. The infected people had all purchased and consumed unlabeled Mexican style soft cheese from local markets or vendors. The vector for these infections was likely unpasteurized milk that was used to make the cheese (CDC, 2001).

Another major outbreak with Mexican style soft cheese occurred in 1984 resulting in 86 illnesses. This outbreak occurred in Los Angeles and Orange County California.

The illnesses were associated with the consumption of cheese that had been sold in a market in the area. Twenty-nine deaths occurred from this outbreak, 13 of which were stillbirths. The company implicated in the outbreak implemented a voluntary recall on the product as well (CDC, 1985).

Also in 2000, an outbreak of listeriosis took place over ten states. 29 total cases were reported (eight of which were perinatal). All of those affected had recently purchased deli turkey products from local delis. This outbreak was investigated by the USDA and the product that was common to all of the delis in which the implicated food had been purchased from had originated from a common source in Waco, Texas. This led to a large recall by the company and a stoppage of shipment of these ready to eat meat products (CDC, 2000).

In late 2002, another multi-state outbreak of listeriosis occurred. This outbreak led to the new USDA-FSIS directive that is discussed in this paper. This outbreak occurred across eight states in the northeastern United States. There were a total of 46 cases, including seven fatalities and three stillbirths. The food associated with the outbreak was a sliceable turkey meat available at delis. Under further investigation, 25 environmental samples from the processing plant where the product originated were positive for *Listeria monocytogenes*. The investigation also showed that there was one positive food sample taken from the plant by the investigators. The company implicated in the contaminated meat products not only suspended operation, but issued a recall in response to the outbreak of 27.4 million pounds of product. This was the largest recall of product by a food company to date (CDC, 2002).

The variability of these outbreaks shows how *Listeria monocytogenes* is a major issue in food safety. It shows the severity of the health impact that contaminated product can have as well as the economic impact it can have on producers in the form of recalls and suspended operations. *Listeria monocytogenes* is a hearty organism that has shown it can contaminate many food products and survive well in the food processing environment. Environmental sampling is an important way to monitor for this pathogen and increase consumer safety and confidence regarding it.

B. *Salmonella enterica*

1. Characteristics and nature of disease

The *Salmonella* species of bacteria are gram negative bacilli. The majority of the subspecies of *Salmonella* are motile with the exception of two serovars (*S. gallinarum* and *S. pullorum*). All *Salmonella* species are non-spore forming (Holt, et al., 1994). *Salmonella* is divided into only two subspecies, and all of the pathogenic forms of *Salmonella* fall under one of the species types, *Salmonella enterica*. The two most well documented disease causing serovars of *Salmonella enterica* are *Salmonella enterica* serovar Typhimurium, and *Salmonella enterica* serovar Enteritidis (IFT, 2004). Additionally, *Salmonella enterica* serovar Typhi is the causative agent of typhoid fever, the most dangerous disease associated with *Salmonella*. *Salmonella* can be found in many sources. The most common natural habitat of the bacterium is the intestinal tract of host animals such as poultry and swine. The bacteria can also be found in many environmental sources such as water, soil and in some cases factory and kitchen surfaces (FDA, 2004a).

Salmonella is a pathogen that is most commonly associated with contaminated food and has been linked to many sources. Some of these sources are raw meats, raw poultry, raw seafood, eggs and egg products (FDA, 2004a). To a smaller extent, chocolate, peanut butter and cocoa have been implicated in some outbreaks involving this organism (IFT, 2004).

The disease caused by *Salmonella enterica* serovar Typhimurium is salmonellosis. This is a food borne infection and not an intoxication (Madigan et al., 2000). The mode of infection usually involves the consumption of contaminated food or water. Symptoms usually occur 8-12 hours after consumption of contaminated foods. The infection leads to symptoms such as headaches, vomiting, nausea, diarrhea and sometimes fever. In some rare cases, bacteremia and shock can occur, but the patient typically improves within 2-3 days (Salyers and Whitt, 2002; Brooks et al., 1998). Another serovar of *Salmonella* that is associated with salmonellosis is *Salmonella enterica* serovar Enteritidis. *Salmonella enterica* serovars Typhimurium and Enterica are the most common ones found in surveillance of human sources (IFT, 2004).

Salmonella enterica serovar Typhimurium has a relatively high infective dose of around 10^5 cells for human infection. After the consumption of the bacteria, the bacterial cells will invade the mucosal cells of the victim's large intestine, or the M cells of the ileum of the victim's small intestine. Once inside this lining, the bacteria then enter the lamina propria. Unlike most bacterial causes of diarrhea, *Salmonella enterica* serovar Typhimurium does not secrete a toxin to produce the diarrhea. Instead, like enteropathogenic *Escherichia coli*, the bacteria release proteins into the underlying intestinal cells that disrupts there typical functions. In response to this, the cells produce

cytokines that attract polymorphonuclear leukocytes to the cell. The release of prostoglandins by these PMNs leads to an increase in cAMP activity in the cell. This increases cAMP presence, leads to a decrease in the absorption of sodium ions and a release of chlorine ions. This causes a loss of water from the tissues leading to diarrhea (Salyers and Whitt, 2002; Brooks et al., 1998).

Salmonella enterica serovar Typhi is also of great concern because it is the causative agent of typhoid fever. The infection may take as long as 2-3 weeks to show symptoms. Symptoms of typhoid fever include high fever, chills, flushing, loss of appetite, convulsions, and in some cases delirium (Salyers and Whitt, 2002). Typhoid fever has caused many deaths throughout the world and still has a high mortality rate in developing countries (Brooks et al. 1998). Another major concern with this species is that people infected with it can spread the bacteria for years as it is shed in their feces. Also, people can become carriers of *Salmonella enterica* serovar Typhi and not show symptoms at all but still spread the disease (Salyers and Whitt, 2002).

Typhoid fever is caused by the consumption of cells usually from contaminated food. The infective dose for this organism is lower than that of *Salmonella enterica* serovar Typhimurium. The infective dose is around 10^3 bacteria (Brooks et al., 1998). After the bacteria are consumed, they enter the lumen of the small intestine and from there enter the local lymphatic system. Colonization of the bacteria usually occurs in the liver and spleen and the bacteria spread to the bloodstream. From these two areas they can spread throughout the body (Brooks et al., 1998). After a period of time that may be a week to a month, the patient becomes symptomatic. The first symptoms are fever, loss of appetite and lethargy. The bacteria can then move on to infect the gall bladder. As the

infected gall bladder releases bile into the intestine, bacteria are shed as well. These bacteria can cause severe ulcerations in the intestine which in some cases may lead to death (Salyers and Whitt, 2002). Most cases of Typhoid fever are recognized and treated quickly in the United States and fatality is rare, however this is not the case in developing countries.

2. Presence in foods and food processing environment

Salmonella spp. is a major issue of concern in the food industry because the organism is present throughout all levels of food production. *Salmonella* may be found in the intestines of many types of animals especially poultry and swine, and can be transferred to food contact surfaces. There are also many environmental sources of *Salmonella* spp. such as water, soil, insects, animal products and factory surfaces (FDA, 2004a).

Salmonella outbreaks have been associated with many different types of foods. These sources range from raw meat products such as beef, poultry or seafood, to prepared foods like cake mixes and salad dressings. There has also been some food outbreaks associated with fermented foods such as cocoa and chocolate. The most common source of *Salmonella* spp. in foods is poultry and egg products (IFT, 2004).

One example of a food borne illness outbreak associated with *Salmonella* spp. occurred in 1994 in Dodge County, Wisconsin. In this incident, doctors reported an unusually high number of people exhibiting symptoms typical of a *Salmonella* infection. These patients had eaten raw ground beef in the three days before they felt sick. By the end of the investigation, the Dodge County Human Services and Health Department and

Wisconsin Division of Health had confirmed 107 cases of salmonellosis and identified and additional 51 probable cases. The meat that had been consumed was linked back to a butcher shop, and further back still to three different beef suppliers. Although environmental samples at the suppliers were negative, a deficiency in the sanitizing of meat grinders at the butcher shop was the likely source of the contamination (CDC, 1995).

Another outbreak occurred in 1995 in Nevada and was associated with a private Thanksgiving dinner that had been hosted. During the days following this dinner, all six persons who had attended the dinner came down with salmonellosis and an additional person who had consumed leftover food from the dinner also became ill. Two people were hospitalized during this outbreak and a third was found at home comatose and later died from dehydration. The outbreak was linked to undercooked turkey and possibly the eggs used in the stuffing in the turkey, as these were the only food products consumed by all of the people affected by illness (CDC, 1996b).

An outbreak specifically associated with *Salmonella* Typhimurium occurred in 2002 and 2003. This outbreak was connected to the consumption of unpasteurized milk from a farm in Ohio. Patients in four states were affected by this outbreak. The unpasteurized milk was served at a local farm that had a snack bar and dairy on site. 62 persons reported illness, 40 of which were customers, 16 employees and six household contacts. Samples from the dairy were also analyzed and five tested positive for *Salmonella* Typhimurium. Stool samples of the animals and environmental samples all came up negative. This outbreak resulted in recommendations from the Ohio Department of Agriculture to eliminate the sale of raw milk products (CDC, 2003).

