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The Effect of Lecithin on Inactivation by Eugenol of *Escherichia coli* O157:H7

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I am submitting herewith a thesis written by Sasha Marie Wilkinson entitled "The Effect of Lecithin on Inactivation by Eugenol of *Escherichia coli* O157:H7." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

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The Effect of Lecithin on Inactivation by Eugenol of *Escherichia coli* O157:H7

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ABSTRACT

Many essential oils and their components are known to have antimicrobial activity. However, their strong aroma, flavor, and hydrophobic nature make them difficult to incorporate into food products. New methods to improve antimicrobial activity at reduced concentrations and/or reduce the influence of food components on antimicrobial activity are needed. In this study, the objective was to combine the emulsifier soy lecithin with eugenol in an attempt to enhance the activity of the latter against the foodborne pathogen *Escherichia coli* O157:H7.

Eugenol was added to a pH 7.2 sodium phosphate buffer (PBS), PBS with 0.03% (v/v) tryptic soy broth (TSB), PBS with 0.3% TSB, PBS with 0.6% TSB or PBS with 0.9% TSB and lecithin solution (0.0025, 0.005, 0.010, or 0.015% (w/v)) and homogenized at 10,000 rpm for 3 min. An overnight culture of *Escherichia coli* O157:H7 was added and survivors enumerated at 0, 1, 3, 6, 12, and 24 h on tryptic soy agar (TSA) incubated for 24 h at 37°C. The effect of varying microbiological media concentrations (0.03-0.09% w/v) on lecithin interaction with eugenol was also evaluated.

In buffer with 550 ppm of eugenol, 0.01% lecithin slightly increased the effectiveness of eugenol ($P<0.05$) while 0.0025, 0.005, and 0.015% samples did not differ from the control. When 600 ppm of eugenol was used, the samples containing 0.0025% and 0.015% lecithin slightly increased the antibacterial activity of eugenol ($P<0.05$) while 0.005, 0.005, and 0.010% lecithin had no significant effect ($P<0.05$). At 650 ppm of eugenol, there was no significant difference ($P<0.05$) between the control

and the lecithin containing samples. In 0.03% TSB, the presence of 0.01% lecithin significantly increased the antibacterial activity of eugenol at both 550 and 650 ppm while 0.1% lecithin significantly decreased the activity. In 0.3% TSB, only minor differences were found while in 0.9% tryptic soy broth (TSB), the antimicrobial activity of eugenol was essentially eliminated except for a 1 log CFU/ml reduction in the presence of 0.01% (but not 0.1%) lecithin. Overall, lower concentrations of lecithin improved the antimicrobial properties of eugenol when media concentrations were low indicating interactions between the lecithin, eugenol and microbiological media.

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CHAPTER I

INTRODUCTION

Foodborne illnesses remain a major issue across the United States despite advances in food safety and processing technologies. The Centers for Disease Control and Prevention report that 1 in 6 Americans suffer from a food related illness every year *(62)*. One potential method for improving food safety would be to add antimicrobials to foods to prevent growth of or inactivate foodborne pathogens. While consumers demand safe and wholesome foods, their acceptance of traditional antimicrobials is declining. As a result, the food industry as a whole is increasingly seeking out label-friendly antimicrobials. A potential solution for the food industry lies in the use of antimicrobials that come from natural sources and do not need to be labeled as synthetic chemical preservatives *(75)*.

Essential oils (EOs) and their components are known to have antimicrobial properties and are considered natural which may be appealing to consumers. Their Generally Recognized as Safe (GRAS) classification makes them desirable to food manufacturers. Because of these properties, EOs have the potential to serve as novel antimicrobial preservatives in food industry. However, in many cases a high concentration, usually over 500 ppm, of the EOs or EO components are needed to inhibit foodborne pathogens *(18)*. These relatively high concentrations make them unsuitable for use in many food products due to strong flavor and aroma characteristics. If the antimicrobial activity of EOs and EO components can be increased, use in the industry as natural food preservatives may be possible.

Eugenol, an EO component of cloves is known to have broad spectrum antibacterial properties. It has been shown by some research that EOs in emulsions may have better activity in foods than free EOs. For example, in a previous study, Li *(46)* found that the antimicrobial activity of eugenol against *Escherichia coli* O157:H7 was increased in the presence of certain concentrations of lecithin. The inactivation rate as measured by D-value (time to decrease the viable population by 90%) in the presence of eugenol decreased from ca. 4.0 min to 1.2 min in the presence of 0.0025% (w/v) lecithin *(46)*. What made the study unique was that at concentrations above 0.0025% lecithin, the D-value increased indicating the potential for an optimum level of lecithin for inactivation. The purpose of the present study was to further investigate if the addition of the emulsifier, soy lecithin, to eugenol was could serve to stabilize the emulsion and increase the antibacterial activity of free eugenol.

The objectives of this study were (1) to determine whether the previous results could be replicated, and (2) what effect time of exposure, lecithin concentration, and concentration of microbiological media had on the antibacterial activity of eugenol against *E. coli* O157:H7.

CHAPTER II

LITERATURE REVIEW

Escherichia coli O157:H7

Background

Escherichia coli are Gram-negative, rod-shaped bacteria that ferment lactose with the production of gas in 48 h at 35°C making them members of the coliform group of bacteria as well as Enterobacteriaceae. There exist strains of *E. coli* that are pathogenic or non-pathogenic. The pathogenic types are enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC), enteroaggregative (EAEC) and enterohemorrhagic (EHEC). Serotype *Escherichia coli* O157:H7 was the first EHEC recognized as a foodborne pathogen when it was isolated in 1982 from four patients in Oregon and Michigan who had eaten undercooked meat from a fast food restaurant chain *(49, 60)*.

The optimum growth temperature for this bacteria is 37°C with an upper limit of 44 to 45°C and a minimum of 8°C *(40)*. It grows well at pH 5.5 to 7.5 but the growth rate declines heavily at lower pH values with a minimum growth pH between pH 4 and 4.5 *(16, 17)*. In food products under the minimum growth pH, *E. coli* O157:H7 can survive for weeks or months with increased survival in refrigeration temperatures *(15)*.

Cattle are a common reservoir of *E. coli* O157:H7 *(74)*. Young cows more frequently carry the bacterial than adult animals. A survey of cattle herds in the United States found an incidence rate of 3.2% among dairy calves *(74)*. The *E. coli* population being shed by the calves was between $<10^2$ and 10^5 CFU/g *(15)*. *E. coli* O157:H7 is

found inside the gastrointestinal tract of cattle, particularly in the fore stomachs, distal ileum, proximal cecum, spinal colon, and descending colon *(15)*. Transmission to humans is most commonly associated with raw beef and milk, but it has been identified in a multitude of different food products including fruit, vegetables, juice, and water *(24, 49, 68)*

Pathogenesis

E. coli's ability to produce Shiga toxins greatly contributes to its ability to cause disease *(47)*. Both Shiga toxin 1 and 2 are produced by *E. coli* O157:H7 but Shiga toxin 2 is more often associated with severe illness *(48)*. This toxin can lead to hemolytic uremic syndrome, hemorrhagic colitis, kidney damage, and death in severe cases *(28, 48)*. The toxin passes through the epithelium of the intestines and disrupts the epithelial cells which line the blood vessels that connect to the kidneys *(69)*. Illness can occur between 1 and 8 days after exposure with an average incubation period of 3 days *(49)*. Symptoms can include diarrhea, vomiting, fever, abdominal pain *(49, 67)*.

