

MICROBIOLOGICAL METHODS

Quantitative Recovery of *Listeria monocytogenes* and Select *Salmonella* Serotypes from Environmental Sample Media

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Environmental sampling has become increasingly important in the food industry for monitoring the presence of specific pathogens such as *Listeria monocytogenes* and *Salmonella enterica*. Several microbiological media are available for storage and transport of environmental samples from the processing plant to the test laboratory. In this study, we quantified the survival of *L. monocytogenes*, *S. Typhimurium*, *S. Enteritidis*, and *S. Typhi* in environmental sampling media over several time and temperature combinations to determine optimum conditions for transport and storage. A cocktail of *L. monocytogenes* strains and *Salmonella* serotypes was separately added to tubes of Dey-Engley (D/E) Neutralizing Broth, Copan SRK solution, and Neutralizing Buffer and incubated at either -4, 4, 10, or 15°C. Counts were made of the bacterial load after 0, 12, 24, and 48 h. Neutralizing Buffer and Copan SRK solution were best at maintaining bacterial concentrations at all temperatures. D/E Neutralizing Broth, at 10 and 15°C, allowed significant bacterial growth. This study helped validate the use of these 3 media for environmental sample transport and storage at cold holding temperatures and demonstrated that, at elevated temperatures (>4°C), it is preferable to use Neutralizing Buffer or Copan SRK solution for quantifying microbial recovery.

Environmental sampling is a very important tool for studying the source, level, and dispersion of microbiological contamination in a defined area or facility. Two major microbial pathogens of concern in the food industry are *Listeria monocytogenes* and *Salmonella* spp. These 2 pathogens can be found in food processing facilities and are major concerns for public health officials. In the food industry, environmental sampling has become a common method for detecting microbial pathogens such as

these in addition to other microorganisms that can serve as indicators of the presence of pathogens. In some cases, this type of sampling minimizes the need for end product testing, which can reduce costs for food processors. It can also give the investigators a good idea of what might be causing product contamination and where the contaminant may have entered the food production line.

Although it is responsible for less than 0.1% of foodborne illnesses per year, *L. monocytogenes* is responsible for nearly 30% of deaths related to foodborne illness. Also, the hospitalization rate of listeriosis is >92% and the mortality rate exceeds 20% (1). Listeriosis is of special concern to immunocompromised populations and pregnant women (2). *L. monocytogenes* is associated with many types of food and is ubiquitous in the environment (3). Because of multiple recent outbreaks of listeriosis throughout the country, and the subsequent public outcry, the U.S. Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS) released an interim final rule on *L. monocytogenes* control in ready-to-eat meat and poultry products. This interim final rule (9 CFR Part 430) establishes what producers of these food products must do to eliminate this pathogen from their products and processes (4). An assessment of the effectiveness of the interim final rule was published by the USDA-FSIS in 2004. Although it made some recommendations about how to implement new procedures more easily to abide by the final rule, it found that overall the rule had increased awareness and safety procedures in processing facilities that produced ready-to-eat meat products (5).

Salmonella spp. are also of great public health concern. Each year in the United States, foodborne disease causes an estimated 76 million illnesses. Of these, an estimated 1.4 million are caused by *Salmonella*, resulting in approximately 16 000 hospitalizations and 580 deaths (1). These numbers may be low estimates, since salmonellosis often goes unreported for various reasons. *Salmonella* can cause a variety of diseases, including typhoid fever (6). Many serotypes of the species *S. enterica* are associated with human disease. The serotypes Typhimurium and Enteritidis represent the 2 strains most readily associated with human salmonellosis; serotype Typhi is considered the strain most dangerous to infected patients. These serotypes may be found

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in the intestines of many types of animals, especially poultry and swine, and can be transferred to food contact surfaces. Environmental sources of *Salmonella* include water, soil, insects, animal products, and factory surfaces (7). Therefore, *Salmonella* may be present throughout all stages of food production.