Salmonella Enteritidis infections have been steadily increasing in number. *Salmonella* Enteritidis isolates increased in proportion of total isolates from 5% to 26% between 1976 and 1994. Also over the ten year period from 1985 to 1995, 24,058 cases of illness were reported involving 582 outbreaks of *Salmonella enteritidis*. Over two thousand hospitalizations and 70 deaths occurred during these outbreaks (CDC, 1996a). This data indicates the increasing importance of this *Salmonella* serotype in food safety.

In 2003 and 2004, an outbreak of *Salmonella* Enteritidis occurred in the United States and Canada. The outbreak was associated with raw almonds that were distributed throughout the northwest United States and in Canada. 29 total patients in 12 states as well as Canada were shown to be infected by the same strain of *Salmonella* Enteritidis. Seven of the patients were sick enough to warrant hospitalization but none died. When investigating the almond producers, and almonds in the patient's homes, no isolates of *Salmonella* Enteritidis were found, but two equipment samples and one environmental sample were positive for *Salmonella* Enteritidis. A voluntary recall was initiated by the distributor of the almonds. This was the second such outbreak of *Salmonella* Enteritidis associated with raw nuts. In 2001, over six months, raw almonds were associated with another outbreak. These almonds were not linked to the almonds associated in this most recent outbreak. PFGE typing was done to analyze the isolates associated with each outbreak (CDC, 2004).

Some other significant outbreaks associated with *Salmonella* Enteritidis occurred in 1994 – 1995. One outbreak was associated with raw shell eggs that were used to prepare hollandaise sauce at a hotel in Washington DC. 56 persons reported symptoms and 20 were hospitalized. In 1995 another outbreak occurred in a nursing home in

Indiana where 70 residents reported symptoms and 39 cases were confirmed. This outbreak was associated with baked eggs that had come from a distributor who used multiple flocks as suppliers. It is likely that undercooking of the eggs may have led to this outbreak. Also in 1995 another outbreak occurred involving Caesar salad dressing served at a wedding reception. The dressing was prepared using raw eggs and was left un-refrigerated for over 8 hours before being served. 28 people fell ill and 26 confirmed cases were reported. Again in 1995, a small outbreak occurred in a single household which was associated with a homemade beverage. The beverage contained raw refrigerated shell eggs as an ingredient. Three people became sick, two of which consumed the drink immediately after refrigeration. The other patient consumed it after it had been refrigerated for 5 hours. The outbreak was confirmed to be from this source when positive cultures were obtained from the beverage and the egg shells of the eggs used (CDC, 1996a).

Although rare in the United States, an outbreak of Typhoid fever can be of significant interest to public health officials because of its high mortality rate. Water and food are the most common vectors for this pathogen to enter humans (Olsen et al., 2003). For these reasons, this organism should be of considerable interest from a biosecurity standpoint. In 1997, a large outbreak of typhoid fever occurred in Tajikistan, a former Russian republic. The outbreak was caused by contamination of the water system in the Dushanbe area. The first indication of an outbreak was an unusually high number of cases of typhoid fever during a two week period (over 2000). After the local government requested help from the CDC, it was noted that 8901 cases of typhoid fever were reported from January through June of 1997. This was 83% of the total reported cases from

January 1996 through July of 1997. Among these 8901 cases, 95 deaths occurred. The major cause of the outbreak was determined to be the water supply in the area. The water treatment plants in the area were not well maintained and measures were taken to help eliminate this risk. (CDC, 1998) This outbreak shows how easily *Salmonella* Typhi can be disseminated throughout a population in a water supply. This should be of major concern to biosecurity officials in any country with the recent terrorist surge throughout the world.

These various outbreaks show the range of products that can become contaminated with *Salmonella* spp. and the effect the consumption of these products can have. It also shows the severity of the health risk involved with this organism and shows its possible affects on food production. The various serotypes of disease causing *Salmonella* that will be tested in this analysis have all been associated with outbreaks and are of concern to both the food industry and public health officials.

C. Monitoring of *Listeria* and *Salmonella* in foods and food processing environments

The USDA-FSIS has recently changed their regulations concerning environmental sampling, and *Listeria monocytogenes*. In 1999, the USDA-FSIS issued an update on the guidelines for *Listeria* in the food industry. In this document, they noted *Listeria monocytogenes* as a pathogen of concern that must be addressed in food safety plans and HACCP plans (FSIS, 1999). It also indicated that their may be in the future, a zero tolerance for *Listeria monocytogenes* in ready to eat (RTE) food products, similar to the regulations on *Escherichia coli* O157:H7 in ground beef. It also gave the food companies

ideas as to how to alter their current safety and HACCP plans to accommodate *Listeria*. In this report, there was also an assessment of various ready to eat food products and the incidence of *Listeria monocytogenes* in them. In this assessment, sliced ham, luncheon meat and small diameter sausages had the highest rate of positive results (FSIS, 1999).

In late 2002, the USDA-FSIS issued an updated *Listeria monocytogenes* directive. This directive answered a lot of questions that had arisen recently about the correct procedures for sampling for *Listeria monocytogenes* and how to analyze and deal with the results that are obtained (FSIS, 2002). This new directive further classified RTE food processing plants by their risk of contamination. It took into consideration, the food products that they produce, the sanitation programs they have in place, their HACCP plan and how available they make their data to FSIS. In the new directive, it is told to RTE food processors that all of their environmental sampling data must be made available to the USDA FSIS. They also laid out the procedures that would be taken in response to positive results for *Listeria monocytogenes* in product sampling or environmental sampling (FSIS, 2002).

The USDA/FSIS has also emphasized reducing *Salmonella* levels in food processing facilities as well. In 1996 they addressed this issue in the Federal Register. They issued a directive called The Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) Systems; Final Rule. This rule took effect over time and created performance standards for reducing *Salmonella* levels. These levels were verified by testing product samples collected on site. By early 2000, the Rule had taken effect in all meat and poultry establishments and collected data has shown that this Rule helped significantly lower *Salmonella* levels. The performance standards set were promulgated

from baseline studies done before the implementation of HACCP programs. An assessment of FSIS regulatory compliance data between 1998 and 2002 shows that while the numbers of samples that tested positive for *Salmonella* have fluctuated over the four years, the numbers have stayed significantly below the performance standards set (FSIS, 2004b). Although the governing agencies do not specifically collect environmental samples for *Salmonella* presence, they are clearly concerned that its presence in food and food processing facilities can gain knowledge about contamination by utilizing environmental sampling plans. An example of this is the use of environmental sampling in hen houses to determine the efficacy of on farm egg safety programs (FDA, 2004b).

Food processing facilities take many steps to reduce or eliminate food pathogens such as *Listeria monocytogenes* and *Salmonella enterica*. Because of its psychrotrophic nature, the elimination of *Listeria monocytogenes* from ready to eat foods that are refrigerated can be more difficult. Sanitation is a very important factor in eliminating the problem. Equipment and storage facilities must be cleaned regularly with sanitizers such as quaternary ammonium compounds and when possible the use of bactericidal compounds during production is recommended. Also, since raw product can be a source of contamination it is important that the production line be linear and compartmentalized to reduce the chance of contamination from one section to another. Training of workers is also important. Simply training workers can help to eliminate the chance of cross contamination throughout a facility. The implementation of HACCP programs has also shown to be effective in reducing *Salmonella* and *Listeria* levels (FSIS, 2004b, Tompkin et al. 1999, Tompkin 2001).

D. Environmental sampling for detection of *Listeria* and *Salmonella*

Environmental sampling has become a favored tool of the food industry recently for a couple of major reasons. One major advantage of environmental sampling is the fact that it greatly reduces end product sampling and its associated costs to the food producer or processor. Another important advantage of environmental sampling is that it can help an investigator determine how and at what point a contaminant is entering the food processing line. This information is very important to determining steps that can be taken to stop contamination and eliminate future problems. These are two very big advantages of environmental sampling that contribute to the increase in its acceptance throughout the food industry.

Research has been conducted comparing many different enrichment techniques to determine the best way to recover *Listeria monocytogenes* from environmental samples. Osborne and Bremer (2001) tested how various enrichment broths optimized recovery of *Listeria monocytogenes* cells under various stresses such as pH, temperature and alcohol content. A similar study was done comparing recovery methods utilizing various enrichment broths to recover stressed cells of *Listeria monocytogenes*, *Salmonella typhimurium* and *Campylobacter coli* (Chang et al., 2002). Another study compared various agars for the recovery of *Listeria monocytogenes* from food and environmental samples (Pinto et al., 2001).

