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THE SURVIVAL OF VARIOUS PATHOGENIC ORGANISMS IN FATS AND OILS

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THE SURVIVAL OF VARIOUS PATHOGENIC ORGANISMS IN FATS AND OILS

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Agriculture Food and Environment at the University of Kentucky

By

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Lexington, Kentucky

Director: Dr. Melissa Newman, Professor of Food Microbiology

2017

ABSTRACT OF THESIS

THE SURVIVAL OF VARIOUS PATHOGENIC ORGANISMS IN FATS AND OILS

The research within this thesis sought to determine the ability of various animal derived fats and plant derived oils to support the survival of several pathogenic cocktails over a multitude of storage times. The *Salmonella* study explored the survival rate of a four strain *Salmonella* cocktail in beef tallow, pig lard, duck fat, coconut oil, and extra virgin olive oil over seven days at 26˚C and 37˚C storage. The animal fats and the coconut oil supported the survival of the bacteria until the conclusion of the study. The Shiga-toxin producing *Escherichia coli* study explored the survival rate of a five strain *STECs* cocktail in extra virgin olive oil over seven days at 26˚C and 37˚C storage. The two *Listeria* studies explored the survival rate of a four strain *Listeria monocytogenes* cocktail in extra virgin olive oil over several time periods with different frequencies of sample mixing. In vitro, all genuses showed a 2.5-log cfu/mL to ≥ 7-log cfu/mL reduction in the extra virgin olive oil by the conclusion of the experiments. Extra virgin olive oil was then applied to cooked pork tenderloin, cheddar cheese snack squares, and turkey lunchmeat in hopes of inhibiting the *L. monocytogenes* cocktail. No reduction was observed.

KEYWORDS: *Salmonella*, *STEC*, *Listeria monocytogenes*, survival, animal fat, extra virgin olive oil

Kelsey Ellen Lamb

April 28th, 2017

THE SURVIVAL OF VARIOUS PATHOGENIC ORGANISMS IN FATS AND OILS

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Dr. Melissa Newman
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April 28th, 2017
(Date)

This thesis is dedicated in honor of my grandfather
Joe Eugene Fuqua (1928-2014)

Love Jesus Christ, love family, and love the College of Agriculture at the University of Kentucky.

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CHAPTER 1

INTRODUCTION

The survival of *Salmonella*, Shiga-toxin producing *E. coli* (*STECs*), and *Listeria monocytogenes* (*LM*) in commercial fats and oils intended for dry pet food usage is not well documented. This is due to the safety that is generally associated with pet food extrusion, fat rendering, and oil production processes. The extrusion process has been acclaimed as one of the most efficient and safest manufacturing processes for developing dry food items intended to be shelf stable with an extensive shelf life.[93,191,195] Extrusion is a high temperature, short time (HTST) process which yields a sterile product.[75,195] Similarly, fat rendering results in a sterile product by exposing the raw materials to high temperatures over prolonged periods of time.[1,120,121] Both methods have been widely accepted for the production of safe shelf-stable dry pet foods and pet food related products. Commercial plant oils are often considered safe products due to the lack of available nutrients for sustainability of various organisms. Since many oils contain several natural antimicrobial agents they are not subjected to heat treatments.[66,88,118,180,201]

Despite the safety associated with these products and their processing methods, the Food and Drug Administration (FDA) has issued 205 recalls from January 2010 to February 2017 concerning pet foods, livestock feeds, pet treats, and other animal specialty items.[59] The risk of possible contamination due to pathogenic microorganisms was the primary reason for domestic pet food related recalls.[59] Of the 205 total recalls, possible contamination due to *Salmonella* was responsible for 108 recalls and recall expansions.[59] Possible contamination due to *Listeria* was responsible for 12 recalls and recall expansions; six of the cases overlapped with possible *Salmonella* contamination recalls.[59] No *E. coli* or Shiga-toxin producing *E. coli* (*STECs*) were implicated in any pet food or pet related products within this time frame.[59] Nemser et al. (2014) recovered non-O157 *STECs* in 10 of the 196 tested commercial raw pet food diets, indicating that raw pet food, more than dry pet foods, could be a potential source of consumer contact with Shiga-toxin producing *E. coli*.[135] *E. coli*, specifically *STECs*, contaminating pet food

products remains a concern within the pet food industry due to the use of raw meats and other products associated with the organism.[60]

It has been suggested that the addition of fats and other nutrient compounds, following the primary cooking process of pet foods, could contribute to the introduction of pathogenic organisms in the final product.[128,191] Fats and other heat sensitive nutrients are either added in excess at the beginning of production or after the primary cooking and drying stages.[128,195] This is done in order to maintain desired nutrient amounts in the final product which may be compromised or destroyed during the intense heat treatments of extrusion. It has been shown that bacteria are more likely to survive in heated environments when lipids are present.[86,177,209] Animal derived fats, specifically beef tallow, pig lard, and duck fat, have not been associated with having antimicrobial properties like coconut oil or olive oil.[118,119,180] Although there remains some debate over the modes of action, medium chain fatty acids of coconut oil may act as mild antimicrobials. Olive oils contain several phenolic compounds that may have antimicrobial action.[118,119,180] The general survival times of *Salmonella*, Shiga-toxin producing *E. coli*, and *Listeria monocytogenes* in beef tallow, pig lard, duck fat, coconut oil, and extra virgin olive oil were not well documented in the available literature. These survival times would be beneficial in establishing the possible risk that each of these products could contribute to the pet food production process, especially if reheating temperatures used to apply these products are insufficient as a kill step for these bacteria.

The thesis is comprised of several studies which explore the ability of selected *Salmonella* strains, Shiga-toxin producing *E. coli* (*STECs*) strains, and *Listeria monocytogenes* (*LM*) strains to survive in commercial fats and oils over time. This research was initially designed to set a baseline survival time for these organisms over a week long time period at ambient (26˚C) and abused (37˚C) storage temperatures. These temperatures were chosen because products could be exposed to these two temperatures during shipment and storage prior to use in the pet food manufacturing process. A week time period was initially proposed to allow for multiple sampling times that would minimize product oxidation due to prolonged storage. A reheating treatment of 10 minutes or less in a 50˚C water-bath was proposed to liquefy the animal fat for sampling.

This was done to mimic a minimal reheating treatment, which would be inadequate as a control point for microorganisms and prevent excessive oxidation within the animal fats. The plant derived oils were never heated since they would remain in a liquid state. These initial survival times were then to act as a baseline for research assessing the minimal reheat treatment necessary to ensure a 7-log reduction within any contaminating bacterial populations. The optimum reheating treatments would then be applied to fat and oil and added to dry pet food products. The research design was later altered after the *Salmonella* study to solely focus on the effects of the extra virgin olive oil on the bacterial populations of the *STECs* and the *LM*. The design was later expanded over several time points for the *LM* with application focuses on several human food items rather than dry pet foods.

CHAPTER 2

LITERATURE REVIEW

2.1. Pet Foods

Pet foods can be described as nutritionally complete or nutritionally complementary.[149] The majority of products labeled as true pet foods are nutritionally complete meals which contain all of the necessary nutrients to support or maintain growth, depending upon the stage of life, without the need for additional supplementation.[149,191] Treats and other supplemental food items are considered complementary items to be used in conjunction with nutritionally complete meals or other complementary components to form a well balanced diet.[149] Complete and complementary diets comprise the two types of pet foods that can be subdivided into various categories.

Currently, the literature and the pet food industry appear to be divided over how to categorize pet foods based upon the characteristics acquired during processing. In the industry, pet foods can be subdivided based on what processing technique was utilized to develop the final product. This may include canned/retorted, baked, extruded, frozen, freeze-dried, air-dried, and refrigerated products.[1,191] The issue with this division is that there is often overlap within many of the product characteristics such as baked and extruded products which both produce dry pet foods. Pet foods may be subdivided based on storage location of the final product. Numerous pet food products are shelf stable at ambient temperatures, while others are refrigerated or frozen. This categorization is highly impractical due to the large variety of products which fall into the shelf stable type. Both dry pet kibble and canned pet foods are considered to be shelf stable products, but lack a common process or product composition. In the literature, Zicker (2008) presented the most favorable categorization, based on processing characteristics, in a topical review of commercial pet foods. Here, pet foods were divided into three types based on water content: moist, semi-moist/soft, and dry.[208] The largest of these three categories is dry pet food, which accounts for the largest weight and monetary value within the pet food industry.[106,195] Moist pet foods contained a range of 60-87% water,

semi-moist/soft pet foods contained a range of 25-35% water, and dry pet foods generally contained 11% water or less.[208] These ranges have also been verified in other works with some variation.[13,18,52,123] Treats are sometimes typed as an additional category; however, they are generally included into these three overarching types.

Within the dry pet food category, Spears and Fahey Jr (2004) reported that extrusion was the production method utilized for 95% of the pet food diets. Semi-moist/soft foods and treats may have also been included in the final percentages since many of these products undergo the same initial extrusion process, but negate the final drying step.[195,208] Without the final drying step, products maintain a softer and chewier texture due to the higher moisture content.

Extrusion technology was first patented by Joseph Bramah in 1797 for the production of seamless lead piping utilizing a piston press.[34,93] The first true patent for a screw driven machine was in 1879, which was followed by the success of German inventor Paul Troester who sold 500 machines from 1892-1912.[34] During the 1860's, pet foods were becoming more commercialized with the development of baked pet food biscuits.[1,36] The first canned pet foods appeared in the consumer market in the 1920's in hermetically sealed retorted containers.[1] At the same time, various manufacturing based products continued to be made using extruder technology throughout the early 1900's. It was not until the 1950's that an extruder was used to produce food based items with the development of the Single screw extruder and the Twin screw extruder.[1] In the mid 1950's, the first pet foods were produced using extrusion technology and rapidly grew to the largest share of the pet food market.[1,106,195,208]

Extrusion is defined as a high temperature, short time (HTST) bioreactor process.[75,128,195] Under these processing conditions, physical and chemical changes affecting the nutrition, structure, and palatability occur as the product is cooked and subsequently extruded.[195] Temperature conditions range from 80-200˚C (typically 110-150˚C) with an average 300g/kg moisture content and 34-37 atm pressure for a relatively short time of 10-270 seconds followed by a varied drying time at 120-160˚C.[49,128,195,208] Raw ingredients are ground and mixed together to form a viscous homogenous dough.[178,208] This dough can then be conditioned with water or it can be added directly

into the extruder barrel which cooks the product under heat from steam and friction caused by the increased pressure experienced by the spiral screws in the chamber.[128,132,162,195] Once the food product has been rendered sterile, due to the temperature, pressure, and added steam combination within the barrel, it is forced through a die, at 3-6 MPa pressure, which forms the ultimate shape of the product.[75,93,191,208] A knife is used to cut the extruded pet food product to the desired length prior to drying to a moisture content of 6-8% and subsequent cooling at ambient temperature.[132,195,208] The extrusion process ultimately results in a sterile product free of microorganisms and their related toxins.[75,191,195,208]

Despite the numerous safety, nutritional, and production benefits of the extrusion process, the oxidation of various fat components, which may lead to rancidity, continues to be a concern within the final products.[110,162] The loss of heat labile nutrients, specifically vitamins and minerals, is also of concern within the industry, especially for products that are formulated to be complete meals.[51,94,195] All pet foods are regulated by the Food and Drug Administration (FDA) through the Federal Food, Drug and Cosmetic Act (FD&C Act), with some overlap with the United States Department of Agriculture (USDA) and Environmental Protection Agency (EPA).[48,58,208] The Association of American Feed Control Official (AAFCO) holds no regulatory authority, but works closely with the FDA and other state governments to provide the necessary safety and nutritional information concerning pet food products.[58] AAFCO formed the Canine Nutrition Expert Subcommittee in 1990-1991 followed by the Feline Nutrition Expert Subcommittee in 1991-1992 to address various nutrient requirements to be met by pet food label claims of complete diets.[48] Many pet food companies had already been adjusting the fat and heat labile nutrients within their products to compensate for losses, during extrusion, but with the establishment of minimum requirements for growth/reproduction-based diets and adult maintenance-based diets the industry faced nutritional accountability for their products.[48] Fats and heat sensitive nutrients can be added in excess at the beginning of the extrusion process to compensate for the loss of some during the processing.[51,94,195] This method of nutrient compensation can be extremely costly if the final nutrient balance deviates from the estimated loss to a point of

deficiency or excess outside of the accepted limits. The addition of excessive fats to the initial dough mixture before extrusion can ultimately influence the cooking process of the extruded product by decreasing the friction within the screw chamber.[110,195] Excessive fat levels at the beginning of production can also result in a product that lacks the proper expansion and structural properties necessary for extruded products.[110,195] Due to these undesirable characteristics, fats and heat sensitive nutrients are typically added as flavors and nutrient enhancements during the drying and cooling phases of pet foods.[128] This can be accomplished by several different methods of spray drying, drop coating, drum drying, and other coating styles with various antioxidant mixes such as tocopherols (Vitamin E).[128,191] The addition of these fats and heat labile nutrients after the primary cooking step and the initial drying process helps to ensure their structural survival during production. It does, however, increase the likelihood of microbial contamination within the final product.

2. 2. Pathogens of Concern

2.2.1 *Salmonella* Various Strains

From the beginning of January 2010 to the end of February 2017, the FDA issued 205 individual recalls for livestock and domestic pet related products.[59] The largest number of these recalls was attributed to suspected or laboratory confirmed bacterial contamination, typically *Salmonella spp*.[59] The following categorization of the FDA recall cases was determined by the writer for use in this thesis. *Salmonella spp.* have been implicated in the contamination of 41 dry pet food products, 21 moist or frozen pet food products, 38 pet treat products, three animal medications, and five specialty pet foods or added ingredients.[59] Two of the dry pet food recalls included associated treat recalls; 13 of the listed 38 pet treat recalls were of animal origins, such as pig ears and cow hooves.[59] The majority of these recalls were precautionary with no formally reported pet or human illness despite some having confirmed laboratory evidence.[59] Minimally processed pet food products, such as raw diets and pet treats of animal origin, are known to increase the risk of human exposure to *Salmonella* via handling contaminated food items or direct animal contact.[24,146,152,171] White et al. (2003) examined the frequency of *Salmonella spp.* in animal derived pet treats available in stores

across the United States. Of the 158 sampled products, 65 (41%) were contaminated with at least one strain of *Salmonella*, with several of the isolates displaying resistance to one (36%) or multiple (13%) antimicrobials.[203] Nemser et al. (2014) reported the analysis of 480 dry and semi-moist pet products yielded a positive *Salmonella* recovery in one dry cat food. In the analysis of 576 raw pet foods, exotic feeds, and jerky-style treats, 15 positive *Salmonella spp*. were recovered from raw pet food samples.[135] Although the recovery of *Salmonella* is less frequent from dry pet foods, there have been a two major outbreaks associated with these products.

From January 1, 2006 to December 31, 2007 the Centers for Disease Control and Prevention (CDC) and FDA traced an outbreak of *Salmonella enterica* ser. Schwarzengrund in 70 patients across 19 states with the majority being reported in the northeastern United States.[27] Of 61 known cases, at the time of the report, the median patient age was three years old, with 24 (39%) of the cases occurring in children less than one year of age.[27] The outbreak was later expanded from December 31, 2007, to September 18, 2008, due to nine associated cases resulting in a total of 79 patients from 21 states.[32] In seven of the nine known cases, at the time of the report, the median age was eight months old, with six of the patients being less than two years old.[32] In both reports, some patients experienced bloody diarrhea and were hospitalized, but no deaths were reported.[27,32] The cause of the outbreak was traced to two brands of dry dog food produced by Mars Petcare US at their Everson, Pennsylvania, location.[27,32] The manufacturing plant was shut down for cleaning and renovations in July of 2007, after the outbreak strain was identified by the FDA in unopened packages of the final products.[27] The plant was later reopened in November of 2007, but with the addition of more outbreak cases in 2008, continuing the outbreak for three years, the Everson plant issued a nationwide recall of all dry cat and dog foods produced at the plant over a five month time frame and officially closed in October 2008.[27,32] This was the first reported human *Salmonella* outbreak associated with dry pet food.[27] Although the source of the bacterial contamination was never definitively identified, it has been hypothesized that contamination may have occurred during the flavoring and enhancement of the products after extrusion.[27]

From February 1, 2012, to July 18, 2012, the CDC reported an outbreak of *Salmonella Infantis* in 47 patients from 20 states and two patients from Canada.[26,29] Upon recovery of *Salmonella* from a routine retail test, conducted by the Michigan Department of Agriculture and Rural Development on April 2, 2012, Diamond Pet Foods recalled 17 brands of dry dog and cat food from its manufacturing plant in Gaston, South Carolina. This particular *Salmonella Infantis*, in addition to a second strain, was linked to the outbreak of human illness attributed to contact with the contaminated pet food or animals that had access to the food.[26] This was the second reported human *Salmonella* outbreak to be associated with dry pet food.[26,29] The source of the contamination was never confirmed; however, the FDA report suggested post-processing contamination of the product due to poor employee hygiene and a lack of microbial analysis for incoming animal fats.[54] It was also noted that an employee touched an in-line fat filter and oil with their bare hands during the plant inspection on April 13, 2012.[54]

Salmonella are generally described as motile bacteria via peritrichous flagella (some non-motile); can produce hydrogen sulfide (varied); can utilize ammonium citrate; fail to liquefy gelatin; fail to hydrolyze urea; are indole and oxidase negative; are catalase positive; produce acid and occasional gas from glucose, sorbitol, mannitol, and maltose; and fail to utilize salicin, adonitol, sucrose, and lactose.[17] These bacteria are facultative aerobes that do not form spores and are typically observed as single 0.6-0.7 microns by 2.0-3.0 microns Gram-negative rods.[17,147] All known serotypes, over 2500, are pathogenic to humans and may be pathogenic in other animals.[17,68,147] The number of bacteria in an infective dose varies with the strain of *Salmonella.* Although most records indicate illness at 5.0-log to 10.0-log cfu/g among human participants, as few as 3.0-log cfu/g to a few hundred cells have been thought to cause *Salmonella* outbreaks among humans.[12,147] *Salmonella* infection from ingestion of contaminated food items or contact with animal carriers can result in fever, diarrhea, and abdominal cramping within 12-72 hours after exposure with symptoms persisting for four to seven days.[31] In the CDC Foodborne Illness Fact Sheet (2000-2008), *Salmonella* was reported as the most common pathogenic bacteria to cause the largest number of food-borne related illnesses (1,000,000 people), hospitalizations (19,000 people), and deaths (380 people) per year.[30,172]

The first human isolate of Salmonella, specifically *Salmonella enterica* subsp. *enterica* ser. Typhi, was attributed to Georg Gaffky in 1884.[68] In 1885, veterinarian Daniel Salmon and microbiologist Theobald Smith isolated *Salmonella*, specifically *Salmonella enterica* ser. Choleraesuis, from swine.[68,147] Salmon was credited with the discovery and the bacteria were subsequently named in his honor.[147] Around 1983, DNA-DNA hybridization revealed only two separate species of *Salmonella, S. enterica* and *S. bongori*; therefore, resulting in the categorization of *S. enterica* into six subspecies.[68,147] *Salmonella* are also categorized by serotypes, or serovars, based on surface structures including flagella (H) and somatic (O) antigens.[147] These serotypes are described in the White-Kauffmann-Le Minor (WKL) scheme and recent supplemental listings.[68,71,82,154] The four strains of *Salmonella* utilized in the current study included: *Salmonella typhimurium* (ATCC 13311), *Salmonella choleraesuis* subsp. *choleraesuis* (ATCC 13312), *Salmonella pullorum* (ATCC 19945), and *Salmonella choleraesuis* subsp. *arizonae* (ATCC 13314). *Salmonella enterica* subsp. *enterica* ser. Typhimurium (ATCC 13311) was originally isolated from a human fecal sample due to food poisoning.[3] *Salmonella choleraesuis* subsp. *choleraesuis* ser. Choleraesuis (ATCC 13312), otherwise identified as *Salmonella enterica* subsp. *enterica* ser. Choleraesuis, was obtained from a quality control strain for Enteric research.[3] *Salmonella enterica* subsp. *enterica* ser. Pullorum (ATCC 19945) was obtained as a bacteriophage host for enteric research.[3] *Salmonella enterica* subsp. *arizonae*, otherwise identified as *Salmonella choleraesuis* subsp. *arizonae*, (ATCC 13314) was obtained for Enteric research.[3]

2.2.2 Shiga-Toxin Producing *Escherichia coli* (*STECs*)

Neither *Escherichia coli* nor Shiga-toxin producing *E. coli (STECs)* strains, including *E. coli O157:H7*, have been associated with any pet food related recalls from January 2010 to February 2017. Nemser et al. (2014) reported that no *STEC* strains were isolated in the 480 dry and semi-moist pet foods assayed. There was also no detection of *STECs* in any of the exotic pet foods or jerky-type treats. There was, however, recovery of non-O157 *STECs* in 10 of the 196 raw pet food diets.[135] This indicated that raw pet food diets have the potential to harbor *STECs* and that there is the possibility for human

exposure to the organisms, if proper hygiene is neglected.[135] Despite the lack of formal recalls associated with these organisms in pet products, there have been recalls due to *E. coli* contamination, including *STECs*, in several human food grade ingredients such as: meats, nut-spreads, flours, cheeses, and leafy greens.[60] All of these products have the potential to be used for manufacturing pet foods. Outbreaks of various *STECs* have been documented in several products ranging from raw and unpasteurized products to cured meats and packaged cookie dough.[33,40,134,159,175] Outbreaks have also been reported to have occurred from contact with livestock and with infected persons.[114,184] *STECs* have also been isolated from numerous mammalian species including: cattle, pigs, sheep, goats, cats, and dogs.[11,102,202] Although it has been proposed that companion animals may be a source of potential contact with *STECs*, the actual source of those organisms to humans remains unclear.[10,102] *E. coli*, specifically *STECs*, remain a prominent concern within the pet food industry due to the severity of the disease and the potential transfer to humans.

The identification of *Escherichia coli (E. coli)* was credited to Theodor Escherich in 1885.[17,56,181] The German pediatrician isolated *Bacterium coli commune* from the human colon, which was later renamed *Escherichia coli* in his honor.[56] Despite *E. coli* belonging to the *Enterobacteriaceae* family with other exclusive pathogens, such as *Salmonella*, it is considered to be an opportunistic pathogen.[17,56] *E. coli* are generally described as motile or non-motile, do not produce hydrogen sulfide, do not utilize ammonium citrate, produce acid and gas from both glucose and lactose, are methyl red and catalase positive, are oxidase negative, fail to hydrolyze urea, and are indole positive.[17,43] These bacteria are facultative aerobes that do not form spores and are typically observed as paired or short chained 0.5 microns by 1.0-3.0 microns Gram-negative rods.[17,43] There are six different pathotypes of *E. coli* including: Diffusely-adherent *E. coli* (DAEC), Enteroaggregative *E. coli* (EAEC), Enterohemorrhagic *E. coli* (EHEC), Enteroinvasive *E. coli* (EIEC), and Enterotoxigenic *E. coli* (ETEC).[43,91,133] The Enterohemorrhagic *E. coli* (EHEC) pathotype are categorized within the larger Shiga-toxin producing *E. coli* (*STEC*) group.[44] Konowalchuk et al. (1977) discovered that certain *E. coli* produced toxins that were cytotoxic to vero cells, thus describing them as

Vero-toxins or Vero-toxigenic *E. coli*.[97,188] O'Brien et al. (1983) discovered that a strain of *E. coli* linked to a hemorrhagic colitis outbreak, *E. coli O157:H7,* produced a Shiga-like toxin.[90,138] The research of the *E. coli* Vero-toxins, Shiga-toxins, and Shiga-like toxins met in the study conducted by Karmali (1985) that showed a significant correlation between patients displaying Hemolytic Uremic Syndrome (HUS) and the recovery of Vero-toxin producing *E. coli* from their fecal samples.[90,92] Here, the terms of Shiga-toxin (Stx), Shiga-like toxin (SLTx), and Vero-toxin (VT) became synonymous in describing various strains of *E. coli* which produced toxins that caused severe illness among human patients including hemolytic uremic syndrome (HUS) or thrombic thrombocytopenic purpura (TTP).[90,92,188] The Shiga-toxin is an A_1B_5 toxin.[50,122] The toxin causes death to various eukaryotic cell types, namely vero cells, by binding to the cell surface with the five B subunits and inserting the active A subunit into the cell.[50] The A subunit disrupts normal protein synthesis within the cell and ultimately leads to cell death.[50,122] General symptoms of infection include watery diarrhea, vomiting, muscle cramps, nausea, variable mild fever, variable blood in stool, and possible progression to the development of HUS or TTP.[23,188,192] The illness typically lasts five to seven days, but can cause permanent damage or death, especially when the individual is immunocompromised.[23,192] The structures, including flagella (H), somatic (O), and encapsulation (K) antigens, help to differentiate the serotypes of *E. coli* from one another.[133] These serotypes are described in the World Health Organization reference collection for *E. coli*, similar to the White-Kauffmann-Le Minor (WKL) scheme used for *Salmonella*.[155,204]

The five strains of *E. coli* utilized in the current study included: *Escherichia coli O157:H7* (ATCC 35150), *Escherichia coli STEC 15AB* (ATCC 99-3311), *Escherichia coli STEC 15AE* (ATCC 2006-3008), *Escherichia coli STEC 15AF* (ATCC 2002-3211), and *Escherichia coli STEC 15AG* (ATCC O111). *Escherichia coli O157:H7* (ATCC 35150) was ser. O157:H7 isolated from a human fecal sample and known to be associated with hemorrhagic colitis.[3] *Escherichia coli* (ATCC BAA-2192: strain designation 99-3311) was a non-motile ser. O145 isolated from human feces in South Dakota.[3] *Escherichia coli* (ATCC BAA-2215: strain designation 2006-3008) was ser. O103:H11 isolated from Idaho.[3] *Escherichia coli* (ATCC BAA-2219: strain designation

2002-3211) was ser. O121:H19 isolated from human feces in Virginia.[3] *Escherichia coli* (ATCC BAA-2440: strain designation O111) was ser. O111 isolated from a human subject.[3]

2.2.3 *Listeria monocytogenes*

Listeria spp. have also been associated with several contaminated pet related foods, but to a lesser extent than *Salmonella spp.* The following categorization of the FDA recall cases was determined by the writer for use in this thesis. *Listeria spp.* were implicated in 12 of the 205 recalls, with the majority being in refrigerated/frozen meals and raw pet food diets.[59] A freeze-dried pet food and a fermented air-dried pet food were also listed among the recalled items due to potential *Listeria* contamination.[59] Half of the *Listeria* recalls were also associated with possible *Salmonella* contamination in the same products.[59] Nemser et al. (2014) reported the analysis of 480 dry and semi-moist pet products that yielded a single positive *Listeria* in one dry cat food, separate from the dry cat food that recovered *Salmonella*. In the same study, 32 positive *Listeria monocytogenes* (*LM*) samples were exclusively isolated from raw pet food samples.[135] An additional 34 positive non-*monocytogenes Listeria* were isolated from other raw pet food samples and a single jerky-style treat.[135]

Unlike *Salmonella* and *E. coli* serotypes, which can reside within certain host animals without causing illness, *Listeria* does not have a defined asymptomatic animal host.[62,98,205] Dairy farms have been implicated as potential reservoirs for *Listeria* to produce biofilms and persist in silage, milk bulk tanks, and ill cattle.[15,130] Several *Listeria* strains have also been shown to survive in various adverse environments and food items for prolonged periods of time. Liao and Shollenberge (2003) showed that 27 *Listeria* strains were recoverable up to three years from storage in sterile phosphate buffer (SPB). *LM*, specifically, survived in SPB until the conclusion of a four week and a 30 week study.[108] *Listeria* has also been shown to survive in spray dried milk with a moisture content of 3.6-6.4% and pork rinds and cracklings at a water activity of 0.27.[45,81] Due to the death of *Listeria* during pasteurization and other processing treatments, it is thought that most contamination occurs post processing.[15,130,193] Although there are no reported outbreaks directly linking human illness from *Listeria spp.* contamination in pet

foods, the industry remains concerned due to the severity of the illness and the recent emergence of the organism in refrigerated/frozen meals and raw pet food diets.[59]

The identification of *Listeria*, initially *Bacterium monocytogenes*, was attributed to Dr. Everitt Murray and colleagues who observed the bacteria upon the seemingly spontaneous deaths of several laboratory rabbits and guinea pigs in 1924.[131,199] *Listeria* were not implicated in human disease until 1929, in Denmark, despite earlier possible isolations from human patients in Germany, 1893, and from France, 1891 and 1921.[70,199] In 1927, Pirie observed the same bacteria in the liver of the African jumping mouse which he named *Listerella hepatolytica*.[70] The genus of the bacteria was later changed to *Listeria* in honor of the English surgeon and bacteriologist Joseph Lister.[17,151] *Listeria* are generally characterized as an invasive bacteria observed as small rounded rods.[17] *Listeria* are motile via peritrichous flagella, are psychotrophic (2.5˚C-25˚C), do not liquefy gelatin, are indole and oxidase negative, do not produce hydrogen sulfide, are catalase positive, are salt (NaCl) tolerant, and fail to produce nitrites.[17,112] They can produce acid without gas from trehalose, salicin, and glucose; occasionally produce acid without gas from glycerol, soluble starch, sucrose, rhamnose, lactose, and melezitose; and lack acid or gas production from inositol, insulin, dulcitol, xylose, or mannitol.[17] These bacteria are facultative aerobes that do not form spores and are typically observed as single, V-shaped paired, or parallel paired 0.4-0.5 microns by 0.5-2.0 microns Gram-positive rods.[17,112,199] *Listeria*, specifically *LM*, can cause a wide array of symptoms from gastrointestinal distress to septicemia, meningitis, spontaneous abortions, and death.[112] Laboratory research and recovery from outbreak samples have suggested a large infective dose (>6.0-log cfu/g) is necessary to induce the typical nausea, vomiting, diarrhea, muscle cramps, and fever associated with gastrointestinal related febrile gastroenteritis.[39,53,112,199] It has also been suggested that outbreaks have been caused by 2.0-log to 4.0-log cfu/g and that precautions should be taken by immunocompromised individuals, especially pregnant women. *LM* are categorized into 14 serotypes with 1/2a, 1/2b, and 4b attributing to most of the human illness.[14] The four strains of *LM* utilized in the current study included: *Listeria monocytogenes* 150C (ATCC 51781), *Listeria monocytogenes* 150D (ATCC 43256), *Listeria monocytogenes* 150E (ATCC 15313), and

Listeria monocytogenes 150F (ATCC 19115). *Listeria monocytogenes* (ATCC 51781) was ser. four isolated from dairy products from Belgium.[3] *Listeria monocytogenes* (ATCC 43256) was isolated from Mexican-style cheese from California.[3] *Listeria monocytogenes* (ATCC 15313) was isolated from a rabbit from Cambridge, England.[3] *Listeria monocytogenes* (ATCC 19115) was ser. 4b isolated from a human.[3]

2.3. Fats and Oils

Contrary to the plethora of recalls and outbreaks related to pet food products, the extrusion process of pet foods has been deemed one of the most efficient and safest processing methods for dry shelf stable food with a prolonged shelf-life.[93,191,195] With the high temperature short time (HTST) process, resulting in a pathogen free pasteurization quality product, it is thought that the majority of bacterial contamination results from additional industrial processes.[103,128,153,191,195] Similar to the thermal treatment of the extrusion process, the rendering process of animal fats uses processing temperatures intended to completely reduce the bacterial load within the final products.[1,2,121,141] The National Renderers Association recognizes treatment of animal fats ranging from 115˚C to 146˚C for an average of 40 minutes or more is a sufficient rendering process.[120,121] Since the thermal treatment experienced by the animal fats during the rendering process will kill any pathogenic bacteria of concern, the source of contamination must come post-processing. Unlike animal fats, which require a thermal treatment, plant based oils are often processed using ambient to cold press methods depending upon the desired characteristics and subsequent price range of the product.[2,66,104,201] Although differences in processing can cause variation within the quality of the products, the overall safety can be insured via sterilizing washes, removal of contaminated skins, and drying treatments similar to other produce items prior to further processing.[66,201] Due to the destruction of microorganisms during rendering and oil production processes, it has been hypothesized that contamination with pathogenic bacteria in these products may be due to post-processing contamination. These contaminated fats and oils may subsequently contaminate dry pet foods when added as flavors and nutrient enhancements after the primary cooking and drying steps in the pet food production process.

