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EVALUATION OF SEPARATION METHOD ADDITIVES FOR THE RECOVERY OF BACTERIA FROM FOOD MATRICES

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in
Biosystems and Agricultural Engineering
in the College of Engineering at the University of Kentucky

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2012

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ABSTRACT OF THESIS

EVALUATION OF SEPARATION METHOD ADDITIVES FOR THE RECOVERY OF PATHOGENS FROM FOOD MATRICES

The microbiological testing of foods is a well-established science. Due to the severity of foodborne pathogen illnesses, the widespread use and implementation of rapid detection methods in food testing labs is increasingly important. The first step for successful testing is sampling. Surfactants have been highly used in food microbiology, but there is not much, if any, published research about the use of fatty alcohols and chemical dispersants as aids in microbial separation. The microbial extraction efficiency of *Escherichia coli* K12 and *Listeria innocua* from hot dogs, spinach, and milk was measured using chemical additives (surfactants, fatty alcohols, and a chemical dispersant) in a buffer solution. Dry matter content was calculated using the oven method to determine how clean the sample was at the end of processing. Tween 80 at 0.01% was found to be the most effective additive for microbial recovery for each food matrix examined. The addition of fatty alcohols to surfactants also showed much promise in aiding separation as well as in minimizing dry matter in the final solution. However, the use of Buffered Peptone Water as the diluting agent resulted in very high recovery percentages without the need for additives.

KEYWORDS: Food Sampling, Foodborne Pathogen, Surfactant, Fatty Alcohol, Chemical Dispersant

Jennifer L. Frederick

July 26, 2012

EVALUATION OF SEPARATION METHOD ADDITIVES FOR THE RECOVERY OF PATHOGENS FROM FOOD MATRICES

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July 26, 2012

I dedicate this work to my sister, Emily, my brother, Luke, and my son, Levi.

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CHAPTER 1. **INTRODUCTION**

In 2011, the Center for Disease Control estimated at least 47.8 million people would become sick in the United States from foodborne illnesses. Of the 47.8 million, over 127,000 would be hospitalized and more than 3,000 would die. While these estimates do reflect a downward trend in foodborne pathogen related illnesses, food safety specialists and regulators still face the challenge of ensuring a safe food supply in the United States and abroad.

The University of Florida's Emerging Pathogens Institute estimates that 14 known pathogens cost our economy 14.1 billion dollars in hospital bills, sick days lost at work, and in secondary illnesses (Batz, 2011). Some foodborne illnesses are thought to have long-lasting effects that cause secondary illnesses such as ankylosing spondylitis, arthropathies, renal disease, cardiac and neurologic disorders, nutritional and other malabsorptive disorders (Lindsay, 1997). Everyone faces the risk of contracting a foodborne illness simply because everyone eats.

Food safety is a great concern in the United States and abroad for many reasons. Each year in the United States, new food products are introduced to the market, increasingly complex in nature and harder to test for pathogens. There is also an increasing demand for fresh, minimally processed or non-processed foods which are more susceptible to contamination than pasteurized or processed foods. Finally, other countries that export their food to the United States may not have food safety programs. In the United States, programs such as Hazard Analysis and Critical Control Point (HACCP) and Good Manufacturing Practices (GMP) are two efforts by the government and food industries to reduce the incidence of foodborne pathogens.

Both the United States Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) are government agencies tasked with keeping our food safe. These agencies have established testing methods and protocols for detecting pathogens in food, however, traditional enrichment processes are used to test for pathogens and require at least 48 hours to complete. According to the FDA, the infectious doses of *Escherichia coli* O157:H7 and *Listeria monocytogones* are unknown, but it is believed that less than 10 cells and 1,000 cells, respectively, can cause illness in humans.

The Food and Drug Administration (FDA) and the United States Department of Agriculture (USDA) have stringent food testing programs set up to prevent the outbreak of foodborne pathogen related illnesses. Food manufacturers must have their product tested before it reaches retailers' shelves. Currently, food manufacturers are increasing their food testing due to increased requirements from the federal government and also because of consumer demand and the manufacturer's own self-interest. A foodborne illness outbreak traced to a certain manufacturer can potentially put the company out of business.

The analysis of foods for pathogen presence is a standard practice to ensure the safety of our food. However, the composition of food matrices can make the analysis quite complicated. Foods include a wide range of ingredients including proteins, carbohydrates, fats, oils, and chemicals (Swaminathan, 1994). Some of these can have adverse effects on the viability of bacteria and can interfere with pathogen detection. Differences in solid versus semi-solid versus liquid foods also pose challenges. The presence of fats and oils and different viscosities create difficulties with obtaining consistent results in analysis. The presence of indigenous microflora can create challenges to detect pathogens that may be present in very small quantities. Finally, food processing techniques to safeguard our foods such as freezing, drying, adding preservatives, and other chemicals can sublethally injure pathogens causing them to be sensitive to growth media and not show up in traditional methods for foodborne pathogen testing even though they still pose a threat in the food. There is a great need for improved methods for foodborne pathogen detection in food matrices.

Because of the risk of foodborne illnesses, a lot of time and effort is spent on improving microbiological methods to detect foodborne pathogens. Due to advances in molecular microbiology, scientists are discovering ways to distinguish one microorganism from another based on metabolic traits, nucleic acid sequences, or structural components, among others (Brehm-Stecher et al., 2009). However, many times these detection methods neglect to include the initial preparation step of the food sample. Instead, they focus more on clinical samples, which are typically much more homogeneous than food samples. Foods pose many challenges to scientists because of their complex make-up and

often low levels of contamination. It is difficult to apply novel detection methods to food matrices without an adequate protocol to prepare the sample to be tested.

The overall goals in sample preparation should be to separate the target cells from the food matrix, concentrate the cells, purify them, and exclude any inhibitory substances (Brehm-Stecher et al., 2009). Developing one protocol to achieve these goals is extremely difficult to achieve because food matrices vary greatly from one to another. A method that works well for tomatoes may not work at all for peanut butter, for example. In addition, there are certain compounds found in foods that are inhibitory to novel detection methods, such as calcium in milk samples for polymerase chain reaction (PCR) detection.

There are currently two methods employed for sample preparation depending on what kind of data is needed: qualitative and quantitative. Qualitative assays aim to detect and identify the presence of a determined microorganism whereas quantitative assays aim to provide an estimate of the amount of a microorganism in a given sample. Microbiological testing of food typically employs qualitative assays to determine the presence or absence of a microorganism, but these assays can take multiple days to perform. Quantitative assays are growing in importance, however, because there is a greater desire from researchers to understand microbial growth kinetics and inactivation in foods, and to estimate populations for surveillance purposes.

1.1. Objectives

Microbiological analysis of food must be able to detect small numbers of bacteria in a complex sample, posing many challenges to microbiologists and engineers. The initial goal of this research was to maximize the microbial recovery from various types of food focusing on the initial food sample preparation and separation steps. The primary goal was to maximize the microbial recovery efficiency through the use of chemical additives while lowering dry matter content.

The preliminary objectives of the project were to :

1) Develop a standardized contamination method for hotdogs, spinach, and whole milk.
2) Create a universal protocol for the separation of microorganisms from food matrices.

Once the preliminary objectives were achieved, the following objectives were addressed:

3) Quantify the bacterial recovery from food matrices with the addition of surfactants, fatty alcohols, and a chemical dispersant to the buffer solution.
4) Determine whether the additives that aided most in bacterial recovery also aided in lowering the overall dry matter content of the samples.

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CHAPTER 2. **LITERATURE REVIEW**

2.1. **PATHOGENIC BACTERIA**

Bacteria are unicellular microbes that lack nuclei (Bauman, 2007). Most bacteria contain cell walls made of a polysaccharide called peptidoglycan. Peptidoglycan consists of covalently linked sugar and peptide units. The peptidoglycan layer is much thicker in Gram positive bacteria (20-80 nm) than in Gram negative bacteria (7-8 nm). In fact peptidoglycan forms around 90% of the dry weight of Gram positive bacteria but only 10% of the dry weight of Gram negative strains. Gram negative bacteria contain an outer membrane of phospholipids and lipopolysaccharides, the latter of which are highly charged resulting in an overall negative charge on the cell wall.

While most bacteria are not harmful to humans, pathogenic bacteria can cause serious illness. Many times the pathogenicity of a bacterial cell can be attributed to certain surface cell structures. Bacterial surface structures serve many functions including acting as permeability barriers allowing for the selective passage of nutrients and exclusion of harmful substances, adhesins used to attach to specific surfaces, enzymes to promote specific reactions on the cell surface, protective structures against phagocytic engulfment, antigenic defenses to prevent the activation of host immune defenses, endotoxins that cause an inflammatory response in the host, and certain proteins that respond to temperature, osmolarity, salinity, light, oxygen, and nutrients, resulting in a molecular signal to the cell that causes the expression of some determinant of virulence (e.g. an exotoxin) (Todar, 2009).

The two bacteria used in this research were *E. coli* K12 and *Listeria innocua*, which represent a Gram negative and a Gram positive strain, respectively. These bacteria were used as non-pathogenic surrogates for *E. coli* O157:H7 and *Listeria monocytogenes* (Pathanibul et al., 2009), which are both highly pathogenic bacteria that have been implicated numerous times in foodborne pathogen outbreaks.

2.1.1. *E. coli* O157:H7

E. coli O157:H7 is one of hundreds of strains of *E. coli*. Most strains of *E. coli* are opportunistic pathogens and can be found in the intestines of animals and humans.

However, O157:H7 produces a shiga toxin that can cause severe illness. Symptoms include bloody diarrhea and abdominal cramps, and in some cases it can cause hemolytic uremic syndrome (HUS) which leads to kidney failure. *E. coli* O157:H7 is a mesophilic, Gram negative rod-shaped bacterium. It has adhesive fimbriae which aid in its attachment to cells, and a cell wall that consists of an outer membrane containing lipopolysaccharides, a periplasmic space with a peptidoglycan layer, and a cytoplasmic membrane (CDC, 2005).

E. coli O157:H7 was first discovered in 1982 from undercooked hamburger meat. Since then, it has been the cause of foodborne pathogen outbreaks in beef, unpasteurized apple juice, contaminated water, spinach, and romaine lettuce. *E. coli* from cattle is the major source for human illness. Animal feces used as fertilizer or contaminated irrigation water may also contain the bacteria which can contaminate fresh produce.

2.1.2. *L. monocytogenes*

L. monocytogenes is a Gram positive rod-shaped bacterium that is ubiquitous in nature. It is a facultative intracellular pathogen that induces its own uptake into nonphagocytic cells and then spreads from cell to cell using an actin-based motility process (Dussurget et al., 2004). *L. monocytogenes* is the cause of a serious disease called listeriosis which may cause gastrointestinal illness, septicemia, or meningitis. People over 50, pregnant women, newborns, and those with compromised immune systems are at a higher risk of illness.

L. monocytogenes was named a foodborne pathogen in 1981. It has been implicated in foodborne outbreaks from pasteurized milk, deli meat, Mexican style cheese made from pasteurized milk, cut celery, and contaminated canteloupes (CDC, 2012). Because the bacteria is ubiquitous in nature and can live and survive in harsh environments, including under refrigeration, it is a big concern in ready-to-eat foods as it can be introduced into the product after pasteurization or other treatments before being packaged. This makes testing foods for pathogens all the more important.

2.2. TRADITIONAL METHODS FOR PATHOGEN TESTING IN FOODS

Conventional methods require that the food sample be pre-enriched in order to grow the bacteria in the food to detectable levels (>10 CFU/ml). The initial pre-enrichment of a food sample allows for resuscitation of physiologically stressed microbes, followed by a period of selective enrichment to enable growth of the target organism. From there, the pathogen, if present, is isolated on selective agar, and purification and confirmation occur using morphological, biochemical, or physiological tests. This process usually takes several days to complete and is labor-intensive.

For example, according to the FDA, the culture based test for *E. coli* detection includes 18 different types of media and reagents. The time required for the enrichment and isolation steps could take a total of six days to confirm a positive result. For *Listeria*, 37 different media and reagents are included for the enrichment and isolation steps, and the total time needed to confirm a positive result is 7 days on average (Hitchins et al., 2011). This time spent waiting on results equates to money lost for food manufacturers.

2.3. RAPID DETECTION METHODS FOR FOODBORNE PATHOGENS

Rapid methods have been introduced to shorten analysis time. “Rapid” typically describes methods that give results in 24 hours. Rapid methods include molecular methods, immunoassays, and spectral methods.

2.3.1. Molecular Methods

Molecular methods use sequences of DNA or RNA and amplification of the strands to identify microorganisms. These methods are rapid, specific in that they can distinguish strains of the same species, and can be automated.

PCR, or polymerase chain reaction, is based on the principle of DNA hybridization. PCR is used to amplify the number of copies of a specific region of DNA thus creating a sample that is adequate to be tested. One main application of this technique is identification of bacteria and viruses. Short fragments of DNA or primers are hybridized to a specific sequence which can then be amplified by DNA polymerase using a thermocycler. The DNA template to be copied is denatured by heating a reaction mix to 94°C which separates the two strands of DNA. A mixture with an excess of DNA

primers, DNA polymerase, and an abundance of the four deoxyribonucleotide triphosphates (A, T, G, and C) are added to the target DNA. The mixture is cooled to 65ºC, which allows the double-stranded DNA to re-form. The temperature is then raised to 72ºC which increases the rate at which DNA polymerase replicates each strand, thus producing more DNA (Bauman, 2007).

Even though PCR can theoretically amplify a single copy of DNA by a million fold in less than two hours, the presence of inhibitors in foods and culture media can prevent the primer from binding and thus amplification is decreased, which creates the need for some cultural enrichment and sample treatment to take place prior to analysis.

Two PCR methods developed for pathogen detection are real-time PCR and multiplex PCR. Real-time PCR is able to combine amplification and detection in a one-step closed tube reaction. Real-time PCR was used by Malorny et al. (2004) to detect *Salmonella* in chicken rinses, minced meat, fish, and raw milk. Total time required for analysis was 24 hours as opposed to 4 or 5 days for traditional culturing steps. Multiplex PCR offers the advantage of detecting multiple pathogens at once. Alarcón et al. (2004) developed a multiplex PCR method coupled with gel electrophoresis to detect *Staphylococcus aureus*, *Listeria monocytogenes*, and *Salmonella* spp.

2.3.2. Immunoassays

Antibody based detection (immunoassay) involves the binding of antibodies to a target antigen, and then the detection of the antigen-antibody complex. It is a biochemical test that measures the concentration of or detects a specific substance in a complex mixture. Because of the specificity of the immunoassay, methods in this category are the most widely used for foodborne pathogen detection (Andrews et al., 2003).

The enzyme-linked immunosorbent assay (ELISA) is the most common antibody assay format used for detecting pathogens in food. In this assay, an antibody is bound to a solid matrix and is used to capture the antigen in enrichment cultures. A second antibody is conjugated to an enzyme, and that is what is used for detection. Most often, microtiter plates with polyvinyl chloride (PVC) wells are used as the solid matrix support. Other solid matrices can include dipsticks, paddles, membranes or pipet tips (Andrews et al.,

2003). The detection limits of immunoassays range between 10^3 and 10^6 colony-forming units (CFU)/mL, with assay times from 10 min to several hours (Magliulo et al., 2007).

Bohaychuk et al. (2005) used ELISA to detect *Salmonella*, *Campylobacter*, *Listeria*, and *E. coli* O157:H7 inoculated in raw and processed meat and poultry products. They noted that ELISA worked better with *Salmonella* than the other methods they tried, including lateral flow immunoprecipitation and PCR, showing 100% sensitivity and specificity.

2.3.3. Spectroscopy Methods

Fourier Transform Infrared (FTIR) spectroscopy is one method employed where infrared radiation is passed through a sample and the resulting spectrum is based on how much of the IR radiation is transmitted through the sample. The spectrum obtained is like a molecular fingerprint of the sample. The peaks in the spectrum correspond to the frequency of the vibration between the atomic bonds that make up the sample. The size of the peaks directly relate to the amount of the particular material present.

Dispersive instruments can be very time consuming with their scans. FTIR employs a method to measure all the infrared frequencies simultaneously and not individually. An optical device called an interferometer was first developed by Michelson in 1891. The interferometer divides a beam of radiation into two paths and then recombines them after a path difference is introduced (Griffiths et al., 2008). Two mirrors are used that are mutually perpendicular to one another. One of the mirrors is fixed while the other is able to move along an axis. Between the two mirrors is a beamsplitter. A beam of radiation is partially reflected to the fixed mirror and partially transmitted to the moveable mirror. The beam of radiation recombines when the paths meet back at the beamsplitter. The beams interfere with one another and the result is partially reflected and partially transmitted. The exiting signal from the interferometer is called the interferogram, and is the result of the two beams interfering with one another. Due to the interference, the intensity of the beams passing to the detector and returning to the source depends on the difference in path of the beams in the two arms of the interferometer (Griffiths et al., 2008). This variation in intensity of the beams as a function of the path difference is what yields the spectral information in an FTIR. The interferometer reduces the time per

sample to just seconds as opposed to minutes. The interferogram signal cannot be interpreted directly, and so the Fourier transformation is performed by the computer and the result is the spectral information that can be used for analysis, which is a plot of the intensity at each individual frequency.

Work by Naumann et al. (1991) revealed that bacterial IR spectra generally show peaks in five main regions: fatty acids (3000-2800 cm^{-1}), amides (1700-1500 cm^{-1}), polysaccharide (1200-900 cm^{-1}), and fingerprint (900-700 cm^{-1}). The fatty acid region (3000-2800 cm^{-1}) is dominated by the --CH_3, >CH_2, and =CH stretching vibrations of the functional groups usually present in the fatty acid components of the various membrane amphiphiles (a chemical compound which possessses both hydrophilic and lipophilic properties). The amide region (1700-1500 cm^{-1}) is dominated by amide I and II bands of proteins and peptides. The polysaccharide region (1200-900 cm^{-1}) is characterized by fingerprint absorption bands of the cell wall's carbohydrates. The fingerprint region (900-700 cm^{-1}) is an area which reveals specific spectral patterns that cannot be assigned to cellular components or functional groups.