Although a lot of research has been done concerning the technique used for taking samples and how best to isolate and identify target organisms from samples, very little research has been done concerning the storage and transport of these samples in between these two steps. This step in the process is important to maintaining cell numbers in the

sample and also is important to the survival of possibly low counts of organisms of interest.

Two of the most common sampling techniques for food contact or other environmental surfaces are swabbing and sponging the target being sampled. Swabs are small and are used to sample hard to reach places such as equipment or drain grills. Sponges are more commonly used to sample surfaces such as conveyor belts or floors. Both of these techniques require that the swab or sponge be wetted and stored in some form of diluent. These diluents serve a few purposes. They help to maintain isotonic conditions for the organism and help to reduce the stress on the cells during the storage and transport of the sample. The diluent also must neutralize any residual sanitizers that may be present on the surfaces or equipment being sampled. There also may be some enrichment products in this diluent that can allow the cells to grow and reproduce in the diluent itself during the transport and storage period (CCFRA, 1999).

Several diluents have been formulated and recommended for food processing environmental sample collection and transport. Most of these contain one or more compounds that are intended to neutralize common sanitizer chemicals that may still be present on the sampled surface. Examples of these environmental sample transport media (diluents) are Letheen broth, Dey / Engley Neutralizing Broth, Nutrient Broth, and Neutralizing Buffer (Zimbro and Power, 2003).

Two transport media commonly used by the food industry (Neutralizing Buffer and Dey / Engley Neutralizing Broth) and one medium recently marketed to the food industry (Copan SRK solution). This medium was initially marketed to medical industries. These three sample diluents offer different combinations of neutralizing

compounds and nutrients. Dey/Engley (D/E) Broth is a purple liquid that is commonly used for environmental sampling because of its universal neutralizing properties. D/E Broth contains neutralizers for every major sanitizer used in food processing facilities. Lecithin is used in the diluent to neutralize quaternary ammonium compounds. Sodium thioglycollate neutralizes mercurials, sodium thiosulfate neutralizes iodine and chlorine compounds, and sodium bisulfite neutralizes formaldehyde and gluteraldehyde. Polysorbate 80 is also present in this diluent to act as a surfactant for the lecithin and also to neutralize phenolics. In addition to the neutralizers present in the medium, D/E Broth also contains enrichment nutrients that allow for the growth and propagation of viable cells. Dextrose is present as the carbon energy source, and casein peptone and yeast extract provide amino acids, nitrogenous compounds, B vitamins and peptides. One other compound present in D/E Broth that is unique among common sampling diluents is Bromcresol purple which changes color as an indicator of pH change in the diluent. This can be used as an indicator of growth in some situations.

Neutralizing Buffer was the second diluent tested during this project. It is also a very commonly used diluent in environmental sampling. It does not contain the growth compounds that are present in D/E Broth but it does contain neutralizers. Monopotassium phosphate is used in the diluent to neutralize the pH. Sodium thiosulfate is also present to neutralize chlorine and iodine compounds, and aryl sulfonate complex is present to neutralize quaternary ammonium compounds (Zimbro and Power, 2003).

The third diluent used in this research was a new diluent that is part of a swab rinse kit formulation produced by Copan USA (Corona, CA). This is a package that includes a swab that is attached to the screw cap of the diluent tube and a neutralizing

diluent to hold the swab in. The diluent included in the package is made to neutralize multiple disinfectants and sanitizers. A Ringers balanced salt solution is in the diluent to neutralize the pH and osmotic pressure. In addition, like D/E Broth, this diluent contains polysorbate 80, lecithin, sodium thioglycollate, sodium thiosulfate and sodium bisulfate. In addition to these compounds, the Copan SRK solution also contains sodium pyruvate which helps to neutralize toxic oxygen compounds, and sodium hexametaphosphate which neutralizes metal ions (Zimbro and Power, 2003).

The temperatures at which microbiological environmental samples are stored and transported are important to total cell survival. It is important to keep samples that are to be refrigerated at 0°C - 4.4°C. Samples that are to be kept frozen should be kept below -20°C (APHA, 2001). Pathogen survival in each transport media at a variety of storage temperatures was examined. One temperature tested was -4°C to simulate a sample that may be kept slightly below optimal refrigeration temperature. Samples were also kept at 4°C and 10°C to simulate an optimal refrigeration temperature and a temperature at the upper limit of acceptable refrigeration. One sample was also kept at 15°C to simulate a sample stressed above refrigeration temperature.

Storage time is also a variable that must be considered in maintaining optimal conditions for the storage and transport of environmental samples. In some situations, a food processing facility may have a lab on site to do their sample processing. In some other situations, they have to pool their samples together and send them out to a local lab at the end of the day. Some other facilities have no local laboratories and must ship their samples for a distance. In many cases, it is impossible, impractical or inconvenient to begin sample enrichment and analysis on the same day as sample collection. Generally,

laboratories recommend that analyses begin within 36 hours of sample collection, but it may be up to 48 hours or more before the samples are tested, especially if they are collected on a Friday. This is why it is important to determine whether or not there is any significant affect on cell survival during the transport and storage of the environmental samples.

Although quantitative studies for pathogen presence in food processing facilities is not common now it will likely become increasingly important as better data is collected determining safe numbers of organisms that can be present in foods. It is also important to quantify because most environmental samples taken in the field will contain small numbers of organism, so even a small reduction in recovery could give false negative results in a qualitative test. Quantitative analysis may also help increase knowledge of how well a particular pathogen may survive when competing with other organisms that are present in environmental samples.

III: Chapter One: Quantitative Recovery of *Listeria monocytogenes* and *Salmonella enterica* from Environmental Sampling Media

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A. Abstract

Environmental sampling is a pathogen monitoring technique that has become increasingly important in the food industry. Many food processing companies have adopted environmental sampling in their processing plants as a way to verify sanitation GMP's and decrease end product testing. Two specific pathogens that are of major concern to the food industry and that are routinely tested for using environmental sampling are *Listeria monocytogenes* and *Salmonella enterica.* In many cases, environmental samples are not analyzed on sight immediately but instead are batched and analyzed at a later time or shipped before processing. There are multiple media on the market today for storage and transport of environmental samples. These various media types along with differences in holding temperatures and time create a lot of variability in transport and holding conditions. In this study, selected time, temperature and media combinations were tested to determine cell survival levels. The objective was to determine optimum conditions for transport and storage of these two pathogens. Analysis for each pathogen tested was done individually. Cocktails of *Listeria monocytogenes* and *Salmonella enterica* were added to sample tubes of the three media. Counts were made of the bacterial load at 0, 12, 24 and 48 hours. After comparison it was determined that the Neutralizing Buffer and Swab Rinse Kits were best at maintaining the bacterial load levels at all temperatures. At higher temperatures (10 and 15 C), growth occurred in Dey / Engley broth. This study helped validate the use of these three media for environmental sample transport and storage at proper holding temperatures and demonstrated that at temperatures outside optimal ranges it is preferable to use Neutralizing Buffer or a Swab Rinse Kit when attempting to quantify results.

B. Introduction

Environmental sampling has become a very important tool in food safety. It is a way for investigators to determine the level of contamination in a site that is being investigated, as well as track how pathogens of concern may enter facilities and contaminate food products. In some cases, this type of sampling minimizes the need for end product sampling, which can reduce costs for the food processors.

Two major pathogens of concern in the food industry are *Listeria monocytogenes* and *Salmonella enterica*. These two pathogens are readily sampled for in food processing facilities and commonly monitored to enhance food safety. *Listeria monocytogenes* is a foodborne pathogen that is associated with many types of food. Listeriosis, the disease associated with an infection with *Listeria monocytogenes* has a high mortality rate, especially among pregnant women and the immunocompromised (Brooks et al., 1998). Because of multiple recent outbreaks of listeriosis throughout the country, and the subsequent public outcry, the United States Department of Agriculture's Food Safety Inspection Service (USDA-FSIS) released a directive in 1999 and updated it in 2002 to assess *Listeria monocytogenes* as a pathogen of concern in the food industry that must be considered when food facility safety procedures and HACCP plans are created (USDA, 1999). In 2002, this directive was updated to further classify the risks associated with various food processing plants and to announce that all environmental sampling data that was collected by a food processing plant would now have to be shared with the USDA-FSIS (USDA, 2002).

Salmonella enterica has long been a concern in the food industry because of its pathogenesis in humans. *Salmonella* can cause a variety of diseases including salmonellosis and typhoid fever (Salyers and Whitt, 2002). The three strains of *Salmonella* used in this research will be referred to by their shorter names. *Salmonella enterica* serovar Typhimurium will be referred to as *Salmonella* Typhimurium. *Salmonella enterica* serovar Typhi will be referred to as *Salmonella* Typhi. *Salmonella enterica* serotype Enteritidis will be referred to as *Salmonella* Enteritidis. These strains are commonly referred to in this manner to simplify comparisons (Salyers and Whitt, 2002).