It has been suggested that various fats and oils can act as vehicles for microbial contamination through buffering capacities experienced during heat treatments.[124,177,209] Burnett et al. (2000) reported that *Salmonella spp.* were capable of surviving in various peanut butters, reduced sugar and reduced sodium peanut butters, and reduced fat spreads for 24 weeks at 5˚C and 21˚C storage. Juneja and Eblen (2000) displayed an increase in the lag time of *Salmonella spp.* within ground beef as the fat content increased, contributing to an overall increase in bacterial survival during heat treatment. This prolonged survival was contrasted with the rapid linear decline of *Salmonella spp.* populations within heated chicken broth (3% fat).[86] Holliday et at. (2003) reported on the survival of *Salmonella spp, E. coli O157:H7,* and *LM* in various butters and yellow spreads over three weeks at 4.4˚C and 21˚C storage. Overall, the three genus of bacteria were reported to have higher surviving counts within the higher fat (>61%) products compared to the lower fat products.[78] The survival of the different bacterial populations was ultimately compromised due to the confounding influence of pH and preservatives within several of the inoculated products.[78] The phenomenon of increased heat resistance and prolonged survival rates in fatty products have been attributed to possible encapsulating and buffering capacities of the fatty acids within the lipid medium, as well as the uneven dissipation of heat through the lipid medium due to reduced water activity.[86,124,176,177,209]

A review of the literature yielded no natural antimicrobial abilities associated with beef tallow, pig lard, or duck fat resulting in a reduction of *Salmonella*, *E. coli*, or *Listeria* populations. Beef tallow fatty acid composition varied within a wide range based on the sex, age, breed, and diet of the animal.[1,2,73] Generally, the saturated fatty acid content of tallow ranged from 30-55% and the unsaturated fatty acid content ranged from 35-64%.[16,38,73,99] The major saturated components included palmitic acid (C16:0) and stearic acid (C18:0).[16,38,73,207] The major unsaturated components included oleic acid (C18:1) and palmitoleic acid (C16:1).[16,38,73,207] Edible tallow was exclusively derived from beef fat and bone sources that had been approved by USDA, or country specific, inspectors for human consumption.[1,2] Beef tallow was described as a light colored hard solid fat (low moisture fat) with a melting point of 40-50˚C.[1,2,38] Beef tallow could be

refined, polished, and deodorized to make it more appealing for human consumption; it could also be left in a crude state for animal consumption.[1,2]

Similar to beef tallow, the fatty acid composition of pig lard varied based on sex, age, breed, geographical location, and diet of the animal.[1,2,73,150] The saturated fatty acid content of the lard typically ranged from 38-45% while the unsaturated fatty acid content ranged from 45-62%.[1,2,73,150] The major saturated components included palmitic acid (C16:0) and stearic acid (C18:0).[2,16,73,161] The major unsaturated components included oleic acid (C18:1), linoleic acid (C18:2), and palmitoleic acid (C16:1).[2,16,73,161] Lard, also referred to as edible grease, was derived from the rendered adipose tissue of pigs that had been approved by the USDA, or country specific, inspector for human consumption.[1,2,161] Lard was described as a light colored soft solid fat with a melting point of 34-44˚C.[1,2,161]

Poultry fat, specifically duck fat, reflected the same variation within the fatty acid composition as noted by the factors effecting the beef tallow and pig lard.[1,2,73,206] The saturated and unsaturated fatty acid compositions of most poultry fat (chicken, turkey, and duck) were summarized together in most of the literature rather than subdivided into their own categories. Duck fat would be a specific category of rendered poultry fat and would have duck as the sole source of fat. The pet food industry is said to utilize anywhere from 10-20% of the entire annual yield of rendered poultry fat.[1] Poultry fat, as a whole, has a melting point of 23-40˚C and had a range in composition of 25-32% saturated fatty acids and 57-75% unsaturated fatty acids.[1,2,73] Witak et al. (2008) described their specific duck fat composition to contain palmitic acid (C16:0) and stearic acid (C18:0) as the primary saturated fatty acids. The unsaturated fatty acids were primarily oleic acid (C18:1), linoleic acid (C18:2), and palmitoleic acid (C16:1).[206]

Coconut oil is fairly common place in human food applications, but it is not a typical additive coating of the pet food market. However, due to recent market place trends searching for possible plant derived oils to replace animal derived fats in pet foods, coconut oil may serve as a desirable substitute. Coconut oil is reminiscent of the animal fats in that it appears solid at room temperature after processing due to the saturated fatty acid composition. Coconut was described to have a typical composition range of 90-96% saturated fatty acids including: lauric acid (C12:0), which encompassed 44-56%, myristic

acid (C14:0), palmitic acid (C16:0), caprylic acid (C8:0), capric acid (C10:0), and stearic acid (C18:0).[16,73,100,104,194] Despite being solid at or below ambient temperature (25˚C), the melting point of coconut oil was considered to be 20-25˚C because it takes very little added heat to liquefy the solidified oil.[104,194] Coconut oil was also unique in that the majority, approximately 51-70%, of the saturated fatty acids are medium chain fatty acids.[100,104] Only babassu, cohune, cuphea, and palm kernel oils contained significant amounts of these medium chain fatty acids similar to those found in coconut oil.[104] There were three types of coconut oil described: virgin coconut oil, unrefined coconut oil, and refined, bleached, and deodorized (RBD) coconut oil.[104] Kumar et al. (2015) showed that Indian virgin coconut oil had the highest composition of saturated fatty acids, the highest composition of medium chain saturated fatty acids, the highest amount of lauric acid (C12:0), the lowest free fatty acid value, and the lowest phenolic composition compared to Indian unrefined coconut oils and Indian RBD coconut oils. Virgin coconut oil has displayed antimicrobial effects associated with the medium chain saturated fatty acids, despite lacking many of the phenolic compounds commonly associated with antimicrobial activity.[88,180]

Olive oil is not commonly used as an additive coating in the pet food industry, despite multiple applications within the human food market, because it remains in a liquid state at ambient temperature. Similar to coconut oil, olive oils present a plant based alternative for additional lipids within the pet diet. As a standard, refined olive oil consisted of 12-14% saturated fatty acids and 86% unsaturated fatty acids on average, with the majority of those unsaturated varieties being monounsaturated (approximately 77%).[16,73,194] Palmitic acid (C16:0) and stearic acid (C18:0) were the primary saturated components, while oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) were the primary unsaturated components, with oleic acid making up approximately 65-80%.[73,194] The reason that refined olive oil is used as the composition standard is that there are three types of olive oil: virgin olive oil, refined olive oil, and pomace olive oil.[118] The total fatty acid content of the oils was reported to be the same with the only true difference being the phenolic compound content.[65,118] Some slight variation within the fatty acid and phenolic content could also be explained due to the olive cultivar and

the geographic location in which the fruit was raised, similar to the effects seen by the previously described fats and oils.[65] Historically, olive oil has been used for medicinal salves, religious offerings, public rituals, daily and ceremonial cleansings, burial preparation, and most importantly as lamp fuel for several centuries across numerous civilizations.[200] Only recently has the olive been cultivated for mass scale consumption rather than practical utilization. The genetic origins of the current olive cultivar *Olea europaea L. var communis* are unknown, although it is assumed to be a distant cultivar of Syrian or sub-Sahara African decent after the expansion of the olive production from the Mediterranean coast.[200] Olive oils, especially virgin olive oils, have been shown to have strong antimicrobial abilities against a large number of microorganisms compared to other fruit and seed derived oils.[118] As noted by Medina et al. (2006), the potency of the phenolic and other antimicrobial compounds within olive oils tend to decrease with dilution and refining of the oil. Virgin oils contain the highest amount of antimicrobial compounds, followed by refined olive oils, which depend on their virgin oil content for the antimicrobial effects, and pomace oil which contain limited amounts of phenolic and other antimicrobial compounds.[65,118,179] Oleuropein was determined to be the major phenolic compound of the olive fruit itself which displayed antimicrobial effects towards fermentative lactic acid bacteria.[19,64] Due to the bitter sensory association, the use of this compound as an antimicrobial agent was limited.[19] More recent research has focused on the presence of α,β-unsaturated aldehydes in olive oils which were thought to cause disruptions within the plasma membrane of various pathogenic bacterial cells.[101] Research concerning the influence of various simple phenolic and other related compounds were also expounded upon. The synergistic action of tyrosol, hydroxytyrosol, oleuropein aglycons, and the dialdehydic form of decarboxymethyl ligstroside were consequently explored.[118,119] Since the antimicrobial component and properties of olive oils are still largely unknown, further research on the potency and the ability of the oil to harbor bacteria needs to be conducted.

CHAPTER 3

THE SURVIVAL OF A *SALMONELLA* COCKTAIL IN VARIOUS FATS AND OILS COMMON TO DRY PET FOOD

3.1. Summary

The purpose of this study was to determine if extra virgin olive oil, coconut oil, duck fat, pig lard, and beef tallow would support the survival of *Salmonella*. Inoculated stocks of each fat/oil were made using a four strain *Salmonella* cocktail and divided into 14 disposable tubes. Each tube received 5mL of stock. The tubes were randomized and held at two different temperatures, 26˚C and 37˚C, for seven days. Each tube was only sampled once. The hypothesis of this study was that these fats and oils would maintain *Salmonella* inoculum levels, now approximately 6-log cfu/mL, over the seven day period. *Salmonella typhimurium* (ATCC 13312), *Salmonella choleraesuis* subsp. *choleraesuis* (ATCC 13311), *Salmonella pullorum* (ATCC 19945), and *Salmonella choleraesuis* subsp. *arizonae* (ATCC 13314) all survived the 7 day period at 26˚C and 37˚C in the duck fat, pig lard, beef tallow, and coconut oil without reduction in the population. The extra virgin olive oil was the only material to not sustain the original *Salmonella* counts. An average 3-log cfu/mL reduction was observed after 24 hours. *Salmonella choleraesuis* subsp. *arizonae* (ATCC 13314) remained the only viable strain within the extra virgin olive oil after seven days at both temperatures. No statistical difference was found between holding samples at 26˚C or 37˚C for any fat/oil type.

3.2. Introduction

Salmonella contamination is a well established concern within the pet food industry since it is ubiquitous in the environment and is found in several living organisms.[5,107] Among pet product related recalls issued by the Food and Drug Administration over the past seven years, *Salmonella* recalls outnumber all mycotoxins, nutrient imbalances, foreign chemicals, physical materials, other microorganisms, lack of sterility, and mislabeling recalls combined. Of the 108 pet related *Salmonella* recalls, listed between January 2010-January 2017, 41 were for dry pet food products, 21 pertained to wet or frozen pet food products, 38 were for pet treat products including 13 from animal parts, three pertained to animal medications, and five were unique pet food

products or ingredients.[59] Although some cases were connected with severe illness in both pets and humans, the majority of these recalls were issued for precautionary measures with no pet or human illnesses formally reported despite confirmed presence of *Salmonella spp*. within a laboratory setting.

It is well documented that *Salmonella* can survive for various amounts of time at refrigerator temperatures,[157] freezing temperatures,[47,95] drying treatments,[77,109] and even freeze-drying treatments.[158] Minimally processed products like raw pet food diets naturally increase the risk of finished products containing *Salmonella* because they do not undergo a cook step which is a primary processing defense against the organism.[103] Animal derived products are also more likely to come into processing facilities with higher numbers of initial organisms, such as *Salmonella*, which could allow enough retention of the pathogens after minimal processing to cause illness.[183,203] Refrigeration and freezing methods can slow or halt the growth of *Salmonella* that is already present on the food product, but organisms have the potential to survive these storage mechanisms and infect a host upon thawing. Extrusion and drying methods carry the risk of *Salmonella* exposure through post-processing contaminations such as crossing raw and finished products and unhygienic practices.[139] Many of the dry pet food recalls issued by the FDA neglected to include an initial source for the *Salmonella* contamination. It has been hypothesized that a potential source for *Salmonella* may be with the addition of heat sensitive nutrients or other products after the primary cooking step.[103,153,169]

The purpose of this study was to determine if extra virgin olive oil (EVOO), coconut oil, duck fat, pig lard, and beef tallow would be viable sources for *Salmonella* contamination in pet food. The hypothesis of this study was that these fats and oils would maintain the initial *Salmonella* inoculum levels over the seven day period incubated at both 26˚C and 37˚C. Baseline data on the survival rates of *Salmonella* species in these fats and oils would benefit food producers who may store and utilize these products in room temperature or temperature abused settings. This information would be an important application in determining the possible sources for contamination specifically associated with pet foods which may be coated in these lipids.

3.3. Materials

3.3.1. Pathogens

Salmonella typhimurium (ATCC 13312), *Salmonella choleraesuis* subsp. *choleraesuis* (ATCC 13311), *Salmonella pullorum* (ATCC 19945), and *Salmonella choleraesuis* subsp. *arizonae* (ATCC 13314) were the four strains of *Salmonella* utilized in the study.

3.3.2. Evaluated Products

Two commercial oils and three commercial animal fats were evaluated on the basis of their ability to harbor pathogens over seven days at 26˚C and 37˚C. These products included extra virgin olive oil (EVOO), coconut oil, duck fat, pig lard, and beef tallow. Primo Gusto©: Italian Foods 100% Italian Extra Virgin Olive Oil (cold pressed) (country of origin: Italy via Gordon Food Service® P.O. Box 1787, Grand Rapids, MI 49501) and Kirkland Signature: Organic Coconut Oil (via Costco 999 Lake Drive, Issaquah, WA 98027) were purchased from common grocery venues. Good Vittles: Hudson Valley Foie Gras & Duck Products Rendered Duck Fat (via Farm Fresh Duck, P.O. Box 373, Hamburg, PA 19526) and Proper Foods for Life: Non-hydrogenated Pure Lard and Non-hydrogenated Beef Tallow (4065 Fox St. Mesa, AZ 85205) were ordered from online stores. These products were selected because they did not include any additives or preservatives which could have interfered with the survival rates of the bacteria within the products.

3.3.3. Medias

The broths utilized in the *Salmonella* study included Bacto™ Brain Heart Infusion (BHI) and BBL™ Lactose broth. Both products were manufactured by Becton, Dickinson and Company BD (7 Loveton Circle, Sparks, MD, 21152) and were made according to the specifications on the label. The BHI was dispensed into Pyrex tubes with a final volume of 7mL each and autoclaved. The lactose broth was dispensed into Pyrex tubes with a final volume of 9mL each and autoclaved. Dilution blanks utilized throughout the study were Sterile Phosphate Buffer dilution blank tubes. These dilution tubes were made from 24mL PO_4 solution, 95mL $MgCl_2$ solution, and 19L of double deionized water. Tween80 was added to the dilution blank mixture at 1mL/1L so that the

final product contained 0.1% Tween80. The dilution mixture with Tween80 was adjusted to a pH between 7.4-7.5. The mixture was then dispensed into Pyrex tubes with a final volume of 9mL and autoclaved. The agars utilized in the *Salmonella* study included BBL™ Trypticase™ Soy Agar: Soybean-Casein Digest Agar (TSA) and Difco™ XLD Agar (XLD). Both products were manufactured by Becton, Dickinson and Company (7 Loveton Circle, Sparks, MD, 21152) and were made according to the product specifications. All agars were poured into sterile 100x15mm polystyrene disposable VWR Petri plates (1050 Satellite Blvd NW, Suwanee, GA 30024).

3. 4. Methods

3.4.1. Fat/Oil Sterility Validation

The fats/oils were transferred using sterile technique from their original containers into sterile 250mL glass bottles and caps. Samples of each fat/oil were plated on TSA and XLD and incubated for 48 hours at 37˚C (and TSA incubated for 48 hours at 26˚C) to check for bacterial background. All fats/oils were confirmed to be commercially sterile with an aerobic count below the level of detection. These fat/oil stock bottles were then wrapped in foil and placed in refrigerator storage to prevent any additional oxidation or contamination.

3.4.2. Inoculum Preparation

A sterilized loop was used to transfer a scrape of the desired *Salmonella spp* strain from a pre-existing refrigerated BHI slant into BHI broth. The culture was incubated for 24 hours at 37˚C. The culture was then streaked for isolation onto XLD agar using sterile technique and incubated for 24 hours at 37˚C. An isolated colony was picked with a sterile loop and placed into a new tube of BHI. This tube was then incubated for 24 hours at 37˚C. The culture was transferred two additional times by placing 0.1mL of the former culture into new BHI broth and incubating for 24 hours at 37˚C.

3.4.3. Bacterial Lawn Preparation

S. typhimurium (ATCC13312), S. choleraesuis subsp. *choleraesuis (ATCC13311), S. pullorum (ATCC19945),* and *S. choleraesuis* subsp. *arizonae (ATCC13314)* were grown individually as formerly described. TSA plates were labeled for each of the four *Salmonella* strains. Each plate received 0.1mL of the designated culture that was then

spread over the entire surface of the plate using a sterile disposable spreader. The plates were inverted and incubated for 24 hours at 37˚C resulting in a bacterial lawn for each of the four *Salmonella* strains. One lawn plate from each of the different *Salmonella* strains was combined to yield a four strain cocktail. This was done for each of the five fat/oil types.

3.4.4. Sample Preparation

All fat/oil stock bottles were obtained from refrigerator storage and warmed in a 50˚C water bath just until the fats/oils became liquid. The four *Salmonella* lawn plates were obtained from the incubator for the desired fat/oil. Using sterile technique in a Labconco Purifier Class II Biosafety Cabinet, 2mL of the desired liquid fat/oil was pipetted onto the surface of each bacterial lawn. Each of the bacterial lawns was then scraped into the fat/oil on the surface of the plate using a sterile disposable spreader. The culture rich fat/oil was then pooled from each of the bacterial lawn plates into a sterile 25mL beaker to make a four strain *Salmonella* cocktail for that specific fat/oil. The 25mL beaker was placed in a sterile 250mL beaker before being placed on a mildly heated, approximately 50˚C, stir plate in order to prevent overheating the culture rich fat/oil and to prevent possible splashing as the culture was mixed. The 8mL total of the four strain *Salmonella* cocktail was kept in a liquid state using the heated stir plate. The coconut oil and EVOO were not heated on the stir plate since they remained liquid at ambient temperature.

Two successive 1:10 dilutions were made in 9mL of sterile liquid fat/oil. After sufficient mixing, 8mL of the final dilution were added to 72mL of sterile liquid fat/oil in a sterile 250mL beaker to create the sample stock. This 250mL beaker was placed into a sterile 1000mL beaker to prevent overheating and splashing as the stock was kept in a liquid state and mixed. This sample stock, now approximately 6-log cfu/mL, was pipetted into 14 sterile disposable culture tubes. Each tube contained 5mL of the sample stock. The tubes were randomized and divided into two groups of seven tubes. The tubes were labeled Day 1-7 for the appropriate fat/oil held at 26˚C or 37˚C incubation. Day-1 samples were plated immediately following the sample set up while the other tubes were stored at their designated temperatures until their sampling time. All of this

was done using sterile technique so that *Salmonella* would be the only bacteria present and TSA could be used as the growth media for the study.

3.4.5. Sample Plating Procedure

Sample tubes for each fat/oil were obtained from the 26˚C and 37˚C incubators at the designated sample times. The fat samples were briefly warmed, approximately 10 minutes or less depending upon the fat type, in a 50˚C water bath until liquefied. This was not applicable for the oils which were already liquid. Dilution blanks and enrichments were warmed for the same amount of time as the samples so that the temperatures were the same when the fat/oils were pipetted into the tubes. Each of the Day-1 samples for the different fats/oils used three sequential dilution blanks. Each of the 1:10 dilution tubes were vortexed until the fat/oil sample was sufficiently mixed. The final dilution tube was poured into a sterile spiral plate cup. The sample was spiral plated in duplicate onto TSA at a 50µL spiral setting of Eddy Jet2. This was done for each of the Day-1 fat/oil samples at both 26˚C and 37˚C. The plates were inverted and incubated for 24 hours at 37˚C. The number of dilution blanks used for each of the following sample days depended upon the spread and overall growth of the bacteria from the previous day. The straight sample was never plated because of pilot studies which indicate that the bacteria present in the sample grew better when separated from the fat/oil. Thus, at least one dilution blank was used as the minimum dilution for each sample instead of the straight fat/oil. This only applied to the EVOO since the other fats/oil maintained their spiral plate readability. This procedure was conducted throughout the seven days that the fats/oils were sampled.

3.4.6. Enrichment Procedure

Enrichments were performed by pipetting 1mL of each day's fat/oil sample into 9mL of sterile lactose broth. The broths were then vortexed, labeled, and placed into the incubator for 24 hours at 37˚C. If the spiral plates showed bacterial growth then the enrichment tubes were discarded. If the spiral plates did not show growth then the enrichments were vortexed and streaked onto XLD which was then incubated at 37˚C for 24 hours. Enrichments were recorded as positive or negative for growth.

3.4.7. Reading Spiral Plate Procedure

Plates of the samples were read after 24 hours incubation at 37˚C. A FlashAndGo automated counter and FlashAndGo 1.0 Computer program were used to count the spiral plate colonies. These items were combined to form the FlashAndGo -Basic Economy Automated Colony Counter through ILU Instruments of NEU-TEC Group Inc. (1 Lenox Avenue, Farmingdale, NY 11735). The dilution factor was adjusted as needed for each of the plated fat/oil samples from that particular day and combined with the counted colonies to algorithmically determine the total bacterial count within the given sample. The duplicate plates were averaged together within Microsoft Excel to give a more accurate total count of the given sample.

3.4.8. Statistical Analysis

Statistical analysis was performed using SAS 9.4 with significance indicated at $p < 0.05$. Data organization and graphical figures were constructed using Microsoft Office Excel 2007. A bacterial reduction of 3-log cfu/mL or greater was noted as a significant reduction within the bacterial population.

3.5. Results

To satisfy the requirements and assumptions of an analysis of variance (ANOVA) the data was transformed to the square-roots. These results were later reverted to their original format, Log10, for interpretation. The Sphericity Tests yielded significance in both Transformed Variates (Pr>ChiSq <0.0001) and Orthogonal Components (Pr>ChiSq <0.0001). This indicated that there was significance in the statistical model. During the MANOVA Test, specifically the Hotelling-Lawley Trace test, the day (Pr>F <0.0001), day*trial (Pr>F 0.0209), day*fat (Pr>F <0.0001), day*fat*trial (Pr>F 0.0013), day*fat*temperature (Pr>F 0.0064), and day*fat with day*fat*trial error (Pr>F <0.0001) effect were all significant. This indicated that in the larger scope of the statistical model these effects and interactions held some potential statistical value across the study. Closer inspection of the repeated measures ANOVA showed that the fat type was significant with Pr>F <0.0001 for a between subjects effect. A repeated measures ANOVA for the within subjects effect showed that day (Pr>F <0.0001), day*trial (Pr>F

0.0496), day*fat (Pr>F <0.0001), and day*fat*trial (Pr>F 0.0024) effects were significant. This indicated that the fat type was the primary differentiating factor in the study. There was also a strong influence from the sample day and a weak influence from the trial, as well as, their potential interaction within the fat type throughout the week. Temperature was not significant in the experimental model overall nor was it significant as a between or within subject effect. Therefore, 26˚C and 37˚C were considered to behave similarly in their influence of the surviving bacterial populations over the seven day sample period.

All of the negative controls for each fat/oil, stored at 26˚C and 37˚C, were negative for growth and subsequent enrichment for each of the seven sample days. This ensured that the *Salmonella* species were the only bacteria present within the fat/oil throughout the experiment. The three positive controls that were performed alongside the fat/oil samples demonstrated the ability of the *Salmonella* species to survive within sterile phosphate buffer (SPB) plus Tween80 (0.1% v/v) over a seven day sample period. A repeated measures ANOVA found no significance in the within effects or between effects of the positive control bacterial counts from Day-1 through Day-7. When the difference between the bacterial counts of Day-1 and Day-7 were compared it was not found to be significant. No true 1-log cfu/mL increase or decrease was observed in the positive controls. This indicated that the *Salmonella* species could survive at a consistent infective level for at least seven days at both 26˚C and 37˚C in SPB plus Tween80 (0.1% v/v).

All of the *Salmonella* species survived all seven days in the duck fat, pig lard, beef tallow, and coconut oil at similar levels to those of the initial sample tested on Day-1. These survival results were confirmed with visual observations of isolation streaks on XLD. Table 3.1. shows the surviving bacterial averages for the duck fat, pig lard, beef tallow, and coconut oil at 26˚C and 37˚C over the seven sample days. It was noted that none of the animal fats or coconut oil reached the 3-log cfu/mL reduction threshold.

TABLE 3.1. Surviving bacterial averages in duck fat, pig lard, beef tallow, coconut oil, and extra virgin olive oil

	Average Surviving *Salmonella* counts in Log10 cfu/mL						
Fat/Oil Type	**Day-1**	**Day-2**	**Day-3**	**Day-4**	**Day-5**	**Day-6**	**Day-7**
Positive C (26˚C)	7.95 [a]	8.04 [a]	7.93 [a]	7.95 [a]	7.94 [a]	7.99 [a]	8.02 [a]
Positive C (37˚C)	7.94 [a]	7.97 [a]	7.98 [a]	7.99 [a]	7.99 [a]	7.86 [a]	8.01 [a]
Duck Fat (26˚C)	7.27 [a]	7.39 [a]	7.02 [a]	7.61 [a]	7.51 [a]	7.27 [a]	7.48 [a]
Duck Fat (37˚C)	6.86 [a]	7.31 [a]	7.57 [a]	6.95 [a]	6.87 [a]	6.60 [a]	6.52 [a]
Pig Lard (26˚C)	6.86 [a]	6.52 [a]	6.88 [a]	7.18 [a]	6.44 [a]	6.90 [a]	6.77 [a]
Pig Lard (37˚C)	6.48 [a]	6.97 [a]	6.87 [a]	6.00 [a]	6.73 [a]	6.58 [a]	6.56 [a]
Beef Tallow (26˚C)	6.76 [a]	7.49 [a]	6.56 [a]	6.80 [a]	6.51 [a]	6.63 [a]	6.28 [a]
Beef Tallow (37˚C)	6.57 [a]	6.88 [a]	6.13 [a]	6.23 [a]	6.11 [a]	6.15 [a]	5.77 [a]
Coconut Oil (26˚C)	7.23 [a]	7.24 [a]	6.96 [a]	6.96 [a]	6.49 [a]	6.23 [b]	6.51 [a]
Coconut Oil (37˚C)	7.70 [a]	7.21 [a]	7.06 [a]	5.57 [b]	6.14 [b]	6.83 [a]	6.76 [a]
EVOO (26˚C)	6.68 [a]	3.36 [c]	2.32 [c]	1.59 [c]	0.82 [c]	0.82 [c]	1.88 [c]
EVOO (37˚C)	6.89 [a]	3.91 [c]	1.00 [c]	1.62 [c]	1.84 [c]	0.82 [c]	3.34 [c]

- All resulting numbers are the LOG10 bacterial counts from the TSA plates counted in cfu/mL
- All enrichments in lactose broth were positive for Duck Fat, Pig Lard, Beef Tallow, and Coconut Oil (data not shown)
- Counts of 1.00 or less indicate a *Salmonella* strain was recovered in the enrichment so the true surviving bacterial counts were <1-log cfu/mL
- (a) represents a lack of statistical significance comparing the bacterial populations of a sample to the initial bacterial populations, (b) represents a 1-log cfu/mL reduction from the initial bacterial population, (c) represents a ≥3-log cfu/mL reduction from the initial bacterial population

The duck fat, pig lard, and beef tallow maintained an average *Salmonella* count throughout the week similar to the initial bacterial population at both 26˚C and 37˚C incubation. Statistical significance for each animal fat was considered for various effects between and within the repeated measures ANOVAs. Differences within the bacterial population were attributed to various samples needing a longer exposure time in the heated water bath to liquefy the fat. Prolonged heating could have compromised some of the surviving bacteria and resulted in lower recoverable counts, thus explaining the slight variation among some days. Significance, for these effects, was eventually discarded because the data variation gave no true 1-log cfu/mL reduction over the seven sample days. Overall none of the animal fats displayed any practical significance of a 3-log cfu/mL reduction as seen in Figure 3.1. This indicated that the *Salmonella* species could survive at a consistent infective level within the duck fat, pig lard, and beef tallow for a period of at least seven days at both 26˚C and 37˚C. This supports the hypothesis that no

change would occur from the initial *Salmonella* inoculum levels over the seven day period within the animal fats.

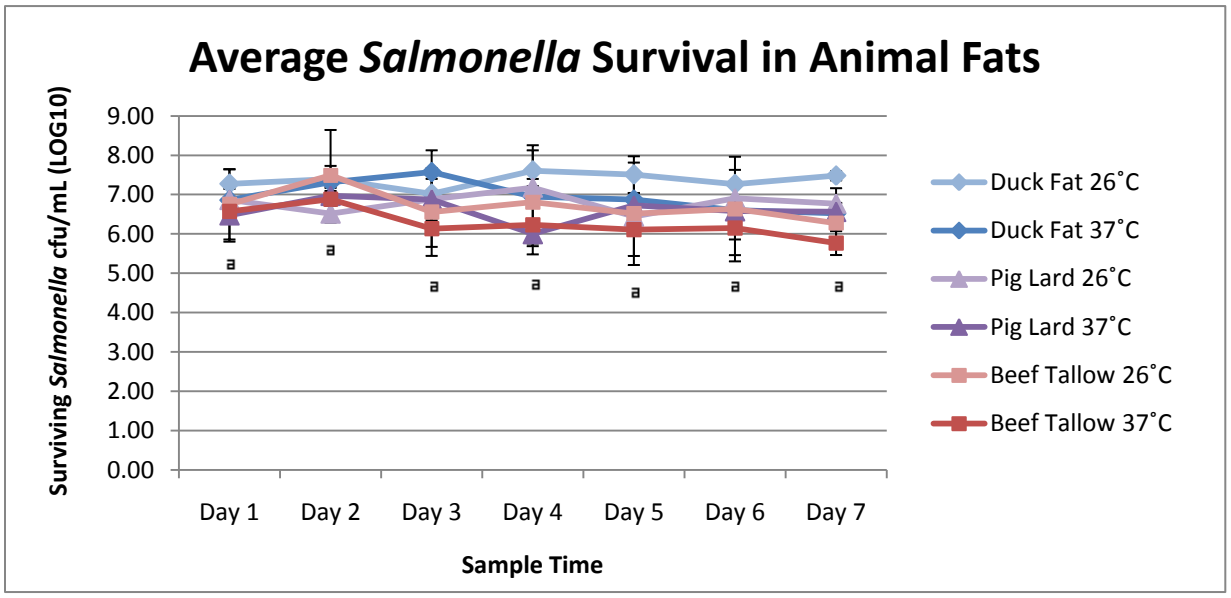

Figure 3.1. The average *Salmonella* survival in duck fat, pig lard, and beef tallow at 26˚C and 37˚C

- (a) represents a lack of statistical significance comparing the bacterial populations of a sample to the initial bacterial populations (PC data not shown)

The coconut oil, displayed in Figure 3.2., had a slight decrease from the average bacterial population of 7-log cfu/mL that was surviving at 26˚C and 37˚C over the seven day period. A repeated measures ANOVA was performed and no significance was found between effects for the coconut oil from Day-1 through Day-7. Statistical significance was only found within a few of the sample days when compared to the initial bacterial population. The average surviving bacterial counts did not experience a true 1-log cfu/mL reduction even though some of the individual trials saw 1-log cfu/mL reductions by the seventh sample day. There was also a lack of consistency in maintaining the 1-log cfu/mL reduction in the samples as seen by the bacterial count rebound in the averages of the coconut oil. Moreover, the coconut oil did not reach the 3-log cfu/mL bacterial reduction threshold. When the bacterial survival counts of Day-1 and Day-7 were compared no significance was shown in coconut oil. This indicated that the *Salmonella* species could survive at a relatively consistent infective level within the coconut oil for at least seven days at both 26˚C and 37˚C. This supports the hypothesis that no change

would occur from the initial *Salmonella* inoculum levels over the seven day period within the coconut oil.

The *Salmonella* species did not all survive for the entire seven day period in the EVOO as seen in Figure 3.2. The bacterial counts within the EVOO were the only counts to experience a 3-log cfu/mL reduction. In contrast to the consistency experienced by the other fats and oil, the *Salmonella* showed a dramatic decline in the average surviving bacterial population at 26˚C and 37˚C over the seven sampling days in the EVOO. A repeated measures ANOVA was performed and found no significance between the effects of the EVOO. Significance of Pr>F <0.0001 was found within the days of the EVOO. This indicated the decrease was significant in the surviving bacterial population over the seven days in the EVOO.

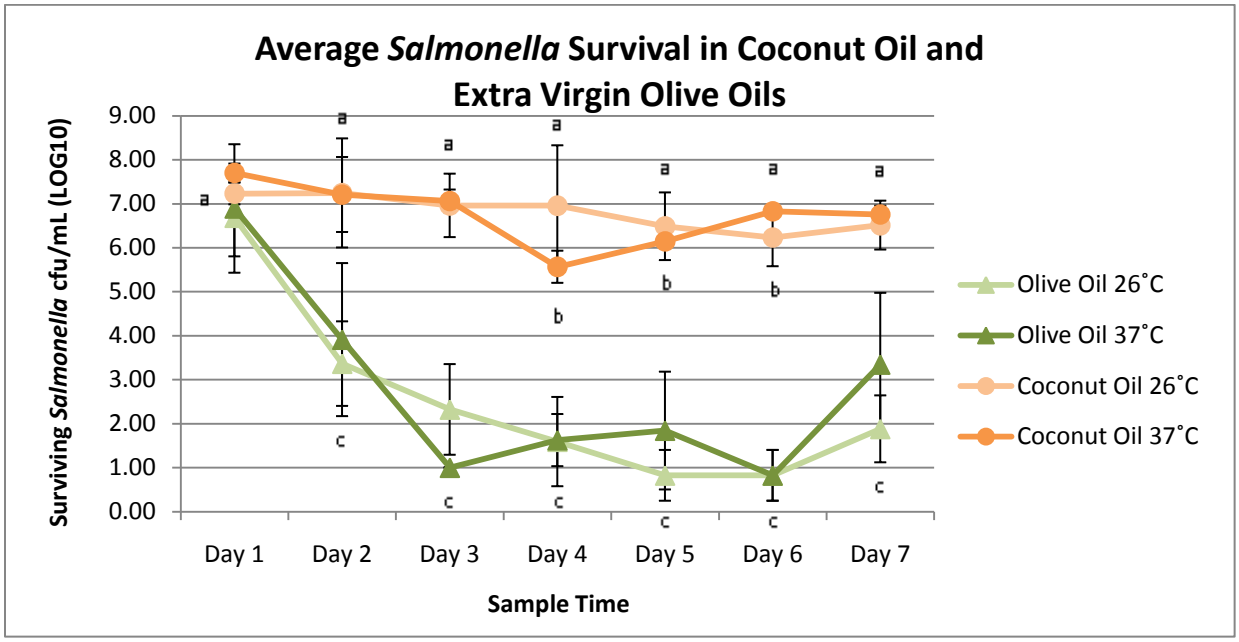

Figure 3.2. The average *Salmonella* survival in coconut oil and extra virgin olive oil at 26˚C and 37˚C

- (a) represents a lack of statistical significance comparing the bacterial populations of a sample to the initial bacterial populations, (b) represents a 1-log cfu/mL reduction from the initial bacterial population, (c) represents a ≥3-log cfu/mL reduction from the initial bacterial population

Surprisingly, the comparison of Day-1 and Day-7 did not produce any statistically significant results when modeled. This lack of significance was attributed to the large errors associated with the recoverability of different trials. It was later determined that a

single *Salmonella* strain had caused the inconsistent recovery which had led to the variability among the trials. Despite this statistical insignificance the EVOO was the only substance to meet the 3-log cfu/mL bacterial reduction. All trials, except Trial-1 at 37˚C which began at a lower initial count than expected, experienced a 3-log cfu/mL reduction or greater within a 24 hour time frame at both 26˚C and 37˚C. This decrease was also displayed in the surviving bacterial averages for the EVOO which decreased from 6-log cfu/mL to 3-log cfu/mL for both 26˚C and 37˚C. Within 48 hours, the surviving bacterial counts were <1-log cfu/mL for each of the samples except Trial-3 at 26˚C which maintained its bacterial counts of 2-log cfu/mL. It was later determined that a single resistant strain of *Salmonella* was the cause of the inconsistent bacterial decline over the seven day period. *Salmonella choleraesuis* subsp. *arizonae* (ATCC 13314), was identified from recovered Day-7 TSA colony picks and enrichments streaked for isolation on XLD. Confirmation was obtained by running the organism through Vitek2. Despite this inconsistency, all samples reached a state of <1-log cfu/mL by Day-6 on the spiral plates. Only Trial-1 (37˚C) Day-5, Trial-2 (26˚C) Day-4, Trial-3 (26˚C) Day-5, Trial-3 (26˚C) Day-6, Trial-3 (37˚C) Day-5, and Trial-3 (37˚C) Day-6 were <1-log cfu/mL cfu/mL for both the spiral plates and the enrichments. These results were very important because they showed that without the presence of the resistant *Salmonella* strain the other strains were susceptible to the EVOO and were damaged below the level of detection (<1-log cfu/mL). Overall, the 3-log cfu/mL reduction experienced by the *Salmonella* indicated that EVOO may not be a viable source for all types of *Salmonella* contamination. There may still be other resistant strains of *Salmonella* in which EVOO may remain a viable source for contamination. For this study, not all *Salmonella* species could survive at a consistent infective level within the EVOO for a period of seven days at both 26˚C and 37˚C. This data rejects the hypothesis that no change would occur from the initial *Salmonella* inoculum levels over the seven day period within the EVOO.

3.6. Discussion

The survival of the *Salmonella* satisfies the purpose of this study in that coconut oil, duck fat, pig lard, and beef tallow stored at room temperature (26˚C) and an abused

temperature (37°C) remain viable sources for *Salmonella* contamination even after a seven day storage time. This bacterial survival has also been shown in other high fat products such as peanut butter, butter, and oil-based spreads. Burnett et al. (2000) reported that during a survival study all reduced sugar/low sodium peanut butters, reduced fat peanut spreads, and traditional peanut butters (except the natural peanut butter) remained positive for *Salmonella* at 21˚C storage for 24 weeks. *Salmonella* was recovered from all products at 5˚C storage.[20] This study supports the ability of *Salmonella* to survive in high fat contents with low water activities for long enough time periods to reach the market and potential consumers. Holliday et al. (2003) found that *Salmonella* species survived for three weeks in sweet whipped salted butter at 21˚C. *Salmonella* also survived in light salted butter, yellow fat spread-1, and light margarine for one week and in sweet whipped salted butter, sweet whipped unsalted butter, and yellow fat spread-2 for three weeks at 4.4˚C.[78] It is thought that temperature stress and preservatives interfered with the survival of the *Salmonella*.[78] Despite this interference, the study supports the ability of *Salmonella* to survive for prolonged periods of time by residing within fat-based products. In contrast to the complex food items presented in the peanut butter and butter studies, the current study revealed the survival ability of *Salmonella* simply within rendered animal fats and plant based oils. This is an important distinction in that no other nutrients were available to the *Salmonella* outside of those naturally occurring within the fats and oils. Coconut oil, duck fat, pig lard, and beef tallow remained viable environments for the survival of the four *Salmonella* species over the seven day study at both 26˚C and 37˚C.