Outbreaks

E. coli O157:H7 is the most commonly isolated enterohemorrhagic serotype of *E. coli* in several countries including the United States *(47)*. It is estimated that *E. coli* O157:H7 infections cause 63,000 illnesses and 20 deaths in the United States annually *(62)*. These infections cost an estimated 255 million dollars annually in 2009 dollars *(38)*. While the annual number of *Salmonella* and *Campylobacter* cases exceed the number of *E. coli* O157:H7 cases annually, the fatality rates and hospitality rates of *E. coli* are much higher *(47)*.

Since 2011, there have been 6 multistate outbreaks of *E. coli* O157:H7 reported by the Centers for Disease Control *(11)*. In 2014, a four state outbreak was traced to a Detroit meat packing plant and caused sickness in at least 12 people in four states *(3, 10)*. Of the 12 confirmed cases, all reported eating a hamburger at a restaurant and 8 stated that they had eaten an undercooked hamburger, rare or medium-rare, days before symptoms began *(3)*. In 2013, an outbreak associated with ready-to eat salads infected at least 33 people in four states *(9)*. Romaine lettuce was determined to be the cause of the outbreak and it was believed the lettuce became contaminated at the farm level through wind transfer or from contaminated farm equipment *(2)*. In 2012, 33 infections were reported in 5 states *(8)*. This outbreak was traced to a single producer but the cause of the contamination is not known *(8)*. In 2011, three multistate outbreaks were reported. The first involved 60 people in 10 states who were affected by an outbreak traced to romaine lettuce *(6)*. The second outbreak affected 14 people in 5 states and was traced to store bought bologna *(5)*. The third multistate outbreak in 2011 involved 8 people in 3 states and was caused by in-shell hazelnuts *(7)*.

Control Measures

Apple cider and other fruit juices are have been associated with *E. coli* O157:H7 infections due to the high acid resistance and low infectious dose of *E. coli (44)*. This pathogen is also able to survive for long periods of time in refrigeration temperatures *(73)*. Juices can become contaminated due to contact with contaminated soil, water, or employees. The Food and Drug Association has a 5-Log Reduction Performance Standard in place for fruit and vegetable juices. According to this standard, juice products must be treated so that the population of the pertinent pathogen, frequently

Escherichia coli O157:H7, is reduced by 5 logs in order to comply with the Hazard Analysis and Critical Control Point (HACCP) requirements *(4)*.

Heat treatment is often used to control *E. coli* O157:H7 in food products. This includes pasteurization of liquid products and cooking of solid products to a temperature of 68.3˚C for 15 seconds *(42)*. However, heat treatment is not an option for all food products and may actually reduce the nutritional quality and flavor of some products such as apple cider *(44)*. Additionally, pasteurization processing and materials are also very expensive. Therefore, non-thermal methods of control could be very beneficial to the food industry.

Essential Oils

Background

Spices are defined as aromatic or fragrant plant products which can be used to impact flavor, aroma, or color to food products *(22, 37)*. Herbs are generally defined as leaves and flowering portions of soft-stemmed plants which are used to season food products *(22)*. Typically, herbs and spices are obtained from the fruit, seeds, rhizomes, roots, leaves, bark, flower, or bulb portion of a plant *(22)*. The principal flavoring components of spices are essential oils *(58)*.

Properties

Essential oils are defined as oily liquids which are obtained from aromatic plant materials *(18)*. Each oil can contain 20 to 60 components in various concentrations with two or three components typically found in high concentrations, between 20 and 70%, and the rest being found in trace amounts *(12)*. The major components of essential oils are generally divided into four categories based on their chemical structures. These

categories are terpenes, terpenoids, phenylpropenes, and other *(39)*. Terpenes are primarily responsible for the aroma of essential oils and consist of 5 carbon isoprene units *(1, 39)*. Terpenoids are partially responsible for the flavor and aroma characteristics of essential oils and they consist of repeating branched five carbon units with an isopentance skeleton *(54)*. Phenylpropenes have a six carbon aromatic phenol group *(39)*. Essential oils are produced by plants to provide protection from microorganisms and insects and are extracted from several different areas of plants including leaves, bark, flowers, and seeds *(51, 57)*.

Many essential oil components have been shown to have antibacterial and antifungal properties. The antimicrobial activity of these components is heavily influenced by their hydrophobicity which improves their ability to solubilize and disrupt the phospholipid bilayers of cytoplasmic membranes *(45)*. The structure of essential oil compounds greatly impacts its ability to disrupt cytoplasmic membranes. The presence of a free -OH group in essential oil compounds, such as carvacrol and thymol, is believed to contribute to the EOs ability to disrupt the cytoplasmic membrane *(45)*. Eugenol's hydroxyl group is believed to bind to and interact with proteins *(39)*. In eugenol, EO components such as phenolic alcohol, aldehydes, or ketones are also effective antibacterial agents *(45)*. Phenolic compounds have the ability to disrupt the cytoplasmic membrane, proton motive force, electron flow, and active transport *(18)*. Phenolic compounds may also cause a microorganism to alter its fatty acid or phospholipid composition in the cytoplasmic membrane which may result in a disruption in nucleic acid synthesis, nutrient uptake, and electron transport *(25)*.

In general, Gram-negative bacteria are less susceptible to EOs than Gram-positive bacteria *(30)*. This is due to the presence of lipopolysaccharides in the outer cell membrane which, because of their hydrophilic nature, provide an increased tolerance for hydrophobic compounds such as those found in essential oils *(39, 52)*.

Extraction Methods

Essential oils can be extracted from plants by several different methods including steam distillation, solvent extraction, and supercritical fluid extraction *(13)*. Steam distillation is the most commonly used commercial method. In this method, selected parts of the plant are heated with hot water or steam. The heat causes the cell structures to break down and releases the essential oils which are volatilized and collected via distillation *(70)*. Solvent extraction is more often used for delicate flower materials or fragile plant components which are not resistant to heat or steam treatment *(70)*. For this process, acetone, hexane, petroleum ether, methanol, or ethanol is added to the selected plant material and it is heated to extract the essential oil. The temperature for this process varies by technique with the accelerated solvent extraction technique requiring temperatures ranging from 50 to 180℃ *(41)*. The filtrate is then concentrated using solvent evaporation. Alcohol is mixed with the concentrate and the mixture is distilled at a low temperature which allows the alcohol to absorb the fragrance *(70)*. When the alcohol evaporates, the absolute oil remains *(70)*. To perform supercritical carbon dioxide extraction, carbon dioxide is exposed to high-pressure, about 72 bar, which causes it to take a liquid form *(41)*. Supercritical carbon dioxide is useful because it can penetrate cellular matrices to extract the EO components which are very soluble in it *(41, 70)*. When this is complete, the pressure is returned to normal

atmospheric conditions, around 1 bar, and the carbon dioxide then reverts to its gas form and evaporates leaving the essential oil compounds *(41, 70)*. Carbon dioxide is a good solvent for this extraction because it has a low toxicity, is easy to separate from the extracted compounds, and has a low cost *(34, 76)*.

