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Establishing a Method for Microbiological Evaluation of Fresh Produce at Risk for *Salmonella* Contamination

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Thesis Committee Chair: Karen Levy, PhD

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Abstract

Establishing a Method for Microbiological Evaluation of Fresh Produce at Risk for *Salmonella* Contamination
By Whitney Pennington

Salmonellosis is one of the leading causes of gastroenteritis in the United States, with incidence of human cases highest in the southeastern states of Alabama, South Carolina, North Carolina, Florida, and Georgia. Of recent concern is the frequency and impact of foodborne outbreaks of *Salmonella* due to contamination of pre- and post-harvest produce, specifically leafy greens, tomatoes, and cantaloupe. While the source of contamination in these produce-related outbreaks is rarely specified, suspected causes include wildlife, proximity to agricultural farms whose wastes infiltrate water supply used for irrigation, and application of feces as a form of fertilizer. Here we will establish methods to evaluate the surface of fresh produce grown on farms using untreated surface water as source water for irrigation for the presence of *Salmonella* bacteria. We describe the standardization, optimization, and validation of a produce wash method that preserves and then resuscitates samples frozen at -80C. We confirm that freezing samples in a non-selective media supplemented with 15% glycerol for preservative followed by a 2-hour reincubation after being thawed is optimal to efficiently recover *Salmonella* from frozen samples. Moreover we show that this method is sufficient at recovering *Salmonella* from the surface of broccoli where the most probable number (MPN) *Salmonella* concentration of irrigation water is 0.26 MPN/100ml.

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INTODUCTION

Foodborne Illness and Food Safety

The number of outbreaks of enteric illnesses related to the consumption of contaminated foods, particularly produce, is on the rise [1, 2]. While increases in surveillance and detection as well as an increase in the consumption of fresh produce play a role, the primary drivers of the increase in foodborne disease outbreaks are thought to be issues related to food production and distribution [1, 3].

One of the challenges in addressing produce safety concerns is tracing illness back to a specific food source. Sprouted seeds, leafy greens, and tomatoes are the most commonly identified vehicles of foodborne outbreaks [3]. Specific to *Salmonella* outbreaks, tomatoes and melons are the most common vehicles [4]. In the last 10 years, the Centers for Disease and Control and Prevention (CDC) has investigated dozens of outbreaks related to produce sources from the Unites States and Mexico including cucumbers, cantaloupe, mangoes, alfalfa sprouts, peppers, and tomatoes [5].

Even more challenging than determining the vehicle of the outbreak is tracing the origins of contamination of these foodstuffs. While the source of contamination in these outbreaks is rarely specified, factors influencing contamination are distinguished as occurring pre-harvest or post-harvest. Post-harvest factors such as packing shed conditions (presence of rodents, quality of storage containers, etc.) and worker hygiene practices have generally received the most attention [2]. Pre-harvest factors influencing contamination include the use of untreated surface water for irrigation, wildlife, application of fecal material as a form of fertilizer, and proximity to agricultural farms or livestock operations whose wastes infiltrate water supplies used for irrigation, all of

which are generally less understood than their post-harvest counterparts [2]. It has been suggested that research investigating these pre-harvest factors at broader scales and with larger scope may enhance the understanding of and ability to prevent produce-related outbreaks [6].

The Food and Drug Administration (FDA) is responsible for overseeing the safety of domestic food both pre- and post-harvest through pathogen monitoring programs. [7]. To specifically address concerns with pre-harvest factors, proposed rule § 112.41 would require that "all agricultural water must be safe and of adequate sanitary quality for its intended use". This rule requires regular testing of the water sources and irrigation distribution equipment to ensure that neither is a potential source of contamination. The rule is specific to foods to which irrigation is directly applied to the harvested portion of the plants (e.g. greens) [8].

Salmonellosis

Salmonellosis is one of the leading causes of gastroenteritis in the United States, with an estimated 1 million people infected each year [9]. CDC national reporting data from 2011 also show that the incidence of human cases is highest in the southeastern states of Alabama, South Carolina, North Carolina, and Georgia [10]. The etiologic agent of salmonellosis is the diverse genus of gram-negative *Salmonella* spp., specifically those of the *enterica* species. With over 2500 known serotypes, *Salmonella enterica* is a ubiquitous pathogen capable of infecting or colonizing the intestines of a diverse set of hosts (human, birds, reptiles, and others) and surviving in environmental reservoirs (water and soil). (Hereafter, *"Salmonella*" will be used in place of "*Salmonella* spp." unless otherwise noted by the name of the specific species or serotype.)

Primarily a foodborne pathogen, *Salmonella* is the second most common etiologic agent of the thirty-one agents currently known to cause foodborne illness [9]. Based on current knowledge, the bacteria is most commonly transmitted to humans through ingestion of foods of infected animal origin (e.g. chicken or eggs) or other foods products (e.g. fruits and vegetables) that have been contaminated either pre- or post-harvest [4, 11] . While animal feces is most often implicated as the source of this contamination, other sources, such as food preparation handlers and processes, have also been confirmed [12].

Worldwide, clinical salmonellosis cases are most often caused by the *Salmonella enterica* serotypes Typhimurium and Enteritidis [13]. Based on epidemiological data from the United States, the majority of severe cases, 74%, are caused by only eight serotypes [14]. This suggests that while all of the 2500 serotypes are considered pathogenic, the number that are clinically relevant is much smaller [15]. Moreover, those illnesses resulting from the ingestion of contaminated animal products are caused by different serotypes than those illnesses resulting from the ingestion of contaminated fresh produce [4]. Specifically, plant-related cases are more commonly caused by serotypes from environmental and amphibian reservoirs whereas those more commonly causing animal-commodity outbreaks are clinically relevant in that animal species [4].

Salmonella can survive outside of a host, particularly in environmental reservoirs such as soil or water, which can lead to the contamination of fresh produce [16]. While ecological studies suggest that survivability differs between serotypes of *Salmonella*, compared to other enterobacteria, such as *Escherichia coli*, genetic factors specific to the broad *Salmonella* genus enhance its survivability against stresses encountered external to a host, such as temperature, pH, and low nutrient availability [3, 16]. While *Salmonella*

contamination of produce as the direct result of the application of contaminated irrigation water or harvest from contaminated soil has not been definitively established, nevertheless these reservoirs are often cited as potential culprits of contamination [3, 15] because of their presence in the source waters used for irrigation [17].

Georgia and the Southeastern Coastal Plain

Between 1998 and 2008, the incidence rate of salmonellosis in Georgia was between 17-26.5 cases per 100,000 people [18], a rate consistently among the highest in the country [10, 19, 20]. Moreover, the South Atlantic region, which includes Florida, Georgia, North Carolina, and South Carolina, has one of the highest incidence rates among any region in the United States [20]. Among the ten states that make up CDC's Foodborne Diseases Active Surveillance Network (FoodNet[1]), Georgia has the highest incidence rate of salmonellosis, with the south-central region of the state having highest incidence [18]. Case control-studies have indicated that potential risk factors for illness in Georgia include foodborne, waterborne, and environmental exposures: consumption of well water, consumption of poultry products (inside or outside of the home), and handling of reptiles or amphibians [21, 22].

The areas of Georgia with especially high incidence of salmonellosis lie in the Southeastern Coastal Plain (SECP), an ecoregion important for vegetable production in the United States. Vegetable producers in the SECP most often utilize constructed surface water ponds as irrigation sources, a practice that allows for high capacity withdrawal and water application. These ponds are created by damming local streams and

[1] FoodNet States include Connecticut, Georgia, Maryland, Minnesota, New Mexico, Oregon, and Tennessee and selected counties of California, Colorado, and New York.

are primarily replenished by these streams and run-off from precipitation events. Water withdrawn from the pond is applied to produce via drip, center pivot, or solid set irrigation systems. In drip irrigation systems, water is withdrawn from the pond and passes through a series of sand filters before entering the irrigation piping. Alternatively, for center pivot and solid set systems, water enters the irrigation piping without being filtered.