The three serovars of *Salmonella* chosen for this project are of major concern to the health care and food industries. *Salmonella* Typhimurium and *Salmonella* Enteritidis will be used because of their prevalence in the food industry and because they are the leading cause of salmonellosis (IFT, 2004). *Salmonella* Typhi will be utilized in this project because of the severity of the disease it can cause. Although rare in western cultures and more easily treatable, it is still a major issue in developing countries (Salyers and Whitt, 2002). All three of these serovars are a major health concern in modernized and especially developing countries.

Also, these two organisms were selected for this investigation because of the differences in cell envelope structure. *Salmonella enterica* is a gram negative organism and has an outer and inner membrane encapsulating a much thinner peptidoglycan layer in its envelope (White, 2000). *Salmonella enterica* and most gram negative organisms have a convoluted outer cell wall which gives it a much higher surface area/volume ratio (Madigan et al., 2000). *Listeria monocytogenes*, in contrast, is a gram positive organism

with a thick outer peptidoglycan layer covering its cell membrane in its cell envelope (White, 2000).

One of the issues of concern with environmental sampling is its reliability. There have been several research projects optimizing sampling techniques and procedures. One aspect of the process that has not been widely studied is the storage and transport of environmental samples between the time they are taken and the time they are processed. These samples may have to be shipped to off site labs and may be in the transport and storage chain for up to 48 hours. This time is critical to the reliability of the tests.

There are many types of transport media that are readily used for environmental sampling. The major purpose of these various media is to neutralize residual sanitizers and maintain a neutral environment for the recovery of desired bacteria. The two common transport broths used in the industry are Dey / Engley Neutralizing Broth (D/E Broth), and Neutralizing Buffer. These will be tested along with a third, recently introduced media, Copan Swab Rinse Kit transport solution.

In some cases, processing facilities have labs on site that and therefore they can test there samples almost immediately. If a facility has a lab on site, they may however wait to batch a group of samples before testing them. Unfortunately not all facilities have these on site labs. In these cases, the facility will have to ship there samples to an off-site test lab. This can take up to two days.

The temperature at which samples are held during storage and transport may vary as well. In most cases, samples are refrigerated for this time, but in some cases this refrigeration temperature may not be maintained throughout the duration. In other cases, the samples may be kept at freezer temperatures.

The above conditions are important to investigate with regards to environmental sampling. It is important to develop an optimum time/temperature/medium combination for the recovery of various microorganisms of interest. It is also important to determine what combinations will maintain cell numbers best for quantitative analyses.

This study provides an analysis of various transport and storage variables. The pathogens of concern that were used for the research are often the target of environmental sampling and offer a public health risk that is important to the food processors. The two different organisms tested offer a variance in cell envelope physiology and therefore are representative of two different classes of bacteria.

C. Materials and Methods

1. *Listeria* inoculum preparation

Four strains of *Listeria monocytogenes* (LCDC, Scott A, D43 and V7) were used to create a cocktail. These strains were obtained from the Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, VA. Cultures were stored frozen at -80 ^{o}C in Tryptic Soy Broth supplemented with 25% glycerol. Prior to use, cultures were thawed and added to 10 ml tubes of Brain Heart Infusion Broth (BHIB, Difco, Franklin Lakes, NJ). Cultures were subcultured into fresh BHIB daily for at least two consecutive days. The cultures were incubated at 33 +/- 2 ^{o}C during growth. Fresh cultures were started from frozen cultures for each test repetition.

Preliminary studies on each of these four strains of *Listeria monocytogenes* determined the average concentration of cells in suspension in a 24 hour culture. A standard plate count method determined that the average concentration of each of the four

strains of *Listeria monocytogenes* was ~1.5 x 10^9 CFU/ml after 24 hours of incubation. Equivalent proportions of each cultured strain were combined prior to dilution in Butterfield's Phosphate Dilution Water (BPDW) (Biotrace International Bioproducts, Bothell, WA).

Culture purity and confirmation of species identification was performed prior to each experiment with the sample collection media. From the final subculture of each of the four strains of *Listeria monocytogenes*, 0.1 mL was streaked onto Brain Heart Infusion Agar (BHIA, Difco). These plates were incubated for 24 hours at 33 +/- 2 ^{o}C. The incubation temperature (33°C) was chosen because it is an optimal growth temperature for both *Salmonella enterica* and *Listeria monocytogenes* and the use of one temperature keeps this test uniform. All colony morphologies were similar on all plates throughout the experiments. At least one isolated colony from each plate was identified with an API *Listeria* biochemical test kit (bioMerieux, Hazelwood, MO). All colony isolates were identified as *Listeria monocytogenes*.

2. *Salmonella* inoculum preparation

Three strains of *Salmonella enterica* (Typhi, Typhimurium, and Enteritidis) were used to create a cocktail for use in the following test. Two of the strains (*Salmonella* Typhimurium and *Salmonella* Enteritidis) were obtained from the Virginia Maryland Veterinary Medical College, Virginia Polytechnic Institute and State University, Blacksburg, VA. The remaining strain (*Salmonella* Typhi) was obtained from the American Type Culture Collection, Manassas, VA. Cultures were stored frozen at -80°C in Tryptic Soy Broth (TSB) supplemented with 25% glycerol. Prior to use in testing,

cultures were thawed and added to 10mL tubes of TSB. Cultures were subcultured into fresh TSB daily for at least two consecutive days. The cultures were incubated at 33 +/- 2°C during growth. Fresh cultures were prepared from frozen stocks for each test repetition.

Preliminary studies on each of these three strains of *Salmonella enterica* determined the average concentration of cells in suspension in a 24 hour culture. A standard plate count method determined that the average concentration of each of the three strains of *Salmonella enterica* varied between strains. Theses varied concentrations were adjusted at the initiation step of the procedure to create an even distribution of the strains in the test. The plate count procedure above was done in triplicate to determine the concentrations.

Culture purity and confirmation of species identification was performed prior to each experiment with the sample collection media. From the final subculture of each of the three strains of *Salmonella enterica*, 0.1 mL was streaked onto Tryptic Soy Agar (TSA). These plates were incubated for 24 hours at 33 +/- 2°C. All colony morphologies were similar on all plates throughout the experiments. The identity of at least one isolated colony from each plate was confirmed with an API 20E biochemical test kit (bioMerieux, Hazelwood, MO).

3. Sample Collection Test Media

Three commercially available media for microbiological sample collection and transport were tested in this experiment. Dey / Engley (D/E) Neutralizing Broth

(Biotrace International BioProducts, Bothell, WA), per manufacturer instructions, was stored at 4°C. Neutralizing Buffer (Difco), per manufacturer instructions, was stored at room temperature (~25°C) until 24 hours before test initiation, and then refrigerated. Swab Rinse Kits (SRK) (Copan USA, Corona, CA), per manufacturer instructions, were stored refrigerated (4°C). Tubes of SRK included a swab attached to the inside top of the tube. The swabs were removed prior to use in the test. The removal was done using stainless steel scissors that had been dipped in 95% ethanol and flamed before use to maintain an aseptic environment in the tube. The ingredients for each medium are listed in Table 1. All three test media were chilled at least 24 hours at 4°C to simulate being refrigerated at time of test initiation. This was done to maintain uniformity among the three media. The Neutralizing Buffer was stored at room temperature and therefore had to be chilled to an identical temperature as the other two media. Refrigeration temperature was utilized as a starting temperature because in practice sampling media would be chilled for storage and use and be at refrigeration temperature at the time samples are taken.

The initial pHs of the three transport media was similar and neutral. The pH of the D/E Neutralizing Broth was the highest at 7.52. The pH of the Neutralizing Buffer was the lowest at 7.04. The pH of the Copan SRK Solution was 7.18. All of these pHs were taken when the media was at 19°C and all are neutral and suitable for growth of each organism.

4. *Inoculation of Test Media with Listeria*

One mL of each of the four strains of *Listeria monocytogenes* was added to 99 mL of sterile Butterfield's Phosphate Dilution Water (BPDW, Biotrace International Bioproducts, Bothell, WA). The initial inoculum was prepared from 24 hour cultures. The culture was further diluted with BPDW to achieve a cell concentration of approximately 1.0 x 10^4 CFU/ml. This method was used to produce a fresh cocktail before each temperature set for each repetition.

One mL of the prepared cocktail (22 +/- 2 ^oC) was aseptically added to each of three tubes of each of three test media (9 tubes total per temperature per repetition). The tubes of test media were held at 4 ^oC at the time of the addition of the cocktail to simulate the preferred use temperature for environmental sample storage. Each tube was shaken by hand after each addition. The cocktail was agitated between each addition as well.

Three tubes of each media (9 total tubes) were incubated at one of four test temperatures (-4^oC, 4^oC, 10^oC or 15^oC) for up to 48 hours. The temperatures of the incubators were checked frequently and monitored by electronic thermometers.

