THE INHIBITION OF POTENTIAL PATHOGENS BY PERSIMMON PUREE AND
SELECTED PHENOLICS

by

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Abstract

Three experiments were conducted to study the antimicrobial effectiveness of persimmon puree and phenolic compounds commonly found in the persimmon and plum. The objectives in experiment 1 were to evaluate the antimicrobial effects of persimmon puree on Bioball[TM] *Listeria monocytogenes* and *Escherichia coli* O157 in a liquid medium. Persimmon puree was added at 1, 3, 5, and 10% wt./vol concentrations to brain heart infusion broth and inoculated with Bioball[TM] *Listeria monoctogenes* and Bioball[TM] *Escherichia coli* 0157. Microbial growth was evaluated at 0, 24, 36 and 72 h. Results indicated that at 24 h, persimmon puree at all concentrations suppressed ($P<0.05$) growth of *L. monocytogenes* compared to the control. Suppressed ($P<0.05$) growth of *L. monocytogenes* continued through 36 and 72 h for all concentrations of persimmon puree tested. However, due to non-pathogenic background Gram-negative micoflora, inhibition of *E. coli* O157 could not be ascertained. The objectives of experiment 2 were to evaluate the antimicrobial effectiveness of selected phenolic compounds (benzoic acid, gallic acid, vanillic acid, chlorogenic acid, and quercetin on *E. coli* O157:H7, *Salmonella* Typhimurium, *Bacillus cereus*, *Yersinia enterocolitica*, *L. monocytogenes*, and *Staphylococcus aureus*. Quercetin, vanillic, and chlorogenic acids were effective against selected pathogens at varying levels, but not as potent as Benzoic or Gallic acid. Results indicated that benzoic acid had the most effect against *E. coli* O157:H7, *S.* Typhimurium and *B. cereus* at concentrations of 452.98, 239.63 and 518.79 µg/ml, respectively. Gallic acid was the most effective against *Y. enterocolitica, L. monocytogenes*, and *S. aureus* at concentrations of 11.01, 29.06 and 22.45 µg/ml, respectively. The objective of experiment 3 were to evaluate the antimicrobial effectiveness of persimmon puree at concentrations of 0, 3, 5 and 10% wt./wt on a five strain cocktail of *L. monocytogenes* in ground beef. There was no suppression of growth at any concentration at 0, 1, 3 or 5 d. However, there was an increase ($P<0.05$) on 5 d for concentrations 5 and 10% persimmon puree when compared to the control. These series of experiments suggest that benzoic and gallic acids may have potential to suppress microbial growth. Persimmon puree appears to be an effective antimicrobial agent against Gram-positive bacteria in a liquid medium. However, incorporation of persimmon puree into ground beef did not yield an antimicrobial effect. Therefore, more research needs to be conducted to validate the

effectiveness of phenolic compounds and persimmon puree as antimicrobial agents in food substances.

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Dedication

I dedicate this Thesis to my Grandparents Larry Robertson and Clair Mader who could not hear about this today, but whose impacts on my life will never be forgotten!

Introduction

CHAPTER 1 - Review of Literature

Foodborne Disease

The Centers for Disease Control and Prevention (CDC) estimates that there are 76 million foodborne diseases every year resulting in an estimated 325,000 hospitalizations and 5,000 deaths. A majority of foodborne illness can be attributed to improperly prepared food ingested by immunocompromised individuals such as the elderly or young infants and children both in the home and in restaurants. Most healthy individuals' immune systems are able to defend against microorganisms. However, the defense systems in the elderly and young children may not be as efficient resulting in serious illness or death. Yet, even healthy individuals are not safe from becoming ill from some foodborne disease.

According to the CDC, foodborne disease can be attributed to many organisms with the most common being *Campylobacter, Salmonella, Escherichia coli* O157:H7, and Norwalk virus. Other pathogens of concern include *Listeria monocytogenes, Staphylococcus aureus, Bacillus cereus, Yersinia enterocolitica, Shigella, Vibrio parahaemolyticus,* hepatitis, *Giardia lambdia,* and *Cryptosporidium.* Unfortunately, significant outbreaks such as the Jack-in-the-Box *E. coli* O157:H7 occurrence in 1993, the spinach *E. coli* O157:H7 recall in 2007, and the *Salmonella* contaminated peanut butter in 2009 with Peanut Corporation of America, which continually focuses public and industry concern on the safety of our food supply. As the food industry continues to grow, so do opportunities for pathogen contamination. This presents the food industry with a critical need to control pathogens in the world's food supply. Furthermore, many foods such as fresh vegetables provide a continual challenge to maintain food quality while implementing technologies that will insure a safe product.

Pathogens of Concern

Escherichia coli O157:H7

 Escherichia coli is an important coliform in the food industry. The generic, non-pathogenic organism is commonly used as an indicator for fecal contamination in food testing. However, the serovar O157:H7 is of great concern due to its virulence, which costs the food industry millions of dollars every year. *Escherichia coli* O157:H7 is well known for contamination of raw meat, which legally is considered an adulterant according to FSIS and results in a loss of that product.   In addition, with the recent outbreaks involving spinach, *Escherichia coli* O157:H7 has gained increased attention in the fresh leafy vegetable sector (FDA, 2006).

 Escherichia coli is a Gram negative, motile, facultative anaerobe with a rod shaped morphology that belongs to the family *Enterobacteriaceae*. *Escherichia coli* is commonly found in the intestines of animals and humans. The human pathogen, *E. coli* O157:H7 has a growth range from $10^{o}C$ to $50^{o}C$ with an optimum growth at $37^{o}C$ and a pH tolerance from 4.5 to 9.0. *Escherichia coli* O157:H7 designates a specific strain by the letter and number after the genus and species. Antigens are denoted by the letters O (heat stabile somatic antigens), K (heat liable somatic antigens) and H (heat liable flagellar antigens) of which there are 164, 100 and 56 serovars, respectively. There are over 200 O serotypes, which may be further divided with the H (flagellar) antigens. *Escherichia coli* O157:H7 may be categorized into five virulence groups: enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohemorrhagic (EHEC), and enteroaggregative (EAggEC) (Jay et al., 2005).

 Primary symptoms of *E. coli* O157:H7 infection includes all those associated with gastroenteritis such as watery diarrhea, vomiting, and nausea. When *E. coli* O157:H7 colonizes the intestines, a toxin is produced after the concentration reaches 10^{6} CFU/ml, that results in illness (Jay et al., 2005). *Escherichia coli* O157:H7 (EHEC) is slightly different in the fact that it will produce veratoxins that may develop into Hemolytic Uremic Syndrome (HUS). Symptoms include vomiting, nausea, cramping, and bloody diarrhea (CDC, 2008). In the elderly, young, and immune-compromised, HUS may develop further symptoms such as fever and neurological complications that may lead to Thrombotic Thrombocytopenic Purpura (TTP). Infectious doses

are relatively unknown, but as few as 10 cells have been associated with outbreaks in immune compromised and in the elderly (Center, 2006).

Enteropathogenic *Escherichia coli*

Enteropathogenic *E. coli* (EPEC) strains usually do not cause severe illness, however they can cause diarrhea in young children. Enteropathogenic *E. coli* has the ability to adhere to tissue culture or agglutinate in a tissue culture. The partial virulence of the EPEC strains is due to adherence plasmids that allow for intestinal attachment. Enterpathogenic *E. coli* strains cause lesions, attachment-effacement (A/E), that are produced by Esps (EPEC secreted proteins) These proteins block phagocytosis and lead to cytoskeletal rearrangement and tyrosine phosphorylation (Tir), thus destroying the brush border of the intestines (Jay et al., 2005).

Enterotoxigenic *Escherichia coli*

Enterotoxigenic *E. coli* (ETEC) is more noted for its ability to cause diarrhea in not only children, but may be associated with adults or Traveler's diarrhea. Virulence with ETEC may be associated with fimbrial colonization factor (CFA). Colonization factors are encoded with the heat stable plasmid and typically does not produce toxins (ST and LT) above 20°C. ETEC strains are thought to need 10^{8} and 10^{10} CFU to cause diarrhea. In addition, ETEC produces two types of toxins, a heat-labile and a heat stable toxin, the first of which are designated as either human and porcine pathogenic strain (Jay et al., 2005).

Enteroinvasive *Escherichia coli*

Enteroinvasive *E. coli* (EIEC) are non-toxin forming strains that colonize epithelial cells that spread adjacently. This infection typically is associated with immunocompromised individuals, and is known for causing traveler's diarrhea and can be spread by person-to-person transmission. The 140-MDa plasmid causes the enteroinvassiveness (pINV) of this strain (Jay et al., 2005).

Enterohemorrhagic *Escherichia coli*

Enterohemorrhagic *E. coli* (EHEC) strains are similar to EPEC in their ability to create lesions due to the chromosomal gene eaeA. Enterohemorrhagic *E. coli* only affects the large intestine by producing Shiga-like toxins. Enterohemorrhagic *E. coli* contain the plasmid 60-MDa which aids with fimbriae attachment and allows for attachment to the epithelial cells but

does not actually invade the cell (Jay et al., 2005). Enterohemorrhagic strains such as *E. coli* O157:H7 with the *Shigella* like veratoxins cause infections in which 5-10% of cases can develop HUS, which ultimately leads to kidney failure (CDC, 2008).

Enteroaggregative Escherichia coli

Enteroaggregative *E. coli* (EAggEC) strains are adherent strains that usually exhibit a "stacked-brick-type" adherence to HEp-2 cells. These strains are not yet implicated with the other known foodborne pathogens. However, some strains are capable of producing a heat stable enterotoxin that causes diarrhea in children for more than fourteen days (Jay et al., 2005).

In order to properly identify and enumerate *E. coli,* several biochemicals and media can be used. When isolating *E. coli,* samples can be enriched with Escherichia coli (EC) medium for 24 hr and plated onto either MacConkey Sorbitol Agar (MSA) or Levine Eosin Methylene Blue agar (L-EMB). Colonies appear as a colorless colony on MSA and purplish colony on L-EMB with a green metallic sheen. After growth on a solid medium, an isolated colony can be Gram-stained to find a Gram negative short rod. If positive, indole Methyl Red Voges-Proskauer Citrate (IMViC) will yield results of biotype 1 (-, +, -, -) or biotype 2 (+, +, -, -). More rapid and conventional methods include API20E (bioMerieux, Hazelwood, MO) or the automated VITEK (bioMerieux, Hazelwood, MO). Further confirmation can be done using autoclaved cells with VIP (BioControl, Seattle, WA) or latex agglutination (FDA, 2006).

Salmonella Typhimurium

Salmonella is a common pathogen contracted from swine and predominantly poultry with all known species being pathogenic (Jay et al., 2005). Sources of contamination include raw meat, feces, any surface possibly touched by the previous, raw seafood and raw poultry (Walderhaug, 1992). *Salmonella* is a Gram negative, motile, facultative anaerobe with a short rod morphology. There are over 2,400 known serovars with only four that infect humans: *Salmonella* Typhimurium, *Salmonella* Typhi, *Salmonella* Paratyphi A, and *Salmonella* Paratyphi C. *Salmonella* can be grouped into three categories when following their epidemiology: human pathogens only, host adapted (Host specific pathogens), and host unadapted, that can be pathogenic to humans and animals. *Salmonella* is commonly found in the intestinal tract of animals and humans, but has been noted in insects (Jay et al., 2005). Transmission occurs when

feces are excreted and come in contact with the feces or influenced areas. Water may be a source of frequent contamination, especially in run-off areas of fecal deposition which are then carried to a stream and continue to flow downstream. In addition, *Salmonella* has been found in the spleen, liver, bile and in lymph nodes of pork (Kampelmacher, 1963).

Identification of *Salmonella* begins by segregating species into groups A, B, or C according to O, H, and K antigens. Groups are created by shared antigens such as C_1 shares O antigens 6 and 7. The C_2 groups are composed of O antigens 6 and 8. Further classification is employed using the flagellar H antigen that may be broken down into phase 1 or 2. Phase 1 is only shared with a few *Salmonella* strains, the rest are associated with phase 2. Serovars are named after the places they were first isolated which is the reason for *S.* London, *S.* Newport and *S.* Miami for example (Jay et al., 2005).

The Centers for Disease Control and Prevention estimates that there are 40,000 cases of Salmonellosis each year in the United States, with 400 resulting in deaths. Non-typhoidal outbreaks of Salmonellosis were implicated in foods such as fresh tomatoes, alfalfa sprouts, cantaloupes, unpasteurized orange juice, raw undercooked shell eggs, raw uncooked ground beef, chocolate, and dry foods such as peanuts (Jay et al., 2005). The most recent and largest outbreak involved *Salmonella* SaintPaul, that were implicated with fresh vegetables from Mexico, such as fresh Serrano and Jalepeno pepper in 2008 (CDC, 2008). *Salmonella* is a very ubiquitous organism that has the ability to exist and thrive in many conditions, thus it is always a possibility for foodborne illness.

Yersinia enterocolitica

Yersinia enterocolitica is a Gram negative rod from the family *Enterobacteriaceae* that is composed of 11 species. *Yersinia enterocolitica* is a facultative anaerobic, non-spore forming bacterium that is motile at 20˚C but not at 37˚C. *Yersinia* biochemically recognized as a glucose fermenter with little or no gas, rhamnose negative, sucrose positive, oxidase negative, and urease positive. *Yersinia enterocolitica* is psychotrophic, but has an optimum growing temperature between 22-29˚C (Jay et al., 2005). *Yersinia enterocolitica* has the ability to tolerate pH extremes from 4.6 to 9.6 at 3˚C and salt tolerances of 3% at 3˚C (Stern et al., 1980). *Yersinia enterocolitica* has a wide range of tolerances. This high tolerance for environmental factors increases the chance of human or animal host acquiring the bacteria.

Yersinia enterocolitica may be found in terrestrial environments as well as in aqueous environments including; lakes, streams and pooled water. *Yersinia enterocolitica* is known to be found in humans and other land mammals, with swine being the most prevalent (Jay et al., 2005). A study conducted by Funk et al. (1998) found that 92% or 95/103 market hogs carried at least one strain of *Y. enterocolitica*, with 98.7% of pathogenic isolates indentified as O:5. In 4,841 stool specimens collected from seven cities in the U.S. between November 1989 to January 1990, 38, 49, 60, and 98 percent contained *Y. enterocolitica*, *Shigella*, *Campylobacter*, and *Salmonella*, respectively (Lee et al., 2001)). *Yersinia enterocolitica* is most commonly found in fresh meats, vacuumed packaged meats and foods, seafood, vegetables, and milk (Jay et al., 2005).

Yersinia enterocolitica produces a heat stable enterotoxin that is capable of surviving 100˚C for 20 minutes (Jay et al., 2005). In order for these toxins to be produced, temperatures must be at or below 30˚C (Pai and Mors, 1978). Symptoms caused by *Y. enterocolitica* include gastroenteritis, pseudoappendicitis, reactive arthritis, colon and neck abscesses (Jay et al., 2005). The first recorded outbreak in the U.S. occurred in 1976, when contaminated chocolate syrup was added to pasteurized milk in New York State (Black et al., 1978). Time of year appears to play a role in *Y. enterocolitica* activity. There are the fewest incidences noted in the spring and the most incidences reported in the fall. Children tend to be the most vulnerable, and it often causes abdominal pain as well as diarrhea. *Yersinia enterocolitica* can be recovered from blood, stools, joint fluid, lymph nodes, and urine. Extended complications from infection rarely occur and often pass within a couple of days (Jay et al., 2005).

Staphylococcus aureus

Staphylococcus aureus and Staphyloccal foodborne illnesses have been studied since the late 1800's. *Staphylococcus aureus* is a Gram positive, facultative anaerobe, with cocci morphology and is typically found in a cluster, similar to the pattern of grape clusters. In fact, it can be quite ubiquitous in nature and humans appear to be a hospitable carrier harboring the organisms in their skin, nose, and hair (Fung, 1999). Moreover, humans are host to half of the known species belonging to the genus *Staphylococcus* (Jay et al., 2005). Most foodborne illnesses can be attributed to human carriers and contamination can occur from sneezing or contact with affected individuals.

Staphylococcus aureus has a wide variety of growth ranges and can survive a range of environmental factors. They appear to require nitrogen from amino acids, C and B vitamins, nicotinic acid, and thiamine to maintain homeostasis. Arginine appears to be the direct nutrient needed for enterotoxin B production. Optimum temperature for growth occurs at between 40˚C and 45˚C, but can survive conditions ranging from 10˚ to 46˚C. Staphylococcus aureus is a halophile, meaning it can tolerate salt stresses. Growth can occur in concentrations up to 20% NaCl in some strains, but also grows well at 7% or less. Furthermore, S. aureus was shown to grow in product with Water Activity as low as 0.83, however 0.86 is considered the gold standard for safety (Jay et al., 2005). All these factors combined together, can affect the organism's ability to proliferate.

Recently S. aureus has made headlines for its Methicillin Resistant Strain or MRSA. Methicillin was a drug first introduced for the treatment of S. aureus in 1959 (Jevons, 1961). Resistance is attributed to the mecA gene, which is not present in susceptible strains (Hiramatsu, 2001). This organism is of major public health concern in hospital and nursing homes. Institutionalized health care settings provide opportunities for this bacterium to continually select for resistance genes (CDC, 1999; Hussain, 2001). Fortunately for food safety, cooking still eliminates most the serious threats that MRSA can present and toxins are still of primary concern.

Symptoms of Staphylococcal foodborne intoxication or poisoning usually occur when the infected food is consumed, after the organism is allowed to proliferate. A concentration of $10^{5\text{-}6}$ CFU/g is the level required for a toxin to be produced. Currently, there are 13 indentified toxins produce by S. aureus. Fifty percent of Staphylococcus isolates from humans in the United States were found to produce Enterotoxin A (Casman et al., 1967). Staphylococcus aureus entertoxin is very heat resistant. Research has shown that Staphyloccocal enterotoxin B (SEB) can resist heating for 16 hours at 60˚C without losing its biological activity (Schantz et al., 1965). Further research has shown that Staphylococcal enterotoxin C (SEC) is more heat stable than SEB. Moreover, SEC has the ability to reactivate, after being inactivated by heat, that makes this toxin very hard to control once in food systems (Fung et al., 1973). The toxin can cause rapid symptoms because it is ingested and taken into the systems quickly unlike an infection where the bacterium has to first colonize the host. Symptoms include nausea, vomiting, abdominal cramps, diarrhea, sweating and prostration about 4 to 6 hours after consumption of contaminated foods.

Symptoms usually last between 24-48 hr (Fung, 1999; Schlievert et al., 1996; Jay et al., 2005). Death is not common and rarely occurs (Jay et al., 2005). Schlievert et al. (1996) reported a death rate as low as 0.03%. Most incidents can be avoided by good hygiene and sanitary practice for at home preparation.

Listeria monocytogenes

Listeria monocytogenes is a Gram positive, facultative anaerobe, non-spore forming rod that is characterized by a unique tumbling or slight turning motility. Interestingly enough, *L. monocytogenes* is motile at room temperatures (between 20˚C-25˚C), but cannot synthesize flagellar proteins at 37˚C (Todars, 2008). Most importantly, *L. monocytogenes* is a psychrotrophic organism capable of growth at refrigerated temperatures. *Listeria monocytogenes* can grow in temperatures as low as 0.5˚C to as high as 45˚C (Jay et al., 2005). The CDC reports that there are an estimated 2,500 cases of Listeriosis each year. However, there was a decreasing trend over from 1989 to 1993 and again from 1996 to 2006 with a 34% and a 36% decline, respectively. *Listeria monocytogenes* is a serious concern for pregnant women and the immune-compromised. Pregnant women should be on high-risk alert as Listeriosis can cause still births and abortions. Ready-to-eat meats and fresh soft cheese should be avoided or boiled because they are a major source of *L. monocytogenes* contamination and growth.