Use in Food Industry

EOs are primarily used in the food industry to add desirable flavors and/or aromas to food products *(53)*. US FDA regulations classify these compounds as generally recognized as safe (GRAS) for use in foods. Because they are GRAS and are considered "natural" additives, the food industry has great interest in EOs for uses other than their contribution to flavors, such as use as antimicrobials and antioxidants. This is because of a growing consumer demand for food products with "less processing" and more "natural" ingredients *(61)*. Thus, EOs have been investigated and actually applied as food preservatives due to their antibacterial and antifungal capabilities *(23, 53)*.

Eugenol as an Antimicrobial Compound

Properties

Eugenol (Figure 1) belongs to the phenylpropanoid class of compounds. It is the major component of several oils including allspice, basil, and clove and makes up approximately 84% of clove EO and 72% of cinnamon leaf EO *(20, 51)*. This oily substance is typically a clear or pale yellow color *(71)*. Eugenol has a molecular weight of 164.2 and is partially hydrophobic *(31, 43)*.

Antibacterial Activity

Eugenol is a particularly effective essential oil against foodborne pathogens such as *E. coli* due to the amount of phenolic compounds it possesses. It has the ability to

penetrate the cellular membranes of bacteria and cause ATP and potassium to leak from the cell *(32, 35, 39, 72)*. It has been found to inhibit amylase and protease production in *Bacillus cereus* and contributed to the deterioration of the cell wall and the inhibition of enzyme activity in *Enterobacter aerogenes (71).* The antimicrobial activity of eugenol against the Gram-negative bacteria *Proteus mirabilis* is due to its ability to bind to the cytoplasmic membrane of the bacterium and make it more permeable *(27)*. Additionally, eugenol can cause pores to form in the plasma membrane of bacteria which causes intracellular proteins to be released *(27)*.

Sensory Characteristics

Essential oils are frequently used in the food industry to impart flavor and aroma characteristics to food products *(39)*. The concentration of essential oils required to act as preservative agents in food, however, is much higher. In order to achieve antimicrobial activity in food products, concentrations over 500 ppm must be used for many products *(18, 66)*. At that concentration, however, major changes to the sensory characteristics of food products will likely make the product unacceptable to consumers. In a recent study, the tolerance limit for carvacrol, thyme, and rosemary essential oils was found to be 20 ppm or less when tested on a hedonic scale in tomato juice and vegetable juice *(29)*. Many spice components, including eugenol, contribute a bitter or pungent taste to food *(36)*.

Enhancing Antimicrobial Effectiveness of Eugenol and other EO components

EO components, while effective against a variety of bacteria, are difficult to incorporate into food products. This is due to their hydrophobic nature and because of the complex nature of food. EO components may react with hydrophobic food

components which reduces their activity. Because of this, a high concentration of EO or EO components is required which may lead to potential sensory problems. Therefore, the effectiveness of EOs must be increased so that a lower, and more commercially acceptable, amount can be used. The use of limiting intrinsic and/or extrinsic factors for foods along with reduced concentration EO treatments could potentially be used as preservation hurdle technologies. These limiting factors may include pH, temperature, atmosphere, combinations of EO components or and the use of compounds such as emulsifiers to enhance antimicrobial activity. Hurdle technology is the combination of two or more preservation parameters which creates maximum control against microorganisms while preserving the sensory characteristics of the product *(64)*.

One potential method to enhance EO effectiveness is to adjust the pH of the environment. The effectiveness of essential oil components in different pH environments has been evaluated. Several essential oils and components were tested at pH 7.2, 4 and 4.5. The minimum inhibitory concentration (MIC) of eugenol was 0.05% v/v against *E. coli* O157:H7 at pH 7.2. When the pH was reduced to 4.5, the MIC decreased to 0.025% and at pH 4 the MIC was reduced to <0.0031% v/v. Additionally, the minimum bactericidal concentration at pH 7.2 was 0.1% and was decreased to 0.05 at pH 4 *(55)*. Temperature may also contribute to the effectiveness of EOs. EOs were found to be more effective at room temperature in one study due to the changes in membrane fluidity *(59)*. At low temperatures, the phospholipids in the membrane are more closely packed together forming a rigid gel structure. At higher temperatures, the phospholipids are less tightly packed and the membrane has a liquid-crystalline structure *(59)*. Combinations of essential oil components have increased the

antibacterial effects against some microorganisms. The minimal inhibitory concentration against *E. coli* CGMCC 1.487 was decreased form approximately 1600 ppm to 400 ppm when treated with a combination of cinnamaldehyde, thymol, or carvacrol *(56)*.

The inclusion of emulsifiers on the activity of EOs has been evaluated extensively, either for their potential enhancement or for producing water soluble micro- or nanoparticles. In one study, researchers combined EOs with lecithin and agar stabilizers. When 0.25% (w/v) lecithin was tested in combination with oregano oil and thyme oil, the antibacterial properties of the EOs against *E. coli* O157:H7 was greatly reduced. It was proposed by the authors that the lecithin interfered with the ability of the essential oils to physically interact with the cells of the bacteria *(19)*. When combined with 0.05% agar, the antibacterial properties of EOs was significantly increased. Researchers believe this is due to less rapid separation of the essential oil components from the water phase or because the ability of the essential oil component to interact with the phospholipids in the outer layer of the bacteria was neutralized by the additional presence of phospholipids in lecithin *(19)*.

Lecithin

Background

Lecithin (Figure 2) is widely used in the food industry as an emulsifier, wetting agent, release agent, anti-spatter agent, and phosphate dispersant *(65)*. It contains a mixture of phospholipids including phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidic acid, phosphatidyl serine, and lysophosphatides

Lecithin can be produced from many sources of living matter including plant, animal, and microbial sources *(63)*. It was first derived from egg yolk but it is now more

commonly recovered from plant sources including cottonseed, peanuts, sunflower seeds, sesame seeds, flax seed, and soybeans *(21)*. For commercial use, lecithin derived from vegetable oil, primarily soybeans, is the most common *(21)*.Soybean lecithin, on average, contains about 35% soybean oil, 18% phosphatidylcholine, 15% phosphatidylethanolamine, 11% phosphatidylinositol, 9% other phosphatides, and 12 % carbohydrates *(50)*. Commercial soybean lecithin is typically obtained as a by-product of vegetable oil production. Several methods can be used to extract lecithin from soybeans including hydraulic pressing, screw pressing, prepress solvent extraction, and direct solvent extraction *(21)*.