The survival of *Salmonella* during the brief heat treatment in the 50˚C water bath, used to liquefy the animal fat for sampling, indicates a potential point of contamination within pet food production line. The brief heat treatment of fat to the point of liquification is insufficient to kill potential organisms being held within the fat. Juneja and Eblen (2000) drew a contrast between the heating time needed to kill *Salmonella* in chicken broth at 3% fat verses ground beef at 7%, 12%, 18%, and 24% fat. The homogenous nature of the chicken broth allowed for even heating and resulted in a predictable linear death rate while increased fat levels within the ground beef increased

the lag period in the survival of *Salmonella* when exposed to various heating levels.[86] It was hypothesized that this survival was due to poor heat transfer attributed to decreased water activity from increased fat levels and the buffering capacity of these fat levels which kept the *Salmonella* from experiencing substantial injury during heating.[74,86] This study supports the idea that increased levels of fat within ground beef products contribute to the temporary extended survival of *Salmonella* species during a heating process. Juneja et al. (2001) later validated and expanded their former findings to explore the survival rate of *Salmonella* in ground chicken (7% fat), turkey (9% fat), and pork (8.5% fat) during cooking. As expected, the higher fat content and more complex composition of the meat resulted in longer lag periods which allowed the *Salmonella* to survive longer in those products than broth.[87] This study also supported the idea that prolonged lag times in *Salmonella* were due to increased fat levels within the ground meat products which contributed to the survival of the bacteria when exposed to various heating levels. A similar effect was seen in the current study where the inoculated animal fats were briefly heated to a liquid state so that they could be sampled. Like the fat effects in the Juneja studies (2000 and 2001), it was thought that some buffering capacity allowed for the gradual heating of the fat to a liquid state without compromising the bacteria. The coconut oil carried a similar risk since it remained in a natural liquid state for the seven day period. Without an additional heat treatment sufficient to kill any *Salmonella* the coconut oil has the same potential as the minimally heated animal fats to carry any existing bacteria onto the food product itself.

Although the current study mimicked a pre-processing contamination of the animal fats and coconut oil, *Salmonella* could potentially cause post-processing contamination by lodging itself within the fat on a finished product. González-Forte et al. (2014) showed the survival of probiotic *Lactobacillus plantarum* in a gelatinized corn starch coating and a calcium alginate coating that were dried onto two formulations of dry dog biscuits. The coatings provided a greater protection level and survival rate for the probiotic bacteria during simulated digestion tests and a month long storage time at 20˚C compared to the probiotic bacteria of control biscuits without coatings.[67] Although these coatings were not fat based they do provide some insight into the possibility of

various bacteria surviving drying conditions through the application of food based coatings. As seen in the previously listed studies of Burnett et al. (2000) and Holliday et al. (2003), *Salmonella* species are more than capable of residing within complex food products that contain high amounts of fat and nutrients. The current study has also confirmed the ability of *Salmonella* to reside in pure fat/oil, with limited access to any nutrients outside of those naturally occurring within the fat/oil itself, for at least a week at both 26˚C and 37˚C. Based on the limitations of this current study it can only be hypothesized that the survival of *Salmonella* species in coconut oil, duck fat, pig lard, and beef tallow over a week long time frame allows for the possible introduction of the pathogen during processing with minimal heat treatments and the possibility of the bacteria residing within the fat on the finished pet food product.

The rapid decrease of *Salmonella* in the EVOO over the study was unexpected. On average, the *Salmonella* species experienced a 3-log cfu/mL reduction within the first 24 hours of inoculation into the EVOO. All samples reached <1-log cfu/mL counts by Day-6, with several samples being below the level of detection on both the spiral plates and in the enrichments from Day-4 to Day-6. *Salmonella typhimurium* (ATCC 13312), *Salmonella choleraesuis* subsp. *choleraesuis* (ATCC 13311), and *Salmonella pullorum* (ATCC 19945) did not survive until Day-7 as confirmed by isolation streaks on XLD. The survival of one resistant *Salmonella* strain, *Salmonella choleraesuis* subsp. *arizonae* (ATCC 13314), was identified by XLD isolation and validated through Vitek2 from Day-seven samples. This *Salmonella* had the ability to form colonies on the spiral plated TSA agar and isolated colonies from the enrichments plated on XLD. This indicates that this particular strain was very robust because it was not solely recovered in the enrichments, but grew large and seemingly undamaged colonies even during spiral plating. Recovery of the strain in the enrichments alone would have shown a greater sense of damage to the bacteria. The variation in the recoverable numbers may be attributed to how much of this particular strain was initially present in each of the randomly filled sample tubes. The other three strains were below the level of detection (<1-log cfu/mL) due to their exposure to the oil. These strains were absent on both the spiral plates and in the enrichments after four to six days. The three strain populations may have decreased to

this level sooner than four days, but the presence of the resistant *Salmonella choleraesuis* subsp. *arizonae* (ATCC 13314) strain in the enrichments confounded the exact time.

Similarly, Medina et al. (2006) observed antimicrobial abilities while comparing the effects of several edible oils on the survival of different pathogens. Overall, the study found that the olive oils had the strongest antimicrobial effects against the bacteria while the sunflower, corn, rapeseed, soybean, and cotton oil had minimal inhibitory effects.[118] The most effective antimicrobial effects were displayed in extra virgin olive oil (EVOO), then refined olive oil, and then pomace olive oil which reflected the potency of the phenolic and lesser secondary components that are thought to be the active antimicrobial agents within the oils.[118,65] The antimicrobial ability of EVOO does however have its limitations, as shown by the resistance of Salmonella *choleraesuis* subsp. *arizonae* (ATCC 13314) in the current study and the lesser effect on *Salmonella enteric* in the Medina study. The lethal effects against the other three *Salmonella* species in this study may indicate lethality towards other *Salmonella* strains as well as other bacterial species. Overall, the EVOO did not remain a viable environment for three *Salmonella* species. It is unlikely that olive oil would be a potential source of contamination for these three susceptible *Salmonella* species; however, other species may display a similar resistance to the oil like Salmonella *choleraesuis* subsp. *arizonae* (ATCC 13314). It is therefore important to note that the use of EVOO within the food industry would best serve as an additional antimicrobial hurdle in the processing procedure to help minimize the likelihood of *Salmonella* contamination.

3.7. Conclusion

Salmonella typhimurium (ATCC 13312), *Salmonella choleraesuis* subsp. *choleraesuis* (ATCC 13311), *Salmonella pullorum* (ATCC 19945), and *Salmonella choleraesuis* subsp. *arizonae* (ATCC 13314) all survived the seven day study in duck fat, pig lard, and beef tallow at 26˚C and 37˚C. All of these animal fats confirmed the experimental hypothesis and remained viable sources for potential *Salmonella* contamination within the pet food industry by maintaining counts equal to those on the first day at both temperatures. With the survival of all four *Salmonella* strains and no consistent 1-log

cfu/mL reduction it was determined that coconut oil held minimal antimicrobial ability like the animal fats. Thus, the coconut oil confirmed the experimental hypothesis and remained a viable source of potential *Salmonella* contamination at both temperatures despite a slight decrease in bacterial counts over the seven day period. The extra virgin olive oil (EVOO) did not support the survival of *Salmonella typhimurium* (ATCC 13312), *Salmonella choleraesuis* subsp. *choleraesuis* (ATCC 13311), or *Salmonella pullorum* (ATCC 19945) at either 26˚C or 37˚C. The EVOO caused an average 3-log cfu/mL reduction within 24 hours with an eventual decrease to <1-log cfu/mL by Day-6. *Salmonella choleraesuis* subsp. *arizonae* (ATCC 13314) proved to be a resistant *Salmonella* strain through its recovery on both the spiral plated TSA counts and the lactose broth enrichments at both 26˚C and 37˚C. Overall the experimental hypothesis was rejected for the EVOO since it failed to maintain its bacterial counts from the first day and reached the reduction threshold of 3-log cfu/mL despite the resistant *Salmonella* strain. No statistical difference between 26˚C and 37˚C was reflected in the survival rates of *Salmonella* within the fats and oils.

CHAPTER 4

THE SURVIVAL OF A SHIGA-TOXIN PRODUCING *ESCHERICHIA COLI* (*STECs*) COCKTAIL IN EXTRA VIRGIN OLIVE OIL

4.1. Summary

The purpose of this study was to determine if extra virgin olive oil would support the survival of Shiga-toxin producing *Escherichia coli* (*STEC*). Inoculated stocks of oil were prepared using a five strain *STEC* cocktail and divided into 5mL samples among 14 disposable tubes. The tubes were randomized and held at two different temperatures, 26˚C and 37˚C, for seven days. Each tube was only sampled once. The hypothesis of the study was that the extra virgin olive oil would cause at least a 3-log reduction in the initial *STEC* inoculum levels, approximately 6-log cfu/mL, over the seven day period at both 26˚C and 37˚C. An average 3.5-log cfu/mL reduction threshold was met after 24 hours. *Escherichia coli STEC 15AB* (ATCC 99-3311) remained the only recoverable strain within the extra virgin olive oil after seven days at both temperatures. No statistical difference was found between holding samples at 26˚C or 37˚C throughout the study.

4.2. Introduction

Shiga-toxin producing *Escherichia coli* (*STECs*) are *E. coli* with genes that encode one or more Shiga-toxins otherwise known as Vero-toxigenic *E. coli*.[188] The Shiga-toxin structure is that of an A_1B_5 toxin, which includes five B subunits that bind to the eukaryotic cell surface and an active A subunit that enters the cell to disrupt protein synthesis.[50] The ability to produce this toxin is the differentiating virulence factor between *STECs* and other *E. coli*. *STEC* serotypes which have somatic antigen (O) 157 and flagellar antigen (H) 7 are most often associated with hemolytic uremic syndrome (HUS).[188] Due to the severity of the gastrointestinal illnesses and associated deaths, the most well known *STEC* is *E. coli O157:H7*.

STECs have been implicated in numerous human illnesses ranging from contaminated greens[85,115,129,186], cured meats[35,175], raw milk [40,72], ground beef [6,41,159], and unpasteurized apple cider.[8,33] There have also been reported cases from person-person

transfer[22,114] and animal-person transfer.[46,184] Some of the animal-person transfers were even thought to be possible transmission of *E. coli* from household pets.[7,10,170] Despite the multitude of recall cases in human foods, the FDA has no current or archived cases of pet food recalls caused by *STECs*, *E. coli O157:H7*, or general *E. coli*.[59] Although some of these implicated human food products such as flours, meats, and spices[60], can be used in pet food products, *Salmonella* and *Listeria* remain the primary organisms of concern in pet foods.

Nemser et. al. 2014, reported that 10 of the 576 randomly sampled raw pet foods were positive for non-*O157 STECs*. No other *STECs* or *O157:H7 E. coli* were detected in any of the exotic foods, jerky-type treats, or the 480 dry/semi-moist pet foods.[135] This study supports the idea that *E. coli*, specifically *STECs*, are not the primary organisms of concern when dealing with dry pet food products; however, there is still cause for safety concerns over the production of raw pet food diets and treats which may be contaminated.

The purpose of this study was to determine if extra virgin olive oil (EVOO) had any inhibitory properties toward various strains of Shiga-toxin producing *E. coli* (*STEC*), including *E. coli O157:H7*, over a seven day period. The hypothesis of the study was that the EVOO would cause at least a 3-log cfu/mL reduction in the initial *STEC* inoculum levels over the seven day period at both 26˚C and 37˚C. More importantly, this study investigates the overall antimicrobial abilities of EVOO against five *STEC* strains, including *E. coli O157:H7*, which may also have implications within the raw pet foods and various ready-to-eat human foods.

4.3. Materials

4.3.1. Pathogens

Escherichia coli O157:H7 (ATCC 35150), *Escherichia coli STEC 15AB* (ATCC 99-3311), *Escherichia coli STEC 15AE* (ATCC 2006-3008), *Escherichia coli STEC 15AF* (ATCC 2002-3211), and *Escherichia coli STEC 15AG* (ATCC 0111) were the five strains of *Escherichia coli* utilized in the study.

4.3.2. Evaluated Product

One commercial extra virgin olive oil (EVOO) was evaluated on its ability to harbor *STECs* at 26˚C and 37˚C over seven days. Primo Gusto: Italian Foods 100% Italian Extra Virgin Olive Oil (cold pressed) (country of origin: Italy via Gordon Food Service® P.O. Box 1787, Grand Rapids, MI 49501) was purchased from a local grocery store. This product was chosen because it did not contain any additives or preservatives which could have hindered the survival rate of the bacteria within the product.

4.3.3. Medias

The broths utilized in the *STEC* study included Bacto™ Brain Heart Infusion (BHI) and BBL™ Lactose broth. Both products were Becton, Dickinson and Company (7 Loveton Circle, Sparks, MD, 21152) and were prepared within the specifications. The BHI was dispensed into Pyrex tubes with a final volume of 7mL each and autoclaved. The lactose broth was dispensed into Pyrex tubes with a final volume of 9mL each and autoclaved. Dilution blanks utilized throughout the study were Sterile Phosphate Buffer dilution blank tubes. These dilution tubes were made from 24mL PO_4 solution, 95mL $MgCl_2$ solution, and 19L of double deionized water. Tween80 was added to the dilution blank mixture at 1mL/1L so that the final product contained 0.1% Tween80. The dilution mixture with Tween80 was adjusted to a pH between 7.4-7.5. The mixture was then dispensed into Pyrex tubes with a final volume of 9mL and autoclaved. The agars utilized in the *STEC* study included BBL™ Trypticase™ Soy Agar: Soybean-Casein Digest Agar (TSA), Difco™ MacConkey Agar (MAC), and Biolog Rainbow Agar O157 (Rainbow agar). Both TSA and MAC were Becton, Dickinson and Company (7 Loveton Circle, Sparks, MD, 21152) and were made according to the product specifications. The Rainbow agar was purchased from Biolog Inc. (21124 Cabot Blvd, Hayward, CA 94545) and was made according to the specifications on the package. All agars were poured into sterile 100x15mm polystyrene disposable VWR Petri plates (1050 Satellite Blvd NW, Suwanee, GA 30024).

4.4. Methods

All of the methodologies are derived from the procedure utilized in the *Salmonella* study (Chapter 3).

4.4.1. Oil Sterility Validation

The oil was transferred using sterile technique from its original container into sterile 250mL glass bottles with caps. Samples of the oil were taken and plated on TSA and MAC and incubated for 48 hours at 37˚C (also TSA incubated for 48 hours at 26˚C) to check for bacterial background. The oil was confirmed to be commercially sterile with an aerobic count below the level of detection. These oil stock bottles were then wrapped in foil and placed in refrigerator storage to prevent any additional oxidation or contamination.

4.4.2. Inoculum Preparation

A scrape of each desired *STEC* strain was obtained from a pre-existing refrigerated BHI slant and was transferred into BHI broth using sterile technique. The culture was incubated for 24 hours at 37˚C. The culture was then streaked for isolation onto MAC agar and was incubated for 24 hours at 37˚C. The morphology of the colonies was observed and an isolated colony was transferred to a new tube of BHI. The culture was then incubated for 24 hours at 37˚C. The culture was transferred twice by placing 0.1mL of the former culture into new BHI and incubating for 24 hours at 37˚C.

4.4.3. Bacterial Lawn Preparation

Escherichia coli O157:H7 (ATCC 35150), *Escherichia coli STEC 15AB* (ATCC 99-3311), *Escherichia coli STEC 15AE* (ATCC 2006-3008), *Escherichia coli STEC 15AF* (ATCC 2002-3211), and *Escherichia coli STEC 15AG* (ATCC 0111) were grown individually as formerly described. TSA plates were labeled for each of the five *STEC* strains. Each plate received 0.1mL of the designated culture spread over the entire surface of the plate using a sterile disposable spreader. The plates were incubated for 24 hours at 37˚C to form a bacterial lawn of each of the five *STEC* strains. One lawn plate from each of the *STEC* strains was used with the EVOO resulting in a five strain cocktail.

4.4.4. Sample Preparation

The oil stock bottles were obtained from refrigerator storage and allowed to come to ambient temperature, 25˚C. The five *STEC* lawn plates were obtained from the incubator. Using sterile technique in a Labconco Purifier Class II Biosafety Cabinet, 2mL of the oil was pipetted onto the surface of each bacterial lawn. Each of the bacterial lawns was then scrapped into the oil on the surface of the plate using a sterile disposable spreader. The culture rich oil was then pooled from each of the bacterial lawn plates into a sterile 25mL beaker to make a five strain *STEC* cocktail. The 25mL beaker was placed in a sterile 250mL beaker before being placed on a stir plate in order to prevent possible splashing as the culture was mixed. The 10mL total of the five strain *STEC* cocktail was sufficiently mixed with a sterile stir rod. Two successive 1:10 dilutions were made in 9mL of sterile liquid oil. After sufficient mixing, 8mL of the final dilution were added to 72mL of sterile oil in a sterile 250mL beaker to create the sample stock. This 250mL beaker was placed into a sterile 1000mL beaker to prevent splashing as the stock was kept in a liquid state and mixed. This sample stock, now approximately 6-log cfu/mL, was pipetted into 14 sterile disposable culture tubes. Each tube contained 5mL of the sample stock. The tubes were randomized and divided into two groups of seven tubes. The tubes were labeled Day 1-7 with the appropriate temperature and were placed at 26˚C or 37˚C incubation. Day-1 samples were plated immediately following the sample set up while the other tubes were stored at their designated temperatures until their sampling time. All of this was done using sterile technique so that *STECs* would be the only bacteria present and TSA could be used as the growth media for the study.

4.4.5. Sample Plating Procedure

Sample tubes from the 26˚C and 37˚C incubators were obtained at the designated sample times. The Day-1 samples were vortexed and then three sequential dilution blanks were used. Each of the 1:10 dilution tubes were vortexed until the oil sample was sufficiently mixed. The final dilution tube was poured into a sterile spiral plater cup and placed in the spiral plater. The sample was spiral plated in duplicate onto TSA at a 50µL spiral setting of Eddy Jet2. This was done for the Day-1 samples at both 26˚C and 37˚C. The plates were inverted and incubated for 24 hours at 37˚C. The number of dilution

blanks used for each of the following sample days depended upon the spread and overall growth of the bacteria from the previous day. If the bacteria appeared to have a consistent and readable spread across the plate then the number of dilution blanks used remained the same. If however, the bacterial counts appeared to be decreasing then the number of dilution blanks used for that sample were decreased to keep the bacteria at readable levels. The straight sample was never plated. This was because of pilot studies which indicate that the bacteria present in the sample grew better when separated from the straight oil (data not shown). Thus, at least one dilution blank was used as the minimum dilution for each sample rather than just plating the straight oil. This procedure was conducted throughout the seven days that the oil was sampled at the two temperatures.

4.4.6. Enrichment Procedure

Enrichments were performed by pipetting 1mL of the sample into 9mL of sterile lactose broth on the sample day. The broths were then vortexed, labeled, and placed into the incubator for 24 hours at 37˚C. If the spiral plates showed bacterial growth then the enrichment tubes were removed from the incubator and discarded. If the spiral plates did not show growth then the enrichments were removed from the incubator, vortexed, and streaked onto MAC and Rainbow agar which was then incubated at 37˚C for 24 hours. Enrichments were recorded at positive or negative for growth.

4.4.7. Reading Spiral Plate Procedure

Plates of the samples were read after 24 hours incubation at 37˚C. A FlashAndGo automated counter and FlashAndGo 1.0 Computer program were used to count the spiral plate colonies. These items were combined to form the FlashAndGo -Basic Economy Automated Colony Counter through ILU Instruments of NEU-TEC Group Inc. (1 Lenox Avenue, Farmingdale, NY 11735). The dilution factor was adjusted as needed for each of the plated oil samples from that particular day and combined with the counted colonies to algorithmically determine the total bacterial count within the given sample. The duplicate plates were averaged together within Microsoft Excel to give a more accurate total count of the given sample.

4.4.8. Statistical Analysis

Statistical analysis was performed using SAS 9.4 with significance indicated at $p < 0.05$. Data organization and graphical figures were constructed using Microsoft Office Excel 2007. A bacterial reduction of 3-log cfu/mL or greater was noted as a significant reduction within the bacterial population.

4.5. Results

A split-Split Plot design was used to analyze the data of this study. A Univariate Student's t Test revealed that there was overall significance of Pr>t <0.0001within the statistical model. When the effects were observed fat was significant with Pr>F 0.0006, day was significant with Pr>F <0.0001, and fat*day was significant with Pr>F <0.0001. Overall, this indicated a significant difference between the surviving *STEC* counts observed in EVOO and those observed in the positive control. The significant difference across the days displayed an overall shift in the surviving bacteria throughout the sample time of the study. The significance of fat*day effect indicated that the interaction of these two variables contributed to the statistical value across the study. The fat type was the primary differentiating factor within the study as displayed through its influence over the surviving bacterial counts across each sample day. Temperature was not significant in the experimental model overall, nor was it significant in any subject interaction; thus, 26˚C and 37˚C were considered to behave similarly in their influence of the surviving bacterial populations over the seven day sample period.

All of the negative controls for the EVOO were negative for growth and enrichment for each of the seven sample days at 26˚C and 37˚C storage. This ensured the *STEC* strains were the only bacteria present within the oil throughout the experiment. The three positive controls that were conducted alongside the oil samples validated the ability of the *STEC* strains to survive within sterile phosphate buffer (SPB) plus Tween80 (0.1% v/v) over a seven day sample period. A split-Split Plot model found no significance in the temperatures, the trials, or the days of the positive control bacterial counts from Day-1 through Day-7. All bacterial counts remained within the same 7-log cfu/mL values from Day-1 compared to Day-7. This indicated that the *STEC* strains

could survive at a consistent infective level for at least seven days at both 26˚C and 37˚C in SPB with Tween80 (0.1% v/v).

The bacterial counts within the EVOO were dramatically decreased in the average surviving bacterial population at 26˚C and 37˚C over the seven sampling days. An average 3.5-log cfu/mL reduction was observed within the *STECs* during the first 24 hours of the study at both temperatures. Following 48 hours in the oil, the bacterial population had reduced by 6-log cfu/mL at both temperatures. From Day-4 onward the bacterial counts were not detectable using the spiral plate method on TSA (<1-log cfu/mL); however, *STEC* strains were still recovered in enrichments. It was later determined that these positive enrichments were due to a single resistant strain. Table 4.1. shows the surviving bacterial averages for the EVOO and positive controls at 26˚C and 37˚C over the seven sample days.

TABLE 4.1. Surviving bacterial averages in extra virgin olive oil and PCs in SPB+Tween80

	Average Surviving *STEC* counts in Log10 cfu/mL						
Medium Type	**Day-1**	**Day-2**	**Day-3**	**Day-4**	**Day-5**	**Day-6**	**Day-7**
Positive C (26˚C)	7.83 [a]	7.76 [a]	7.79 [a]	7.85 [a]	7.87 [a]	7.89 [a]	7.68 [a]
Positive C (37˚C)	7.81 [a]	7.85 [a]	7.80 [a]	7.71 [a]	7.85 [a]	7.75 [a]	7.73 [a]
EVOO (26˚C)	7.25 [a]	3.60 [b]	1.20 [b]	1.00 [b]	0.75 [b]	0.50 [b]	0.50 [b]
EVOO (37˚C)	7.26 [a]	3.72 [b]	1.24 [b]	1.00 [b]	0.75 [b]	0.50 [b]	0.25 [b]

- All resulting numbers are the LOG10 bacterial counts from the TSA plates counted in cfu/mL
- Counts of 1.00 or less indicate an *STEC* strain was recovered in the enrichment so the true surviving bacterial counts were <1-log cfu/mL
- (a) represents a lack of statistical significance comparing the bacterial populations of a sample to the initial bacterial populations, (b) represents a ≥3-log cfu/mL reduction from the initial bacterial population

A split-Split Plot model was performed for the EVOO. No significance was found in the temperatures or the trials of the oil. Significance was found within the days of the EVOO with Pr>$|t|$<0.0001. This was shown by comparing the surviving bacterial counts of Day-2 through Day-7 to the initial bacterial counts of Day-1. All surviving bacterial counts from the other sample days showed a significance of Pr>$|t|$ <0.0001 when compared to Day-1. All surviving bacterial counts from the other sample days also showed a significance of Pr>$|t|$ <0.0001 when compared to Day-2. The surviving bacterial counts of Day-6 and Day-7 remained significant when compared to the bacterial counts of Day-3 with Pr>$|t|$ 0.0339 and Pr>$|t|$ 0.0134 respectively. By Day-4 no

significant difference was found between the surviving bacterial counts because the counts remained at 1.00 cfu/mL or less. This dramatic bacterial reduction and subsequent plateau was displayed in Figure 4.1. The significant of the fat effect was displayed most prominently when the fat*day interactions of the EVOO and the positive control were compared. There was no significance between the initial bacterial counts of the Day-1 positive control and the initial bacterial counts of the Day-1 EVOO. This indicated that the average initial bacterial counts began within a comparable range for the two fat types as shown by all the values being 7-log cfu/mL. The positive control maintained this 7-log cfu/mL range throughout the study and no significant difference was observed between Day-1 and Day-7. This was validated by the lack of a significant difference between the initial bacterial counts of the Day-1 EVOO and the final bacterial counts of the Day-7 positive control. This allowed for an accurate comparison of the changing bacterial counts within the EVOO.

Within the EVOO itself, a significant difference of $Pr>|t| <0.0001$ was observed when comparing the initial bacterial counts of Day-1 to the final bacterial counts of Day-7. As visualized in both Table 4.1. and Figure 4.1., the *STECs* experienced a rapid decline from 7-log cfu/mL to <1-log/mL over the seven sample days. This statistical difference was also observed when comparing the final bacterial counts of Day-7 in the EVOO to both the initial bacterial counts of the Day-1 positive control ($Pr>|t| <0.0001$) or to the final bacterial counts of the Day-7 positive control ($Pr>|t| <0.0001$). As shown practically and statistically, the EVOO met the 3-log cfu/mL bacterial reduction threshold and continued its bactericidal effects at a surviving bacterial level of 1-log cfu/mL or less.

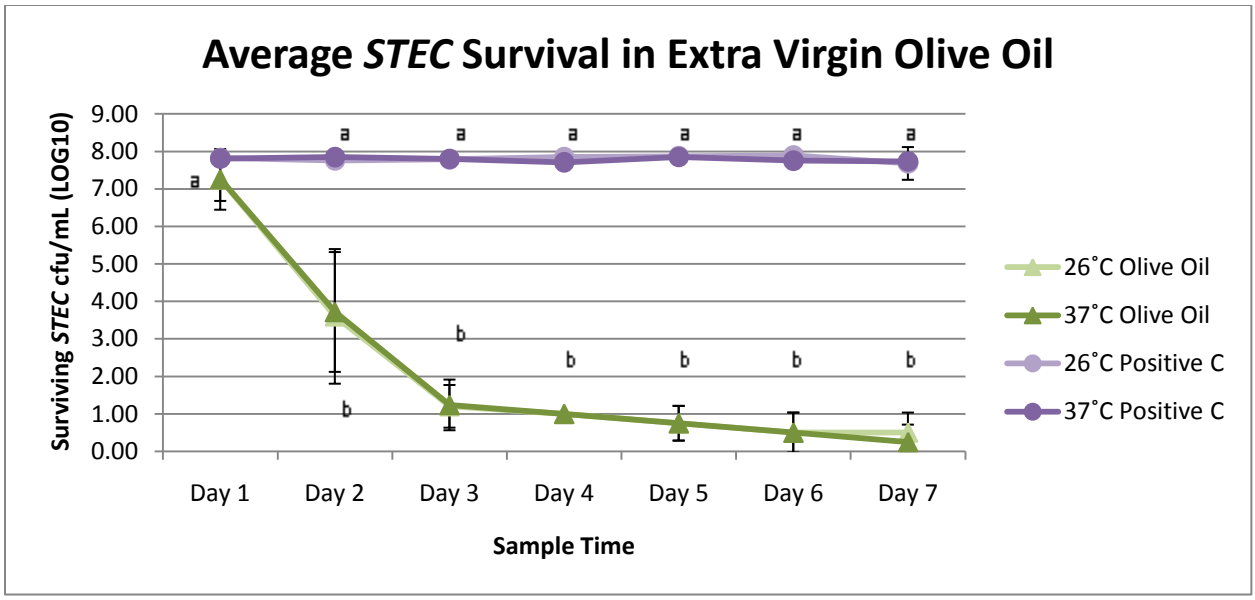

Figure 4.1. The average *STEC* survival in positive controls and extra virgin olive oil at 26˚C and 37˚C

- (a) represents a lack of statistical significance comparing the bacterial populations of a sample to the initial bacterial populations, (b) represents a ≥3-log cfu/mL reduction from the initial bacterial population

It was concluded that a single strain of *STEC* was the cause of the recovered bacteria within the enrichments over the seven day period. *Escherichia coli STEC 15AB* (ATCC 99-3311) was identified from recovered Day-4 through Day-7 enrichments streaked for isolation on MAC. Confirmation was obtained by picking three isolated colonies from each MAC plate, growing them for 24 hours at 37˚C in separate BHI tubes, and streaking them onto a divided Rainbow Agar plate. The resulting streaks were then compared to the known standard streaks of each *STEC* strain. Vitek2 was not conducted since it would not differentiate between the four different *STEC* strains.

All samples reached a state of <1-log cfu/mL by Day-4. Only Trial-1 (26˚C) Day-5, Trial-1 (26˚C) Day-6, Trial-1 (26˚C) Day-7, Trial-4 (26˚C) Day-6, Trial-4 (26˚C) Day-7, Trial-1 (37˚C) Day-7, Trial-3 (37˚C) Day-6, Trial-3 (37˚C) Day-7, Trial-4 (37˚C) Day-5, Trial-4 (37˚C) Day-6, and Trial-4 (37˚C) Day-7 were <1-log cfu/mL on both the spiral plates and the enrichments. These results were vital because they showed that without the presence of the resistant *STEC* strain the other strains were susceptible to the EVOO and were damaged below the level of detection (<1-log cfu/mL). It also showed the possibility of *STEC 15AB* (ATCC 99-3311) being damaged below the level of

detection (<1-log cfu/mL) on the two selective agars used after enrichment. Overall, the >3-log cfu/mL reduction experienced by the *STECs* indicated that EVOO may not be a viable source for contamination. There may still be other resistant strains of *E. coli* which can survive the antimicrobial effects of EVOO similar to or better than *STEC 15AB* (ATCC 99-3311). For this study, not all *STEC* strains could survive at a consistent infective level within the EVOO for a period of seven days at both 26˚C and 37˚C. This data supports the hypothesis that the EVOO would cause at least a 3-log reduction in the initial *STEC* inoculum levels over the seven day period at both 26˚C and 37˚C.

4.6. Discussion

In light of the minimal reporting of *STECs* within the dry pet food industry, the discussion of the current research was shifted from targeting the fat application as a potential contamination area within the dry pet food industry to observing the general antimicrobial properties of the EVOO and hypothesize its potential use in raw pet food and human food industries. EVOO did not support the growth or survival of *STECs* throughout the seven day study at either 26˚C or 37˚C. The EVOO displayed an extremely potent antimicrobial effect across the five *STEC* species. This was best displayed in the Day-7 enrichments of Trial-1 and the Day-6 and Day-7 enrichments of Trial-4 in which all five of the species were unrecoverable at both 26˚C and 37˚C.

Although the literature is lacking for many of the *STEC* species, EVOO has been shown to have antimicrobial effects against several *E. coli* including *E. coli O157:H7.* Medina et al. (2006) tested the antimicrobial abilities of 15 virgin, three refined, and three pomace olive oils against several food pathogens including *E. coli* (CECT 434). After one hour of constant mixing, the *E. coli* experienced a 1.76 cfu/mL reduction in the virgin olive oil, a 1.22 cfu/mL reduction in the refined olive oil, and a 0.72 cfu/mL reduction in the pomace olive oil from an initial 5-log cfu/mL inoculum.[118] It was noted that the *E. coli* and *Shigella sonnei* were more resistant to all the olive oils and *S. enterica* was resistant to the pomace olive oil over the hour long exposure. The authors hypothesized that the olive oils were less active against the Gram-negative bacteria than they were against the Gram-positive bacteria.[118] Even though the *E. coli* did not experience a

dramatic decrease, compared to the Gram-positive bacteria, this study supports the susceptibility of *E. coli* to olive oils within a mere hour of exposure. The current study saw a 3.5-log cfu/mL decrease in the *STECs* from an initial 7-log cfu/mL count within 24 hours. The samples were only mixed immediately before the sample time.

Rounds et al. (2012) found that when ground beef with an olive extract, added to a concentration of 5% (w/w), was inoculated with *E. coli O157:H7* (ATCC 35150) and cooked, the bacterial counts were below detectable limits (<1-log cfu/g). This study was later expanded by Rounds et al. (2013) in which the addition of an olive extract to a concentration of 3% (w/w), but not 1% (w/w), within the ground beef was found to reduce the bacterial counts of *E. coli O157:H7* (ATCC 35150) to below the detectable limit (<1-log cfu/g). The Rounds et al. studies support the antimicrobial abilities of olive derived compounds as well as the in vivo application of them against *E. coli O157:H7* (ATCC 35150). The current study used EVOO to inhibit *E. coli O157:H7* (ATCC 35150) and four other *STECs* in vitro. The Rounds studies also highlighted the importance of the concentration used with the olive compounds. Shah et al. (2013) found that virgin olive oil was ineffective against *S. enteriditis* and *E. coli* (25922) at its lowest dilution of 1/40 (v/v). Medina et al. (2006) attributed the difference in antimicrobial abilities among the various olive oils to their phenolic compound content. The virgin olive oils had the highest percent of phenolic compounds which decreased in refined olive oils and was the lowest in pomace olive oils.[118] This correlates with the survival of the Gram-negative bacteria, namely *E. coli*, within the various olive oils. Parenti et al. (2008) claimed that the overall phenolic content also increased within olive oil that was cold pressed. Similarly, the current study utilized a cold pressed EVOO to challenge the *STECs* survival.

4.7. Conclusion

The extra virgin olive (EVOO) oil did not support the survival of *Escherichia coli O157:H7* (ATCC 35150), *Escherichia coli STEC 15AE* (ATCC 2006-3008), *Escherichia coli STEC 15AF* (ATCC 2002-3211), and *Escherichia coli STEC 15AG* (ATCC 0111) at either 26˚C or 37˚C. The EVOO caused an average 3.5-log cfu/mL reduction within 24

hours and an average 6-log cfu/mL reduction within 48 hours. By Day-4 no counts were detectable on TSA, indicating that any recovered counts were <1-log cfu/mL. *Escherichia coli STEC 15AB* (ATCC 99-3311) proved to be a more resistant *STEC* strain through its recovery in lactose broth enrichments at both 26˚C and 37˚C. *STEC 15AB* (ATCC 99-3311) was not recovered for Trial-1 (26˚C) Day-5, Trial-1 (26˚C) Day-6, Trial-1 (26˚C) Day-7, Trial-4 (26˚C) Day-6, Trial-4 (26˚C) Day-7, Trial-1 (37˚C) Day-7, Trial-3 (37˚C) Day-6, Trial-3 (37˚C) Day-7, Trial-4 (37˚C) Day-5, Trial-4 (37˚C) Day-6, and Trial-4 (37˚C) Day-7. These samples indicated that even *STEC 15AB* (ATCC 99-3311) was, at times, subject to damage caused by the EVOO. Overall, the experimental hypothesis was supported for the EVOO since it exceeded the reduction threshold of 3-log cfu/mL despite the persistent *STEC* strain. No statistical difference between 26˚C and 37˚C was reflected in the survival rates of *STECs* within oil.

CHAPTER 5

THE SURVIVAL OF A *LISTERIA MONOCYTOGENES* COCKTAIL IN EXTRA VIRGIN OLIVE OIL DURING VARIOUS TIME FRAMES

5.1. Summary

The purpose of this study was to determine if extra virgin olive oil had any inhibitory properties towards a four strain cocktail of *Listeria monocytogenes* (*LM*). Experiment A was designed to determine the survival rate of the *LM* in sterile phosphate buffer (SPB) with and without the addition of Tween80 at both 26˚C and 37˚C for seven days. The samples were spiral plated on TSA and incubated for 24 hours at 37˚C. The hypothesis that no significant change would occur was ultimately rejected because of the average 2.9-log cfu/mL reduction experienced by the 37˚C samples by Day-7. Experiments B, C, and D were all designed with single source tubes of *LM* inoculated extra virgin olive oil stored at 26˚C and 37˚C over different time periods. The samples were spiral plated on TSA and had UVM enrichments on MOX which were all incubated for 24 hours at 37˚C. All of the hypotheses, predicting a minimum 3-log cfu/mL reduction by the conclusion of the time period, were supported by these experiments. All of the bacterial populations in these experiments experienced a ≥7-log cfu/mL reduction by the conclusion of the sampling time. Experiments E, F, and G were all designed to determine the influence of the mixing frequency on the survival of the *LM* populations. All of these samples were spiral plated on TSA and had UVM and BHI enrichments on MOX split-plates which were all incubated for 48 hours at 37˚C. The hypotheses, predicting a minimum 3-log cfu/mL reduction by the conclusion of the time period, were supported by Experiments E and G, in which both bacterial populations experienced ≥7-log cfu/mL reductions by the conclusion of the sampling time. This hypothesis was ultimately rejected for Experiment F which observed an average 2.5-log cfu/mL reduction in the *LM* populations of the single mix 6-Hour samples. The general conclusions of the study were that the frequency of mixing contributed to the rapid decline of the *LM* populations and that this bacterial decline within the extra virgin olive

oil was due to the direct and constant contact with the oil, which elicited a strong antimicrobial effect.