A previous study of the natural surface waters in the south central region of Georgia revealed surface water sources to be consistently contaminated with *Salmonella* [23]. Thirteen serotypes were isolated from river tributaries of over a one-year sampling period, with *Salmonella* arizonae being most prevalent. Interestingly, none of the notable clinical strains commonly causing human illness in the area were isolated from the waters. The absence of these clinical strains suggests that intraspecies variation exists that confers increased water survivorship in particular *Salmonella* serotypes and that the clinically relevant serotypes of the area may not have that capability [23].

Closing the Gap

In a recent study carried out by Dr. Karen Levy (Emory University), Dr. George Vellidis (University of Georgia) and other colleagues, measurable amounts of *Salmonella* have been recovered regularly from these types of irrigation ponds in and around Tifton, Georgia. While these particular ponds are not known to have caused any produce contamination, gaps in produce safety knowledge exist that result in contaminated source waters generally being assumed as the source of crop contamination without adequate confirmation [17]. A new study by Drs. Levy and Vellidis seeks to close this gap using

produce wash techniques to evaluate the surfaces of vegetables irrigated with water from ponds where *Salmonella* has been detected.

The study presented here describes the development and validation of produce wash methods, focusing on sampling of broccoli. This includes determining the methods with which to wash the produce given limitations induced by freezing, standardizing the amount of produce to be washed, and determining the upper and lower limits of concentrations of *Salmonella* in irrigation water where the pathogen is still recoverable from the produce wash rinseate. The conditions determined to be most optimal for recovery will be used as part of a new study by Drs. Vellidis and Levy to determine if irrigation ponds contaminated with *Salmonella* pose a threat to the microbiological quality of produce.

METHODS

Overview of Produce Washing Methods

Produce wash methods are used to evaluate the surfaces of fruits and vegetables for microbial contamination. The methods involve rinsing a standard amount of produce in media followed by the enumeration of microorganisms from that rinseate media [24]. The specific microorganism(s) to be detected determines the exact protocol from rinse to enumeration—the types of medias used, direct plating techniques, and incubation times and temperatures [24].

Review of the literature reveals that produce washes are generally standardized by the amount of surface area of the specific food product being washed [24-30]. Standardizing by surface area is ideal because it estimates the presence of microorganisms as a function of how much of the exterior of the plant is exposed to potential sources of contamination. Because of differences in size, shape, and morphological features, standardization by surface area is not always possible [24]. In particular, broccoli presents a unique challenge to the produce wash procedure, as the complex floret nature of broccoli makes the true surface area of broccoli extremely large. Whereas other types of produce are standardized by surface area, in the literature broccoli was always standardized according to weight [25-31].

An additional consideration to be made in the development of this particular produce wash method is the requirement of a 90-day period of freezer storage of produce rinseates to secure the participation of vegetable growers. This must be done in order to avoid regulatory reporting issues for growers if *Salmonella* is found in any sample while the produce is still in the market. Analysis of samples after harvest and circulation in the

market through this holding technique is the only way growers will cooperate with researchers to test fresh produce.

In a review of microbiological injury and recovery methods in food, Wu [32] describes the three consequences of stress on microbiological organisms: survival, sub-lethal injury, or death. In this particular case we are concerned with these consequences as they are related to cold-stress since rinseates will be frozen at -80C for 90 days. Cell death as the result of freezing may be prevented using preservation methods and recovery of the remaining viable cells may be facilitated by resuscitation through incubation in enrichment media. Experiments aimed at determining the most efficient recovery of *Salmonella* from frozen produce rinseates should therefore experimentally determine the optimal volume concentration of preservation media, the best media for resuscitation, as well as the optimal length of time samples are resuscitated (Figure 1).

Phase 1

Most Probable Number Enumeration

The need to freeze our produce rinseates for 90 days before they are cultured forces us to make considerations generally not required by standard Most Probable Number (MPN) culture techniques. Generally the MPN technique for *Salmonella* involves incubation of samples in a non-selective media, followed by incubation in a selective media, and finally plating onto selective agar. The aim of this project was to determine whether *Salmonella* was recoverable from frozen water samples using this technique and if so, establish the preservation and resuscitation techniques that facilitated the most efficient recovery after freezing. To maintain consistency with the enumeration techniques used for the irrigation pond sampling, we sought to augment the MPN

protocol already validated and in use by collaborators at UGA-Tifton described below and in Figure 2.

To determine the concentration of *Salmonella* in irrigation ponds, two liters of water are collected from each pond in 1L size plastic bottles and large Whirl-Pak® bags. These samples are stored on ice and transported to the laboratory where they are refrigerated overnight. The next day, samples are shaken and poured into three replicates of each of the following volumes for a total of nine replicates: 500ml, 100ml, and 10ml. One additional 10 ml replicate is poured. To each of the 10 replicates of sample, an equal volume of non-selective enrichment media is added: sterile double strength lactose broth (LB). The additional 10ml replicate serves as a positive control and is spiked with one plastic 1µl loopful of a lab-maintained *Salmonella* inoculum. Samples are then incubated at 37C for 24 hours. Following this incubation, 1ml of each of sample is added to 10ml of sterile tetrathionate broth, a media selective for *Salmonella* microorganisms, and incubated for 24 hours at 37C. The next day, one 1µl loopful of each sample is streaked onto Xylose Lysine Tergitol-4 agar, agar specific for selection of *Salmonella* species. Plates are inverted and incubated at 37C. After 20 hours, plates are checked for the presence of black presumptive *Salmonella* colonies. After an additional 4 hours, plates are checked for additional growth. Up to three black colonies from each presumptively positive replicate are streaked onto ChromAgar, another *Salmonella* selective agar. Plates are inverted and incubated for 24 hours at 37C. Presumptive purple *Salmonella* colonies are then preserved in Luria Broth at room temperature before undergoing PCR confirmation. Once colonies have been confirmed as *Salmonella*, growth or the absence

of growth for each replicate is recorded and an MPN is calculated based on methods adapted from Jarvis et al. [33].

Resuscitation Media and Optimal Resuscitation Length

When exposed to cold stress, some microorganisms will die, and those that survive may do so with injury to the cytoplasmic membranes, limiting their ability to multiply as well as withstand future stress [32]. This future stress includes the exposure to selective agents they would otherwise have resistance to. However, the restoration of these lost capabilities is possible through a process termed *resuscitation*: a period of incubation under ideal conditions that allows for cellular repair [34]. After repair, cells once again confer resistance to selective agents as well as the ability to multiply.

As described by Wu [32], resuscitating bacteria for most probable number (MPN) counts requires special attention such that injured bacteria are resuscitated but surviving cells do not multiply, inflating their concentration within a sample. Generally, literature describes reincubation of bacteria in a non-selective media for 1-5 hours as the best practice. This time period allows the total population of pathogens to be resuscitated before using selective media to target the species of interest [32].

In order determine which media already used in the MPN method, non-selective LB or selective tetrathionate, was a better resuscitation media, a total of 18 water samples were collected from three surface ponds near Tifton, GA in September 2013. These water samples were taken to the UGA-Tifton laboratory where they were processed according to the MPN methods described above. After the incubation of samples with sterile double strength LB, a 10ml aliquot of each of the eighteen 500ml sample-LB replicates was

transferred into an empty conical vial and capped. Additionally, a 1ml aliquot of each of the eighteen 500ml sample-LB replicate was transferred into a conical vial containing 10ml of tetrathionate broth. Both sets were then driven at room temperature for three hours to the Emory laboratory in Atlanta, GA. Once at Emory, these samples were frozen at -80C for 20 days.

During this freezing time, the Tifton laboratory completed culture and isolation of *Salmonella* from the original water samples according to the standard protocol. After PCR confirmation, it was determined that 22 of the 36 replicates frozen in each set at Emory came from water samples where *Salmonella* was recovered in the Tifton laboratory.