Listeria monocytogenes can be expected to exist anywhere that the lactic acid bacterium, *Brochothrix* and coryneform bacterium occur (Jay et al., 2005). They have a growth range from as low as 1˚C up to 45˚C, which allows them to be fairly ubiquitous. Anywhere there are feces, sewage, silage, water or decaying vegetation, *L. monocytogenes* can be harbored. These sources of contamination can carry over into many foods such as cheese, milk, fresh meats, frozen meats, ready-to-eat meats, vegetables and seafood. On October 17, 2008, The Public Health Agency of Canada reported over 53 cases, four suspected cases, and 22 deaths, of which, 20 were confirmed Listeriosis. The confirmed *L. monocytogenes* cases had no underlying causes or complications that were not associated with infected product from Maple Leaf Foods. In other words, the individuals who were confirmed were not ill due to pre-existing conditions, but were confirmed ill because of ingesting contaminated product. Maple Leaf recalled all breakfast products, meats and cheeses that were produced on lines eight and nine of the plant (Canada, 2009).

Unfortunately, Maple Leaf Foods has become an example of what can happen when *L. monocytogenes* contamination occurs.

Listeria monocytogenes was first discovered in 1911 by Hülphers (Junttila et al., 1988). Mainly found in humans, it is a known pathogen to a variety of animals including ticks, fish, fowl, and over 50 mammalian species. Reported cases had appeared to be dropping in the 1990's. However, for Australia, Canada, Denmark, the United Kingdom, and the United States 2, 2-4, 4-5, 2-3 and about 4 million cases have been reported, respectively (Jay et al., 2005). USDA's Food Safety and Inspection Service (FSIS) currently has a zero tolerance regulation in place in the United State for *L. monocytogenes* in Ready-to-Eat (RTE) products. Some countries have different levels of severity, such as France with a zero-tolerance policy in raw product versus Canada who has three categorical levels of safety. The three levels include: Category one are products linked to outbreaks, Category 2 are products with shelf-life over 10 days, and Category 3 are products with shelf-life under 10 days (Jay et al., 2005). There is still not a universal conclusion on the way products should be treated in regards to *L. monocytogenes,* leaving a lot of room for future research and regulation.

Bacillus cereus

Bacillus cereus is an aerobic, Gram positive, spore-forming rod that is very ubiquitous in soil, water and dust. Other strains of *Bacilli* include *Bacillus anthracis, Bacillus subtilis* and *Bacillus thuringiensis.* *Bacillus cereus* is a pathogen responsible for gastroenteritis and has the ability to grow at temperatures as low as 4˚C and as high as 50˚C (Jay et al., 2005). Growth can occur at a pH range between 4.9 to 9.3 (Goepfert et al., 1972).

Bacillus cereus gastroenteritis is caused by the production of an extracellular toxin and enzymes (Jay et al., 2005). The toxin causes gastroenteritis with diarrhea. However, the individual toxin has been identified as, hemolysin BL, and the components can be etiologically linked to the diarrheal condition (Beecher et al., 1995). There are two types of intoxication diarrheal and emetic. However, symptoms of both may span 8-16 hours, which include diarrhea, stomach pain, nausea, and vomiting (emetic). Emetic type food poisoning is classified as more severe than the diarrheal condition and requires up to 2.0×10^9 cells (Gilbert, 1979; Turnbull et

al., 1979). Sources of *B. cereus* include starchy products such as corn, cornstarch, potatoes, vegetables, meat, rice dishes, and soups.

Spore formation usually occurs with onset of adverse conditions. Spores are a way for *B. cereus* to protect itself from negative environmental conditions. Spores may regenerate when environmental conditions are more suitable for the organisms' proliferation. Spores may be a problem because of their resistance. Even when a vegetative cell is destroyed, spores can survive. Spores have been shown to resist sterilization at 121˚C for 30 min. In addition, spores were shown to have an adhesion factor which can lead to contamination of improperly sanitized processing facilities. Research by Faille et al. (2007) showed that the environmental conditions (i.e., heat and physical stress) in which the spores are produced can negatively impact their ability to adhere to surfaces. Clearly the best way to avoid this condition is through prevention with proper sanitation and Good Manufacturing Practices. Further research, was conducted through the use of bacteriophages to control vegetative cells and spore germination in *B. anthracis*. However, this has only been documented in lab conditions and has not been applied to pathogenic spore germination (Walter, 2003).

Antibiotics and Antimicrobials in the Food Industry

Introduction

Food safety is of paramount importance in the food industry. Beginning in 1996, Hazard Analysis and Critical Control Points (HACCP) were introduced to address food safety concerns. Developed by NASA and the Pillsbury Company, industry uses this model directly and indirectly to find points in their process where control can be applied to significantly reduce or eliminate foodborne pathogens.   Some of the first antimicrobial work can be attributed to Nicolas Appert who created a thermal process for sealed containers, preserving food to feed Napoleon's armies. The study of antimicrobials may be attributed to Louis Pasteur and his work in the early 1800's. Pasteur developed the, "Germ Theory," where any liquid, even an easily spoiled one, will remain sterile if protected from these germs. Pasteur became even more famous with his ability to show that wine could be protected from disease when heated to 55˚C (Fung, 1995). This heating process that destroys microorganisms was later coined, "Pasteurization," thus creating the first industrial process for the intentional destruction of microorganisms in food.

In 1929, after Alexander Flemming went on vacation, he came back to notice contamination on one of his inoculated plates. He noticed a ring of inhibition around a mold colony that prevented bacterial growth, which upon further analysis became the first antibiotic, penicillin (Poupard, 1994). Antibiotics are traditionally defined as, "Any substance produced by one organism against another organism" (Fung, 2004). Antibiotics are commonly employed in animal feeds to increase animal health and efficiency. Antibiotics are not currently implemented into commercial food systems amid public fears of chemical tainting and more importantly due to the increasing concern of antimicrobial resistance. Microbes have adapted resistance to different environmental stresses, nutrient availability and antibiotics. Some argue that microorganisms are not evolving directly from adaptation to antimicrobials, instead we are selecting for the microorganisms that are more resistant through our continual use of these substances (Apley, 2008). The U.S. alone was responsible for over 1 million pounds of antibiotic production in the 1950's (McEwen, 2006). That number according to the CDC, now exceeds 50 million pounds, with almost half going to animal agriculture and welfare (Nawaz, 2001; Shea, 2004). Antimicrobials play a major role in keeping food products from spoiling, extending shelf-life and decreasing food waste, but most importantly they keep our food safe.

Intrinsic and Extrinsic Factors

Current practices initiated in the food industry to control microorganisms range from extrinsic and intrinsic factor manipulation. Intrinsic factors such as pH, moisture content, nutrient content, oxidation/reduction potential and biological structure can affect growth of bacteria in food products. United States Food and Drug Administration (FDA) classify low acidified foods as a pH of 4.6 or less. This definition was introduced to control the growth of *Clostridium botulinum* in canned foods.   Most organisms grow best around a pH of 7. However, molds can grow as low as a pH of 2 and are able tolerate as high as 11 (Jay et al., 2005). Most organisms will not grow with a pH below 4, nor over 9, with the exception of *Vibrio parahaemolyticus*, that grow beyond pH of 9. Yet, pH can alter the heat needed to inactivate microorganisms that positively alter food quality in rice cereal and salami matrices (Clavero and Beuchat, 1996; Jay et al., 2005; Yun et al., 1998).

Extrinsic factors of growth include time, temperature, relative humidity, the presence of gases, and physical stress. Bacteria grow at a wide range of temperatures, usually between 0 to

45˚C and can be inactivated by extremes. The $Q_{10}=2$ method describes reactions relationships to temperature changes in that for every 10˚C increase the reaction will increase 2 fold. If the reaction decreases 10˚C, according to the $Q_{10}=2$ method it will slow down 2 fold (Jay et al., 2005).   In optimal temperature ranges bacteria double every 10 to 30 min. In order to avoid bacterial problems foods should be kept below 4˚C and above 60˚C (Garden-Robinson, 2007). United States Department of Agriculture (USDA) mandates under 21 CFR 110.80, processors of cold foods must be conducted under 7.2˚C to reduce possible bacterial problems (Administration, 2008).

D-values are used to determine the time and temperature required to reduce bacteria by 1 log or 90%. The D-value becomes much more complex and the food matrix must be considered when using heating methods to destroy bacteria. Heat shock has been shown to increase D-values of *E.coli* O157:H7. In order to simulate improperly processed hamburgers, D-values increased 37, 68 and 50% in 54, 58 and 62˚C, respectively, in a liquid system. Unfortunately, the theory was not able to be significantly replicated in the hamburger matrix (Williams and Ingham, 1997).

In addition to the time and temperature combinations that work together in D-value combinations, the relative humidity may also provide an added antimicrobial effect. Water activity can play a significant role in allowing microorganisms the free moisture need to grow. Relative humidity can alter food products free water and make it more available for uptake by bacteria. Due to this reason, storage conditions should be kept relatively free of humidity to prevent enhanced microbial growth conditions.   Knowledge of the products being stored must be taken into account to ensure they are not contributing to excess moisture migration. Negative quality issues can arise when higher water activity foods are kept in dry conditions causing moisture to migrate out of the product. Antimicrobial effectiveness can be enhanced when water activity is kept as low as the product can allow while maintaining quality.

Food integrity must be monitored closely when changing temperature, time or humidity processing, especially once a product is packaged. Atmospheric gases can impact microbial proliferation, as bacteria growth can occur from aerobic to anaerobic conditions. Several gases are important in microbial control such as carbon dioxide and ozone (Clark and Lentz, 1973; Parekh and Solberg, 1970). Modified Atmosphere Packaging or MAP packaging was shown to improve both food safety and quality in fresh red meats; however, careful consideration of the

benefits and disadvantages should be considered (Cornforth and Hunt, 2008; Grossbauer, 2003). Moreover, use of CO_2 was shown to reduce microorganisms at refrigerated temperatures in seafood as well (Rutherford et al., 2007). Jay et al. (2005) cautions the use of ozone, as extreme oxidation will occur. Alternatively, carbon dioxide may reduce bacterial pathogens but can hide indicators of food quality and visual measures of doneness in cooked meats (Clark and Lentz, 1973; Jay et al., 2005).

Further extrinsic factors that aid in microbial control include physical stress, such as acid washes. Acid washes have been proven to reduce the bacterial load on carcasses during slaughter and have even been recommended as a critical control point (CCP) (Kerth and Braden, 2007). Physical stresses are limited to what industry can add to food and still be safe for human consumption. National sales trends favor the consumption natural or organically produced foods (Norwood, 2004). To meet these demands industry is constantly striving to find more effective, more sustainable, and more consumer appealing methods. Naturally occurring plant phenolics may be a solution to meet these demands. Phenolics like those found in the plum and the persimmon have exhibited antimicrobial properties and would be a naturally safe additive in food that can enhance quality and most importantly ensure a safer food supply.

Irradiation

Irradiation is defined as the treatment of foods by exposing them to ionizing radiation, also call ionizing energy, to achieve certain technical objectives (Loaharanu, 2003). Ionizing radiation comes in three forms including: X-rays, gamma rays, microwaves, ultraviolet rays and beams of high-energy electrons which are produced by electron accelerators (Loaharanu, 2003; Jay 2007). Irradiation is a big concern for many consumers now days who do not fully understand its uses with and on food. It can be a way of sterilizing food or only reduces a few organisms for extended shelf life. However, it can also cause negative effects such as rancidity and physical degradation if to high of a dose is given.

Irradiation is the process of using energy such as joules or rads as units of measurement. Radiation is measured in Curie's or the dose of radiation 3.7×10^{10} disintegrations (Jay et al., 2005). Radiation can also be measured in absorption values such as rads or grays. Each can be expressed in terms of krad or kGy depending on the amount absorbed. The Gray is the newer unit for rads commonly expressed and 1 Gy=100 rads = 11 joule/kg; 1 kGy = 10^5 rads. UV light

is very effective agent due to short wavelengths of 2,600 Å where it mutates DNA of the bacterial cell causing in ability to function, thus causing cell death (Jay et al., 2005). Gamma Rays are the cheapest form of radiation because the primary source is a by product of atomic fission which has great penetration vs. other methods typically yielded by ^{60}Co or Cs ^{137}. X-Rays have high velocity electrons that have similar properties as Gamma Rays (Jay et al., 2005) and electron accelerators work in similar ways and have similar properties as Gamma Rays. Electron Accelerators and Gamma Rays are the most typical types of irradiation probably due several factors including financial considerations and penetration depth into the product being irradiated.

The FDA first approved irradiation in the US for spices and dried vegetables in 1983 (Loaharanu, 2003). Its was then quickly adopted for *Trichiinella* in pork 1985, insect infestations and shelf life extensions in 1986, poultry and red meat 1990 and 97, respectively and shell eggs and sprouting in 2000 (Loaharanu, 2003). Foods that are FDA approved are fresh non-processed pork, fresh foods, foods, dry or dehydrated enzymes, spices, fresh or frozen poultry, frozen packaged meats (NASA), uncooked meat, fresh shell eggs, seeds for sprouting, and fresh or frozen molluscan shellfish with associated doses of .3kGy min-1kGy max, 1 kGy max, 1 kGy max, 10kGy max, 30 kGy max, 3 kGy max, 4.4 kGy max, 4.5 kGy max, 7 kGy max, 3.0kGy max, 8.0kGy max, and 5.5 kGy max, respectively (FDA, 2007). Guidelines are set up to produce the maximum effectives for the intended purpose and maintain product integrity.

Radiations characteristics that prevent spoilage, kill pathogens, extend shelf life and inhibit spoilage, however irradiation is accompanied by disadvantages such as production deterioration, vitamin loss and rancidity. Product deterioration can occur if the dose is too high. The same gamma waves that mutate DNA also have the ability to lethally injury the cell wall or cause lysis, however this is the most extreme cases. Much in the same way as to much sun can cause a sun burn and damage the epidermal cells of you skin. In a more common concern has to do with vitamin loss where doses as low as 1 kGy can reduce vitamin content slightly, especially with vitamins A, B1, E and K (Loaharanu, 2003). Doses between 1-10 kGy will cause a medium loss and high doses >10 kGy will cause a major loss in vitamins with the exception of vitamin D, riboflavin and niacin (Loaharanu, 2003). In addition irradiation will leave the major components like your fats, proteins and carbohydrates minimally altered (Loaharanu, 2003). Rancidity is often another major problem and side effect in some irradiated foods. Products should be irradiated in a frozen state or as cool as possible to eliminate free radical and oxygen species that

may be made available for oxidation and peroxide formation (Jay et al., 2005). James Jay also notes in his book Modern Food Microbiology that a lack of oxygen will actually enhance a microorganisms ability to resist radiation. Free water can also aid in the creation of peroxide which is another reason to keep samples to be irradiated in a frozen state. The more fat there is, the more of an opportunity there is for oxidation and similarly with the higher dose comes higher oxidation potential.

Destruction of microorganisms specifically led to the development of radappertization, radicidation and radurization to define certain doses. Radappertization is the same concept as "commercial sterility," in such industry's such as canning. Radicidation can be compared to pasteurization in milk where the main goal is to reduce the number of viable, but not spore forming pathogens and virus. Radurization refers to just pasteurization in general sense in order to extend shelf life and reduce spoilage organisms (Jay et al., 2005). D-value refers to the dose of radiation to achieve a one log reduction of organisms. So in order to achieve a 4 log reduction for *Salmonella* you would simply take D-value (0.45 kGy – 0.60 kGy) times four. Gram (-) organisms are typically less resistant than Gram (+) with cocci being the most resistant while the exact science behind it is not exactly understood (Jay et al., 2005).

Phenolics in Food

Introduction

Phenolics have been used in food traditionally as antioxidants and more recently as antimicrobials. Phenolics are structural OH functional groups attached to a benzene ring. They are naturally found in their esterified or glycosidic form (Raccach, 1984). Antioxidants are not classified as antimicrobials as there are separate mechanisms of action. Phenolics used in the food industry play a valuable role now and could continue to be developed to play a crucial role in the future of food protection.

Two major categories of phenolics are hydrobenzoic acids and hydrocinnamic acids (Figure 1). Hydrobenzoic acids consist of a benzene ring with three available attachment sites and a carboxylic acid. These structures are commonly found in their bound form and make up the structure of tannins. Tannins can be grouped into either hydrolysable or non-hydrolysable (condensed) structures. The hydrolysable groups contain a D-glucose molecule at the core, with

gallic acid, gallotannins and ellagitannins being the main structures (Shahidi and Naczk, 1995). Non-hydrolysable groups are known for proanthocyanidins which have been linked to decreased heart disease and lower incidence of artherosclerosis (Donovan et al., 1998; Lee et al., 2007). Benzoic acids and its derivatives are typically found in lower concentrations; however, the gallic acids and its derivatives are the most abundant in the benzoic series and can be found in fruits. The most common derivatives of the series include: p-hydroxybenzoic acid, protocatechuic acid, vanillic acid, syringic acid, and gallic acid (Shahidi and Naczk, 1995).

Hydroxycinnamic acids are the second major series of phenols. Major derivatives of the hydroxycinnamic's are p-coumaric acid, caffeic acid, ferulic acid, sinapic, and chlorogenic acids. Vynl-substituted phenols are formed through the decarboxylation of the hydroxycinnamic and are approved in food use. Caffeic acid makes up 75% of the derivatives present in stone fruits with p-coumaric being the most identifiable in citrus fruits. Similar to the benzoic series, cinnamic's are typically found in the bound form, but can be released with through processing (Shahidi and Naczk, 1995). Although hydroxycinnamic acids and the benzoic acid series are the most common phenolic groups, they are not all inclusive.

Two other major phenolic groups commonly found in foods are coumarins and flavanoids (Figures 2 and 3). Coumarins are lactones or from the cis-O-hydroxycinnamic acids that are present in foods in a free form or as a glycoside and can be isolated in the highest concentrations from bark and leaves (Shahidi and Naczk, 1995). Currently, coumarins are banned for use as additives in human foods (CFR, 2008). Flavanoids are present as glycosides in food systems. Quercetin is a derivative classified which is classified as a flavanol. Quercetin is a flavonal that is linked causing discoloration of fruits and vegetables. Catechins and chlorogenic acids are known to speed up the browning oxidation action of Quercetin. Many of these phenols are not approved addition into foods when most are found naturally in fresh products (Shahidi and Naczk, 1995). Currently, phenols that are approved for use include: Rutin, vanillic acid and Tannic acid, subsequently gallic acid an easily oxidized derivative from tannic acid (CFR, 2008).