5. *Inoculation of Test Media with Salmonella*

Because of the varying concentrations of each organism used for the cocktail, the cocktail was prepared as follows. A portion (3.2 mL) of a 24 hour culture of *Salmonella* Typhimurium was added to a 99mL sterile dilution blank containing BPDW. In addition, a portion (2mL) of a 24 hour culture of *Salmonella* Enteritidis was added to the same BPDW blank. Also, in addition, a portion (4ml) of a 24 hour culture of *Salmonella* Typhi. The culture was further diluted with BPDW to achieve a cell concentration of

approximately 1.0 x 10^4 CFU/ml. This method was used to produce a fresh cocktail before each temperature set for each repetition.

One mL of the prepared cocktail (22 +/- 2 ^oC) was aseptically added to each of three tubes of each of three test media (9 tubes total per temperature per repetition). The tubes of test media were held at 4 °C at the time of the addition of the cocktail to simulate the preferred use temperature for environmental sample storage. Each tube was shaken by hand after each addition. The cocktail was agitated between each addition as well.

Three tubes of each media (9 total tubes) were incubated at one of four test temperatures (-4^{o}C, 4^{o}C, 10^{o}C or 15^{o}C) for up to 48 hours. The temperatures of the incubators were checked frequently and monitored by electronic thermometers. At Day 0 of the testing procedure, the test was initiated for all four of the temperatures to be tested.

6. *Enumeration Procedures*

An initial *Listeria monocytogenes* concentration (time zero) was determined for each inoculated media tube as described below. All tubes were sampled again after 12, 24 and 48 hours when stored at one of the four test temperatures. All tubes were returned to their respective incubator within 20 minutes after sampling. Each tube was agitated and 0.1 mL was spread onto duplicate plates of BHIA using aseptic technique. Additionally, 1 mL of the test medium was diluted tenfold in BPDW, agitated, and 0.1 mL was spread plated on BHIA using aseptic technique. In some cases (e.g. 15^{o}C media incubation) additional dilutions with BPDW were required before plating. All plates were then incubated at 33 +/- 2 ^oC for 24 hours before being counted. Any plates that appeared to be contaminated were discarded and not used.

An initial *Salmonella enterica* concentration (time zero) was determined for each inoculated media tube as described below. All tubes were sampled again after 12, 24 and 48 hours when stored at one of the four test temperatures. All tubes were returned to their respective incubator within 20 minutes after sampling. Each tube was agitated and 0.1 mL was spread onto duplicate plates of TSA using aseptic technique. Additionally, 1 mL of the test media was diluted tenfold in BPDW, agitated, and 0.1 mL was spread plated on BHIA using aseptic technique. In some cases (e.g. 15^{o}C media incubation) additional dilutions with BPDW were required before plating. All plates were then incubated at 32 +/- 2 ^{o}C for 24 hours before being counted. Any plates that appeared to be contaminated were discarded.

7. Data Analysis

For each microorganism, three tubes of each transport media were inoculated for each of the incubation temperatures and sampling times. The mean concentration of three tubes from each test condition was determined. These experiments were performed in triplicate. Data were analyzed with the Proc Mixed procedure of version 6.12 of Statistical Analysis System software (SAS Institute, Cary, NC) and subjected to analysis of variance to determine the effect of media, temperature and time on pathogen recovery. A least significant difference test was performed to determine differences among the treatments ($\alpha = 0.05$).

D. Results and Discussion

1. *Listeria monocytogenes* survival

The mean log CFU/ml recovery of *L. monocytogenes* over time was significantly different ($\alpha<0.05$) for only two storage conditions- D/E Neutralizing Broth held at either 10°C or 15°C. As seen in Figure 1, *Listeria monocytogenes* maintained its population relatively well in D/E Broth at 4°C. At -4°C, there was a slight decrease in number of detectable cells after 24 hours. At 10°C, there was significant overall growth after 24 hours. At 15°C, the mean cell count increased from 3.80 log CFU/mL at time zero, to 4.16 log CFU/mL after only 12 hours. This overall increase over 48 hours was the most dramatic increase in cell concentration for all of the media and incubation combination variables for each organism.

The *Listeria monocytogenes* count remained constant over the 48 hour test period for all four temperatures tested with the Neutralizing buffer (Figure 2). There was no significant growth or cell death under these conditions. In the SRK solution, the *Listeria monocytogenes* concentration also remained constant (Figure 3). Over the 48 hour test period there was very little fluctuation in cell concentration at any of the four temperatures tested. The standard deviation for all data sets (n=9) was < 0.2 log CFU/mL with two exceptions. In the D/E Neutralizing Broth, when the samples were held at 15°C for 24 hours the count was 5.35 log CFU/ml +/- 0.57 and after 48 hours the final count was 7.43 log CFU/ml +/-1.10.

2. *Salmonella enterica* survival

The mean log CFU/ml recovery of *S.* enterica over time was significantly different ($\alpha<0.05$) for only three storage conditions- Copan SRK solution held at -4°C, and D/E Neutralizing Broth held at either 10°C or 15°C. Detectable numbers of *Salmonella enterica* remained constant over the 48 hour test period for when the samples were kept at -4°C and 4°C (Figure 4). At 10°C, the cell number showed a slight increase of about 0.5 log CFU/mL. There was a dramatic increase in growth in D/E broth after 12 hours at 15°C. When *Salmonella enterica* was incubated in Neutralizing Buffer the cell concentration was maintained at a relatively constant concentration throughout the test period of 48 hours for all temperatures tested; there was no dramatic cell growth or decrease in detectable cells (Figure 5). The *Salmonella enterica* cell concentrations remained relatively constant throughout the 48 hour test period when held at 4°C, 10°C and 15°C in the Copan SRK solution (Figure 6). At -4°C there was a decrease in detectable cells of about log 0.3 which is more than can be seen under other conditions. This equates to an approximated 45% reduction in cell concentration over 48 hours and is the most dramatic decrease seen in the test. The standard deviation for all data sets (n=9) was less than 0.2 log CFU/mL with three exceptions. In the D/E Neutralizing Broth when the samples were held at 10°C for 48 hours the final count was 4.21 log CFU/ml +/- 0.28. Also in the D/E Neutralizing Broth, when the samples were held at 15°C for 24 hours the count was 4.76 log CFU/ml +/- 0.38 and after 48 hours the final count was 6.82 log CFU/ml +/-0.33.

3. Transport media incubation time and temperature

Listeria monocytogenes is able to grow in the D/E Broth at 10 and 15°C. This growth can be attributed the presence of growth compounds in the D/E Broth that is not present in the other two diluents. The carbon source and nitrogen and vitamin providers promote cell growth which would be expected. D/E Broth can also be used as a growth media for testing sanitizers. *Listeria monocytogenes* is also a psychrotrophic organism and therefore can grow at lower temperatures. This would lead to the growth at the two higher temperatures. If the test were to be continued for a longer duration, growth could be expected at the 4°C temperature as well.

Salmonella enterica grew at the higher test temperatures in D/E Broth. It grew well at 15°C and slightly at 10°C. The reason for this growth as mentioned above was the growth compounds present in the diluent. *Salmonella* can grow at these temperatures but at a slower rate than *Listeria monocytogenes* which is why the growth at 10°C was much slighter and the growth at 15°C was not as great either.

Under certain conditions there was an initial decline in the cell count. In these situations, the population returned to equivalent of the initial concentration after a time period but there was a definite initial population decrease. This can be seen in Figures 5 and 6. The reasoning for this is likely an initial shock to the cells which may make them harder to recover. This initial shock may be due to the lack of growth compounds in the two media associated with these results. The cells could be stressed initially by the temperature change and become easier to recover as they adapt to the environment at the later sampling times.

Under most of the test conditions, the recovered population decreased no more than 10% from the initial concentration over the total 48 hour time period. The greatest exception was the *Salmonella enterica* which decreased in concentration about 45% in the Copan SRK Solution after 48 hours. This shows that the majority of testing combinations were able to minimize cell death over the course of 48 hours.

Since the preferred storage temperature for environmental samples tested was 4°C it is important to note that the cell concentrations decreased less that 10% for the *Salmonella enterica* in the Neutralizing Buffer. The *Salmonella enterica* concentration decreased 13-19% in the D/E Broth and 20-23% in the Copan SRK Solution. The concentrations also decreased less that 10% for *Listeria monocytogenes* in the D/E Broth, Neutralizing Buffer and Copan SRK Solution. The *Salmonella enterica* concentration decreased 13-19% in the D/E Broth and 20-23% in the Copan SRK Solution.

The data also suggest that while D/E Broth may have a high neutralizing ability and is good for sampling damaged or stressed cells because of the growth compounds that are present in the media it may not be ideal for quantitative testing since significant growth can occur in this media for the two organisms tested.

In conclusion, if a qualitative test is being done and there is no issue of quantifying the bacterial load for any particular sample, D/E Broth may be the most ideal medium to be used because it can help to recover damaged and stressed cells and may be able to maintain a minimum amount of cells. If there is a concern about quantifying the samples, the Neutralizing Buffer or Copan SRK Solution may be more effective because it maintains the initial cell concentration but does not allow for growth.