Use in Food Industry

Lecithin obtained from vegetable sources has GRAS regulatory status in the United States *(63)*. With modification, lecithin can be used in both oil-in-water and water-in-oil emulsions *(26)*. The emulsification properties of lecithin are largely due to the presence of phospholipids, the main component of lecithin. These phospholipids are partly hydrophobic (non-polar) and partly hydrophilic (polar) *(33)*. The hydrophilic portion is soluble in water while the hydrophobic portion is soluble in oil *(14)*. To create an emulsion, the emulsifier orients at the boundary between two immiscible liquids with the hydrophobic portion in the oil phase and the hydrophilic portion in the water phase *(14)* thereby stabilizing it.

CHAPTER III

MATERIALS AND METHODS

Bacterial Strain

E. coli O157:H7 strain ATCC 43889 was obtained from the Department of Food Science at the University of Tennessee. This was grown in tryptic soy broth (TSB; Difco, Sparks, MD) at 37°C for 24 h, combined with glycerol, and kept at 0°C. Working cultures were created by inoculating 10 ml of TSB and incubating for 24 h at 37°C.

Eugenol-Lecithin Preparations

Eugenol (4-allyl-2-methoxyphenol, 99%) was obtained from Acros Organics (Fairlawn, NJ) and lecithin (soybean lecithin, >99%) was obtained from Fisher Scientific (Fairlawn, NJ).

To replicate the original experiments on which this study was based, the concentration of eugenol was 800 ppm, the concentrations of lecithin used ranged from 0.001 to 0.015% w/v, the suspension medium was 0.5% TSB (based upon carryover of medium from the culture), and the time of exposure was 0 to 30 min.

For the initial experiments, (0.5% w/v TSB), lecithin concentrations ranged from 0.001% w/v to 0.015% w/v. Lecithin was added to 0.1M sodium phosphate buffer (pH 7.2) which had been sterilized at 121°C for 15 min using an autoclave. The lecithin in buffer mixture was stirred continuously while heating to boiling (ca. 100°C) for 5 min to dissolve the lecithin. Distilled water was added to the solution to replace the water lost during boiling (about 3% of total volume). To prepare emulsions, 800 ppm eugenol was added to stock lecithin buffer solutions in 125 ml Erlenmeyer flasks and samples were

then homogenized at 10,000 rpm using a Polytron PY 10/35 homogenizer (Kinematica, Inc., Bohemia New York) for 3 min at room temperature *(46)*.

For the next set of experiments in 0.03%, 0.3%, 0.6% and 0.9% (w/v) TSB, lecithin was added at concentrations ranging from 0.001% w/v to 0.015% w/v. Lecithin was added to 0.1M sodium phosphate buffer (pH 7.2) which had been sterilized at 121°C for 15 min using an autoclave. The lecithin in buffer mixture was stirred continuously while heating to boiling (ca. 100°C) for 5 min to dissolve the lecithin. Distilled water was added to the solution to replace the water lost during boiling (about 3% of total volume). To prepare emulsions, 550, 600 or 650 ppm eugenol was added to stock lecithin buffer solutions in 125 ml Erlenmeyer flasks and samples were then homogenized at 10,000 rpm using a homogenizer (Polytron, Kinematica) for 3 min *(46)*.

Inactivation Experiments

A 24 h culture of *E. coli* O157 that had been grown at 37°C was then added to each flask containing eugenol-lecithin mixtures. Flasks were incubated statically at 25°C and a sample was removed from each flask at 0, 1, 3, 6, 12, and 24 h to enumerate *E. coli* O157. Samples were serially diluted using 0.1% peptone water (Difco) and spread plated in duplicate on tryptic soy agar (TSA; Difco). TSA plates were incubated for 24 h at 37°C and the log CFU/ml was calculated for each sample.

For samples containing 0.03% w/v media, 99 ml of the lecithin, eugenol, and phosphate buffer solution were added to a flask and 1 ml of an overnight (24 h) culture of *E. coli* O157:H7 was added. For samples containing 0.3% w/v media, 90 ml of the lecithin, eugenol, and phosphate buffer solution was added to the flask and 10 ml of an overnight (24 h) culture of *E. coli* O157:H7 was added. For samples containing 0.6%

w/v media, 80 ml of the lecithin, eugenol, and phosphate buffer solution was added to the flask and 10 ml of an overnight (24 h) culture of *E. coli* O157:H7 was added. Then 10 ml of sterile TSB broth was added. For samples containing 0.9% w/v media, 70 ml of the lecithin, eugenol, and phosphate buffer solution was added to the flask and 10 ml of an overnight (24 h) culture of *E. coli* O157:H7 was added. Then 20 ml of sterile TSB broth was added.

Effect of Time

In the present experiments, the maximum incubation times evaluated initially were 30 min but were lengthened to 24 h for subsequent experiments.

Statistics

Each experiment was repeated three times. Analysis of variance was conducted using version 9.4 of Statistical Analysis Software. Least significant differences were used to compare treatments and significant difference was defined as $P<0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

In initial testing, 800 ppm of eugenol was combined with 0 to 0.015% lecithin in buffer containing 0.03% TSB and *E. coli* O157:H7 was exposed for 30 min (Fig. 3). Lecithin (0.001, 0.0025, 0.005, 0.005, and 0.015% w/v) did have a significant effect on increasing the activity of eugenol compared to the control ($P< 0.05$) after 30 min but reductions were only ca. 1 log CFU/ml. The concentration of lecithin did not appear to have any relationship to the extent of inhibition as was reported in a previous study where 0.0025% lecithin was found to be an "optimum" level for inactivation *(46)*. Since the findings of Li *(46)* could not be replicated, the effect of eugenol and lecithin concentrations, time, and microbial media concentration were investigated further.

The first thing evaluated was the antimicrobial effect of lecithin itself. The addition of soy lecithin alone (0.0025 to 0.015%) in 0.3% TSB had no antimicrobial effect on *E. coli* O157:H7 after 24 h ($P<0.05$) (Fig. 4). There also was no significant reduction ($P<0.05$) in the population of *E .coli* O157:H7 after 24 h in the control buffer.