(a)

R_1 — (benzene ring) — COOH, with R_2 and R_3 substituents
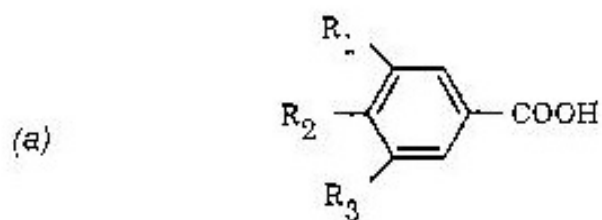
Acid	R_1	R_2	R_3
p-Hydroxybenzoic	H	OH	H
Protocatechuic	H	OH	OH
Vanillic	CH_3O	OH	H
Syringic	CH_3O	OH	CH_3O
Gallic	OH	OH	OH

(b)

R_1, R_2, R_3 — (benzene ring) — CH=CH—COOH
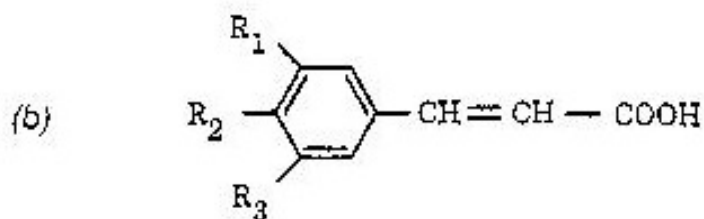
Acid	R_1	R_2	R_3
p-Coumaric	H	OH	H
Caffeic	H	OH	OH
Ferulic	CH_3O	OH	H
Sinapic	CH_3O	OH	CH_3O

Figure 1 A) Benozoic Acids   B) Hydroxycinammic Acid

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(a)

Coumarin	$R_1 = R_2 = H$
Umbelliferone	$R_1 = OH$, $R_2 = H$
Aesculetin	$R_1 = R_2 = OH$
Scopoletin	$R_1 = OH$, $R_2 = OCH_3$

(b)

Psoralen	$R_1 = R_2 = H$
Xanthoxin	$R_1 = H$, $R_2 = OCH_3$
Bergapten	$R_1 = OCH_3$, $R_2 = H$
Bergaptol	$R_1 = OH$, $R_2 = H$
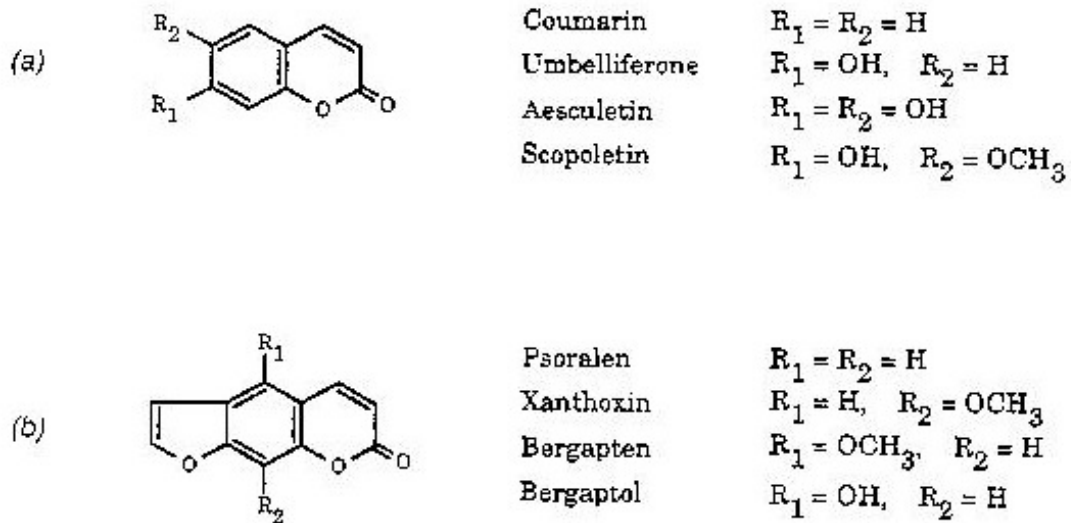
Figure 2 Basic Coumarin Structures
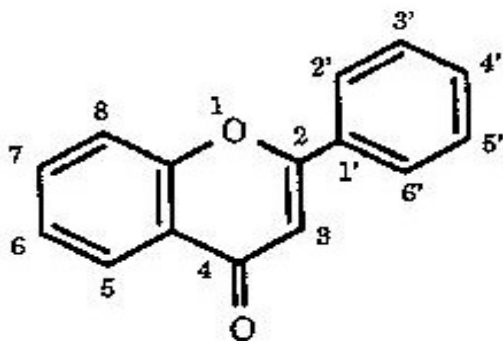
Figure 3 Basic Flavonoid Structure

Phenolic Antioxidants

"Antioxidants are compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions" (Karou et al., 2005). Gailani and Fung (1984) further define antioxidants in foods as any substance that is added to fats to retard oxidation and prolong wholesomeness and stability. An antioxidant should be able to stop oxidation; however it must also keep food quality and wholesomeness in mind. Antioxidants are introduced to a food system to react with the free radicals before they react naturally with the fats in the food matrix causing rancidity. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) propyl gallate (PG) and tertiary butylhydroquinone (TBHQ) are commonly used in foods to prevent autoxidation (Gailani and Fung, 1984; Lindsay, 1996). Fung et al. (1985) state that for an antioxidant to be ideal, seven qualities need to be met:

1) Must have no harmful physiological effects
2) Must have no contribution to off flavors, odor, and color to food
3) Must be effective in small concentrations
4) Must be fat soluble
5) Must last through processing to provide protection to food
6) Must be readily available
7) Must be cost efficient

Antioxidants work by interrupting the oxidation chain reaction during the initiation and propagation stage. The oxidation chain reaction occurs in three stages (Fung et al., 1985). Propagation is the first stage where the formation of the free radicals occur and di-radicals are formed from the Hydrogen and R groups. The second stage is propagation and is where the free radicals react with oxygen molecules to form peroxides, peroxy radicals, and additional hydrocarbon radicals. Lastly, the chain does not stop until two radicals react (termination) and are returned to normal state (Fung et al., 1985). Antioxidants help mediate and stop the reaction as they readily accept the free radicals produced without contributing to the chain reactions, thereby alleviating the formation of more uncontrolled free radicals. Antioxidants can be

powerful tools in the food industry and for that reason they are under the strict oversight of the Food and Drug Administration.

United States Food and Drug Administration, has oversight over the food industry with the exception of meat, poultry and egg products, which belong in the jurisdiction of the United States Department of Agriculture (USDA). The Code of Federal Regulations (CFR) states that BHA, BHT and TBHQ must have limited use in food products. Ranges for BHA and BHT include, as low as 2 ppm are allowed in beverages up to 1000 ppm in active dry yeasts (Administration, 2008). BHA, BHT and TBHQ are regulated to a maximum of 0.02% in oil, emulsions and essential oils to prevent rancidity. Alkyl gallates are another phenolic approved in food. Fung et al. (1985) reported many studies on the effects of BHT, BHA, TBHQ and PG in extreme, high and low levels, which were confirmed through legislation to be safe in the average diet. Legal limits already in place do not allow for the destruction of bacteria and will require combinations with other mechanisms to achieve effectiveness as well as comply with legislation (Fung et al., 1985; Raccach, 1984). Effects of these phenolics are generally recognized as safe and their effects on bacteria *Staphylococcus aureus, Salmonella, Clostridium, Pseudomonas* and *Escherichia coli* seemed to be thoroughly discussed and studied (Fung et al., 1985).

Phenolic Antimicrobials

Antimicrobials are any substance that influences a microorganism in a negative way. Antimicrobials can be classified in many ways such as bactericidal, bacteriostatic, antiseptics, disenfectants, and sanitizers. These can range from quaternary ammonia compounds, iodophores, acid washes, and more recently phenolics (Fung et al., 1985; Jeon and Schmidt, 2008). With antimicrobial resistance on the rise, 70% of bacteria are found resistant to at least one drug (National, 2006). "Natural products that are a source of antimicrobials are penicillins, tetracyclines and glycopeptides" (Silver and Bostian, 1990). Phenolic flavonoids are being called on for heavy development that are able to work on new active sites not able to be reached with conventional antibiotics (Kimberlin and Whitley, 1996). Thus, alternative ways to control bacteria are on the rise and other options that were not a high priority are getting a second look, such as phenolic compounds.

Phenolic compounds used as antimicrobials are not a new idea. Plants have used phenolics as a natural defense mechanism for thousands of years. Phenolic antimicrobial

effectiveness in food systems can be linked to the use of antioxidants which dates back to the 1950's (Fung et al., 1985; Nickerson and Starr, 1960). Phenolic antioxidants as antimicrobials are particularly good to study because of their previous record of use in food and vast amounts of research available. In addition, phenolic antioxidants are soluble in non-polar solvents like ethanol or propylene glycol. Due to their relatively non-polar nature, it is necessary to use alternative solvents because water is polar and thud not an effective solvent. The more hydroxyl groups there are on the phenol the more non-polar or less soluble they become. One hydroxyl group present allows for about 50% solubility, however if more than one hydroxyl group is present the solubility decreases to 1-10% (Raccach, 1984). Much of the current research known about phenolic mechanisms of action exist from the use and study of antioxidants.

Many factors should be considered when determining the antimicrobial activity of phenolic antioxidants such as: species/strains of bacteria, physical stress, concentration and phenolic type, microbial load, synergistic effects of phenols with other antimicrobials, temperature and food additive combination, the food matrix and the phenolic carriers, or the method of addition (Raccach, 1984). Gram positive species have been shown to be more susceptible than Gram negative species when treated with phenolic antioxidants. One study shows the Minimum Inhibitory Concentration (MIC) for Gram positive to be 125-1000 µg/ml concentration in contrast to Gram negative where MIC's maintained levels from 2000-5000 µg/ml (Kabara, 1980). Raccach (1984) is quick to point out the inconsistencies in this paper that neglect to take into account of the strains being used which are highly variable in susceptibility, regardless of Gram reaction. *Staphyloccus aureus* LD_{90} is in the range of 290-300 µg/ml; however when extremes are shown two strains are as low as 100 µg/ml (Post, 1982). Post (1982) found similar variation in *Pseudomonas fragi* and *P. fluorescens,* from 347 µg/ml to 117 µg/ml, respectively. Studies by Gailani (1981) and Racaccach (1982) conclude similar results as Post.

Stress may have a significant effect on how microorganisms are affected by phenolic antioxidants. Stress such as heat or cold, which are beyond the optimum growth range and into the extremes may significantly alter their susceptibility to phenolic effectiveness. Heat stress in combination with TBHQ exhibited an increased effectiveness against bacteria (Ray, 1979). Other studies confirm similar results with *Aspergillus flavus* (Beuchat and Jones, 1978) and decreased water availability with *Saccharomyces cerevisiae* (Eubanks and Beuchat, 1982).

Stress to the bacteria may affect the membrane allowing a synergistic effect that would otherwise not be present with out the stress.

The type of antioxidant, in addition to the concentration being used will result in varying levels of antimicrobial effectiveness. Antioxidants have varying degrees of steric hindrance which can impact their antimicrobial effectiveness. However, even with steric hindrance an increase in concentration of the phenolic antioxidant will result in a higher mortality rate in bacterial cells (Raccach, 1984). A study with BHA and BHT (Increased hinderance) found that BHA could be as effective at 200 ppm as BHT at 500 ppm (Trelease and Tompkin, 1976).

Synergism can also occur in non-stressed systems as the phenolics work on different active sites around the bacterial membrane. Increased effectiveness was concluded when BHA was added in combination to TBHQ at 100 ppm than by themselves at 100 ppm against aflatoxin growth (Lin and Fung, 1983). Similarly, when BHA and BHT are combined if BHA is reduced a much higher concentration is needed to achieve the same inhibition during glucose fermentation of *Pseudomonas pentosaceus*. When compared to rancidity of BHT, an addition of BHA that does not exceed that of the removed BHT is required to achieve the same effect. However, the inhibition was higher than BHA alone and lower than BHT used alone (Raccach, 1980).

Adding an antioxidant alone in a system does not act upon all organisms the same. The higher the bacterial concentration the lower the antimicrobial effectiveness (Raccach and Henningsen, 1982). Raccach (1982) examined the need for an additional 5 µg/ml increase in MIC for a one log climb from $10^5 - 10^6$. Results do not go on to discuss the possibility of exponential MIC increase for 1 log growth where for every 1 log growth there is an equal 5 µg/ml increase.

Concentration will have no effect if there is not a method of addition in place that allows phenolic absorption. Currently, there are three main methods of phenolic addition. The three methods of phenolic addition are direct addition, dipping, and spraying. Raccach (1984) reported that there were no real noted differences in these methods, however there was one concern with the spray method. Spraying does not allow for the control of phenolic aggregation, which does not provide even surface concentration. During aggregation the phenolic is allowed to concentrate which may pass legal statutes. Carriers are used to assure dispersion of phenolic antioxidant in the food matrices. Citric acid, propylene glycol, sorbitan and troleate are common

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carriers, with the latter three acting specifically with the fatty portion of matrix, thus decreasing effectiveness (Raccach, 1984).

In order to comply with some of the current legislation, phenolics are used in combination with other additives that allow for lower concentrations of phenolic used to be effective. Lowering the temperature plus phenolic addition is one way to increase effectiveness of the phenol. Citric acid was shown to decrease phenolic antioxidant addition while increasing effectiveness in fermentative sausages (Raccach, 1980). Lower temperatures have been shown to increase the hydrophobicity of the cell membrane which allows for easier phenolic penetration and action against the cell membranes (Singer and Wan, 1977). Raccach (1984) showed that a few cases resulted in higher effectiveness than would be needed alone, which may allow for lower preservative use. In a NaCl preservative system, increased effectiveness was shown over the control, but not enough to create an additive effect (Raccach, 1984). In addition most phenolic extracts are not soluble in water so it is necessary to use a solvent. Solvents such as dimethyl sulfoxide (DMSO), methanol, ethanol and pyridine are all used; however, it is important to know their regulation as well. All of these substances can be toxic to bacteria as well as human so care must be taken for their use. DMSO and ethanol were shown to have effects on bacteria at 16%, but used in moderation 2-4%, show no statistical evidence of injuring bacteria (Muthuswamy and Rupasinghe, 2007).

Another class of phenols, flavanoids, which are well known from the extraction from natural sources, provides a rich source for antimicrobial activity. Flavanoids contain compounds such as quercetin and rutin (Flavanones), catechin, epicatechin gallate and epigallocatechin (Flavon-3-ols); however, the toxicity must be further researched (Tsuchiya et al., 1996). Many research groups have selectively identified flavanoids that possess antimicrobial ability including: epigallocatechin gallate and its derivatives, quercetin, and derivatives, chalcones, kaempferol and derivatives, galangin, pinocembrin, ponciretin, genkwanin, and naragin are just a few (Cushnie and Lamb, 2005c). In guinea pigs oral capsules were shown effective against *Shigella* (Vijaya and Ananthan, 1996). To build on this research positive results were shown through injection and oral prophylaxis in pigs with these phenolic compounds, as well (Cushnie and Lamb, 2005a).

Mechanisms of Action

Free radical chain reaction is fairly well understood and accepted, however the mechanism of antimicrobial action in phenolics and phenolic antioxidants is not as clear. Many theories exist with some being more accurately documented, thus leading to some being more acceptable than others. Theories in place for microbial destruction include: 1) imitation with loss of biological activity; 2) enzyme disruption; and 3) membrane disruption, which is the most documented. Decreased biological activity can occur when phenolic substances imitate substances used for a vital role in the bacterial cell. When the cell incorporates the mock molecule for the intended structure, it renders the molecule biologically dead. BHA is an analogue of one of the five ring structures in Aflatoxin. Butylated hydroxyanisole can be incorporated in place of existing structures or during synthesis, which will inhibit aflatoxin synthesis (Fung et al., 1977).

Another mechanism is through enzyme disruption causing loss of organ function, lipid inhibition or protein (including DNA, RNA) inhibition. "BHT in beef sperm reduced the oxidative decarboxylation of pyruvate before entering the TCA cycle, lowering O_2 consumption (Hammerstedt et al., 1976). Carbon incorporation in lipids and protein amino acids was decreased in *Tetrahymena pyriformis* (Surak, 1977; Surak et al., 1976b). In another study by Raccach et al. (1982), the ability of phenolic antioxidants was found to inhibit DNase in *Staphylococcus* species. "Inhibition of DNA and RNA protein synthesis suggest the interaction with genetic material and the interaction with the enzymes is responsible for inhibiting their synthesis (Raccach and Henningsen, 1982). Butylated hydroxyanisole and TBHQ inhibited the synthesis of DNA/RNA in *T. pyriformis* or exhibited nucleid acid synthesis inhibitors (Metcalfe, 1971). Flavanoids have shown the ability to disrupt the energy chain (ETC, Electron Transport Chain) of bacterial cell by preventing oxygen consumption for Gram positive, not Gram negative bacteria. NADH-cytochrome C reductase was inhibited, but not cytochrom C oxidase or NADH CoA reductase, thus disrupting ETC (Haraguchi et al., 1998). A gradient is essential for Adenosine Triphosphate synthesis, transport and motility, therefore gradient disruption often results in cell running out of energy and are unable to complete energy synthesis reactions (Cushnie and Lamb, 2005b).

Membrane disruption at the membrane-water interface seems to be the most widely supported theory. General theory behind the membrane disruption is the non-polar hydroxyl

molecules interact with the phospholipid bilayer in the membrane, and the more polar end interacts with the water. According to Singer et al. (1977) to interact with the membrane the phenol must contain at least one hydroxyl group to be lipid soluble. In addition this will allow for membrane attachment and disruption from normal organization. "The reactive portion of a phenolic antimicrobial is directly related to the free OH group" (Prindle and Wright, 1977). Steric hindrance also seems to play a major role in lower membrane-water interface reaction in effect creating a loss antimicrobial activity. One example for this uses 2,6 ditertiary butyl phenol which has an MIC of 250 ppm. Alternatively non-hindered 2,5 ditertiary butyl phenol yielded an MIC of 15.6 ppm (Kabara, 1981). If not for steric hindrance BHA and BHT should be equally effective (Singer and Wan, 1977). Butylated Hydroxyanisole has a single tertiary structure, where BHT contains two tertiary structures; BHA is therefore more effective than BHT. Less steric hindrance allows for a stronger membrane-water interface interaction, which is responsible for BHA and other phenolic enhanced antimicrobial activity (Cushnie and Lamb, 2005b; Kabara, 1980). Antimicrobial ability to prevent fermentation or disrupt membrane function in bacteria can be expressed by TBHQ>BHT>BHA>PG (Raccach, 1980).

Membrane disruption through the membrane-water interface is thought to cause death by intracellular leakage. Butylated hydroxyanisole was shown to cause cellular leakage by Ultra Violet light (260 nm) detection with as little as 100 µg/ml (Degre et al., 1983). Similar results were found in *Pseudomonas* and *T. pyriformis* with BHA/BHT's ability to cause cellular leakage (Davidson and Branen, 1980; Singer and Wan, 1977; Surak et al., 1976a). Another cause of intercellular leakage may be caused by lipid alterations in the membrane. Davidson and others (1980) examined PG in *Pseudomonas* and found that it lowered phosphatidyl ethanolamine. This can disrupt membrane function and order, possibly causing cellular leakage or autolysis due to reduced ability to resist osmotic pressure (Cushnie and Lamb, 2005c; Davidson and Branen, 1980; Sato et al., 1997). In an effort to adjust and correct to phospholipid disruption, fatty acid ratio's of unsaturated fats versus saturated fats showed an increase in the ratio with *Pseudomonas fragi* and a decrease in the ratio for *Pseudomonas fluorescens* (Davidson and Branen, 1980). Epigallocatechin was shown to cause cellular leakage through membrane fusion or catechin penetration of the Gram positive cell membrane (Ikigai et al., 1993). Electron microscopy has confirmed membrane confirmation changes exhibiting pseudomulticellular aggregates. "As

mechanisms are understood different analogues can be created to develop a more potent natural antimicrobial," concerning phenolic flavanoids (Cushnie and Lamb, 2005c).

Fruit derived Antimicrobial Potential

Introduction

Production of safe and natural foods is of major concern to consumers and the food industry, fruit phenolics can help ensure microbial hazards are controlled as naturally as possible. Typical fruit phenolics consist of flavanoids as a major phenolic constituent in addition to various other polyphenols. Polyphenols are secondary metabolites produced in higher plants for plant defense, odor, flavor and pigmentation. These also provide very strong evidence of antimicrobial characteristics. Natural reserves of polyphenols are found in all fruits such as apples which include catechin and chlorogenic acid (Muthuswamy and Rupasinghe, 2007). Puupponen-Pimia et al., (2001) have proposed that fruit phenols are not inhibitory to Gram negative, but only to Gram positive. Natural fruit polyphenols such as gallic acid and caffeic acid have been shown to mimic the amino acid proline. Phenols are substituted in proline dehydrogenase, thus disrupting the ETC (Kwon et al., 2007). Fruits effective against bacteria include lemon balm, cranberry, plums, bergamot peel, cloudberry, and raspberry (Kwon et al., 2007; Mandalari et al., 2007; Puupponen-Pimia et al., 2005; Vattem, 2003). Vattem et al. (2003) enhanced phenolic (gallic acid) concentration in cranberry pomace by *Rhizopus oligosporus* fermentation. Puupponen et al. (2005) showed cloudberry (*Rubis triflorus)* and raspberry (*Rubis chamaemorus)* to be effective inhibitors of *Staphylococcus* and *Salmonella* which contrast earlier studies showing Gram negatives may not be inhibited. Synergism has been seen when 25% cranberry was added to 75% oregano mixture which optimized bactericidal effects on *Listeria monocytogenes* (Lin et al., 2004). Fruit phenolics were shown cause no inhibitory effects of probiotic bacterium such as *Lactobacillus rhamnosus,* hinting little threat towards colon intestinal microflora (Muthuswamy and Rupasinghe, 2007). Fruit phenolics present a solution to an industrial problem that provides an antimicrobial effect as well as erasing concerns of synthetics or chemical alternatives being used (Muthuswamy and Rupasinghe, 2007).