Of these 22, 15 were randomly selected to be thawed and cultured in the Emory laboratory. After 20 days, the randomly selected replicates were removed from the freezer and thawed at room temperature for 2-3 hours. For those samples that had been frozen in lactose broth, we selected three resuscitation times to experimentally test: two hours, five hours, and 24 hours. A review of microbiological recovery literature by V.C.H. Wu suggested that for most organisms, resuscitation time of 1-5 hours in non-selective media is sufficient for repair, though some organisms or stresses (e.g. cold, hot, pH) may require a longer period of time [32]. Thus two and five hours were selected as representative of the minimum and maximum values of this range and 24 as a longer period and thawed samples were then randomly assigned one of these time periods. After this reincubation, 1ml of each sample was added to 10ml of tetrathionate, incubated for 24 hours at 37C, and then streaked onto XLT-4 consistent with the standard MPN method.

For those samples that had been frozen in tetrathionate broth, we only selected one reincubation time period: 24 hours. In the standard MPN methods, once transferred to tetrathionate broth, samples are incubated for 24 hours before being streaked onto XLT-4 agar such that non-*Salmonella* organisms are selected against and do consume resources available in the agar used for enumeration. We felt that to incubate samples for less than 24 hours would not be long enough to eliminate these competing organisms and thus would limit the growth of *Salmonella*. Thus after thawing, all 15 *Salmonella* positive samples that had been frozen in tetrathionate were reincubated for 24 hours at 37C before being streaked onto XLT-4 agar.

After 20 and 24 hours incubated at 37C, the re-growth of *Salmonella* bacteria was recorded for each replicate in order to establish a recovery efficiency each of the four experimental conditions, calculated as the fraction of total samples cultured in each condition that showed recovery after freezing and resuscitation. The two most efficient conditions were then used in the next experiments. As PCR had been performed on the same samples at the UGA-Tifton laboratory to confirm the presence of *Salmonella*, the colonies grown in this experiment were not purified on ChromAgar nor was PCR preformed.

Volume Concentration of Preservative and Optimal Resuscitation Length

In a second experiment, we chose the two best performing conditions from the previous experiment and tested how the addition of a preservative to these medias before freezing increased recovery. In 1887, T.M. Prudden first noticed the deleterious effects of freezing on bacterial survivability [35]. In order to combat these effects of freezing, preservatives are used. Specifically, Hollander and Nell (1954) found that the addition of

15% glycerol before freezing increased the survivability of *Treponema pallidum*, *E. coli*, and *Diplococcus pneumonia* during freezing [36]. Howard found the same to be true of *Salmonella* [37]. We specifically chose to compare this 15% volume concentration with two alternatives: 0% and 50%. Zero was chosen to test whether preservative was in fact necessary for this particular protocol and 50% was selected because it is the current concentration of preservative used by the UGA-Tifton laboratory for the long-term storage of *Salmonella* isolates recovered from irrigation pond.

In order to determine which concentration of preservation facilitated the most efficient recovery in conjunction with optimal resuscitation media, four 100ml samples of tap water were each inoculated with a different strain of *Salmonella* derived from a specific pond location in the previous experiment. To ensure that the concentration of these samples was within the detectable range of the MPN method, a total of 10 colonies was added to each water sample. After inoculation, water samples were refrigerated for 24 hours to be consistent with irrigation pond sampling where samples are refrigerated overnight before processing. Twenty-four hours later, 100ml of double strength LB was added to each sample, and samples were incubated for 24 hours at 37C. The following day for each sample, three 1ml aliquots were added to 10ml of tetrathionate broth, six 10ml aliquots were added to an empty conical vial (0% glycerol by volume), six 8.5ml aliquots were added to 1.5ml glycerol (15% glycerol by volume), and six 5ml aliquots were added to 5ml glycerol (50% glycerol by volume) (see Figure 3). The replicates containing tetrathionate broth then followed the standard MPN protocol to plating on XLT-4 agar to confirm the presence of *Salmonella* in the original samples. The replicates that did not contain tetrathionate (0%, 15%, and 50% glycerol samples) were then mixed

well and placed in the -80C freezer. In order for all samples to be completely frozen to the conditions expected in a 90 day freeze, samples were remained in the freezer for 4 days. The samples were then removed from the freezer and thawed for 2-3 hours on the benchtop. Three replicates of each glycerol concentration for each sample were then reincubated for 2 hours at 37C and three for each glycerol condition and sample were reincubated for 5 hours at 35C. Following this reincubation, 1ml, 1.15ml, and 2ml of, respectfully, the 0%, 15%, and 50% was added to 10ml of Tetrathionate broth and incubated for 24 hours at 37C. The following day, each replicate was plated on XLT-4 agar and incubated at 37C. After 20 and 24 hours, the re-growth of bacteria was recorded, and the recovery efficiency for each condition (6 total) was calculated as described previously.

To further determine the optimal combination of preservation media and resuscitation time, a third experiment proceeded exactly as the second with a few minor modifications. Five 100ml volumes of distilled water samples were inoculated one of five *Salmonella* strains: one of the four used in the previous experiment or the Tifton laboratory positive control strain. This experiment was conducted twice. Again, to ensure that the concentration of these samples was within the detectable range of the MPN method, a total of 5 colonies was added to each water sample for the first trial and a total of 1 colony was added to each water sample for the second trial.

Each of these water samples was then refrigerated for 24 hours, and the experiment proceeded according to the methods for experiment two, creating samples in 0%, 15%, or 50% glycerol and reincubated for 2 or 5 hours. We determined that including the five different strains was a sufficient form of replication that unlike the

previous experiment, for each experimental condition only one replicate was cultured whereas the previous experiment cultured three.

Since the second and third experiments tested the same experimental conditions, the results of these two experiments were pooled together to determine which of the six experimental conditions had the highest recovery efficiency and would be chosen as the optimal resuscitation and recovery method.

Phase 2

Once we determined the ideal culture protocol for our produce rinseates, we completed the produce wash procedure by standardizing the mass of broccoli in each wash. Then to determine the range of concentrations of irrigation water where recovery from the rinseate is possible, we inoculated store-bought produce in the lab and washed it according to our protocol (Figure 4). We were interested in identifying the MPN of *Salmonella* inoculum applied to broccoli whose washes resulted in MPN methods (1) with 8 of 9 replicates producing growth (upper limit of detection) and (2) with only 1 of 9 of replicates producing growth (lower limit of detection).

Standardizing the Produce Wash

To determine the amount of broccoli to be used in a produce wash, store-bought broccoli crowns were placed into 123 ounce Whirl-Pak® bags. These bags were selected based on their capacity to hold 2L of liquid, the approximate volume needed for the existing MPN culture methods. The amount of produce to be added was standardized by mass, specifically by multiple ½ cup, 35g serving sizes of raw broccoli [38]. To determine this number, crowns were individually weighed and added to a Whirl-Pak® bag until the bag reached capacity. Two liters of water was poured into the bag, and it

was shaken for 30 seconds, massaged for 60 seconds, and shaken for an additional 30 seconds. The total mass of the produce placed in the bag was divided by 35g to determine how many serving sizes the wash contained. This number was rounded to the nearest whole number serving size and used as the standard amount in every future broccoli wash.

Creating Nalidixic Acid Resistant Strain of Salmonella

In order to create a strain of *Salmonella* that would be differentiable from any microorganisms existing on store-bought produce, the previously mentioned four *Salmonella* strains derived from pond sampling and laboratory positive control strain were plated onto XLT-4 agar, inverted, and incubated for 24-48 hours at 37C until a at least 10 *Salmonella* colonies had grown on each plate. Three to six colonies of each strain were then streaked onto XLT-4 agar supplemented with nalidixic acid to a concentration of 0.0001%. The plates were inverted and incubated at 37C for 24-48 hours. If enough colonies became resistant to this concentration of antibiotic to successful multiply and form more colonies, three to six of these new colonies of each strain were then streaked onto XLT-4 agar supplemented with nalidixic acid to a concentration of 0.0005%. The plates were inverted and incubated at 37C for 24-48 hours. This process proceeded in the same way with three to six colonies being plated on to XLT-4 agar supplemented with nalidixic acid to a concentration that was 150% that of the previous plating until at least one of the strains was resistant to 0.01% nalidixic acid.