Next the effects of varying concentrations of eugenol (550-650 ppm) and the presence of microbiological medium (0.03-0.9%) in the phosphate buffer on the antimicrobial activity against *E. coli* O157:H7 was determined (Fig. 5-7). In the experiments performed by Li *(46)*, 10 ml of an overnight *E. coli* culture in TSB was added to 45 ml of lecithin and phosphate buffer solution. This resulted in a total TSB concentration of 0.5% w/v in each sample. In order to determine whether microbiological media concentration had an effect on the antibacterial properties of eugenol and lecithin, 0.03% w/v, 0.3% w/v, 0.6% w/v, and 0.6% w/v media were added

to the lecithin-eugenol mixtures and the inactivation experiment was repeated. At 550 ppm eugenol, there was no reduction (< 1 log CFU/ml) in viable *E. coli* O157:H7 nor any significant differences between 0.6 and 0.9% media ($P<0.05$) (Fig. 5). When the media concentration was reduced to 0.3%, *E. coli* O157:H7 was significantly lower ($P<0.05$) than 0.6 and 0.9% media but the average log reduction was only 1.72 log CFU/ml. At 0.03% media, a smaller volume of culture reduced the initial count by 1 log but the reduction after 24 h was much greater at ca. 4.9 log CFU/ml compared to 0.6 and 0.9%. Similarly to 550 ppm, at 600 ppm eugenol there was no reduction (< 1 log CFU/ml) in viable *E. coli* O157:H7 nor any significant differences between 0.6 and 0.9% media (Fig. 6). When the media concentration was reduced to 0.3%, *E. coli* O157:H7 counts were significantly lower ($P<0.05$) than 0.6 and 0.9% media with an average log reduction of 1.83 log CFU/ml. At 0.03% media, a smaller volume of culture reduced the initial count by 1 log. After 24 h of incubation there was a very large decrease in viable counts of ca. 7 log CFU/ml compared to the 0.6 and 0.9% media. At 650 ppm eugenol, there was no reduction (< 1 log CFU/ml) in viable *E. coli* O157:H7 nor any significant differences between 0.6 and 0.9% media (Fig. 7). When the media concentration was reduced to 0.3%, *E. coli* O157:H7 showed an average log reduction of 4.8 log CFU/ml which was significantly lower ($P<0.05$) than 0.6 and 0.9% media. At 0.03% media, the reduction after 24 h was ca. 7 log CFU/ml compared to 0.6 and 0.9% media. Overall, it was evident that TSB had significantly negative effect on the antimicrobial activity of eugenol against *E. coli* O157:H7.

Next, the interactive effects of varying concentrations of eugenol (550, 600, and 650 ppm) with lecithin (0.0025, 0.005, 0.01, and 0.015% w/v) in phosphate buffer on the

antimicrobial activity of eugenol against *E. coli* O157:H7 was determined (Fig. 8-10). At 550 ppm eugenol, there was no significant difference between 0.0025, 0.005, and 0.015% lecithin compared to 0% lecithin (Fig. 8). All showed an average of 1.7 log CFU/ml reduction ($P<0.05$). When the lecithin concentration was 0.010% w/v, *E. coli* O157:H7 counts were significantly lower ($P<0.05$) with an average reduction of 3.07 log CFU/ml after 24 h. At 600 ppm, there was no significant difference in viable *E. coli* O157:H7 counts between 0.005 and 0.01% lecithin compared to 0% lecithin (1.8 log CFU/ml reduction) ($P<0.05$) (Fig. 9). At 0.0025 and 0.015% w/v lecithin, *E. coli* counts were significantly lower ($P<0.025$) than 0% lecithin with an average log reductions of 2.80 log CFU/ml and 3.51 log CFU/ml, respectively, after 24 h. 650 ppm eugenol with no lecithin was lethal to the *E. coli* O157:H7 with a reduction of nearly 5 log CFU/ml after 24 h (Fig. 10). The addition of soy lecithin (0.0025, 0.005, 0.010, and 0.015% w/v) had no additional antimicrobial effect on *E. coli* O157:H7 and, in fact, 0.0025% was antagonistic. Therefore, while higher concentrations of eugenol caused greater reduction of viable *E. coli* O157:H7, the lecithin either had no effect or the effect was not related to concentration.

The influence of media concentration on interaction between eugenol and lecithin was evaluated by reducing the concentration of TSB to 0.03%. Eugenol was tested at 550 or 650 ppm and lecithin was tested at 0.01 or 0.1% w/v (Fig.11-12). With 550 ppm eugenol and no lecithin, the reduction in viable counts of ca. 4 log CFU/ml after 24 h in 0.03% TSB (Fig. 11) was much larger than that the 1.5 log CFU/ml reduction after 24 h seen in 0.3% TSB (Fig. 8). When the lecithin concentration was 0.1% w/v, viable *E. coli* O157:H7 were significantly higher ($P<0.05$) than the 0% lecithin control with an average

log reduction of ca. 2.3 log CFU/ml. When the lecithin concentration was reduced to 0.01%, at 24 h viable cells of *E. coli* O157:H7 were significantly reduced by an average 6.73 log CFU/ml compared to time 0 which was significantly lower ($P<0.05$) than 0% lecithin. Increasing the eugenol concentration to 650 ppm enhanced inactivation under all test conditions compared to 550 ppm but the pattern was very similar with 0.01% lecithin dramatically increasing the inactivation of eugenol while 0.1% had an antagonistic effect (Fig. 12). With 0.1% w/v lecithin, *E. coli* O157:H7 counts were significantly higher ($P<0.05$) than 0% lecithin at 24 h. After 24 h, the populations of *E. coli* O157:H7 in both the 0% and 0.01% lecithin samples were below the detection limit of 1 log CFU/ml. Thus, it was again evident that TSB concentration had a very large impact on the antimicrobial activity of eugenol and lecithin with higher concentrations reducing activity. In addition, at lower TSB concentrations, higher concentrations of lecithin negatively impacted eugenol antimicrobial activity against *E. coli* O157:H7. This was further confirmed by evaluating the compounds in 0.9% TSB (Fig. 13-14). Here the effect of 550 or 650 ppm eugenol 0.01 or 0.1% lecithin were evaluated for their antimicrobial activity against *E. coli* O157:H7. As with 0.3% media (Fig. 8), neither 550 ppm eugenol alone or in combination with either concentration of soy lecithin had any antimicrobial effect on *E. coli* O157:H7 after 24 h ($P<0.05$) (Fig. 13). Similarly, at 650 ppm eugenol had no antimicrobial activity on *E. coli* O157:H7 after 24 h by itself nor with addition of 0.1% w/v lecithin ($P<0.05$) (Fig. 14). When the concentration of lecithin was decreased to 0.01% w/v, viable *E. coli* O157:H7 was significantly reduced ($P<0.05$) compared to the 0 and 0.1% lecithin with an average log reduction of 1.45 log CFU/ml.

Overall, a reduction of the antimicrobial activity of eugenol in the presence of 0.1% lecithin was consistent with the findings of Burt and Reinders (*19*). They showed that the addition of 0.25% lecithin greatly reduced the antibacterial properties of oregano, thyme oil light and thyme oil red against *E. coli* O157:H7 *(19)*. This reduction was likely due to the lecithin physically impeding contact of the oil with the bacteria or because the EO activity against the phospholipids in the bacterial cell membrane was limited due to the interaction of the EO with the phospholipids present in soy lecithin *(19)*.