Plum (*Prunus domestica*) Phenolic Potential

Introduction

Total dried plum production in the northern hemisphere (United States and France) for one marketing year (MY) was estimated at 192,955 metric tons in 2002-2003. This was estimated to increase to 206, 852 metric tons for the MY 2003-2004, with global production estimated at 238,850 metric tons. Including the United States, France and Chile, accounts for ninety percent of world production typically for export grossing close to 220 million dollars, with the top importers being Japan, Germany and the UK (Atlas, 2004). The California Agricultural Statistics Service estimates the five year average production in the U.S. to be around 137,748 metric tons with the 2008 crop coming in around 120,000 metric tons. This is up from the unusually low 2007 crop of 80,000 metric tons (National Agriculture Statistics Service, 2008). The dried plum industry in the U.S. is best represented by the California Dried Plum Board (CDPB). The CDPB promotes products such as pitted dried plums, whole dried plums, prune juice, fresh plum juice and fresh prune juice with a wide range of health benefits.

The amounts and types of food additives introduced to food vary widely and having an additive that could serve dual function such as phenolic antioxidants, spices, flavorings, phosphates and lactates would eliminate the need for costly toxicological studies to ensure the safety of the food (Raccach, 1984; Thompson, 2001). Thompson (2001) showed similar results in a sensory study conducted at Texas A&M University showed no off-flavors in ground beef patties up to 5% and actually improved moistness upon reheating of the hamburger by acting as a humectants. Furthermore, results showed antimicrobial effectiveness increased with increasing concentration of added dried plum puree. Optimum effectiveness for functionality and microbial destruction was between three to six percent addition of dried plum puree (Thompson, 2001).

Plum phenolics are essentially the same between specie; however, they vary in amounts depending on the variety. Specie of plum can span a range of total phenolic concentration from 685.5 µg/ml to 173.9 µg/ml (Chun et al., 2003; Kim et al., 2003). In all studies on phenolic composition the variety Belteville Elite showed the highest phenolic concentration of any other plum variety follow by Cacak Best, Long John and Empress (Chun et al., 2003; Kim et al., 2003). Phenolic composition averaged 2385 µg/ml on the skin where the fruit would naturally need the most protection, versus the flesh with 430 µg/ml (Cevallos-Casals et al., 2006). Most

studies agreed showing neo-chlorogenic acid as the main phenol, followed by chlorogenic, rutin, catechin and trace amounts of caffeic (Donovan et al., 1998). Additional studies confirmed the presence of rutin, ferulic, and p-coumaric acid, along with caffeic in higher concentrations that exhibited by Donovan et al (1998), possibly due to variation in specie (Lombardi-Boccia et al., 2004; Tomaslorente et al., 1992).

Persimmon (*Diospyros kaki)* Phenolic Potential

Introduction

Persimmon fruits (*Diospyros kaki)* are usually a yellow-orange to orangish-red color and are typically about the size of a common tomato. R.J. Collins et al. (1998) reported an annual production of 4,000 metric tons in the US, 287,000 metric tons in Japan, and over 567,750 metric tons in China. The Food and Agricultural Organization (2008) recently estimated world production to be as high as 2.5 million metric tons in 2005 for the Japanese persimmon *Diospyros kaki*. In addition, China recorded 1,837,000 metric tons, Korea produced 250,000 metric tons, Japan produced 230,000 metric tons, and the United States produced 4,000 metric tons (FAO, 2008; Growers, 1996). Moreover, according to Agricultural and Food Agency (AFA) of Taiwan showed persimmons can range in size from 120 g to 250 g depending on the variety. One study reported sizes ranging from 46 to 301 g and diameters of 42 to 91 mm as an overall average of many varieties (Celik, 2007). Persimmon fruit is not as commonly consumed in the United States but its importance oversees can be seen in their overall production versus the United States.

Persimmons are typically eaten in the raw, in a pureed food and added as an ingredient to food in many ways. Some typical uses include persimmon salsa, dried and sliced, or as a jerky type product known as fruit leather. Persimmons can be incorporated into many products just like a tomato or any dried fruit. In research conducted by Celik and Ercisli (2007) water content at time of harvest for the Hachiya persimmon was 80.94% with dimensions of 63.85, 69.20, and 64.96 mm for the length, width and thickness respectively. According to Rahman et al. (2002) from a nutritional point of view Diospyros kaki or the Japanese persimmon is the most valuable or healthful. Nutritional properties include 0.60 g protein, 5.40 pH, 27 mg phosphorus, 203 mg potassium, 16 mg calcium, 11 mg magnesium, 10 mg sodium, 12.00 mg/100 g Vitamin C and 3.15 mg/100 g of tannins (Celik, 2007). Persimmon puree has a wide variety of vitamins and

minerals, but also has other compounds that have been linked with beneficial properties such as phenolic compounds.

Tannins hold a particular interest when looking at the physical make-up of persimmons and persimmon phenolics because of the many roles they play. Tannins are used as a measure of astringency in persimmon fruits or the bitterness which in nature can act as a retardant or a self defense mechanism. Tannins are a type of polyphenols derived from plant sources. Factors that affect astringency are persimmon variety, stage of maturity, packaging and commercial processing, all of which correspond to tannin level. Due to the utilization of persimmon in the raw form, processing to reduce astringency is on the forefront. However, deastringency treatments tend to lower total phenolic content, specifically the phenolic tannins which are responsible for the astringency factor to begin with. Some studies showed that with a nitrogen treatments in Modified Atmosphere Packaging and storage atmosphere, decreased total phenolics to 0.5% to 0.2% formula weight (FW), from 2% to 1% FW, respectively (Bibi et al., 2007). Satter et al. (1992) reported a linear relationship with significant loss of phenolics when compared to maturity time. Salvador et al. (2007) reported a significant ($p=0.05$) decrease in tannin concentration from .78% to .36% FW over maturity time and down to .01-.02% FW for carbon dioxide treated persimmon. In addition, fruits in experimental conditions show tannin reduction during ripening due to decreased polymerization; however, shelf life was enhanced (Chaudry et al., 1998).

Persimmons (Diospyros Kaki Thunb.) are traditionally grown in China, but since have migrated to Korea, Japan and eventually the US around the 1800's (Growers, 1996). The most common types of persimmons are the Hachiya (More astringent) and the Fuyu (Less astringent) persimmon. Astringent varieties include the Hachiya, Eureka, Honan Red, Tamopan, Tanenashi, Triumph, and Saijo. Non-astringent varieties include Fuyu, Gosho/Giant Fuyu, Imoto, Izo, Jiro, Maekawajiro, Okugosho, and Suruga (Growers, 1996). Further cross-pollinations may produce hybrid fruits that are seedless, but may lean towards the astringent side (Growers, 1996). Phenolic composition of the persimmon exhibited the presence of gallic acid, catechin, epi-gallocatechin, epicatechin, chlorogenic acid and caffeic acid (Chen et al., 2008; Gorinstein et al., 1994). Trace amounts of the phenolics ferulic and vanillic acid were also found (Gorinstein et al., 1994).

These strong bitter compounds are now being recognized for more than just their bitter taste as they are being looked at for heart health and as a potential antimicrobial. The peel of the persimmon contains proanthocyanidins which are important polyphenols that have been linked to improved heart health especially in red wines. Lee et al. (2007) found that when rats were fed the proanthocyanidins from the persimmon peel it reduced the oxidative stress induced by diabetes by reducing serum glucose levels. Moreover, powdered persimmon leaves were added to a high fat rations being fed to rats and showed a significantly lowered plasma total cholesterol and triglyceride concentrations with elevated ratio of HDL-C/total-C and an improved artherogenic index (Lee et al., 2007). The persimmons' health effects were exhibited in scientific studies in rats and are being linked to beneficial health effects for humans. Phenolics were shown to act as an effective antimicrobial in many situations and persimmons phenolics may have the potential to yield the same effect.

Previous Persimmon Research

Little research using persimmon phenolics as antimicrobials exist, especially in the U.S. where production is very limited comparatively to the rest of the world. Research has also been conducted showing heart healthy benefits in rats and persimmon tannin from the leaves (Chun et al., 2003). There also exist extreme phenolic variation when comparing astringent with non-astringent varieties (Celik, 2007). Persimmon tannin, tannic acid and epi-gallocatechin show MIC's with *Streptococcus mutans* of 62.5, 15 and 62.5 µg/ml, respectively. Results with *Streptococcus sobrinus* were similar having 125, 31.3 and 1000 µg/ml MIC's, respectively (Yoshioka et al., 2005). Due to the limited knowledge available in this area concerning persimmons, much research still needs to be conducted to determine its contribution to the world of antimicrobials.

CHAPTER 2 - **The Antimicrobial Effects of Persimmon Puree on Bioball^{TM}** *Listeria monocytogenes* **and Bioball^{TM}** *Escherichia coli* **O157 in Brain Heart Infusion Broth.**

Introduction

One event that focused the national spotlight on food safety was the Jack-In-The-Box incident in 1993 where four children died and over 700 persons were infected from eating hamburgers that were contaminated with a strain of *Escherichia coli* O157:H7 (FDA, 2001). Since the Jack-In-The-Box occurrence many companies have been trying to improve their pathogen detection methods. In addition to detection, many companies are taking a preventive approach by initiating programs such as HACCP. Developed for NASA by the Pillsbury Company and mandated by the FDA for seafood and then juice products, is being adopted by the meat industry as well (FDA, 2001).

Consumers are concerned with the methods that the industries are using for preventing contamination and how it may affect their health or environments. Consumers are interested in preventative organics or more natural products because of their perceived better environmental and health effects. Thompson (2001) reported that plum concentrate was effective at concentrations from 1-10% at reducing microbial populations in liquid medium, ground sausage and ground beef. In addition, an adjunct study was conducted at Texas A & M showing the plum puree had no unacceptable off flavors and made the hamburger patties more moist upon reheating (Thompson, 2001). Similar to the plum puree, persimmon fruit could be a solution that both consumers and the industry that are looking for alternative methods to ensure food safety.

The objective of this study was to determine the effects of persimmon puree on Bioball^{TM} *Listeria monocytogenes* and Bioball^{TM} *Escherichia coli* O157. We hypothesized the if persimmon puree was added, then an antimicrobial effect would be seen. The null hypothesis would state that there will be no differences in microbial growth when persimmon puree is added ($H_{o:}$ $\mu 1 = \mu 2 = \mu 3 = \mu 4$) (p=0.05).

Materials and Methods

Pathogen Tested

Listeria monocytogenes (NCTC) 11944

Escherichia coli O157   (NCTC) 12900

Bioball

BioballTM (bioMerieux. Hazelwood, MO) Listeria monocytogenes (NCTC 11944) and BioballTM (bioMerieux. Hazelwood, MO) Escherichia coli O157 (NCTC 12900) were the cultures used for this experiment. Each Single Shot BioballTM contained 30 ± 2 CFU which allowed for close monitoring of microbial inhibition. The BioballTM provides unique opportunity to know the exact quantity of organism or CFU's being used. BioballTM was used for this experiment to know the exact CFU in each concentration of persimmon puree, which eliminates some of the random variation with inoculation. Samples could then be monitored and analyzed from the same starting point.

Sample Preparation and Inoculation

Persimmon puree (LaVinge Organics, Riverside, CA) arrived in 2 lb hermetically sealed, frozen and zip locked bags. Persimmon puree, 0, 1, 3, 5 and 10 g, was thawed and aseptically added to sterile stomacher bags containing sterile Brain Heart Infusion (BHI, Difco, Detroit, MI) broth with 100, 99, 97, 95 and 90 ml, respectively. Samples were then stomached (Stomacher Mix 1 Lab Blender, Microbiology International, Frederick Maryland) for 1 min to evenly homogenize the sample. Two sets of persimmon and BHI mixtures were made at each concentration. One Bioball^{TM} (bioMerieux. Hazelwood, MO) for L. *monocytogenes* or *E. coli* O157 was aseptically added to each stomacher bag for each concentration of persimmon puree. Samples in stomacher bags were then placed in incubators at $37^{o}C$ for 24 h for *E. coli* O157 and 48 hrs for *L. monocytogenes*. This process was considered one replication and was repeated three times for replication.

Enumeration and Isolation

Escherichia coli O157 samples were plated on MacConkey Sorbitol Agar (MSA, Difco, Detroit, MI) and *L. monocytogenes* on Modified Oxford Medium (MOX, Difco, Detroit, MI). At 0, 6, 12, 24, 36, 48, and 72 h of incubation at 37˚C, samples were taken for bacterial enumeration. Samples were gently and aseptically hand-massaged for 1 minute to homogenize the bacteria before sampling. Colonies were isolated at 37˚C using a 1:10 dilution of the sample in 0.1% Peptone Water, with 1 ml of the sample being diluted in 9 ml of 0.1% peptone water. Samples were spread plated in duplicate with 0.1 ml of the sample.

Statistical Analysis

All data presented are averages of the three replications. Analysis was performed using the MIXED procedure of SAS version 9.1 and finding the Least Squared Means. Logarithmic transformation of counts was conducted for statistical analysis. All significance values were determine by 95% confidence or a ά = 0.05.

Results and Discussion

The effects of persimmon puree concentration in BHI liquid medium over time on Bioball[TM] *L. monocytogenes* and Bioball[TM] *E. coli* O157 and other Gram-negatives are summarized in Tables 1-2 and Figures 4-5. Total Bioball[TM] *E. coli* O157 counts were combined with total Gram-negatives because background microflora were interfering with proper Bioball[TM] *E. coli* O157 identification on MacConkey Sorbitol Agar. Total background microflora from a randomized three repetition sample resulted in 1.80 CFU/ml total aerobic counts. Organisms were isolated and identified in persimmon puree included: *Moellerella wisconsensis* (Enterotube II), *Pasteurella pneumotropica* (API 20E), *Pantoea spp.* (API 20E), and *Leuconostoc mesenteroides* (BBL Pos).

Significant fixed effects were found for concentration and time (p=0.05). However, there was a significant interaction between concentration and time, thus interactions are the only results being reported (p=0.05).

At time 0 h, no significant differences were seen between persimmon puree concentrations (1, 3, 5, and 10) and the control for *L. monocytogenes* (Figure 4). There was a significant increase in *E. coli* O157 growth at 5% persimmon puree concentration compared to

the control (Figure 5). However, there was no significant difference between the 5% concentration and other concentrations tested. It is unlikely that this difference was due to any experimental factors. Most likely, this could be caused by the unintended high amounts of the background microflora.

At 6 h, there were no significant differences for *L. monocytogenes* growth between any concentrations of persimmon puree when compared to the control. There was a significant increase in growth of Gram-negatives and *E. coli* O157 at persimmon concentrations 5 and 10% when compared to the control. This was more likely due to the growth of the non-pathogenic background microflora. However, this is only an assumption as *E. coli* O157 could not be independently enumerated. This is also unlikely an effect of treatments because six hours does not provide substantial time for growth.

At time 12 h, there were no significant differences for *L. monocytogenes*, Gram-negatives, or *E.* coli O157 growth at any concentration of persimmon puree when compared to the control. A sample that was not completely homogeneously mixed may provide a reason for the significant *E. coli* O157:H7 growth at 6 h, but a absence of this significant growth at 12 h.

At time 24 h, there was significant suppression of *L. monocytogenes* at all concentrations (1, 3, 5, and 10%) persimmon puree when compared to the control. Moreover, there was significant suppression of *L. monocytogenes* at concentrations 3, 5, and 10% when compared to 1%. This suggests that as concentration of persimmon puree is increased, there may be an increased ability for suppression. Alternatively, there was a significant growth of total Gram-negatives and *E. coli* O157 at 1% persimmon puree when compared to the control.

At time 36 h, there continued to be significant suppression of *L. monocytogenes* growth at all concentrations (1, 3, 5, and 10%) persimmon puree when compared to the control. In addition, there was significantly greater suppression of *L. monocytogenes* at 5 and 10% persimmon puree when compared to 1% concentration. At 3% persimmon puree concentrations there was significant suppression of Gram-negatives and *E. coli* O157 when compared to the control. However, at 5 and 10% persimmon puree concentration there was significant growth of Gram-negatives and *E. coli* O157, when compared to the control.

At time 72 h, there continued to be significant suppression of *L. monocytogenes* at all concentrations (1, 3, 5, and 10%) persimmon puree when compared to the control. There was also significantly greater suppression of *L. monocytogenes* at 5% when compared to 1%

(p=0.05). Moreover there was further significant growth when 10% persimmon puree was compared to 1 or 3% concentrations. In addition, there was significant suppression of *L. monocytogenes* from 24 h to 72 h with increased concentrations of persimmon when compared to the control, which supports that the persimmon may be effective at suppressing *L. monocytogenes* growth. In contrast, significant suppression of Gram-negatives and *E. coli* O157 was not present at 1, 3, and 5% persimmon puree when compared to the control. However, there was significant suppression of Gram-negatives and *E. coli* O157 at 10%. This is likely due to growth of bacteria hitting the death phase of their lifecycle as growth was continually increasing up to 10.02 CFU/ml Gram-negatives and *E. coli* O157 at 36 h, which dropped off significantly to 7.00 CFU/ml Gram-negatives and *E. coli* O157 at 72 h (Refer to Table 2). True effect on Bioball^{TM} *E. coli* O157 could not be determined due to inability to independently separate pathogenic colonies from background microflora on MacConkey Sorbitol Agar.

Suppression of *L. monocytogenes* was significantly greater at the 10% persimmon puree concentration, when compared to 1 and 3% persimmon puree at 72 h. Suppression of *L. monocytogenes* was also statistically significant for 5% when compared to 1 persimmon puree at 72 h. This suggests that as persimmon puree concentration increases so does the suppression of *L. monocytogenes*.

These results similar to previous research by Thompson (2001), who found the Plum puree to be an effective suppress of *L. monocytogenes* at 1, 3, 5, and 10% plum puree. However, she also found it to be effective against *E. coli* O157, where this study did not. This was most likely due to lower concentration of total polyphenols than were present in the plum. Background microflora were not a problem in enumeration and isolation with the plum puree either. This could be due to the use of organic persimmon; however, no bacteria is a good bacteria in terms of providing a higher quality product even those these bacteria were not pathogenic. Sachiko et al. (2005) found the minimum inhibitory concentrations of persimmon tannin to be effective on *Streptococcus mutans* at 62.5 μg/ml and *Streptococcus sobrinus* at 125 μg/ml. The results of this study did not yield complete inhibition as those in Sachiko et al. study. However, they were not pure compounds as were used in their study. The amount of total phenols present in our solution was not measured, but phenolics present in this study most likely much more diluted than those concentrated tannins used in Sachiko et al. research. According to these results our null hypothesis could be rejected (p=0.05).

A small pilot study was conducted to eliminate the background microflora by irradiation. Persimmon puree from four packages was sent to Iowa State to undergo irradiation at 1, 3, and 5 kg. Three replications were plated onto Tryptic Soy Agar for each treatment. Background microflora counts were found to be 3.76(0.38), 2.51(.46), and 1.24 (0.09) $\log_{10}$ CFU/g total aerobic count, respectively.

Conclusions

1. In this experiment, it was found that persimmon puree could be effective against the Gram positive bacteria *L. monocytogenes*.

2. This experiment showed the possibility that persimmon puree may be an effective antimicrobial against other Gram positive bacteria.

3. Persimmon puree may contain the phenolic compounds needed to been an effective Gram positive suppressor.

4. Persimmon puree was not found to be effective against Gram negative bacteria at any concentration for twenty four hours.

5. Further research needs to be conducted to determine if the background microflora present in the Gram negative trial, were the result of the Gram positive decline and to help build causal case for phenolics.

6. Irradiation is was not an effective means of eliminating Gram-negative microflora at the levels tested in this thesis.