Limit of Detection Experiments

Trial 1

In order to determine the limits of detection, an inoculum was first prepared by adding one black 0.01% nalidixic acid resistant *Salmonella* colony to 400ml of sterile 1% peptone water. This inoculum was incubated at 37C. After 18-20 hours, a serial dilution series of this inoculum was created.

The objective of trial one was to determine the dilutions of inoculum where the MPN culture of the produce wash methods met the limits of detection. In order to do this, the incubated inoculum was diluted down to 10^{-10} of the original concentration, with dilutions 10^{-1}-10^{-4} and 10^{-8}-10^{-10} each prepared to a total of volume of 360ml.

To confirm that the nalidixic acid resistant *Salmonella* was present in the inoculum dilutions, an additional 1µl of each dilution 10^{0}-10^{-6} was streaked onto 0.01% nalidixic acid XLT-4 agar, inverted and incubated at 37C for 48 hours.

Broccoli was purchased from a local grocer and weighed out into samples of 630g each. Each sample was divided into fourths and then seeded with the prepared inoculum. Briefly, each fourth of the sample was placed crown up into a 1000ml beaker. If necessary, florets were removed from the perimeter of the broccoli head and placed in the bottom of the beaker until the whole head rested inside the lip of the beaker. Then 90ml of a specific dilution of inoculum was dripped over each beaker using a pipet. We specifically applied 90ml to simulate the standard irrigation event where one-half inch of water is applied. Additionally, a negative control was seeded with sterile 1% peptone water. All broccoli crowns were allowed to dry for 30 minutes and then placed into a Whirl-Pak® bag with wooden skewers.

To each Whirl-Pak® bag, 2L of sterile 1% peptone water was added and the bag was tightly sealed. The bag was shaken by hand for 30 seconds, massaged lightly for 60

seconds, and then shaken for another 30 seconds. This peptone water wash was then poured out of the top of the bag into a media storage bottle containing an equal volume of sterile double strength LB-- 500ml, 100ml, and 10ml--in triplicate, and cultured according to the MPN methods described above with two minor modifications: First, during this phase, 0.01% nalidixic acid XLT-4 agar was used to culture microorganisms; second, after streaking on these plates, the presence or absence of colonies was noted at 24 and 48 hours These time points were chosen based on observation during creation of the nalidixic acid resistant strains that suggested that these strains took longer than 24 hours to form black colonies.

Trial 2

Once results of Trial 1 revealed which specific inoculum dilutions elucidated the limit of detection, the limit of detection experiment was repeated to determine the MPN of *Salmonella* of each applied dilutions. Using the same methods as Trial 1, an inoculum solution was prepared, and a serial dilution series of the inoculum was created down to the relevant dilutions determined in Trial 1. Each dilution was prepared to a total volume of 2.36L.

Broccoli was then seeded with this inoculum, allowed to dry, and washed using the methods described for trial one. Additionally, to determine the MPN of each of the inoculum dilutions applied, the remaining volume of each applied dilution was also cultured according to the MPN method described previously with the same modifications as described above.

Once all plates had been checked for the presence of *Salmonella* and MPN calculated for each produce wash and its applied dilution, the results of these two were

compared to determine the MPN range of water samples where it was possible to recover *Salmonella* from the surface of broccoli irrigated with this water.

RESULTS

Phase 1

Resuscitation Experiments

Results of experiment one, where frozen samples came from irrigation ponds, are shown in Table 1. Each of those conditions where samples were frozen and reincubated in LB had higher recovery efficiencies than those samples frozen and reincubated in tetrathionate broth. *Salmonella* recovery from the frozen LB samples was between 42-60% depending on reincubation time, while *Salmonella* was recovered from 27% of the frozen tetrathionate samples. When reincubated for 2 or 5 hours, the most efficient recoveries, 60% and 53% respectively.

When the experiment was repeated using water inoculated in the lab with concentrations of 10 colonies/100ml to determine the volume of preservative that best facilitates recovery, 4 of the 6 experimental conditions produced 100% recovery efficiency of *Salmonella*. The only conditions that did not have 100% recovery were conditions 15% glycerol with 2 hour reincubation (92% recovery) and 50% glycerol with 5 hour reincubation (83% recovery) (Table 1).

At an initial concentration of 5 colonies/100ml, again 4 of 6 experimental conditions produced 100% recovery efficiency. Zero percent glycerol and 15% glycerol preservation facilitated 100% *Salmonella* recovery regardless of reincubation time (Table 1). When starting concentrations were 1 colony/100ml, addition of 15% glycerol at both 2-hours and 5-hours were the only two conditions to facilitate 100% recovery (Table 1).

Table 1 shows the overall recovery efficiency for each of the eight experimental conditions. While recovery of *Salmonella* was possible under each condition, the overall efficiencies ranged from 27% recovery when frozen in a selective media to 100%

recovery when frozen in a non-selective media with 15% glycerol and then reincubated for 5 hours. While the latter condition was the only to facilitate 100% recovery across all experiments, the same freezing conditions with a reincubation time of 2 hours show a similar high efficiency of recovery, 95% (Table 1).

Phase 2

Standardizing the Produce Wash

Three large-sized store bought broccoli crowns was found to be the maximum amount of produce that could fit into the 123-ounce Whirl-Pak® bags. This was found to be equivalent to approximately 633g. Dividing by the 35g serving size of raw broccoli revealed that the bag contained approximately 18 servings. This mass allowed for 2L of liquid to be added and the whole bag massaged so that liquid was able to flow inside the bag to make potential contact with all surfaces of the produce inside.

Limit of Detection

Results of trial one of the limit of detection experiment are shown in Table 2. After culturing by the MPN method, colonies formed on each of the nine replicate agar plates for each of the four highest dilutions (10^{-1}-10^{-4}). For the 10^{-8} dilution, colonies formed on 6 replicate plates for an MPN of 1.5 MPN/100ml/630g broccoli. Colonies formed on 3 of the 10^{-9} plates for an MPN of 0.26 MPN/100ml/630g broccoli. No colonies formed on any of the 10^{-10} replicate plates.

Results of trial two are shown in Table 3. For the 10^{-8} produce wash, colonies formed on 3 of nine replicates while for the 10^{-8} applied water they formed on 9 of nine. This equates to MPN calculations of 0.21 MPN/100ml/630g broccoli and ≥11 MPN/100ml respectively, for an efficiency of recovery of applied MPN of 2%. For the

10^{-9} produce wash, colonies were present on 1 plate, 0.055 MPN/100ml/630g. For the 10^{-9} applied water, colonies formed on 7 plates, 4.6 MPN/100ml. The recovery efficiency of this dilution was 1%. Colonies were present on 2 of the 10^{-10} produce wash replicate plates, 0.13 MPN/100ml, and there was growth on 3 of the 10^{-10} applied plates, an MPN of the dilution of 0.26 MPN/100ml and 50% MPN recovery efficiency.

DISCUSSION

We carried out a series of experiments to examine the best protocol for recovery of *Salmonella* from the frozen reinstates of produce samples, and the limit of detection (LOD) for this protocol. We determined that the optimal protocol freezes these rinseates in a non-selective media that is 15% glycerol by volume and then reincubated samples for 2 hours after being thawed. Below we discuss and justify each of these components in further detail. We also determined the LOD for the produce washing method to be at an applied water concentration of 0.26 MPN/100ml, and discuss this further below as well. This series of experiments provide critical information for produce sample collection and processing going forward.