L. monocytogenes is a psychrotrophic organism which can grow and survive at refrigeration temperatures and may grow at temperatures as low as 0°C (8). This gives *L. monocytogenes* a competitive advantage over other pathogenic or spoilage bacteria present in foods and in food processing and storage environments. *L. monocytogenes* and *S. enterica* also differ in their cell envelope structure. *L. monocytogenes* is a Gram-positive organism with a thick outer peptidoglycan layer covering its cell membrane in its cell envelope. *S. enterica* is Gram-negative and has an outer and inner membrane encapsulating a much thinner peptidoglycan layer in its envelope (9). Also, *S. enterica* and most Gram-negative organisms have a convoluted outer cell wall which gives them a much higher surface area-to-volume ratio than a Gram-positive cell wall (10). These properties may lead to differing growth responses to storage times and temperatures.

The reliability and consistency of environmental sampling protocols is a concern to many in the food industry. Environmental sampling protocols may not specify a particular collection or transport media, sample size, or sample storage conditions. Furthermore, protocols may recommend a maximum rather than an optimum holding time or temperature for environmental samples before analysis. 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 (11). Other researchers have demonstrated differences in the ability of buffers to recover bacteria from environmental surface samples based on a buffer's ability to counteract the effects of residual sanitizers (12, 13). Appropriate diluents must also help maintain isotonic conditions for the organism and help reduce the stress on the cells during the storage and transport of the sample. Some diluents contain enrichment ingredients that can allow the cells to grow and reproduce during storage and transport (14).

Storage practices for environmental samples between collection time and the time they are processed (e.g., transferred to enrichment broth or plated onto microbiological media) can vary widely. In many cases, environmental samples are shipped to off-site laboratories and may be in the transport and storage chain for up to 48 h. This delay is critical to the reliability of the tests. The time between when environmental samples are taken and when they are processed in a laboratory can vary, depending on the facility being tested and the distance between the facility and the laboratory where it will be tested. In some cases, processing facilities have laboratories on site for immediate sample analysis; however, there may be a delay due to the need for batching samples

before testing. In other cases, a plant may not have a laboratory on site and will need to ship samples to an off-site test laboratory. The subsequent analysis of these samples may not begin until at least 1 or 2 days after sample collection. The importance of storage time on quantitative and qualitative bacterial recovery is apparent from a study by Arbiqúe et al. (15). In their study of 4 commercial transport media for the survival of *Neisseria gonorrhoea*, they reported that an 80% decrease in recovered populations occurred after just 6 h at 4°C storage. 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).

An optimum time/temperature/medium combination should be developed to recover microorganisms of interest from an environmental sample. 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. Even though an attempt to quantify may not accurately determine low levels of some pathogenic microorganisms, their presence or absence could still be determined. Improved quantitative analysis of microbial pathogens from environmental and food samples may enhance the effectiveness of antimicrobial interventions and increase the value of food safety microbiological risk assessments. The purpose of this research was to improve environmental sampling protocols by determining optimal sample storage media and storage conditions. The pathogens studied are often the target of environmental sampling and present a public health risk that is important to the food processors.

Experimental

Listeria Inoculum Preparation

Four strains of *L. monocytogenes* (LCDC, Scott A, D43, and V7) were used to create a cocktail. These strains were obtained from the Virginia Tech Department of Food Science and Technology, Blacksburg, VA. Single-strain cultures were stored at -80°C in Tryptic Soy Broth (TSB) supplemented with 25% glycerol. Before 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 2 consecutive days. The cultures were incubated at 33 ± 2°C during growth. The mean initial concentration of each of the 4 strains of *L. monocytogenes* was about 1.5 × 10⁹ colony-forming units (CFU)/mL. Equivalent proportions of each cultured strain were combined before dilution in Butterfield's Phosphate Dilution Water (BPDW; Biotrace International Bioproducts, Bothell, WA).

Culture purity and confirmation of species were performed before each experiment with the sample collection media. From the final subculture of each of the 4 strains of *L. monocytogenes*, 0.1 mL was streaked onto Brain Heart Infusion Agar (BHIA) plates and incubated for 24 h at 33 ±

2°C. 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 (bioMérieux, Hazelwood, MO). All colony isolates were identified as *L. monocytogenes*.