5.2. Introduction

Listeria monocytogenes (*LM*) continues to be a growing threat within the food industry because of its ubiquitous nature and its ability to survive for prolonged periods in the production environment.[76,144,167] In the *Listeria* Annual Summary of 2014, the Centers for Disease Control and Prevention (CDC) reported 675 cases of human listeriosis across 47 states and the District of Columbia. Of these reports, 564 cases, excluding pregnant mothers, were considered invasive listeriosis resulting in 485 patient hospitalizations and 107 associated adult deaths.[28] At the time of the report, 18 fetal deaths had occurred and five other infant deaths had occurred after birth.[28] The CDC estimates that approximately 260 deaths occur among the projected 1,600 annual patients that develop listeriosis.[25] It is because of this illness severity and stark mortality rate that *LM* remains a high risk pathogen. In 2015, the FDA issued five separate recalls and one recall expansion for various pet food products that were potentially contaminated with *Listeria*.[59] This year remains the highest recall year for pet food products, contributing six of the 12 *Listeria* related recalls from January 2010-January 2017.[59] Several food products in both the human and pet food industries remain viable candidates for *LM* through post processing contamination.[167] Several essential oils and natural compounds have been proposed as potential antimicrobials against *Listeria*.[42,80,142,143,185] The following research is interested in the effects of extra virgin olive oil (EVOO) as a natural bacterial inhibitor intended to strengthen post processing hurdle technology.

The purpose of this study was to determine if EVOO had any inhibitory properties toward various strains of *Listeria monocytogenes* (*LM*) over time. The hypothesis of Experiment A was that there would not be a significant difference in the number of surviving *LM* between the positive controls, SPB +0.1%Tween80 or SPB without Tween80, over seven days at either 26˚C or 37˚C. The hypothesis of Experiment B was that the EVOO would cause at least a 3-log reduction in the initial *LM* inoculum levels over the seven day period at both 26˚C and 37˚C. The hypothesis of Experiment C was that the EVOO would cause at least a 3-log reduction in the initial *LM* inoculum levels

over the 24 hour period at both 26˚C and 37˚C. The hypothesis of Experiment D was that the EVOO would cause at least a 3-log reduction in the initial *LM* inoculum levels over the six hour period at both 26˚C and 37˚C. The hypothesis of Experiment E was that the EVOO would cause at least a 3-log reduction in the initial *LM* inoculum levels over the four hour period at 26˚C. The hypothesis of Experiment F was that the EVOO would cause at least a 3-log reduction in the initial *LM* inoculum levels over the six hour period at 26˚C for both mixing styles. The hypothesis of Experiment G was that the EVOO would cause at least a 3-log reduction in the initial *LM* inoculum levels over the 48 hour period at 26˚C for both mixing styles. This information could be helpful in assessing the likelihood of EVOO being a source of *LM* contamination. Moreover, this study investigates the overall antimicrobial abilities of EVOO against four strains of *LM* which may have natural preventative implications within the human and pet food industries.

5.3. Materials

5.3.1. Pathogens

Listeria monocytogenes 150C (ATCC 51781), *Listeria monocytogenes 150D* (ATCC 43256), *Listeria monocytogenes 150E* (ATCC 15313), and *Listeria monocytogenes 150F* (ATCC 19115) were the four strains of *Listeria* utilized in the study.

5.3.2. Evaluated Product

One commercial extra virgin olive oil (EVOO) was evaluated on its ability to inhibit *Listeria monocytogenes* (*LM*) over several different timeframes at one or two temperatures, 26˚C or 37˚C. Primo Gusto: Italian Foods 100% Italian Extra Virgin Olive Oil (cold pressed) (country of origin: Italy via Gordon Food Service® P.O. Box 1787, Grand Rapids, MI 49501) was purchased from a local grocery store. This product was chosen because it did not contain any additives or preservatives which could have hindered the survival rate of the bacteria within the product.

5.3.3. Medias

The broths used in the *LM* challenges included Bacto™ Brain Heart Infusion (BHI) and Difco™ UVM Modified *Listeria* Enrichment Broth (UVM). Both products

were from Becton, Dickinson and Company (7 Loveton Circle, Sparks, MD, 21152) and were prepared according to the directions. The BHI, intended for culture growth, was dispensed into Pyrex tubes with a final volume of 7mL each and autoclaved. The BHI, intended to be used as enrichments, had 1mL of Tween80 added per liter of broth so that the final mixture contained 0.1% (v/v). The BHI +Tween80 was then dispensed into Pyrex tubes with a final volume of 9mL each and autoclaved. The UVM, intended to be used as enrichments, also had Tween80 added to a concentration of 0.1% (v/v). The UVM +Tween80 was then dispensed into Pyrex tubes with a final volume of 9mL each and autoclaved. Dilution blanks used throughout the study were Sterile Phosphate Buffer, SPB, dilution blank tubes. These dilution tubes were made from 24mL PO_4 solution, 95mL $MgCl_2$ solution, and 19L of double deionized water. Tween80 was added to the dilution blank mixture at 1mL/1L so that the final blanks contained 0.1% Tween80 (v/v). The dilution mixture with Tween80 was adjusted to a pH between 7.4-7.5. The mixture was then dispensed into Pyrex tubes with a final volume of 9mL and autoclaved. Several dilutions blanks were also made without Tween80 for the positive control study. This dilution mixture, without Tween80, was also adjusted to a pH between 7.4-7.5. They were then dispensed into Pyrex tubes with a final volume of 9mL and autoclaved. A half liter of each SPB mixture, SPB with Tween80 and SPB without Tween80, was kept as a stock to make the positive controls. These half liters were dispensed into autoclave safe containers and were autoclaved with the dilution blank tubes. The agars utilized in the *LM* study included BBL™ Trypticase™ Soy Agar: Soybean-Casein Digest Agar (TSA) and Difco™ Modified Oxford (MOX) from Difco™ Oxford Medium Base and Difco™ Modified Oxford Antimicrobic Supplement. Both TSA and MOX were Becton, Dickinson and Company products (7 Loveton Circle, Sparks, MD, 21152) and were made according to the product specifications. All agars were poured into sterile 100x15mm polystyrene disposable VWR Petri plates (1050 Satellite Blvd NW, Suwanee, GA 30024).

5.4. Methods

5.4.1. Oil Sterility Validation

The oil was transferred from its original container into a sterile 250mL glass bottle with a cap. Samples of the oil were taken and plated on TSA and MOX and incubated for 48 hours at 37˚C to check for bacterial background. Oil samples were also plated on TSA and incubated for 48 hours at 26˚C to validate sterility. The oil was confirmed to be commercially sterile with an aerobic count below the level of detection. The oil stock bottle was then wrapped in foil and placed in refrigerated storage to prevent any additional oxidation or contamination.

5.4.2. Inoculum Preparation

A scrape of each desired *LM* strain was obtained from a pre-existing refrigerated BHI slant and was transferred into BHI broth using sterile technique. The culture was incubated for 24 hours at 37˚C. The culture was then streaked for isolation onto MOX agar and was incubated for 24 hours at 37˚C. The morphology of the colonies was observed and an isolated colony was transferred to a new tube of BHI. The culture was then incubated for 24 hours at 37˚C. The culture was transferred twice by placing 0.1mL of the former culture into new BHI and incubated for 24 hours at 37˚C. The final transfer of *LM 150C* (ATCC 51781), *LM 150D* (ATCC 43256), *LM 150E* (ATCC 15313), and *LM 150F* (ATCC 19115) were then pooled into a sterile 50mL Flacon Tube. The cocktail was vortexed.

5.4.3. Sample Preparation

The bacterial lawn method utilized in the *Salmonella* and *STEC* studies (Chapter 3 and Chapter 4) could not be used in any of the following *LM* studies because of the inconsistent plating results observed in pilot studies. *LM 150C* (ATCC 51781), *LM 150D* (ATCC 43256), *LM 150E* (ATCC 15313), and *LM 150F* (ATCC 19115) were grown individually in BHI broth and pooled together as formerly described.

5.4.3.a. Experiment A: Positive Control 7 Day: Multiple Source Tubes

The half liter bottles of SPB without Tween80 and SPB with Tween80 were taken from their storage area at 26˚C and placed in a Labconco Purifier Class II Biosafety Cabinet. The four strain *LM* cocktail was made as formerly described. Beginning with

the SPB without Tween80, 1mL of the *LM* cocktail was added to 9mL of SPB in a sterile 25mL beaker. The 25mL beaker was placed in a sterile 250mL beaker before being placed on a stir plate in order to prevent possible splashing as the culture was mixed. This 1:10 dilution was sufficiently mixed and 1mL of this dilution was added to 9mL of SPB in a second sterile 25mL beaker. This 1:100 dilution was also sufficiently mixed and 8mL of this latest dilution were added to 72mL of SPB in a sterile 250mL beaker to create the sample stock. This 250mL beaker was placed into a sterile 1000mL beaker to prevent splashing as the stock was mixed. This sample stock, now approximately 6-log cfu/mL, was pipetted into 14 sterile disposable culture tubes. Each tube contained 5mL of the sample stock. The tubes were randomized and divided into two groups of seven tubes. The tubes were labeled Day-1 through Day-7 with the appropriate temperature and were placed at 26˚C incubation or 37˚C incubation. Day-1 samples were plated immediately following the sample set up while the other tubes were stored at their designated temperatures until their sampling time. The same procedure was used for the SPB with Tween80. All of this was done using sterile technique so that *LM* would be the only bacteria present and TSA could be used as the growth media for the study.

5.4.3.b. Experiment B: Extra Virgin Olive Oil 7 Day: Single Source Tube

Due to inconsistent plating results observed in pilot studies, it was decided that a pooled broth inoculum would be used to make the inoculated oil stock. It was also decided that a single source tube would be used to minimize inconsistencies across multiple tubes. The oil stock bottles were obtained from refrigerated storage and warmed in a 50˚C water bath just until the oil became liquid. The oil stock bottles were then placed in a Labconco Purifier Class II Biosafety Cabinet. The four strain *LM* cocktail was made as formerly described and 2mL were added to 28mL of sterile oil in a sterile 50mL beaker. This 50mL beaker was placed into a sterile 250mL beaker to prevent splashing as the stock was mixed. This sample stock, now approximately 6-log cfu/mL was divided into two separate sterile 50mL Falcon tubes. Each tube contained 15mL of the sample stock. The tubes were randomized and were labeled with a 26˚C or 37˚C. The Day-1 samples were plated immediately following the sample set up. The tubes were then placed at the incubation temperature that coordinated with their label. All of

this was done using sterile technique so that *LM* would be the only bacteria present and TSA could be used as the growth media for the study.

5.4.3.c. Experiment C: Extra Virgin Olive Oil 12 Hour: Single Source Tube

The procedure described in 5.4.3.b., Experiment B, was utilized for this experiment. The 0-Hour samples were plated immediately following the sample set up. All of this was done using sterile technique so that *LM* would be the only bacteria present and TSA could be used as the growth media for the study.

5.4.3.d. Experiment D: Extra Virgin Olive Oil 6 Hour: Single Source Tube

The procedure described in 5.4.3.b., Experiment B, was utilized for this experiment. The 0-Hour samples were plated immediately following the sample set up. All of this was done using sterile technique so that *LM* would be the only bacteria present and TSA could be used as the growth media for the study.

5.4.3.e. Experiment E: Extra Virgin Olive Oil 4 Hour: Multiple Source Tubes

Due to the possibility of mixing as a confounding variable, it was decided that multiple source tubes would be used to minimize the influence of this variable. The oil stock bottles were obtained from refrigerated storage and warmed in a 50˚C water bath just until the oil became liquid. The oil stock bottles were then placed in a Labconco Purifier Class II Biosafety Cabinet. The four strain *LM* cocktail was made as formerly described and 4mL were added to 56mL of sterile oil in a sterile 100mL beaker. This 100mL beaker was placed into a sterile 400mL beaker to prevent splashing as the stock was mixed. This sample stock, now approximately 7-log cfu/mL was divided into five separate sterile 50mL Falcon tubes. Each tube contained 12mL of the sample stock. These tubes were only stored at 26˚C because the *LM* appeared less stressed at this temperature in previous experiments. The tubes were randomized and were labeled with 0-Hour through 4-Hour. The 0-Hour sample was plated immediately following the sample set up. The other samples were all stored at 26˚C incubation until their designated sample time. Each tube was only mixed and sampled once throughout the experiment. All of this was done using sterile technique so that *LM* would be the only bacteria present and TSA could be used as the growth media for the study.

5.4.3.f. Experiment F: Extra Virgin Olive Oil 6 Hour: Single/Multiple Mix Tubes

To determine the influence of the confounding variable, mixing, both multiple mix and single mix tubes were used. The oil stock bottles were obtained from refrigerated storage and warmed in a 50˚C water bath just until the oil became liquid. The oil stock bottles were then placed in a Labconco Purifier Class II Biosafety Cabinet. The four strain *LM* cocktail was made as formerly described and 2mL were added to 28mL of sterile oil in a sterile 50mL beaker. This 50mL beaker was placed into a sterile 250mL beaker to prevent splashing as the stock was mixed. This sample stock, now approximately 7-log cfu/mL was divided into two separate sterile 50mL Falcon tubes. Each tube contained 15mL of the sample stock. These tubes were only stored at 26˚C because the *LM* appeared less stressed at this temperature in previous experiments. The tubes were randomized and were labeled Multiple Mix 6-Hours and Single Mix 6-Hours. The Multiple Mix sample was plated as the 0-Hour immediately following the sample set up. After plating, it was stored at 26˚C incubation with the Single Mix 6-Hour sample until the designated sample time. All of this was done using sterile technique so that *LM* would be the only bacteria present and TSA could be used as the growth media for the study.

5.4.3.g. Experiment G: Extra Virgin Olive Oil 48 Hour: Single/Multiple Mix Tubes

To determine the influence of the confounding variable, mixing, both multiple mix and single mix tubes were used. The oil stock bottles were obtained from refrigerated storage and warmed in a 50˚C water bath just until the oil became liquid. The oil stock bottles were then placed in a Labconco Purifier Class II Biosafety Cabinet. The four strain *LM* cocktail was made as formerly described and 2mL were added to 42mL of sterile oil in a sterile 50mL beaker. This 50mL beaker was placed into a sterile 250mL beaker to prevent splashing as the stock was mixed. This sample stock, now approximately 7-log cfu/mL, was divided into three separate sterile 50mL Falcon tubes. Each tube contained 15mL of the sample stock. These tubes were only stored at 26˚C because the *LM* appeared less stressed at this temperature in previous experiments. The tubes were randomized and were labeled Multiple Mix, Single Mix 24-Hour, and Single Mix 48-Hour. The Multiple Mix sample was plated as the 0-Hour immediately following

the sample set up. After plating, it was stored at 26˚C incubation with the Single Mix samples until the designated sample times. All of this was done using sterile technique so that *LM* would be the only bacteria present and TSA could be used as the growth media for the study.

5.4.4. Sample Plating Procedure

All samples were spiral plated in duplicate onto TSA at the 50µL spiral setting of Eddy Jet2. The number of dilution blanks used for each of the following sample times depended upon the spread and overall growth of the bacteria from the previous day. If the bacteria appeared to have a consistent and readable spread across the plate, then the number of dilution blanks used remained the same. If however, the bacterial counts appeared to be decreasing, then the number of dilution blanks used for that sample were decreased to keep the bacteria at readable levels. The straight sample was never plated. This was because of pilot studies which indicate that the bacteria present in the sample grew better when separated from the straight oil. Thus, at least one dilution blank was used as the minimum dilution for each sample rather than just plating the straight oil. All dilution blanks, unless otherwise indicated, contained 0.1% Tween80 (v/v).

5.4.4.a. Experiment A: Positive Control 7 Day: Multiple Source Tubes

SPB without Tween80 and SPB with Tween80 sample tubes from the 26˚C and 37˚C incubators were obtained at the designated sample times. The Day-1 samples were vortexed and then used an average of three sequential dilution blanks. The SPB without Tween80 samples used the dilution blanks without Tween80. The final dilution tube was poured into a sterile spiral plater cup and placed in the spiral plater. The sample was spiral plated in duplicate onto TSA at a 50µL spiral setting of Eddy Jet2. This was done for the Day-1 samples at both 26˚C and 37˚C. The plates were inverted and incubated for 24 hours at 37˚C. This procedure was conducted throughout the seven days that the SPB was sampled at the two temperatures adjusting the dilution blank number as needed.

5.4.4.b. Experiment B: Extra Virgin Olive Oil 7 Day: Single Source Tube

The sample tubes from the 26˚C and 37˚C incubators were obtained at the designated sample times. All samples from this experiment were taken from these two tubes. The 0-Hour samples were vortexed and then used three sequential dilution blanks.

The final dilution tube was poured into a sterile spiral plater cup and placed in the spiral plater. The sample was spiral plated in duplicate onto TSA at a 50µL spiral setting of Eddy Jet2. This was done for the Day-1 samples at both 26˚C and 37˚C. The plates were inverted and incubated for 24 hours at 37˚C. This procedure was conducted for Day-1, Day-2, Day-3, and Day-7 at the two temperatures adjusting the dilution blank number as needed. Day-4, Day-5, and Day-6 were not spiral plated, but an enrichment was performed for each of those sample times at the two temperatures.

5.4.4.c. Experiment C: Extra Virgin Olive Oil 12 Hour: Single Source Tube

The 5.4.4.b., Experiment B, procedure was conducted for this experiment. The 0-Hour, 6-Hour, 12-Hour, and 24-Hour samples were plated for the 26˚C and 37˚C, adjusting the dilution blank number as needed. The plates were inverted and incubated for 24 hours at 37˚C.

5.4.4.d. Experiment D: Extra Virgin Olive Oil 6 Hour: Single Source Tube

The 5.4.4.b., Experiment B, procedure was conducted for this experiment. The 0-Hour, 1-Hour, 2-Hour, 3-Hour, 4-Hour, 5-Hour, and 6-Hour samples were plated for the 26˚C and 37˚C; adjusting the dilution blank number as needed. The plates were inverted and incubated for 24 hours at 37˚C.

5.4.4.e. Experiment E: Extra Virgin Olive Oil 4 Hour: Multiple Source Tubes

Sample tubes from the 26˚C incubator were obtained at the designated sample times. Each sample tube was only mixed and sampled once through the experiment. The 0-Hour samples were vortexed and then used three sequential dilution blanks. The final dilution tube was poured into a sterile spiral plater cup and placed in the spiral plater. The sample was spiral plated in duplicate onto TSA at a 50µL spiral setting of Eddy Jet2. The plates were inverted and incubated for 48 hours at 37˚C. This procedure was conducted for 0-Hour, 1-Hour, 2-Hour, 3-Hour, and 4-Hour at 26˚C; adjusting the dilution blank number as needed.

5.4.4.f. Experiment F: Extra Virgin Olive Oil 6 Hour: Single/Multiple Mix Tubes

The 0-Hour samples were plated from the Multiple Mix sample tube. This tube was vortexed and then used three sequential dilution blanks. The final dilution tube was poured into a sterile spiral plater cup and placed in the spiral plater. The sample was

spiral plated in duplicate onto TSA at a 50µL spiral setting of Eddy Jet2. The plates were inverted and incubated for 48 hours at 37˚C. The tube was then stored in the 26˚C incubator with the Single Mix 6-Hour sample tube until the six hour sample time. At six hours, both tubes were retrieved from the incubator. The Single Mix 6-Hour sample was vortexed once and plated using a first dilution and a third dilution. The Multiple Mix sample was vortexed for the second time and plated at the same dilutions as the Single Mix 6-Hour sample. The plates were inverted and incubated for 48 hours at 37˚C.

5.4.4.g. Experiment G: Extra Virgin Olive Oil 48 Hour: Single/Multiple Mix Tubes

The 0-Hour samples were plated from the Multiple Mix sample tube. This tube was vortexed and then used three sequential dilution blanks. The final dilution tube was poured into a sterile spiral plater cup and placed in the spiral plater. The sample was spiral plated in duplicate onto TSA at a 50µL spiral setting of Eddy Jet2. The plates were inverted and incubated for 48 hours at 37˚C. The tube was then stored in the 26˚C incubator with the Single Mix 24-Hour and Single Mix 48-Hour sample tubes until the designated sample times. At 24 hours, the Single Mix 24-Hour sample tube and the Multiple Mix sample tube were retrieved from the incubator. The Single Mix 24-Hour sample was vortexed once and plated using a first dilution. The Multiple Mix sample was vortexed for the second time and plated at the same dilution. The plates were inverted and incubated for 48 hours at 37˚C. The Multiple Mix sample tube was returned to the 26˚C incubator after sampling. At 48 hours, the Single Mix 48-Hour sample tube and the Multiple Mix sample tube were retrieved from the 26˚C incubator. The Single Mix 48-Hour sample was vortexed once and plated using a first dilution. The Multiple Mix sample was vortexed for the third time and plated at the same dilution. The plates were inverted and incubated for 48 hours at 37˚C.

5.4.5. Enrichment Procedure

Enrichments were performed by pipetting 1mL of the sample into 9mL of sterile UVM +0.1% Tween80 (v/v). The broths were then vortexed, labeled, and placed into the incubator for 24 hours at 30˚C. If the spiral plates did not show growth then the enrichments were removed from the incubator, vortexed, and streaked onto MOX which was then incubated at 37˚C for 48 hours. If the spiral plates showed bacterial growth,

then the enrichment tubes were plated as additional confirmation. Enrichments were recorded as positive or negative for growth. Experiments E, F, and G also had BHI (with Tween80) enrichments in addition to the UVM (with Tween80) enrichments.

5.4.6. Reading Spiral Plate Procedure

Plates of the samples from Experiments A, B, C, and D were read after 24 hours incubation at 37˚C. Plate of the samples from Experiments E, F, and G were read after 48 hours incubation at 37˚C. A FlashAndGo automated counter and FlashAndGo 1.0 Computer program were used to count the spiral plate colonies. These items were combined to form the FlashAndGo -Basic Economy Automated Colony Counter through ILU Instruments of NEU-TEC Group Inc. (1 Lenox Avenue, Farmingdale, NY 11735). The dilution factor was adjusted as needed for each of the plated oil samples from that particular day and combined with the counted colonies to algorithmically determine the total bacterial count within the given sample. The duplicate plates were averaged together within Microsoft Excel to give a more accurate total count of the given sample.

5.4.7. Statistical Analysis

Statistical analysis was performed using SAS 9.4 with significance indicated at $p < 0.05$. Data organization and graphical figures were constructed using Microsoft Office Excel 2007. A bacterial reduction of 3-log cfu/mL or greater was noted as a significant reduction within the bacterial population.

5.5. Results

A split-Split Plot ANOVA was used to analyze the data from Experiment A. There was overall significance in the model based on the temperature (Pr>F 0.0157), day (Pr>F <0.0001), and temperature*day (Pr>F 0.0036) effects. No significance was seen for the fat effect or its interactions. This indicated that there was no significant difference in the survival rate of the *LM* in SPB with 0.1% Tween80 (v/v) or in SPB without Tween80. As displayed in Figure 5.1., there was a noticeable decrease in the surviving

LM counts over the seven sampling days.

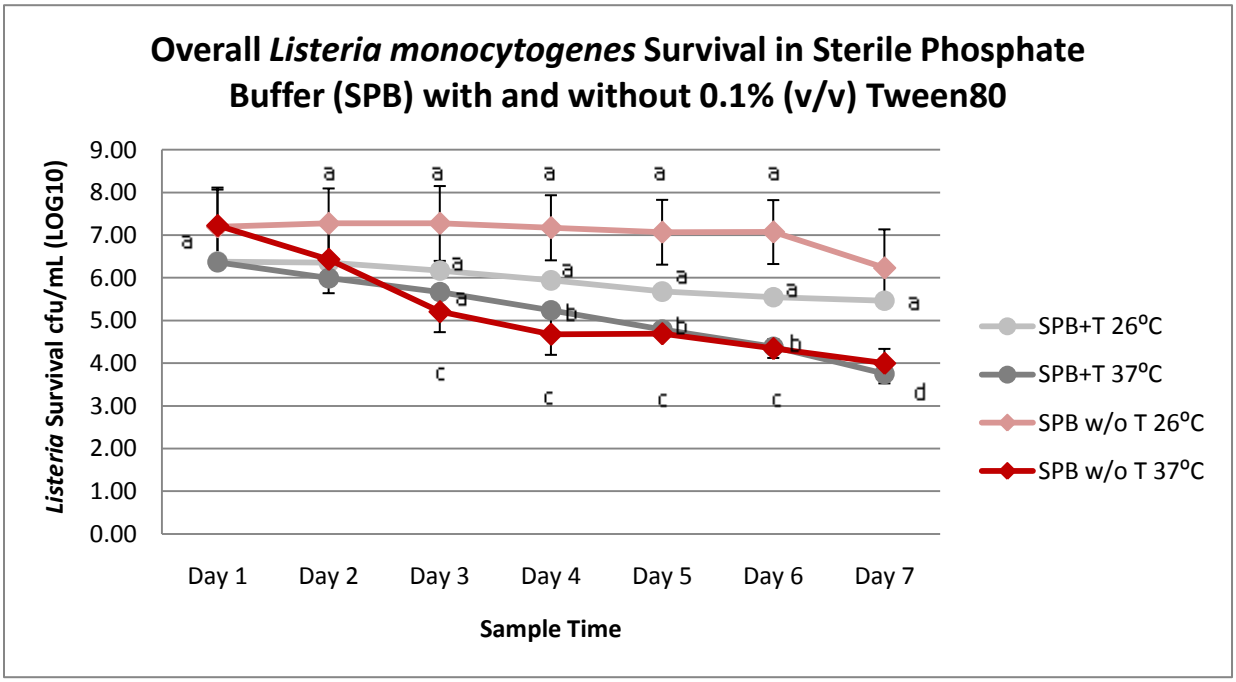

Figure 5.1. The average *Listeria monocytogenes* survival in SPB with and without Tween80 at 26˚C and 37˚C over 7 days

- (a) represents a lack of statistical significance comparing the bacterial populations of a sample to the initial bacterial populations, (b) represents a 1-log cfu/mL reduction from the initial bacterial population, (c) represents a 2-log cfu/mL reduction from the initial bacterial population, (d) represents a ≥3-log cfu/mL reduction from the initial bacterial population

This decrease was more pronounced at 37˚C than at 26˚C. Day-1 for both temperatures was not significant, indicating that the initial *LM* population was approximately the same. When Day-1 and Day-7 were compared, at 26˚C, a significant decrease was indicated (Pr> $|t|$ 0.026). This significance was noted as impractical since the decrease was less than a 1-log cfu/mL reduction. A similar observation was made when comparing Day-7 at 26˚C with Day-1 at 37°C, which showed significance of Pr> $|t|$ 0.0254, but was noted as impractical because the decrease was less than 1-log cfu/mL. Statistical significance was observed when comparing Day-1 at 26˚C with Day-7 at 37˚C (Pr> <0.0001), Day-7 at 26˚C with Day-7 at 37˚C (Pr> 0.0004), and Day-1 at 37˚C with Day-7 at 37˚C (Pr> <0.0001). The decrease experienced by the Day-7 samples at 37˚C compared to those of Day-1 at both 26˚C and 37˚C was nearly a 3-log cfu/mL reduction. A 2-log cfu/mL gap separated the samples on Day-7 at 26˚C and 37˚C, with the decrease

resulting from the declining population in the 37˚C samples. This indicated that the *LM* was sensitive to the higher temperature of 37˚C over prolonged storage times. Due to the lack of a true 1-log cfu/mL the samples stored at 26˚C were said to be unchanged over the seven sample days. The hypothesis that all of the positive controls would maintain their initial *LM* levels over the seven day period was ultimately rejected because of the average 2.9-log cfu/mL reduction experienced by the 37˚C samples by the seventh day.

A Repeated Measures ANOVA was used to analyze the data of Experiment B. Overall significance within the model was attributed to the day effect at Pr>F <0.0001. The temperature and temperature*day effects were not significant in the model, indicating that temperature was not an influential factor over the experiment. The Least Square Means for day revealed that Day-1 held significance (Pr> $|t|$ <0.0001) over Day-2, Day-3, and Day-7. When Day-1 was individually compared to Day-2, Day-3, and Day-7 a significance of Pr> $|t|$ <0.0001 was found for each comparison. None of these other sample days showed any significance when compared to each other because no surviving *LM* counts were detected after Day-1, as seen in Figure 5.2.

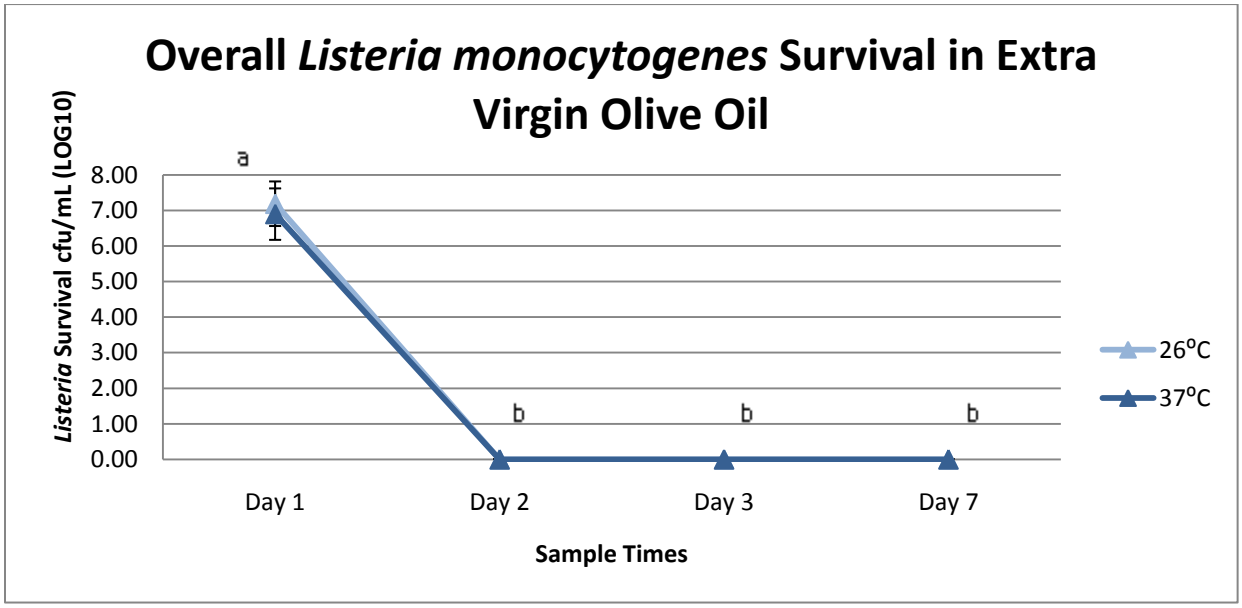

Figure 5.2. The average *Listeria monocytogenes* survival in extra virgin olive oil at 26˚C and 37˚C over 7 days

- (a) represents a lack of statistical significance comparing the bacterial populations of a sample to the initial bacterial populations, (b) represents a ≥3-log cfu/mL reduction from the initial bacterial population

None of the Day-2, Day-3, or Day-7 TSA spiral plates or UVM enrichments on MOX plates at 26˚C or 37˚C showed any growth after being incubated for 24 hours at 37˚C. The enrichments of Day-4, Day-5, and Day-6 also did not show any recovery of *LM*. Exposure to the EVOO for 24 hours, in a single source tube, resulted in a 7-log cfu/mL reduction in the *LM* population. This supported the hypothesis that the EVOO would cause at least a 3-log cfu/mL reduction in the initial *LM* inoculum levels over the seven day period at both 26˚C and 37˚C.

A Repeated Measures ANOVA was also used to analyze the data of Experiment C. Overall significance within the model was ascribed to the hour effect at Pr>F <0.0001. The temperature and temperature*hour effects were not significant in the model, signifying that temperature was not a prominent factor in the experiment. The Least Square Means for hour revealed that 0-Hour held significance (Pr> $|t|$ <0.0001) over 6-Hour, 12-Hour, and 24-Hour. When 0-Hour was compared to 6-Hour, 12-Hour, and 24-Hour individually, a significance of Pr> $|t|$ <0.0001 was found for each comparison. None of these other sample times displayed any significance when compared to each other since no surviving *LM* counts were detected after 0-Hour, as shown in Figure 5.3.

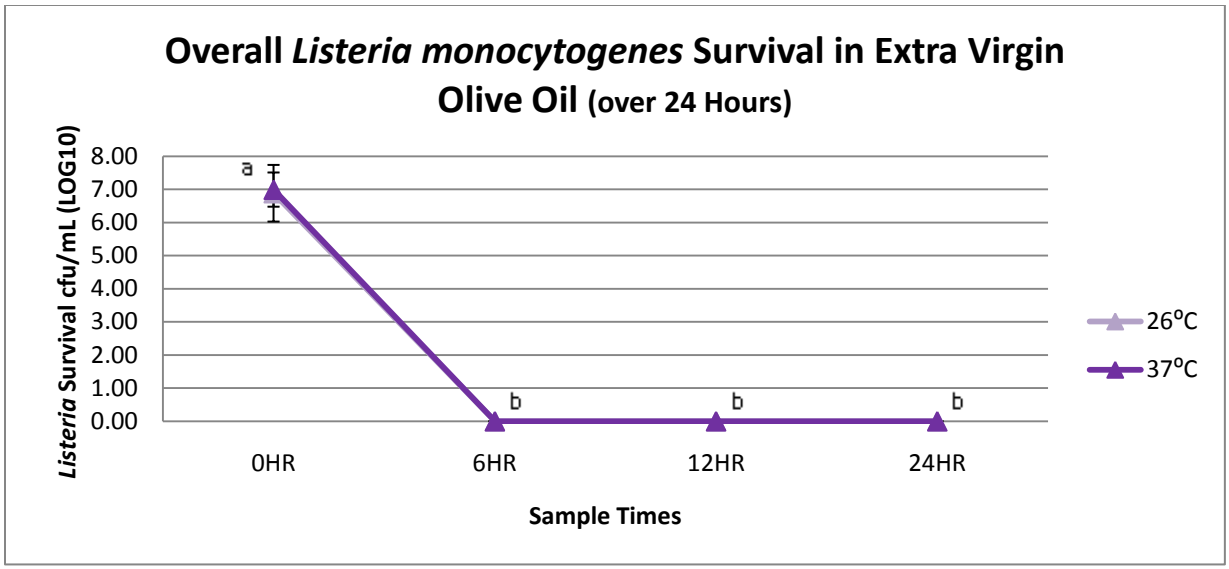

Figure 5.3 The average *Listeria monocytogenes* survival in extra virgin olive oil at 26˚C and 37˚C over 24 hours

- (a) represents a lack of statistical significance comparing the bacterial populations of a sample to the initial bacterial populations, (b) represents a ≥3-log cfu/mL reduction from the initial bacterial population

None of the 6-Hour, 12-Hour, or 24-Hour TSA spiral plates or UVM enrichments on MOX plates at 26˚C or 37˚C showed any bacterial growth after being incubated for 24 hours at 37˚C. Exposure to the EVOO for six hours, in a single source tube, resulted in a 7-log cfu/mL reduction in the *LM* population. This supported the hypothesis that the EVOO would cause at least a 3-log cfu/mL reduction in the initial *LM* inoculum levels over the 24 hour period at both 26˚C and 37˚C.

A split-Split Plot design was used to analyze the data from Experiment D. Overall significance within the model was attributed to the temperature effect at Pr>F 0.0424, the hour effect at Pr>F <0.0001, and the temperature*hour interaction at Pr>F 0.0414. This implied both the variables and their interaction were influential in the experiment, but the hour effect was the primary differentiating factor. The Least Square Means for temperature*hour revealed that 0-Hour and 1-Hour, both at 26˚C and 37˚C, all displayed significance of Pr> $|t|$ <0.0001. As shown in Figure 5.4., the initial counts of 26˚C and 37˚C at 0-Hour were similar and were therefore not significant.