Phase 1

15% Glycerol Preservation

The results of our study validate previous studies that demonstrated that the addition of glycerol to a concentration of 15% facilitates efficient recovery of bacteria, specifically *Salmonella*, after freeze at -80C [35, 36] . Depending on the reincubation time post-freeze, the 15% solution facilitated between 95-100% recovery. While moderate recovery was possible without the addition of glycerol, we chose to include this step in our protocol as it is cost-effective (glycerol is not overly expensive) and does not add a significant time to the length of the protocol.

According to T. M. Prudden [35], cellular damage and death during freezing is caused by the crystallization of the liquid these microorganisms are stored in. Others attribute this death to mechanical crushing that occurs as the results of water expanding when frozen [36]. Hollander and Nell theorize that because glycerol has antifreeze

properties, solutions preserved in glycerol freeze progressively. This slower freeze could result in the arrangement of crystals in a form that produces less internal stress or allows bacteria to freeze before the surrounding solution thus reducing their susceptibility to compression [36]. Overall addition of moderate amounts of glycerol appears to result in a greater number of cells surviving the freezing process.

Non-Selective Media

To establish selective versus non-selective media, in our first experiment we froze a suite water samples from irrigation ponds in both the selective (tetrathionate) and non-selective (LB) medias used in the standard MPN method. After thawing, the samples were resuscitated in these same media. Even under a range of experimental resuscitation times, all samples cultured from freeze and reincubated in the lactose broth experienced a higher recovery efficiency of *Salmonella* than those samples frozen and reincubated in tetrathionate. While tetrathionate facilitated 27% recovery, the best overall recovery from the non-selective lactose broth was more than twice as efficient, 64% (Table 1).

This result is expected in light of what is known about the physiological and biological processes that occur as the result of stress on microorganisms. Without this resuscitation period, direct plating of stressed microorganisms onto selective media would result in selection against target pathogens but also selection against sub-lethally injured cells of the species of interest. In this study, the greater recovery facilitated by incubation in lactose broth suggests that this media allows for resuscitation of the injured microorganisms. Before freezing, all samples contained viable *Salmonella*, culturable via our MPN methods. However, as the result of freezing, not all cells remained viable and some remained technically viable but not culturable according to our conditions. During

incubation in the non-selective lactose broth, those viable but not culturable cells underwent resuscitation to become culturable by the MPN method and resistant to the selective media used in the next step of the culture protocol. Meanwhile, those cells incubated in tetrathionate that were viable but not culturable after freezing were not resistant to the selective properties of the media and likely thus become not culturable. This resulted in a greater number of culturable microorganisms present in the non-selective media and thus more efficient recovery.

2-Hour Resuscitation Time

Although it is established that a resuscitation process is necessary for recovery of microorganisms in general, optimal conditions exist for specific individual species. These ideal conditions include specific non-selective media as well as resuscitation time [32]. Because the goal of this protocol development was to optimize based on the existing MPN culture method, as previously discussed, we were limited to the specific selective and non-selective medias already in use in that method. However, resuscitation time needed to be optimized.

The results of the first experiment, where three time periods (2, 5, and 24 hours) were tested, revealed that recovery from the 2 and 5-hour conditions was greater than that of the 24 hour conditions (Table 1). As a result, the 24-hour condition was not tested in the second set of resuscitation experiments.

Based on the results from the second set of resuscitation experiments, where resuscitation time was considered in conjunction with the concentration of preservative, resuscitation appears to be optimized at 5 hours. After 5 hours, *Salmonella* was recovered from 100% of frozen samples (Table 1). Despite this fully efficient recovery, for our

produce wash methods we chose to resuscitate samples for 2 hours, which showed 95% recovery (Table 1). While not as efficient, we believe the 2-hour resuscitation is sufficient for recovery while optimizing laboratory timing.

The optimal resuscitation period must balance the repair of the target and non-target microorganisms. Resuscitation must be sufficiently long to allow an ample quantity of the target microorganisms to be repaired and re-confer resistance to the selective media used in the next step of the protocol [32]. However, in a non-selective media, repair will occur for all microorganisms present in the sample. While longer periods of resuscitation may induce greater repair in the target organisms, in our case *Salmonella*, longer periods can be detrimental if non-target organism recover quicker and outcompete target organisms.

We attribute the low recovery efficiency seen in the 24-hour resuscitation to competition induced by the repair of non-target organisms in addition to *Salmonella* in the pond water samples. A study by Lawley et al. [39] established that in the gut of a host *Salmonella* is particularly susceptible to competition with other microorganisms, becoming prominently established only when introduced directly following antibiotic regimens that eliminate other bacteria. While the present study focuses on environmental strains of the pathogen, we do not find it unreasonable to assume that similar relationships between *Salmonella* bacteria and its competitors may play out under the enriched environment of non-selective media. Accordingly we would expect competitors to limit the presence of *Salmonella* as resuscitation time increases.

The results from the first experiment support this hypothesis as recovery was least efficient for the 24-hour resuscitation and incrementally improved as the length of

resuscitation shortened. Water samples from that experiment were taken from irrigation ponds where microorganisms other than *Salmonella* would be present. Thus in the first experiment, resuscitation time periods accounted for competition that might limit the proliferation and enumeration of *Salmonella*, further validating our selection of an optimal 2-hour resuscitation time.

Phase 2

Standardizing the Produce Wash

After rounding, our methods suggested that the standardized mass of broccoli was equal to eighteen 35g servings, or 630g total.

Limit of Detection Experiments

For this experiment, we are defining the limit of detection as it pertains to the MPN of the irrigation water we applied broccoli heads. The lower LOD for the water assay was determined by our collaborators to be 0.055 MPN/100ml, and thus we were interested in determining the lowest concentration of *Salmonella* that could be applied to the broccoli where the water assay used could still detect the pathogen. Defining this value will allow us to examine if our produce wash procedures are sensitive enough to capture contamination that may be occurring at our sampling sites.

With colonies growing on all 9 replicates of the 10^{-1}-10^{-4} dilutions, these produce washes were all above the upper limit of detection of the water assay. The 10^{-8} and 10^{-9} dilutions produce washes fell within the detectable range of the assay while the 10^{-10} dilution wash, with no *Salmonella* recovered, fell below detectable range of the assay. Specifically, this shift at the 10^{-9} to 10^{-10} dilutions from the possibility of recovery to no

recovery was determined to be the lower limit our of interest and thus the range of dilutions to focus on in trial two. While this particular trial was not designed to quantify this limit of detection, it confirmed that the method established is able to recover the bacteria from the surface of broccoli.

In trial one, we did not see the same transition from recovery to no recovery. Based on our definition of the lower limit of detection, the concentration of dilution applied where colonies were present on only one of nine replicate agar plates from the produce wash rinseate, the result of trial two would suggest that this occurred when we applied the 10^{-9} dilution, 4.6 MPN/100ml. However, we were still able to recover *Salmonella* from the 10^{-10} produce wash and actually recovered more than we did from the 10^{-9} wash (Table 3).

While the MPN of the produce wash rinseates did not uniformly decrease with consecutive dilutions as expected based on the results of trial one, our MPN values for the dilutions themselves did (Table 3). Because these dilutions did follow the expected trend in MPN, we believe the factors affecting the fluctuations in produce wash MPN to have occurred after the dilutions were created and thus occurred during the inoculating of produce wash process as the result of human error.

The limit of detection experiments were constrained overall by the laboratory environment where concerns for safety and limited space prevent procedures from perfectly replicating true irrigation events in the field. Because of these constraints, the particular way in which broccoli was arranged in beakers when inoculated as well as the technique used to transfer the inoculated broccoli into Whirl-Pak bags once inoculated had the potential to introduce more microorganisms into the produce washing rinseate.

Specifically, the variability in broccoli morphology meant that while resting in the beakers, certain heads were consistently exposed to the volume of inoculum that filled the bottom of the beaker. Moreover, when these heads were transferred to Whirl-Pak bags safety procedures requiring the minimization of liquid spills limited our ability to remove or shake-off these excess microorganisms, resulting in the MPN estimate of each rinseate to be artificially inflated.