7. The objectives of this study were met and we can reject the null hypothesis in favor of the alternative (p=0.05).

Table 1 Total CFU/ml *L. monocytogenes* in Liquid Media Treated with Persimmon Puree

Time (h) Concentration % Wt. / Vol.	0	6	12	24	36	72
Control (0%)	0.35^{abc}	1.29^{abc}	3.94^{ef}	9.39^{j}	10.31^{j}	8.93^{j}
1%	0.00^{a}	1.56^{abcd}	4.27^{efg}	6.71^{i}	5.67^{ghi}	6.99^{i}
3%	0.23^{ab}	1.21^{abc}	3.93^{ef}	4.19^{efg}	4.86^{fgh}	5.99^{hi}
5%	0.23^{ab}	1.47^{abc}	4.36^{efg}	3.99^{ef}	3.07^{de}	5.14^{fgh}
10%	0.00^{a}	1.45^{abc}	4.00^{ef}	3.02^{cde}	1.89^{bcd}	4.19^{efg}

a-j= Data with same superscripts represents data that is not significantly different from each other ($p = 0.05$)

Figure 4 Effect of Persimmon Puree on Average Total *Listeria monocytogenes* CFU/ml in Brain Heart Infusion Broth Over Time

Table 2 Total CFU/ml Gram-Negatives and *E. coli* O157 in Liquid Media Treated with Persimmon Puree

Time (h) Concentration Wt. / Vol.	0	6	12	24	36	72
Control (0%)	0.0^a	3.25^{bc}	5.69^e	8.42^g	9.18^{hik}	8.69^{hik}
1	1.01^{ab}	3.30^{cd}	5.99^e	9.32^{ik}	9.12^{hik}	9.03^{hik}
3	1.07^{ab}	3.25^{cd}	6.42^{ef}	8.65^{ghi}	7.71^{fg}	9.52^{ik}
5	1.71^b	3.36^d	6.40^{ef}	8.08^g	9.75^k	10.01^k
10	1.64^{ab}	3.47^d	6.42^{ef}	8.22^g	10.02^k	7.00^{efg}

a-k= Data with same superscripts, represents data that is not significantly different from each other ($p = 0.05$)

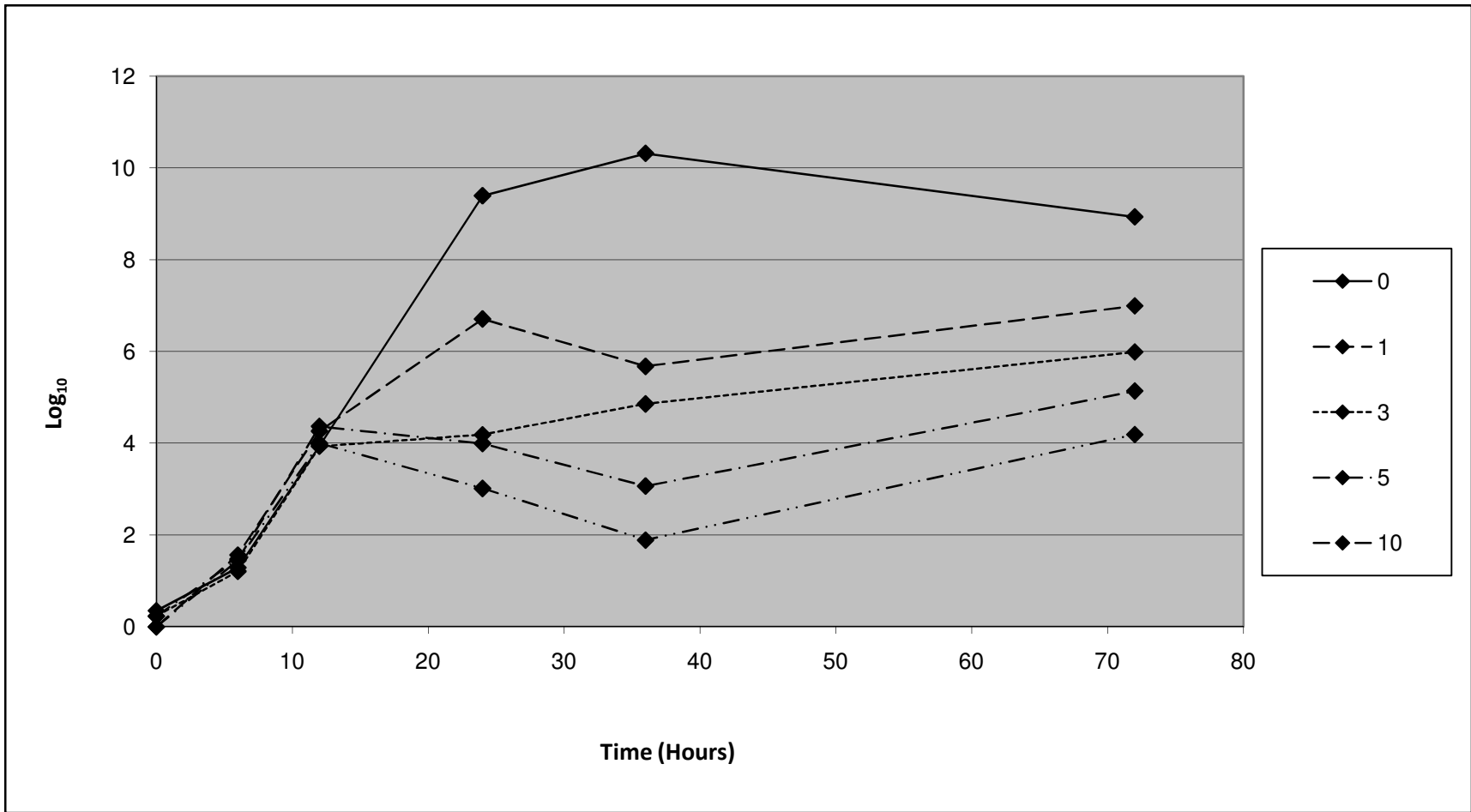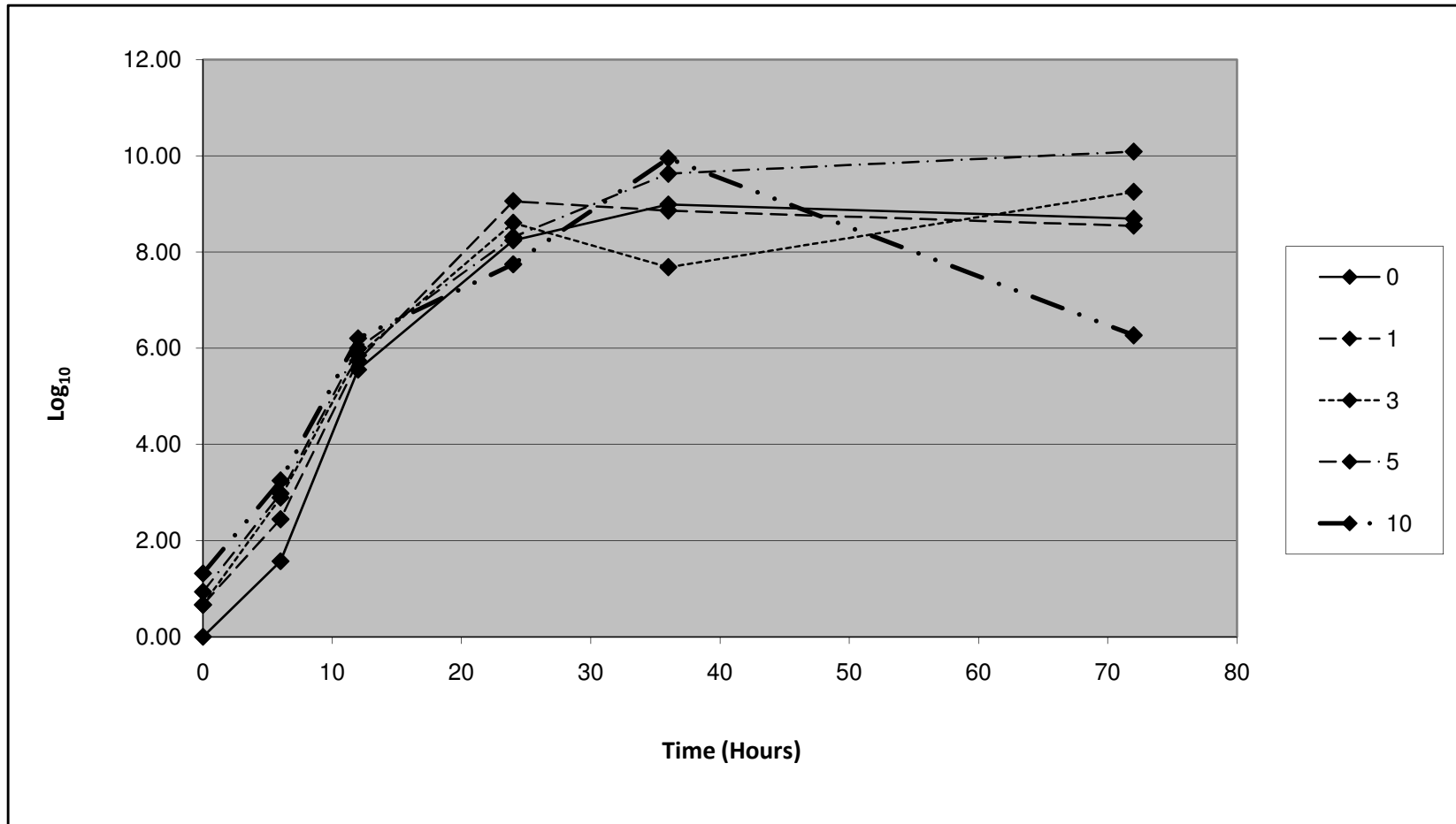**Figure 5 Effect of Persimmon Puree on Average Total *Escherichia coli* O157 and Gram-Negative CFU/ml in Brain Heart Infusion Broth Over Time**

CHAPTER 3 - **The Effects of Select Persimmon and Plum Phenolics on Minimum Inhibitory Concentrations of Common Foodborne Pathogens**

Introduction

Recent trends in the utilization of natural and organic foods are on the rise. At the same time there seems to be an ever increasing rate and size of recalls from pathogen contamination in the United States. Many natural foods such as tea, plums, and cranberries, have been shown when added to a larger food matrix to have antimicrobial properties (Kim, 2003; Lin et al., 2004; Thompson, 2001; Vettam D.A., 2004). Most of these antimicrobial properties are attributed to compounds known as polyphenols. The mechanism are not specifically known, however many are hypothesized. The most accepted theory centers on membrane disruption. The first step in further understanding the antimicrobial mechanisms of phenolic compounds is to understand which phenolics are responsible for the antimicrobial effect or if it takes multiple phenolics working together. Persimmon and plum are commercially available for consumption and contain many polyphenols. Therefore, the objective of this research is to determine if the four most abundant phenols found in persimmon and plum have antimicrobials properties. In addition the phenolic acid, Benzoic acid, from which most phenolics are derived from will be tested for antimicrobial properties. We hypothesized the if phenolic was added, then an antimicrobial effect would be seen. The null hypothesis would state that there will be no differences in microbial growth when a particular phenolic is added ($H_{o:}$ $\mu1 = \mu2 = \mu3 = \mu4 = \mu5$) (p=0.05).

Materials and Methods

Pathogen Observed

Escherichia coli O157:H7 ATCC 43895

Salmonella Typhimurium ATCC 13311

Yersinia enterocolitica   ATCC 23716

Staphylococcus aureus   ATCC 25923

Listeria monocytogenes   ATCC 2249

Bacillus cereus   ATCC 11778

      All cultures were obtained from MicroBioLogics Inc. 217 Osseo Avenue North Saint Cloud, MN 56303. Cultures were stored at $4^{o}C$, prior to use and then sub-cultured twice for 24 hours at $35^{o}C$, prior to use.

Minimum Inhibitory Concentration

Phenolic Preparation

      Phenolics were purchased from Sigma-Aldrich® (St. Louis, MO 63103) included Vanillic Acid (CAS 121-34-6), Chlorogenic Acid-Hemihydrate (CAS 6001-76-9), Benzoic Acid (CAS 65-85-0), Quercetin Dihydrate (CAS 6151-25-3), and Gallic Acid (CAS 149-91-7). Each was accompanied with Certificate of Analysis certifying the chemicals met >95% purity. The phenolic acids were solubolized using di-methyl sulfoxide, NaOH, or water, according to manufacturer recommendations. Phenolics were prepared at an initial concentration of 2500 µg/ml and stored in sterile 250 ml bottles at $25^{o}C$. Using the equation from Clinical and Laboratory Standards Institute (CLSI M31-A3): $Volume\ (ml) = \frac{Weight\ (\mu g) \times Potency\left(\frac{\mu g}{ml}\right)}{Conentration\left(\frac{\mu g}{ml}\right)}$, initial concentration volume to weight could be calculated. Phenolics were serially diluted in a 1:2 ratio from the highest concentration 1,250 µg/ml, to the lowest concentration of 9.76 µg/ml.

Microtiter Preparation

      Sterile, 96 well, flat bottom Falcon microtiter plates were obtained from Becton Dickinson Labwares, Franklin Lakes, NJ, USA. Each well had a capacity volume of 370µl. Each 96 well plate contained 12 rows by 8 columns or 8 rows by 12 columns (Refer to Figure 6.0). This allowed for the option of 12 different samples by 8 dilutions or 8 different samples by 12 dilutions and can be oriented to suit the purpose (Refer to Figure 6.0). Two micropipettes were used, the VWR 8-channel micropipette (VWR, West Chester, PA 19380) and a Wheaton Acura 821 single channel micropipette 50-250 µl (Millville, NJ 08332-2038, USA).

Using the 12 column by 8 row configuration, the 12 columns were divided into 5 phenolics and one control (Refer to Figure 6.0). Using the Acura 821 single channel micropipette, well one (row A, column 1) was dispensed with 0.1ml or 100 µl of phenolic stock solution containing 2,500 µg/ml. The same procedure was followed for the remaining phenolics as needed in row A, column X (x=column number). Then using the VWR multichannel micropipette, 0.05 ml or 50 µl of distilled sterile water was added to rows B through H. Then, 50 µl were transferred from row 1 of 2,500 µg/ml to row B combining 50 µl of diluents to 50 µl phenolic providing a 1:2 dilution, thus a final concentration in row B, column 1 of 1,250 µg/ml. Between dilution transfers each well was mixed three times by withdrawing solution into the micropipette and then re-dispensing the sample into the same well. Microtiter plates were then covered by sterile lids until inoculation at 25°C. Three repetitions were performed containing six repeated measures within each repetition. (Refer to Figure 6.0)

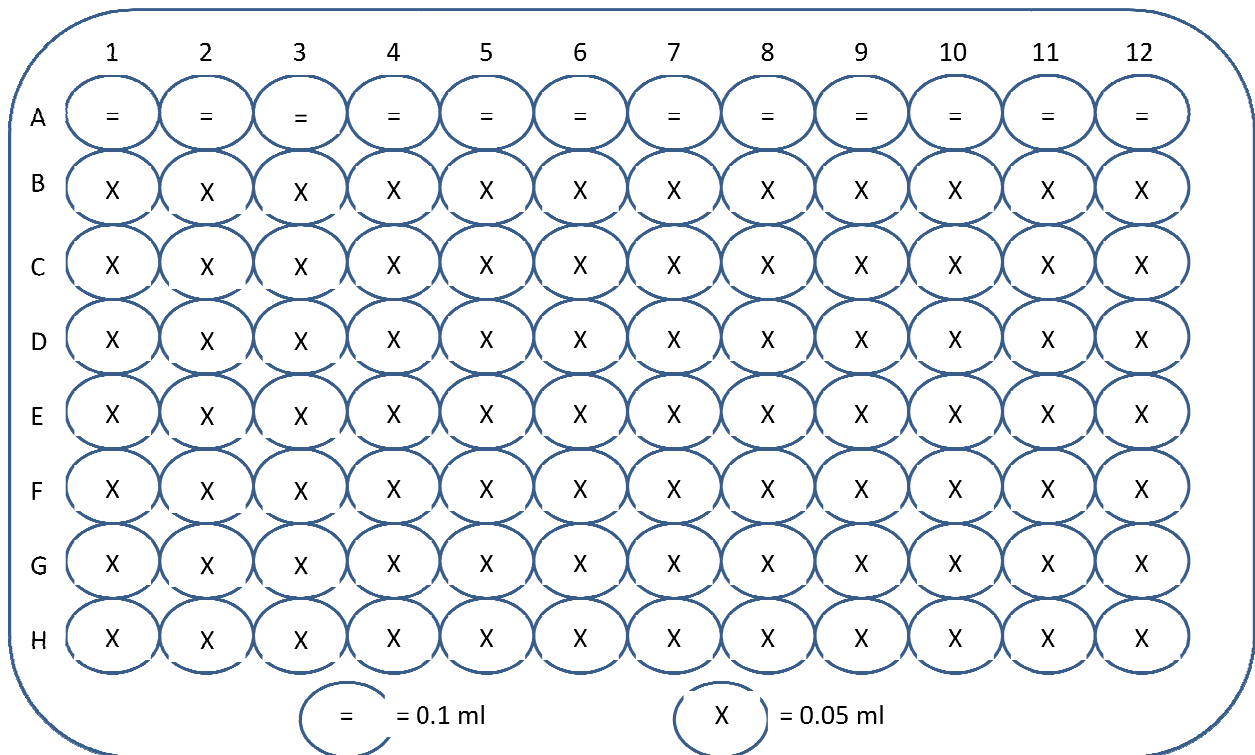|	1	2	3	4	5	6	7	8	9	10	11	12
A	=	=	=	=	=	=	=	=	=	=	=	=
B	X	X	X	X	X	X	X	X	X	X	X	X
C	X	X	X	X	X	X	X	X	X	X	X	X
D	X	X	X	X	X	X	X	X	X	X	X	X
E	X	X	X	X	X	X	X	X	X	X	X	X
F	X	X	X	X	X	X	X	X	X	X	X	X
G	X	X	X	X	X	X	X	X	X	X	X	X
H	X	X	X	X	X	X	X	X	X	X	X	X

= = 0.1 ml   X = 0.05 ml

Figure 6 A Representative Microtiter Plate Format for Minimum Inhibitory Concentration Used to Assess the Phenolics

Microtiter Inoculation and Reading

Cultures (Refer to beginning of materials and methods) were standardized according to CLSI M31-A3 to 0.5 McFarland Standard. Using the VWR 8 channel micropipette 50 µl of culture was aseptically transferred to each well. Due to adding another 50 µl of inoculum to the wells resulted in a further 1:2 dilution yielding the final concentration of the phenolics to 1,250 µg/ml in row A, column 1, to 9.76 µg/ml in row H, column 1. Plates were incubated at 37°C for 24 hr before and analyzed using a microtiter reading mirror (Cooke Engineering Company, Alexandria, Virginia). Growth was designated as heavy turbidity present or sediment. Minimum inhibitory concentrations were represented by the average concentration at which no growth occurred and the last well concentration at which growth occurred and data was recorded. This procedure was considered to be one observation and was repeated six times. One rep was classified as one preparation of phenolic chemical with one sub-cultured strain of bacteria for all the repeated measures. Three replications were performed.

Data Analysis

All data were the analyzed as the result of three replications with six repeated measures. Prediction equations were developed with 95% confidence intervals. After development of the prediction equation, concentrations needed for complete inhibition were modeled with 90% confidence. Data was analyzed using the GENMOD procedure for logistic regression for binary probabilities in SAS 9.1.

Results and Discussion

Antimicrobial susceptibility or Minimum Inhibitory Concentrations (MIC) are a common procedure in veterinary medicine. Microtiter plates are the method of choice to test microbial susceptibility/resistance. Most of these plates come prepared and all that is required is the addition of a particular sample/inoculum. Prepared plates often have a dehydrated media or drug so when the inoculum is deposited, plates can be sealed and incubated as normal. Inoculum is commonly deposited using machines like the Sensititre® by Trek Diagnostic Systems 982 Keynote Circle, Suite 6, Cleveland, Ohio 44131, USA. This procedure required the use of non-prepared media or common pharmaceutical agents, thus this research followed original microtiter

methods developed by Fung and Kraft (1968), dilution methods used by Kim (2003), but in accordance with Clinical Laboratory and Standards Institutes updated MIC procedures.