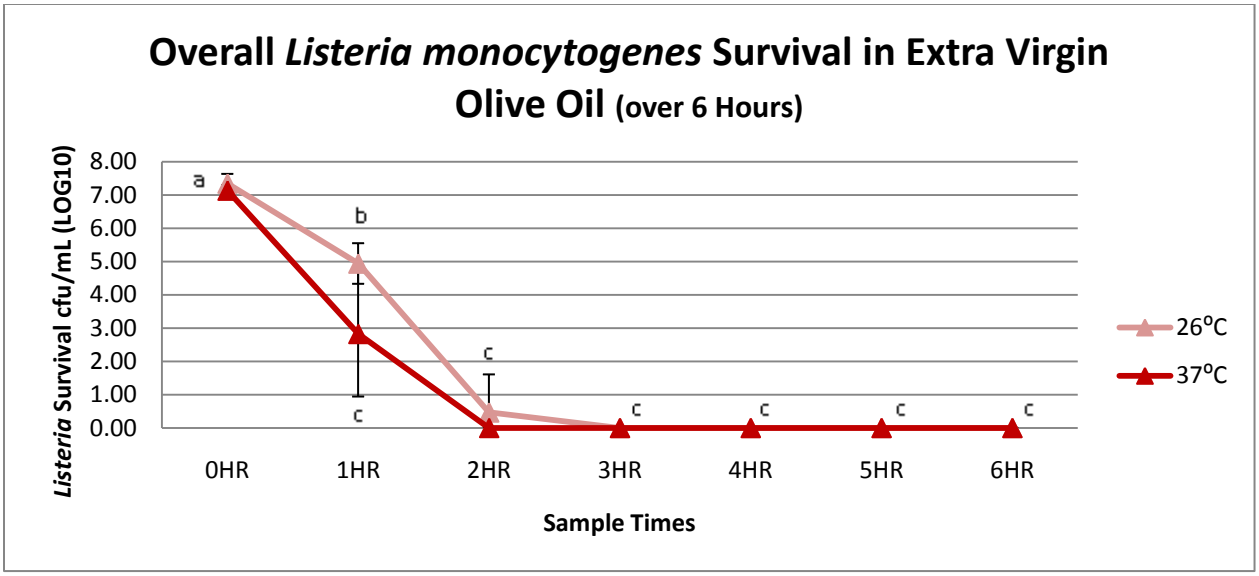

Figure 5.4. The average *Listeria monocytogenes* survival in extra virgin olive oil at 26˚C and 37˚C over 6 hours

- (a) represents a lack of statistical significance comparing the bacterial populations of a sample to the initial bacterial populations, (b) represents a 2-log cfu/mL reduction from the initial bacterial population, (c) represents a ≥3-log cfu/mL reduction from the initial bacterial population

The 0-Hour samples, at 26˚C, were statistically significant at Pr> $|t|$ <0.0001 to all of the other 26˚C experimental time points. The 0-Hour samples, at 26˚C, were also statistically significant at Pr> $|t|$ <0.0001 to all of the 37˚C experimental time points, except the 0-Hour samples at 37˚C. The 1-Hour samples at 26˚C were statistically significant at Pr> $|t|$ <0.0001 to all of the other 26˚C and 37˚C experimental time points. The 2-Hour, 3-Hour, 4-Hour, 5-Hour, and 6-Hour samples at 26˚C were not significant when compared to one another or to any of the 37˚C time points other than the 0-Hour and 1-Hour samples which all showed significance of Pr> $|t|$ <0.0001. The 0-Hour samples at 37˚C and the 1-Hour samples at 37˚C were statistically significant at Pr> $|t|$ <0.0001 to all of the other 37˚C experimental time points. The 2-Hour, 3-Hour, 4-Hour, 5-Hour, and 6-Hour samples at 37˚C were not significant when compared to one another or any of the corresponding 26˚C samples. There was a lack of bacterial recovery from any of the TSA spiral plates and UVM enrichments on MOX plates after the 2-Hour sample at 37˚C and 3-Hour sample at 26˚C. Exposure to the EVOO for two hours at 37˚C and three hours at 26˚C, in a single source tube, resulted in a 7-log cfu/mL reduction in the *LM* population. This supported the hypothesis that the EVOO would cause at least a 3-log

cfu/mL reduction in the initial *LM* inoculum levels over the six hour period at both 26˚C and 37˚C.

A Repeated Measures ANOVA was implemented to analyze the data of Experiment E. Overall significance within the model was credited to the hour effect at Pr>F <0.0001. Temperature was not an effect because all samples were held at 26˚C. The Least Square Means for hour revealed that all the time points held significance of Pr> <0.0001. When 0-Hour was compared to all of the other time points, individually, a significance of Pr> <0.0001 was found for each comparison. 1-Hour also showed a significance of Pr> <0.0001 when compared to all sample times except 2-Hour which showed a significance of Pr> 0.0053. 2-Hour also showed significance of Pr> $|t|$ <0.0001 and Pr> 0.0002 when compared to 3-Hour and 4-Hour respectively. The 3-Hour and 4-Hour did not show any significance when compared. Figure 5.5. displays this gradual decline of the *LM* population, ending with its ultimate survival at the completion of the four hour time period.

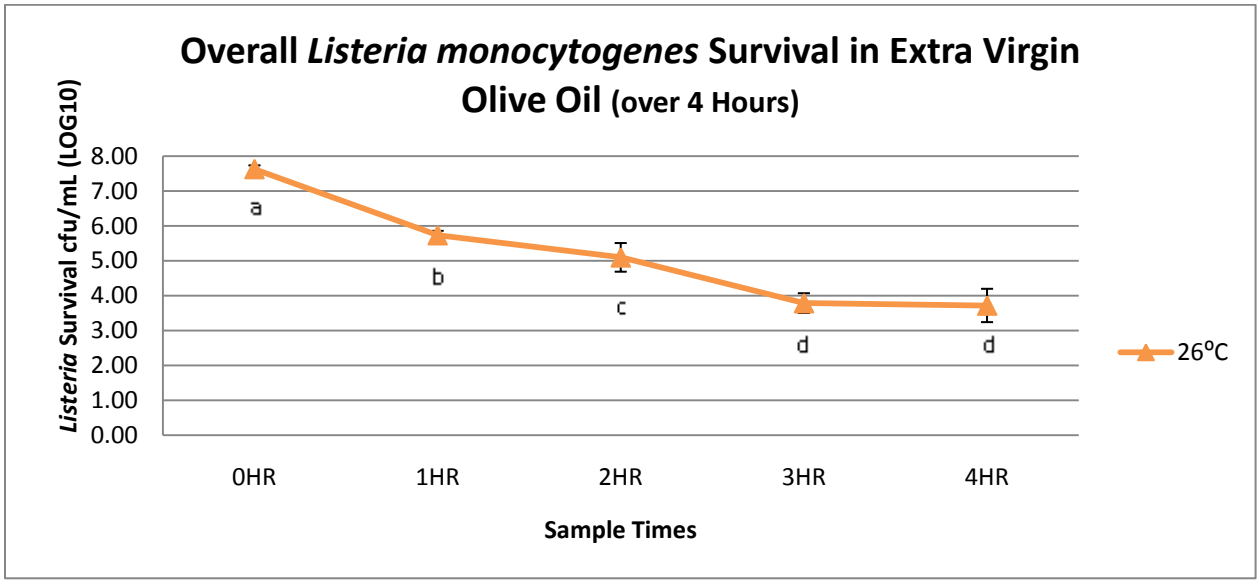

Figure 5.5. The average *Listeria monocytogenes* survival in extra virgin olive oil at 26˚C over 4 hours

- (a) represents a lack of statistical significance comparing the bacterial populations of a sample to the initial bacterial populations, (b) represents a 1-log cfu/mL reduction from the initial bacterial population, (c) represents a 2-log cfu/mL reduction from the initial bacterial population, (d) represents a ≥3-log cfu/mL reduction from the initial bacterial population

This experiment was vital in revealing the influence that a single mixing and sampling had on the surviving *LM* populations. The statistical significance indicated by some of sample comparisons was not practical because they were less than a 1-log cfu/mL reduction, while several other comparisons ranged from a 1 to 2-log cfu/mL reduction. Practically, the reductions observed at 3-Hour and 4-Hour, from the initial 0-Hour samples, reached the 3-log cfu/mL threshold. *LM* was recovered on all TSA spiral plates and all BHI enrichments on the MOX split-plates. *LM* was also recovered from all of the UVM enrichments on the MOX split-plate except the 4-Hour sample (Trial-1), 3-Hour sample (Trial-2), 3-Hour sample (Trial-3), and 4-Hour sample (Trial-3). This was thought to be due to damage sustained from exposure to the oil and stress from the selective medium. Despite the overall survival of the *LM* population, as compared with the findings of previous experiments, the bacteria did experience an average 3.9-log cfu/mL reduction over the four hour sampling period. This experiment merely reveals the flawed design of the single source sample tubes as previously described. Overall, the hypothesis that the EVOO would cause at least a 3-log cfu/mL reduction in the initial *LM* inoculum levels over the four hour period at 26˚C was supported.

A Repeated Measures ANOVA was used to analyze the multiple mix data from the single source tube of Experiment F. Overall significance within the model was attributed to the hour effect at Pr>F <0.0001. Temperature was not an effect because all samples were held at 26˚C. The Least Square Means for hour revealed that the 0-Hour was significance at Pr> $|t|$ <0.0001. No significance was observed for the 6-Hour because no *LM* were recovered on the spiral plates or enrichment plates. When 0-Hour was compared to 6-Hour, a significance of Pr> $|t|$ <0.0001 matched the overall significance of the model, since there were only two comparable time points. A One-Way ANOVA was utilized to analyze the single mix data from the multiple source tubes of Experiment F. Overall significance within the model was attributed to the hour effect at Pr>F 0.0287. Temperature was not an effect because all samples were held at 26˚C. The Least Square Means for hour revealed that the two time points, 0-Hour and 6-Hour, held significance of Pr> $|t|$ 0.0021 and Pr> $|t|$ 0.0048 respectively. When 0-Hour was compared to 6-Hour, a significance of Pr> $|t|$ 0.0287 matched the overall significance of

the model, since there were only two comparable time points. Figure 5.6. displays the contrast experienced by the *LM* as they were exposed to a single or multiple mixing procedure.

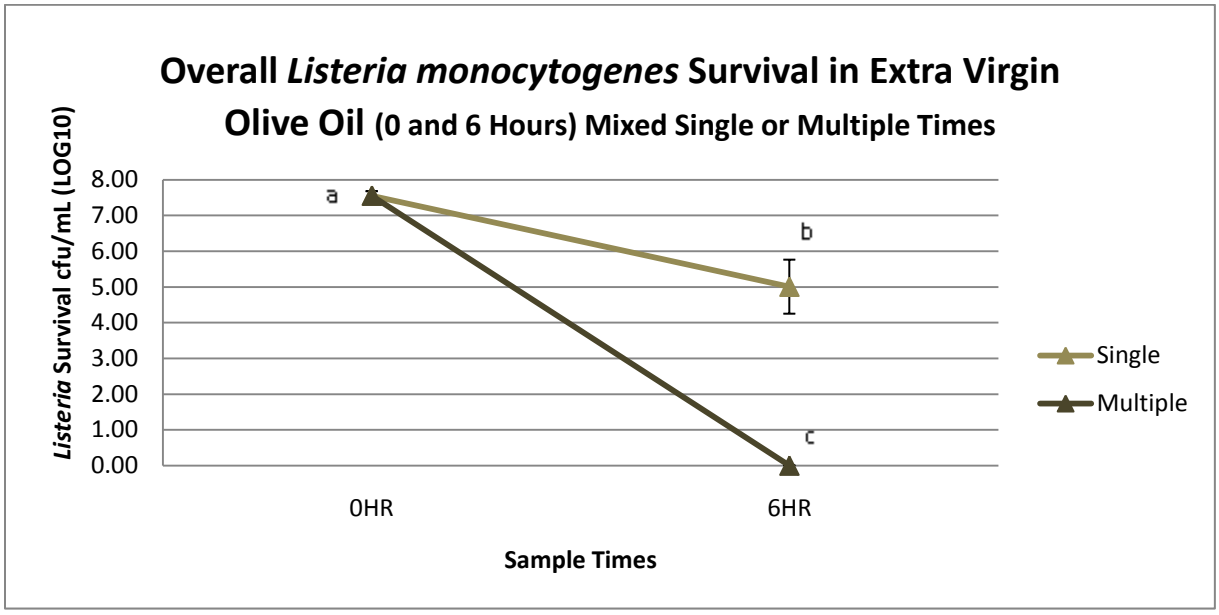

Figure 5.6. The average *Listeria monocytogenes* survival in extra virgin olive oil at 26˚C in single mix and multiple mix tubes over 6 hours

- (a) represents a lack of statistical significance comparing the bacterial populations of a sample to the initial bacterial populations, (b) represents a 2-log cfu/mL reduction from the initial bacterial population, (c) represents a ≥3-log cfu/mL reduction from the initial bacterial population

The Least Squares Means of both the single mix 0-Hour samples and the multiple mix 0-Hour samples were significance of Pr> $|t|$ <0.0001 when compared. However, the overall model comparison was not significant for 0-Hour. This is because the 0-Hour results were the exact same initial values, taken from the multiple mix single source tube, recorded as the 0-Hour value for both the single mix and multiple mix samples. This significance was therefore disregarded. There was overall significance in the comparison of the single mix 6-Hour samples and the multiple mix 6-Hour samples at Pr> 0.0094. The Least Squares Means for 6-Hour, indicated that the single mix 6-Hour from multiple tubes was significant at Pr> 0.0047. The *LM* was recovered from all TSA spiral plates and all BHI enrichments on MOX split-plates for the single mix 6-Hour samples; however, no growth was observed for any of the UVM enrichments on the MOX split-plates. This was thought to be due to damage sustained from exposure to the oil and

stress from the selective medium. The multiple mix 6-Hour, from a single source, was not significant. This was because no *LM* was detected on any of the TSA spiral plates or the MOX split-plates with BHI or UVM enrichments. This indicated that the multiple mix samples from a single source tube experienced a 7.5-log cfu/mL reduction over the six hour time period. The 2.5-log cfu/mL reduction experienced by the *LM* in the single mix from multiple source tubes continued to support the idea that mixing was a confounding variable which negatively influenced the survival rate of the bacteria. Despite the 7-log cfu/mL reduction experienced by the single source tube samples, the hypothesis that the EVOO would cause at least a 3-log reduction in the initial *LM* inoculum levels over the six hour period at 26˚C in both mixing styles was ultimately rejected because the samples from multiple source tubes did not reach the 3-log cfu/mL reduction threshold.

A Repeated Measures ANOVA was used to analyze the multiple mix data from the single source tube of Experiment G. Overall significance within the model was ascribed to the hour effect at Pr>F <0.0001. Temperature was not an effect because all samples were held at 26˚C. The Least Square Means for hour revealed that the 0-Hour was significance at Pr> $|t|$ <0.0001. No significance was observed for the 24-Hour or 48-Hour samples because no *LM* was recovered on the TSA spiral plates or the MOX split-plates with BHI or UVM enrichments. When compared to the 24-Hour and 48-Hour samples individually, the 0-Hour was significance at Pr> $|t|$ <0.0001. No significance was seen when comparing the surviving *LM* counts of 24-Hour and 48-Hour since no bacteria were recovered from any of those spiral plates or enrichment plates. This indicated a 7.75-log cfu/mL reduction. A One-Way ANOVA was utilized to analyze the single mix data from the multiple source tubes of Experiment G. Overall significance within the model was credited to the hour effect at Pr>F <0.0001. Temperature was not an effect because all samples were held at 26˚C. Again, the Least Square Means for hour revealed that the 0-Hour was significance at Pr> $|t|$ <0.0001. No significance was observed for the 24-Hour or 48-Hour samples because no *LM* was recovered on the TSA spiral plates or the MOX split-plates with BHI or UVM enrichments. When 0-Hour was compared to the 24-Hour and 48-Hour samples, a significance of Pr> $|t|$ <0.0001 was

observed for both. No significance was seen when comparing the surviving *LM* counts of 24-Hour and 48-Hour since no bacteria were recovered from any of those spiral plates or enrichment plates. This indicated a 7.75-log cfu/mL reduction. Figure 5.7. displays this drastic decline experienced by the *LM* as they were exposed to a single or multiple mixing procedure.

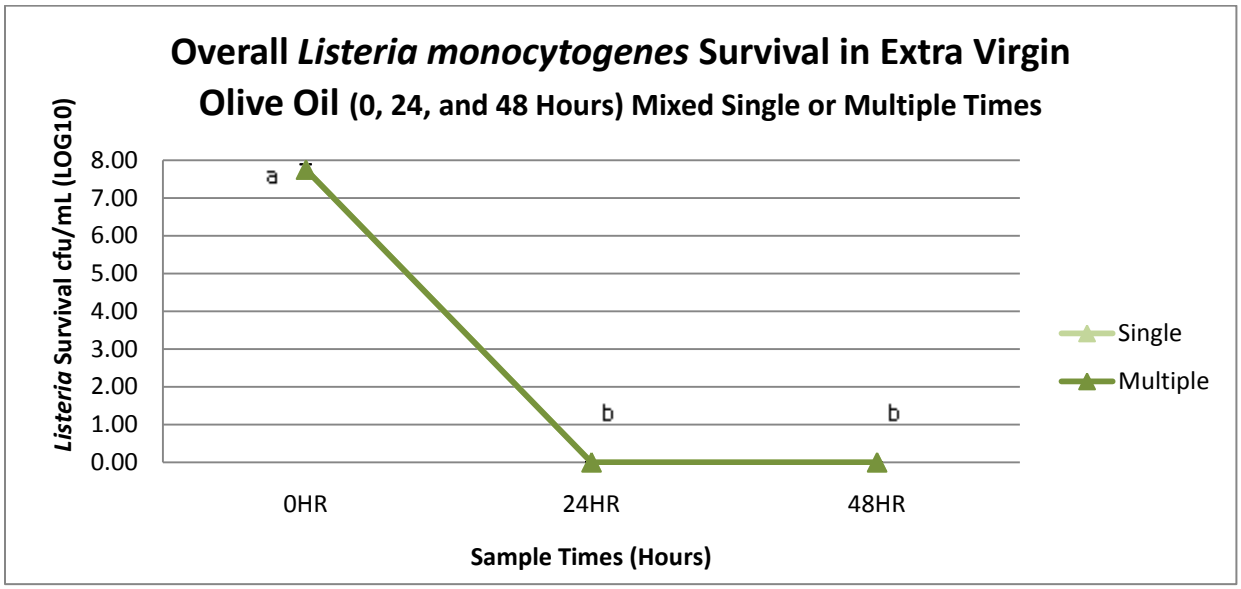

Figure 5.7. The average *Listeria monocytogenes* survival in extra virgin olive oil at 26˚C in single mix and multiple mix tubes over 48 hours

- (a) represents a lack of statistical significance comparing the bacterial populations of a sample to the initial bacterial populations, (b) represents a ≥3-log cfu/mL reduction from the initial bacterial population

This experiment validated the results of Experiment B and showed that the *LM* population was susceptible to the antimicrobial effects of the EVOO at 24 hours regardless of the mixing style. The 7-log cfu/mL reduction in both the multiple mix in single source tube and the single mix in multiple source tubes was observed at both 24 and 48 hours of exposure to the oil. The hypothesis that the EVOO would cause at least a 3-log reduction in the initial *LM* inoculum levels over the 48 hour period at 26˚C for both mixing styles was supported.

5.6. Discussion

In Experiment A, it was determined that either SPB with 0.1% Tween80 (v/v) or SPB without Tween80 could be used as the dilution fluid for the *LM* experiments. Since

the addition of Tween80 at 0.1% (v/v) had no inhibitory effect on the survival rate of the bacteria, but allowed for a longer suspension time of oil in the SPB, the SPB +0.1% Tween80 was utilized as the sole dilution solution for the remainder of the *LM* experiments. Due to the increased sensitivity experienced by the *LM* population at 37˚C in SPB, it was hypothesized that the bacteria might be more susceptible to the antimicrobial effects of EVOO at that temperature. Exposure to this higher temperature for prolonged periods of time was, therefore, noted as a potential influence to any decreased bacterial populations in EVOO over the same time frame. Overall, the *LM* populations did survive until the conclusion of the seven days with an averaged 0.94-log cfu/mL reduction for the SPB with 0.1%Tween80 and the SPB without Tween80 at 26˚C. The *LM* populations also survived the conclusion of the experiment time with an averaged 2.9-log cfu/mL reduction for the SPB with 0.1%Tween80 and the SPB without Tween80 at 37˚C. This indicated that the *LM* could survive at both 26˚C and 37˚C for at least seven days in SPB. The ability of *LM* to survive for prolonged periods of time in SPB at 25˚C was also observed by Liao and Shollenberger in 2003. Of the 35 *Listeria spp.* used, 27 were recovered from the SPB after three years of storage; with *LM* surviving to the conclusion of a four week and 30 week study.[108] The survival of the *LM* in SPB for at least seven days in the current study acted as a positive control when comparing the bacterial survival rate in EVOO in later experiments. Any ≥3-log cfu/mL reduction in the oil, before the conclusion of the studies, could arguably be attributed to the antimicrobial properties within the EVOO.

Experiments A and B were run simultaneously, but could not be statistically compared because of the single source tube design of Experiment B. In Experiment B, it was determined that exposure to EVOO for 24 hours, at 26˚C or 37˚C in a single source tube, resulted in a 7-log cfu/mL reduction in the *LM* population. This extreme decline within the bacterial counts was unexpected since previous experiments, with Gram-negative bacteria, took several days before a significant decline in the bacterial counts were observed (Chapter 3 and 4). As Gram-positive bacteria, it was thought that the thicker peptidoglycan layer might act as a buffer[182] against the oil and prolong the survival of *LM* in the EVOO. Instead, the *LM* appeared to be far more vulnerable to the

antimicrobial activity of the oil as shown by its rapid decline within a short time frame. This idea has been supported by various studies with olive based products which have found Gram-positive organisms to be more sensitive in vitro and in food applications.[101,118,119] Upon further investigation, it was noted that many of these studies also reported a wide reduction range of 3-logs cfu/mL to levels below detection over various time frames. The current study was therefore expanded to include the following experiments.

In Experiment C, it was determined that exposure to EVOO for six hours, at 26˚C or 37˚C in a single source tube, resulted in a 7-log cfu/mL reduction in the *LM* population. It is important to note that later experiments disprove this dramatic reduction rate at the six hour time point due to the influence of mixing, a confounding variable. For this experimental design, a single source tube which was mixed and sampled multiple times, the results of a 7-log cfu/mL reduction by the 6-Hour sample time remain valid. Experiment D, determined that exposure to EVOO for three hours at 26˚C or two hours at 37˚C, in a single source tube, resulted in a 7-log cfu/mL reduction in the *LM* population. Similar to Experiment C, later experiments disprove this dramatic reduction rate occurring under three hours due to the influence of mixing as a confounding variable. It was also determined that the incubation time of the TSA spiral plates should be modified to 48 hours at 37˚C to better reflect the FDA incubation procedures in the Bacteriological Analytical Manual (BAM).[56] Incubation procedures of plated enrichments were also modified to 48 hours at 37˚C based on the procedures described in the USDA Laboratory Guidebook concerning *Listeria monocytogenes*.[198] For this experimental design, a single source tube which was mixed and sampled multiple times; the results of a 7-log cfu/mL reduction by 2-Hour at 37˚C and 3-Hour at 26˚C sample time remain valid.

In contrast to Experiments B, C, and D, which were all designed using a single source tube of inoculated oil that was mixed and sampled from multiple times, Experiment E was designed using multiple source tubes of inoculate oil that were only mixed and sampled once. This design revealed the number of times the sample was mixed to be a confounding variable, which contributed to the rapid decline of the *LM* in the EVOO. This was why the below detection bacterial count observed for the *LM* strain

in Medina et al. (2006) was never accomplished in the current study within an hour time frame. The Medina et al. (2006) study utilized a GLF 3005 orbital shaker to continually mix the samples for one hour at 32˚C. Experiments B, C, and D of the current study were only mixed prior to sampling; during storage the samples were immobile within their designated incubators. Without the constant mixing motion, the samples were allowed to settle and temporarily avoid direct and constant contact with the EVOO. It is hypothesized the *LM* survival rate discrepancies between the Media et al. (2006) study and this current study may be due to a lack of oil exposure or resulting membrane stress prompted by the mixing motion. To test this hypothesis Experiments F and G were designed to include a sample which was blended multiple times and samples which were only blended once.

The design of Experiment F compared surviving *LM* populations within samples blended multiple times, in single source tubes, and samples blended a single time, in multiple source tubes. This experiment supported the idea that the number of mixings experienced by a sample was a confounding variable which contributed to the rapid decline of the *LM* in the EVOO. With only an average 2.5-log cfu/mL reduction of the *LM* population by the sixth hour in the single mix sample, compared to the 7.5-log cfu/mL reduction in the multiple mix sample, it was obvious that mixing was an extremely influential factor that negatively affected the survival rate. The survival rate of *LM* in single mix tubes of Experiment F cannot truly be compared to the results of Experiment E due to differences in experimental design. The discrepancies between these two experiments cannot be attributed to the frequency of mixing as with Experiment C; rather, they must be attributed to the differences in the final sample volumes. It is thought that the smaller sampling volume present in the Experiment E tubes, 12mL, allowed for lower levels of settling to occur within the inoculated oil. With less separation of the oil and water-based (BHI) inoculum, the culture would have had a more complete exposure to the oil for the sampling times of Experiment E. This would, again, suggest that increasing the direct exposure to the EVOO increases the rate of reduction experienced by the *LM*.

The recovery of *LM*, from the single mix 6-Hour TSA spiral plates in Experiment F, was also validated by the BHI enrichments, but not the UVM enrichments on the MOX split-plates. Failure to recover in the selective UVM enrichments and on the selective MOX split-plate was also noted for some of the single mix 3-Hour and 4-Hour samples in Experiment E. This failed enrichment recovery supported the idea that despite the survival of the *LM*, the injury to the cells was severe enough to inhibit their growth in selective medias. This inhibition confirmed the potent antimicrobial actions of EVOO against the *LM* even when a 3-log cfu/mL reduction was not accomplished in the sample. UVM, MOX, and other inhibitory media are standard for attempted environmental and sample recovery of *Listeria*.[56,198] The injury from the oil and the additional stress of the selective media may have resulted in the lack of bacterial growth, despite apparent recovery in general growth medias. This phenomenon has also been seen for a variety of damaged bacterial cells which were recovered on general media, but inhibited on selective types.[21,79,173,174] The increased sensitivity to additional stressors experienced by the *LM* attest the possible damage from exposure to the EVOO.

Experiment G validated the results of Experiment B and showed that, at 24 hours of exposure to the EVOO, the frequency of mixing was negligible because the *LM* were not recovered on the TSA spiral plates. This was validated by the negative results of both the UVM and BHI enrichments on the MOX plates. The experiment supports the idea that less frequent mixing, experienced by the bacteria in the EVOO, equals less direct exposure to the oil. To compensate for this inconsistent exposure, the time must be increased to produce dramatic bacterial reduction in the oil. Medina et al. (2006) did not disprove the findings of the current study; rather, the results supported the influence of the mixing frequency and the need for constant exposure to the oil to elicit a rapid antimicrobial effect. The findings of Experiment G suggest that at 24 hours of exposure to the EVOO the *LM* population will experience an averaging 7-log cfu/mL reduction, without any additional mixing.

5.7. Conclusions

The antimicrobial abilities of an extra virgin olive oil (EVOO) towards a four strain cocktail of *Listeria monocytogenes* (*LM*) was highlighted over several challenge experiments across time. The hypothesis of Experiment A for the *LM* populations in the positive controls, at 26˚C or 37˚C over seven days, was ultimately rejected due to a 2.9-log cfu/mL reduction experienced by the 37˚C samples by the seventh day. The hypothesis of Experiments B, C, D, E, and G were all supported because exposure to the EVOO resulted in a minimal 3-log cfu/mL reduction in the initial *LM* inoculum levels over their allotted sample periods. Experiments B, C, and D all saw reductions of their *LM* populations below detectable limits before the conclusion of their time frames. With the completion of these experiments, it was suggested that the frequency of mixing the single source samples could be a confounding variable. The designs of Experiments E, F, and G were adjusted to determine the influence of this factor. The hypothesis for Experiment F was ultimately rejected because the average *LM* reduction of 2.5-log cfu/mL in the single mix 6-Hour samples did not meet the desired 3-log cfu/mL reduction. The results of Experiment G validated the results of Experiment B; suggesting that at 24 hours of exposure to the EVOO the influence of the mixing frequency could be negated. The overall conclusions of the study were that the frequency of mixing experienced by a sample was an influential factor that contributed to the rapid decline of the *LM* populations within the EVOO and that this bacterial decline was due to the direct and constant contact with the oil which elicited a strong antimicrobial effect.

CHAPTER 6

THE SURVIVAL OF A *LISTERIA MONOCYTOGENES* COCKTAIL IN EXTRA VIRGIN OLIVE OIL DURING AN HOUR WITH MULTIPLE MIXINGS

6.1. Summary

The purpose of the study was to determine the influence that the mixing frequency had on the four strain *Listeria monocytogenes* (*LM*) cocktail in extra virgin olive oil (EVOO) over a one hour time frame. The 5-Minute Mix samples were vortexed every five minutes, the 10-Minute Mix samples were vortexed every 10 minutes, the Hand Mixed samples were mixed by inversion every 20 minutes, and the Mechanically Mixed samples were vortexed every 20 minutes. The four mixing types were sampled every 20 minutes over a one hour time frame at 26˚C. The overall hypothesis, that all of the mixing types would experience a ≥7-log cfu/mL reduction by the completion of the study, was supported. The reduction was accomplished at 20 minutes by the 5-Minute Mix samples, 40 minutes by the 10-Minute Mix samples, and 60 minutes by the Hand Mixed and Mechanically Mixed samples. This showed that increasing the mixing frequency of the sample further contributed to the inhibition of the *Listeria monocytogenes* (*LM*) in the extra virgin olive oil (EVOO). This could be due to the direct and constant physical contact of the bacteria with the antimicrobial compounds within the extra virgin olive oil.

6.2. Introduction

It is has been proposed that the majority of the antimicrobial action of extra virgin olive oil (EVOO) resides with its simple phenolic compound content. In the early 1990's, Montedoro et al. performed a series of HPCL evaluations on virgin oil from Moraiolo cultivar olives identifying various phenolic compounds and their hydrolyzed counterparts.[125-127] However, the idea that only simple or even secondary compounds contributed to the antimicrobial action of the oil seemed to negate other prevalent compounds. Kubo et al. (1995) tested various steam-distilled compounds from olive fruits and leaves against various microorganisms. The antimicrobial activity was attributed to the presence of α,β-unsaturated aldehydes within the olive distillates.[101] A

previous study also suggested that the antimicrobial activity depended on the length of the hydrophobic alkyl tail; which was said to cause disorder in the plasma membrane surface proteins and channels.[101] These disruptions within the plasma membrane could lead to cell leakage and eventual cell death. Medina et al. (2006) analyzed the phenolic compounds in a plethora of virgin olive oils, refined olive oils, and pomace olive oils. The antimicrobial activity of the individual compounds was significantly increased when several of the compounds were recombined.[118] In 2007, Medina et al. supported the claim that the synergistic action of phenolic compounds, such as tyrosol, hydroxytyrosol, the dialdehydic form of decarboxymethyl ligstroside, and oleuropein aglycons, from a previous study[118], showed a broad antimicrobial effect towards several microorganisms. Ironically, the three phenolic compounds, with the highest concentration in the oil, tyrosol, glycoside oleuropein, and hydroxytyrosol, are structurally similar.[196] Despite the progress in isolating various components within olive products and assaying their antimicrobial abilities, it is still unclear which compounds provide the maximum bactericidal affect. This uncertainty can be attributed to the variations among oil types and the apparent synergistic effects of the various components within the oil. Other factors, such as the mixing frequency of the samples, have been marginalized in many studies in order to keep the cultures suspended in the oils and oil extracts.

The purpose of this study was to investigate the influence of the mixing frequency on the reduction of four strains of *Listeria monocytogenes* (*LM*) in extra virgin olive oil (EVOO) over a one hour time frame. The overall hypothesis of the study was that all of the samples would experience a ≥7-log cfu/mL reduction by the completion of the one hour time frame. This study would further previous in-vitro *Listeria* research (Chapter 5) and determine the influence of mixing frequency in addition to the antimicrobial properties of EVOO. This information would be beneficial in developing dip applications for various food products associated with *Listeria* contamination.

6.3. Materials

6.3.1. Pathogens

Listeria monocytogenes 150C (ATCC 51781), *Listeria monocytogenes 150D*

(ATCC 43256), *Listeria monocytogenes 150E* (ATCC 15313), and *Listeria monocytogenes 150F* (ATCC 19115) were the four strains of *Listeria* utilized in the study.

6.3.2. Evaluated Product

One commercial extra virgin olive oil (EVOO) was evaluated on its ability to inhibit *Listeria monocytogenes* (*LM*) over several different time frames at one or two temperatures, 26˚C or 37˚C. Primo Gusto: Italian Foods 100% Italian Extra Virgin Olive Oil (cold pressed) (country of origin: Italy via Gordon Food Service® P.O. Box 1787, Grand Rapids, MI 49501) was purchased from a local grocery store. This product was chosen because it did not contain any additives or preservatives which could have hindered the survival rate of the bacteria within the product. The pH was recorded as 3.88 and the water activity was recorded as 0.39.

6.3.3. Medias

The broths used in the *LM* challenge included Bacto™ Brain Heart Infusion (BHI) and Difco™ UVM Modified *Listeria* Enrichment Broth (UVM). Both products were manufactured by Becton, Dickinson and Company (7 Loveton Circle, Sparks, MD, 21152) and were prepared according to the product specifications. The BHI, intended for culture growth, was dispensed into Pyrex tubes with a final volume of 7mL each and autoclaved. The BHI, intended to be used as enrichments, had 1mL of Tween80 added per liter of broth so that the final mixture contained 0.1% (v/v). The BHI +Tween80 was then dispensed into Pyrex tubes with a final volume of 9mL each and autoclaved. The UVM, intended to be used as enrichments, also had Tween80 added to a concentration of 0.1% (v/v). The UVM +Tween80 was then dispensed into Pyrex tubes with a final volume of 9mL each and autoclaved. Dilution blanks used throughout the study were Sterile Phosphate Buffer (SPB) dilution blank tubes. These dilution tubes were made from 24mL PO_4 solution, 95mL $MgCl_2$ solution, and 19L of double deionized water. Tween80 was added to the dilution blank mixture at 1mL/1L so that the final blanks contained 0.1% Tween80 (v/v). The dilution mixture with Tween80 was adjusted to a pH between 7.4-7.5. The mixture was then dispensed into Pyrex tubes with a final volume of 9mL and autoclaved. The agars utilized in the *Listeria* study included BBL™

Trypticase™ Soy Agar: Soybean-Casein Digest Agar (TSA) and Difco™ Modified Oxford (MOX) from Difco™ Oxford Medium Base and Difco™ Modified Oxford Antimicrobic Supplement. Both TSA and MOX were from Becton, Dickinson and Company (7 Loveton Circle, Sparks, MD, 21152) and were prepared according to the product specifications. All agars were poured into sterile 100x15mm polystyrene disposable VWR Petri plates (1050 Satellite Blvd NW, Suwanee, GA 30024).

6.4. Methods

6.4.1 Oil Sterility Validation

The oil was transferred from its original container into sterile 250mL glass bottles with caps. Samples of the oil were taken and plated on TSA and MOX and incubated for 48 hours at 37˚C to check for bacterial background. Oil samples were also plated on TSA and incubated for 48 hours at 26˚C to validate sterility. The oil was confirmed to be commercially sterile with an aerobic count below the level of detection. The oil stock bottle was then wrapped in foil and placed in refrigerated storage to prevent any additional oxidation or contamination.