Salmonella Inoculum Preparation

Salmonella Typhimurium and *Salmonella* Enteritidis were obtained from the Virginia Maryland Regional College of Veterinary Medicine, Blacksburg, VA. *Salmonella* Typhi (ATCC No. 9993) was obtained from the American Type Culture Collection, Manassas, VA. Cultures were stored at -80°C in TSB supplemented with 25% glycerol. Before use in testing, cultures were thawed and added to 10 mL tubes of TSB, and incubated at 33 ± 2°C. These cultures were subcultured into fresh TSB daily for at least 2 consecutive days. The mean initial concentration of each of the 3 serotypes was about 1.1 × 10⁹ CFU/mL. Equivalent proportions of a 24 h culture of each strain were combined before dilution in BPDW. Fresh cultures were prepared from frozen stocks for each test repetition.

Culture purity and confirmation of species identification was performed before each experiment with the sample collection media. From the final subculture of each of the 3 serotypes of *Salmonella*, 0.1 mL was streaked onto Tryptic Soy Agar (TSA). These plates were incubated for 24 h 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 (bioMérieux).

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) was stored at 4°C per manufacturer's instructions. Neutralizing Buffer (Difco), per manufacturer's instructions, was stored at 25°C until 24 h before test initiation, and then refrigerated. Swab Rinse Kits (SRK; Copan USA, Corona, CA) per manufacturer's instructions, were stored refrigerated (4°C). Tubes of SRK included a swab attached to the inside top of the tube. The swabs were aseptically removed before media use. Function of ingredients in each transport media: *D/E Neutralizing Broth*.—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; bromocresol purple: pH color indicator. *Neutralizing Buffer*.—Monopotassium phosphate: neutralizes pH of buffer; sodium thiosulfate: neutralizes iodine and chlorine; aryl sulfonate complex: neutralizes quaternary ammonium compounds. *Copan SRK solution*.—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. All 3 test media were chilled at least 24 h at 4°C before use. Refrigeration temperature was used as a starting temperature because, in practice, sampling media would be chilled before sample collection. The room temperature pH values of these media were 7.52, 7.04, and 7.18 for D/E Neutralizing Broth, Neutralizing Buffer, and Copan SRK solution, respectively.

Inoculation of Test Media

For *L. monocytogenes*, 1 mL of the prepared cocktail (22 ± 2°C), diluted to ca 1.0 × 10⁴ CFU/mL, was aseptically added to each of 3 tubes of each of 3 test media (9 tubes total per temperature per repetition, and 9 mL per tube) and shaken by hand. Three tubes of each media were incubated at one of the 4 storage temperatures (-4, 4, 10, or 15°C) for up to 48 h. These inoculation procedures were duplicated for the *Salmonella* serotypes in fresh media.

Enumeration Procedures

An initial *L. monocytogenes* concentration (time zero) was determined for each inoculated media tube as described below. All tubes were sampled again after 12, 24, and 48 h incubation at one of the 4 test temperatures. All tubes were returned to their respective incubation temperature within 20 min after sampling. Each tube was agitated, and 0.1 mL was spread onto duplicate plates of BHIA. Additionally, at least one 10-fold dilution was spread-plated onto BHIA. All plates were incubated at 33 ± 2°C for 24 h before counting. These procedures were duplicated for *Salmonella*, except TSA was used in place of BHIA. All plates were then incubated at 33 ± 2°C for 24 h before counting.

Data Analysis

For each microorganism, 3 tubes of each transport media were inoculated for each of the incubation temperatures and sampling times. The mean concentration of 3 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 ($\alpha = 0.05$).

Results and Discussion

L. monocytogenes Survival

The mean log CFU/mL recovery of *L. monocytogenes* over time was significantly different ($\alpha < 0.05$) for only 2 storage

conditions: D/E Neutralizing Broth held at either 10 or 15°C. As seen in Figure 1, *L. monocytogenes* populations were maintained relatively well in D/E Broth at -4 and 4°C. At 10°C, there was significant overall growth after 24 h. 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 h.