Burgula et. al (2009) were able to detect *E. coli* 0157:H7 based on amide II peak areas in the 1581-1471 cm^{-1} region. FTIR was used to discriminate *E. coli* 0157:H7 from non-pathogenic *E. coli* K12 in various fruit juices and to estimate sensitivity estimates for pathogen detection in apple juice. Two pathogen separation methods were used to extract bacteria from the juices: filtration and immunomagnetic beads. Bacteria in broth were centrifuged and the pellets were then washed twice with deionized (DI) water. The bacteria suspensions were then placed in the fruit juices and then underwent filtration and immunomagnetic bead separation. It was determined that both filtration and magnetic bead separation were successful for concentrating the bacteria from fruit juices and for subsequent FTIR analysis. The bacteria on filters were examined by a Nexus 670 Multi-bounce flat plate Attenuated Total Reflectance (MATR) accessory while the bacteria bound to the magnetic beads was examined with the Continuµm® IR-microscope. Both concentration approaches improved spectra data collection with regards to reducing background interference, removing enrichment media, and improving absorbance values of spectra with respect to a previous study conducted by Yu et al. (2004). Yu et al. placed

inoculated apple juice directly onto the ZnSe crystal which required background samples to be subtracted from the inoculated juice sample spectrum.

Davis et. al (2010) conducted an experiment with inoculated ground beef and separated *E. coli* from the food matrix through filtration and immunomagnetic beads. Ground beef samples were inoculated with 2.5 ml of different dilutions of *E. coli* 0157:H7 and then mixed in a stomacher. Then 225 ml of *E. coli* (EC) broth with novobiocin was added to the inoculated beef samples and stomached again. 100 ml of the sample was then incubated at 37ºC for one hour. A 10^{-1} dilution of the sample was prepared in 90 ml of peptone water and this was subsequently used for both filtration and immunomagnetic separation.

Filter surfaces were examined with a Nexus 670 Avatar Smart Multibounce Horizontal Attenuated Total Reflectance (HATR) accessory attached to the spectometer with a liquid nitrogen cooled Mercury Cadmium Telluride (MCT) detector and KBr beam splitter. The immunomagnetic beads were examined with the Continuμm® IR-microscope. Second derivative spectra were used for the analysis. According to Yu and Irudayaraj (2004), the normalization of spectra eliminates path length variation and also reduces differences between each single measurement of the same sample. According to Al-Qadiri et al. (2008), the second derivative transformation also reduces replicate variability, corrects baseline shift , and resolves overlapping peaks thus reducing the effect of band overlap. After the mathematical processing, it was possible to differentiate between contaminated and uncontaminated ground beef samples at an inoculum level of 10^5 or more CFU/g. These differences were most obvious in the spectra in the wavenumber region of 1600-700 cm^{-1}.

Wang et. al (2010) looked at *E. coli* K12 internalized in baby spinach and subsequent detection with FTIR. Bacteria was injected into the tissues of the spinach by scratching the surface of the leaf and then applying the tip of a syringe with no needle onto the scratched area and slowly pushing the piston to allow the bacterial solution to go into the tissue. The leaves were then rinsed three times to remove any bacteria from the surfaces, and then placed under a biological hood for 24 hours at room temperature. Then the spinach leaves (3 g) were placed in 30 ml of sterilized 0.1% peptone water and pulverized

in a Stomacher. After they were left to sit for six min, the spinach extracts were filtered through Whatman number 200 filter paper and immediately analyzed in the FTIR.

The Nicolet 670 used was equipped with a deuterated triglycine sulphate detector and a Smart attenuated total reflectance kit (ARK) which included an HATR accessory. Four ml of sample extracts were loaded onto a ZnSe crystal. Once the spectra were obtained, calibration equations were obtained from original spectra, and the first and second derivative spectra were compared to determine *E. coli* concentrations in the spinach. The limit of detection was found to be approximately 100 CFU/ml.

2.4. **METHODS TO SEPARATE BACTERIA FROM FOODS**

Many advances have been made in detection and identification of microorganisms, however, many of these methods do not fully consider the food matrix. The initial sample preparation is perhaps the most important and crucial step of the process (Brehm-Stecher 2009). Food matrices present many challenges to food microbiologists when it comes to pathogen separation due to the diversity of food samples and potentially low contamination levels present within the sample. There must be a way to prepare a food sample such that it can be successfully incorporated into the novel assays for detection and identification of pathogens. To do this, various goals should be kept in mind to produce a homogeneous sample: separate target cells from food, increase their concentration while reducing the volume of sample, remove any extraneous material and exclude inhibitory substances for further downstream processing and identification (Mandal, 2010). Separation methods can be divided into three main groups: biological, chemical, and physical.

2.4.1. Food Inoculation

Solid food samples in microbiology are typically taken and diluted to 1:10 dilution. One gram of food is equal to 1 milliliter of sample for ease of calculation of dilution factors in microbiological manipulations (Brehm-Stecher et al., 2009). For research purposes, the food sample must be inoculated with a known amount of bacteria so that a recovery percentage can be calculated for a given separation method. Dipping, spraying, and surface inoculation are three procedures used to inoculate foods. With dipping and

spraying a food sample, however, it is unknown for sure how many cells are adhering to the surface of the food (Beuchat et al., 2001). The benefit of surface or spot inoculation is that a known amount of bacteria can be inoculated onto the food sample so that when recovery steps are completed, a known recovery rate can be calculated. The sample is then homogenized before further dilutions for analysis are taken. A Waring blender can be used to blend the food sample in a buffer solution. Other alternatives include a Stomacher or a Pulsifier. After homogenization, methods for separating bacteria from the food matrix can be implemented.

2.4.2. Biological Methods

Biological methods to separate bacteria from a complex matrix take advantage of proteins or antibodies to attach selectively or non-selectively to bacteria. The most used biological method is immunomagnetic separation.

Immunomagnetic separation (IMS) is a technique that makes use of magnetic particles, often spheres, and coats them with an antibody or protein to selectively bind cells from a sample suspension. Although originally developed for separation of blood cells, the technique is now used to separate many biological materials, including pathogens from food matrices. IMS is a rapid technique to separate pathogens from a sample, however, because pathogens are often present in very small numbers, an initial enrichment step is still necessary in many cases.

Coated beads are placed in a sample and allowed to incubate. The target is then separated from the sample by the use of a magnetic particle concentrator. Detection limits are generally 10^3 CFU/ml and lower if IMS is preceded by cultural enrichment (Stevens and Jaykus, 2004). Many times IMS is used in conjunction with another detection method such as PCR or ELISA.

Wright et al. (1994) used IMS to separate *E. coli* from minced beef samples. Dyna Beads (Invitrogen) were used that were coated with an antibody for *E. coli* O157 and placed in the homogenized beef sample. Yang et al. (2010) used IMS coupled with PCR to detect *C. perfringens* in different meat samples, and IMS was ued for rapid detection of *Salmonella* from meat samples by Notzon et al. (2006). IMS is a highly specific

separation technique and is ideal for use with small sample sizes (Stevens and Jaykus, 2004).

2.4.3. Physical Methods

Physical methods to separate bacteria from food matrices involve no chemical changes to the sample. Two widely used physical methods are centrifugation and filtration.

Centrifugation is a separation method that produces centrifugal force by rotating a rotor about a fixed axis. Particles suspended in a liquid medium are sedimented as a result of the centrifugal force, and the Stokes equation can be used to describe this phenomenon:

$$V_g = d^2 (Þ_p - Þ_1) / 18\mu \times G \qquad \text{Equation 2.1}$$

Where V_g = sedimentation velocity

d = particle diameter

$Þ_p$ = particle density

$Þ_1$ = liquid density

G = gravitational acceleration

μ = viscosity of liquid

Thus, the rate of settling is related to particle diameter, particle density, solution density, volume, angle, and the speed of rotation. Typically with foods, a low speed centrifugation step is used to separate out the larger food particles from the solution and a high speed centrifugation is used to spin out the bacteria.

Centrifugation was used by Fukushima et al. (2007) to separate and concentrate pathogens from a food slurry. Food samples were added to a stomacher bag with 1 ml of a bacterial solution and 225 ml of buffered peptone water and tween 20. After homogenizing for 1 min, the sample was centrifuged at 1,880 x g for 5 min at room temperature. The upper portion was then removed and centrifuged at 16,000 x g for 5 min at room temperature. The upper portion was removed and the pellet was suspended in a

salt solution and then centrifuged again at 14,500 x g for 5 min at room temperature. After the supernatant was discarded, a 0.5 ml pellet of bacteria remained. For the low speed centrifugation, bacteria recovery varied depending on the type of bacteria between 5.9 and 98.2%. For the high speed centrifugation step, recovery ranged between 3.2 and 56.8%.

Centrifugation can have an effect on the ability of bacteria to adhere to surfaces. Bell (2005) showed that centrifuging bacteria can alter external cell wall components such as the lipopolysaccharides, extracellular polysaccharides, and proteins. This would depend on the species of bacteria.

Filtration is the separation of solid particles from a fluid. Depending on the pore size of the filter, bacteria will either pass through the filter or become trapped on the surface of the filter. A major problem filtration poses with food analysis is that the filters can be fouled by food particles or other components of the matrix. It is also possible that bacteria cells can be adsorbed onto the filter surface, when theoretically they should pass through, thus limiting cell recovery (Brehm-Stecher et al., 2009). Filtration methods, however, are advantageous in that they remove many food components that could interfere with pathogen detection. Other advantages are that filtration is usually rapid, inexpensive, simple, and non-specific (Stevens et al. 2004).

The Iso-Grid method (Neogen Corp., Lansing, MI) is a dual filtration method that has received approval by the Association of Analytical Communities (AOAC). In this method, the food sample is homogenized and then passed through a 5 µm filter to remove larger food particles. The sample is then passed through a 0.45 µm hydrophobic and gridded filter. The design of the filter prevents the spread of colonies and the grid facilitates counting the colonies after incubation on an agar plate (Payne and Kroll, 1991).

Many times, filtration is used in conjunction with other methods to separate pathogens from a food matrix. Wang et. al (1992) homogenized 25 g of a food sample with 225 ml of phosphate buffered saline (PBS) with 0.2% Triton X-100 for 5 min. The sample was then passed through a Whatman #4 filter to remove large food particles from both meat and cheese samples. The filtrate was then centrifuged and later heated to lyse the cells

and release the nucleic acids for PCR detection. This method was successful for detecting <10 CFU/g of *L. monocytogenes* from contaminated meat products, but not from soft cheese samples.

Fernandez-Astorga et al. (1996) pre-treated milk samples with trypsin and Triton X-100 and then passed the sample through an Isopore polycarbonate 0.2 µm black membrane filter. The filter was stained with a fluorescein-labeled anti-O157 polyclonal antibody and then examined by epifluorescense microscopy. The assay time took under one hour to complete. The results obtained were difficult to interpret, however, because other extraneous matter was concentrated onto the filter's surface in addition to the bacteria.

2.4.4. Chemical Methods

Chemical methods to separate bacteria from food matrices may involve altering the chemistry of certain components of the homogenized samples. Desorption, also known as elution, is one such method that is the process of detaching adsorbed substances from the surface of a solid matrix. Some of the physiochemical interactions to do this include Van der Waal's forces, electrostatic interactions, hydrophobic interactions, and hydrogen bonding (Stevens and Jaykus, 2004). Cell wall components, such as teichoic acids, proteins, and carbohydrate moieties influence bacteria attachment to food surfaces. By altering the physiochemical interactions, desorption can occur due to disruption of chemical forces. Payne and Kroll (1991) concluded that differences in bacterial cell wall composition could help in the development of separation methods based on differential adsorption and desorption.

For this research, seven chemical additives were used in buffer solution to test their efficacy at aiding in the separation of bacteria from foods: Tween 20, Tween 80, Brij 35, NP 40, Hexanol, Decanol, and Sodium Polyphosphate.

2.4.4.1. Surfactants

Tween 20, Tween 80, Brij 35, and NP 40 are all non-ionic surfactants. Surfactant molecules are of interest because they have hydrophilic and hydrophobic regions that orient at surfaces in such a way that modifies hydrophobic surfaces to become more

hydrophilic (Hill et al., 2005). Surfactants have been used in microbial laboratory techniques previously where the goal was to minimize microbial adhesion to surfaces.

Tween 20 is an emulsifier with the molecular formula $C_{58}H_{114}O_{26}$ and is commonly used as a washing agent in immunoassays and in pharmaceutical applications to emulsify essential oils in distilled water. Miller et al. (2011) reported that the addition of Tween 20 to a saline buffer solution significantly increased *Salmonella* recovery from contaminated lettuce and tomatoes. Fukushima et al. (2007) added 0.02% Tween 20 to Buffered Peptone Water (BPW) to emulsify fat in food samples as part of the sample preparation before homogenization.

Tween 80 ($C_{64}H_{124}O_{26}$) is an emulsifier that is a viscous, soluble yellow liquid commonly used in foods. Lukasik et al. (2001) found that in order to elute bacteria from the surface of seeded strawberries and tomatoes, it was necessary to disrupt the hydrophobic and electrostatic interactions between the bacteria and produce surface. The addition of 0.1% Tween 80 to Phosphate Buffered Saline (PBS) increased bacterial recovery an average of two-fold compared to PBS by itself.

Brij 35 ($C_{58}H_{118}O_{24}$) is used as a component of cell lysis buffers or as a surfactant in High-Performance Liquid Chromatography (HPLC) applications. Brown et al. (2001) showed that the use of Brij 35 enhanced the bacteria transport through a column of porous media. Garcia et al. (2001) showed that the presence of Brij 35 suppressed the contact of bacteria with a teflon surface.

Nonyl phenoxypolyethoxylethanol (NP 40) is used in paper and textile processing, paints and coatings, and agrochemicals. It was used in addition to lactic acid as a disinfecting rinse for canteloupes. The addition of NP 40 at 0.3% to a solution of lactic acid at 35ºC was shown to enhance the removal of *E. coli* 0157:H7 cells from the canteloupe rind (Materon, 2003).

2.4.4.2. Fatty Alcohols

Fatty alcohols are aliphatic alcohols (non-aromatic) that consist of a chain of 8-22 carbon atoms. They are produced by bacteria, plants, and animals as a source of metabolic water and energy, and buoyancy in some cases. These alcohols are used in the production of

detergents and surfactants and are used as emulsifiers and thickeners in the cosmetics and food industry.

1-Decanol is a fatty alcohol with molecular formula $C_{10}H_{21}OH$. Hamilton-Kemp et al. (2002) showed that certain long chain alcohols, including 1-Decanol, are produced by enteric Gram negative, including *E. coli*. Neumann et al. (2006) determined that cells of *Pseudomonas putida* that were grown in 10% (vol/vol) 1-Decanol had enhanced cell hydrophobicity and more negative cell surface charges than cells grown without 1-Decanol. However, they had a 10% reduced growth rate and 48% reduced growth yield than cells grown without 1-Decanol. While this study showed the ability of certain bacteria to adapt to the presence of this solvent, 1-Decanol at high enough concentrations had a lethal effect on other bacteria. 2-Ethylhexanol, $C_8H_{17}OH$, is a fatty alcohol with eight carbon atoms.

2.4.4.3. Chemical Dispersants

Sodium Polyphosphate (NaPP) compounds are a type of chemical dispersant. They are highly negatively charged chemicals that have been used to decrease bacterial adhesion to soil (Sharma et al., 1985). Sharma et al. showed that for two common species of bacteria (*Bacillus subtilis* and *Pseudomonas fluorescens*) the addition of NaPP to media drastically changed the extent of bacterial adhesion to soil samples. NaPP compounds work as dispersants by changing the surface charge of microbes, particles, and filter surfaces. They significantly reduce the zeta potential of suspended microbes (Hill et al., 2005). NaPP compounds are produced in various phosphate chain lengths.

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CHAPTER 3. **MATERIALS AND METHODS**

3.1. **BACTERIA AND GROWTH CONDITIONS**

E. coli K12 (ATCC 11775) and *Listeria innocua* (ATCC 33091) were obtained from the Department of Food Science, University of Kentucky. Both bacteria were stored on a slant of Brain Heart Infusion agar (BHIA) [BD Diagnostics, Franklin Lakes, NJ] and inoculated from the slant to a test tube of Brain Heart Infusion broth (BHI) [BD Diagnostics, Franklin Lakes, NJ] the day before experimentation with each food matrix started. For the remainder of experimentation, 1 ml of bacteria in broth was inoculated into fresh BHI and incubated for 18 h for the next day's use. All bacteria samples were incubated for 24 h at 35°C. Each day, 2 ml each of *E. coli* and *Listeria* in BHI were combined into one test tube and vortexed for 15 s before being inoculated onto the food matrix.

3.2. **ENUMERATION OF BACTERIA**

For bacteria enumeration, decimal dilutions of the food homogenate or supernatant were prepared using peptone water 0.1% (PW) [BD Diagnostics, Franklin Lakes, NJ]. A 1 ml sample from the food homogenate was pipetted into a test tube of 9 ml PW to obtain a 10^{-1} dilution. This sample was vortexed for 10 s and a 1 ml sample was then taken from that test tube and pipetted into another test tube of PW to obtain a 10^{-2} dilution. This process continued one more time to obtain a 10^{-3} dilution. One ml samples of the 10^{-3} dilution were pipetted onto *E. coli* Petrifilm (3M, St. Paul, MN) or into petri dishes for *Listeria* counts. Three plates/films were prepared from the 10^{-3} dilution. *Listeria* selective PALCAM agar (Oxoid Limited, England) was then poured into the petri dishes and agitated clockwise, counterclockwise, up and down, and side to side for 20 counts each. After media solidified, plates were inverted and incubated along with Petrifilm for 24 h at 35°C.

Agar plates and Petrifilm were counted manually. The average of the plate counts for a given step was taken and divided by the corresponding dilution factor (10^{-3}) used to result in plates with counts between 25 and 250 colony forming units (CFU). This number was then multiplied by the sample's volume to obtain the total bacteria in the sample.

Twenty microliter samples of bacteria were placed in a fixed volume of BPW depending on what volume was used for each food matrix (200 ml for spinach and hotdog samples and 50 ml for milk). This sample was then plated and recovery efficiencies for each bacteria were calculated from the resulting plate counts; the amount of bacteria that was expected to be recovered if there were 100% recovery. The recovery efficiency, shown by Equation 3.1, was calculated by dividing the total number of bacteria at each step of the experiment (from plate counts taken after blending/mixing and centrifuging) by the total number in the initial buffer/bacteria sample. This number was then multiplied by 100 to get a recovery percentage.

$$\text{Recovery Percentage} = \frac{\text{Recovery Efficiency (CFU)}}{\text{100\% Recovery Value (CFU)}} \times 100\% \qquad \text{Equation 3.1}$$

3.3. DRY MATTER CONTENT

The dry matter content of a sample was determined by recording weights of aluminum trays and then pipetting a 1 ml aliquot of sample into the tray. The samples were weighed using an analytical balance having a resolution of 0.1 mg ± 0.2 mg (AE260, Mettler-Toledo, Inc., OH, USA). Trays were then placed in a convection oven at 100°C for 24 h. After that time, trays were weighed and recorded. Each sample was analyzed in triplicate.