6.4.2. Inoculum Preparation

A scrape of each desired *LM* strain was obtained from a pre-existing refrigerated BHI slant and was transferred into BHI broth using sterile technique. The culture was incubated for 24 hours at 37˚C. The culture was then streaked for isolation onto MOX agar and was incubated for 24 hours at 37˚C. The morphology of the colonies was observed and an isolated colony was transferred to a new tube of BHI. The culture was then incubated for 24 hours at 37˚C. The culture was transferred twice by placing 0.1mL of the former culture into new BHI and incubated for 24 hours at 37˚C. The final transfer of *LM 150C* (ATCC 51781), *LM 150D* (ATCC 43256), *LM 150E* (ATCC 15313), and *LM 150F* (ATCC 19115) were then pooled into a sterile 50mL Flacon Tube. The cocktail was vortexed.

6.4.3. Sample Preparation

The broth method utilized in the first *LM* study (Chapter 5) was also used for this study. *LM 150C* (ATCC 51781), *LM 150D* (ATCC 43256), *LM 150E* (ATCC 15313),

and *LM 150F* (ATCC 19115) were grown individually in BHI broth and pooled together as formerly described.

6.4.3.a. Experiment A: Extra Virgin Olive Oil 1 Hour: Hand verses Mechanically Mixed Tubes

To determine the influence of the mixing types on *LM* in EVOO, Hand Mixed and Mechanically Mixed tubes were sampled from single source tubes every 20 minutes. The oil stock bottles were obtained from refrigerated storage and warmed in a 50˚C water bath, just until the oil became liquid. The oil stock bottles were then placed in a Labconco Purifier Class II Biosafety Cabinet. The four strain *LM* cocktail was made as formerly described and 2mL were added to 28mL of sterile oil in a sterile 50mL beaker. This 50mL beaker was placed into a sterile 250mL beaker to prevent splashing as the stock was mixed. This sample stock, now approximately 7-log cfu/mL, was divided into two separate sterile 50mL Falcon tubes. Each tube contained 15mL of the sample stock. These tubes were only stored at 26˚C because, in previous experiments, the *LM* appeared less stressed at this temperature. The tubes were randomized and labeled Hand Mixed and Mechanically Mixed. The Hand Mixed sample tube was mixed by fully inverting the falcon tube two times, approximately five seconds, before sampling. The Mechanically Mixed sample tube was mixed using the Vortex Genie-2 for five seconds. These single source tubes were both mixed and sampled every 20 minutes for one hour. The 0-Minute samples were taken immediately following the sample set up. After plating, the two tubes were stored at 26˚C incubation until the next designated sample time. All steps were accomplished using sterile technique so that *LM* would be the only bacteria present and TSA could be used as the growth media for the study.

6.4.3.b. Experiment B: Extra Virgin Olive Oil 1 Hour: 5-Minute verses 10-Minute Mix Tubes

To determine the influence of the mixing frequency on *LM* in EVOO, single source tubes were mixed every five minutes and every 10 minutes before being sampled every 20 minutes. The oil stock bottles were obtained from refrigerated storage and warmed in a 50˚C water bath, just until the oil became liquid. The oil stock bottles were then placed in a Labconco Purifier Class II Biosafety Cabinet. The four strain *LM*

cocktail was made as formerly described and 2mL were added to 28mL of sterile oil in a sterile 50mL beaker. This 50mL beaker was placed into a sterile 250mL beaker to prevent splashing as the stock was mixed. This sample stock, now approximately 7-log cfu/mL, was divided into two separate sterile 50mL Falcon tubes. Each tube contained 15mL of the sample stock. These tubes were only stored at 26˚C because the *LM* appeared less stressed at this temperature in previous experiments. The tubes were randomized and were labeled 5-Minute Mix and 10-Minute Mix. The 5-Minute Mix sample tube was mixed, approximately five seconds, using the Vortex Genie-2 every five minutes. The 10-Minute Mix sample tube was mixed, approximately five seconds, using the Vortex Genie-2 every 10 minutes. These single source tubes were both mixed accordingly and were then sampled every 20 minutes for one hour. The 0-Minute samples were taken immediately following the sample set up. After plating, the two tubes were kept at 26˚C and mixed according to their schedule. All steps were accomplished using sterile technique so that *LM* would be the only bacteria present and TSA could be used as the growth media for the study.

6.4.4. Sample Plating Procedure

All samples were spiral plated in duplicate onto TSA at the 50µL spiral setting of Eddy Jet2. The number of dilution blanks used for each of the following sample times depended upon the spread and overall growth of the bacteria from the previous day. If the bacteria appeared to have a consistent and readable spread across the plate, then the number of dilution blanks used remained the same. If, however, the bacterial counts appeared to be decreasing, then the number of dilution blanks used, for that sample, was decreased to keep the bacteria at readable levels. The straight sample was never plated. This was due to pilot studies which indicated that the bacteria present in the sample grew better when separated from the straight oil. Therefore, at least one dilution blank was used as the minimum dilution for each sample, rather than just plating the straight oil.

6.4.4.a. Experiment A: Extra Virgin Olive Oil 1 Hour: Hand verses Mechanically Mixed Tubes

Sample tubes from the 26˚C incubator were obtained at the designated 20 minute sample times. Each sample tube was mixed, as indicated by its label, and sampled. The

0-Hour samples were mixed and used three sequential dilution blanks each. The final dilution tube for each was poured into a sterile spiral plater cup and placed in the spiral plater. The sample was spiral plated in duplicate onto TSA at a 50µL spiral setting of Eddy Jet2. The plates were inverted and incubated for 48 hours at 37˚C. This procedure was conducted for the 0-Minutes, 20-Minutes, 40-Minutes, and 60-Minutes samples at 26˚C. The dilution blank number was adjusted to one tube after plating the 0-Minute samples.

6.4.4.b. Experiment B: Extra Virgin Olive Oil 1 Hour: 5-Minute verses 10-Minute Mix Tubes

The sample tubes were kept at 26˚C and were mixed according to their schedule. The 5-Minute Mix tubes were mixed every five minutes and sampled every 20 minutes. The 10-Minute Mix sample tubes were mixed every 10 minutes and sampled every 20 minutes. The 0-Hour samples were mixed and used three sequential dilution blanks each. The final dilution tube for each was poured into a sterile spiral plater cup and placed in the spiral plater. The sample was spiral plated in duplicate onto TSA at a 50µL spiral setting of Eddy Jet2. The plates were inverted and incubated for 48 hours at 37˚C. This procedure was conducted for the 0-Minutes, 20-Minutes, 40-Minutes, and 60-Minutes samples at 26˚C. The dilution blank number was adjusted to one tube after plating the 0-Minute samples.

6.4.5. Enrichment Procedure

Enrichments were performed by pipetting 1mL of the sample into 9mL of sterile UVM +0.1% Tween80 (v/v) and 9mL of sterile BHI +0.1% Tween80 (v/v). The broths were then vortexed, labeled, and placed into the incubator for 24 hours at 30˚C. If the spiral plates did not show growth then the enrichments were removed from the incubator, vortexed, streaked onto MOX, and then incubated at 37˚C for 48 hours. If the spiral plates showed bacterial growth, then the enrichment tubes were plated as additional confirmation. Enrichments were recorded as positive or negative for growth.

6.4.6. Reading Spiral Plate Procedure

Spiral plate counts were read after 48 hours incubation at 37˚C. A FlashAndGo automated counter and FlashAndGo 1.0 Computer program were used to count the spiral

plate colonies. These items were combined to form the FlashAndGo -Basic Economy Automated Colony Counter through ILU Instruments of NEU-TEC Group Inc. (1 Lenox Avenue, Farmingdale, NY 11735). The dilution factor was adjusted as needed for each of the plated oil samples from that particular sample time and combined with the counted colonies to algorithmically determine the total bacterial count within the given sample. The duplicate plates were averaged together within Microsoft Excel to give a more accurate total count of the given sample.

6.4.7. Statistical Analysis

Statistical analysis was performed using SAS 9.4 with significance indicated at $p < 0.05$. Data organization and graphical figures were constructed using Microsoft Office Excel 2007. A bacterial reduction of 3-log cfu/mL or greater was noted as a significant reduction within the bacterial population.

6.5. Results

To simplify the statistical analysis, the data of Experiment A and Experiment B were compared within the same statistical model. This was done because the experimental designs were the same and both were run simultaneously. A Repeated Measures ANOVA was used to analyze the data from the experiments. The statistical model indicated overall significance with the mix (Pr>F 0.0093), minute (Pr>F <0.0001), and mix*minute (Pr>F <0.0001) effects. It was determined that the mix effect, representing the frequency at which the samples were mixed, was the primary influential factor of the experiments. This was displayed in the significance of the minute effect and the mix*minute interaction which reflected how the *LM* population was affected over the multiple mixings. The Least Squares Means for the mix*minute interaction revealed that 0-Minutes was significant at $Pr>|t| <0.0001$ for every sample type. The 20-Minute samples from the Hand Mixed tubes ($Pr>|t| <0.0001$), the Mechanically Mixed tubes ($Pr>|t| <0.0001$), and the 10-Minute Mix ($Pr>|t| 0.0121$) were also significant. The only 40-Minute sample that was significant was the from the Hand Mixed tube at $Pr>|t|$ 0.0002. The primary reason for the insignificance of the other samples was because many of the samples were below the point of bacterial detection (<1-log cfu/mL). All of

the 0-Minute samples across the four different mixing frequency types were insignificant, indicating they all had a similar initial bacterial count, 7-log cfu/mL. These observations were displayed below in Figure 6.1.

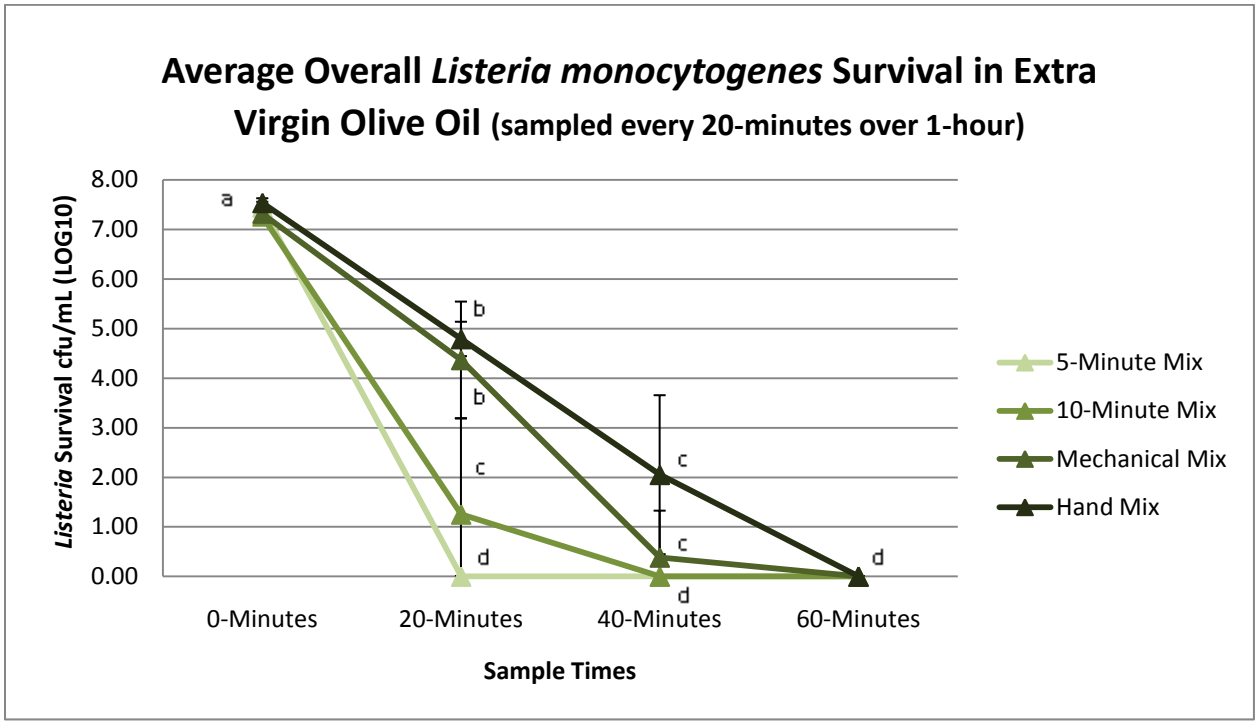

Figure 6.1. The average *Listeria monocytogenes* survival in extra virgin olive oil with different mixing frequencies over 1 hour at 26˚C

- The 5-Minute Mix samples are mechanically mixed every five minutes and sampled every 20 minutes, the 10-Minute Mix samples are mechanically mixed every 10 minutes and sampled every 20 minutes, the Mechanical Mix samples are mechanically mixed and sampled every 20 minutes, the Hand Mix samples are mechanically mixed and sampled every 20 minutes
- (a) represents a lack of statistical significance comparing the bacterial populations of a sample to the initial bacterial populations, (b) represents a 2-log cfu/mL reduction from the initial bacterial population, (c) represents a ≥3-log cfu/mL reduction from the initial bacterial population, (d) represents a ≥7-log cfu/mL reduction from the initial bacterial population

None of the 20-Minute, 40-Minute, or 60-Minute TSA spiral plates or enrichment plates showed any growth for the 5-Minute Mix samples. A ≥7-log cfu/mL reduction was observed by the 20-Minute sample. This was supported by the statistics which showed the 0-Minute samples, from the 5-Minute Mix tubes, were significant ($Pr>|t|$ <0.0001) when compared to all of the 20-Minute, 40-Minute, and 60-Minute samples from the 5-Minute Mix, 10-Minute Mix, Hand Mixed, and Mechanically Mixed tubes. The only exception was the 20-Minute Hand Mixed sample, at $Pr>|t|$ 0.0004, which was still significant. This supported the hypothesis that the 5-Minute Mix samples would experience a ≥7-log cfu/mL reduction by the completion of the one hour time frame. The

5-Minute Mix samples experienced the fastest bacterial reduction of all the sample types, at 20 minutes.

The 20-Minute sample results, of the 10-Minute Mix tubes, were inconsistent. Two trials observed a ≥7-log cfu/mL reduction by 20 minutes, while the other trial simply experienced a little over a 3.5-log cfu/mL reduction. This could have been due to inconsistent mixing or better survival of *LM* in that particular sample. The average *LM* count decrease of the 20-Minute samples was a 6-log cfu/mL reduction. A ≥7-log cfu/mL, was observed by the 40-Minute sample. None of the 40-Minute or 60-Minute TSA spiral plates or enrichments showed any growth for the 10-Minute Mix samples. This was supported by the statistics which indicated that the 0-Minute samples, from the 10-Minute Mix tubes, were significant ($Pr>|t|$ <0.0001) when compared to all of the 20-Minute, 40-Minute, and 60-Minute samples from the 5-Minute Mix, 10-Minute Mix, Hand Mixed, and Mechanically Mixed tubes. The only exceptions were the 20-Minute Hand Mixed sample at $Pr>|t|$ 0.0009 and the 20-Minute Mechanically Mixed sample at $Pr>|t|$ 0.0002 which were still significant. This supported the hypothesis that the 10-Minute Mix samples would experience a ≥7-log cfu/mL reduction by the completion of the one hour time frame. The 10-Minute Mix samples experienced the second fastest bacterial reduction of all the sample types, at 40-minutes.

The *LM* population within the Mechanically Mixed samples did not officially experience a ≥7-log cfu/mL reduction until the 60-Minute sample. This was due to the presence of a single *LM* colony on one of the 40-Minute sample spiral plates in the third trial. This *LM* could have been an airborne contaminant from another plate or the environment. It could have also been a surviving bacterium within the actual sample. Due to the uncertainty of its origin, this *LM* was counted as a viable colony on the spiral plate. No other *LM* was recovered from either the BHI or UVM enrichments of that sample. All other spiral plated samples and enrichments were consistent, except for one of the 20-Minute samples. Despite having readable TSA spiral plate counts and growth on the MOX plate from the BHI enrichment, this sample was negative for growth on the MOX plate from the UVM enrichment. An approximate 3-log cfu/mL reduction was observed with every 20 minute mixing and sampling period. The first average reduction

in the bacterial population was 2.96-log cfu/mL observed at 20 minutes, followed by an average 3.98-log cfu/mL reduction at 40 minutes, and then the residual reduction at 60 minutes. This was supported by the statistics which indicated that the 0-Minute samples, from the Mechanically Mixed tubes, were significant (Pr>$|t|$ <0.0001) when compared to all of the 20-Minute, 40-Minute, and 60-Minute samples from the 5-Minute Mix, 10-Minute Mix, Hand Mixed, and Mechanically Mixed tubes. The only exception was the 20-Minute 10-Minute Mix sample at Pr>$|t|$ 0.0002 which was still significant. This supported the hypothesis that the Mechanically Mixed samples would experience a ≥7-log cfu/mL reduction by the completion of the one hour time frame.

Similar to the Mechanically Mixed samples, the *LM* population within the Hand Mixed samples did not experience a ≥7-log cfu/mL reduction until the 60-Minute sample. Inconsistent recovery in the UVM enrichments was also noted in the Hand Mixed samples. Trial-1 observed a lack of growth on the MOX plates from the UVM enrichments of the 20-Minute and 40-Minute samples. These negative results were unusual because the samples had readable TSA spiral plate counts and growth on the MOX plates from the BHI enrichments. The only other conflicting enrichment was the 40-Minute sample of Trial-2 which experienced the same phenomenon as the 40-Minute sample of Trial-1. All other UVM enrichments reflected the BHI enrichments; despite the tendency to have less overall growth comparatively. An approximate 2.7-log cfu/mL reduction was observed with every 20 minute mixing and sampling period. The first average reduction in the bacterial population was 2.75-log cfu/mL observed at 20 minutes, the second was an average 2.74-log cfu/mL reduction at 40 minutes, and the third was the residual 2.05-log cfu/mL reduction at 60 minutes. This was supported by the statistics which indicated that the 0-Minute samples, from the Hand Mixed tubes, were significant (Pr>$|t|$ <0.0001) when compared to all of the 20-Minute, 40-Minute, and 60-Minute samples from the 5-Minute Mix, 10-Minute Mix, Hand Mixed, and Mechanically Mixed tubes. The only exceptions were the 20-Minute Hand Mixed sample at Pr>$|t|$ 0.0002, the 20-Minute 5-Minute Mix sample at Pr>$|t|$ 0.0002, and the 20-Minute 10-Minute Mix sample at Pr>$|t|$ 0.0009 which were still significant. This

supported the hypothesis that the Hand Mixed samples would experience a ≥7-log cfu/mL reduction by the completion of the one hour time frame.

6.6. Discussion

In general, all of the mixing types could be statistically compared because they were of the exact same experimental design and were conducted simultaneously. The 5-Minute Mix samples experienced an average 7.44-log cfu/mL reduction by 20 minutes. The 10-Minute Mix samples experienced an average 7.26-log cfu/mL reduction by 40 minutes. The Mechanically Mixed samples experienced an average 7.32-log cfu/mL reduction by 60 minutes. The Hand Mixed samples experienced an average 7.54-log cfu/mL reduction by 60 minutes. All mixing types observed a ≥7-log cfu/mL reduction by the conclusion of the one hour study; thus, supporting the overarching hypothesis of the study.

The inconsistencies experienced by the UVM enrichments were also observed in a previous *Listeria* study in extra virgin olive oil (Chapter 5). Modified *Listeria* Enrichment Broth (UVM), manufactured by Becton, Dickinson and Company, contains nalidixic acid to inhibit the growth of Gram-negative organisms and acriflavine hydrochloride to inhibit unwanted Gram-positive bacteria.[4] Although these ingredients are helpful in preventing the recovery of other undesirable bacteria within the sample or environment, they may also have inhibitory effects towards damaged *Listeria* cells. Difco™ Oxford Medium Base (OX) contains lithium chloride and a high sodium chloride content contributes to the inhibitory properties of the media base.[4] Difco™ Modified Oxford Antimicrobic Supplement contains moxalactam and colistin methane sulfonate, or colistin sulfate, as inhibitory compounds.[4] This supplement is added to the media base to produce Difco™ Modified Oxford (MOX). This media also presents a similar problem to the recovery of damaged *Listeria* cells similar to that of UVM. This study did not compare the recovery rate of potentially damaged *Listeria* cells from UVM on selective and non-selective agars. Therefore, it is uncertain whether the inhibition of the damaged *Listeria* occurred in the UVM itself or with the multiple exposures to selective agents in UVM and MOX. However, this study did compare the recovery rate of potentially

damaged *Listeria* cells within a selective and non-selective enrichment broth. The non-selective BHI enrichments consistently reflected the presence or absence of the *LM* on the TSA spiral plates. The only exception was the one 40 minute spiral plate from the Mechanically Mixed sample. In contrast, the UVM failed to recover *LM* from several Mechanically Mixed and Hand Mixed samples of different trials, which showed growth on TSA spiral plates and in BHI enrichments. Across all mixing types, the recovery of *LM* from the UVM tubes was never as prolific as the recovery from BHI tubes. This may be attributed to a slower recovery in the media or to the possible death of injured cells in a selective environment. Regardless, the UVM enrichments, either alone or in combination with MOX, were not as reliable in displaying the surviving *LM*. A number of studies have also observed a similar inhibition of stressed and damaged bacterial cells by selective media.[21,89,173] This phenomenon was helpful in indicating the possible damage inflected by the EVOO on the *LM* populations.

The reduction in the *LM* population within the EVOO was most prominent in the 5-Minute Mix samples followed by the 10-Minute Mix samples. These reductions were extremely rapid; unlike the Mechanically Mixed and Hand Mixed samples, which experienced a more linear decline in the bacterial populations over the course of the study. These samples also displayed the inconsistent recovery by the UVM enrichments on MOX. This highlighted the possible damage experienced by the EVOO. The EVOO utilized in the study had a pH of 3.88. The typical pH promoting the growth of *LM* ranges from 4.4 to 9.6.[4,165] By logical progression, it could be concluded that the low pH of the EVOO could not be tolerated by the *LM*. This idea was refuted by numerous studies that have recorded bacterial survival in fruit juices[9,37,118], sodas[119], acidic cheeses[160] below this 4.4 pH threshold. The EVOO was also inoculated with a stationary phase *LM*. Stationary phase *Listeria* have been shown to be resistant to acid challenges of pH 3.5 via heightened expression of the *prfA*-gene which influences various virulence factors.[140] This further refutes the idea that the pH of the EVOO was the sole reason for the decline within the initial *LM* populations.

The EVOO utilized in the study had a water activity of 0.39. The minimum water activity required for the growth of *Listeria spp*. typically ranges from 0.90-0.92

depending upon the media and other substrates.[137,148] The survival of *Listeria* varies over a wide range of water activities in products such as 0.90-0.8 in dried sausages[81,136], spray dried milk (moisture content 3.6-6.4%)[45], and 0.27 in pork rinds and cracklings[81]. This information refutes the idea that water activity alone could result in the bacterial reductions observed in this study. The idea that the *LM* may have decreased due to a lack of oxygen was also proposed. Medina et al. (2006) refuted this idea by studying the effects of *Listeria* within several different edible oils. None of these oils, except the olive oils, showed any significant inhibition of the *Listeria*.[118] Lungu et al. (2009) reviewed the survival of *Listeria* in various low oxygen and anaerobic conditions including the human body and various foods. The facultative nature of this bacteria allows it to adjust across a wide range of oxygen gradients.[113] This indicated that suffocation within the EVOO was also an unlikely factor in the reduction of the bacterial population.

Based on the inability of these factors to cause the rapid decline in the *LM* populations, the ≥7-log cfu/mL reduction observed in this study, can indeed be attributed to antimicrobial properties in the EVOO. This reduction was aided by the increased mixing frequency experienced by the samples. The EVOO utilized in the study was categorized as cold pressed. It has been suggested that oil made from cold pressed olives increases the total phenolic content of the oil.[84,145] It is hypothesized that the more frequent the mixing, the more direct contact the bacteria had with the antimicrobial agents within the EVOO. This was best displayed over the different mixing frequencies. The Mechanically Mixed and Hand Mixed samples were only mixed and sampled every 20 minutes. This allowed for a certain amount of settling to occur within the sample. As the amount of time allowed for settling increased, the overall time needed to accomplish bacterial reduction also increased. As the mixing frequency was increased, the amount of time allowed for settling of the sample decreased, so did the overall time needed to observe a ≥7-log cfu/mL reduction. With less settling, the samples were exposed to the antimicrobial agents of the oil for longer periods of time. If any of these antimicrobial components disrupt the plasma membranes of the bacteria[101], then it follows that the mixing component of this study may have contributed a great deal of stress on the damaged organisms. It is thought that the external stress from this mixing may have

overwhelmed the internal pressure of the bacteria resulting in leakage through the compromised membrane eventually leading to cell lyses.

6.7. Conclusions

This study confirmed the idea that the mixing frequency, experienced by the *Listeria monocytogenes* (*LM*) cocktail in EVOO, contributed to the bacterial decline over a one hour time frame. The more mixing the bacteria were exposed to, while in the oil, the faster the rate of reduction. All of the sample types, 5-Minute Mix, 10-Minute Mix, Hand Mixed, and Mechanically Mixed tubes, experienced a ≥7-log cfu/mL reduction by the one hour conclusion of the study. The fastest ≥7-log cfu/mL bacterial reduction was that of the 5-Minute Mix samples, which were unrecoverable at 20 minutes. This was followed by the bacterial reduction of the 10-Minute Mix samples at 40 minutes and the reductions of the Mechanically Mixed and Hand Mixed samples at 60 minutes. It is thought that the more often a sample is mixed, the more exposure the bacteria have to the active antimicrobial compounds within the olive oil. With this increased exposure it is thought that the rate of reduction experienced by the bacteria also increases contributing to a ≥7-log cfu/mL reduction in less time.

CHAPTER 7

THE SURVIVAL OF A *LISTERIA MONOCYTOGENES* COCKTAIL ON PORK TENDERLOIN SPREAD WITH EXTRA VIRGIN OLIVE OIL (MINI STUDY)

7.1. Summary

The purpose of this study was to determine if extra virgin olive oil could reduce the *Listeria monocytogenes* populations on the surface of tenderloin medallions. The method consisted of 0.1mL of a 7-log cfu/mL *Listeria monocytogenes* (*LM*) cocktail inoculum dispensed and spread onto the surface of an approximate 25g cooked pork tenderloin medallion. This was followed by 0.1mL of extra virgin olive oil spread across the surface of the meat. Samples were taken from 26˚C storage at 0 minutes, 30 minutes, and 60 minutes. It was hypothesized that exposure to the extra virgin olive oil would result in a 3-log cfu/g reduction of the *LM* population within one hour. This hypothesis was rejected since no reduction was observed in any of the *LM* strains in this experiment. The dryness of the meat, the lack of oil volume, the lack of oil spreading, and the limited exposure time were thought to contribute to the bacterial survival.

7.2. Introduction

There have been several successful studies concerning the incorporation of olive based inhibitors into food products. Radford et al. (1991) attributed the reduction of *Salmonella enteritidis* in egg mayonnaise, within 48 hours, to the acidity and phenolic compounds within virgin olive oil (EVOO). Similarly, Medina et al. (2007) found that greater reduction rates of *Salmonella enteritidis* within egg mayonnaise occurred with the addition of virgin olive oil held at 30 minutes and to a lesser extent 10 minutes. It was also determined that the addition of virgin olive oil in milk mayonnaise reduced the *Listeria monocytogenes* (*LM*) below detectable levels within 30 minutes.[119] *LM* was likewise reduced beyond the point of detection in lettuce with the addition of virgin olive oil which was occasionally mixed for 30 minutes.[119] Tassou and Nychas (1994) found that the addition of 0.5% and 1% (w/v) olive extract increased the lag period of *Staphylococcus aureus* in milk by three hours before exponential growth occurred.

Concentrations of 1.5% and 2% (w/v) olive extract reduced the viable *S. aureus* counts within the milk to 1.5-logs (cfu/mL) less than the control.[189] The antimicrobial activity of extra virgin olive oil (EVOO) is not as well documented in meat products because of the complexity of the protein and fat matrix and the difficulty of establishing a proper procedure. More studies have been conducted using olive products as additional hurdles in combination with modified atmospheres and packaging of meat products.[190] This multi-hurdle approach makes it difficult to attribute the antimicrobial effects towards one specific factor, such as the addition of virgin olive oils. In 2012 and 2013, Rounds et al. applied a powdered olive extract to ground beef in order to reduce the bacterial counts of *E. coli O157:H7* under detectable levels (<1-log cfu/g) after a milder cook treatment. These studies indicated that the inhibitory effects of olive products could be the primary factor in influencing bacterial reduction within meat products.

This mini study was designed to determine if extra virgin olive oil (EVOO) would have any antimicrobial effects against four strains of *LM* on the surface of cooked pork tenderloin medallions. The purpose of the study was to see if EVOO could reduce the *LM* populations on the surface of tenderloin medallions over an hour time frame at 26˚C storage. A drop and spread technique was used to inoculate the meat surface and to add the oil. Based on the coinciding research conducted with *LM* in extra virgin olive oil (Chapter 5 and Chapter 6) it was hypothesized that a 3-log cfu/g reduction might be observed in the surviving bacterial populations when exposed to the oil on the surface of the meat. This information would be beneficial in determining the effectiveness of EVOO as a natural antimicrobial on complex food items such as cooked meats. This information may inspire further exploration into preventative sprays and coatings derived from natural antimicrobials such as extra virgin olive oils.

7.3. Materials

7.3.1. Pathogens

Listeria monocytogenes 150C (ATCC 51781), *Listeria monocytogenes 150D* (ATCC 43256), *Listeria monocytogenes 150E* (ATCC 15313), and *Listeria*

monocytogenes 150F (ATCC 19115) were the four strains of *Listeria* utilized in the study.

7.3.2. Evaluated Product

One commercial extra virgin olive oil (EVOO) was evaluated on its ability to inhibit *Listeria monocytogenes* (*LM*) on cooked pork tenderloin medallions at 26˚C. Primo Gusto: Italian Foods 100% Italian Extra Virgin Olive Oil (cold pressed) (country of origin: Italy via Gordon Food Service® P.O. Box 1787, Grand Rapids, MI 49501) was purchased from a local grocery store. This product was chosen because it did not contain any additives or preservatives which could have hindered the survival rate of the bacteria within the product.

One pork tenderloin was purchased from a local grocery venue. This tenderloin was selected because it was labeled as minimally processed and contained no added phosphates, nitrates/nitrites, or injected water. No spices or salt were added to the tenderloin. The pork tenderloin was cooked for forty minutes at 350˚F using conventional oven to an internal temperature of 200˚F.

7.3.3. Medias

The broths utilized in the *LM* challenge included Bacto™ Brain Heart Infusion (BHI) and Difco™ UVM Modified *Listeria* Enrichment Broth (UVM). Both products were manufactured by Becton, Dickinson and Company (7 Loveton Circle, Sparks, MD, 21152) and were prepared within the specifications. The BHI was dispensed into Pyrex tubes with a final volume of 7mL each and autoclaved. Tween80 was added to the UVM at 1mL/1L so that the final product contained 0.1% Tween80. The UVM was dispensed into Pyrex tubes with a final volume of 9mL each and autoclaved. Dilution blanks utilized throughout the study were Sterile Phosphate Buffer dilution blank tubes. These dilution tubes were made from 24mL PO_4 solution, 95mL $MgCl_2$ solution, and 19L of double deionized water. Tween80 was added to the dilution blank mixture at 1mL/1L so that the final product contained 0.1% Tween80. The dilution mixture with Tween80 was adjusted to a pH between 7.4-7.5. The mixture was then dispensed into Pyrex tubes with a final volume of 9mL and autoclaved. The agars utilized in the *Listeria* study included BBL™ Trypticase™ Soy Agar: Soybean-Casein Digest Agar (TSA) and Difco™

Modified Oxford (MOX) from Difco™ Oxford Medium Base and Difco™ Modified Oxford Antimicrobic Supplement. Both TSA and MOX were Becton, Dickinson and Company products (7 Loveton Circle, Sparks, MD, 21152) and were made according to the product specifications. All agars were poured into sterile 100x15mm polystyrene disposable VWR Petri plates (1050 Satellite Blvd NW, Suwanee, GA 30024).

7.4. Methods

7.4.1. Oil Sterility Validation

The oil was transferred from its original container into a sterile 250mL glass bottle with a cap. Samples of the oil were taken and plated on TSA and MOX and incubated for 48 hours at 37˚C to check for bacterial background. Oil samples were also plated on TSA and incubated for 48 hours at 26˚C to validate sterility. The oil was confirmed to be commercially sterile with an aerobic count below the level of detection. The oil stock bottle was then wrapped in foil and placed in refrigerator storage to prevent any additional oxidation or contamination.

7.4.2. Inoculum Preparation

A scrape of each desired *LM* strain was obtained from a pre-existing refrigerated BHI slant and was transferred into BHI broth using sterile technique. The culture was incubated for 24 hours at 37˚C. The culture was then streaked for isolation onto MOX agar and was incubated for 24 hours at 37˚C. The morphology of the colonies was observed and an isolated colony was transferred to a new tube of BHI. The culture was then incubated for 24 hours at 37˚C. The culture was transferred twice by placing 0.1mL of the former culture into new BHI and incubating for 24 hours at 37˚C. The final transfer of *LM 150C* (ATCC 51781), *LM 150D* (ATCC 43256), *LM 150E* (ATCC 15313), and *LM 150F* (ATCC 19115) were then pooled into a sterile 50mL Flacon Tube. The cocktail was vortexed and diluted (1:100) in sterile phosphate buffer (SPB) without Tween80 to give the final inoculum.

7.4.3. Sample Preparation

A conventional oven was used to cook the tenderloin in a rimmed tray for forty minutes at 350˚F. The pork tenderloin reached an internal temperature of 200˚F in the

foil tent. The tenderloin was aseptically wrapped in the foil and placed inside a Labconco Purifier Class II Biosafety Cabinet. The tenderloin was allowed to cool to room temperature, 26˚C. Using sterile technique, the meat was sliced into approximately 25g medallions. The medallions were then placed into tared sterile Petri plates. A total of seven sample medallions were cut: two negative controls, two positive controls, and three with EVOO added.

The two negative control samples were sealed and set aside to prevent any contamination. Each of the remaining pork tenderloin samples were inoculated with 0.1mL of the four strain *LM* cocktail at 7-log cfu/mL. The inoculum was dropped onto the surface of the samples in five separate locations which mimicked the five side of a die. The inoculum was to be spread across the surface of the sample with a sterile spreader, but the meat absorbed the inoculum almost immediately. The two positive control samples were sealed and set aside to prevent any contamination. The final three tenderloin samples received 0.1mL of EVOO in addition to the *LM* inoculum. The oil was spread across the surface of the meat using a sterile spreader. The three samples were then labeled 0-Minutes oil, 30-Minutes oil, and 60-Minutes oil. The 30-Minute oil sample and 60-Minute oil sample were placed in the 26˚C incubator until their sampling time.

7.4.4. Sample Plating Procedure

At the designated sample time, the samples were removed from the Petri dishes using sterile forceps and placed into a tared sterile stomacher bag. The sample was weighed and a 1:10 dilution (w/v) was made using either 0.1% (w/v) sterile peptone water or UVM +0.1% (v/v) Tween80. The samples were placed in the Stomacher 400 Circulator (Seward Laboratory Systems Inc. USA. 574 NW Mercantile Place, Unit 107, Port Saint Lucie, FL 34986 USA) and mixed for 60 seconds at 230 rpm. Additional dilutions were made as needed using sterile phosphate buffer +0.1% (v/v) Tween80 dilution blanks. The samples were then spiral plated in duplicate onto TSA and/or MOX at a 50µL spiral setting of Eddy Jet2.