L. monocytogenes counts remained constant over the 48 h test period for all 4 temperatures tested with the Neutralizing Buffer (Figure 2). There was no significant growth or cell death under these conditions. In the Copan SRK solution, *L. monocytogenes* concentrations also remained constant (Figure 3). The mean log CFU/mL recovery standard deviation for all data sets ($n = 9$) was <0.2 log CFU/mL, with 2 exceptions. In the D/E Neutralizing Broth, when media was held at 15°C for 24 h, the count was 5.35 log CFU/mL ± 0.57 , and after 48 h, the final count was 7.43 log CFU/mL ± 1.10 .

Salmonella Survival

The mean log CFU/mL recovery of the *Salmonella* serotypes over time was significantly different ($\alpha < 0.05$) for only 3 storage conditions: Copan SRK solution held at -4°C, and D/E Neutralizing Broth held at either 10 or 15°C. Detectable numbers of *Salmonella* remained constant over the 48 h test period when D/E Neutralizing Broth was held at -4 and 4°C (Figure 4). After 48 h at 10°C, the cell number showed a slight increase of about 0.5 log CFU/mL. A dramatic increase in growth was observed in D/E Neutralizing Broth after 24 h at 15°C. When *Salmonella* cells were incubated in Neutralizing Buffer, the cell concentration was maintained throughout the test period of 48 h for all temperatures tested (Figure 5). *Salmonella* cell concentrations remained relatively constant throughout the 48-h test period when inoculated Copan SRK solution was incubated at 4, 10, and 15°C (Figure 6). However, during -4°C storage, there was a decrease in detectable cells of ca log 0.3. This reduction in cell concentration over 48 h was the greatest observed in this study. The mean log CFU/mL recovery standard deviation for all data sets ($n = 9$) was <0.2 log CFU/mL, with 3 exceptions. In D/E Neutralizing Broth held at 10°C for 48 h, the mean counts were 4.21 log CFU/mL ± 0.28 . In the D/E Neutralizing Broth held at 15°C, the mean count was 4.76 log CFU/mL ± 0.38 after 24 h, and 6.82 log CFU/mL ± 0.33 after 48 h.

Transport Media Incubation Time and Temperature

Although D/E Broth may have a high ability to neutralize sanitizers or resuscitate injured cells, this study suggests that this media may not be ideal for quantitative testing because significant growth can occur in this media for the 2 organisms tested. *L. monocytogenes* populations increased in the D/E Broth at 10 and 15°C. This growth could be attributed to the presence of compounds (e.g., casein peptone, dextrose, yeast extract) in the D/E Broth that are not present in the other 2 diluents (see *Sample Collection Test Media*). *L. monocytogenes* is also a psychrotrophic organism and therefore can grow at lower temperatures. If these tests were continued beyond 48 h, growth could be expected at the 4°C

temperature as well. The *Salmonella* serotypes also showed growth at the higher test temperatures in D/E Broth. They grew well at 15°C, and populations increased slightly at 10°C, probably due to the presence of the compounds mentioned above. *Salmonella* growth rates would be expected to be slower than that of *L. monocytogenes* at these temperatures (10–15°C).

Under certain conditions, a slight, initial decline in cell count was observed (Figures 5 and 6), probably because of 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 2 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 during 48 h incubation. The greatest population decrease (about 45%) was for *Salmonella* serotypes stored in Copan SRK solution for 48 h. Because the preferred storage temperature for environmental samples is 4°C (16), it is important to note that, at this temperature, the cell concentrations decreased $<10\%$ for *Salmonella* in Neutralizing Buffer, 13–19% in D/E Neutralizing Broth, and 20–23% in Copan SRK solution. Cell concentrations decreased $<10\%$ for *L. monocytogenes* in D/E Neutralizing Broth, Neutralizing Buffer, and Copan SRK solution after 48 h.