Bacillus cereus was resistant against three of the selected phenolics at the concentrations tested. Gallic acid, quercetin, and chlorogenic acid were ineffective against *B. cereus* at all concentrations tested (> 937.5 μg/ml). However, benzoic and vanillic acid proved to be effective. Benzoic acid was able to inhibit growth with 90% confidence at 518.79 μg/ml. Vanillic acid was able to inhibit growth with 90% confidence at 739.94 μg/ml (Table 3 and Figure 7).

Escherichia coli O157:H7 was resistant against four selected phenolics at the concentrations tested. Only the phenolic acid, Benzoic acid was effective at 452.98 μg/ml with 90% confidence (Table 4 and Figure 8). Gallic, quercetin, chlorogenic, and vanillic were all ineffective at inhibiting *E. coli* O157:H7 at all concentrations tested.

Listeria monocytogenes was only resistant against one phenolic at the concentrations tested. Gallic acid was the most effective phenolic tested at 29.06 μg/ml with 90% confidence. Quercetin was the second most effective at a concentration of 214.86 μg/ml with 90% confidence. Benzoic acid was next at 470.36 μg/ml with 90% confidence. Finally, vanillic acid was effective at 903.92 μg/ml. Chlorogenic acid was the only phenolic tested that was not effective against *L. monocytogenes* at the concentrations tested (Table 5 and Figure 9).

Salmonella Typhimurium was resistant to only one phenolic, which was chlorogenic acid at the concentrations tested. Benzoic acid was the most effective only requiring a concentration of 239.63 μg/ml to inhibit growth, with 90% confidence. Gallic acid was the next most effective at 372.64 μg/ml to inhibit growth, with 90% confidence. The third most effective phenolic was Quercetin at a concentration of 643.27, μg/ml with 90% confidence. Vanillic acid was effective at 760.58 μg/ml with 90% confidence. (Table 6 and Figure 10)

Staphylococcus aureus was resistant only to chlorogenic acid at the concentrations tested. Gallic acid was the most effective phenolic with a concentration of 22.45 μg/ml needed to inhibit growth with 90% confidence. Quercetin was the next most effective phenolic, requiring a concentration of 69.81 μg/ml to inhibit growth with 90% confidence. The third most effective phenolic was benzoic acid needing a concentration of 775.07 μg/ml for 90%

confidence. Vanillic acid was the final phenolic effective at a concentration of 915.09 µg/ml with 90% confidence. (Table 7 and Figure11)

Yersinia enterocolitica was susceptible to all the phenolics tested. Gallic acid was the most effective phenolic, inhibiting growth at concentration of 11.01 µg/ml with 90% confidence. Quercetin was the second most effective phenolic requiring only a concentration of 69.81 µg/ml to inhibit growth with 90% confidence. The third most effective phenolic was benzoic acid, which inhibited growth at 205.39 µg/ml with 90% confidence. Chlorogenic was the fourth most effective phenolic inhibiting growth at a concentration of 287.37 µg/ml with 90% confidence. Vanillic acid was the least effective of the phenolics tested, but still inhibited *Y. enterocolitica* at a concentration of 485.96 µg/ml with 90% confidence. (Table 8 and Figure 12)

Results showed gallic acid to be the most effective phenolic, followed closely by benzoic acid. Gallic acid was shown to be the most effective against *Yersinia enterocolitica, L. monocytogenes,* and *Staphylococcus aureus,* with concentrations of 11.01, 29.06, and 22.45 µg/ml, respectively. Benzoic acid was shown to be the most effect against *E. coli* O157:H7, *Salmonella* Typhimurium, and *B. cereus* with concentrations of 452.98, 239.63, and 518.79 µg/ml, respectively.

Benzoic acid, is the phenolic through which all of the common persimmon phenolics (gallic, catechin, and epi-gallocatechin) can be derived (Chen et al., 2008; Gorinstein et al., 1994; Shahidi and Naczk, 1995). Gallic acid is formed when benzoic acid attaches OH groups at positions R1, R2, and R3 (Shahidi and Nacsk, 1995). Gallic acid proving to be more effective with selected pathogens is consistent with the most widely accepted mechanism that phenolics disrupt cellular membranes for lethality. Lethality is thought to be correlated to free OH groups and their ability to disrupt cell membranes, which is supported by the more effective gallic acid against the selected pathogens (Kabara, 1981; Racacch, 1982; Raccach, 1984; Singer et al., 1997; Singer and Wan, 1997).

The potential cause of lethality with benzoic acid is not as clear. It is possible that carbonic acid side chain is responsible for disrupting carbon incorporation into cellular components causing lethality. Benzoic acid was effective on all selected pathogens in the concentrations tested and this may explain why it was more widely effective. The mechanism of action may have been more targeted on basic needs of the pathogen as opposed to physical non-

polar disruption of the membrane. This would suggest that *E. coli* O157:H7 and *B. cereus* are considerably more equipped to handle environmental diversity, which is beyond the scope of this research.

Effectiveness of quercetin and chlorogenic acid is not fully understood. Quercetin (contains five OH groups) and chlorogenic (contains four OH groups) should chemically have the potential to be more effective than gallic acid (three OH groups). However, results did not support this theory. Vanillic acid only contains two OH groups, thus supporting research suggests it should be less effective than gallic acid. Raccach (1984) discusses the effectiveness of antimicrobials as highly variable on the strain as well as genus of the bacteria being studied and suggests that a "Silver Bullet" may not be possible. Research in natural antimicrobials, especially in fruits may hold much future interest as many contain multiple phenolics, which could be used in multiple combinations to obtain effective antimicrobials in foods. Further research needs to be conducted to properly ascertain any correlation between hydroxyl groups and increase lethality through membrane disruption.

Conclusions

1. All phenolics were not effective on all bacteria; however all phenolics were effective at varying levels depending on bacteria

2. Gallic acid and Benzoic acid were the most effective inhibitors of certain bacteria.

3. Phenolics tested in this study have the potential for inhibition of pathogenic bacteria in a liquid medium

4. The objectives of this study were met and we can reject the null hypothesis in favor of the alternative ($p=0.05$).

Table 3 *Bacillus Cereus* **Inhibitory Probability vs. Concentration for Benzoic Acid and Vanillic Acid**

Concentration (µg/ml) Phenolic	7.32	14.65	29.30	58.59	117.19	234.38	468.75	518.79*	739.94**	937.50
Benzoic	0.985	0.985	0.983	0.978	0.965	0.913	0.605	0.504*		0.032
Vanillic	0.999	0.999	0.998	1.000	0.996	0.999	0.978		0.514**	0.388

* = 90% Confidence Concentration and Probability for Benzoic Acid
** = 90% Confidence Concentration and Probability for Vanillic Acid
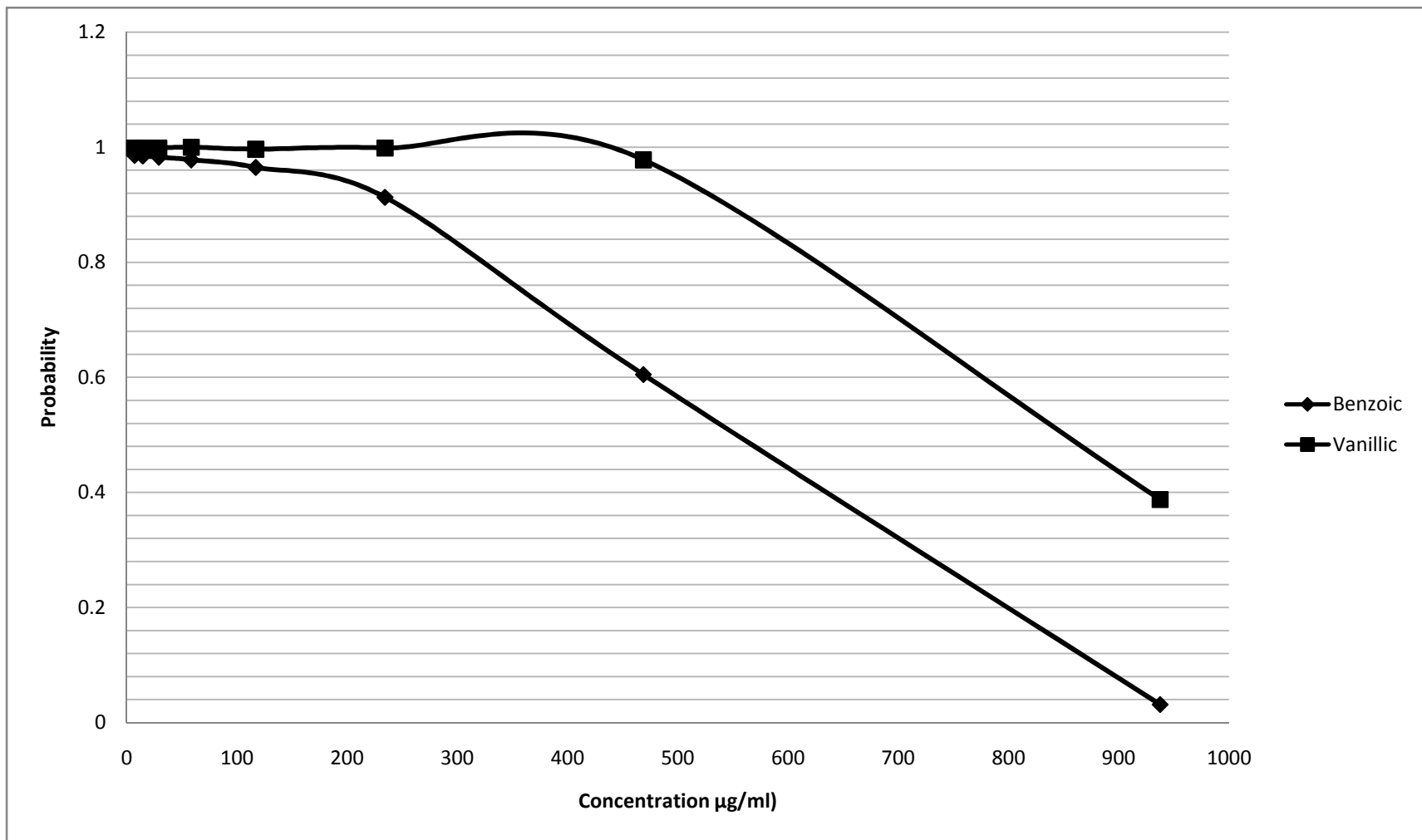
Figure 7 *Bacillus cereus* **Death Curve Probability for Benzoic Acid and Vanillic Acid**

Note: Actual values do not exist above 1 or below 0, these are results of line smoothing, which provides a more accurate prediction

probability curve.

Table 4 *Escherichia coli* O157:H7 Benzoic Acid Inhibitory Probability vs. Concentration

Concentration (µg/ml)	7.32	14.65	29.3	58.59	117.19	234.38	452.98*	468.75	937.5
Phenolic									
Benzoic	0.988	0.987	0.985	0.981	0.966	0.898	0.503*	0.464	0.008

* = 90% Confidence Concentration and Probability for Benzoic Acid

Figure 8 *Escherichia coli* **O157:H7 Death Curve Probability for Benzoic Acid**
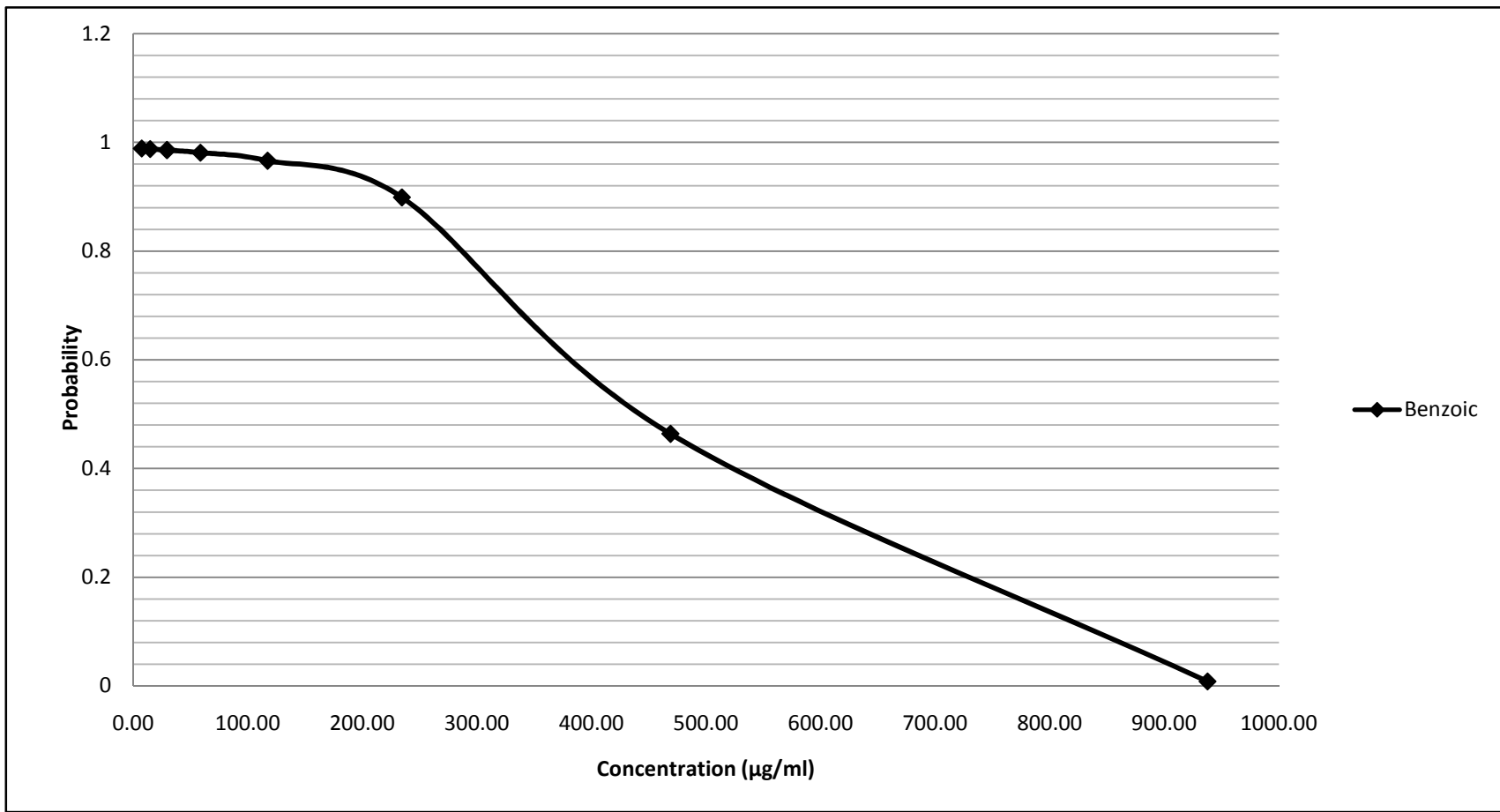
Table 5 *Listeria monocytogenes* **Inhibitory Probability vs. Concentration for Vanillic Acid, Benzoic Acid, Gallic Acid, and Quercetin**

Concentration (µg/ml) Phenolic	7.32	14.65	29.06*	29.3	58.59	117.19	214.87**	234.38	468.75	470.36***	903.92****	937.5
Vanillic	1.000	1.000		1.000	1.000	1.000		1.000	1.000		0.528****	0.028
Benzoic	1.000	1.000		1.000	1.000	1.000		1.000	0.611	0.566***		0.000
Gallic	0.792	0.717	0.530*	0.527	0.178	0.008		0.000	0.000			0.000
Quercetin	0.950	0.944		0.933	0.902	0.802	0.508**	0.440	0.029			0.000

* = 90% Confidence Concentration and Probability for Gallic Acid
** = 90% Confidence Concentration and Probability for Quercetin
***= 90% Confidence Concentration and Probability for Benzoic
****= 90% Confidence Concentration and Probability for Vanillic Acid
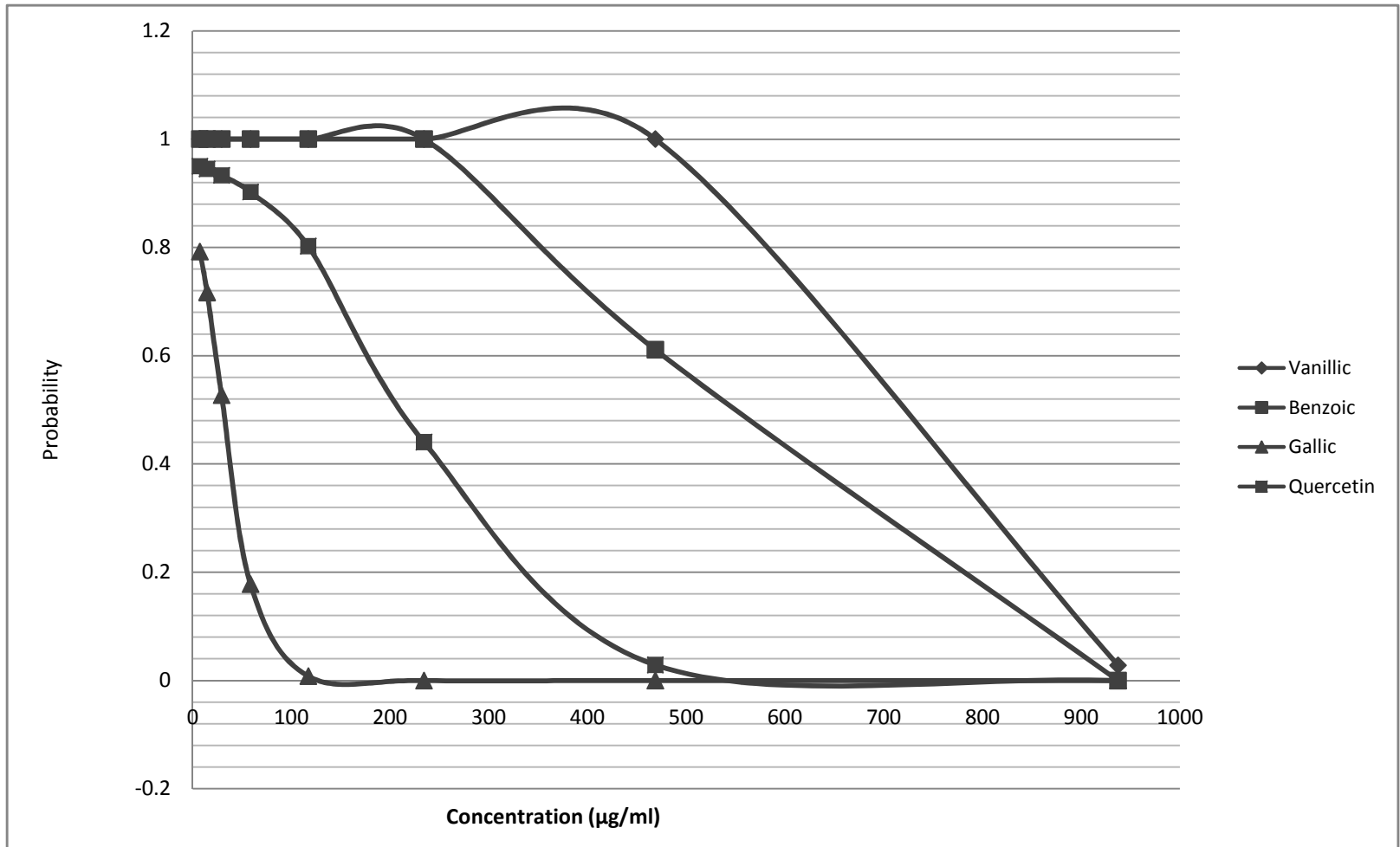
Figure 9 *Listeria monocytogenes* **Phenolic Death Curve Probability Vanillic Acid, Benzoic Acid, Gallic Acid, and Quercetin**
Note: Actual values do not exist above 1 or below 0, these are results of line smoothing, which provides a more accurate
prediction probability curve.