Even though these human errors may artificially increase the presence of *Salmonella* in the produce wash rinseates, they are equally likely to act on each replication of the wash. Therefore, based on the results of this study, our best estimate of the lowest concentration of irrigation water for which *Salmonella* is present and detectable on the surface of broccoli is 0.26 MPN/100ml and is based on the results of application of the 10^{-10} dilution; however we acknowledge that this value may be greater than the true value of the limit of detection we were interested in determining.

The range of *Salmonella* concentrations sampled from the irrigation ponds used to irrigate these fields spans the whole range of detection for the MPN method: 0.058 to 11 MPN/100 ml. The limit of detection we determined of the wash method falls near the lower end of that range and is unable to adequately detect contamination with the same sensitivity as the water assay. Should irrigation water have the capacity to cause broccoli contamination at levels $\geq$0.26 MPN/100ml, this method has the ability to capture it, however we cannot capture contamination occurring when irrigation water MPN is less than 0.26 MPN/100ml. More thorough investigation of the results of irrigation pond sampling is required to determine whether this method is sensitive enough for this particular system or whether a more sensitive method should be developed.

Limitations

Resuscitation Experiments

These experiments were not without limitation. Limited by time, the samples in these experiments were not frozen for the complete 90-day period that produce wash samples will be frozen. We chose to truncate this frozen storage period to 4-20 days on the assumption that the negative effects of cold storage occur during the freezing process and thus will be seen in any length of freezing time so long as samples are allowed to freeze completely. This assumption may be flawed as Prudden [35], and others noticed empirically that damage and die-off of frozen microorganisms continued to occur throughout the length of cold storage, however greater impact on those microorganisms occurred when they were stored at warmer temperatures (e.g. -20C as opposed to -80C [35, 36, 40]. Moreover, Howard [37] confirmed 15% glycerol preserves *Salmonella* for up to 5 months, a period of time longer than our cold storage, so its addition is a conservative attempt to protect our samples from potential damage as the result of long-term storage at -80C.

Moreover, the second group of resuscitation experiments was performed using water inoculated in the lab as opposed to environmental samples. The experiments did not fully take into account the complicated dynamics of interspecies competition and taken alone, biased the optimization results towards longer resuscitation periods because longer resuscitation provided greater time for *Salmonella* to multiply and increase to concentrations detectable by recovery methods. However, this limitation was addressed by interpreting these results in light of the first experiments where samples were environmentally derived and balanced resuscitation of both target and non-target microorganisms.

Limit of Detection Experiments

In these particular experiments, we did not freeze the produce wash rinseates in determining the LOD as we would freeze the true samples. The freezing process may cause the death of some cells resulting in recovery being diminished and thus the inability to detect *Salmonella* on the surface of broccoli irrigated with water whose *Salmonella* concentration was as low as 0.26 MPN/100ml. However, the resuscitation experiments revealed that our methods were able to recover *Salmonella* from our frozen samples with 95% efficiency, thus we predict that the loss of cells during the freezing process to be negligible and our lower LOD to reflect its true value.

To seed the broccoli in these experiments, we used a serial dilution series and were thus limited in our ability determine the true limit of detection. In this study we estimated the lower limit to occur for the 10^{-10} produce wash, where colonies formed on 3 of nine replicates; however, we do not know if the MPN we applied is the absolute lowest MPN for which we could recover *Salmonella* because we did not apply water to samples at an MPN lower than 0.26MPN/100ml. The LOD that we defined is then a conservative estimate of the LOD as it may be possible to seed with progressively more dilute inoculums and still recover *Salmonella* from just one of the nine replicates. To determine the true lower limit of detection, we would want to alter the concentration of the inoculum applied on a finer scale, say in 5-fold increments as opposed to 10.

There was also noticeable difference between the broccoli crop from the Tifton farms and what we purchased in the grocery store for inoculation. Because of the harsh winter conditions of January and February 2014, broccoli crop was subject to freezing and thus the stems were hallowed out. Whereas inoculation of 630g in the lab could be

done with 3-4 heads, 630g samples collected for the actual project consisted of 4-5 heads. Thus the crop sampled for Drs. Levy's and Vellidis' new study had more surface area and thus greater opportunity surface for contamination. While one alternative would have been to inoculate broccoli that was harvested at the same time as broccoli sampled for the project, ultimately we do not believe this limitation will negatively bias the results of the real project. Having sampled a larger surface area than inoculated in the limit of detection assays, we have the potential to detect more contamination than we estimated in limit of detection experiments and thus the true contamination will still fall above the lower LOD we derived.

Overall, the complex morphological structure of broccoli is a challenge to standard produce wash procedures. The vegetable has extremely large surface area that is impossible to calculate and also compare across different individual broccoli heads. This forces the non-ideal standardization of all produce washes based on weight, which because of the structure and density of different produce types, does not equivocate with risk of exposure to contaminants and thus risk contamination. The purpose of this study was to account for such complications induced by broccoli, and we feel these limitations cannot be overcome completely but that we have created a procedure to minimize them to the best of our ability.

Future Directions

In order to overcome the limitations of human-induced error, the limit of detections should be replicated to estimate the true lower limit of detection with better accuracy and precision. Work still to be done for this particular limit of detection study

includes the elucidation of the upper limit of detection of the assay. This would include inoculating broccoli with the dilutions just above those focused on in the second trial, 10^{-7} and 10^{-6} and determining the maximum concentration of the applied dilution where colonies form on 8 of the nine plate replications. Moreover, future limit of detection experiments should also incorporate a freezing period into culture techniques to estimate the LOD for the complete method: produce wash and recovery.

The primary future direction of this work is to apply the same standardization and limit of detection techniques to each of the other produce types grown and sampled throughout the length of the study by Drs. Vellidis and Levy. These crops may include mustard greens, cantaloupe, eggplant, or tomatoes.

Conclusion

Based on published methods from the literature as well as personal communication with researchers who have performed these resuscitation methods, we expected that the most efficient recovery of *Salmonella* from the frozen samples would occur when the samples were frozen in non-selective media, 15% glycerol solution and resuscitated for 2 hours following removal from the freezer and before the addition of selective media. The results of our study confirmed these conditions to be optimal for recovery in our lab, and we consider this method appropriate for the recovery of *Salmonella* bacteria from frozen produce washes. Moreover we were able to determine that the lower limit of detection for the method, 0.26 MPN/100ml, is able to adequately capture broccoli contamination resulting from irrigation with contaminated pond water at or greater than that concentration. Future work should focus on replicating these experiments to determine a more accurate and precise estimate of the LOD and determine

if this LOD signifies if the method is sensitive enough to capture produce contamination as the result of irrigation with contaminated pond water should it be occurring in this system.