Summary

Several microbiological media are available for storage and transport of environmental samples to a testing laboratory. Differences in the media formulations, along with inconsistencies in incubation conditions (time and temperature) can lead to variability in cell populations. In this study, Neutralizing Buffer and Copan SRK solution maintained consistent bacterial populations at all test temperatures. At 10 and 15°C, D/E Neutralizing Broth supported bacterial growth of both *L. monocytogenes* and select *Salmonella* serotypes (*S. Typhimurium*, *S. Enteritidis*, and *S. Typhi*). Subsequent studies could examine other strains or serotypes of these pathogens and quantify microbial concentrations of individual strains or serotypes. Another consideration for further study is a determination of the impact of competing microorganisms on the relative populations of the pathogens studied. In this case, temperature control of the collection media may prove to be especially critical. This study validates the use of D/E Neutralizing Broth, Neutralizing Buffer, and Copan SRK solution for environmental sample transport and storage at proper (cold) holding temperatures. If samples are held at temperatures $>4^\circ\text{C}$, Neutralizing Buffer or Copan SRK solution should be used for quantifying microbial recovery.

Listeria monocytogenes in D/E Neutralizing Broth

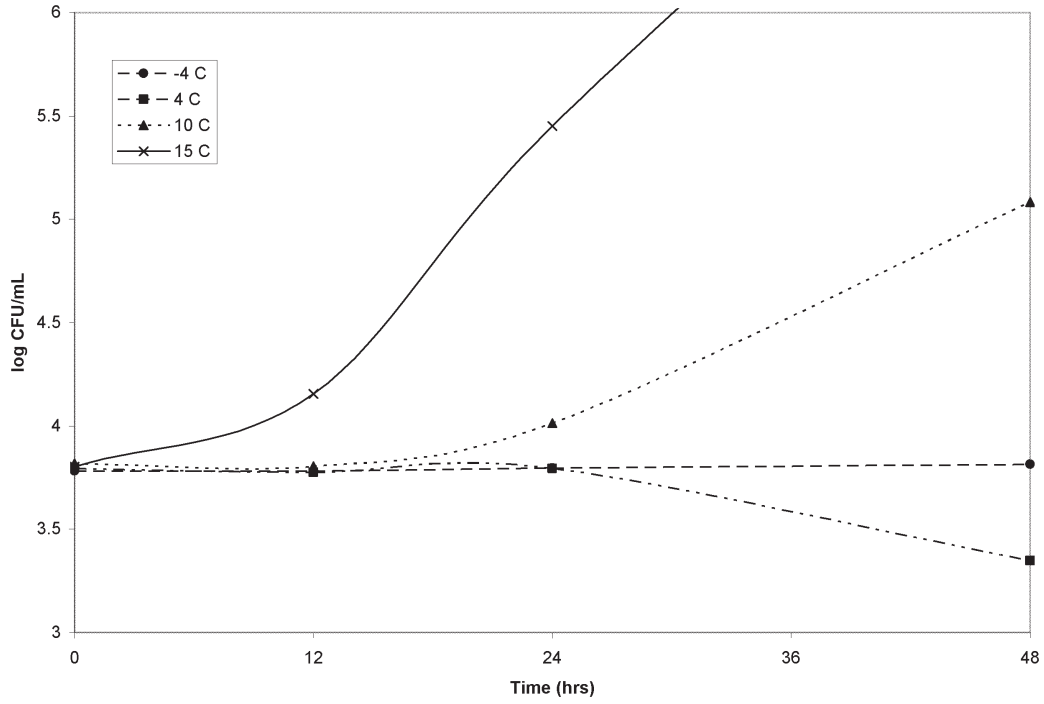


Figure 1. Mean log CFU/mL recovery of *L. monocytogenes* from D/E Neutralizing Broth over time at different storage temperatures ($n = 9$ for each temperature except 15°C where $n = 6$ because limit of detection exceeded on 3 samples).