Another important factor that should be considered before one chooses the transport diluent is what conditions they expect the sample to go through before being processed or plated. If the samples are going to be frozen or there is any chance of the sample dropping to below freezing temperatures it is best to avoid using the Copan SRK Solution because some cell death was shown with the *Salmonella enterica*. If it is anticipated that the sample will be difficult to maintain at or below refrigeration temperatures, however it would be best to avoid using D/E Broth because of possible overgrowth of your sample. Neutralizing Buffer may be the most consistent transport diluent and the best to use if it is uncertain how the samples will be treated before testing.

Future testing in this area may quantify the recovery of other organisms such as spore forming or acid fast microorganisms that vary in physiology from the organisms tested. This may be applicable especially with the new environmental sampling methods being used to test for *Bacillus anthracis* for biosecurity reasons. It also may be of interest to examine survival of various pathogenic organisms in the presence of other species or how an organism may survive in the presence of certain sanitizers or an organic load.

E. Tables and Figures

D/E Neutralizing Broth	
Ingredient	**Function**
Casein Peptone	provides amino acids, nitrogenous compounds, B vitamins
Yeast Extract	provides amino acids, nitrogenous compounds, B vitamins
Dextrose	carbon energy source
Sodium Thioglycollate	neutralizes mercurials
Sodium Thiosulfate	neutralizes iodine and chlorine
Sodium Bisulfite	neutralizes formaldehyde and gluteraldehyde
Polysorbate 80	surfactant and neutralizer of phenolics
Lecithin	neutralizes quaternary ammonium compounds
Bromcresol Purple	pH color indicator
Neutralizing Buffer	
Ingredient	**Function**
Monopotassium Phosphate	neutralizes pH of buffer
Sodium Thiosulfate	neutralizes iodine and chlorine
Aryl Sulfonate Complex	neutralizes quaternary ammonium compounds
Copan SRK Solution	
Ingredient	**Function**
Ringers Balanced Salt Solution	neutralizes pH and osmotic pressure of buffer
Polysorbate 80	surfactant and neutralizer of phenolics
Lecithin	neutralizes quaternary ammonium compounds
Sodium Thiosulfate	neutralizes iodine and chlorine
Sodium Thioglycollate	neutralizes mercurials
Sodium Bisulfite	neutralizes formaldehyde and gluteraldehyde
Sodium Pyruvate	neutralizes toxic oxygen compounds
Sodium Hexametaphosphate	neutralizes metallic ions

Table 1. Function of ingredients in each transport media.

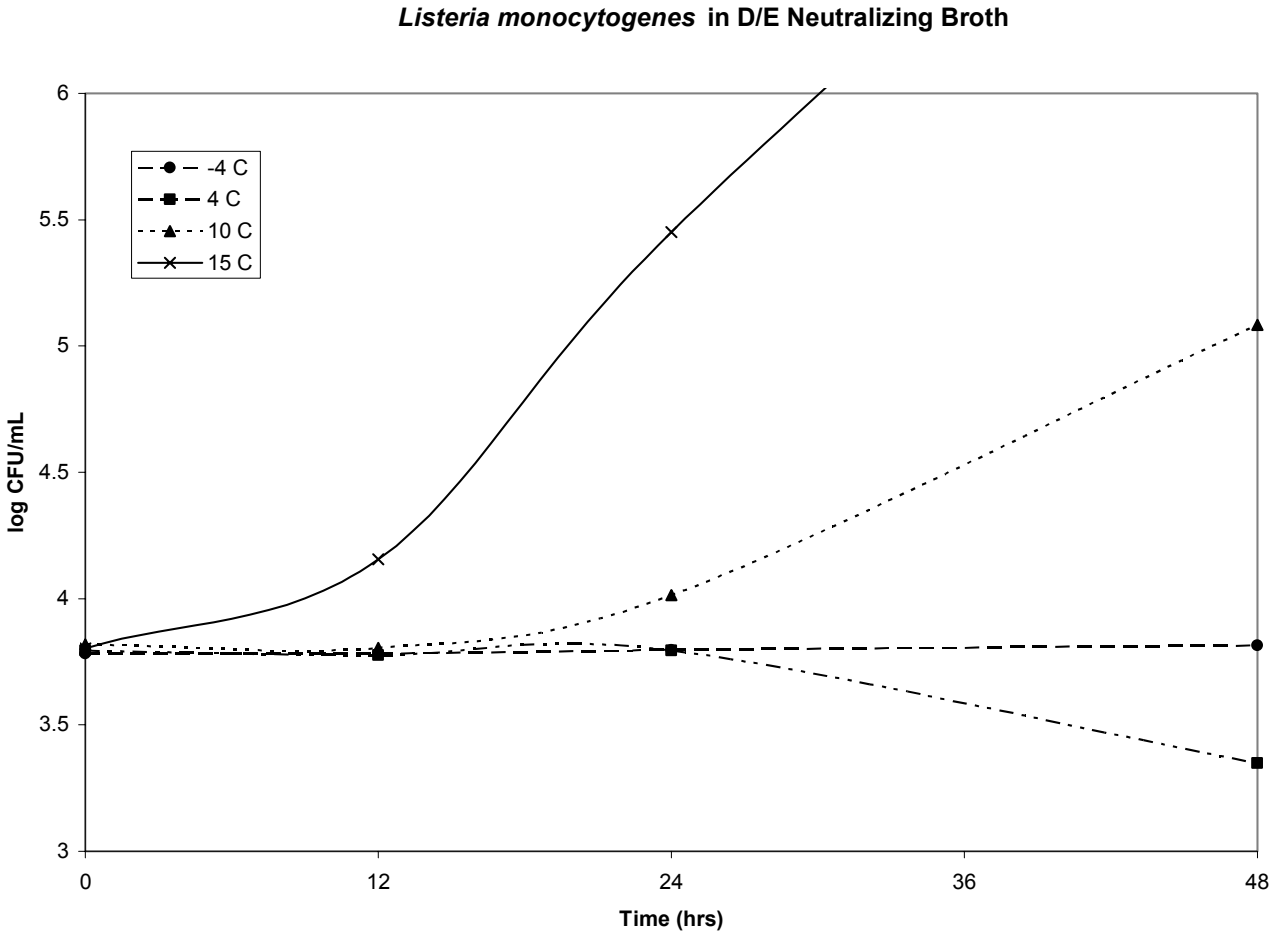

Figure 1. Mean log CFU/ml recovery of *L. monocytogenes* from D/E Neutralizing Broth over time at different storage temperatures (n=9 for each temperature except 15° where n=6 because of limit of detection on 3 samples).