It was apparent that media concentration affected the antimicrobial activity of the eugenol and the eugenol-lecithin combinations. For example, 0.01% lecithin significantly reduced the effectiveness of eugenol in the presence of 0.9% media, had almost no effect in the 0.3% media samples, and significantly increased the effectiveness of eugenol in 0.03% media after 24 h. Based on the results of this study, enhancement of eugenol antimicrobial activity against *E. coli* O157:H7 by an optimum concentration of lecithin was demonstrated as was reported by Li (*46*). This may be due to differences in TSB lots. As it was demonstrated in the present study, microbiological medium concentration has a large influence on the antimicrobial activity of eugenol and lecithin, and differences in the media could affect the interaction of lecithin and eugenol. Additionally, differences in the *E. coli* culture handling and errors measuring such small lecithin concentrations used by Li *(46)* may have influenced the results.

CHAPTER V

CONCLUSIONS

The ability of soy lecithin to enhance the antimicrobial activity of eugenol appears to be dependent on media concentration of the sample. In low media conditions, 0.01% lecithin greatly increased the antibacterial activity. Therefore, lecithin is a promoter of eugenol activity in limited situations. This is likely due to lecithin increasing the stability of the emulsion which can slow the separation of the oil and the water phase and improve the ability of eugenol to inhibit *E. coli (19).* In order to improve effectiveness, the use of lecithin may be combined with other treatments to further increase the antibacterial properties of essential oils.

Tryptic soy broth was found to have a significant effect on the antimicrobial activity of eugenol and lecithin. Further studies should therefore determine how the structure and nutrient availability of food products affects the effectiveness of eugenol and lecithin in the absence or near absence of TSB.

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APPENDIX

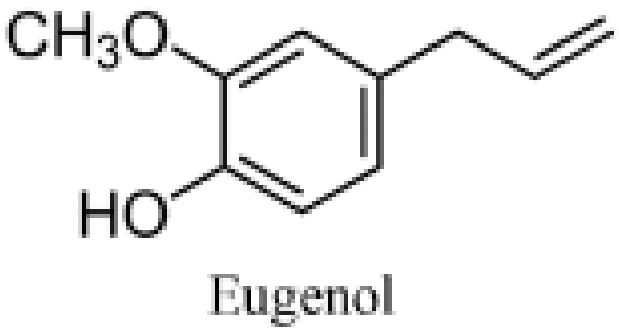

Figure 1 Structural Formula of Eugenol (from Hyldgaard and others (40))

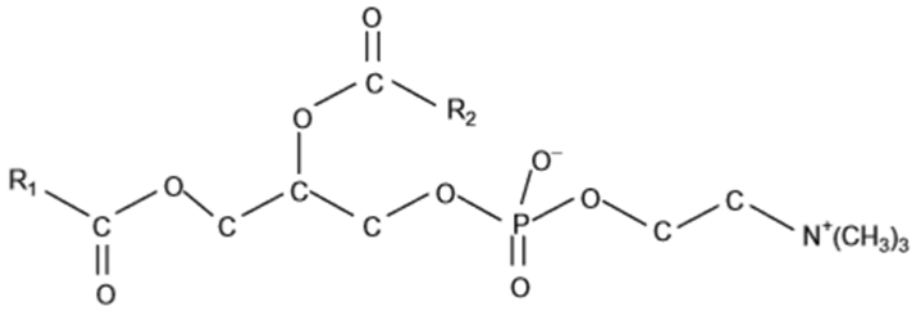

Figure 2 Molecular structure of phosphatidylcholine. R1 and R2 are alkyl chains (From Weland and Hartel (75))

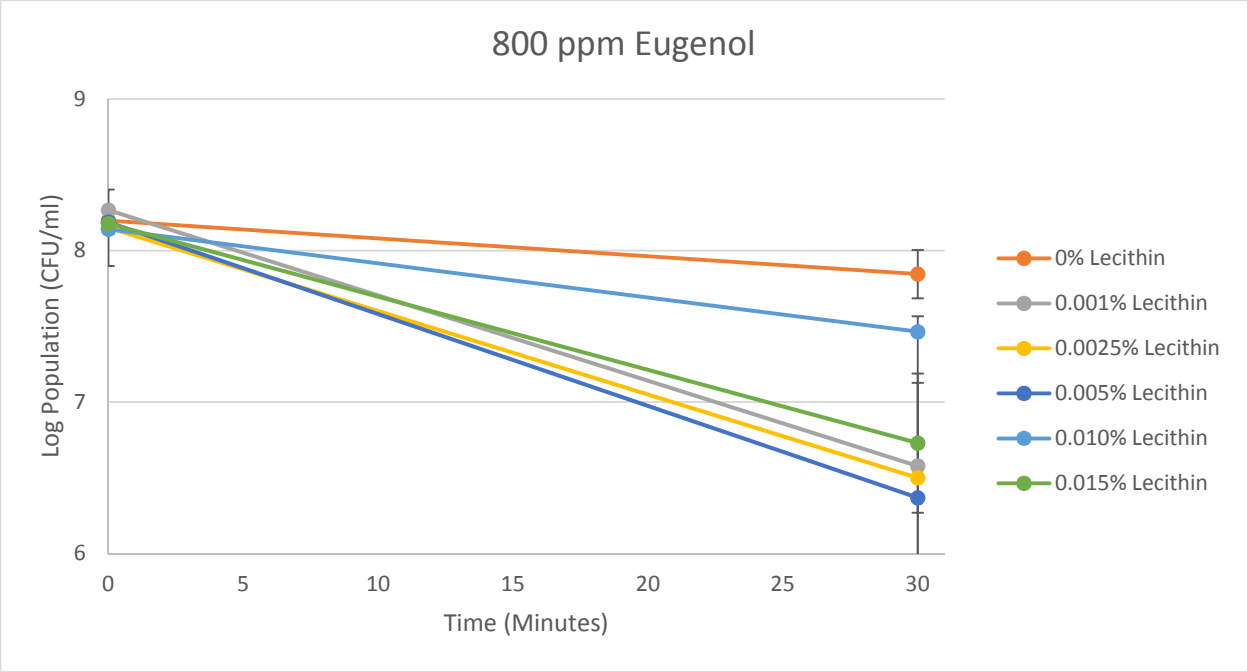

Figure 3 Average log population of E. coli O157:H7 with 0.5% w/v media, 800 ppm of eugenol, and varying lecithin concentrations in phosphate buffer after 30 min.