The dry matter content was calculated by taking the final mass of the sample (final tray weight from oven minus tray weight with no sample) and dividing it by the initial mass of the sample (initial tray weight with sample minus tray weight with no sample) and multiplying by 100 to get a percentage (Equation 3.2).

$$\text{Dry Matter Content (\%)} = \frac{m_f}{m_i} \times 100 \qquad \text{Equation 3.2}$$

where m_f equals the mass of the dried sample, and m_i equals the mass of the sample before drying.

This test was used as another tool to measure the overall success of the separation processes. The ideal protocol would reduce the greatest amount of solids without compromising microbial recovery.

3.4. PREPARATION OF MEDIA

Buffered Peptone Water was the buffer solution used for each of the food matrices based on initial test results outlined in Appendix A.

Surfactants used were: Tween 80 (VWR International, West Chester, PA), Tween 20 (Amresco, Solon, OH), Brij 35 (Alfa Aesar, Ward Hill, MA), and Tergitol NP-40 (Spectrum Chemical, Gardena, CA).

One chemical dispersant and two fatty alcohols were also tested: Sodium Polyphosphate (NaPP) [Spectrum Chemical, Gardena, CA], 1-Decanol (Alfa Aesar, Ward Hill, MA) and 2-Ethyl-1-Hexanol (Acros Organics, Pittsburgh, PA).

All media were made with deionized (DI) water (Barnstead International, .Dubuque, IA) and stored in a dry cabinet after being autoclaved. Surfactants, chemical dispersant, and fatty alcohols were added directly to BPW before being autoclaved at 121°C and 15 psi for 20 min.

3.5. EXPERIMENTAL METHODOLOGY

Hotdogs (Oscar Meyer Naturals), fresh spinach, and whole milk, were purchased from a local grocery store. Oscar Meyer Naturals were chosen as the brand of hotdog to use because they do not contain preservatives commonly found in packed hotdogs (nitrates and nitrites). Unpackaged spinach was also chosen to ensure that no preservatives were used. Kroger whole milk was used as opposed to 1% or skim milk because of its higher fat content, which has been shown to harbor microorganisms (Valerio, 2010), however, all milk utilized was pasteurized.

The overview of the procedure followed for spinach and hotdog experimentation can be seen in Figure 3.1. The total number of viable microflora was estimated by a plate count on Aerobic Plate Count Petrifilm (3M, St. Paul, MN). Hotdog and spinach samples were weighed (20±0.1 g) and spot inoculated with 20 µl of both *E. coli* and *Listeria* in broth. The sample was allowed to sit for 5 min before being further processed. A buffer solution with an additive of volume 180 ml was placed in a laboratory blender (Waring, Torrington, CT) with the inoculated hotdog or spinach sample. Samples were blended at approximately 22,000 rpm for 2 min. A 1 ml sample was then pipetted out and a plate

count completed on selective media. The sample in the blender next underwent a low-speed centrifugation (2000 rcf) for 3 min. A plate count was taken of the supernatant.

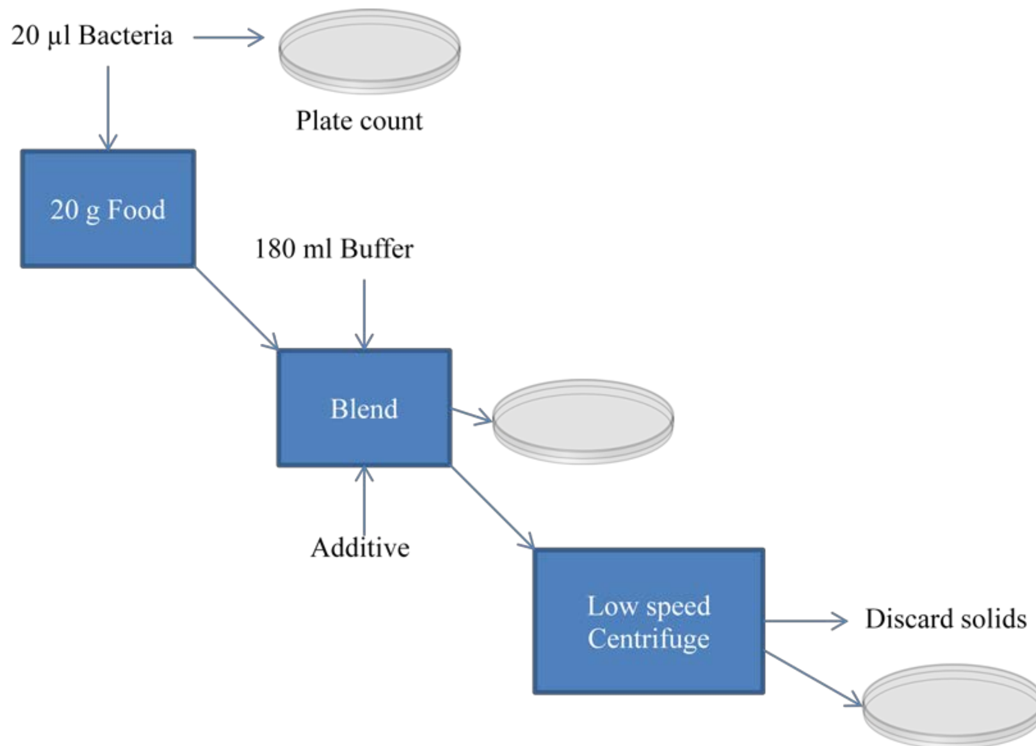

Figure 3.1Single additive experiment overview of procedure followed for hotdogs and spinach.

Figure 3.2 shows an overview of the procedure followed for milk. The process is only different in the ratio of food sample to buffer which changed from 1:9 (typical of food sampling) to 2:3, and blending was not necessary as milk is already homogenized.

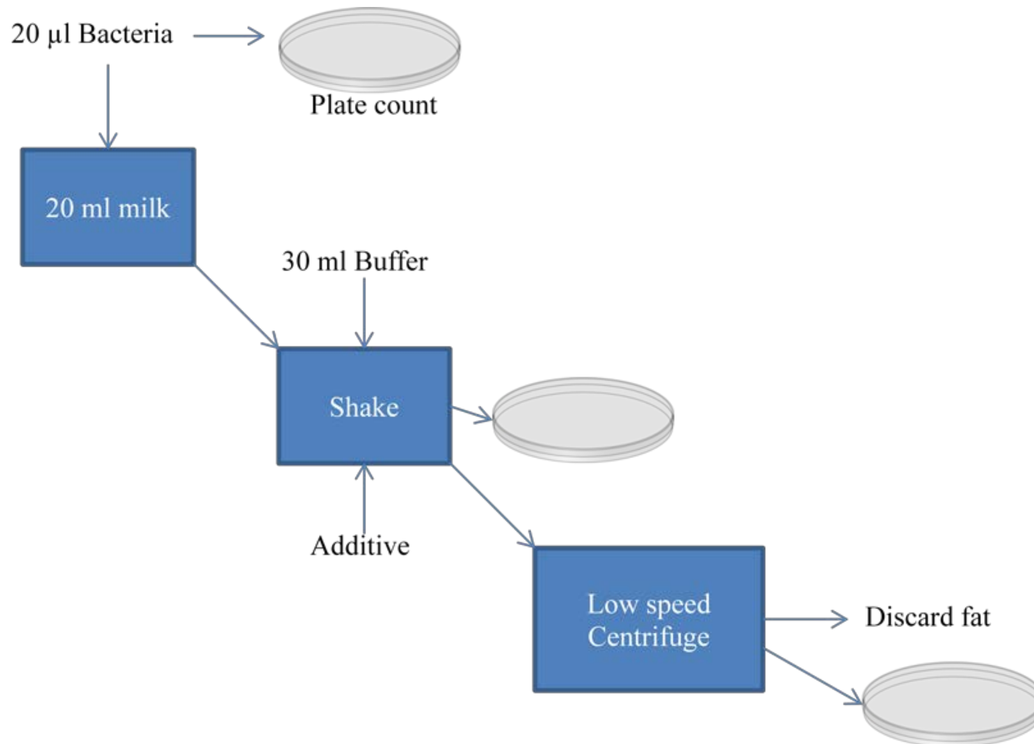

Figure 3.2 Single additive experiment overview of procedure followed for milk..

3.6. Single Additive Experimental Design

A full factorial experimental design was implemented for three factors: food matrix, additive, and concentration. Seven additives at three concentrations each were used for each food matrix. Each food matrix had a total of 66 trials (3 replications per additive/concentration combination) including a control where no additive was added to BPW. The order of trials was randomized such that no additive and concentration combination was tested more than once in each 22 trial block. Table 3.1 shows the foods, additives, and concentrations that were used.

Table 3.1 Additive experiment factorial design showing foods, additives, and concentrations used.

FACTOR	LEVELS		
Food Matrix	Hotdogs Spinach Milk		
Additive	*Surfactant*	*Chemical Dispersant*	*Fatty Alcohols*
	Tween 80 Brij 35 Tween 20 NP-40	Sodium Polyphosphate	*Hexanol *Decanol
Concentration	Low	Medium	High
	0.01%	0.1%	1.0%
	*0.001%	*0.01%	*0.1%

3.7. ADDITIVE COMBINATION EXPERIMENTAL DESIGN

Data collected from each food matrix were examined to determine which additive at which concentration worked best from each class, which was based on which additive/concentration combination had the highest percent recovery for both *E. coli* and *Listeria.* Table 3.2 shows the testing plan that was followed for the additive combination experiment, and Table 3.3 shows the additives and concentrations that were selected for each food matrix. Testing order was randomized each day for four days of experimentation for each food matrix.

Table 3.2 Combination experiment setup including the treatments and number of replications for each.

No.	*Treatment*	*Replications*
1	Surfactant	4
2	Fatty Alcohol	4
3	Chemical Dispersant	4
4	Surfactant + Fatty Alcohol	4
5	Surfactant + Chemical Dispersant	4
6	Fatty Alcohol + Chemical Dispersant	4
7	Surfactant + Fatty Alcohol + Chemical Dispersant	4
8	Control	4
	Total Experiments	*32*

Table 3.3 Additives and concentrations used in additive combination experiment.

	Hotdog	Spinach	Milk
Surfactant	Tween 80 0.01%	Tween 80 0.1%	Brij 35 1%
Fatty Alcohol	Decanol 0.001%	Hexanol 0.001%	Hexanol 0.001%
Chemical Dispersant	NaPP 1%	NaPP 0.01%	NaPP 1%

3.8. TWEEN 80 + NAPP AND TWEEN 80 + HEXANOL EXPERIMENTAL DESIGN

Based on the results from the additive combination experiment, two additive combinations were selected for further study. The experimental procedure was changed to minimize any error due to day to day variations. First, Tween 80 and NaPP were used together and compared to a control with no additives. This experiment consisted of three controls and three trials with Tween 80 0.01% and NaPP 0.1% added to the buffer solution for all three food matrices: hotdogs, spinach, and milk. Figure 3.3 shows the procedure followed. Food samples were homogenized in buffer and then divided into six 50 ml sample containers. BPW was then added to the control samples, and the combination of two additives and BPW were added to the other samples to reach 50 ml and the proper concentration of additives. Each sample was blended again for 60 s before undergoing the low speed centrifuge step as outlined in section 3.5. After the supernatant was poured off, a plate count was taken and dry matter analysis was performed. The same procedure was followed using Tween 80 0.01% and Hexanol 0.001%.

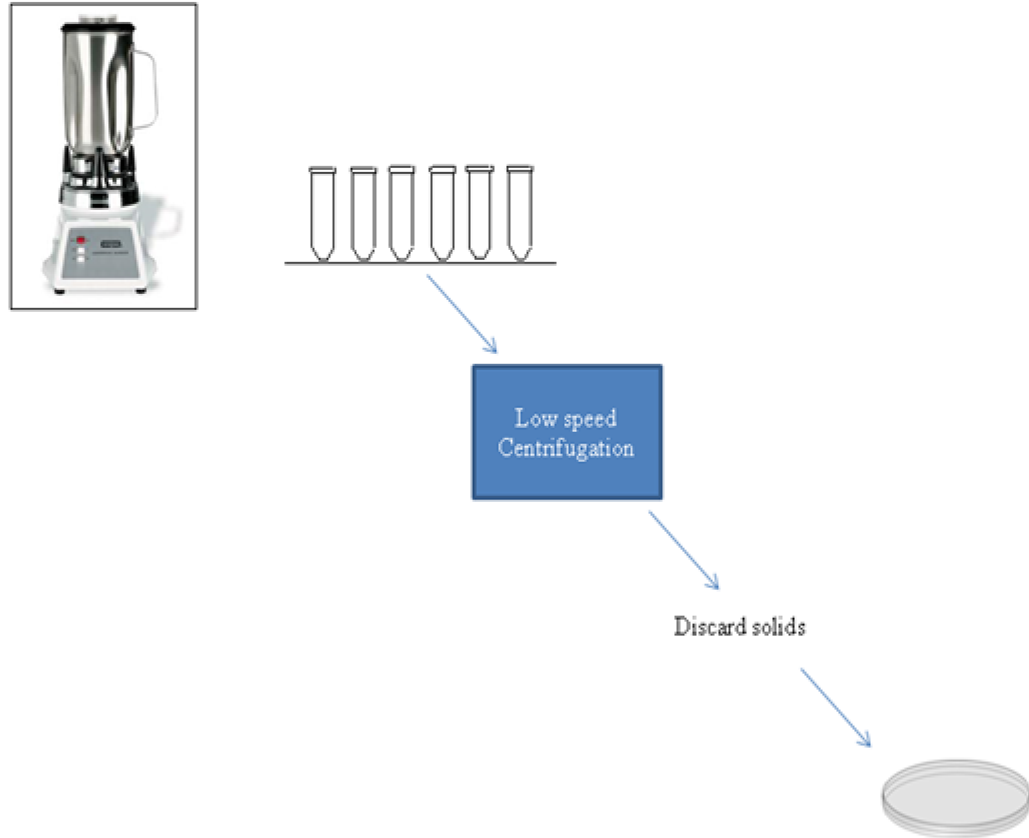

Figure 3.3 Experimental procedure for additive combination experiments to take place in one day.

3.9. Statistical Analysis

After all the data were collected from the first experiment, a general linear model was created in SAS statistical software (version 9.2, Cary, NC) to determine whether the additive and concentration effects were significant ($p<0.05$) for each food matrix. The analysis of variance (ANOVA) table summarizes the variability in the observations from the experiment. It includes the source of variation, degrees of freedom, sum of squares, mean square, F value, and p-value. The general linear model relates the response variable, y, to the design variables, x_1, x_2, …, x_k, through a set of parameters, β_0, β_1, β_2, …,

β_k, so that it is linear in the set of parameters, which for this research were additive and concentration. The general linear model is described by Equation 3.3.

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + ... + \beta_k x_k + e \qquad \text{Equation 3.3}$$

In addition, averages were taken from the three replications of each additive/concentration combination and subtracted from the average of the control replications. The results were then plotted to see which additive/concentration combinations resulted in a higher recovery.

A Dunnett test was conducted to compare the recovery results from each additive at each concentration to the control. The Dunnett test is a multiple comparison test that can be used to determine if there are significant differences between a single control group mean and the treatment group mean (Dunnett, 1955). The Dunnett test compares each treatment mean, $\overline{y_i}$, with the control treatment mean, $\overline{y_c}$. The Dunnett criterion to compare k treatments to the control is given by Equation 3.4,

$$D(k, \propto_E) = d_{\propto,k,v} \sqrt{\frac{2s^2}{r}} \qquad \text{Equation 3.4}$$

where k is equal to t -1 comparisons, $\propto_E$ is the experimentwise Type I error, $d_{\propto,k,v}$ is the tabled statistic for one-sided comparisons for an experimental error rate of $\propto_E$, with v degrees of freedom for the estimate of experimental error variance, s is the standard deviation, and r is the number of replications.

The simultaneous two-sided confidence interval estimates for the differences between the individual treatment means and the control means μ_i - μ_c is given by Equation 3.5.

$$\overline{y_i} - \overline{y_c} \pm D(k, \alpha_E) \qquad \text{Equation 3.5}$$

For the two-sided test, to test the null hypothesis that the treatment mean is equal to the control mean, $H_0: \mu_i = \mu_c$, versus the alternate hypothesis, $H_a: \mu_i \neq \mu_c$. The null hypothesis is rejected if:

$$|\overline{y_i} - \overline{y_c}| > D(k, \alpha_E)$$ Equation 3.6

In the event there was a significant p-value in the ANOVA but the difference was not evident from the Dunnett test results comparing the treatment means to the control mean, a Tukey method was performed to find where the significant difference was in the data. The Tukey method is a pairwise comparison also known as the Honestly Significant Difference (HSD). The HSD can be computed by Equation 3.7:

$$\text{HSD}\ (k, \propto_E) = q_{\propto,k,v}\sqrt{\frac{s^2}{r}}$$ Equation 3.7

where $q_{\propto,k,v}$ is the Studentized range statistic for a range of k treatment means in an ordered array.

Two treatment means are declared not equal (μ_i - $\mu_j \neq 0$) if:

$$|\ \overline{y_i} - \overline{y_j}|\ > \text{HSD}(k, \propto_E)$$ Equation 3.8

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CHAPTER 4. **RESULTS AND DISCUSSION**

4.1. **Preliminary Objectives for Development of Protocol Results**

Preliminary experiments were conducted to adress the preliminary objectives laid out in 1.1. The following procedures were tested in order to develop an optimized protocol for separating microorganisms from foods.

1. Food sampling and contamination method (A.1)
2. Buffer solution to be used (A.2)
3. Filtration or centrifugation (A.3)
4. Additives and concentrations to use (A.4)

Based on the results, outlined in Appendix A, it was determined that solid foods should be sampled in Buffered Peptone Water at a ratio of 1:9 (food to buffer). Food and buffer samples can then be homogenized in a Waring blender for two minutes after which they undergo a low speed centrifugation (2000 rcf) for three minutes. After centrifugation, the supernatant can be poured off and bacteria can then be enumerated by a plate count.

4.2. **Results for Single Additives at Varying Concentrations**

Seven additives were added to BPW at three different concentrations each. Four surfactants were tested: Tween 20, Tween 80, NP 40, and Brij 35. Two fatty alcohols were tested: Hexanol and Decanol. Finally, one chemical dispersant, Sodium Polyphosphate, was used. The concentrations that were tested for surfactants and the chemical dispersant were 0.01, 0.1, and 1%. For fatty alcohols, 0.001, 0.01, and 0.1% were the concentrations used.