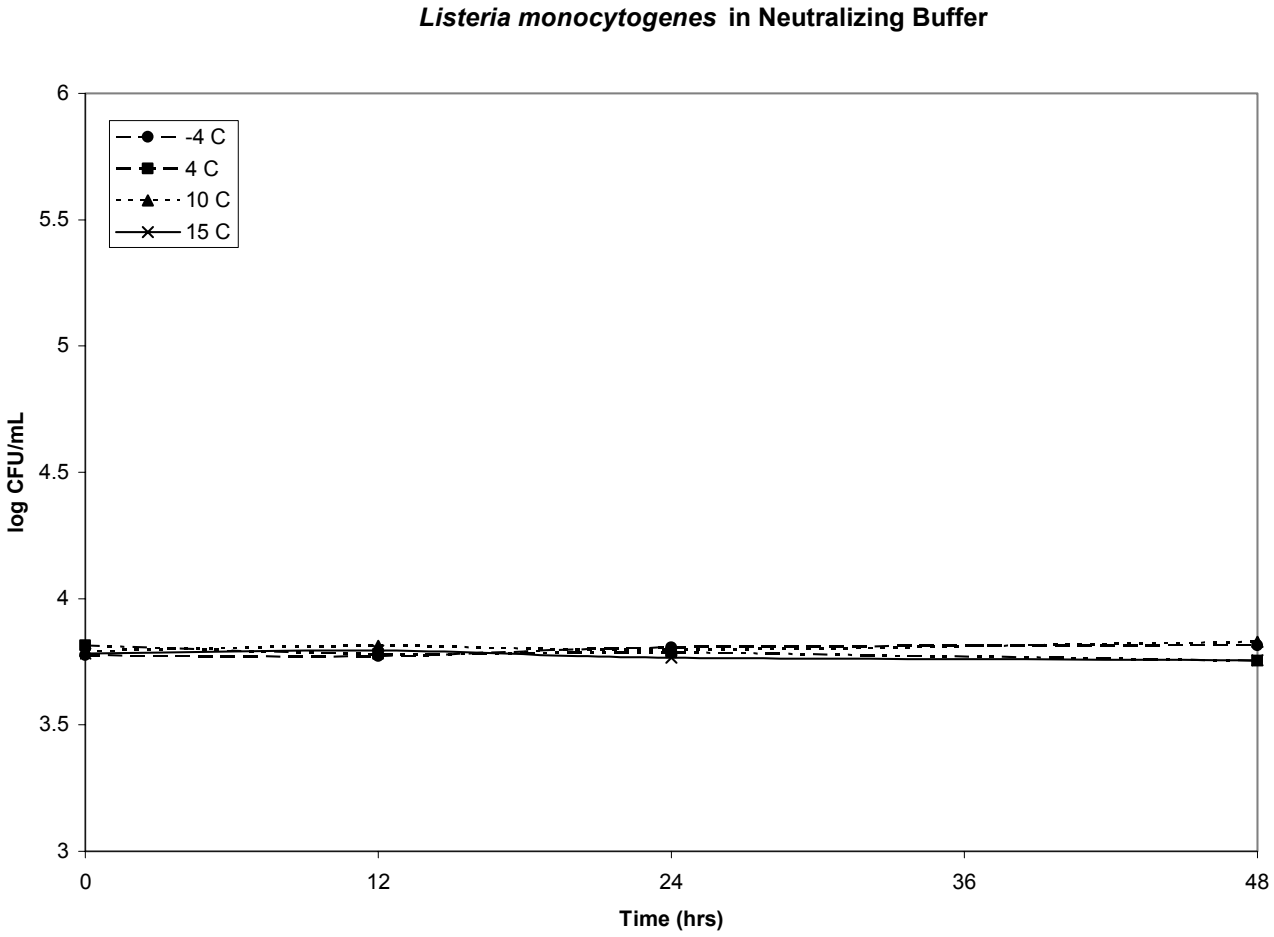

Figure 2. Mean log CFU/ml recovery of *L. monocytogenes* from Neutralizing Buffer over time at different storage temperatures (n=9 for each temperature).

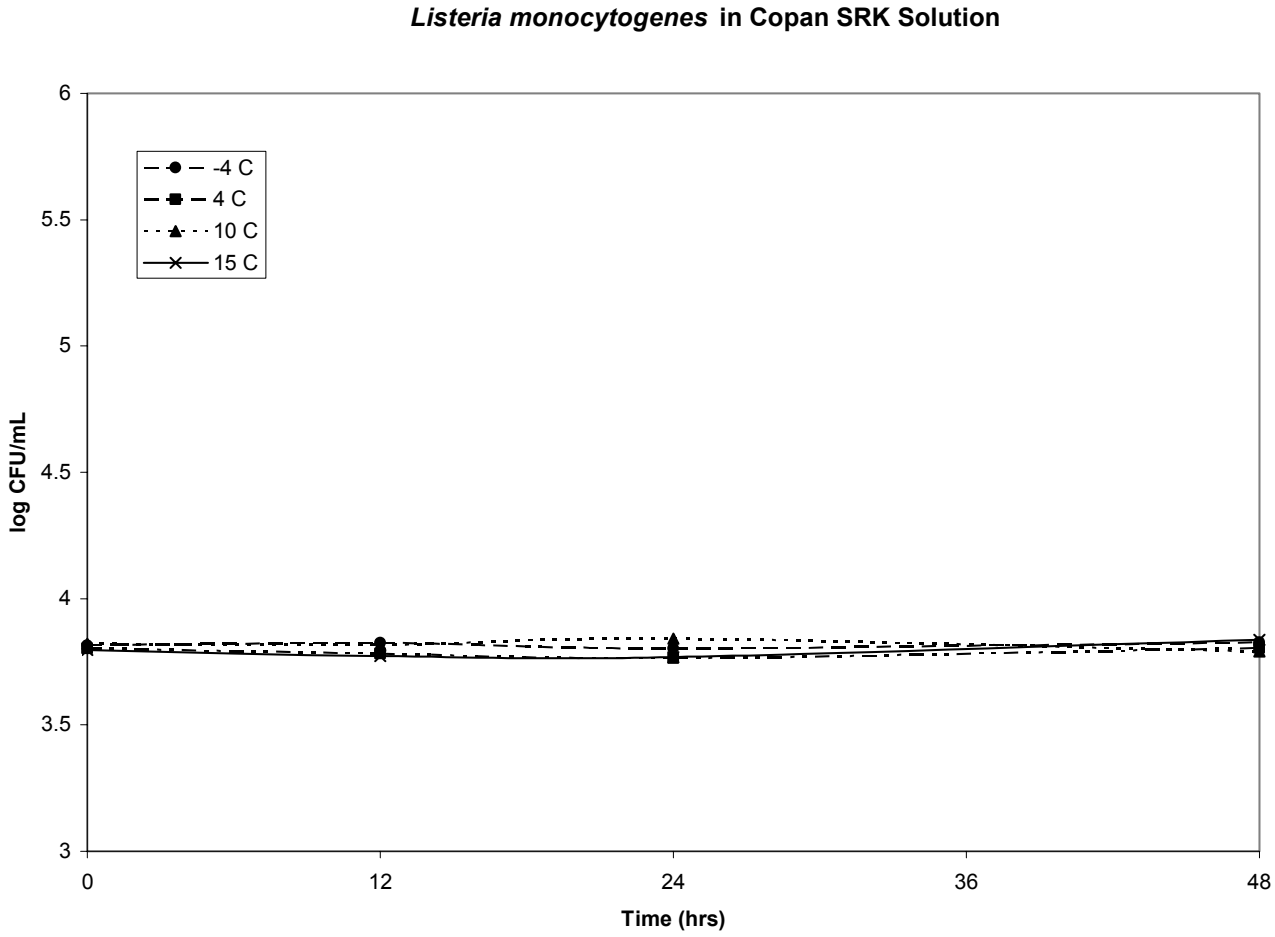

Figure 3. Mean log CFU/ml recovery of *L. monocytogenes* from Copan SRK Solution over time at different storage temperatures (n=9 for each temperature).

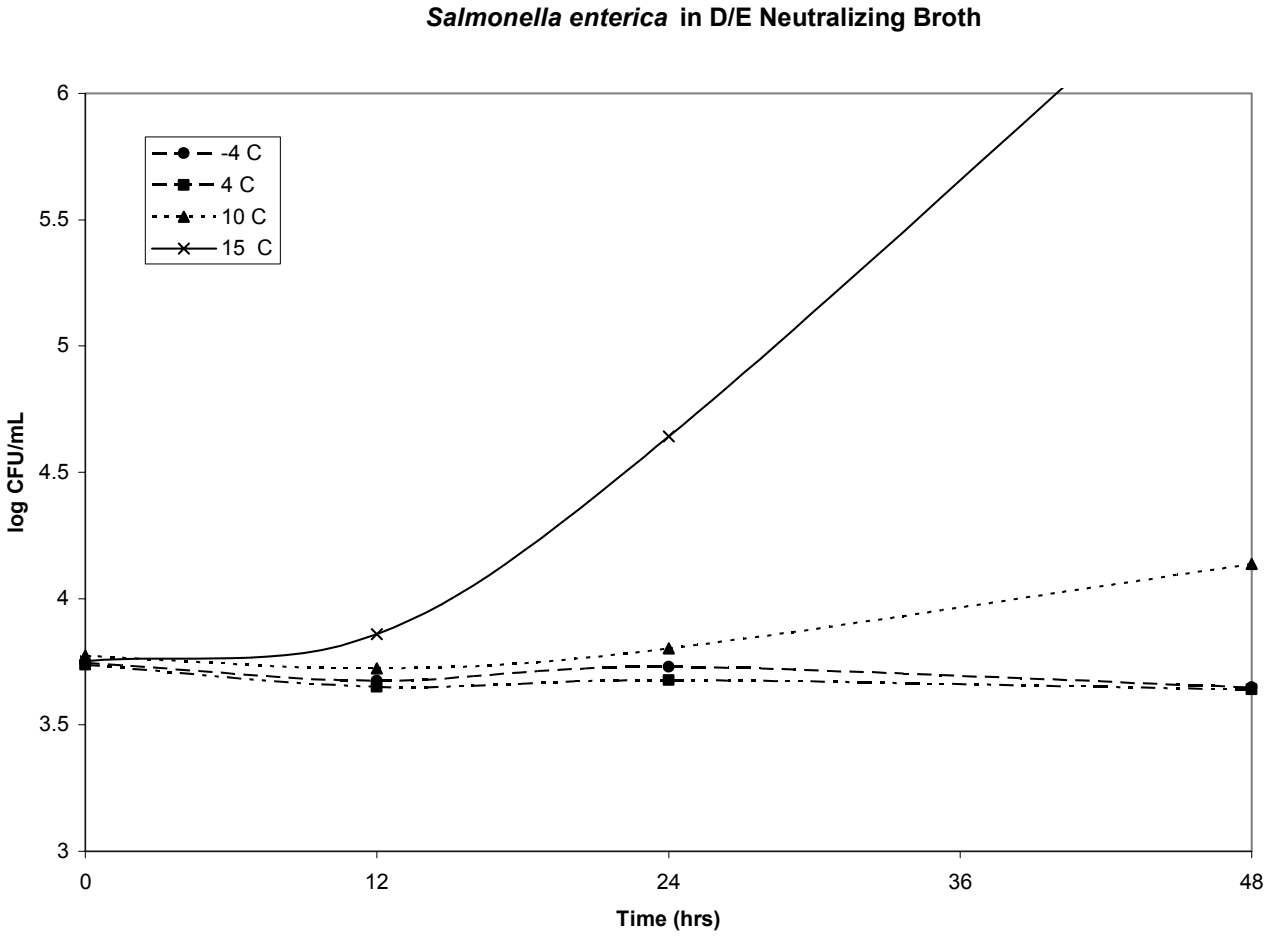

Figure 4. Mean log CFU/ml recovery of *S. enterica* from D/E Neutralizing Broth over time at different storage temperatures (n=9 for each temperature).