Listeria monocytogenes in Neutralizing Buffer

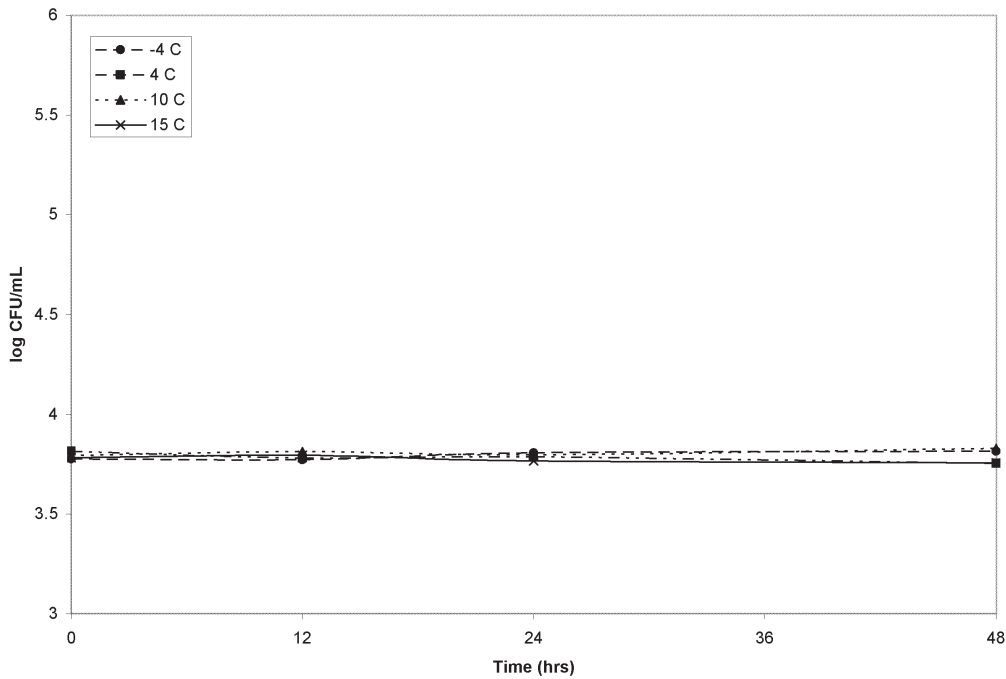


Figure 2. Mean log CFU/mL recovery of *L. monocytogenes* from Neutralizing Buffer over time at different storage temperatures ($n = 9$ for each temperature).

Listeria monocytogenes in Copan SRK Solution

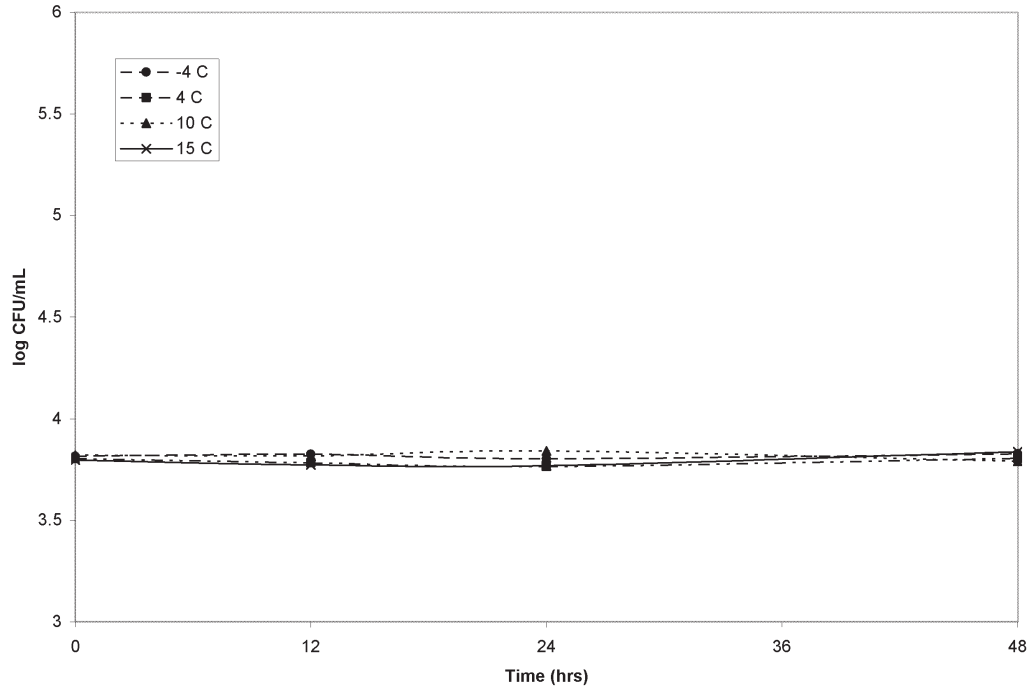


Figure 3. Mean log CFU/mL recovery of *L. monocytogenes* from Copan SRK solution over time at different storage temperatures ($n = 9$ for each temperature).