Table 6 *Salmonella* **Typhimurium Inhibitory Probability vs. Concentration for Quercetin, Gallic Acid, Benzoic Acid, and Vanillic Acid**

Concentration (µg/ml) Phenolic	7.32	14.65	29.3	58.59	117.19	234.38	239.63*	372.64**	468.75	643.27***	760.58****	937.5
Quecetin	0.999	0.999	0.999	0.998	0.997	0.988			0.868	0.504***		0.042
Gallic	0.958	0.955	0.950	0.936	0.899	0.766		.501**	0.306			0.008
Benzoic	1.000	1.000	1.000	1.000	1.000	0.833	0.620*		0.000			0.000
Vanillic	0.995	0.994	0.994	0.992	0.989	0.975			0.884		0.507****	0.233

 * = 90% Confidence Concentration and Probability for Benzoic Acid
** = 90% Confidence Concentration and Probability for Gallic Acid
***= 90% Confidence Concentration and Probability for Quercetin
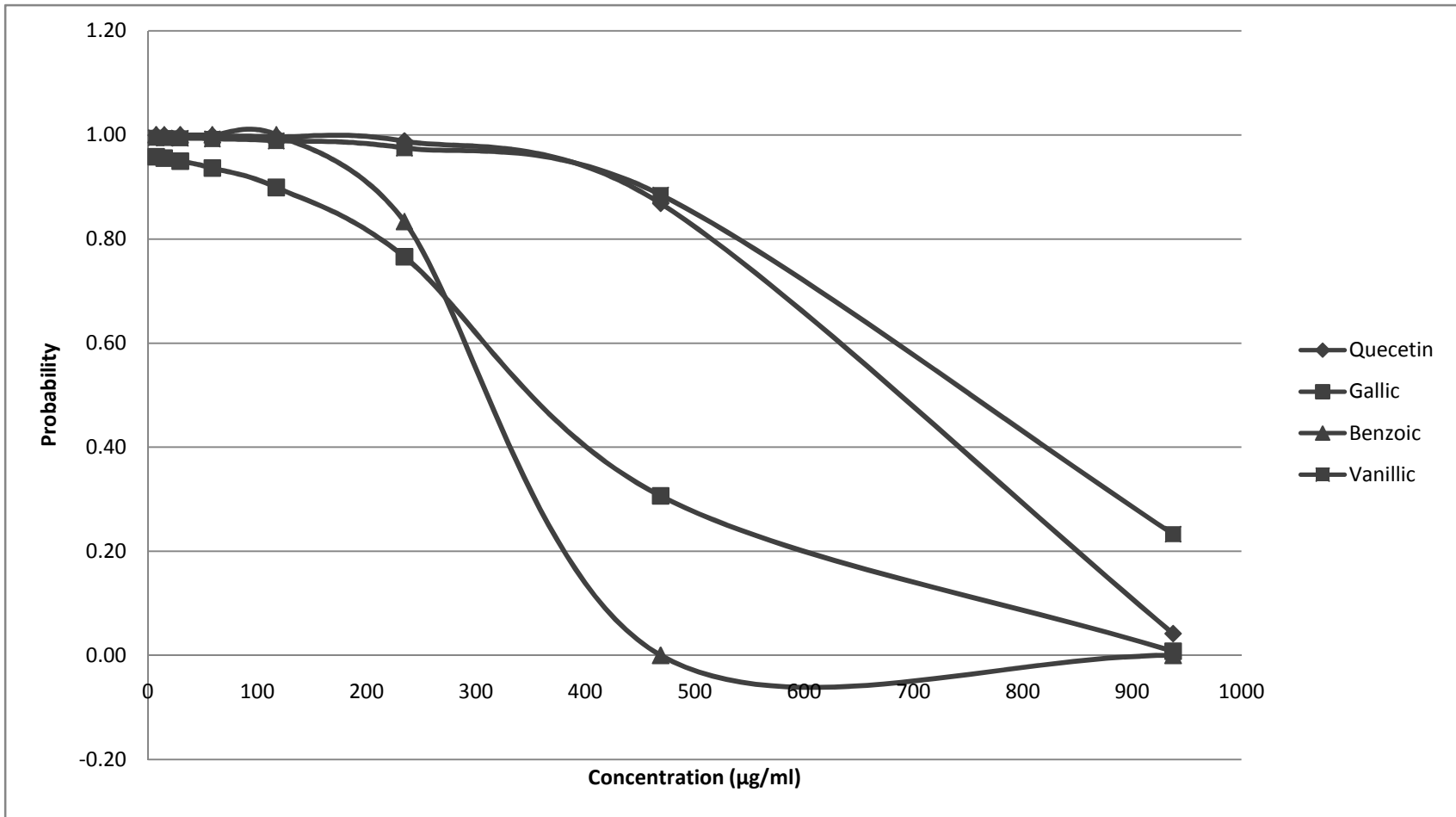****= 90% Confidence Concentration and Probability for Vanillic Acid

Figure 10 *Salmonella* **Typhimurium Phenolic Death Curve Probability for Quercetin, Gallic Acid, Benzoic Acid, and Vanillic Acid**
Note: Actual values do not exist above 1 or below 0, these are results of line smoothing, which provides a more accurate prediction probability curve.

Table 7 *Staphylococcus aureus* Inhibitory Probability vs. Concentration for Quercetin, Gallic Acid, Benzoic Acid, and Vanillic Acid

Concentration (µg/ml) Phenolic	7.32	14.65	22.45*	29.3	58.59	112.79**	117.19	234.38	468.75	775.07***	915.09****	937.5
Quercetin	0.995	0.993		0.985	0.940	0.474**	0.474	0.003	0.000			0.000
Gallic	0.680	0.607	0.524*	0.450	0.186		0.018	0.000	0.000			0.000
Benzoic	1.000	1.000		0.999	0.999		0.999	0.996	0.957	0.500***		0.162
Vanillic	1.000	1.000		1.000	1.000		1.000	1.000	1.000		0.531****	0.222

* = 90% Confidence Concentration and Probability for Gallic Acid
** = 90% Confidence Concentration and Probability for Quercetin
***= 90% Confidence Concentration and Probability for Benzoic Acid
****= 90% Confidence Concentration and Probability for Vanillic Acid
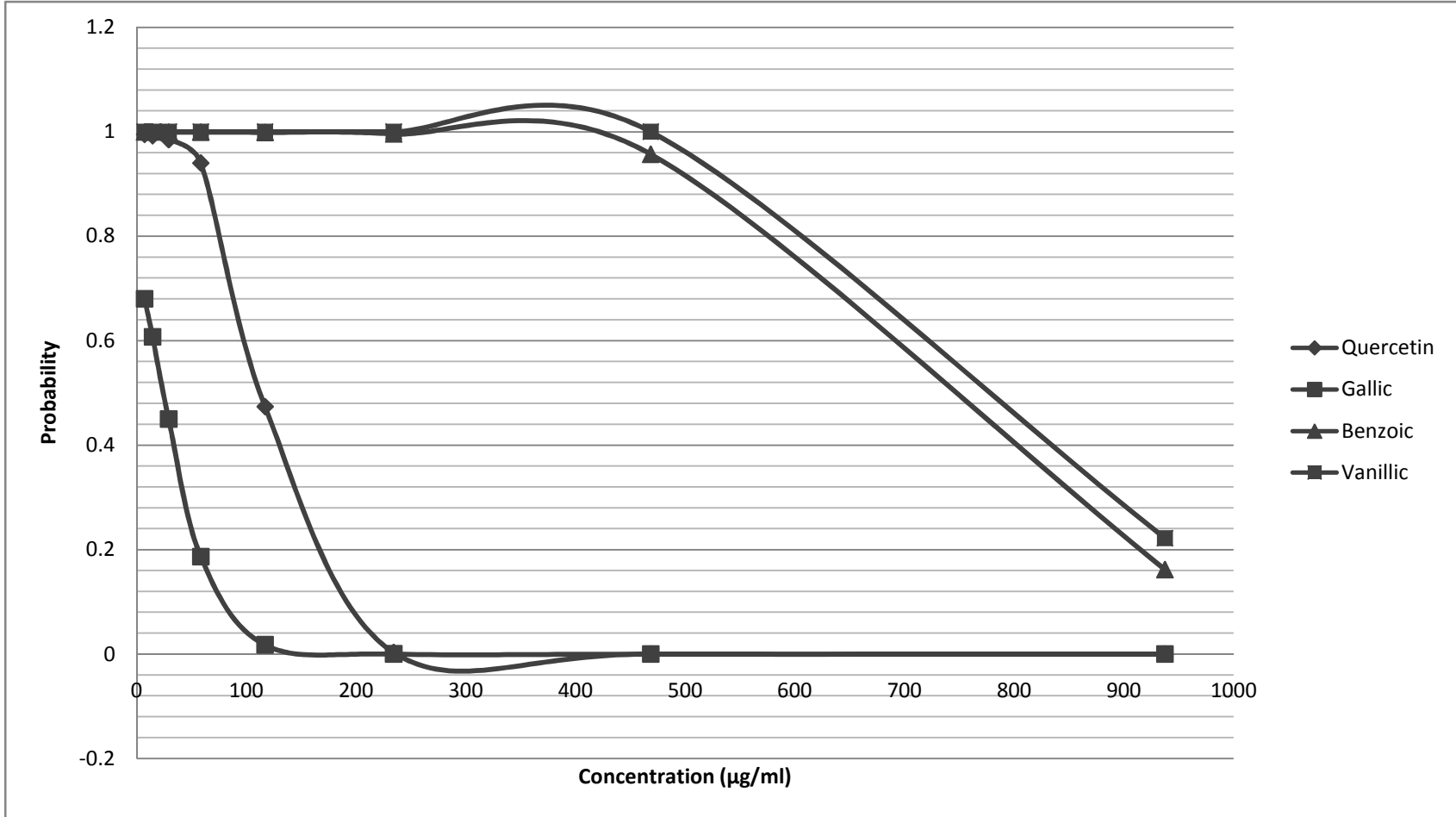
Figure 11*Staphylococcus aureus* **Phenolic Death Curve Probability for Quercetin, Gallic Acid, Benzoic Acid, and Vanillic Acid**
Note: Actual values do not exist above 1 or below 0, these are results of line smoothing, which provides a more accurate prediction probability curve.

Table 8 *Yersinia enterocolitica* **Inhibitory Probability vs. Concentration for Quercetin, Chlorogenic Acid, Gallic Acid, Benzoic Acid, and Vanillic Acid**

Concentration (µg/ml) Phenolic	7.32	11.01*	14.65	29.3	58.59	69.81**	117.19	205.39***	234.38	287.37****	468.75	485.96*****	937.5
Quercetin	0.994		0.990	0.968	0.744	0.543**	0.027		0.000		0.000		0.000
Chlorogenic	0.929		0.924	0.914	0.891		0.828		0.624	0.506****	0.165		0.003
Gallic	0.945	0.743*	0.332	0.000	0.000		0.000		0.000		0.000		0.000
Benzoic	0.997		0.996	0.994	0.987		0.932	0.516***	0.315		0.001		0.000
Vanillic	0.994		0.993	0.992	0.990		0.981		0.936		0.550	0.504*****	0.031

* = 90% Confidence Concentration and Probability for Gallic Acid
** = 90% Confidence Concentration and Probability for Quercetin
***= 90% Confidence Concentration and Probability for Benzoic Acid
****= 90% Confidence Concentration and Probability for Chlorogenic Acid
*****=90% Confidence Concentration and Probability for Vanillic Acid
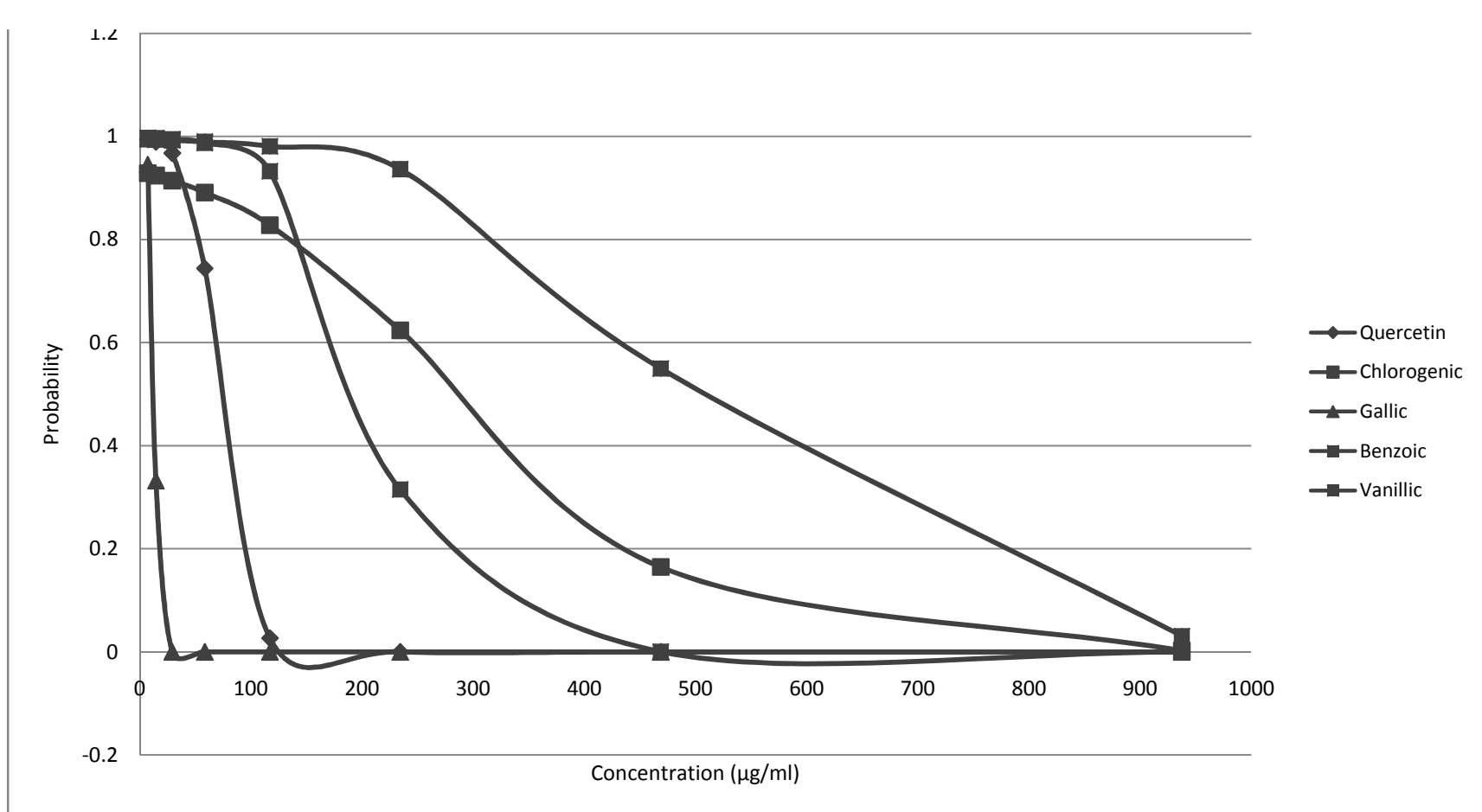
Figure 12 *Yersinia enterocolitica* **Death Curve Probability for Quercetin, Chlorogenic Acid, Gallic Acid, Benzoic Acid, and Vanillic Acid**
Note: Actual values do not exist above 1 or below 0, these are results of line smoothing, which provides a more accurate prediction probability curve.

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CHAPTER 4 - **The Antimicrobial Effects of Persimmon Puree on Five Strain Cocktail of *Listeria monocytogenes* in Ground Beef.**

Introduction

As exhibited in study 1, persimmon puree can be an effective antimicrobial against *Listeria monocytogenes* in a liquid system. In study 2, data shows that persimmon phenolics when used in isolation may be effective antimicrobials. To really determine the utility of phenolic destruction of pathogens, persimmon phenolics need to be tested in a ground beef matrix. The objective of this study is to determine the effects of persimmon puree on a five strain cocktail of *Listeria monocytogenes* in a raw uncooked ground beef. We hypothesized the adding persimmon puree would have an antimicrobial effect. The null hypothesis would state that $(H_{o:} \mu1 = \mu2 = \mu3 = \mu4)$ (p=0.05)

Materials and Methods

Listeria monocytogenes ATCC 7644
Listeria monocytogenes ATCC 19113
Listeria monocytogenes ATCC 19115
Listeria monocytogenes ATCC 19118
Listeria monocytogenes ATCC 2249

Inoculum Preparation

The inoculum used in this study consisted of 5 strains of *Listeria monocytogenes*. The strains were *L. monocytogenes* ATCC 7644, ATCC 19113, ATCC 19115, ATCC 19118, and ATCC 2249. All cultures were obtained from MicroBioLogics Inc. 217 Osseo Avenue North Saint Cloud, MN 56303. Cultures were tested for purity using Gram stain and Gram-positive ID (BBL, Becton Dickinson, Sparks, MD). The five strain cocktail was prepared by growing each strain separately in 9 ml sterile Tryptic Soy Broth (TSB) tubes and then incubating at 35°C for 24 h. Cultures were then vortexed for homogenation and then using sterile pipettes transferring 2

ml of each strain into a sterile 13 X 100 test tube. After each of the five strains were combined, cultures were vortexed again for homogenation.

Preparation of Uncooked Ground Beef

Persimmon puree (LaVinge Organics, Riverside, CA) arrived in 2 lb hermetically sealed, frozen and zip locked bags. Ground beef for this experiment was purchase from a local retailer the day the mixtures were made. Following basic procedures previously used by Thompson (2001), 0, 3.75, 6.25, and 12.5 g of persimmon puree was combined with 125, 121.25, 118.75, and 112.5 g of ground beef (20% fat), respectively. Mixtures were then mixed for 2 min, by sterile gloved hands, to yield final concentrations of 0, 3, 5, and 10 percent persimmon puree in the ground beef mixture. As proportion of persimmon added was increased, the fat percentage of the mixture will decrease slightly. The meat mixture was inoculated by adding 1.25 ml of the 5 strain cocktail. Inoculated mixtures were mixed by hand, sterile gloved, for 5 min. Target concentration for the pathogen was 3 log CFU/g. After mixing, samples were allowed to rest for 1 hr before proceeding. Mixtures were separated into 25 g samples and placed into sterile filter stomacher bags (Spiral Biotech, model SFB-410, Bethesda, MD) for further testing and storage. Samples were stored at 4°C until sampled.

Sampling Methods

After samples set for 1 hr and were divided or pulled from storage, 225 ml of 0.1% peptone water were added to 25 g inoculated samples. Samples were stomached (Lab Blender Stomacher 400, model BA 612, A.J. Seward, London) for 2 min and were diluted by pipetting 1 ml of stomached sample into 9 ml sterile 0.1% peptone water. Samples were serially diluted out to the seventh tube to ensure detectable levels. Then, 0.1 ml of sample was spread plated onto Tryptic Soy Agar (TSA) (DIFCO Laboratories, Detroit, MI) and Modified Oxford Medium (MOX) (DIFCO Laboratories, Detroit, MI) for the detection of total plate count and total enumeration of *L. monocytogenes* in duplicate. Plates were allowed to dry for 30 min, inverted, and placed into the incubator at 35°C for 24 hr. After incubation, plates were counted for typical colonies and recorded as CFU/g. The same procedure was followed for sampling times 1 and 3. Typical colonies on MOX were defined as white w/ black zone around colony. This procedure makes up one repetition and three repetitions were performed.

Statistical Analysis

All data presented are the averages of three replications performed. Experimental design was a completely randomized block with a split-plot. Analysis was performed using the MIXED procedure of SAS 9.1 and finding the Least Squared Means (p=0.05). Logarithmic transformation of counts was conducted for statistical analysis. All significance values were determine by 95% confidence or a ά = 0.05.

Results and Discussion

Persimmon puree significantly inhibited (p = 0.05) the growth of *L. monocytogenes* in Brain Heart Infusion liquid medium. Liquid medium exhibits the simplest of environments. It provides all the nutrients necessary for growth in excess without the complexity of physical environment and possible barriers to those nutrients. Food systems are much more complex than liquid mediums and therefore treatments successful in liquid medium may not always translate to food systems. Potential barrier for effectiveness in food systems include the interactions that may take place between the persimmon puree (phenolics) and the fats, proteins, and the ability to evenly homogenize the puree in the ground beef.