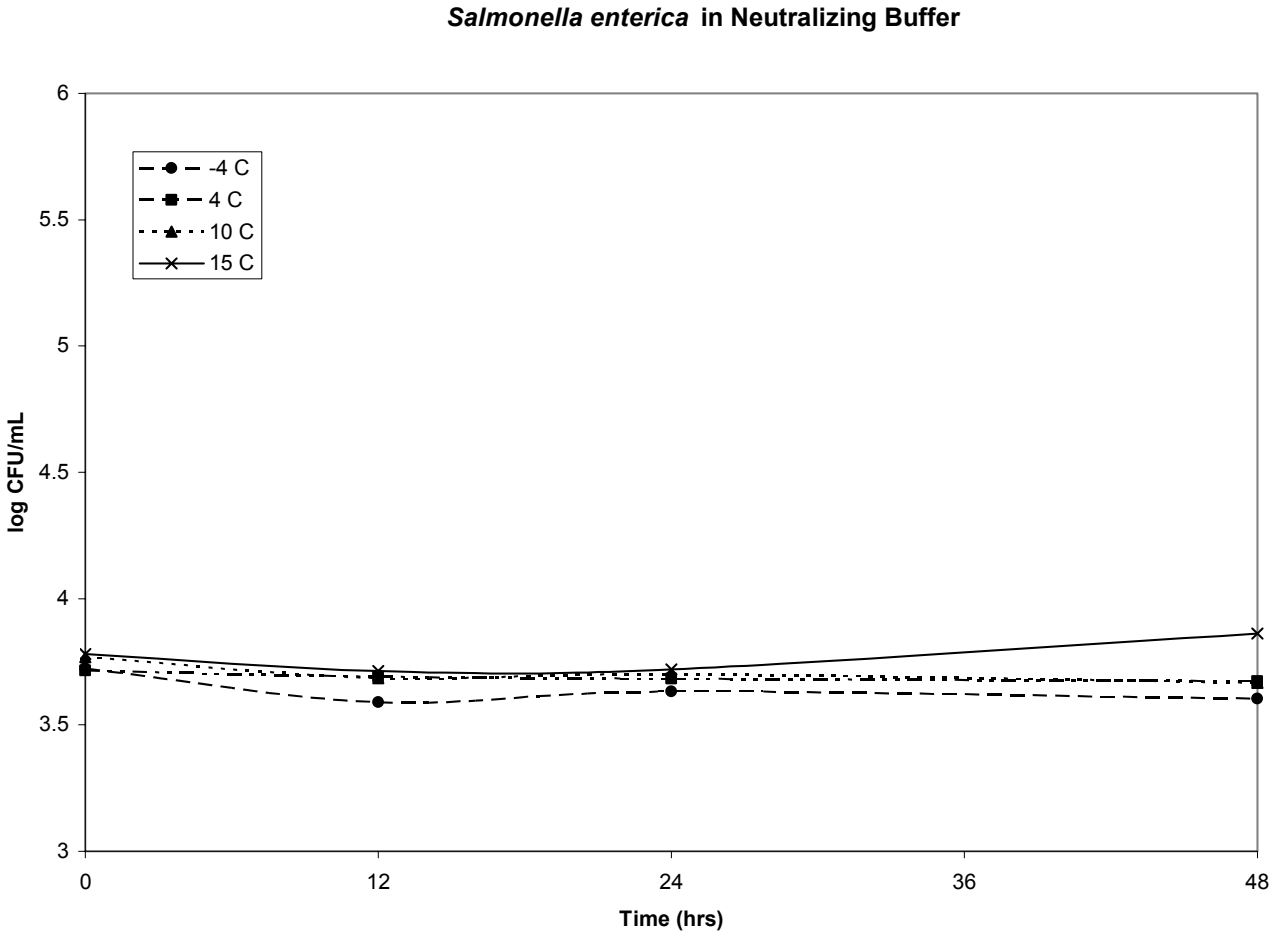

Figure 5. Mean log CFU/ml recovery of *S. enterica* from Neutralizing Buffer over time at different storage temperatures (n=9 for each temperature).

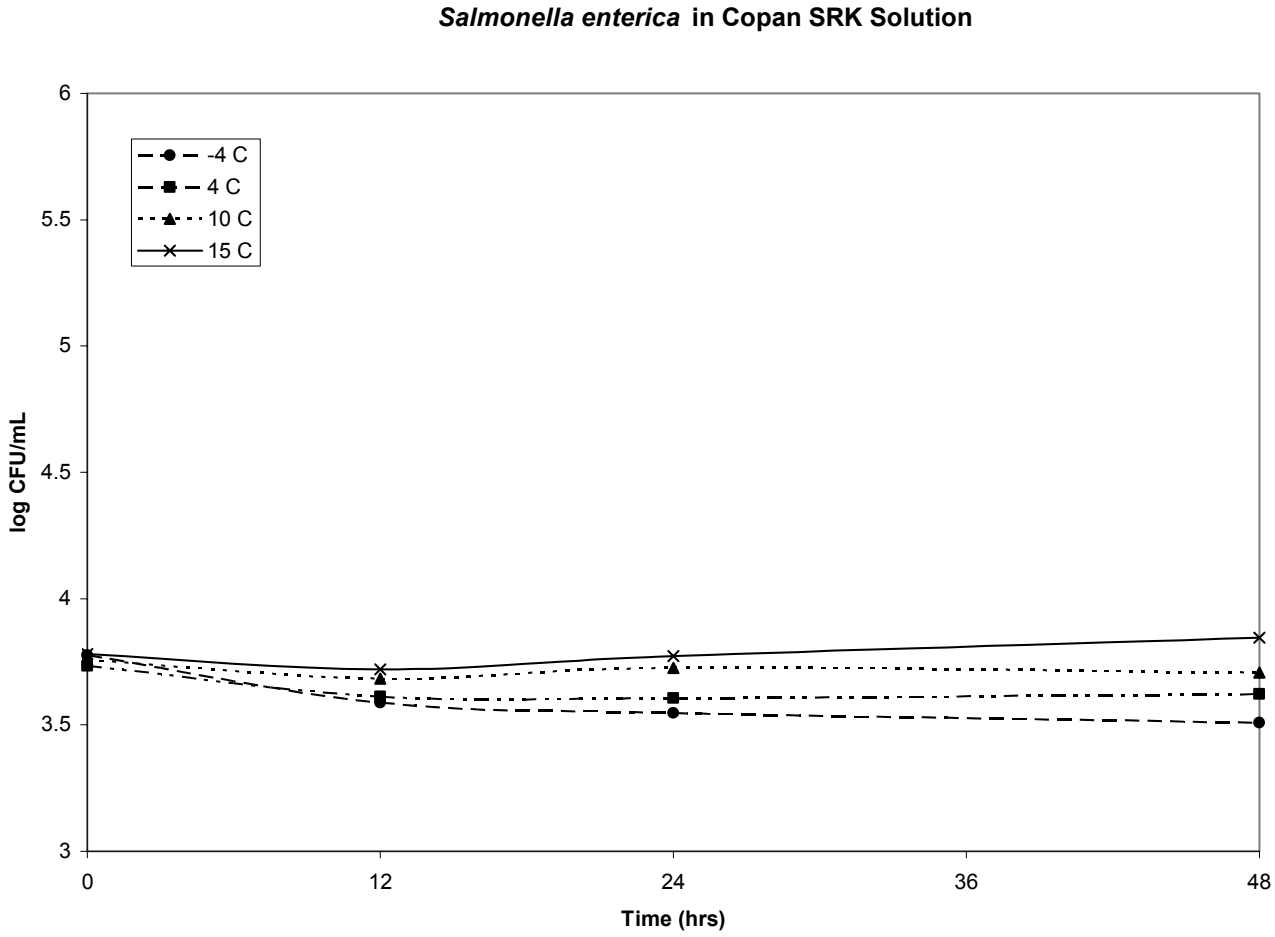

Figure 6. Mean log CFU/ml recovery of *S. enterica* from Copan SRK Solution over time at different storage temperatures (n=9 for each temperature).

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V. Vita

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