Salmonella enterica in D/E Neutralizing Broth

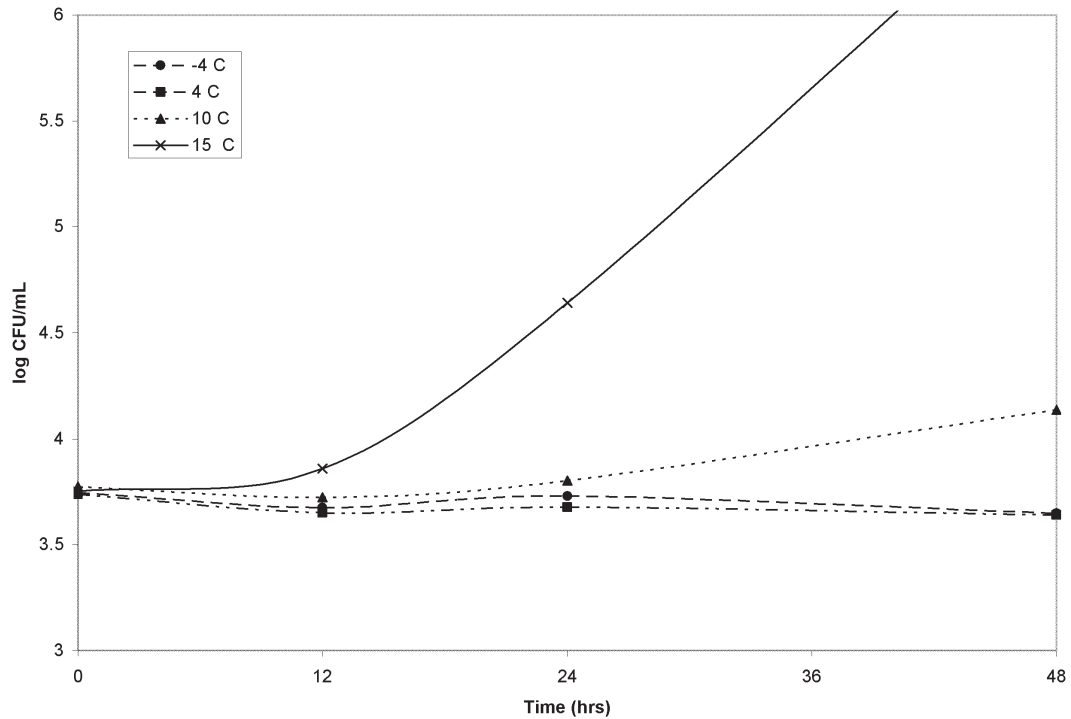


Figure 4. Mean log CFU/mL recovery of select *Salmonella* serotypes from D/E Neutralizing Broth over time at different storage temperatures ($n = 9$ for each temperature).