FIGURES

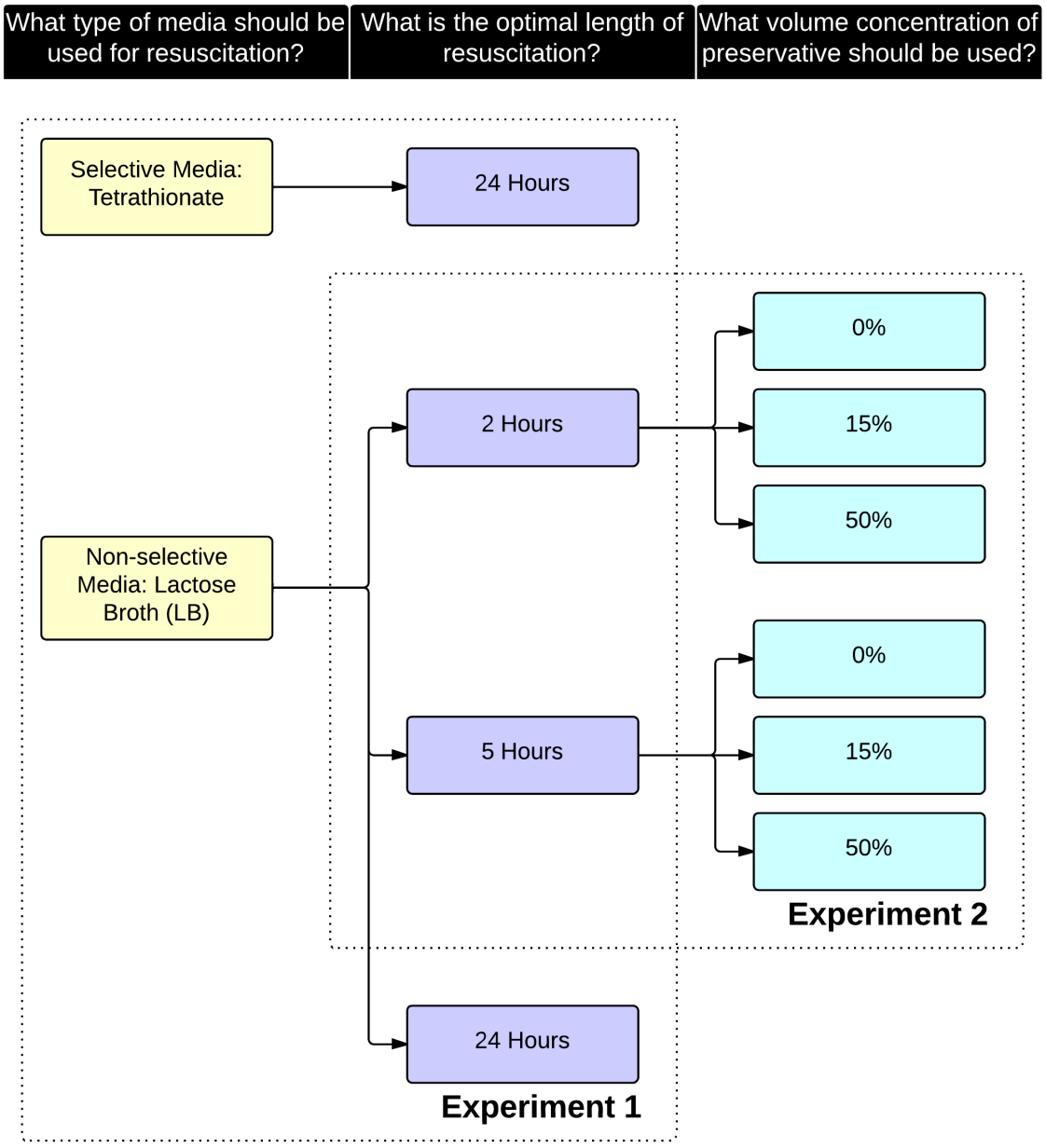

Figure 1: Overview of experiments to determine the most efficient recovery of *Salmonella* from frozen water samples

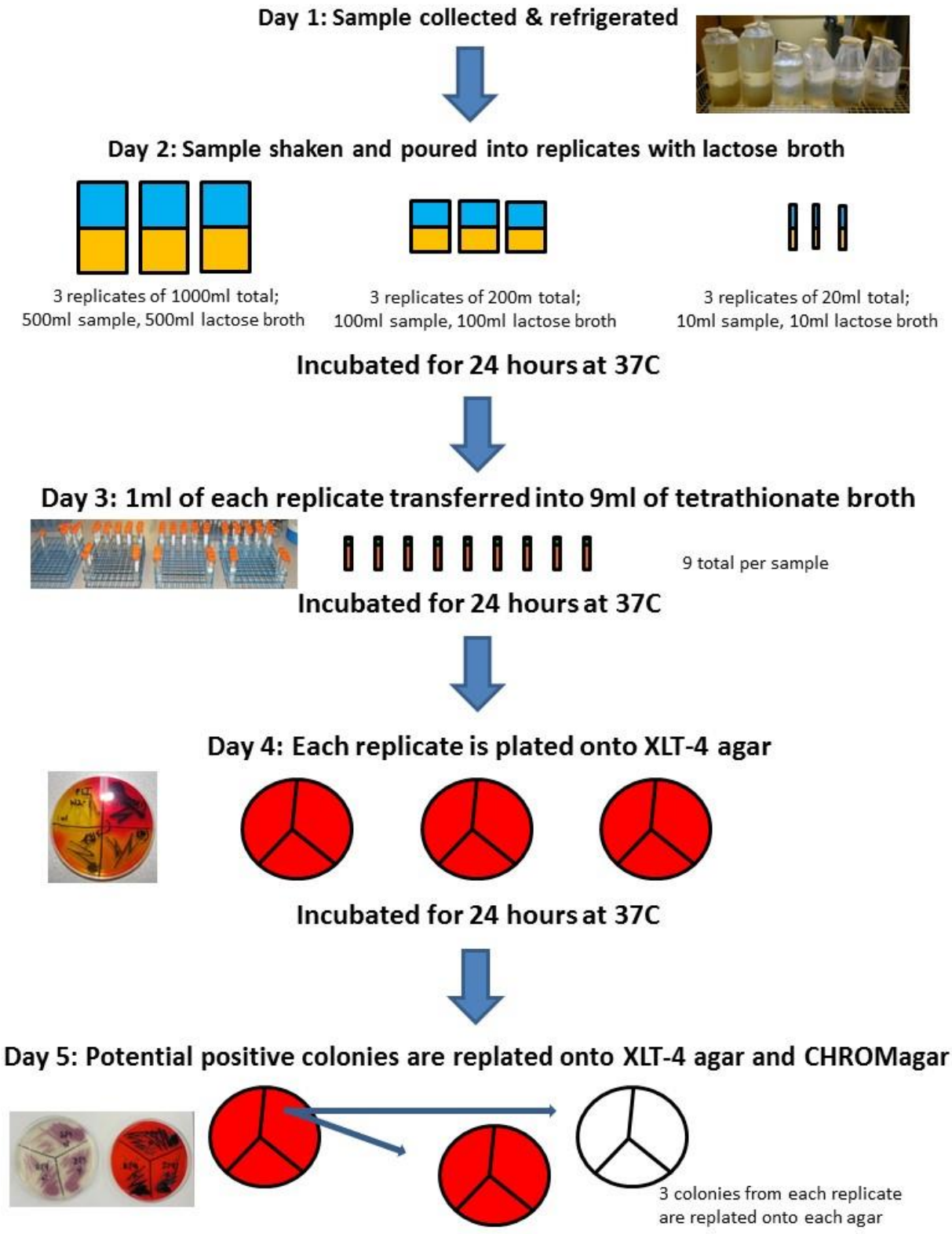

Figure 2: The standard Most Probable Number (MPN) protocol used by collaborators at the UGA-Tifton laboratory to determine the concentration of *Salmonella* in irrigation ponds on local produce farms