4.2.1. Hotdog Results

The results from the hotdog experiment reveal that the highest recovery of *E. coli* from hotdogs was achieved using Decanol at 0.01%, which resulted in an average recovery of 13% over the control. At the low concentration of all additives, the recovery of *E. coli* was greater than the control (Table 4.1). In addition, additives Tween 20, Decanol, Hexanol, and Sodium Polyphosphate resulted in greater recovery than the control at all three concentrations used. The plot of results for Brij 35 reveals a decrease in results from

low to high concentration, whereas the plot of Tween 20 shows increased results between low, medium, and high concentrations (Figures 4.1 and 4.3). NP 40, Tween 80, Hexanol, and NaPP each had the lowest recovery at the medium concentration level.

The highest recovery of *Listeria* from hotdogs was achieved using Decanol at 0.001% which resulted in an average recovery 13% higher than the control. *Listeria* recovery from the control was higher than from *E. coli*, 73% versus 47% respectively. Only the following additive/concentration combinations resulted in higher recovery percentages compared to the control: NP40 1%, Tween 80 0.01%, Decanol 0.001 and 0.01%, and Hexanol 0.01%. Decanol results show a negative trend in results from low to high concentrations, and no positive trends can be noted for the three concentrations tested for any of the additives (Figure 4.2).

Table 4.1 Hotdog recovery results (n=3) normalized with control (treatment recovery % - control recovery %). *E. coli* recovery averaged 47% and *Listeria* recovery averaged 72%.

E. coli Recovery from Hotdogs			
	Low	Medium	High
Brij 35	6%	4%	*-6%*
NP40	4%	*-11%*	6%
Tween 20	2%	6%	12%
Tween 80	11%	*-8%*	*-7%*
Decanol	10%	13%	4%
Hexanol	4%	1%	4%
Sodium Polyphosphate	12%	7%	12%
Listeria Recovery from Hotdogs			
	Low	Medium	High
Brij 35	*-8%*	*-13%*	*-12%*
NP40	*-12%*	*-22%*	7%
Tween 20	*-10%*	*-3%*	*-4%*
Tween 80	10%	*-16%*	*-10%*
Decanol	13%	6%	*-20%*
Hexanol	0%	6%	*-3%*
Sodium Polyphosphate	*-8%*	*-9%*	0%

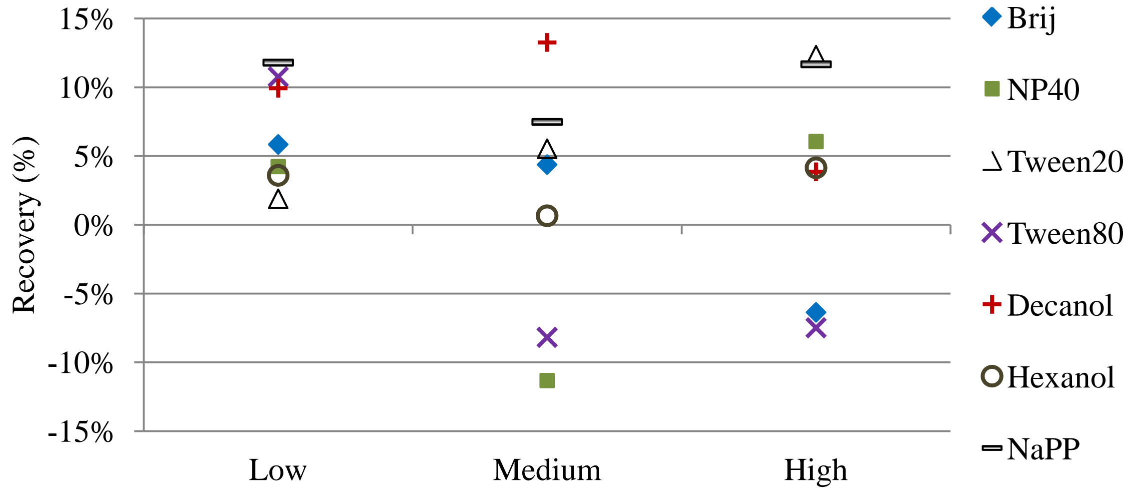


Figure 4.1 Hotdog results: *E. coli* recovery normalized with control for all additives at three concentration levels.

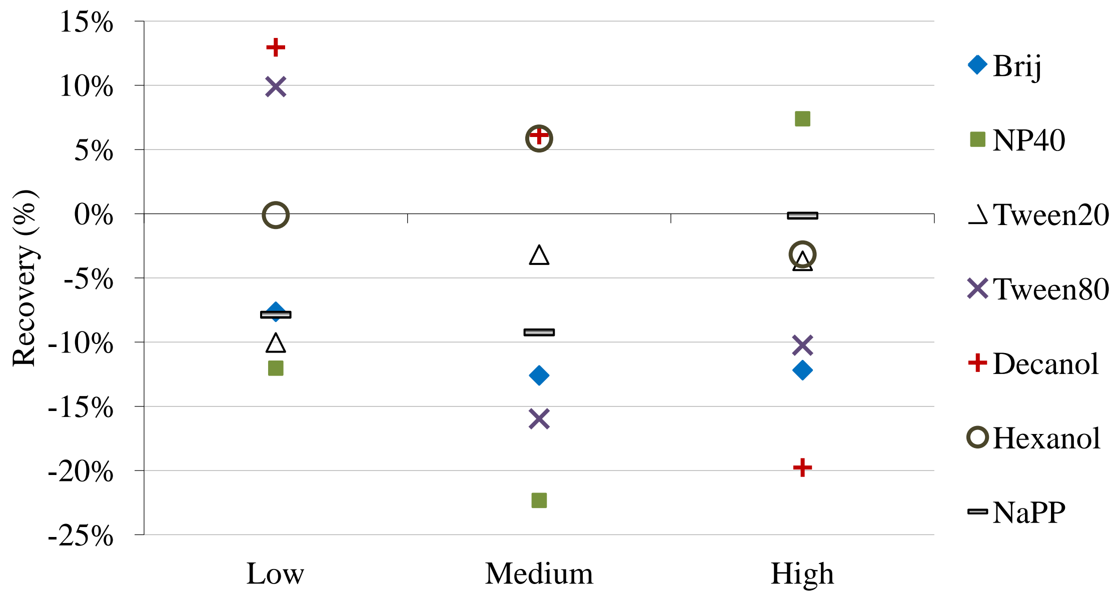


Figure 4.2 Hotdog results: *Listeria* recovery normalized with control for all additives at three concentration levels.

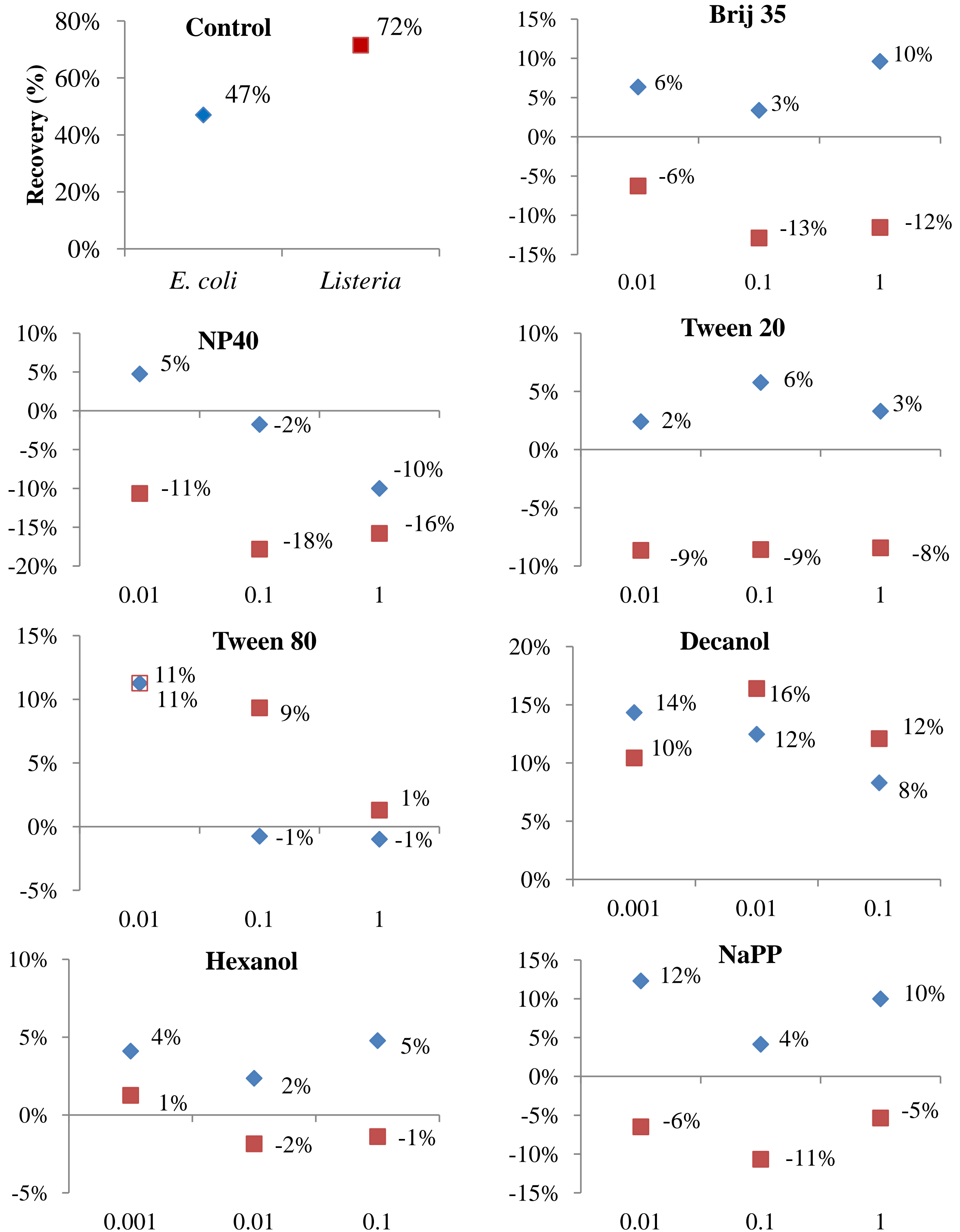


Figure 4.3 Graphs showing the control data from hotdogs for both *E. coli* and *Listeria* and then the effects of each of the additives (from Table 4.1).

A general linear model was run on the hotdog data. The results revealed that bacteria was a significant factor in the model ($p<0.0001$) so the data were divided by bacteria in the

model. The ANOVA obtained for *E. coli* and *Listeria* reveal that the additive and concentration were not significant in this experiment (Tables 4.2 and 4.3). The Dunnett test also showed no significant differences between additives and the control.

Table 4.2 ANOVA for *E. coli* recovery from hotdogs where the classes in the model were additive and concentration.

Source	SS	DF	MS	F	p
Model	0.1472	9	0.0164	1.34	0.2388
Error	0.6844	56	0.0122		
Corrected Total	0.8316	65			

Additive Comparison with Control	Difference Between Means	95% Confidence Limits	
Brij 35	-0.0224	-0.21097	0.16616
NP40	-0.03872	-0.22728	0.14984
Tween 20	0.03058	-0.15798	0.21914
Tween 80	-0.05163	-0.24019	0.13693
Decanol	0.05488	-0.13368	0.24344
Hexanol	-0.00726	-0.19582	0.18130
NaPP	0.06787	-0.12069	0.25643

Table 4.3 ANOVA for *Listeria* recovery from hotdogs where the classes in the model were additive and concentration.

Source	SS	DF	MS	F	p
Model	0.1446	9	0.0161	0.75	0.6621
Error	1.200	56	0.0214		
Corrected Total	1.3447	65			

Additive Comparison with Control	Difference Between Means	95% Confidence Limits	
Brij 35	-0.09431	-0.32329	0.13467
NP40	-0.07614	-0.30512	0.15284
Tween 20	-0.04252	-0.27150	0.18646
Tween 80	-0.04064	-0.26962	0.18834
Decanol	0.01147	-0.21751	0.24045
Hexanol	0.03821	-0.19513	0.27155
NaPP	-0.04382	-0.27280	0.18516

4.2.2. Spinach Results

The results from the spinach additive experiment showed that the highest recovery came from the use of Tween 80 at 0.1% (Table 4.4). The highest recovery of *E. coli* from spinach was 13% over the control from Tween 80 at 0.01%, Brij 35 at 0.1%, and NP40 at 0.1%. Brij 35 and Tween 80 were the only additives that resulted in greater recovery than the control at all three concentrations. Tween 20 had recoveries that increased from low to high concentrations, whereas Tween 80, Hexanol and NaPP had decreasing results (Figures 4.4 and 4.6). Brij 35 and NP40 each performed best at the medium concentration. It is also interesting to note that Hexanol had a significant effect on the recovery of *E. coli* at 0.1% causing a 0% recovery.

The highest recovery of *Listeria* from spinach was >100%, or 16% above the control value, from Tween 80 at 0.1%. The only additive/concentration combinations that resulted in a higher recovery than the control were Tween 80 at 0.01 and 0.1%, and NaPP at 0.01% (Figure 4.5). Tween 20, Decanol, and Hexanol's recoveries were below the control at all concentrations. It is interesting to note here that Decanol and Hexanol both had a significantly negative effect on *Listeria* at the high concentration of 0.1% resulting in a 0% recovery.

Table 4.4 Spinach recovery results (n=3) normalized with control (treatment recovery % - control recovery %). *E. coli* recovery averaged 67% and *Listeria* recovery averaged 84%.

E. coli Recovery from Spinach			
	Low	Medium	High
Brij 35	7%	13%	9%
NP40	*-6%*	13%	8%
Tween 20	*-8%*	*-4%*	3%
Tween 80	13%	9%	3%
Decanol	*-4%*	*-7%*	3%
Hexanol	3%	1%	*-67%*
Sodium Polyphosphate	12%	3%	*-2%*
Listeria Recovery from Spinach			
	Low	Medium	High
Brij 35	0%	*-2%*	*-10%*
NP40	*-2%*	*-9%*	0%
Tween 20	*-20%*	*-15%*	*-8%*
Tween 80	6%	18%	*-9%*
Decanol	*-18%*	*-8%*	*-79%*
Hexanol	*-16%*	*-20%*	*-84%*
Sodium Polyphosphate	1%	*-12%*	*-7%*

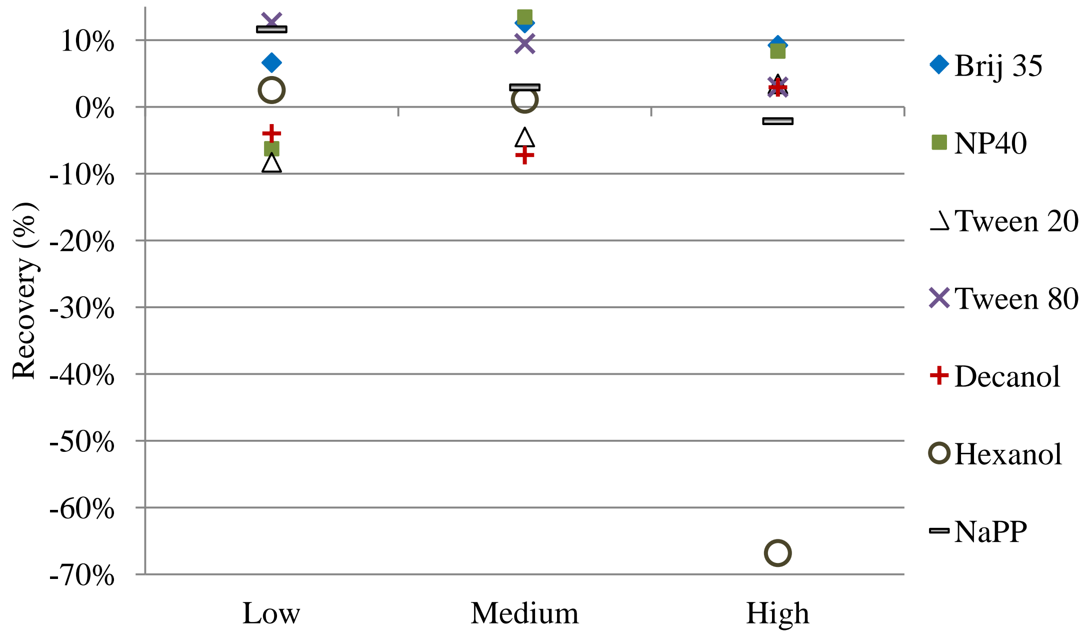


Figure 4.4 Spinach results: Normalized *E. coli* recovery from all seven additives used at three levels of concentration.

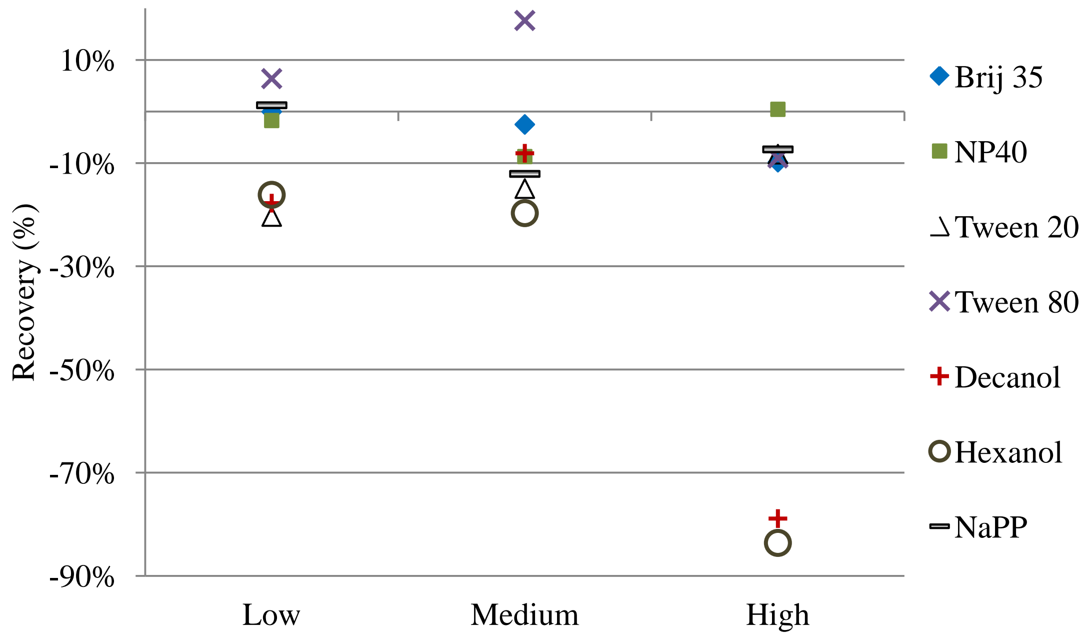


Figure 4.5 Spinach results: Normalized *Listeria* recovery from all seven additives used at three levels of concentration.