The first positive control and the first negative control were made into a 1:10 dilution with peptone water. The negative control in peptone water (NC+pw) was spiral

plated at the 1:10 dilution on TSA. This was to verify that the meat was sterile from the cooking process. The positive control in peptone water (PC+pw) was spiral plated at the 1:10 dilution on TSA and the 1:1000 dilution on MOX. This was to verify that the *LM* was present on the inoculated samples and to compare counts with those in UVM. The second positive control and the second negative control were all made into a 1:10 dilution with UVM +0.1% (v/v) Tween80. The negative control in UVM (NC+UVM) was spiral plated at the 1:10 dilution on both TSA and MOX. This was to verify that there was not *LM* present on the meat prior to inoculation. The positive control in UVM (PC+UVM) was spiral plated at the 1:10 dilution on TSA and the 1:1000 dilution on MOX. This was to verify the presence of the inoculated *LM*. Each of the time dependent samples with EVOO were also made into 1:10 dilutions using UVM +0.1% (v/v) Tween80. The 0-Minute oil sample was spiral plated at the 1:10 dilution on TSA and the 1:1000 dilution on MOX. The MOX plates were utilized at the higher dilution for the 0-Minute oil sample because of the initial inoculation. The 30-Minute oil sample was spiral plated at the 1:10 dilution on MOX and the 1:1000 dilution on TSA. The 60-Minute oil sample was spiral plated at the 1:10 dilution on MOX and the 1:1000 dilution on TSA. The MOX plates were utilized at the lower dilution for the 30-Minute and 60-Minute oil samples because it was thought that the surviving bacterial counts may have decreased within that time frame. All additional dilutions from the initial 1:10 were performed using sterile phosphate buffer (SPB) plus 0.1% (v/v) Tween80. All plates were incubated for 48 hours at 37˚C.

7.4.5. Enrichment Procedure

The initial 1:10 dilutions in UVM +0.1% (v/v) Tween80 were kept as enrichments. These enrichment bags were folded, clipped, and incubated at 30˚C for 48 hours. They were to be removed from the incubator after 48 hours, mixed, and streaked onto MOX for a positive or negative result if the spiral plates did not show any growth. If growth was observed on the spiral plates, the enrichments were merely discarded.

7.4.6. Reading Spiral Plate Procedure

Plates of the samples were read after 48 hours incubation at 37˚C. A FlashAndGo automated counter and FlashAndGo 1.0 Computer program were used to count the spiral

plate colonies. These items were combined to form the FlashAndGo -Basic Economy Automated Colony Counter through ILU Instruments of NEU-TEC Group Inc. (1 Lenox Avenue, Farmingdale, NY 11735). The dilution factor was adjusted as needed for each of the plated samples and combined with the counted colonies to calculate the total bacterial count within the given sample. The duplicate plates were averaged together within Microsoft Excel to give a more accurate total count of the given sample.

7.4.7. Data Analysis

Data organization and graphical figures were constructed using Microsoft Office Excel 2007. A bacterial reduction of 3-log cfu/mL or greater was noted as a significant reduction within the bacterial population. Formal statistics with SAS 9.4 were not conducted for this experiment.

7.5. Results

Contrary to the potent antimicrobial effects observed in previous studies (Chapter 5 and Chapter 6), the EVOO did not cause any reduction within the bacterial population of the *LM* despite an hour of exposure. Table 7.1. shows that the surviving bacterial counts of the samples exposed to 0.1mL of EVOO (0-Min oil, 30-Min oil, and 60-Min oil) were comparable to both the initial positive controls in the UVM+0.1% (v/v) Tween80 and in the peptone water. All of the samples which received oil and all of the positive controls remained in the approximate 5-log cfu/g range of surviving *LM* counts. The negative controls in both UVM+0.1% (v/v) Tween80 and peptone water read at counts <1-log cfu/g. This indicated that there were few, if any, other organisms present on the surface of the tenderloin medallions throughout the study.

TABLE 7.1. Surviving bacterial averages on pork tenderloin plated on TSA and MOX

Average Surviving *Listeria* on Tenderloin Log10 cfu/g			
Sample		TSA	MOX
	0-Min oil	5.20 [a]	5.15 [a]
	30-Min oil	4.99 [a]	5.12 [a]
	60-Min oil	5.41 [a]	5.07 [a]
	PC	5.23 [a]	4.96 [a]
	PC+pw	5.20 [a]	4.96 [a]
	NC	<1.00	<1.00
	NC+pw	<1.00	-

- All resulting numbers are the LOG10 bacterial counts from the spiral plates in cfu/g at 26˚C
- All samples were initially diluted using UVM +0.1% (v/v) Tween80 unless otherwise indicated (pw=peptone water)

The TSA plates and MOX plates showed minimal difference in the number of *LM* recovered from the tenderloin samples. MOX was utilized to ensure that other bacteria were not recovered from the tenderloin samples. MOX was also used alongside the TSA to see if any of the *LM* had been damaged by the EVOO to the point of susceptibility to the antimicrobial agents in the selective agar. If enough damage was inflicted by the oil there could have been a lack of recoverability on the MOX plates specifically. The seemingly uninhibited recoverability on both agars reiterated the lack of damage experienced by the *LM* as displayed in Figure 7.1.

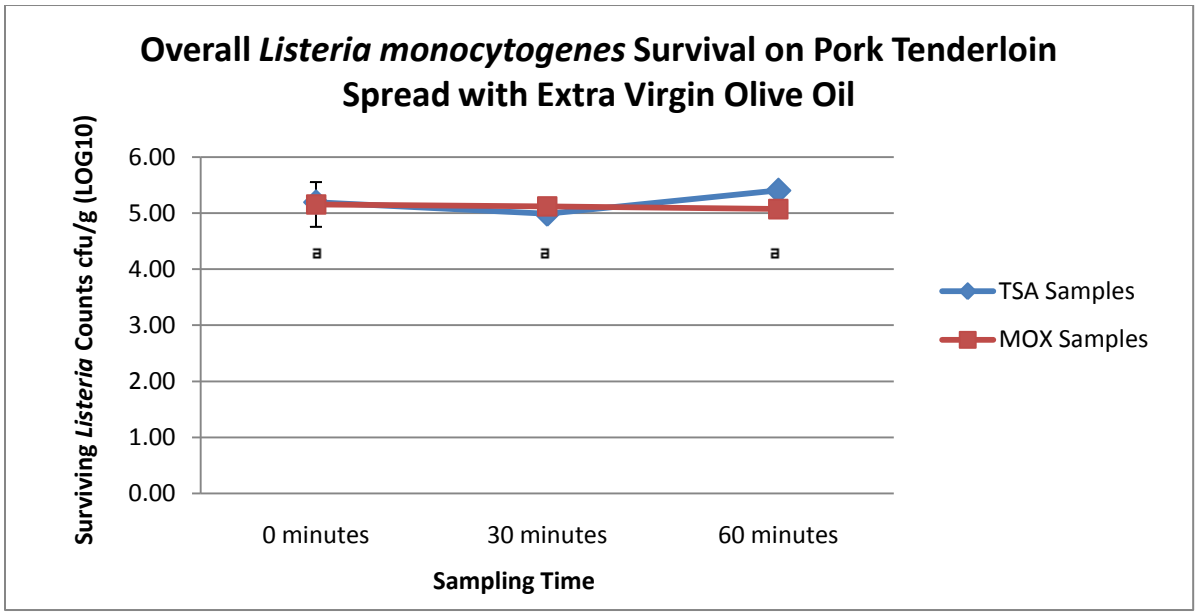

FIGURE 7.1. The average *Listeria monocytogenes* survival exposed to extra virgin olive oil plated on TSA and MOX

As seen in the figure, the recovered *LM* counts from the tenderloin remained fairly consistent throughout the sampling hour. There was also no noticeable difference between the counts recovered on TSA verses those recovered on MOX. The three tenderloin samples exposed to EVOO (0-Min oil, 30-Min oil, and 60-Min oil) did not experience a 3-log cfu/g bacterial reduction; rather, they remained comparable to the initial positive control counts of 5-log cfu/g throughout the hour study on both TSA and MOX. Since no decrease in the bacterial population was detected for any sample, enrichments were discarded without being plated.

7.6. Discussion

It was determined that the drop and spread technique implemented during this mini project was not a viable method for observing the antimicrobial effects of EVOO on pork tenderloin medallions inoculated with *LM*. The hypothesis that a 3-log reduction within the bacterial population would be observed within one hour after the addition of 0.1mL of EVOO was ultimately rejected. No reduction of any kind was observed for the *LM* counts on the tenderloin medallions at 26˚C. The methodology was thought to fail because of several factors including the dryness of the meat, the lack of oil coverage, the lack of constant spreading, and the limited exposure time.

The dryness of the meat proved to be a detrimental factor in the ability to spread the *LM* inoculum evenly across the entire surface of the tenderloin medallions. The dryness of the meat contributed to the development of crevasses on the surface of the meat which may have allowed the bacterial inoculum to absorb into the meat. This rapid absorption not only prevented the even spread of bacteria across the meat, but it may have allowed the bacteria to escape exposure to the EVOO by sinking into the surface crevasses. Without exposure to the oil, the *LM* would be able to survive, uninhibited, within the tenderloin crevasses and thus avoid the antimicrobial effects of the oil. This issue could have been resolved by cooking the meat to a lower internal temperature (165˚F) and adding some extra moisture to the meat pan during cooking. A different resolution may be to use a different meat medium, such as a lunch meat product, which would contain more moisture and fewer crevasses for harboring the bacteria. Lunch meat

would also offer a more homogenous and consistent surface in order to study the possible antimicrobial effects of the EVOO against *LM* on a complex food item.

A second explanation of the failed methodology would be that there was not enough EVOO to obtain the full antimicrobial effect. The study utilized 0.1mL of oil per inoculated tenderloin sample so as to mimic the amount that might be sprayed onto the surface of a meat product within an industrial type setting. The oil was to be spread across the surface of the meat with a sterile spreader to ensure an even coating. As previously stated, the dryness of the cooked meat was rather detrimental to the methodology of the experiment. Not only did the meat absorb the *LM* inoculum due to dryness, but the EVOO did not spread across the entire surface of the meat. The oil remained on the surface of the tenderloin samples and did not soak into the crevasses as effectively as the inoculum. The lack of EVOO appeared to be a combination of the dryness of meat and the limited volume of oil available for use. Without sufficient oil across the surface and within the crevasses of the meat, there was no way to know if the bacteria were actually in contact with the oil. It was noted that being in the vicinity of the EVOO, even as close as the surface and the crevasses, did not elicit any noticeable antimicrobial effects against the *LM*. Based on this observation, it was hypothesized that the EVOO must be in direct contact with the bacteria in order to have any antimicrobial effects. Simply being in close proximity to the oil does not appear to have any negative impact on the survival of *LM*. This issue could have been resolved by adding more EVOO to the meat in order to ensure the saturation of the bacteria with the oil. This increased oil volume may be less practical for finished meats, such as tenderloins and steaks, but may be useful in applying coatings to ready to eat meats, like sausages and lunch meats.

A third explanation of the failed methodology would be the lack of constant spreading. As observed in earlier studies, it took more time for the EVOO to elicit the same reduction in *LM* when it was not mixed (Chapter 5) compared to when it was mixed (Chapter 6). The EVOO was not consistently spread across the surface of the inoculated tenderloins. The oil was spread immediately after placement onto the meat, but because of the dryness of the meat and the lack of available oil the EVOO was not continuously

spread until the designated sample times. This lack of spreading meant that the bacteria were not exposed to a constant supply of EVOO and therefore would not have a rapid reduction in bacterial counts like those of Chapter 5. A sufficient volume of oil should have been added to the inoculated meat and the oil should have been continuously spread across the surface to ensure even coverage and constant exposure to the oil. Since this constant spreading motion would be rather impractical in many food industry settings, a prolonged exposure time to the oil may be a better method to ensure a more effective antimicrobial effect.

The fourth explanation of why the methodology failed closely resembled the third explanation in that there was not enough exposure time given during the mini study to observe any possible antimicrobial effects. Without enough exposure to the EVOO, from either a lack of oil volume or a lack of spreading, more time would be required to prolong the exposure of the bacteria to the available oil. The time frame would more closely reflect the time of the non-mixed oil exposure studies described in Chapter 5. Therefore, it is unsurprising that no antimicrobial results were observed from the tenderloin samples because the *LM* would not have had enough exposure time to elicit the antimicrobial effects of the oil within an hour sampling time. The inconsistent surface of the tenderloin medallion offered various areas to escape exposure to the oil, as well as abundant nutrients to promote bacterial survival. This issue could have been remedied by either continuously spreading a sufficient amount of EVOO across the surface of the tenderloin medallions or by increasing the exposure time of the *LM* to the oil. In an industrial setting, it may be more practical to add EVOO as a coating or marinade during packaging and have the antimicrobial effect take place during shipment and storage.

Overall, the methodology of the mini study failed due to the complexity of the meat product and various confounding variables which restricted the four *LM* strains from continuous exposure to the EVOO. It is thought that this lack of exposure due to insufficient oil volume and a limited time frame contributed to the survival of the bacteria on the surface of the tenderloin medallions. A finished meat product, such as a tenderloin, may not be the best model to study the antimicrobial effects of EVOO on meat because of its complex physical structure. Sensory may also be noticeably

influenced on such a meat product with the addition of more oil to elicit a greater antimicrobial effect. This methodology may be better suited for a moist meat, such as lunch meat, or a less porous product, such as sliced cheese. Future research should focus on a more effective application procedure for EVOO onto meat products in order to study their antimicrobial effects on complex food items. Spraying or film methods for EVOO should be explored for inhibiting bacterial viability.

7.7. Conclusion

The mini study that was designed to determine if EVOO had any antimicrobial effects against four strains of *Listeria monocytogenes* (*LM*) on the surface of cooked pork tenderloin medallions failed due to various confounding factors. The dryness of the meat, the lack of oil volume, the lack of spreading contact, and the limited exposure time were all viable explanations as to why the study failed. Lack of exposure to the oil and the harboring of the bacteria within the minimally treated meat appeared to negate any antimicrobial effects held by the oil. The hypothesis that a 3-log cfu/g reduction might be observed in the surviving bacterial populations when exposed to the oil after one hour at 26˚C was rejected. No reduction, in any of the *LM* strains, was seen from the addition of 0.1mL of EVOO spread onto the surface of the meat within one hour. Direct and constant contact appears to be necessary for EVOO to be an effective antimicrobial; neither of which were accomplished in the mini study. Future research may be best suited for studying the antimicrobial effects of EVOO on moist meat surfaces, like various lunch meats.

CHAPTER 8

THE SURVIVAL OF A *LISTERIA MONOCYTOGENES* COCKTAIL ON SLICED CHEDDAR CHEESE SPREAD WITH EXTRA VIRGIN OLIVE OIL (MINI STUDY)

8.1. Summary

The purpose of this study was to determine if extra virgin olive oil could reduce the *Listeria monocytogenes* (*LM*) populations on the surface of cheddar cheese snack squares. The method consisted of 0.1mL of a 7-log cfu/mL *LM* cocktail inoculum dispensed and spread onto the surface of an approximate 22g cheddar cheese snack square. This was followed by 0.1mL of extra virgin olive oil spread across the surface of the cheese. Samples were taken from 26˚C storage at zero hours, three hours, and six hours. It was hypothesized that exposure to the extra virgin olive oil would result in a 3-log cfu/g reduction of the *LM* population within six hours. This hypothesis was rejected since no reduction was observed in any of the *LM* strains in this experiment. The separation of the inoculum and oil, the pooling effect of the oil, the interference from background bacteria, and the lack of exposure time were thought to contribute to the bacterial survival.

8.2. Introduction

Listeria monocytogenes (*LM*) has been a common concern within the dairy industry because of the ubiquitous nature of the organism and its ability to reside within both raw and finished products. In the summer of 1983, a *Listeria* outbreak in Massachusetts was attributed to sick dairy cows and possible post-contamination of pasteurized whole and two percent milk.[63] Another large outbreak of *Listeria* occurred in California, in 1985, and was traced to a Mexican-style cheese which was contaminated with raw milk during processing, in addition to possible environmental contaminates.[111] Similarly, a Mexican-style cheese was found to be the source of a five state *Listeria* outbreak from October 2008 to January 2009.[83] Koch et al. (2010) surveyed one of the largest *Listeria* outbreaks across Germany (Oct 2006-February 2007) concerning Harzer Käse which is a soft textured cheese curdled using lactic acid bacteria in pasteurized milk

ripened with *Brevibacterium linens* commonly referred to as a red smear. Although *Listeria* outbreaks appear to be more common in milks and soft cheeses, research has been conducted to explore the vulnerabilities of harder cheeses. Larson et al. (1999) observed the ability of *LM* to survive over two hundred days in numerous commercial provolone and brick brines at 4˚C. Ryser and Marth (1988) displayed the ability of *Listeria* to survive in non-acidified cold-pack cheddar cheese products for 142 days with 0.3% sodium propionate and 130 days with 0.3% sorbic acid. With the addition of lactic acid, acetic acid, and a combination of both acids the survival rate of the *Listeria* decreased to 118 days, 103 days, and 98 days respectively for the 0.3% sodium propionate cheese and 112 days, 93 days, and 74 days respectively for the 0.3% sorbic acid.[168] This study expanded the findings of previous research which explored the ability of *LM* in the manufacturing process of cheddar cheese.[166] It was found that *Listeria* did not increase during the manufacturing process, but appeared to have an extended lag phase due to acid development and competition with the lactic culture. [166] The *Listeria* then experienced a slight population increase during the first two to three weeks of ripening followed by a decline past the required 60 day ripening period.[166] This extended survival of the *Listeria* past the standard ripening period was of special relevance to the current mini study which was interested in enhancing the hurdle technology of various food products through the natural antimicrobial abilities of extra virgin olive oil (EVOO).

This mini study was designed to determine if extra virgin olive oil (EVOO) would have any antimicrobial effects against four strains of *LM* on the surface of cheddar cheese snack squares. The purpose of the study was to see if EVOO could reduce the *LM* populations on the surface of the cheese squares over a six hour time frame at 26˚C storage. A drop and spread technique was used to inoculate the cheese surface and to add the oil. Based on the coinciding research conducted with *LM* in extra virgin olive oil (Chapter 5, Chapter 6, and Chapter 7) it was hypothesized that a 3-log cfu/g reduction might be observed in the surviving bacterial populations on the surface of the cheese when exposed to the oil for six hours at 26˚C. This information would be beneficial in determining the effectiveness of EVOO as a natural antimicrobial when added to complex food items. This information may promote further exploration into preventative

films, sprays, and marinades derived from natural antimicrobials, such as extra virgin olive oils.

8.3. Materials

8.3.1. Pathogens

Listeria monocytogenes 150C (ATCC 51781), *Listeria monocytogenes 150D* (ATCC 43256), *Listeria monocytogenes 150E* (ATCC 15313), and *Listeria monocytogenes 150F* (ATCC 19115) were the four strains of *Listeria* utilized in the study.

8.3.2. Evaluated Product

One commercial extra virgin olive oil (EVOO) was evaluated on its ability to inhibit *Listeria monocytogenes* (*LM*) on cheddar cheese snack squares at 26˚C. Primo Gusto: Italian Foods 100% Italian Extra Virgin Olive Oil (cold pressed) (country of origin: Italy via Gordon Food Service® P.O. Box 1787, Grand Rapids, MI 49501) was purchased from a local grocery store. This product was chosen because it did not contain any additives or preservatives which could have hindered the survival rate of the bacteria within the product.

Kroger Mild Cheddar Cheese Snack Squares were purchased from a local grocery store (sell by 10 Jan 2017, PF59 05:17, 10 count net weight 7.5oz /212g). The cheese was individually wrapped (approximately 22g each) and came in 10 count packages. These cheese squares were selected because they were labeled as natural cheese and did not contain any artificial flavors or preservatives.

8.3.3. Medias

The broths utilized in the *LM* challenge included Bacto™ Brain Heart Infusion (BHI) and Difco™ UVM Modified *Listeria* Enrichment Broth (UVM). Both products were provided by Becton, Dickinson and Company (7 Loveton Circle, Sparks, MD, 21152) and were prepared within the specifications. The BHI was dispensed into Pyrex tubes with a final volume of 7mL each and autoclaved. Tween80 was added to the UVM at 1mL/1L so that the final product contained 0.1% Tween80. The UVM was dispensed into Pyrex tubes with a final volume of 9mL each and autoclaved. Dilution blanks

utilized throughout the study were Sterile Phosphate Buffer dilution blank tubes. These dilution tubes were made from 24mL PO_4 solution, 95mL $MgCl_2$ solution, and 19L of double deionized water. Tween80 was added to the dilution blank mixture at 1mL/1L so that the final product contained 0.1% Tween80. The dilution mixture, with Tween80, was adjusted to a pH between 7.4-7.5. The mixture was then dispensed into Pyrex tubes with a final volume of 9mL and autoclaved. The agars utilized in the *Listeria* study included BBL™ Trypticase™ Soy Agar: Soybean-Casein Digest Agar (TSA) and Difco™ Modified Oxford (MOX) from Difco™ Oxford Medium Base and Difco™ Modified Oxford Antimicrobic Supplement. Both TSA and MOX were Becton, Dickinson and Company products (7 Loveton Circle, Sparks, MD, 21152) and were made according to the product specifications. All agars were poured into sterile 100x15mm polystyrene disposable VWR Petri plates (1050 Satellite Blvd NW, Suwanee, GA 30024).

8.4. Methods

8.4.1. Oil Sterility Validation

The oil was transferred from its original container into a sterile 250mL glass bottle with a cap. Samples of the oil were taken and plated on TSA and MOX and incubated for 48 hours at 37˚C to check for bacterial background. Oil samples were also plated on TSA and incubated for 48 hours at 26˚C to validate sterility. The oil was confirmed to be commercially sterile with an aerobic count below the level of detection. The oil stock bottle was then wrapped in foil and placed in refrigerator storage to prevent any additional oxidation or contamination.

8.4.2. Inoculum Preparation

A scrape of each desired *LM* strain was obtained from a pre-existing refrigerated BHI slant and was transferred into BHI broth using sterile technique. The culture was incubated for 24 hours at 37˚C. The culture was then streaked for isolation onto MOX agar and was incubated for 24 hours at 37˚C. The morphology of the colonies was observed and an isolated colony was transferred to a new tube of BHI. The culture was then incubated for 24 hours at 37˚C. The culture was transferred twice by placing 0.1mL

of the former culture into new BHI and incubating for 24 hours at 37˚C. The final transfer of *LM 150C* (ATCC 51781), *LM 150D* (ATCC 43256), *LM 150E* (ATCC 15313), and *LM 150F* (ATCC 19115) were then pooled into a sterile 50mL Flacon Tube. The cocktail was vortexed and diluted (1:100) in sterile phosphate buffer (SPB) without Tween80 to give the final inoculum.

8.4.3. Sample Preparation

A total of 30 sterile Petri plates were grouped in sets of three plates in the Labconco Purifier Class II Biosafety Cabinet. Sets were labeled accordingly: NC 0-Hour (A,B,C), PC 0-Hour (A,B,C), Spread 0-Hour (A,B,C), PC 1-Hour (A,B,C), Spread 1-Hour (A,B,C), PC 3-Hour (A,B,C), Spread 3-Hour (A,B,C), NC 6-Hour (A,B,C), PC 6-Hour (A,B,C), Spread 6-Hour (A,B,C). Where PC represented positive controls, NC represented negative controls, and Spread represented the samples which were spread with 0.1mL of EVOO. Each sterile Petri plate received one 22g cheese square. The cheese was transferred to the plate using sterile technique and was covered with a lid to prevent contamination. The NC cheese squares were sealed in their containers and placed in the 26˚C incubator until their designated sample time. Each of the PC and Spread cheese squares were inoculated with 0.1mL of the four strain *LM* cocktail at 7-log cfu/mL. The inoculum was dropped onto the surface of the samples in five separate locations which mimicked the five side of a die. The inoculum was spread on the surface of the cheese using a sterile spreader for each square. The inoculum was allowed to dry on the surface of the cheese for 10 minutes. The PC were then covered and placed in the 26˚C incubator until their designated sample times. Beginning with the 6-Hour samples, all of the Spread samples had 0.1mL of EVOO added to the surface in the same pattern of the inoculum. A new sterile spreader was used to spread the oil over the surface of each cheese sample, excluding the NC and PC samples. After completing the 6-Hour, 3-Hour, and 1-Hour Spread samples, all were labeled and placed in the 26˚C incubator until their designated sample time. Finally, the 0-Hour Spread samples had the EVOO added and spread onto the surface of the cheese squares. The 0-Hour Spread samples were immediately transferred to tared sterile stomacher bags using sterile forceps.

8.4.4. Sample Plating Procedure

The stomacher bags received a 1:10 (w/v) dilution of UVM + 0.1% (v/v) Tween80 based on the cheese square weight. No additional dilutions were utilized in this study. The cheese was mixed slightly by hand through the bag and was then placed in the Stomacher 400 Circulator (Seward LaboratorySystems Inc. USA. 574 NW Mercantile Place, Unit 107, Port Saint Lucie, FL 34986 USA) and mixed at 230rmp for one minute. A sterile pipette was used to gather 2mL of the slurry and place it in a sterile spiral platting cup. The slurry was then spiral plated, in duplicate onto MOX, using the 50µL spiral setting of Eddy Jet2. Once all three of the 0-Hour Spread samples were finished, the MOX spiral plates were placed in the 37˚C incubator for 48 hours. This dilution, mixing, and plating process was repeated for the 0-Hour PCs on MOX. The 0-Hour NCs received the same dilution and mixing process, but were also plated on TSA, in addition to the MOX. This was to check for other bacterial background on the cheese which might be inhibited on MOX. These plates were also incubated for 48 hours at 37˚C. The process was then repeated for the 1-Hour PCs and Spread samples and the 3-Hour PCs and Spread samples. No NCs were done for the one hour or three hour time points. The NCs, PCs, and Spread samples were all sampled at the six hour time period. The plates were all incubated for 48 hours at 37˚C.

8.4.5. Enrichment Procedure

Due to the fact that they were diluted with UVM +0.1% (v/v) Tween80, the sample filled stomacher bags were kept as enrichments. The bags were clipped together and stored at 30˚C for 24 hours. If no bacterial growth occurred on the spiral plates, then 0.1mL of the enrichments would have been spread onto a split MOX plate and incubated for 48 hours at 37˚C. The plates would have been read as positive or negative for growth after that time. If growth was visible on the initial spiral plates the enrichments were discarded.

8.4.6. Reading Spiral Plate Procedure

Plates of the samples were read after 48 hours incubation at 37˚C. A FlashAndGo automated counter and FlashAndGo 1.0 Computer program were used to count the spiral plate colonies. These items were combined to form the FlashAndGo -Basic Economy

Automated Colony Counter through ILU Instruments of NEU-TEC Group Inc. (1 Lenox Avenue, Farmingdale, NY 11735). The dilution factor was adjusted as needed for each of the plated samples and combined with the counted colonies to calculate the total bacterial count within the given sample. The duplicate plates were averaged together, using Microsoft Excel, to give a more accurate total count of the given sample.

8.4.7. Data Analysis

Data organization and graphical figures were constructed using Microsoft Office Excel 2007. A bacterial reduction of 3-log cfu/mL or greater was noted as a significant reduction within the bacterial population. Formal statistics with SAS 9.4 were not conducted for this experiment.

8.5. Results

The EVOO did not cause any reduction within the *LM* counts, even after six hours of exposure. These results resembled those found in the Tenderloin mini study (Chapter 7), rather than the studies which placed the four strain *LM* cocktail into EVOO directly (Chapter 5 and Chapter 6). Table 8.1. shows that the surviving *LM* counts exposed to the EVOO were comparable to those of the positive controls (PC) over the six hour sample time. A mixed bacterial background was found when negative controls were plated on TSA during the initial check of the cheese products. These organisms were assumed to be the cheese cultures and possibly spoilage organisms. TSA was not used as the primary media within the mini study because of this background. Due to its ability to inhibit the growth of other bacterial strains present in the cheese snack squares, MOX was utilized as the spiral plate and enrichment media. MOX did not inhibit the growth of the *LM*, therefore, it remained a suitable media for the cocktail. The negative controls (NC) of the study were read at counts of <1-log cfu/g, below the level of detection, on the MOX spiral plates.

TABLE 8.1. Surviving bacterial averages in extra virgin olive oil, PCs in SPB+Tween80, and NCs

Average Surviving *Listeria* on Cheddar Cheese Snack Squares Log10 cfu/g					
		0 Hour	**1 Hour**	**3 Hour**	**6 Hour**
Sample	Spread with Oil	5.03 [a]	4.99 [a]	4.99 [a]	4.97 [a]
	Positive Control (PC)	5.01 [a]	5.03 [a]	5.00 [a]	4.99 [a]
	Negative Control (NC)	< 1.00	-	-	<1.00
	NC on TSA	TNTC non-*Listeria*	-	-	TNTC non-*Listeria*

- All resulting numbers are the LOG10 bacterial counts from the spiral plates in cfu/g at 26˚C diluted using UVM +0.1% (v/v) Tween80
- No NC samples were taken during the one hour and three hour time points

Both the positive controls and the samples spread with EVOO maintained around a 5-log cfu/g bacterial population throughout the experiment as seen in Figure 8.1. Despite the extended time frame of six hours, the *LM* did not appear to be damaged based on their recovery on the MOX spiral plates.

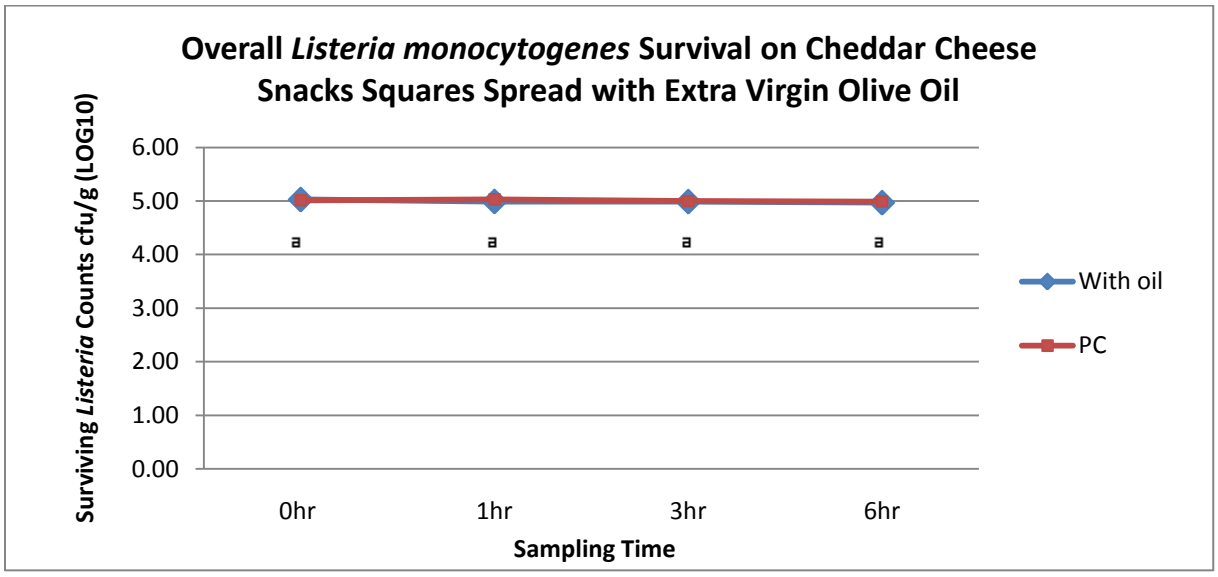

FIGURE 8.1. The average *Listeria monocytogenes* survival in positive controls and extra virgin olive oil on cheese surface

As seen in the figure, the recovered *LM* counts from the cheese snack remained fairly consistent throughout the sampling time. The samples exposed to EVOO did not experience a 3-log cfu/g bacterial reduction at any of the time points. Instead, the samples spread with oil remained comparable to the initial positive control counts. Since

no decrease in the bacterial population was detected for any sample, enrichments were discarded without being plated.

8.6. Discussion

It was determined that the drop and spread technique applied during this mini project was not a viable method for observing the antimicrobial effects of EVOO on cheddar cheese snack squares inoculated with *LM*. The hypothesis that a 3-log reduction within the *Listeria* counts would be observed within six hours at 26˚C after the addition of 0.1mL of EVOO was ultimately rejected. No reduction, of any magnitude, was observed for the bacterial population on the cheese snack squares. The failed methodology was hypothesized to be due to various confounding factors including the separation of the inoculum from the oil, the pooling effect of the oil, interference from the bacterial background, and the lack of exposure time.

One of the downfalls of the previous *Listeria monocytogenes* on pork tenderloin (Chapter 7) mini study was that the meat was too dry and therefore absorbed the inoculum down into the crevasses of the meat thus escaping exposure to the oil. To avoid this problem, cheddar cheese snack squares were utilized because of their waxy surface. This surface would be less likely to instantly absorb the inoculum; this increased the likelihood of the *LM* having contact time with the EVOO. As predicted, the waxy surface of the cheese allowed for minimal absorption of the *LM* culture which would allow for optimal oil exposure. Unfortunately, because of this low absorption rate the inoculum remained present in small droplets even after a 10 minute drying period. When the EVOO was added these droplets did not fully mix with the oil despite vigorous spreading. These inoculum droplets often separated from the oil resulting in individual oil and inoculum droplets across the entire surface of the cheese square. This phenomenon was concerning because it was previously hypothesized that the *LM* needed to have direct contact with the EVOO in order for any antimicrobial action to occur. Being in close proximity to the oil would not be sufficient exposure to elicit the desired 3-log reduction cfu/g, if any antimicrobial effect, on the bacterial population. This issue may have been resolved with the addition of more EVOO to cover the surface of the

cheese square. More oil would prevent this inoculum separation and force the bacteria to be in direct contact with the oil at all times. The separation problem may have also been resolved by re-spreading the oil and inoculum several times throughout the experiment. This re-spreading would be to increase the amount of direct exposure the *LM* would have to the EVOO in hopes of obtaining an antimicrobial effect.

In addition to the droplet separation, the EVOO experienced a pooling effect across the surface of the cheese snack squares. This pooling action consisted of several oil droplets fusing together to cause puddles of oil across the surface of the cheese. Instead of having a consistent spread of oil across the surface of the cheese as intended, the pooling effect left both the inoculum and the cheese surface itself unexposed to the oil. This pooling was moderately noticeable at the one hour sample time. Several smaller puddles had already started forming to the neglect of the inoculum and surface of the cheese in various places. This pooling was extremely pronounced by the third and sixth hour sample times. The puddles which formed drew to the edges of the cheese squares and left large areas of the cheese and isolated inoculum droplets unexposed to the oil. Despite this dramatic pooling, the oil was not re-spread across the surface of the cheese squares. This was so that the results reflected a single spread exposure, which would be more typical in industry, rather than a multiple spread exposure. This pooling created a stark separation of the inoculum from the EVOO. It was therefore unsurprising that the *LM* counts remained so high throughout the entire experiment since there was minimal direct exposure to the oil even by the hour time point. This issue may have also been resolved with the addition of more EVOO to cover the entire surface of the cheese squares. With more oil this pooling effect would not have occurred and the *LM* would have had direct exposure to the antimicrobial effects of the oil. Similarly, re-spreading the oil throughout the experiment may have allowed for more exposure to the oil and minimized the pooling effect while increasing direct contact with the bacteria, despite the impracticality in an industry setting.

Another interesting explanation for the failed methodology may be attributed in part to interference from the bacterial background. It is not clear if these background organisms had any influenced on the antimicrobial effects of the EVOO. It is possible,

although not explored in this mini study, that the bacterial background on the cheese snack squares may have acted as a buffer between the antimicrobial effects of the oil and the *LM*. The oil may have actually been eliciting an antimicrobial effect, but displaced it towards the background bacteria rather than the bacteria of concern, the *LM*. With the antimicrobial compounds of the oil bound in reactions with the background bacteria this may have allowed the *LM* to escape the effects of the oil until the pooling phenomenon completely separated the inoculum from any exposure to the oil itself. This explanation seems rather obscure since it is not clear whether the antimicrobial compounds in the oil actually bind with the effected bacteria or if they are altered or destroyed after a number of interactions. It also seems highly improbable that the oil would selectively elicit an antimicrobial effect towards one bacteria while negating the same effect towards another bacteria in the same area. Neither the biochemistry of the specific oil compounds nor the reduction of the background bacteria were explored in this mini study. Therefore, it can only be hypothesized that the background bacteria present on the cheese snack squares could have had some potential influence in inhibiting the antimicrobial effect of the oil on the *LM*. The issue presented by the bacterial background is largely unavoidable since most food products are not sterile and could contain various amounts of ubiquitous organisms. Cheese, in particular, presents a challenge since it often contains a number of necessary bacterial cultures and is prone to several spoilage organisms. If the EVOO was utilized as a natural antimicrobial within the food industry the influence of other organisms present on the food items must be taken into account in addition to the target pathogens. Future studies concerning the addition of EVOO on food products should consider the presence of a complex and diverse microflora.