There were significant (p = 0.05) fixed effects for both concentration and day for total aerobic counts. There was a significant increase (p = 0.05) in growth of total aerobic bacteria in 10% persimmon puree concentration in ground beef when compared to control (Figure 15). This suggests that the persimmon did not actually suppress growth, but provided conditions to enhance it. Additionally, fixed effects for day showed a significant (p = 0.05) increase in growth of total aerobic count at day 5 when compared to all other days (0, 1, 3) (Figure 16). Persimmon puree may have provided an antimicrobial effect on days 1 and 3, but had no effect on day 5. Moreover, there was a trend (p = 0.0703) towards an interaction with concentration by day.

No significant (p = 0.05) results were found between concentrations. However, there were significant (p<0.05) day fixed effects. Day 5 had significantly increased growth of total aerobic count CFU/g, when compared to day 0, 1, and 3. Growth significantly increased from 5.69, 5.63, and 5.59 for days 0, 1, and 3, respectively, to 6.02 CFU/g total aerobic count on day 5. Significant growth from on the fifth day is interesting, considering numerical values had been

declining from 0, 1, and 3 days. If the persimmon concentration had an effect it may have worn off by day five, which explains the jump in growth. However, it more likely that after allowing for attachment time after inoculation and the refrigerated temperatures that log phase was slightly suppressed. Further research should be conducted to determine the full extent of this effect.

At day 5, there was a trend (p = 0.0703) in suppression of growth *L. monocytogenes* at 3% persimmon puree concentration when compared to the control. This was the only point that had any separation graphically and occurs at the highest concentration (10%) persimmon puree tested. This is most likely due to a more extreme data point that was not consistent with the other data points. However, it is a possibility that a potential ceiling exist with persimmon puree when added into ground beef before it is effective.

However, overall the data suggests that the greater the concentration of persimmon puree (1, 3, 5 and 10%) may actually increase the growth of total aerobic bacteria CFU/g in ground beef, instead of suppressing growth. Lower concentration of persimmon puree in ground beef could be protective against growth of bacteria when compared to ground beef with persimmon added. There are many complexities to consider on why the puree in ground beef may not have worked similar to those in the study with liquid medium.   Kim (2003) suggested that phenolics need temperatures above 7°C and closer to 25°C to be the most biologically active. Therefore, if persimmon puree phenolics were allowed to work at room temperature, suppressions of *L. monocytogenes* may be achieved as reported by previous studies (Chapter 2). However, it is not practical to store ground beef at room temperature. In addition, pathogen growth would most likely only grow and product quality deterioration would be accelerated.

Previous studies using only fruit phenolics have shown plum to be suppressive against pathogens at 3% or greater Thompson (2001). Results may differ from this experiment for several reasons. Raccach (1984) explains the complexities of adding antimicrobial substances to food, which is further supported by these findings. Moreover, plums have been shown to have a much higher phenolic concentration than that of the persimmon (Thompson, 2001). Furthermore, the Fuyu persimmon is of the least astringent varieties and accordingly contains a smaller concentration of natural phenolics compared to more astringent varieties.

Conclusions

1. Persimmon puree (*Diospyros kaki* c.v. Fuyu) is not an effective antimicrobial against *L. monocytogenes* or aerobic bacteria, when added to ground beef at concentrations up to 10%.

2. The objectives of this study were met, however, the original hypothesis was incorrect and we can accept the null hypothesis in favor of the alternative (p=0.05).

Future Research

1 More astringent persimmon varieties should be used

2 Persimmon phenolic isolation and quantification

3 Straight addition of phenolic to liquid medium and solid medium

4 Minimum inhibitory concentrations with extracted phenolic should be conducted

5 There needs to be a greater emphasis placed on understanding the mechanism behind phenolic lethality.

Table 9 Average Total Aerobic Counts CFU/g by Concentration Persimmon Puree and Time in Ground Beef

Time (Days) Concentration Wt. / Wt.	0	1	3	5
0%	5.62[a]	5.57[a]	5.47[a]	5.53[a]
3%	5.71[a]	5.60[a]	5.50[a]	5.87[a]
5%	5.71[a]	5.56[a]	5.74[a]	6.13[a]
10%	5.73[a]	5.64[a]	5.82[a]	6.55[b]

a-b= Data with a similar superscript within the same row are not different from each other (p = 0.0703)
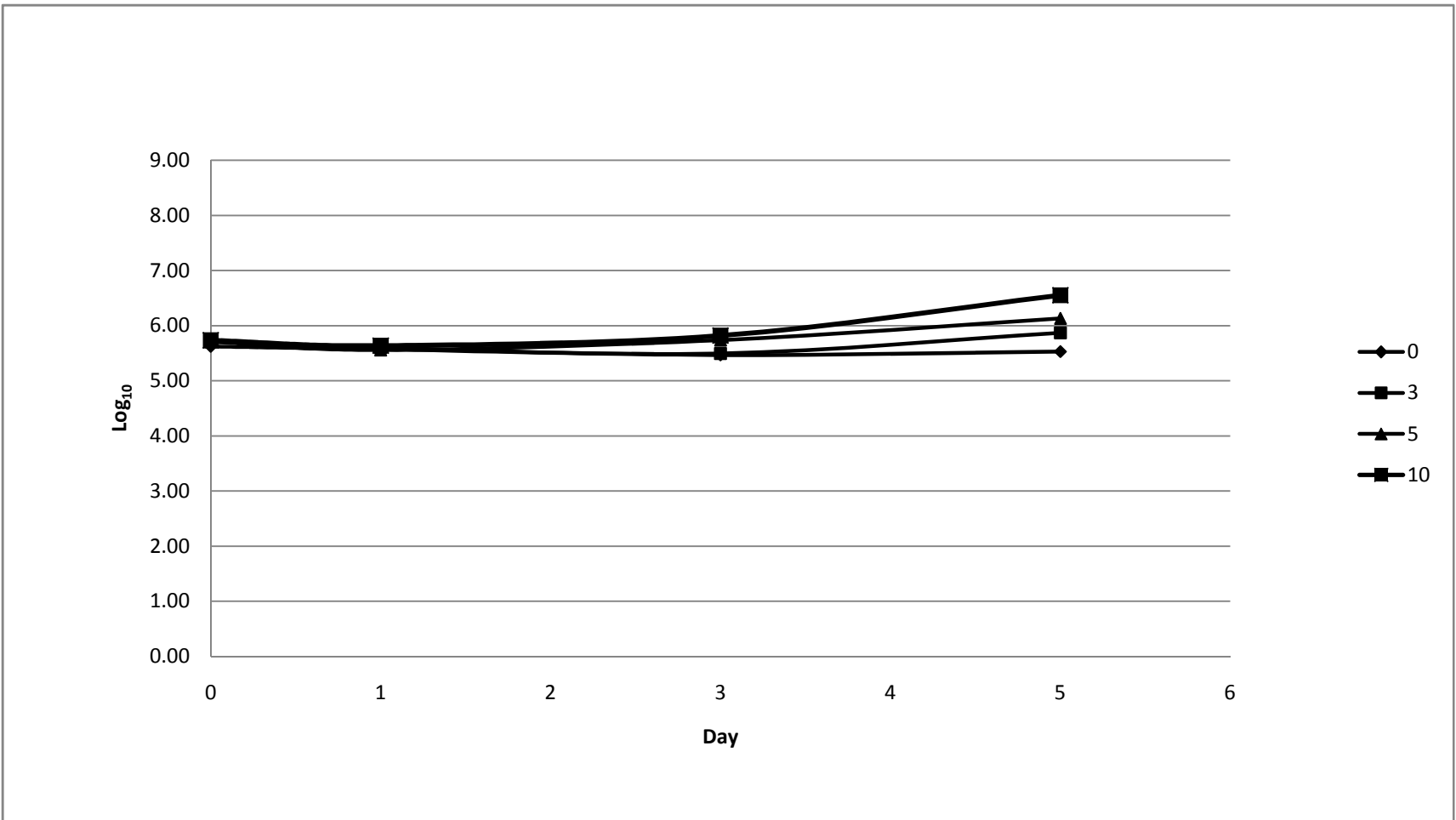
Figure **13 Average Total Aerobic Count CFU/g in Ground Beef with Persimmon Puree on Tryptic Soy Agar**

Table 10 Average *Listeria monocytogenes* CFU/g by Concentration Persimmon Puree and Time in Ground Beef

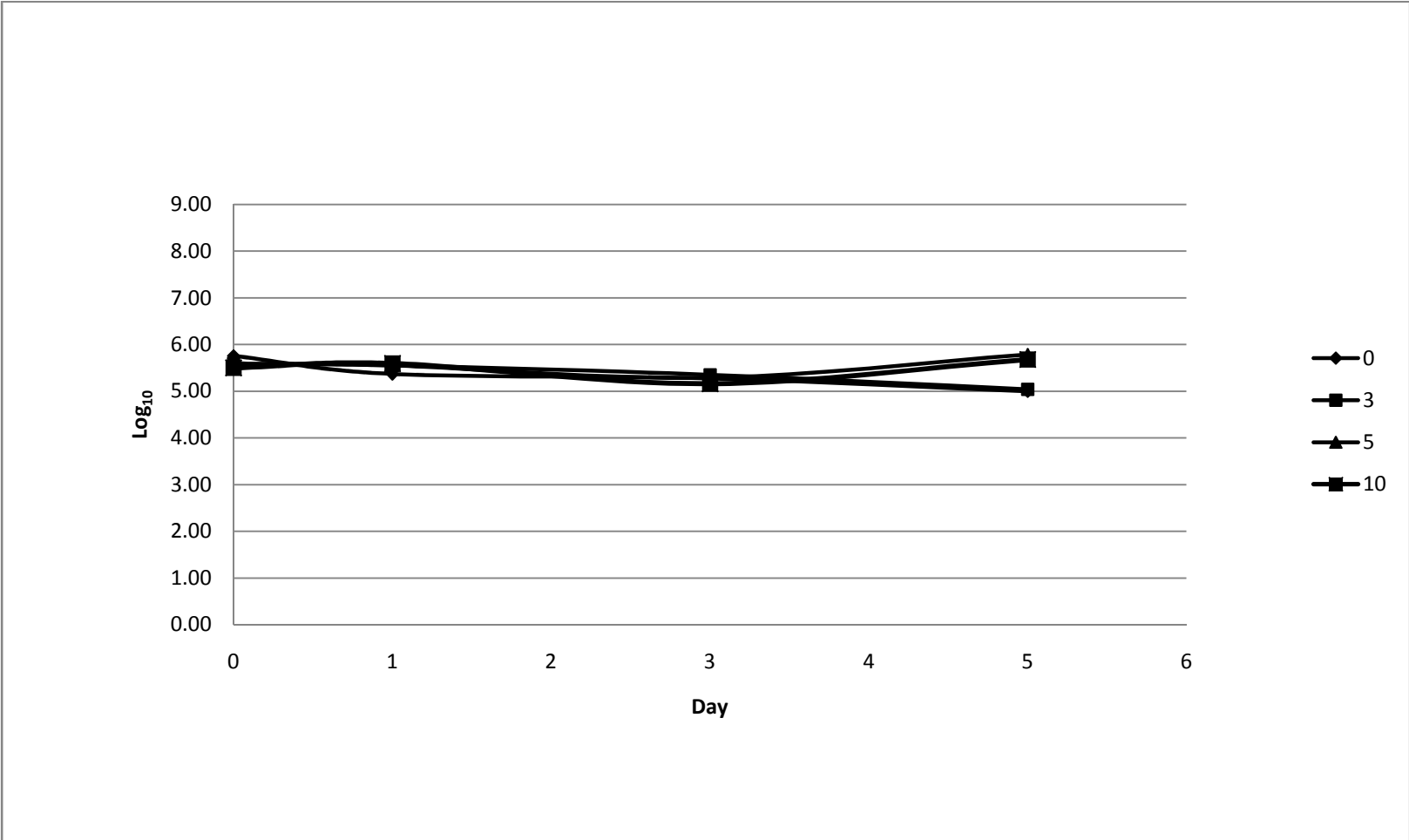| Time (Days) / Concentration Wt. / Wt.	0	1	3	5
0%	5.76	5.37	5.28	5.00
3%	5.60	5.56	5.36	5.03
5%	5.58	5.55	5.29	5.79
10%	5.50	5.59	5.16	5.68

Figure 14 Total *Listeria monocytogenes* CFU/g in Ground Beef by Persimmon Puree on Modified Oxford Medium
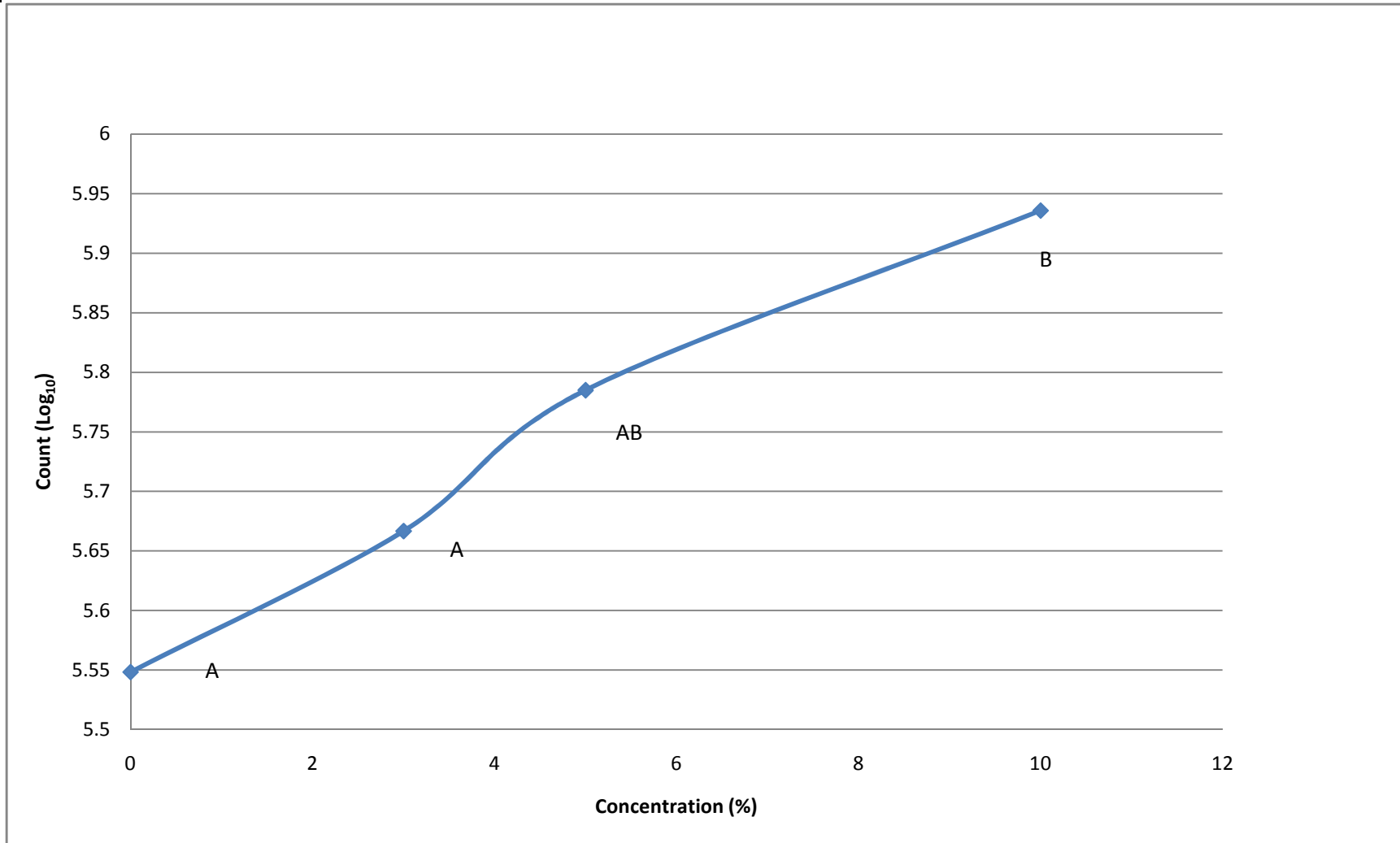
Figure 15 Persimmon Puree Concentration Effects on Total Aerobic Counts CFU/g on Tryptic Soy Agar

a-b= Data with the same letter under each organism represents data that is not significantly different from each other (p = 0.05)
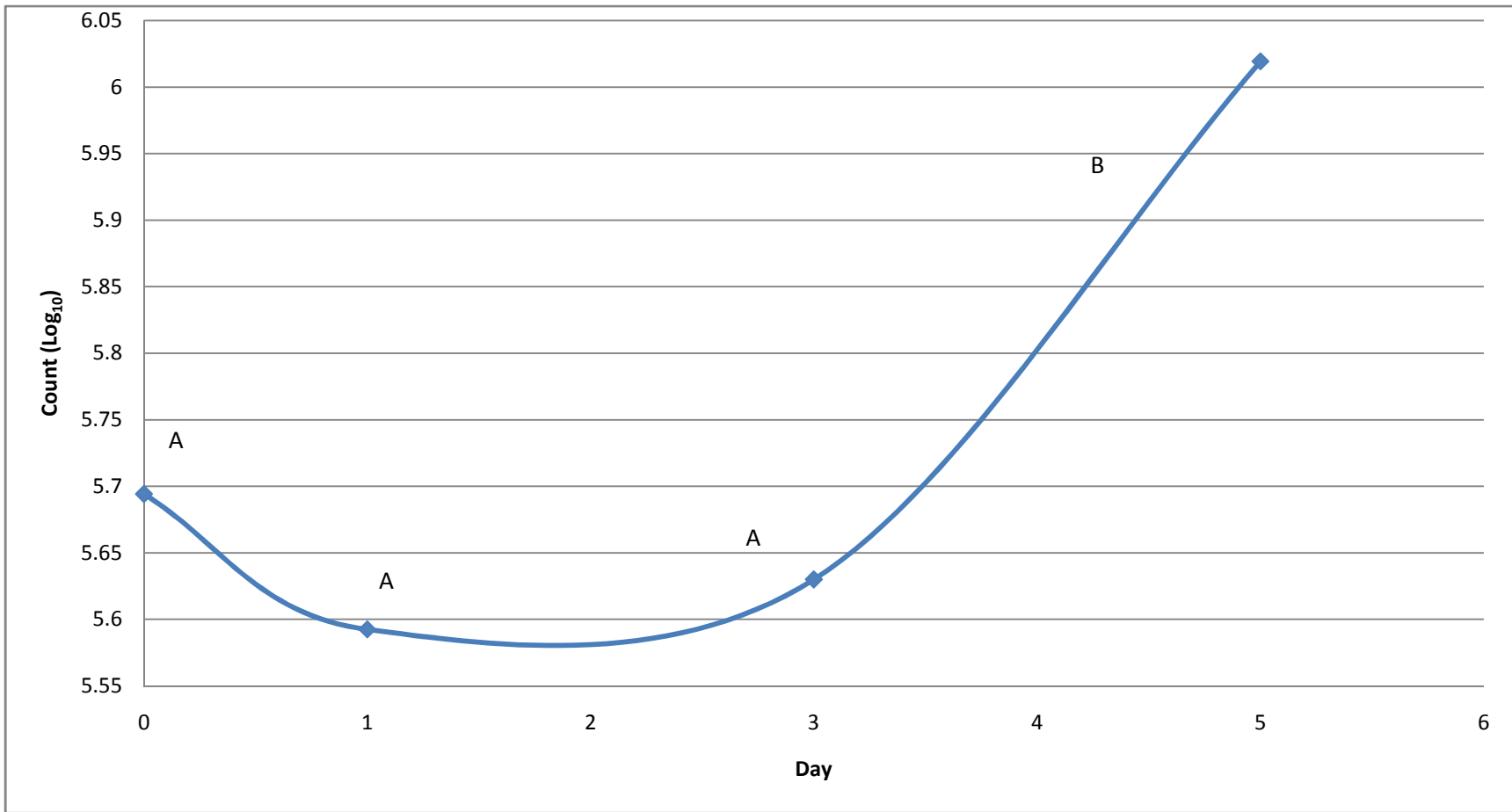
Figure 16 Persimmon Puree in Ground Beef Time Effects on total aerobic CFU/g on Tryptic Soy Agar

a-b= Data with the same letter under each organism represents data that is not significantly different from each other (p = 0.05)

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