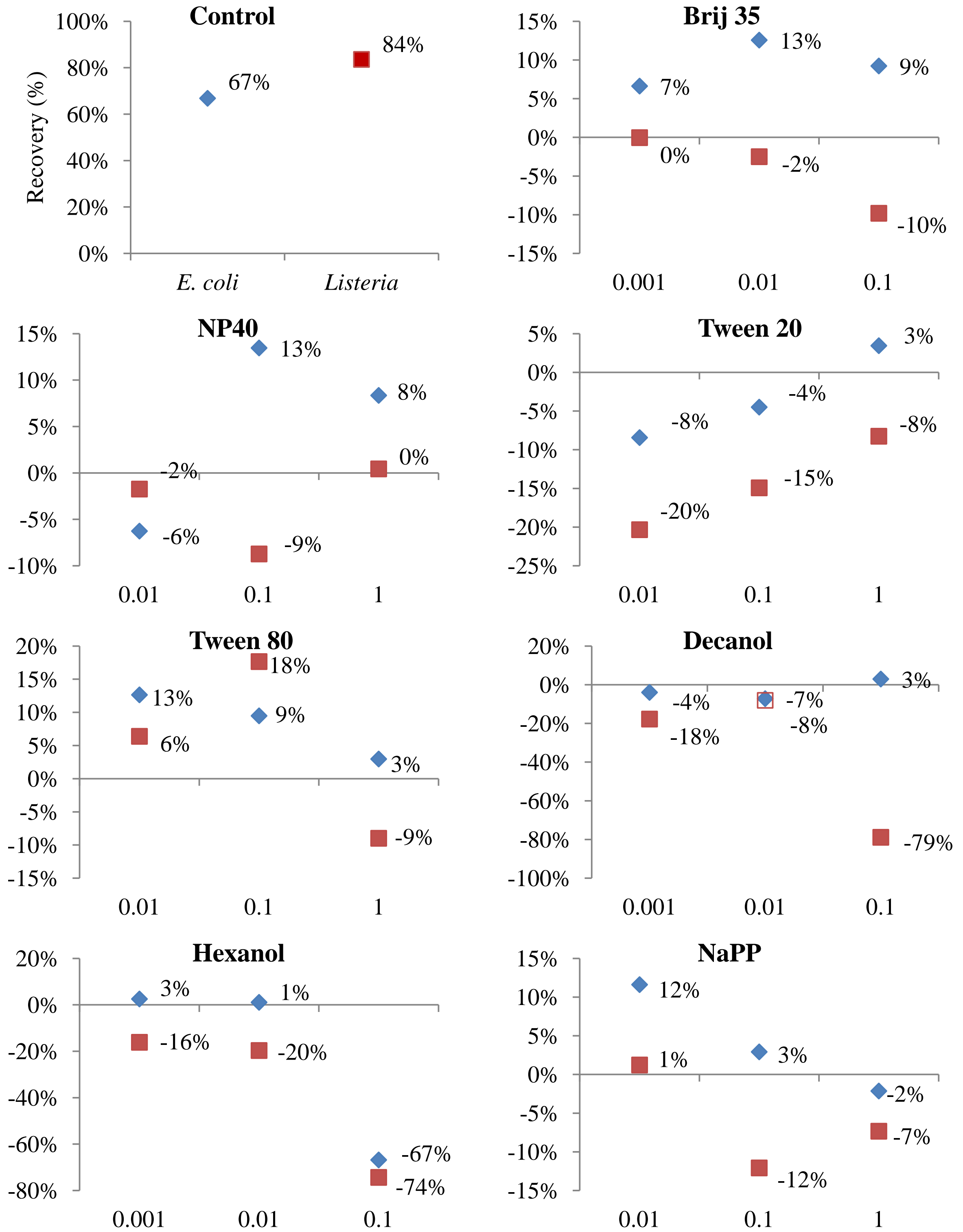


Figure 4.6 Graphs showing the control data from spinach for both *E. coli* and *Listeria* and then the effects of each of the additives (from Table 4.4).

For the statistical analysis, a general linear model was run on the spinach data, which was once again divided up by bacteria type. The ANOVAs obtained for *E. coli* and *Listeria* reveal that the additive and concentration were significant in this experiment (Tables 4.5, 4.6). However, the Dunnett test for *E. coli* data showed no significant differences between additives and the control. A Tukey test was run on the *E. coli* data and the significant differences were found to be between Hexanol and the following additives: NP 40, Brij 35, and Tween 80, where the use of the latter three resulted in significantly greater results over the use of Hexanol. The Dunnett test for *Listeria* data revealed that the significant differences were between the control and the use of the fatty alcohols, Decanol and Hexanol.

Table 4.5 ANOVA and Dunnett Test results obtained for *E. coli* recovery from spinach.

Source	SS	DF	MS	F	p
Model	0.6483	9	0.0720	2.49	0.018
Error	1.619	56	0.0289		
Corrected Total	2.2675	65			

Additive Comparison with Control	Difference Between Means	95% Confidence Limits	
Brij 35	0.09473	-0.19529	0.38476
NP40	0.10071	-0.18931	0.39074
Tween 20	-0.03161	-0.32164	0.25841
Tween 80	0.09239	-0.19764	0.38241
Decanol	-0.02748	-0.31750	0.26255
Hexanol	-0.1802	-0.47023	0.10982
NaPP	0.04875	-0.24128	0.33877

Table 4.6 ANOVA obtained for *Listeria* recovery from spinach.

Source	SS	DF	MS	F	p
Model	1.9339	9	0.2149	5.19	<0.0001
Error	2.3206	56	0.0414		
Corrected Total	4.2545	65			

Additive Comparison with Control	Difference Between Means	95% Confidence Limits		
Brij 35	-0.04117	-0.38838	0.30604	
NP40	-0.11120	-0.45841	0.23600	
Tween 20	-0.14507	-0.49227	0.20214	
Tween 80	0.05017	-0.29704	0.39737	
Decanol	-0.34906	-0.69627	-0.00186	*
Hexanol	-0.37751	-0.72472	-0.03031	*
NaPP	-0.06063	-0.40784	0.28657	

* denotes a statistical difference at a 0.05 level.

4.2.3. Milk Results

E. coli recovery from whole milk was the greatest with the use of Hexanol at 0.001% and was 17% greater than the control (Table 4.7). Hexanol and Decanol did not have a lethal effect on the bacteria in milk like they did in spinach. Decanol at the high concentration (0.1%) resulted in the second greatest recovery with 15% above the control. Brij 35, Tween 20, and NaPP each had increasing recovery percentages from low to high concentrations, while Tween 80 and Hexanol had decreasing results (Figures 4.7 and 4.9). Most all additive/concentration combinations showed an increase in recovery over the control except for Tween 80 at the medium and high concentrations, and Decanol at the medium concentration.

Listeria recovery from whole milk was 41% above the control with the use of NaPP at 1%. Decanol and NaPP resulted in increased recoveries from low to high concentration and Tween 20 resulted in decreasing recoveries (Figure 4.8). Only the use of Brij 35 0.1% and NP40 0.01% resulted in recovery percentages less than the control. NP40 and

Hexanol had the greatest effect at the medium concentration level, and Brij 35 and Tween 80 had their lowest results at that level.

Table 4.7 Milk recovery results (n=3) normalized with control (treatment recovery % - control recovery %). *E. coli* recovery averaged 81% and *Listeria* recovery averaged 59%.

E. coli Recovery from Milk			
	Low	Medium	High
Brij 35	6%	7%	12%
NP40	1%	12%	0%
Tween 20	1%	10%	11%
Tween 80	4%	*-1%*	*-2%*
Decanol	3%	*-2%*	15%
Hexanol	17%	10%	8%
Sodium Polyphosphate	3%	5%	8%
Listeria Recovery from Milk			
	Low	Medium	High
Brij 35	11%	*-1%*	20%
NP40	*-7%*	5%	2%
Tween 20	23%	20%	14%
Tween 80	16%	1%	31%
Decanol	5%	9%	13%
Hexanol	15%	17%	11%
Sodium Polyphosphate	7%	13%	41%

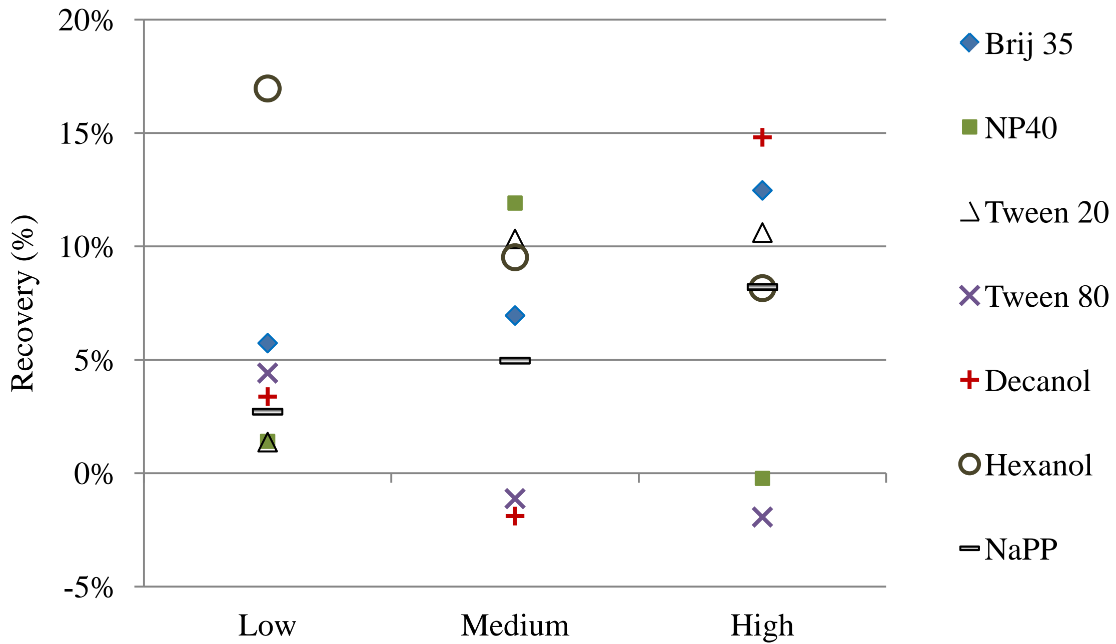


Figure 4.7 Milk results: *E. coli* recovery from the use of all additives at each concentration normalized with control.

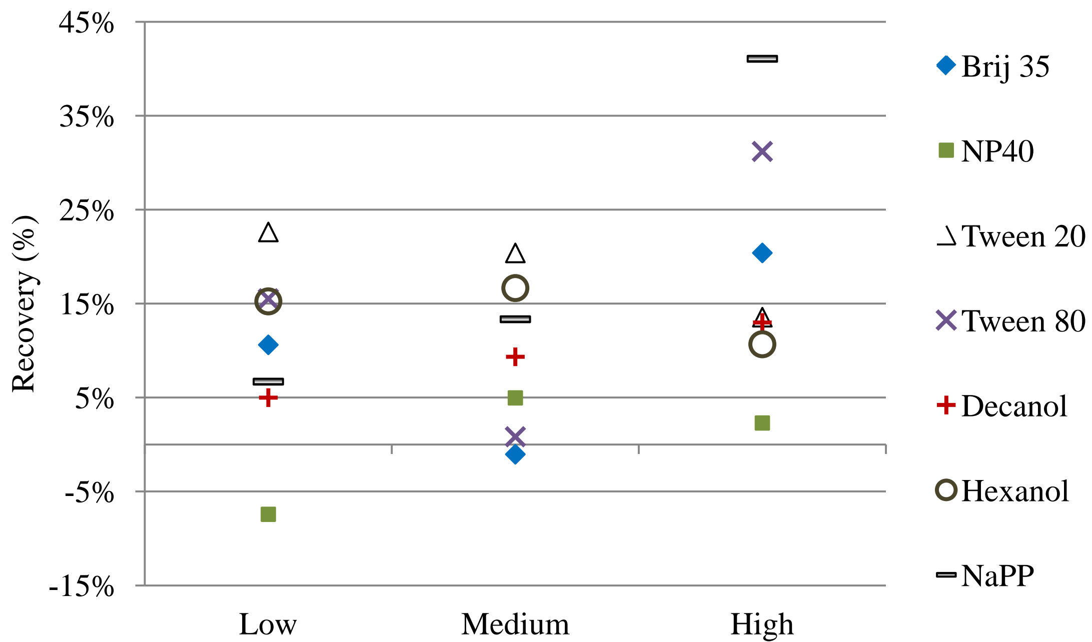


Figure 4.8 Milk results: *Listeria* recovery from the use of all additives at each concentration normalized with control.

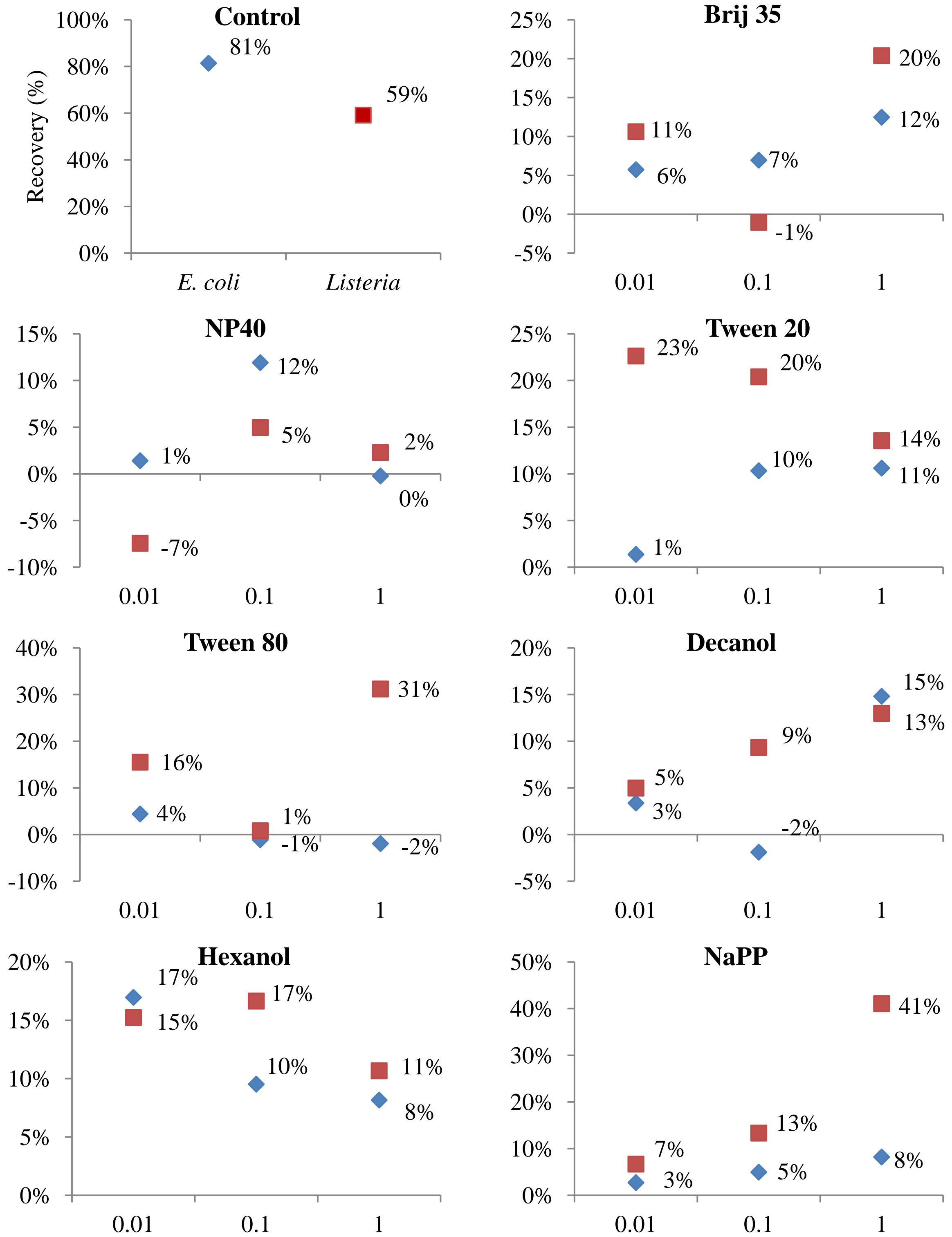


Figure 4.9 Graphs showing the control data from milk for both *E. coli* and *Listeria* and then the effects of each of the additives (from Table 4.7).

The general linear model analysis revealed that bacteria was a significant factor in the model ($p<0.0001$) so the data was divided by bacteria in the model. The ANOVA obtained for *E. coli* revealed that the additive and concentration were not significant in this experiment (Table 4.8). The Dunnett test also showed no significant differences between additives and the control for *E. coli* recovery.

Table 4.8 ANOVA for *E. coli* recovery from milk where the model parameters were additive and concentration.

Source	SS	DF	MS	F	p
Model	0.0854	9	0.0095	0.68	0.7275
Error	0.8009	56	0.0141		
Corrected Total	0.8864	65			

Additive Comparison with Control	Difference Between Means	95% Confidence Limits	
Brij 35	0.08386	-0.10128	0.26901
NP40	0.04363	-0.14151	0.22878
Tween 20	0.07438	-0.11077	0.25952
Tween 80	0.00455	-0.18060	0.18969
Decanol	0.05433	-0.13082	0.23947
Hexanol	0.11549	-0.06965	0.30064
NaPP	0.05295	-0.13220	0.23809

Additive and concentration were, however, significant for *Listeria* recovery from milk (see Table 4.9). No significant differences were found between the control and each additive , so a Tukey test was performed to find any significant difference. It was determined that the use of NaPP at 1% resulted in significantly higher recoveries than from the use of NP40 at 0.01%.

Table 4.9 ANOVA and Dunnett Test results for *Listeria* recovery from milk where the model parameters were additive and concentration.

Source	SS	DF	MS	F	p
Model	0.4465	9	0.0496	2.07	0.0481
Error	1.3690	56	0.0240		
Corrected Total	1.8154	65			

Additive Comparison with Control	Difference Between Means	95% Confidence Limits	
Brij 35	0.09984	-0.14222	0.34189
NP40	0.00065	-0.24271	0.24141
Tween 20	0.18856	-0.05349	0.43062
Tween 80	0.15842	-0.08363	0.40048
Decanol	0.09101	-0.15105	0.33306
Hexanol	0.14183	-0.10023	0.38389
NaPP	0.20357	-0.03848	0.44563

4.2.4. Additive Experiment Discussion

The aim of the additive experiment was to develop a microbial separation protocol that could be used for different food matrices and optimizes the recovery of bacteria. The normalized results obtained from the use of additives for all three food matrices at each concentration for *E. coli* and *Listeria* are presented in Table 4.10. The control data statistics are presented in Appendix C.