Salmonella enterica in Neutralizing Buffer

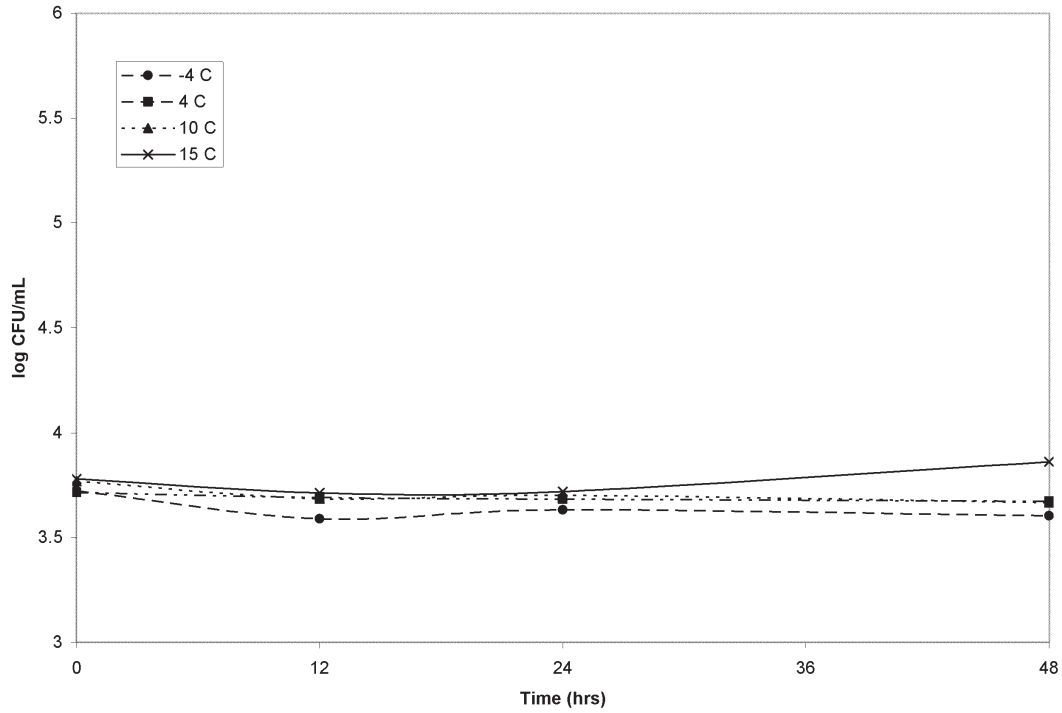


Figure 5. Mean log CFU/mL recovery of select *Salmonella* serotypes from Neutralizing Buffer over time at different storage temperatures ($n = 9$ for each temperature).

Salmonella enterica in Copan SRK Solution

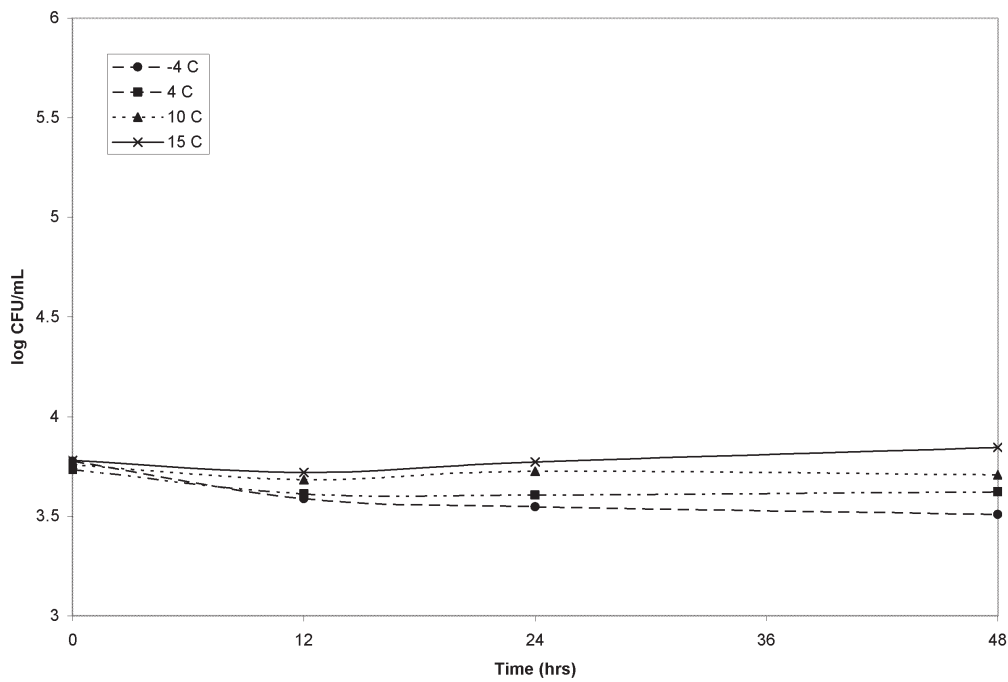


Figure 6. Mean log CFU/mL recovery of select *Salmonella* serotypes from Copan SRK solution over time at different storage temperatures ($n = 9$ for each temperature).

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