The final explanation for the failed methodology of the current mini study was that there was simply not enough exposure time to the oil to cause any observable antimicrobial effect. As noted in various works, bacterial reduction required extended exposure time to the EVOO depending upon the complexity of the food item, the concentration of oil available, and whether the products were mixed in order to experience any antimicrobial action.[119,163,164] Despite the inoculum separation and the pooling oil, the sampling time frame may not have been sufficient for the antimicrobial

compounds to cause an effect. This would explain why the *LM*, even those with direct exposure to the oil puddles across the surface of the cheese, still did not seem to be affected by the presence of the oil. This observation led to a reinvestigation of the six hour time frame experiment conducted in Chapter 5. The resulting four hour, zero to six hour, and 48 hour time frame experiments were recorded in Chapter 5 and validated the need for prolonged exposure to the oil when samples were mixed only once. The mini study did not offer sample times past six hours which limited the observable antimicrobial activity to that time frame. This issue could have been resolved by expanding the sample time points to include a 24 hour or even a 48 hour time point. Based on the findings of the current mini study and the pork tenderloin study of Chapter 7, it appears that EVOO is not useful as a fast acting antimicrobial substance. Future research should be conducted with longer exposure times to the oil in order to determine the anti-*Listerial* action it may have on food products. It may be that EVOO would be better suited as a marinade or coating additive during packaging. These applications would encourage direct contact with the oil and allow for a long exposure time to the food product during storage and shipment.

8.7. Conclusion

The mini study designed to determine if extra virgin olive oil (EVOO) had any antimicrobial effects against a four strain *Listeria monocytogenes* (*LM*) cocktail on the surface of cheddar cheese snack squares failed due to several confounding variables. The separation of the inoculum from the oil, the pooling effect of the oil, interference from the bacterial background, and the lack of exposure time were all potential explanations as to why the study failed. The separation of the inoculum and the pooling effect of the EVOO appeared to be detrimental to the antimicrobial effects of the oil by limiting its direct exposure to the bacteria. The hypothesis that a 3-log cfu/g reduction might be observed in the surviving *LM* counts when exposed to the EVOO after six hours at 26˚C was rejected. No reduction, in any of the *LM* strains, was seen from the addition of 0.1mL of oil spread onto the surface of the cheese squares for any of the sample times. Direct and constant contact appears to be a vital key for the antimicrobial effectiveness of

EVOO. This exposure was not accomplished in this mini study. Future research may be best suited for studying the antimicrobial effects of EVOO on other food surfaces, such as various lunch meats, using either spraying or marinating techniques. Additional research should also include time points ranging from 24 to 48 hours to accommodate the slow acting antimicrobial effects of the EVOO against bacterial strains.

CHAPTER 9

THE SURVIVAL OF A *LISTERIA MONOCYTOGENES* COCKTAIL ON TURKEY LUNCHMEAT SPREAD OR SPRAYED WITH EXTRA VIRGIN OLIVE OIL (MINI STUDY)

9.1. Summary

The purpose of this study was to determine if extra virgin olive oil could reduce the *Listeria monocytogenes* (*LM*) populations on the surface of the lunchmeat. The method consisted of 0.1mL of a 7-log cfu/mL *LM* cocktail inoculum dispensed and spread onto the surface of an approximate 17g slice of turkey lunchmeat. This was followed by 0.1mL of extra virgin olive oil that was either sprayed directly onto the surface or was dispensed and spread across the surface of the lunchmeat. Samples were taken from 26˚C storage at 0 hours and 48 hours. It was hypothesized that exposure to the extra virgin olive oil would result in a 3-log cfu/g reduction of the *LM* population within 48 hours. Overgrowth of contaminating background bacteria on the 48 hour samples made it impossible to differentiate the colonies on the MOX plates. The study was ultimately inconclusive and it could only be assumed that all of the *LM* strains survived with the contaminating background microflora. Based on this assumption, the hypothesis of the mini study was rejected; no reduction was observed in the *LM* population using either the spray method or drop and spread method.

9.2. Introduction

In the 2013 Food Code, the United States Food and Drug Administration defined ready-to-eat foods as products in a form which are edible without additional preparation for food safety or as products that are raw or partially cooked in which the consumer is advised concerning the safety. These items include numerous products such as processed raw animal foods, washed fruits and vegetables, various plant substances (including sugars, seasonings, and spices), bakery items, thermally processed low-acid foods, and various dried, cured, cooked, or fermented meat and poultry products.[61] Despite a wide array of products categorized under the ready-to-eat mantra, the RTE label is commonly limited to describing meats and meat products found in the general food market. Ready-

to-eat products, especially those of meat or dairy origins, have commonly been associated with the risk of *Listeria monocytogenes* (*LM*). The United Kingdom and the Republic of Ireland reported several cases linked to two strains of *LM* associated with pâté from 1985-1990.[116] From January 1989 to July 1999, frankfurters were connected to one of the largest *Listeria* outbreaks in the United States.[117] This outbreak involved 24 states, 108 infected people, 18 deaths, and 35-million pounds of recalled product which eventually led to the decline of the outbreak.[117] This outbreak resulted in the development and modification of regulations concerning ready-to-eat products and their relation to *Listeria.*[117,197] In 2000, 11 states were involved in an outbreak of *LM* which was identified using pulse-field gel electrophoresis and *Eco*RI ribotyping in deli turkey meat.[144] A similar instance occurred with nine states in 2002, which reported an outbreak of *LM* in pre-prepared deli turkey meat.[69]

In 2003, a collaborative Quantitative Assessment, concerning *LM* among selected ready-to-eat foods, was performed by several United States governmental agencies. The risk assessment surveyed the potential risk of contact with *LM* in 23 ready-to-eat food products potentially implicated with *Listeria* cases.[57] Deli meats were ranked as the highest risk items per serving and per annum cases concerning *LM*. Unheated frankfurters followed suit in this high risk category along with several dairy based foods.[57] Pâté and meat spreads carried a high risk per serving and moderate risk for per annum cases while reheated frankfurters and dry/semi-dry fermented sausages ranked as low risk items for both categories.[57] Due to high contamination rates, prolonged storage times, the ability to support the growth of *Listeria* under refrigeration conditions, and the regular consumption of these products, deli meats and unheated frankfurters were designated as the highest risk ready-to-eat products out of this food selection.[57] The desire to expand hurdle technology surrounding ready-to-eat products has increased due to the severity of the illness caused by the organism, as well as its ubiquitous nature in the environment. One potential hurdle to use during the post processing procedure would be to add a natural antimicrobial agent, such as extra virgin olive oil, to the meats during packaging.

This mini study was designed to determine if extra virgin olive oil (EVOO) would have any antimicrobial effects against four strains of *LM* on the surface of oven roasted deli turkey lunchmeat. The purpose of the study was to see if EVOO could reduce the *LM* populations on the surface of sliced turkey lunchmeat over a 48 hour time frame at 26˚C storage. A drop and spread technique was used to inoculate the lunchmeat surface. A spray technique and a drop and spread technique were used to add the EVOO to the surface of the turkey lunchmeat. Based on the coinciding research conducted with *LM* in extra virgin olive oil (Chapter 5, Chapter 6, Chapter 7, and Chapter 8), it was hypothesized that both methods would cause a 3-log cfu/g reduction within the bacterial populations after 48 hour at 26˚C storage. This information would be beneficial in understanding the antimicrobial properties of EVOO when added to ready to eat meats, such as lunchmeat. This information may encourage further research into EVOO as a *LM* preventative in ready to eat products.

9.3. Materials

9.3.1. Pathogens

Listeria monocytogenes 150C (ATCC 51781), *Listeria monocytogenes 150D* (ATCC 43256), *Listeria monocytogenes 150E* (ATCC 15313), and *Listeria monocytogenes 150F* (ATCC 19115) were the four strains of *Listeria* utilized in the study.

9.3.2. Evaluated Product

One commercial extra virgin olive oil (EVOO) was evaluated on its ability to inhibit *Listeria monocytogenes* (*LM*) on oven roasted deli turkey lunchmeat at 26˚C. Primo Gusto: Italian Foods 100% Italian Extra Virgin Olive Oil (cold pressed) (country of origin: Italy via Gordon Food Service® P.O. Box 1787, Grand Rapids, MI 49501) was purchased from a local grocery store. This product was chosen because it did not contain any additives or preservatives which could have hindered the survival rate of the bacteria within the product.

Two packages of Hormel Natural Choice: oven roasted deli turkey lunchmeat were purchased from a local grocery store (use or freeze by 2 Nov 2016, S0058103-01

19:48 P-199-0, package L1-1 and L1-3 gluten free/fully cooked/sliced, net weight 8oz /227g). Each slice weighed approximately 17g. The meat was contained within a resealable zipper package and was visible through a viewing area of the outer cardboard package. This turkey lunchmeat was selected because it was labeled as minimally processed and 100% natural without any artificial ingredients or preservatives. The meat was also labeled as turkey raised without added hormones. No nitrates or nitrites were added, except those naturally occurring in the cherry powder and cultured celery powder that were added to the meat. Although the lunchmeat was not free of antimicrobial influences from other ingredients, it was thought that this meat might provide a favorable surface environment to study the effects of the EVOO on *LM*.

9.3.3. Medias

The broths utilized in the *LM* challenge included Bacto™ Brain Heart Infusion (BHI) and Difco™ UVM Modified *Listeria* Enrichment Broth (UVM). Both products were provided by Becton, Dickinson and Company (7 Loveton Circle, Sparks, MD, 21152) and were prepared within the specifications. The BHI was dispensed into Pyrex tubes with a final volume of 7mL each and autoclaved. The UVM was dispensed into Pyrex tubes with a final volume of 9mL each and autoclaved. Dilution blanks utilized throughout the study were Sterile Phosphate Buffer dilution blank tubes. These dilution tubes were made from 24mL PO_4 solution, 95mL $MgCl_2$ solution, and 19L of double deionized water. The dilution mixture was adjusted to a pH between 7.4-7.5. The mixture was then dispensed into Pyrex tubes with a final volume of 9mL and autoclaved. The agars utilized in the *Listeria* study included BBL™ Trypticase™ Soy Agar: Soybean-Casein Digest Agar (TSA) and Difco™ Modified Oxford (MOX) from Difco™ Oxford Medium Base and Difco™ Modified Oxford Antimicrobic Supplement. Both TSA and MOX were Becton, Dickinson and Company products (7 Loveton Circle, Sparks, MD, 21152) and were made according to the product specifications. All agars were poured into sterile 100x15mm polystyrene disposable VWR Petri plates (1050 Satellite Blvd NW, Suwanee, GA 30024).

9.4. Methods

9.4.1. Oil Sterility Validation

The oil was transferred from its original container into a sterile 250mL glass bottle with a cap. Samples of the oil were taken and plated on TSA and MOX and incubated for 48 hours at 37˚C to check for bacterial background. Oil samples were also plated on TSA and incubated for 48 hours at 26˚C to validate sterility. The oil was confirmed to be commercially sterile with an aerobic count below the level of detection. The oil stock bottle was then wrapped in foil and placed in refrigerated storage to prevent any additional oxidation or contamination.

9.4.2. Inoculum Preparation

A scrape of each desired *LM* strain was obtained from a pre-existing refrigerated BHI slant and was transferred into BHI broth using sterile technique. The culture was incubated for 24 hours at 37˚C. The culture was then streaked for isolation onto MOX agar and was incubated for 24 hours at 37˚C. The morphology of the colonies was observed and an isolated colony was transferred to a new tube of BHI. The culture was then incubated for 24 hours at 37˚C. The culture was transferred twice by placing 0.1mL of the former culture into new BHI and incubated for 24 hours at 37˚C. The final transfer of *LM 150C* (ATCC 51781), *LM 150D* (ATCC 43256), *LM 150E* (ATCC 15313), and *LM 150F* (ATCC 19115) were then pooled into a sterile 50mL Flacon Tube. The cocktail was vortexed and diluted (1:100) in sterile phosphate buffer (SPB) to give the final inoculum.

9.4.3. Sample Preparation

Two packages of the turkey lunchmeat were purchased the day before the experiment and placed in refrigerated storage. The cardboard packaging of the lunchmeat was discarded after retrieving them from refrigerator the next morning. The re-sealable zipper packaging was sterilized with 70% ethanol and allowed to dry inside the Labconco Purifier Class II Biosafety Cabinet. A total of 24 large sterile glass Petri plates were also placed in the Biosafety Cabinet. The packages of lunchmeat were opened using a sterile knife and forceps and 12 slices were randomly selected from each of the two packages. This resulted in a total of 24 samples. Sterile forceps were used to

remove a single slice of the turkey lunchmeat from the package and place it into one of the large sterile glass Petri plates. Each plate was covered with the corresponding lid to prevent contamination and set to the side. After all 24 lunchmeat slices were placed into the Petri plates the dishes were randomized and split into eight groups of three plates.

These plate sets were labeled accordingly: NC 0-Hour (A,B,C), PC 0-Hour (A,B,C), Spread 0-Hour (A,B,C), Spray 0-Hour (A,B,C), NC 48-Hour (A,B,C), PC 48-Hour (A,B,C), Spread 48-Hour (A,B,C), and Spray 48-Hour (A,B,C). Where NC represented the negative controls, PC represented the positive controls, Spread represented the samples which were spread with 0.1mL of EVOO, and Spray represented the samples which were sprayed with 0.1mL of EVOO. The NC turkey slices were sealed in their containers and placed in the 26˚C incubator until their designated sample time. Each of the PC, Spread, and Spray turkey slices were inoculated with 0.1mL of the four strain *LM* cocktail at 7-log cfu/mL. The inoculum was dropped onto the surface of the samples in five separate locations which mimicked the five side of a die. The inoculum was spread on the surface of the meat using a sterile spreader for each slice. The inoculum was allowed to dry on the surface of the turkey for 10 minutes. The PC plates were then covered and placed in the 26˚C incubator until their designated sample times.

Beginning with the 48-Hour samples, all of the Spread samples had 0.1mL of EVOO added to the surface in the same pattern of the inoculum. A new sterile spreader was used to spread the oil over the surface of each turkey slice, excluding the NC and PC samples. The Spray samples were misted with a 0.1mL coating of EVOO across the surface. The Spray samples did not undergo a spreading step, but relied on the spraying ability of the misting bottle to receive an even distribution. All of the labeled 48-Hour samples were placed in the 26˚C incubator until their designated sample time. The same procedure was applied to the 0-Hour samples for both the Spread and Spray treatments. The inoculated 0-Hour Spread samples received 0.1mL of oil which was dropped and spread onto the meat surface using sterile technique. The inoculated 0-Hour Spray samples received 0.1mL of oil misted across the surface of the meat. The 0-Hour Spread

and Spray samples were immediately transferred to individual tared sterile stomacher bags using sterile forceps. The weights of each sample are listed below in Table 9.1.

TABLE 9.1. Weights of oven roasted deli turkey lunchmeat slices

Sample Weights (g) of Turkey Lunchmeat Slices			
Sample		**0 Hour**	**48 Hour**
	A Spread with Oil	17.64	16.74
	A Spray with Oil	18.13	16.85
	A Positive Control (PC)	18.53	15.92
	A Negative Control (NC)	16.92	15.46
	B Spread with Oil	17.74	15.89
	B Spray with Oil	18.09	15.79
	B Positive Control (PC)	17.14	15.58
	B Negative Control (NC)	17.10	14.16
	C Spread with Oil	17.15	16.58
	C Spray with Oil	16.49	16.41
	C Positive Control (PC)	16.96	16.00
	C Negative Control (NC)	18.59	15.79

All turkey slice weights were measured to the second decimal place in grams. The samples were all diluted according to their exact weight.

9.4.4. Sample Plating Procedure

The stomacher bags received a 1:10 (w/v) dilution of UVM based on the turkey slice weight. No additional dilutions were utilized in this study because it was thought that the *LM* counts would decrease over the study and remain readable at the 1:10 dilution. The turkey slice was mixed slightly by hand through the bag and was then placed in the Stomacher 400 Circulator (Seward LaboratorySystems Inc. USA. 574 NW Mercantile Place, Unit 107, Port Saint Lucie, FL 34986 USA) and mixed at 230rmp for one minute. A sterile pipette was used to gather 2mL of the slurry and place it in a sterile spiral plating cup. The slurry was then spiral plated, in duplicate onto MOX, using the 50µL spiral setting of Eddy Jet2. Once all three of the 0-Hour Spread samples and 0-Hour Spray samples were finished, the MOX spiral plates were placed in the 37˚C incubator for 48 hours. This dilution, mixing, and plating process was repeated for the 0-Hour NCs and the 0-Hour PCs on MOX. These plates were also incubated for 48 hours at 37˚C. The process was then repeated for the 48-Hour NCs, 48-Hour PCs, 48-Hour

Spread samples, and the 48-Hour Spray samples once their time point occurred. These plates were also incubated for 48 hours at 37˚C.

9.4.5. Enrichment Procedure

Since the samples were diluted with UVM, the sample filled stomacher bags were kept as enrichments. The bags were clipped together and stored at 30˚C for 24 hours. If no bacterial growth occurred on the spiral plates, then 0.1mL of the enrichments would have been spread onto a split MOX plate and incubated for 48 hours at 37˚C. The plates would have been read as positive or negative for growth after that time. If growth was visible on the initial spiral plates the enrichments were discarded.

9.4.6. Reading Spiral Plate Procedure

Plates of the samples were read after 48 hours incubation at 37˚C. A FlashAndGo automated counter and FlashAndGo 1.0 Computer program were used to count the spiral plate colonies. These items were combined to form the FlashAndGo -Basic Economy Automated Colony Counter through ILU Instruments of NEU-TEC Group Inc. (1 Lenox Avenue, Farmingdale, NY 11735). The dilution factor was adjusted as needed for each of the plated samples and combined with the counted colonies to calculate the total bacterial count within the given sample. The duplicate plates were averaged together, using Microsoft Excel, to give a more accurate total count of the given sample.

9.4.7. pH and Water Activity Analysis

Two large glass Petri plates were sterilized and placed in the Labconco Purifier Class II Biosafety Cabinet. Two slices of the turkey lunchmeat were removed from their packaging as described above in the sample preparation. Each Petri plate received one slice of lunchmeat before being covered with the corresponding lid. The 48-Hour sample was placed in 26˚C storage for 48 hours with the other samples until the sample time. The pH and water activity of the 0-Hour sample was taken immediately. The pH of the lunchmeat was taken using a Foodcare HI 99161 pH Meter (Hanna Instruments, Highland Industrial Park, 584 Park East Drive, Woonsocket, RI 02895). The water activity of the lunchmeat was taken using a Pa_wkit water activity meter (Decagon Devices Inc. 2365 NE Hopkins Court, Pullman, WA 99163). Both instruments were calibrated according to the instruction manuals.

9.4.8. Data Analysis

Data organization and graphical figures were constructed using Microsoft Office Excel 2007. A bacterial reduction of 3-log cfu/mL or greater was noted as a significant reduction within the bacterial population. Formal statistics with SAS 9.4 were not conducted for this experiment.

9.5. Results

Neither spreading nor spraying 0.1mL of EVOO on the surface of the oven roasted deli turkey resulted in a decrease of the *LM* population on the lunchmeat. The 0-Hour samples and controls performed as expected. The negative controls did not grow any colonies on the MOX plates which were recorded as below the level of detection (<1-log cfu/g). The 0-Hour positive control plates, the Spread sample plates, and the Spray sample plates were all found to have *LM* around 5-log cfu/g after 48 hours of incubation at 37˚C. After the 0-Hour samples were read, the 48-Hour samples were all plated in the same manner as the 0-Hour samples. Despite the use of sterile technique, massive contamination was found on the 48-Hour MOX plates for nearly every sample after incubating at 37˚C for 48 hours. This contamination was noted in Table 9.2. as TNTC, too numerous to count, because it overran the MOX plates and made it impossible to get any reliable count from the *LM*.

TABLE 9.2. Surviving bacterial averages in extra virgin olive oil, PCs in SPB, and NCs

Average *Listeria* Survival on Turkey Lunchmeat Slices Log cfu/g			
Sample		**0 Hour**	**48 Hour**
	Spread with Oil	5.17	TNTC
	Spray with Oil	5.10	TNTC
	Positive Control (PC)	5.17	TNTC
	Negative Control (NC)	< 1.00	< 1.00 to TNTC

- All resulting numbers are the LOG10 bacterial counts from the spiral plates in cfu/g at 26˚C diluted using UVM
- The NC TNTC cfu/g was due to the overgrowth of a foreign bacteria on the MOX plates

It was thought that the contamination was due to either environmental or product contamination. A single bacterial colony present on one of the 48-Hour negative control plates displayed the same morphology as the *LM* used in the inoculum. A Gram stain of this colony revealed a short fat rod which looked similar to the four *LM* Gram stains of

the stock cultures. This lone colony was thought to be due to airborne environmental contamination during the experiment. This was validated by a negative result for *LM* in the negative control UVM enrichment. There was only one set of 48-Hour negative control plates, set B, which did not show any contamination on the MOX plates or in the UVM enrichment. The other 48-Hour negative controls were so overgrown with the contaminating bacteria that the plates could not be read at the typical 1:10 dilution. The contamination was also recovered out of these negative control enrichments. Unlike the lone colony, assumed to be a *LM*, these foreign bacteria could not be attributed to random environmental contamination. When Gram stained, these bacteria presented themselves as long slender Gram-positive rods in chains. Numerous colonies were Gram stained directly from the MOX plates while others were grown in BHI tubes for 24 hours at 37˚C and Gram stained from this broth. The exact same staining pattern was observed for every contaminant bacteria that was selected. It was also noted that the colonies which were grown in the BHI formed a film coating on the top of the broth typical of a *Bacillus* culture. Isolation streaks of the cultured BHI broth on TSA yielded colonies typical of *Bacillus*. Further swabs and samples of the laboratory environment and the experimental materials revealed the Turkey lunchmeat as the source of this bacterial contaminant. Swabs of the juice within the package, and additional samples of the meat, grew the same bacteria, assumed to be a *Bacillus*, incubated at 37˚C for 48 hours after mild temperature abuse at 26˚C. Upon inspection of the ingredients it was noted that the celery powder, used as a natural source of nitrates/nitrites in the lunchmeat, was described as "cultured". The storage of experimental samples at 26˚C for 48 hours allowed for this background culture to grow on the surface of the lunchmeat. That is why this contamination was not observed in any of the 0-Hour samples, including the negative controls which were negative for plate and enrichment growth. It is uncertain whether this background bacteria was intended to survive on the finished products as an active culture or if it was truly a contaminant from the manufacturing process.

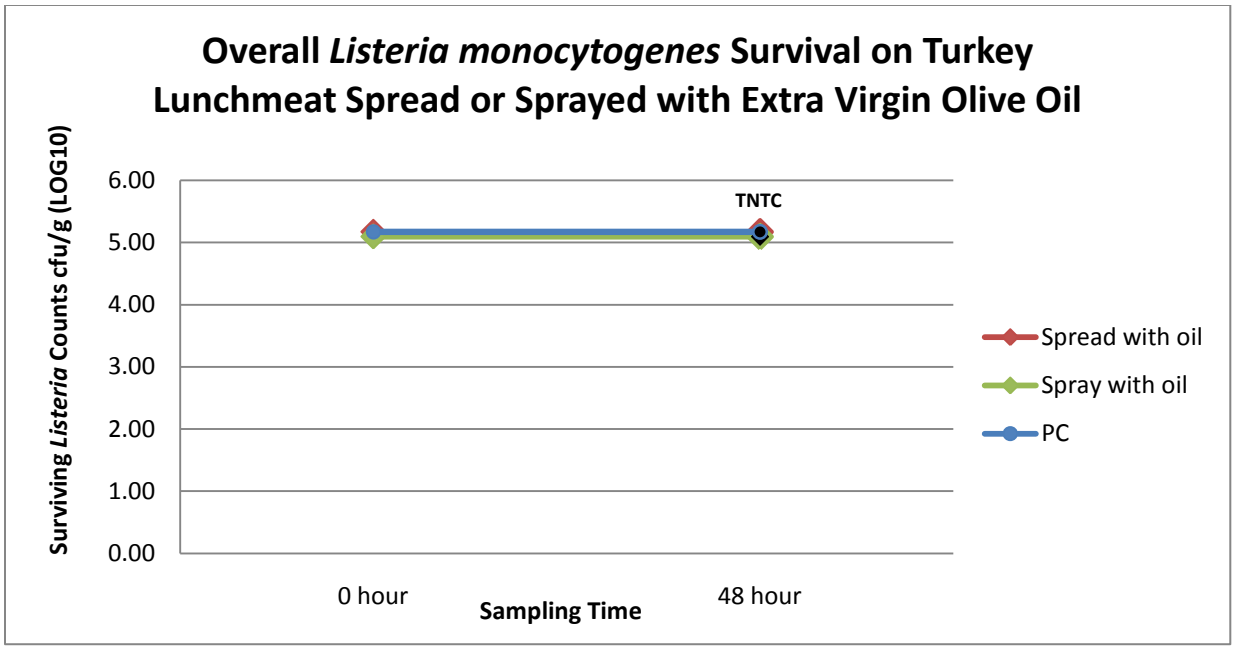

Figure 9.1. The average *Listeria monocytogenes* survival in positive controls and extra virgin olive oil on turkey lunchmeat

- All resulting numbers are the LOG10 bacterial counts from the spiral plates in cfu/g at 26˚C diluted using UVM
- All 48-Hour samples were TNTC from background contamination, *Listeria* counts indicated as the same as initial

Regardless of the intentions concerning the bacterial background, the fact remained that the extensive overgrowth of this contaminant prevented any enumeration of the surviving *LM* on the 48-Hour samples. Figure 9.1. shows the initial counts of the *LM* on the 0-Hour samples and the assumption that the *LM* may still be present in the equal numbers on the 48-Hour samples. Despite the increased number of background contamination, it cannot be assumed that the *LM* would also have increased. Nor can it be assumed that the *LM* counts decreased in the 48-Hour samples. The solid growth of the contaminating bacteria obscured the valid identification of *LM* both visually and numerically. The antimicrobial abilities of the EVOO on the *LM* using the spread and spray techniques were therefore indeterminable. The pH of the lunchmeat remained fairly consistent from the 0-Hour sampling (6.58) to the 48-Hour sampling (6.59). The water activity decreased slightly from 0.96 to 0.94 over the 48 hour storage.

9.6. Discussion

It was determined that neither the drop and spread technique nor the spraying technique applied during this mini project were viable methods for observing the antimicrobial effects of EVOO on slices of turkey lunchmeat inoculated with *LM*. The hypothesis that a 3-log cfu/mL reduction within the *LM* counts would be observed within 48 hours at 26˚C, after the addition of the oil via the drop and spread method, was ultimately rejected. The hypothesis that a 3-log cfu/mL reduction within the *LM* counts would be observed within 48 hours at 26˚C, after the addition of the oil using the spray method, was also rejected. No reduction, of any magnitude, was observed for the bacterial population on the sliced turkey lunchmeat. The failed methodology was hypothesized to be due to various confounding factors, including interference from the bacterial background, drying effects experienced by the meat, and the overall lack of exposure to the EVOO.

The most influential confounding variable during the mini experiment was the presence of the bacterial background on the lunchmeat slices. These bacteria overwhelmed the 48-Hour MOX plates, including some of the negative controls, and made it impossible to distinguish the contaminants from possible *LM*. As described in the results, the contaminant was isolated from several plates and was eventually categorized as a *Bacillus.* The source of the bacteria was found to be the turkey lunchmeat. It was thought that the bacteria were from the cultured celery powder or from an actual environmental contaminant within the finished product. The growth of the bacteria required temperature abuse in order to grow to detectable numbers. This is why only the samples which were stored at 26˚C for 48 hours grew these contaminants on their MOX plates. The 0-Hour samples remained free of any signs of contamination with these organisms. The problem with the background bacteria was three fold. The contaminating bacteria grew at such an alarming rate that within 48 hours they had completely overwhelmed the MOX spiral plates. The solid growth across the plates inhibited the identification of *LM* colonies on any of the 48-Hour agars. This made counting the presumed surviving culture completely impossible. The contaminating bacteria could have also competed with the *LM.* This could potentially cause the *LM* to

diminish across the surface of the lunchmeat since it is not a strong competitor with other organisms. This could have skewed any counts of recovered *LM* if higher dilutions had been utilized in the 48-Hour Spread and Spray samples. It would have been unclear whether any decrease within the *LM* population was due to exposure to the EVOO or exposure to the competing bacteria. Also the contaminating bacteria did not appear to be phased by the presence of the EVOO on either the Spread samples or the Spray samples. This may have been due to the overwhelming number of cells present after the 48 hour storage at 26˚C. It may also have been due to some sort of resistance to the antimicrobial properties of the oil. Further research should be conducted to determine the effects of EVOO on a *Bacillus* cocktail. Due to the negative results of the 0-Hour negative control samples, the issue of the background bacteria was unexpected and therefore unavoidable by the 48-Hour sampling time. This background contaminant may have been avoided entirely by selecting a different lunchmeat product. Temperature abusing some control samples under the same storage conditions of the experiment prior to beginning the study may have also indicated the presence of background bacteria. This would have shown a need for a higher dilution to be used when plating the 48-Hour samples in order to differentiate and count the bacteria. Storing the samples at 4˚C for 48 hours, instead of at 26˚C, may have also continued to discourage the growth of the background bacteria, similar to the results of the 0-Hour time point. The 26˚C storage temperature was to encourage the survival, if not growth, of *LM* on the meat in a temperature abuse situation. Obviously, it was overlooked that other background bacteria may also survive or even thrive under the same conditions.

Another confounding factor experienced during the mini experiment was that the turkey lunchmeat dried slightly while being stored at 26˚C. The 48-Hour samples were noticeably dry around the edges, despite being sealed in the glass Petri dishes. The center of the lunchmeat, where the inoculum would have been in the other samples, was still fairly moist. That is why the water activity did not appear to vary much (0.96 to 0.94) over the 48 hours despite the drying edges of the lunchmeat. Despite the mild numerical difference, this decrease in water activity may have added a confounding stressor to the *LM* populations on the surface of the meat. The drying of the meat indicates less water

available for biochemical reactions and subsequent growth or general survival of the bacteria. This reasoning seems unlikely since the center of the meat, where the inoculum was located, remained moist over the 48 hours and the growth rate of the contaminating bacteria did not appear to be impeded. The drying did cause a problem with the development of crevasses across the surface of the meat. In a typical product setting, the lunchmeat would remain moist within the packaging and thus avoid drying conditions. The meat in the glass Petri plates was sealed from additional air exchange from outside the container; however, there was a sufficient amount of air within the dish itself to allow for drying. Crevasses within the meat are thought to provide an area of escape for the *LM* from exposure to the limited volume of EVOO, thus avoiding the antimicrobial effects. A previous mini study (Chapter 7) found a similar phenomenon with pork tenderloin which was too dry. This drying issue and subsequent crevasse development in the meat could be avoided by transferring the slices of lunchmeat to the stomacher bags after the initial inoculation and addition of oil. The stomacher bags could then be folded and clipped to prevent any potential drying caused by air movement or general air exposure. This would also better simulate the packaging environment typically experienced by lunchmeats in the food market rather than the environment in the glass Petri plates.

The final confounding factor contributing to the failure of the mini study was the general lack of exposure to the EVOO. This can be attributed to the lack of available oil. The volume of oil continued to be an issue throughout all the application mini studies (Chapters 7, 8, and 9). The 0.1mL of EVOO that was applied to the turkey lunchmeat did not experience as many separation issues from the inoculum as seen in Chapter 8. Nor did the inoculum immediately escape into crevasses within the meat as seen in Chapter 7. The turkey lunchmeat appeared to have the right amount of moisture to support the equal mixing and absorption of the inoculum and oil. Unfortunately, it was unclear from the 48-Hour sample plates if this volume of oil was sufficient to elicit an antimicrobial response. The overgrowth of the contaminating background bacteria made it impossible to count any theoretical *LM* colonies. A better application of the EVOO would be to explore marinades and coatings. Direct exposure to the EVOO is vital when acquiring the antimicrobial action of the oil. If there is a lack of oil, it is more likely that a lesser or

negligible antimicrobial effect will be observed regarding any existing bacteria. The lack of oil may also have been due to interactions with the other bacteria present on the lunchmeat. As stated in Chapter 8, it is unclear what influence the interactions of other microflora on the surface of the product have towards the antimicrobial effectiveness of the EVOO. It is also unclear how these antimicrobial compounds interact and potentially change when exposed to the food product itself. Having more available oil would increase the chances of bacterial exposure, specifically *LM*, to the oil. This would effectively increase the likelihood of interactions with the various antimicrobial components and eliciting a bacteriostatic or bactericidal effect.

9.7. Conclusion

The mini study designed to determine if EVOO had any antimicrobial effects against a four strain *Listeria monocytogenes* (*LM*) cocktail on the surface of oven roasted deli turkey lunchmeat was inconclusive. The overgrowth of background bacteria on the 48-Hour lunchmeat samples made it impossible to differentiate and count colonies on the MOX plates. It can only be assumed that the *LM* survived alongside the contaminating background microflora. Based on that assumption, the hypothesis that a 3-log cfu/g reduction might be observed in the surviving *LM* counts when exposed to the EVOO after 48 hours at 26˚C was rejected. This would indicate that no reduction, in any of the *LM* strains, was observed from the addition of 0.1mL of oil spread or sprayed onto the surface of the turkey lunchmeat. This mini study validated the need for direct and constant contact to elicit the antimicrobial action of EVOO. This exposure was not accomplished in this mini study due to the overwhelming background bacteria. Future research may be best suited for studying the antimicrobial effects of EVOO on other self-made lunch meats to minimize the presence of nitrates/nitrites and background microflora. Marinating and coating techniques may be a better application for EVOO, since a prolonged and direct exposure time appears to be necessary for effectiveness. Additional research should also explore the effectiveness of EVOO against a cocktail of different genus to observe the possible interactions of various bacteria when exposed to the oil together.

CHAPTER 10

OVERALL CONCLUSIONS

The overall conclusions for the *Salmonella* study were that the beef tallow, pig lard, duck fat, and coconut oil supported the survival of a four strain *Salmonella* cocktail over a seven day period at either 26˚C or 37˚C incubation. On the seventh day, all samples contained bacterial populations similar to the initial populations. These results validated the hypothesis that the animal derived fats and coconut oil could be potential sources of post-processing contamination if utilized within the pet food industry. The extra virgin olive oil (EVOO) did not support the survival of the four strain *Salmonella* cocktail, the five strain Shiga-toxin producing *E. coli* (*STEC*) cocktail, or the four strain *Listeria monocytogenes* (*LM*) cocktail during their respective studies over the seven day period at either 26˚C or 37˚C incubation. All organisms experienced an average ≥3-log cfu/mL reduction within their bacterial populations within the first 24 hours of storage in the EVOO at both temperatures.

The reduction of the *Salmonella* cocktail ranged from an average 3-log cfu/mL reduction to below the level of detection (<1-log cfu/mL), despite the resistance of *Salmonella choleraesuis* subsp. *arizonae* (ATCC 13314). The reduction of the *STEC* cocktail ranged from an average 3.5-log cfu/mL reduction to below the level of detection (<1-log cfu/mL). The reduction of the *LM* cocktail maintained a reduction below the level of detection (<1-log cfu/mL) after the first 24 hours in the EVOO. In addition, the EVOO did not support the survival of the *LM* in any of the other experimental time frames. An average 2.5-log cfu/mL reduction to below the level of detection (<1-log cfu/mL) was observed for all of the bacterial populations of the *LM* experiments.

Manipulation of the mixing frequency, experienced by the *LM* in the EVOO, contributed to the rapid reduction of the bacterial population. A mixing frequency of every five minutes resulted in an average reduction of the *LM* population to below the level of detection (<1-log cfu/mL) by the first 20 minute sample time. The other mixing frequencies, every 10 minutes and every 20 minutes, had prolonged bacterial survival times, but were all below the level of detecion (<1-log cfu/mL) at the

60 minute sample time. These results supported the idea that direct physical contact with the EVOO was necessary to elicit a strong antimicrobial effect. The pork tenderloin, cheddar cheese snack squares, and turkey lunchmeat application studies displayed no reduction in the inoculated *LM* populations exposed to EVOO with the current methodologies.

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VITA

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