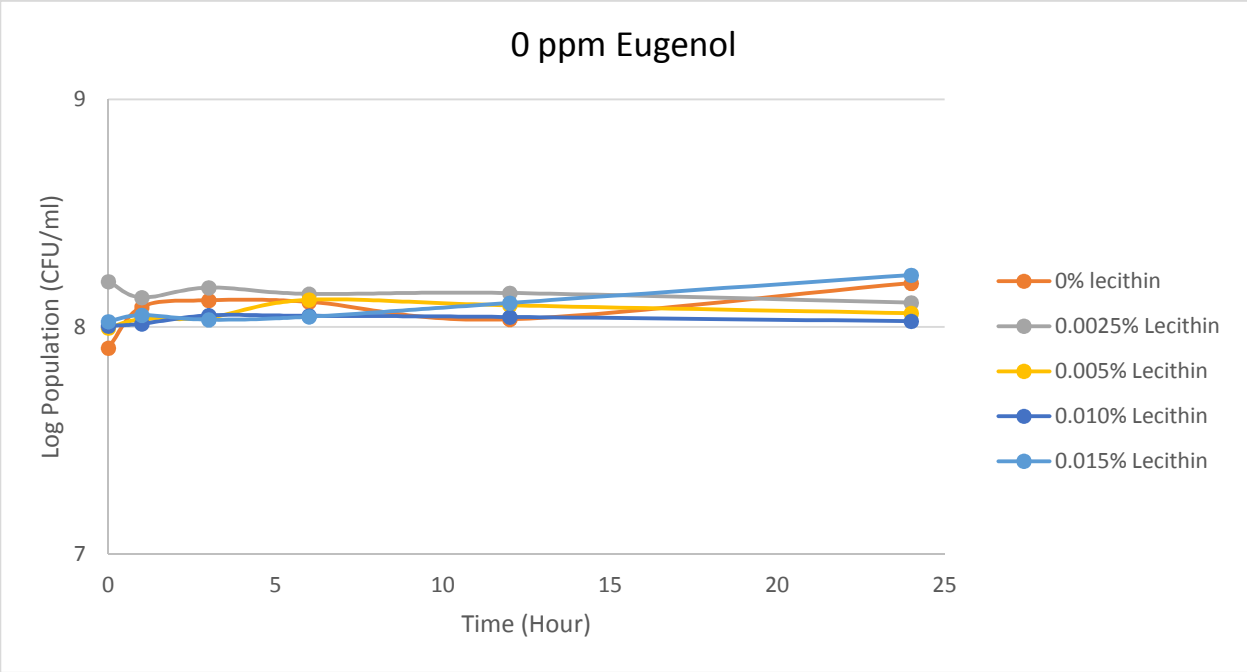

Figure 4 Average log population of Escherichia coli O157:H7 in sodium phosphate buffer (pH 7.2) with varying amounts of soy lecithin and 0.3% w/v media

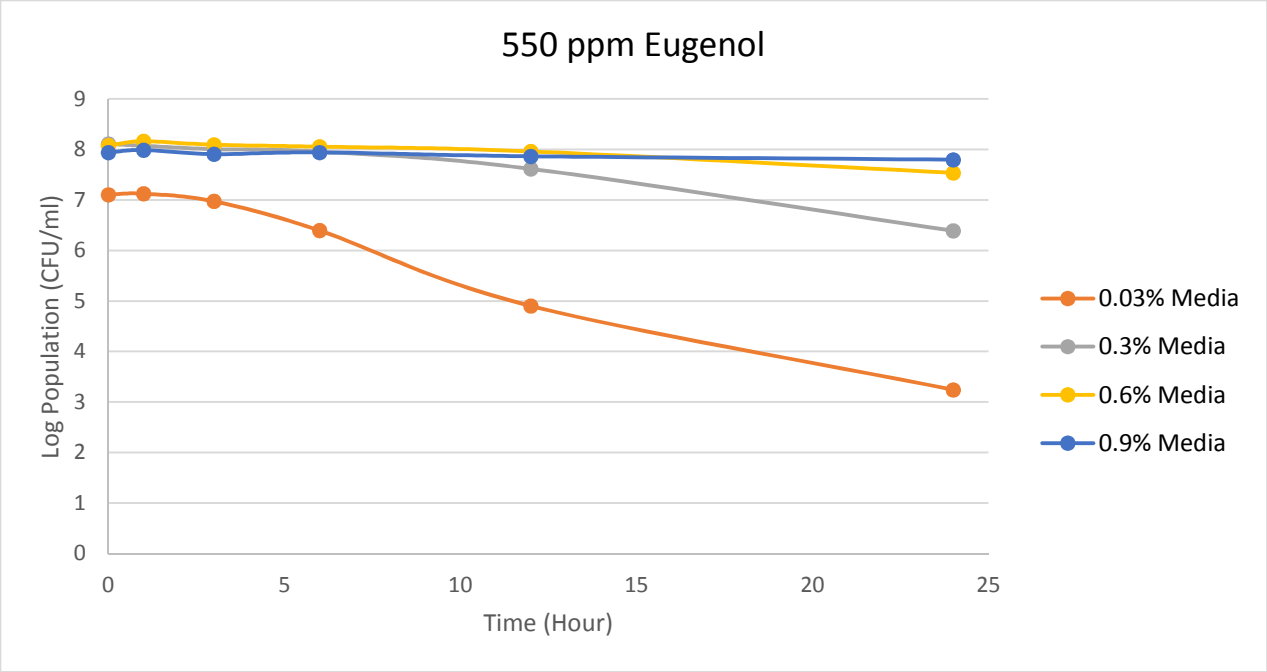

Figure 5 Average log population of Escherichia coli O157:H7 in sodium phosphate buffer (pH 7.2) with varying amounts of media and 550 ppm eugenol v/v