Table 4.10 Normalized recovery efficiencies for *E. coli* and *Listeria* from hotdogs, spinach, and whole milk using three classes of additives at three concentrations.

Additive		Mean Recovery ± SD (%)	
		E. coli	*Listeria*
Brij 35	**0.01**	6 ± 6	0 ± 11
	0.1	8 ± 13	-6 ± 9
	1	5 ± 10	-1 ± 18
NP 40	**0.01**	0 ± 12	-16 ± 24
	0.1	5 ± 16	-10 ± 14
	1	5 ± 14	2 ± 11
Tween 20	**0.01**	-2 ± 10	-1 ± 21
	0.1	4 ± 11	0 ± 16
	1	9 ± 9	0 ± 15
Tween 80	**0.01**	9 ± 9	13 ± 16
	0.1	0 ± 12	0 ± 22
	1	-2 ± 10	3 ± 24
Decanol	**0.001**	3 ± 13	-1 ± 17
	0.01	2 ± 11	2 ± 16
	0.1	7 ± 13	-29 ± 41
Hexanol	**0.001**	8 ± 10	-1 ± 17
	0.01	4 ± 8	0 ± 20
	0.1	-18 ± 37	-24 ± 42
NaPP	**0.01**	9 ± 12	2 ± 18
	0.1	5 ± 5	-4 ± 16
	1	6 ± 10	10 ± 23

Based on the protocol followed, the addition of Tween 80 at 0.01% to BPW resulted in a higher recovery percentage, on average, over the use of no additives for hotdogs, spinach, and whole milk for both *E. coli* K12 and *Listeria innocua*. The results of using Tween 80 at 0.01% normalized with the average control values can be seen in Figure 4.10. The average of all 18 results using Tween 80 0.01% was 11.22% above the control values. The 95% confidence interval was calculated to be 11.22% ± 5.79%, which is greater than zero at its lower bound.

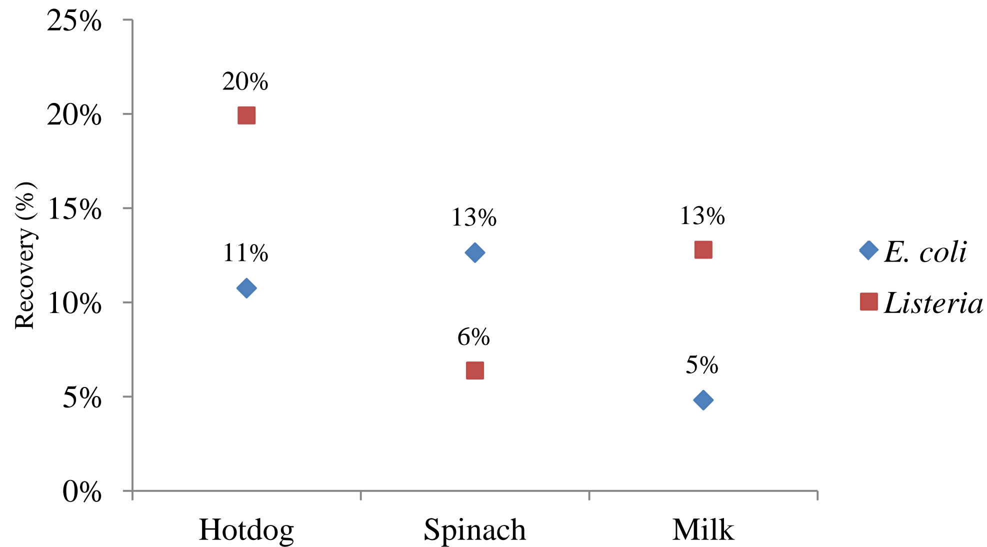


Figure 4.10 Averaged results from the use of Tween 80 0.01% above the control. Tween 80 0.01% resulted in positive recovery values for both bacteria used and with all three food matrices tested.

The use of fatty alcohols could not be found in the literature as having been used as agents for bacterial separation from foods. From this study, it can be concluded that these alcohols at a concentration of 0.1% have a lethal effect on *Listeria* in foods with no fat content. This can be shown by the fact that these alcohols were not lethal to either bacteria on hotdogs or milk, which both have a high fat content, yet were lethal to *Listeria* on spinach, a matrix with no fat. Only Hexanol had a negative effect at 0.1% to *E. coli* cells. Decanol at the same concentration did not result in significantly lower recoveries.

It is also interesting to note that *Listeria* control recoveries were higher than *E. coli* control recoveries with spinach and hotdogs, but the opposite was true with whole milk where *E. coli* control recoveries were higher.

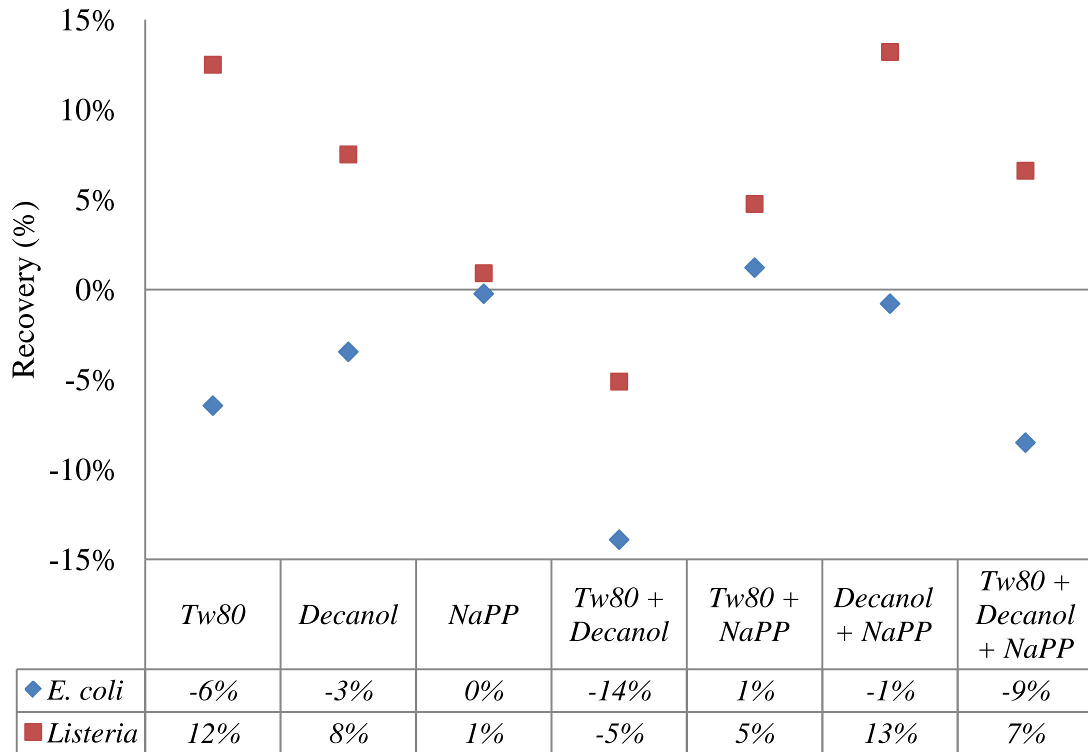
4.3. Additive Combination Experiment Results

Based on the results from 4.2, an additive and concentration combination from each additive class (surfactants, fatty alcohol, chemical dispersant) was chosen based on which one had the highest recovery percentage. Each food matrix was tested using the three

additives chosen from each class individually, and then in combination with one another four times over four days. Eight trials were run each day including one control. When the recovery percentage was calculated, the control's recovery percentage for that day was subtracted from the single additive or additive combination trial values to obtain a normalized value of recovery (above or below the control).

4.3.1. Hotdog Additive Combination Results

The additives tested for hotdogs were Tween 80 0.01%, Decanol 0.001%, and NaPP 1%. Unlike the initial additive experiment, recovery values were higher for *Listeria* than for *E. coli* (see Figure 4.11). On average, the only combination that gave positive recoveries for both *E. coli* and *Listeria* was Tween 80 and NaPP. Considering that these additives and concentrations were chosen based on the initial additive experiment, it is surprising that so many of the combinations resulted in recoveries lower than the control.



	Tw80	Decanol	NaPP	Tw80 + Decanol	Tw80 + NaPP	Decanol + NaPP	Tw80 + Decanol + NaPP
E. coli	-6%	-3%	0%	-14%	1%	-1%	-9%
Listeria	12%	8%	1%	-5%	5%	13%	7%

Figure 4.11 Hotdog additive combination results normalized with the control.

The general linear model analysis of the normalized data revealed that the additive combination was not significant for either *E. coli* or *Listeria* (see Tables 4.11 and 4.12). However, the day on which the data were collected was significant for both ($p<0.0001$ and $p=0.0002$). The day to day variation in results was too great to be able to draw any significant conclusions, therefore, none of the means was statistically significant at a 0.05 level.

Table 4.11 ANOVA and Tukey tables for *E. coli* recovery from the use of single additives and additive combinations.

Source	SS	DF	MS	F	p
Model	0.0447	6	0.0075	0.40	0.8693
Error	0.3896	21	0.0186		
Corrected Total	0.4344	27			

Tukey Grouping	Mean	N	Additive
A	-0.0004	4	Tween 80
A	-0.0815	4	Decanol
A	-0.1475	4	NaPP
A	-0.1766	4	Tw80 + Dec
A	-0.109	4	Tw80 + NaPP
A	-0.0245	4	Dec + NaPP
A	-0.0906	4	Tw80 + Dec + NaPP

(Additives with the same Tukey grouping letter are not statistically different.)

Table 4.12 ANOVA and Tukey tables for *Listeria* recovery from the use of single additives and additive combinations.

Source	SS	DF	MS	F	p
Model	0.0942	6	0.0157	0.18	0.9798
Error	1.8503	21	0.0881		
Corrected Total	1.9445	27			

Tukey Grouping	Mean	N	Additive
A	-0.0004	4	Tween 80
A	-0.0815	4	Decanol
A	-0.1475	4	NaPP
A	-0.1766	4	Tw80 + Dec
A	-0.109	4	Tw80 + NaPP
A	-0.0245	4	Dec + NaPP
A	-0.0906	4	Tw80 + Dec + NaPP

(Additives with the same Tukey grouping letter are not statistically different.)

A dry matter analysis was also conducted for this part of experimentation. The data were entered into SAS and analyzed using a general linear model (Table 4.14). It was determined that the additive was significant and from the Dunnett test it could be seen

that NaPP and any combination with NaPP 1% resulted in a significantly higher dry matter content when compared to the control.

Table 4.13 ANOVA table and Dunnett Test results for hotdog additive combination dry matter content. Each sample contains 2% dry matter from BPW.

Source	SS	DF	MS	F	p
Model	0.00052139	7	0.00007448	36.06	<0.0001
Error	0.00003305	16	0.00000207		
Corrected Total	0.00055444	23			

Additive	% Dry Matter	
Control	2.885%	
Tween 80 0.01%	2.957%	
Decanol 0.001%	2.796%	
NaPP 1%	3.898%	*
Tween 80 + Decanol	2.930%	
Tween 80 + NaPP	3.793%	*
Decanol + NaPP	3.667%	*
Tween 80 + Decanol + NaPP	3.886%	*

* denotes statistical differences at a 0.05 level when compared with the control.

4.3.2. Spinach Additive Combination Results

The additives tested for spinach were Tween 80 0.1%, Hexanol 0.001%, and NaPP 0.01%. The results obtained after recovery percentages from the control trials were subtracted from the recovery percentages of the additive and additive combination trials, and then averaged (Figure 4.12). On average, the only combination that gave positive results was Hexanol and SP.

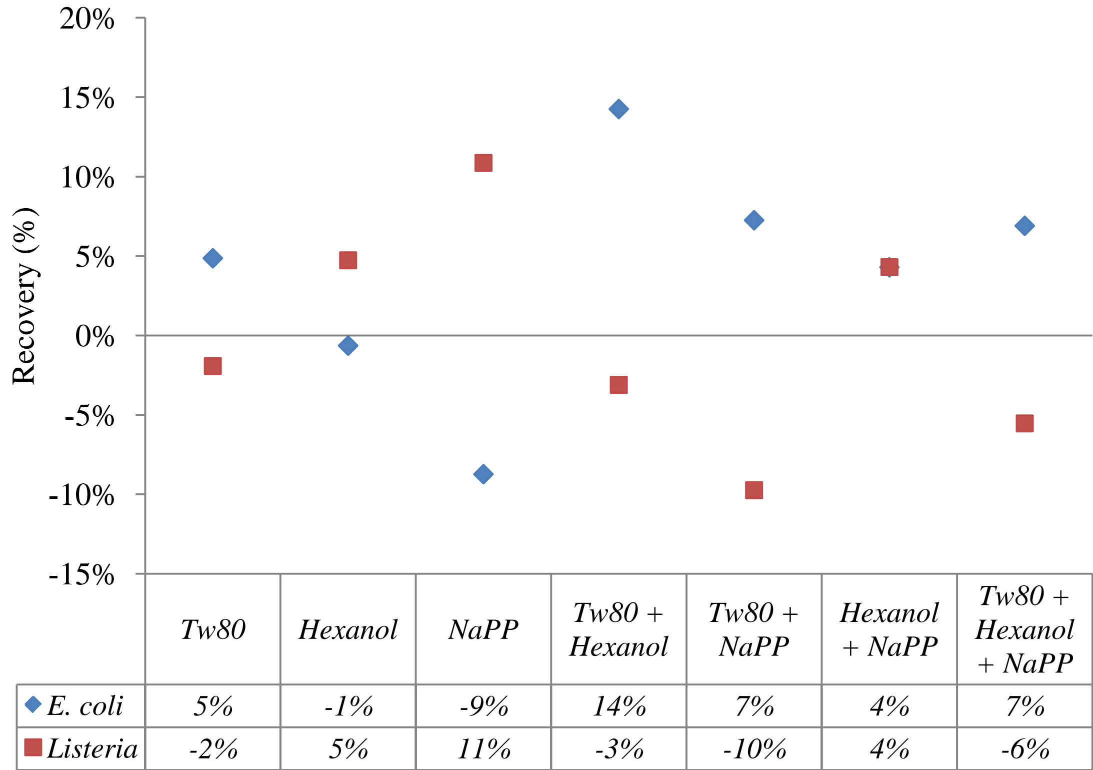


Figure 4.12 Spinach additive combination results normalized with the control and averaged from four days.

Data values were entered into SAS and the additive combinations were analyzed using a general linear model. The additive combination was not significant for either *E. coli* or *Listeria* (Tables 4.15 and 4.16), and the day was not significant (p=0.0627 and p=0.3544). Based on the Tukey test, there were no significant differences between means.

Table 4.14 ANOVA and Tukey test tables from additive combination experiment's *E. coli* recovery from spinach.

Source	SS	DF	MS	F	p
Model	0.1235	6	0.0206	1.33	0.2893
Error	0.3259	21	0.0155		
Corrected Total	0.4493	27			

Tukey Grouping	Mean	N	Additive
A	0.04858	4	Tween 80
A	-0.0065	4	Decanol
A	-0.0874	4	NaPP
A	0.14253	4	Tw80 + Dec
A	0.07252	4	Tw80 + NaPP
A	0.0429	4	Dec + NaPP
A	0.06898	4	Tw80 + Dec + NaPP

Table 4.15 ANOVA and Tukey test tables from additive combination experiment's *Listeria* recovery from spinach.

Source	SS	DF	MS	F	p
Model	0.0872	6	0.0145	0.73	0.6333
Error	0.4203	21	0.02		
Corrected Total	0.5075	27			

Tukey Grouping	Mean	N	Additive
A	0.0257	4	Tween 80
A	0.1216	4	Decanol
A	0.1817	4	NaPP
A	0.0370	4	Tw80 + Dec
A	0.1018	4	Tw80 + NaPP
A	0.1024	4	Dec + NaPP
A	0.0163	4	Tw80 + Dec + NaPP

The averaged percent dry matter from spinach samples is shown in Table 4.16. Dunnett Test results reveal no statistical differences between any of the additives/combinations compared with the control.

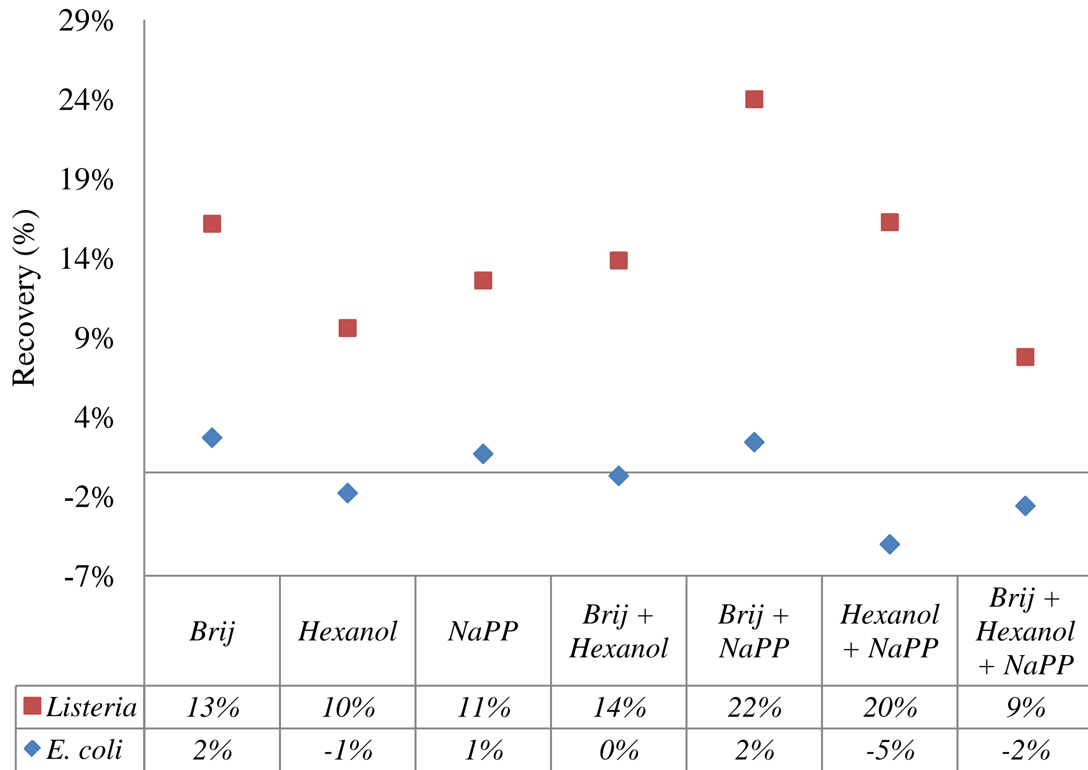
Table 4.16 ANOVA and table of dry matter content of spinach samples after low speed centrifugation step. Each sample contains 2% dry matter from BPW.