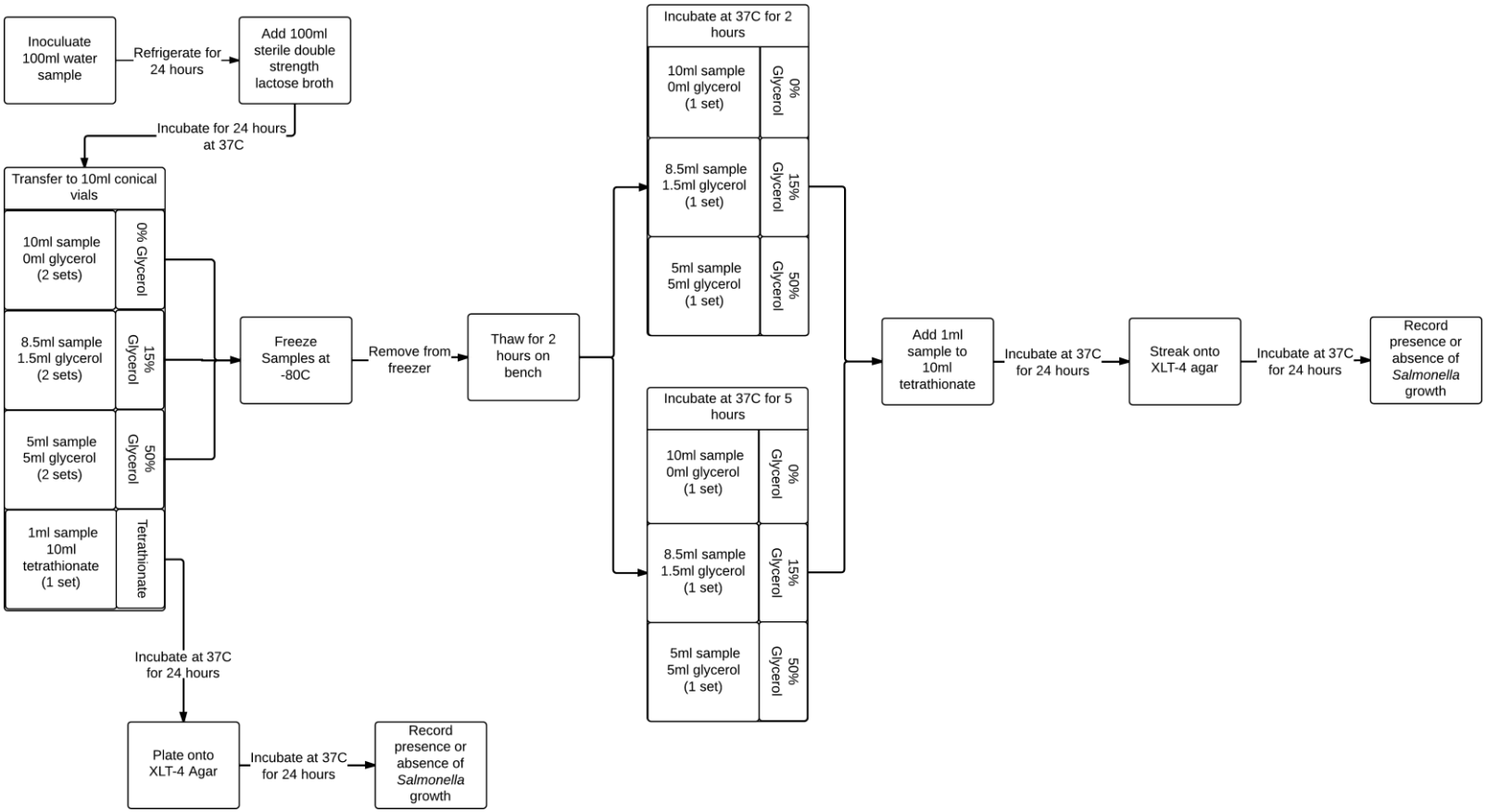

Figure 3: Schematic of protocol used in Emory laboratory to determine the optimal volume of glycerol and resuscitation time for facilitation of *Salmonella* recovery from frozen water samples.

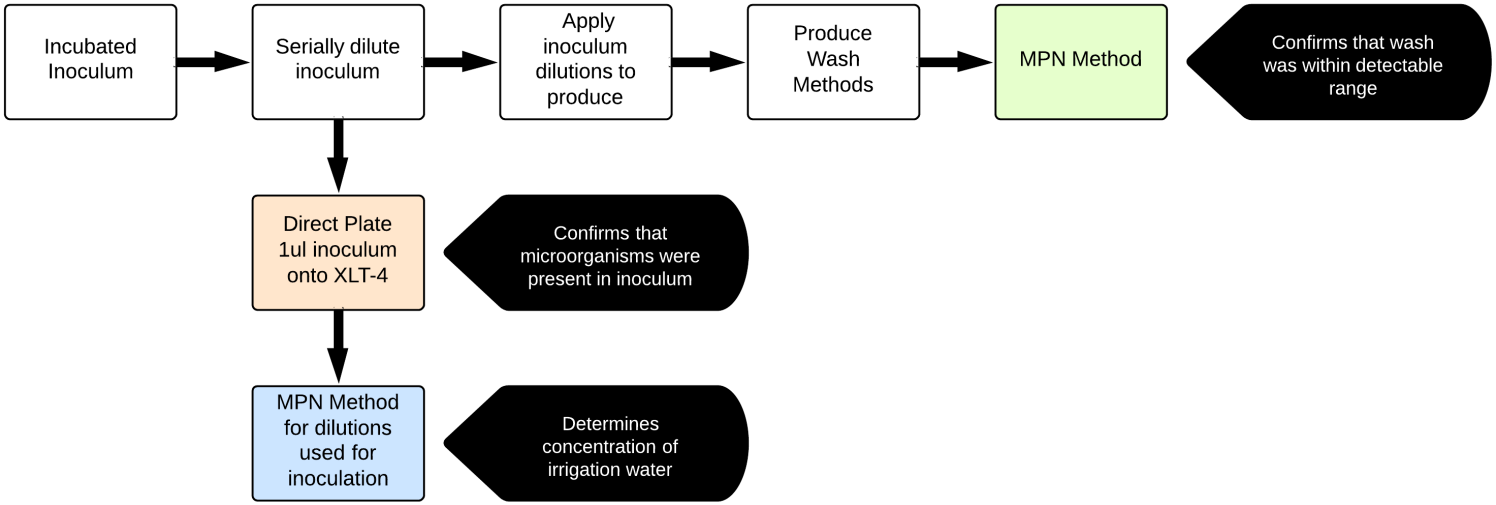

Figure 4: Overview of a seeding experiment used to determine the limit of detection for a broccoli produce wash procedure.

Table 1: Overview of the efficiency of recovery from each of the eight conditions tested in an experiment aimed at determining the best procedure for recovering *Salmonella* from frozen water samples.

Resuscitation Media	Resuscitation Time	Volume Concentration Preservative	Efficiency of Pond Derived Samples (N)	Efficiency of 1 colony/100ml Samples (N=5)	Efficiency of 5 colony/100ml Samples (N=5)	Efficiency of 10 colony/100ml Samples (N=12)	Overall Efficiency (N)
Tetrathionate	24 Hours	0%	0.27 (15)	-	-	-	0.27 (15)
Lactose Broth	2 Hours	0%	0.60 (15)	0.80	1.00	1.00	0.81 (37)
		15%	-	1.00	1.00	0.92	0.95 (22)
		50%	-	0.00	0.40	1.00	0.64 (22)
	5 Hours	0%	0.53 (15)	0.80	1.00	0.80	0.78 (37)
		15%	-	1.00	1.00	1.00	1.00 (22)
		50%	-	0.83	0.00	0.00	0.45 (22)
	24 Hours	0%	0.42 (12)	-	-	-	0.42 (12)

Table 2: The *Salmonella* recovery in MPN/100ml/630g broccoli washed during trial one a seeding experiment attempting to determine the lower limit of detection of a broccoli produce wash method.

Dilution Applied	# plates with growth	Salmonella MPN/100ml of Produce Wash
10^{-1}	9	Over upper limit
10^{-2}	9	Over upper limit
10^{-3}	9	Over upper limit
10^{-4}	9	Over upper limit
10^{-5}	-	Not sampled
10^{-6}	-	Not sampled
10^{-7}	-	Not sampled
10^{-8}	6	1.5
10^{-9}	3	0.26
10^{-10}	0	Not Detected

Table 3: The *Salmonella* MPN/100ml of inoculum applied to broccoli and the corresponding MPN/100ml/630g broccoli recovered from the produce wash of these applications.

		# of Plates with growth	MPN/100 ml			# of plates with growth	MPN/100ml	Efficiency of recovery
Applied Water	10^{-8}	9	≥11	Produce Wash Water	10^{-8}	9	0.21	2%
	10^{-9}	7	4.6		10^{-9}	7	0.055	1%
	10^{-10}	3	0.26		10^{-10}	3	0.13	50%

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