Source	SS	DF	MS	F	p
Model	1.79E-06	7	2.56E-07	0.77	0.6255
Error	2.64E-06	8	3.30E-07		
Corrected Total	4.43E-06	15			

Additive	Dry Matter
Control	2.160%
Tween 80 0.1%	2.196%
Hexanol 0.001%	2.175%
NaPP 0.01%	2.236%
Tween 80 + Hexanol	2.168%
Tween 80 + NaPP	2.245%
Hexanol + NaPP	2.247%
Tween 80 + Hexanol + NaPP	2.186%

4.3.3. Milk Additive Combination Results

The additives tested for milk were Brij 1%, Hexanol 0.001%, and NaPP 1%. Figure 4.13 shows the results obtained after recovery percentages from the control trials were subtracted from the additive and additive combination trials' recovery percentages and then averaged. On average, the use of Brij by itself, NaPP by itself, and Brij and NaPP used together resulted in positive recovery percentages for both *E. coli* and *Listeria*.



	Brij	Hexanol	NaPP	Brij + Hexanol	Brij + NaPP	Hexanol + NaPP	Brij + Hexanol + NaPP
Listeria	*13%*	*10%*	*11%*	*14%*	*22%*	*20%*	*9%*
E. coli	*2%*	*-1%*	*1%*	*0%*	*2%*	*-5%*	*-2%*

Figure 4.13 Milk additive combination results normalized with the control and averaged from four days.

Data values were entered into SAS and analyzed using a general linear model. The additive combination was not significant for either *E. coli* or *Listeria* (Tables 4.17 and 4.18) but the day the data were collected was significant ($p<0.0001$ and $p=0.0013$). From the Dunnett Test results, no combination was significantly different than the control.

Table 4.17 ANOVA and Tukey tables for *E. coli* recovery from the milk additive combination experiment.

Source	SS	DF	MS	F	p
Model	0.0141	6	0.0023	0.04	0.9997
Error	1.2464	21	0.0594		
Corrected Total	1.2605	27			

Tukey Grouping	Mean	N	Additive
A	-0.0583	4	Tween 80
A	-0.0931	4	Decanol
A	-0.0685	4	NaPP
A	-0.0823	4	Tw80 + Dec
A	-0.0611	4	Tw80 + NaPP
A	-0.1254	4	Dec + NaPP
A	-0.1012	4	Tw80 + Dec + NaPP

Table 4.18 ANOVA and Tukey tables for *Listeria* recovery from the milk additive combination experiment.

Source	SS	DF	MS	F	p
Model	0.0564	6	0.0094	0.14	0.9894
Error	1.4233	21	0.0678		
Corrected Total	1.4797	27			

Tukey Grouping	Mean	N	Additive
A	-0.0273	4	Tween 80
A	-0.0582	4	Decanol
A	-0.0529	4	NaPP
A	-0.0265	4	Tw80 + Dec
A	0.05385	4	Tw80 + NaPP
A	0.04073	4	Dec + NaPP
A	-0.0683	4	Tw80 + Dec + NaPP

The results of the percent dry matter of milk samples after they underwent the low-speed centrifugation step are presented in Table 4.19. A Dunnett Test was conducted on the

data collected, and just like with hotdog samples, the statistical differences resulted from the use of NaPP at 1%.

Table 4.19 ANOVA and table of dry matter content of milk samples after low speed centrifugation step. Each sample contains 2% dry matter from BPW.

Source	SS	DF	MS	F	p
Model	0.00021894	7	0.00003128	74.16	<0.0001
Error	0.00000675	16	0.00000042		
Corrected Total	0.00022569	23			

Additive	% Dry Matter
Control	6.05%
Brij 1%	6.03%
Hexanol 0.001%	6.06%
NaPP 1%	6.69% *
Brij + Hexanol	5.96%
Brij + NaPP	6.61% *
Hexanol + NaPP	6.68% *
Brij + Hexanol + NaPP	6.48% *

* denotes a significant difference compared to the control at a level of 0.05.

4.3.4. Additive Combination Experiment Discussion

The goal of this experiment was to determine if recovery percentages were higher from the combination of two or more additives. Although statistically no differences could be found, it was determined that the surfactant and chemical dispersant combination worked the best for both bacteria in hotdog and milk samples (Figures 4.11 and 4.13). For spinach, the fatty alcohol and chemical dispersant worked the best (Figure 4.12). From the milk data, it can be seen that the two individual additives, Brij and NaPP, and the combination of those two provided positive recovery percentages when compared with the control data for both *E. coli* and *Listeria*.

Although NaPP at 1% aided in higher recovery percentages when used in hotdogs and whole milk samples, these samples also resulted in significantly higher dry matter content when compared to the control value.

Up to this point, two different experimental plans have been used. In the singular additive experiment, the data for each replication for the additive and concentration combinations took different periods of time to collect, from three to five days due to varying circumstances (contaminated samples or running out of materials needed, for example). Also, a control was not taken for each day that experiments were run. In contrast, the additive combination experiment was set up differently such that one replication could be done in one day, and each replication included a control. Due to the above mentioned factors, and from the statistical analyses performed, it was determined that the day and replication were significant for most of the data collected. A protocol was thus developed to eliminate any day to day variability based on differences in bacteria or food samples.

4.4. **TWO ADDITIVE COMBINATION EXPERIMENTS**

Two additional experiments were designed to compare the combinations of two additives and perform the experiment in one day. This eliminated any variation in data due to time. Three replications with no additives would be compared to three replications with additives for each of the three food matrices. Dry matter content was also taken for each replication.

4.4.1. Tween 80 0.01% and NaPP 0.1%

Tween 80 0.01% and NaPP 0.1% were tested in combination on the first day. These were chosen to be used because of the success of Tween 80 0.01% from the initial additive experiment, and because of the effect NaPP had on both the surfactants and fatty alcohol from the additive combination experiment. However, the concentration was chosen to be 0.1% because the addition of NaPP at 1% significantly increased the dry matter content in hotdog and milk samples, and the goal of the developed protocol was to increase bacterial recovery while lowering overall dry matter content.

The results showed that the use of the additives resulted in recovery percentages that were significantly less than the control recovery for *E. coli* on hotdogs (Table 4.20 and 4.21, Figure 4.14). There were no statistical differences for the recovery of *E. coli* from spinach or milk or between the additives and controls for *Listeria* recovery from any of

the food matrices (Table 4.22), even though the average of the recovery means was higher from the use of the additives over the control.

Table 4.20 Averaged recovery percentages for controls and additives (Tween 80 0.01% plus NaPP 0.1%) trials for each bacteria and food matrix, plus or minus the standard deviation.

	Hotdog		Spinach		Milk	
	Control	Additives	Control	Additives	Control	Additives
E. coli	94 ± 1	76 ± 3 *	84 ± 13	97 ± 13	93 ± 4	102 ± 8
Listeria	53 ± 8	59 ± 9	103 ± 17	110 ± 29	95 ± 7	98 ± 13

Statistical differences at a 0.05 level are denoted by *.

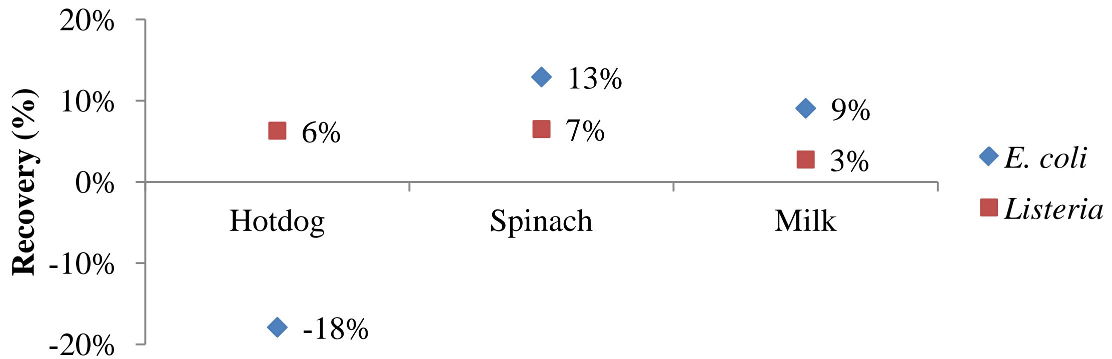


Figure 4.14 Results from Table 4.20 represented graphically where *Listeria* and *E. coli* values are normalized with control values.

Table 4.21 ANOVA and Dunnett Test tables for *E. coli* recovery hotdog samples from controls and use of additives NaPP and Tween 80.

HOTDOG	SS	DF	MS	F	p
Model	0.0481	1	0.0481	126.3900	0.0004
Error	0.0015	4	0.0004		
Corrected Total	0.0496	5			

Additives Compared to Control	Difference Between Means	95% Confidence Limits		
Tween 80 0.01% + NaPP 0.1%	-0.17898	-0.2232	-0.13478	*

Table 4.22 ANOVAs for *E. coli* recovery from spinach and milk, comparing Tween 80 0.01% and Hexanol NaPP 0.1% to the control.

SPINACH	SS	DF	MS	F	p
Model	0.0251	1	0.0251	1.5	0.2876
Error	0.6670	4	0.0167		
Corrected Total	0.0918	5			

MILK	SS	DF	MS	F	p
Model	0.0123	1	0.0123	3.23	0.1469
Error	0.0153	4	0.0038		
Corrected Total	0.0276	5			

Table 4.23 ANOVAs for *Listeria* recovery from hotdogs, spinach, and milk, comparing Tween 80 0.01% and NaPP 0.1% to the control.

HOTDOG	SS	DF	MS	F	p
Model	0.0060	1	0.0060	0.93	0.3902
Error	0.0258	4	0.0064		
Corrected Total	0.0318	5			

SPINACH	SS	DF	MS	F	p
Model	0.0063	1	0.0063	0.11	0.7541
Error	0.2259	4	0.0565		
Corrected Total	0.2322	5			

MILK	SS	DF	MS	F	p
Model	0.0011	1	0.0011	0.10	0.7656
Error	0.0449	4	0.0112		
Corrected Total	0.0460	5			

Dry matter data is presented in Table 4.24. The use of Tween 80 0.01% and NaPP 0.1% resulted in statistically higher dry matter content when compared with the control. Based on this data, it appears that NaPP at 0.1%, like at 1%, significantly increased the dry matter content of samples.

Table 4.24 Averaged percent dry matter for controls and additives (Tween 80 0.01% and NaPP 0.1%) samples after low-speed centrifugation step.

	Hotdog	Spinach	Milk
Control	3.155%	2.398%	5.878%
Additives	3.583% *	2.576% *	6.066% *

* denotes a statistical difference compared to the control at a 0.05 level.

4.4.2. Tween 80 0.01% and Hexanol 0.001%

The last combination to be tested was that of Tween 80 0.01% and Hexanol 0.001%. It had previously been determined that the use of fatty alcohols slightly lowered the overall dry matter content in centrifuged samples when compared to the control, so the goal of this experiment was to see if this combination would maximize recovery and decrease the dry matter as well.

From the data collected, no significant differences between recovery percentages were found (Table 4.25 and 4.26). Despite this, it was still observed that the recovery means from the use of additives were greater than the control means (Table 4.25).

Table 4.25 Averaged recovery percentages for controls and additives (Tween 80 0.01% plus Hexanol 0.001%) trials, plus or minus the standard deviation.

	Hotdog		Spinach		Milk	
	Control	Additives	Control	Additives	Control	Additives
E. coli	53 ± 9	56 ± 8	62 ± 2	69 ± 8	82 ± 4	84 ± 6
Listeria	78 ± 12	87 ± 9	86 ± 8	95 ± 9	71 ± 7	74 ± 8

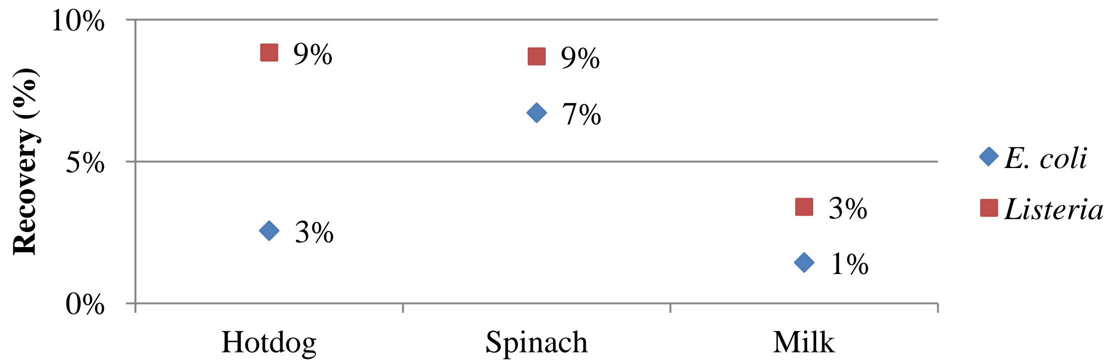


Figure 4.15 Data from Table 4.25 represented graphically where results are normalized with control values.

Table 4.26 ANOVAs for *E. coli* recovery from hotdogs, spinach, and milk, comparing Tween 80 0.01% and Hexanol 0.001% to the control.

HOTDOG	SS	DF	MS	F	p
Model	0.0010	1	0.0010	0.13	0.7398
Error	0.0310	4	0.0078		
Corrected Total	0.0320	5			

SPINACH	SS	DF	MS	F	p
Model	0.0068	1	0.0068	2.15	0.2161
Error	0.0126	4	0.0031		
Corrected Total	0.0194	5			

MILK	SS	DF	MS	F	p
Model	0.0003	1	0.0003	0.12	0.7496
Error	0.0106	4	0.0027		
Corrected Total	0.0110	5			

Table 4.27 ANOVAs for *Listeria* recovery from hotdogs, spinach, and milk, comparing Tween 80 0.01% and Hexanol 0.001% to the control.

HOTDOG	SS	DF	MS	F	p
Model	0.0117	1	0.0117	1.03	0.3669
Error	0.0454	4	0.0114		
Corrected Total	0.0571	5			

SPINACH	SS	DF	MS	F	p
Model	0.0114	1	0.0114	1.48	0.2906
Error	0.0307	4	0.0077		
Corrected Total	0.0421	5			

MILK	SS	DF	MS	F	p
Model	0.0011	1	0.0011	0.19	0.689
Error	0.0240	4	0.0060		
Corrected Total	0.0251	5			

The dry matter data was also analyzed and no significant differences were found among any of the food matrices between the control samples and those where the additives had been used (Table 4.28).

Table 4.28 Averaged percent dry matter for controls and additives (Tween 80 0.01% and Hexanol 0.001%) samples after low-speed centrifugation step.

	Hotdog	Spinach	Milk
Control	5.994%	2.477%	3.141%
Additives	5.995%	2.494%	3.186%

4.4.3. Discussion of Final Experiments

The only statistical differences that were found during this part of experimentation were from the additives Tween 80 0.01% and NaPP 0.1%. It was determined that their use significantly lowered the recovery of bacteria from hotdog samples, and significantly increased the dry matter content for all three food matrices (hotdogs, spinach, and milk). However, the use of Tween 80 0.01% with Hexanol 0.001% resulted in recoveries that were greater than the control values. Even though the results were not statistically

significant, the combination of Tween 80 and Hexanol would benefit microbial recovery for each of the food matrices examined. The use of NaPP is not recommended due to increased dry matter content.

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CHAPTER 5. **CONCLUSIONS**

Based on all data collected, a protocol can be recommended to optimize the recovery of bacteria from various food matrices. A general protocol was successfully developed and tested that resulted in a high microbial recovery rate using Buffered Peptone Water as the diluting agent. Solid foods can be sampled in Buffered Peptone Water at a ratio of 1:9 (food to buffer). Food and buffer samples can then be homogenized in a Waring blender for two minutes. The homogenized sample should then undergo a low speed centrifugation (2000 rcf) for three minutes. After centrifugation, the supernatant can be poured off and sampled by a plate count or another detection method. The data collected for this research shows that BPW alone, without the need for additives, is sufficient to aid in the separation of bacteria from food surfaces.

A sampling experimental procedure can be recommended for the testing of different buffer solutions and additives and their effects on microbial separation and recovery. Due to the high day to day variability encountered in this research, a method was proposed to limit the variability and error present in the microbiological testing of foods. If meaningful significant differences are to be found between control samples and treatment samples, they must all come from the same batch of inoculated homogenized slurry. Then, the additives or buffer being investigated can be added to achieve the proper concentration and then further processed. At least three trials should be run for each treatment, and all trials should be completed in one day.

Of the additives tested, the surfactant Tween 80 was the most effective for the recovery of both *E. coli* and *Listeria*. Fatty alcohols, Decanol and Hexanol, were found to be effective for separation and for lowering dry matter content of samples, however, at a concentration of 0.1% they significantly lowered the bacterial recovery. The most effective concentration was found to be 0.001%.

NaPP was found to be effective at aiding in microbial recovery. However, at 0.1 and 1%, it significantly altered the dry matter content of samples, and thus is not recommended for use at those concentrations.

Various combinations of additives were tested to determine if there was a synergistic effect of using more than one additive in BPW. The results varied, however, one combination was found that resulted in higher recoveries compared to the control each time the experiment was conducted: Tween 80 0.01% and Hexanol 0.001%. This combination worked well for all three foods examined and also kept the dry matter content very close to that of the controls'.

In conclusion, homogenizing food samples in Buffered Peptone Water was found to be very effective in the separation of microorganisms from various food matrices. Chemical additives were found to aid in the recovery, however, the microbial extraction enhancement from the use of chemical additives was not uniformly measured over different conditions and thus, for a general protocol, only Buffered Peptone Water is recommended as a diluting agent. Future work should examine more closely the use of surfactants and fatty alcohols in combination as microbial separation agents.

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APPENDIX A. SUPPORTING INFORMATION

To efficiently address the objectives laid out in the introduction, screening experiments were conducted first to select between centrifugation and/or filtration methodologies and also to screen additives for selection of appropriate additives and their concentration levels for further testing. Screening experiments were also useful in developing effective sampling procedures.

A.1. SAMPLING AND CONTAMINATION

Based on traditional microbiological testing of foods, it was determined that for solid food matrices a ratio of 1:9 would be used (sample to buffer ratio) (Andrews et al., 2003). Spinach and hotdog were spot inoculated as was determined to be the best method for contamination based on inoculation procedures outlined by the FDA (USDA, 2001). Foods were inoculated in two places with 10 µl of bacteria in broth and left to sit for 5 min based on previous experimentation that showed that there is no significant difference between foods left sitting for 5 min versus 24 h in the refrigerator. For ease of experimentation and time constraints, 5 min was decided upon.

A.2. BUFFER SOLUTION

Different buffer solutions were used in an initial experiment and compared to DI water to determine which resulted in the highest recovery efficiencies. Buffered Peptone Water (BPW), Phosphate Buffered Saline (PBS), and Phosphate Buffer with $MgCl_2$ (PB $MgCl_2$) were tested based on the literature review conducted. Fukushima et al. (2007) used BPW for the preparation of a variety of inoculated food samples, Brewster (2008) used PBS for the preparation of inoculated ground beef samples, and the *Standard Methods for the Examination of Dairy Products* (1992) recommends the use of Phosphate Buffer with $MgCl_2$ to aid in the recovery of organisms that may be metabolically injured. Initial tests showed that there were no significant differences ($p>0.05$) in the recovery obtained by using the three mentioned buffer solutions, but the average recovery percentage was slightly higher with the use of BPW, so it was decided to use BPW for the remainder of experiments (Table A.1).

Table A.1 Recovery percentages for *E. coli* in four diluting agents.

Buffer Solution and Recovery %			
PBS	PB $MgCl_2$	BPW	DI Water
65%	55%	73%	65%
54%	60%	65%	60%
60%	61%	71%	59%

After the buffer was chosen, different pH values of the buffer were tested to determine the optimum pH. Values tested were 5, 7, and 9. The pH of the buffer was altered before hotdog samples were blended. Plate counts were taken of the blended sample and the supernatant from the low-speed centrifugation sample. Results showed that pH of around 7.0 would be the best pH for microbial recovery (Table A.2). The ANOVA results show a significant difference between the pH values (Table A.3). The pH of 9.0 resulted in significantly less microbial recovery than the pHs of 5.0 and 7.0. For ease of experimentation, the pH 7.0 was chosen since BPW is naturally at that pH value.

Table A.2 Recovery of *E. coli* from BPW at three different pH values.

	Recovery (%)	
pH	**Blended**	**Supernatant**
4.70	81%	68%
4.76	83%	37%
4.90	85%	39%
6.91	89%	69%
6.83	83%	55%
6.85	83%	43%
8.92	67%	18%
8.92	81%	19%
8.93	59%	10%

Table A.3 ANOVA table for pH of BPW results.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	196.15	1	196.15	128.79	4.6E-09	4.49
Within Groups	24.37	16	1.523			
Total	220.52	17				

A.3. Filtration versus Centrifugation

The addition of surfactants changes the filterability of certain foods. Experiments were conducted to see how certain concentrations of surfactant affected filtration through the VWR 417 filter (40 µm retention). Surfactant concentrations used were 1% or 0.1%. Filtration of homogenized hotdogs could not be achieved for all concentrations of surfactant used through the VWR filter. Because at least two steps of filtration would have to be used, it was decided to move on to centrifugation so as to not risk reduced microbial recovery due to filtration problems.

Low-speed centrifugation was then implemented to settle out the larger food particles. Based on research by Fukushima et. al (2007), 2000 rcf was the speed used for 3 min to achieve initial separation.

A.4. Additives

For the first experiment, additives were tested individually: four surfactants, two heavy alcohols, and one chemical dispersant. Concentrations of 0.01, 0.1, and 1% were used for surfactants and the chemical dispersant based on preliminary testing and on previously published research (Fukushima 2007; Hill 2005; Stevens 2004).

Decanol was tested to see at what concentration it would become lethal to *E. coli*. These preliminary experiments determined that concentrations of 0.1% showed loss in vitality compared to the control (no fatty alcohol) after 60 min, while a concentration of 0.01% showed no difference compared to the control. However, for the first 30 min there was no loss in vitality at any of the concentrations (Table A.4). Therefore, concentrations of 0.001, 0.01, and 0.1% were selected for this study since the bacteria would not be in contact with the heavy alcohol for more than 30 min.

Table A.4 Microbial counts for *E. coli* in Decanol at varying concentrations.

	Time		
Treatment	Initial	15 min	30 min
Control	1.33E+04	-	1.50E+04
Decanol 0.001%	1.01E+04	1.34E+04	1.41E+04
Decanol 0.01%	1.38E+04	1.76E+04	1.68E+04
Decanol 0.1%	1.16E+04	1.25E+04	1.40E+04

Milk was treated differently since it is a liquid matrix. Sample to buffer ratio was decided to be 2:3 which resulted in a solids content of nearly 5%, similar to that of hotdogs and spinach in buffer. A volume of 10 µl of bacteria in broth was injected directly into the milk and allowed to sit for 5 min before further processing to be consistent with the protocol developed for solid food matrices.

APPENDIX B. RAW DATA

Table B.1 Raw data from hotdog additive experiment, including recovery percentages for *E. coli* and *Listeria* from blending and centrifugation steps.

Hotdog microbial recovery from Blending (Step 1), Centrifugation (Step 2)		*E. coli* Recovery		*Listeria* Recovery	
Additive	Concentration (%)	Step 1	Step 2	Step 1	Step 2
Brij 35	0.01	89%	53%	105%	70%
		104%	47%	86%	66%
		80%	60%	77%	60%
	0.1	98%	66%	89%	70%
		62%	44%	76%	51%
		64%	45%	68%	61%
	1	52%	40%	82%	67%
		79%	43%	88%	65%
		72%	41%	66%	50%
NP40	0.01	80%	53%	105%	73%
		88%	71%	82%	56%
		54%	31%	72%	54%
	0.1	63%	33%	89%	51%
		71%	47%	91%	62%
		60%	28%	65%	38%
	1	94%	48%	98%	101%
		67%	57%	88%	72%
		93%	56%	86%	68%
Tween 20	0.01	96%	57%	97%	79%
		82%	57%	113%	64%
		70%	34%	78%	46%
	0.1	93%	67%	97%	79%
		85%	50%	106%	64%
		84%	43%	93%	66%
	1	73%	55%	90%	83%
		114%	64%	91%	66%
		84%	60%	84%	58%
Tween 80	0.01	59%	48%	90%	87%
		64%	66%	92%	72%
		104%	61%	98%	90%
	0.1	86%	30%	88%	66%
		99%	60%	113%	66%
		67%	28%	73%	39%
	1	84%	53%	128%	91%

		51%	35%	71%	51%
		70%	31%	82%	46%
Decanol	0.001	107%	73%	109%	93%
		96%	46%	123%	71%
		103%	54%	126%	93%
	0.01	65%	59%	119%	91%
		86%	63%	99%	66%
		93%	60%	134%	81%
	0.1	94%	69%	86%	70%
		50%	36%	53%	36%
		104%	48%	71%	53%
Hexanol	0.001	118%	55%	101%	84%
		88%	45%	90%	72%
		88%	53%	93%	63%
	0.01	49%	43%	117%	89%
		79%	50%	100%	74%
		105%	52%	118%	73%
	0.1	55%	45%	94%	61%
		104%	57%	127%	85%
		122%	53%	130%	63%
Sodium Polyphosphate	0.01	118%	77%	126%	81%
		61%	39%	61%	43%
		92%	63%	87%	72%
	0.1	80%	52%	84%	68%
		87%	56%	102%	59%
		96%	57%	107%	64%
	1	83%	50%	82%	73%
		86%	63%	75%	58%
		97%	65%	95%	87%
Control		59%	45%	97%	80%
		93%	53%	94%	72%
		84%	56%	88%	74%

Table B.2 Raw data from spinach additive experiment, including recovery percentages for *E. coli* and *Listeria* from blending and centrifugation steps.

Spinach microbial recovery from Blending (Step 1), Centrifugation (Step 2)		*E. coli* Recovery		*Listeria* Recovery	
Additive	Concentration (%)	Step 1	Step 2	Step 1	Step 2
Brij 35	0.01	87%	69%	98%	95%
		91%	83%	99%	84%
		100%	69%	118%	72%
	0.1	84%	83%	100%	75%
		95%	67%	124%	80%
		104%	88%	139%	88%
	1	98%	75%	92%	77%
		99%	76%	88%	77%
		119%	77%	128%	68%
NP40	0.01	90%	57%	131%	84%
		100%	56%	123%	78%
		147%	68%	115%	84%
	0.1	111%	87%	117%	84%
		94%	77%	121%	69%
		101%	77%	115	71%
	1	89%	63%	128%	85%
		117%	87%	155%	88%
		85%	75%	135%	80%
Tween 20	0.01	74%	58%	145%	59%
		123%	60%	125%	71%
		134%	58%	133%	60%
	0.1	81%	58%	100%	69%
		91%	67%	95%	75%
		95%	62%	98%	62%
	1	108%	77%	105%	82%
		105%	78%	107%	84%
		100%	56%	90%	60%
Tween 80	0.01	97%	80%	107%	83%
		106%	70%	130%	82%
		108%	79%	105%	105%
	0.1	95%	70%	119%	113%
		90%	79%	160%	72%
		91%	80%	90%	118%
	1	108%	83%	154%	89%
		80%	61%	130%	68%
		91%	65%	125%	70%

Decanol	0.001	81%	49%	88%	73%
		88%	68%	113%	65%
		90%	71%	95%	60%
	0.01	81%	62%	78%	83%
		90%	47%	18%	66%
		103%	70%	95%	77%
	0.1	74%	68%	23%	6%
		75%	68%	16%	8%
		82%	74%	0%	0%
Hexanol	0.001	87%	82%	97%	81%
		100%	61%	113%	62%
		100%	66%	117%	59%
	0.01	82%	71%	104%	78%
		93%	64%	102%	57%
		113%	69%	100%	56%
	0.1	0%	0%	0%	0%
		0%	0%	0%	0%
		0%	0%	0%	0%
Sodium Polyphosphate	0.01	92%	73%	103%	81%
		93%	77%	147%	70%
		126%	85%	122%	103%
	0.1	96%	69%	103%	80%
		103%	78%	117%	84%
		113%	62%	88%	51%
	1	89%	63%	114%	86%
		103%	56%	95%	69%
		115%	75%	97%	74%
Control		99%	73%	99%	81%
		107%	70%	118%	79%
		109%	58%	119%	91%

Table B.3 Raw data from milk additive experiment, including recovery percentages for *E. coli* and *Listeria* from blending and centrifugation steps.

Milk microbial recovery from Blending (Step 1), Centrifugation (Step 2)		*E. coli* Recovery		*Listeria* Recovery	
Additive	Concentration (%)	Step 1	Step 2	Step 1	Step 2
Brij 35	0.01	92%	86%	59%	81%
		103%	83%	109%	57%
		113%	93%	98%	71%
	0.1	62%	69%	54%	49%
		78%	109%	73%	62%
		118%	87%	80%	64%
	1	84%	88%	90%	77%
		105%	104%	104%	62%
		107%	89%	120%	99%
NP40	0.01	93%	81%	91%	58%
		93%	82%	102%	46%
		100%	85%	59%	51%
	0.1	96%	80%	84%	76%
		105%	114%	80%	52%
		105%	86%	76%	65%
	1	75%	61%	64%	54%
		93%	108%	87%	56%
		105%	75%	90%	74%
Tween 20	0.01	73%	70%	64%	59%
		93%	89%	90%	83%
		108%	89%	110%	103%
	0.1	82%	92%	114%	79%
		107%	80%	100%	89%
		118%	102%	105%	70%
	1	83%	95%	95%	87%
		90%	81%	74%	57%
		98%	100%	88%	74%
Tween 80	0.01	82%	79%	90%	79%
		98%	96%	97%	64%
		105%	82%	95%	81%
	0.1	83%	78%	75%	58%
		95%	79%	104%	73%
		106%	84%	53%	50%
	1	88%	75%	115%	93%
		96%	79%	100%	84%
		97%	84%	117%	94%

Decanol	0.001	75%	68%	59%	58%
		91%	88%	115%	80%
		109%	98%	52%	54%
	0.01	82%	76%	73%	61%
		84%	80%	95%	96%
		108%	83%	51%	48%
	0.1	83%	83%	98%	88%
		93%	112%	60%	55%
		109%	93%	80%	74%
Hexanol	0.001	95%	92%	91%	88%
		101%	104%	67%	55%
		108%	99%	88%	79%
	0.01	94%	92%	102%	96%
		95%	103%	98%	57%
		106%	78%	95%	74%
	0.1	89%	79%	102%	64%
		97%	100%	101%	57%
		112%	90%	99%	88%
Sodium Polyphosphate	0.01	92%	78%	70%	61%
		97%	81%	104%	89%
		110%	93%	49%	48%
	0.1	91%	83%	88%	85%
		94%	83%	74%	58%
		107%	93%	78%	74%
	1	94%	93%	97%	96%
		98%	80%	98%	105%
		100%	96%	108%	100%
Control		100%	88%	90%	88%
		92%	72%	95%	47%
		110%	106%	96%	48%

Table B.4 Raw data from hotdog additive combination experiment. Recovery percentages for both *E. coli* and *Listeria* are from the supernatant after the low-speed centrifugation step.

	Recovery	
Additive / Combination	*E. coli*	*Listeria*
Control	45%	43%
	51%	62%
	68%	43%
	61%	55%
Tween 80 0.01%	50%	71%
	62%	62%
	40%	63%
	47%	57%
Decanol 0.001%	46%	58%
	54%	64%
	53%	78%
	58%	33%
NaPP 1%	61%	57%
	72%	27%
	39%	83%
	52%	40%
Tween 80 + Decanol	31%	46%
	52%	45%
	45%	41%
	41%	51%
Tween 80 + NaPP	57%	67%
	59%	58%
	64%	48%
	50%	49%
Decanol + NaPP	46%	61%
	60%	54%
	63%	88%
	53%	53%
Tween 80 + Decanol + NaPP	38%	61%
	43%	55%
	55%	50%
	56%	63%

Table B.5 Raw data from spinach additive combination experiment. Recovery percentages for both *E. coli* and *Listeria* are from the supernatant after the low-speed centrifugation step.

	Recovery	
Additive / Combination	*E. coli*	*Listeria*
Control	77%	79%
	78%	81%
	82%	70%
	85%	103%
Tween 80 0.1%	77%	81%
	78%	91%
	91%	87%
	95%	67%
Hexanol 0.001%	67%	81%
	84%	94%
	84%	79%
	84%	110%
NaPP 0.01%	66%	77%
	71%	98%
	72%	108%
	78%	105%
Tween 80 + Hexanol	80%	84%
	86%	72%
	95%	103%
	118%	88%
Tween 80 + NaPP	72%	88%
	75%	74%
	101%	89%
	103%	105%
Hexanol + NaPP	77%	95%
	81%	95%
	81%	79%
	100%	105%
Tween 80 + Decanol + NaPP	76%	88%
	82%	88%
	84%	82%
	107%	80%

Table B.6 Raw data from milk additive combination experiment. Recovery percentages for both *E. coli* and *Listeria* are from the supernatant after the low-speed centrifugation step.

	Recovery	
Additive / Combination	*E. coli*	*Listeria*
Control	95%	68%
	114%	95%
	115%	87%
	110%	100%
Brij 1%	93%	82%
	116%	91%
	134%	103%
	100%	128%
Hexanol 0.001%	99%	93%
	104%	72%
	131%	97%
	94%	130%
NaPP 1%	93%	71%
	111%	102%
	126%	106%
	108%	115%
Brij + Hexanol	78%	69%
	128%	100%
	130%	102%
	97%	133%
Brij + NaPP	85%	85%
	125%	123%
	127%	107%
	104%	122%
Hexanol + NaPP	80%	73%
	106%	117%
	120%	101%
	110%	141%
Brij + Decanol + NaPP	104%	80%
	113%	101%
	116%	82%
	92%	124%

Table B.7 Raw data from experiment comparing Tween 80 0.01% plus NaPP 0.1% versus control for all three food matrices and both *E. coli* and *Listeria*.

	***E. coli* Recovery**		***Listeria* Recovery**	
	Control	Additives	Control	Additives
Hotdog	94%	73%	56%	57%
	94%	76%	59%	69%
	95%	79%	44%	52%
Spinach	69%	84%	84%	86%
	88%	97%	117%	101%
	95%	109%	108%	142%
Milk	90%	94%	97%	84%
	91%	102%	87%	99%
	97%	110%	101%	110%

Table B.8 Raw data from experiment comparing Tween 80 0.01% plus Hexanol 0.001% versus control for all three food matrices and both *E. coli* and *Listeria*.

	***E. coli* Recovery**		***Listeria* Recovery**	
	Control	Additives	Control	Additives
Hotdog	43%	49%	88%	78%
	57%	52%	65%	90%
	60%	65%	82%	94%
Spinach	60%	60%	83%	85%
	63%	72%	96%	97%
	64%	75%	80%	103%
Milk	77%	77%	67%	68%
	83%	84%	80%	71%
	86%	89%	67%	84%

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APPENDIX C. CONTROL DATA STATISTICS

Table C.1 Control data statistics for each food matrix and each bacteria, for each phase of experimentation.

Hotdog

E. coli	Single Additive	Combinations	Final 1	Final 2
Mean	0.47	0.56	0.94	0.53
Standard Deviation	0.09	0.10	0.00	0.09
CL (95%)	0.15	0.16	0.01	0.23
Listeria				
Mean	0.72	0.51	0.53	0.78
Standard Deviation	0.08	0.10	0.07	0.12
CL (95%)	0.13	0.15	0.19	0.31

Spinach

E. coli	Single Additive	Combinations	Final 1	Final 2
Mean	0.67	0.81	0.84	0.62
Standard Deviation	0.08	0.04	0.13	0.03
CL (95%)	0.19	0.06	0.33	0.05
Listeria				
Mean	0.84	0.83	1.03	0.86
Standard Deviation	0.07	0.14	0.17	0.09
CL (95%)	0.16	0.22	0.43	0.21

Milk

E. coli	Single Additive	Combinations	Final 1	Final 2
Mean	0.86	1.08	0.93	0.82
Standard Deviation	0.21	0.09	0.04	0.04
CL (95%)	0.33	0.15	0.10	0.10
Listeria				
Mean	0.69	0.88	0.95	0.71
Standard Deviation	0.20	0.14	0.07	0.07
CL (95%)	0.31	0.22	0.18	0.18

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