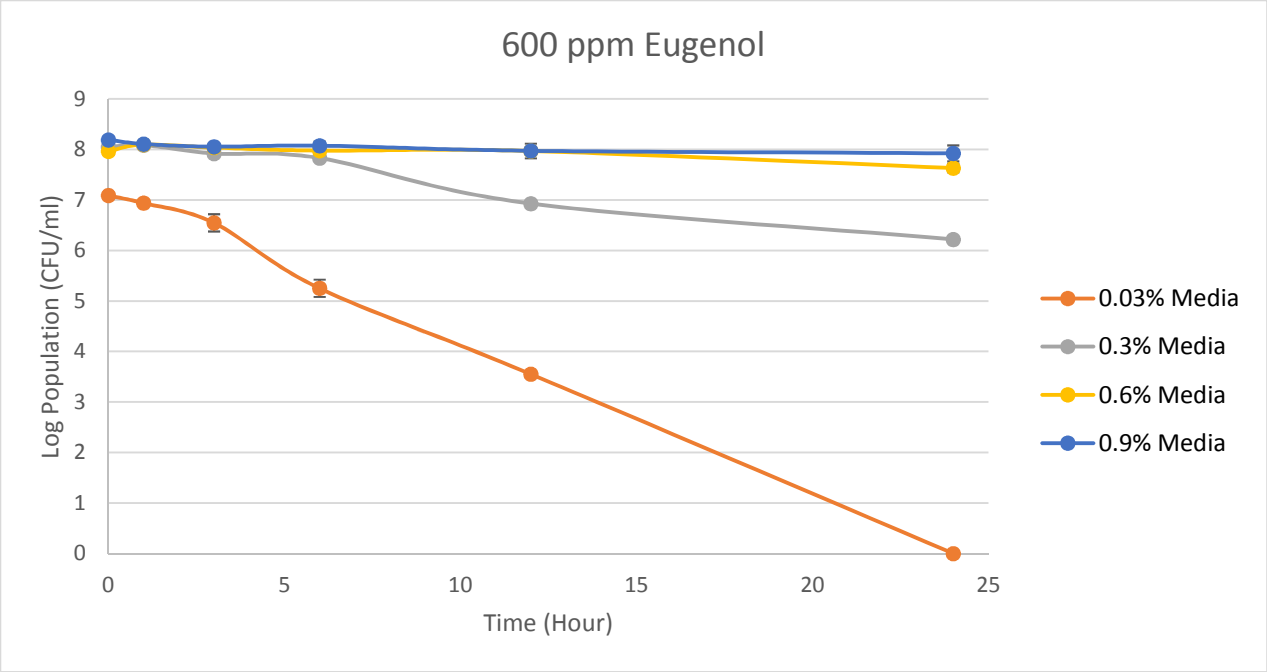

Figure 6 Average log population of Escherichia coli O157:H7 in sodium phosphate buffer (pH 7.2) with varying amounts of media and 600 ppm eugenol v/v

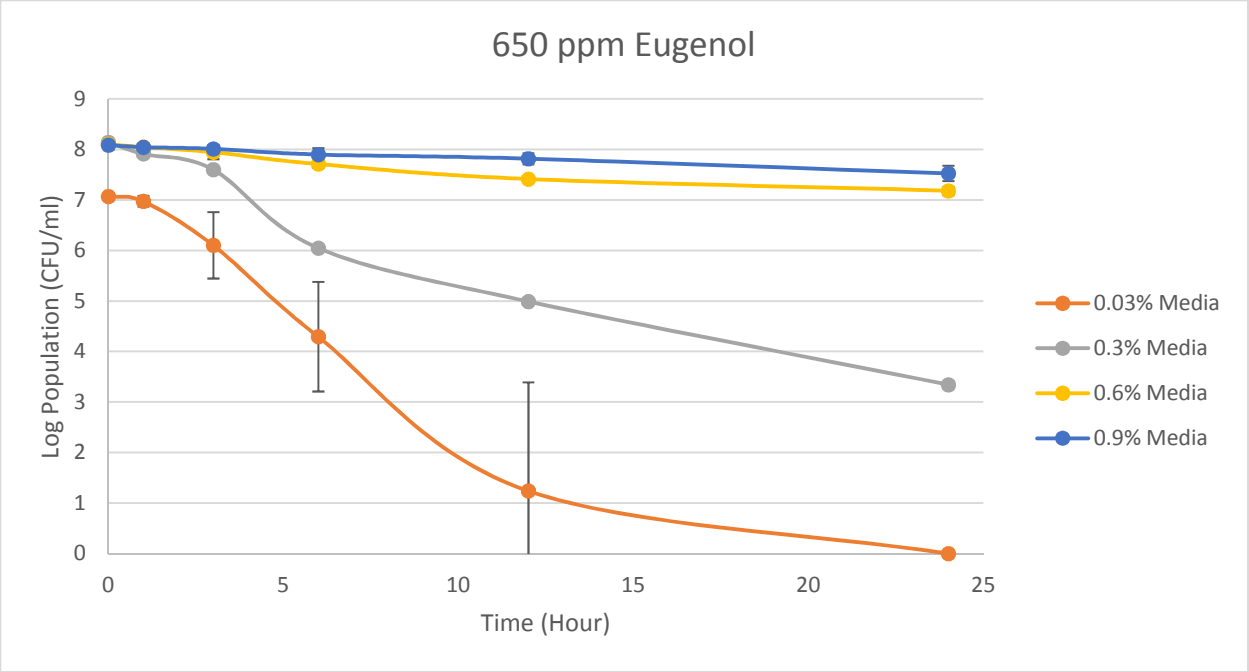

Figure 7 Average log population of Escherichia coli O157:H7 in sodium phosphate buffer (pH 7.2) with varying amounts of media and 650 ppm eugenol v/v

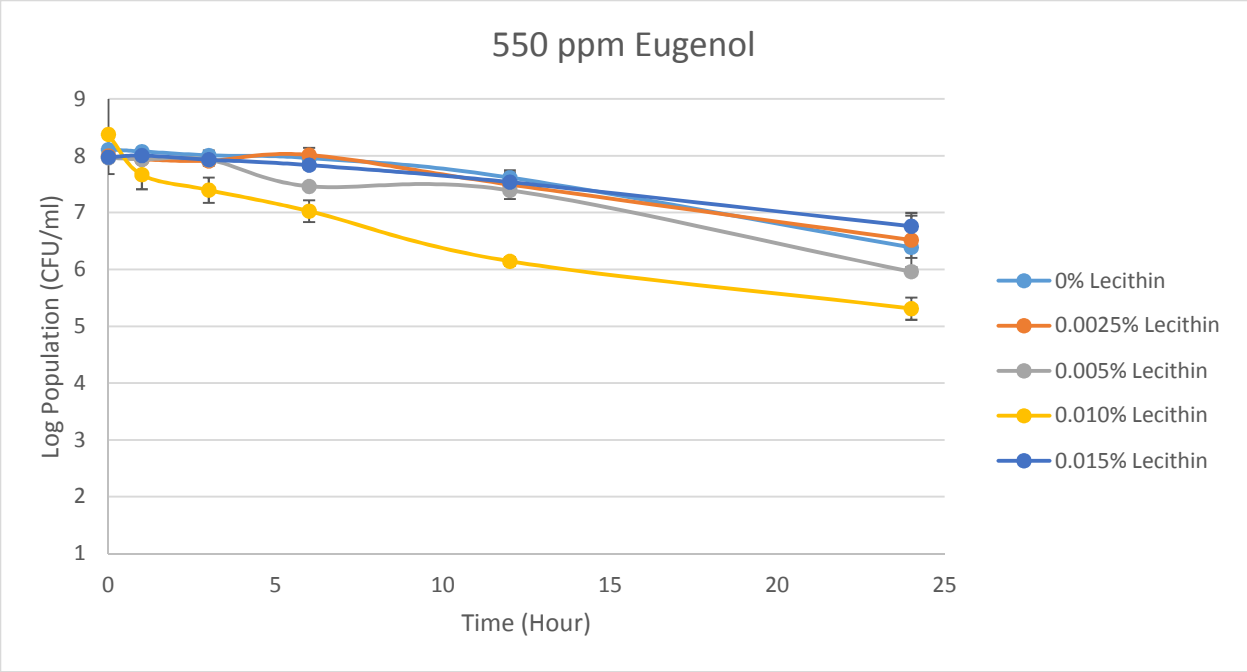

Figure 8 Average log population of Escherichia coli O157:H7 in sodium phosphate buffer (pH 7.2) with varying amounts of soy lecithin, 0.3% w/v media, and 550 ppm eugenol v/v

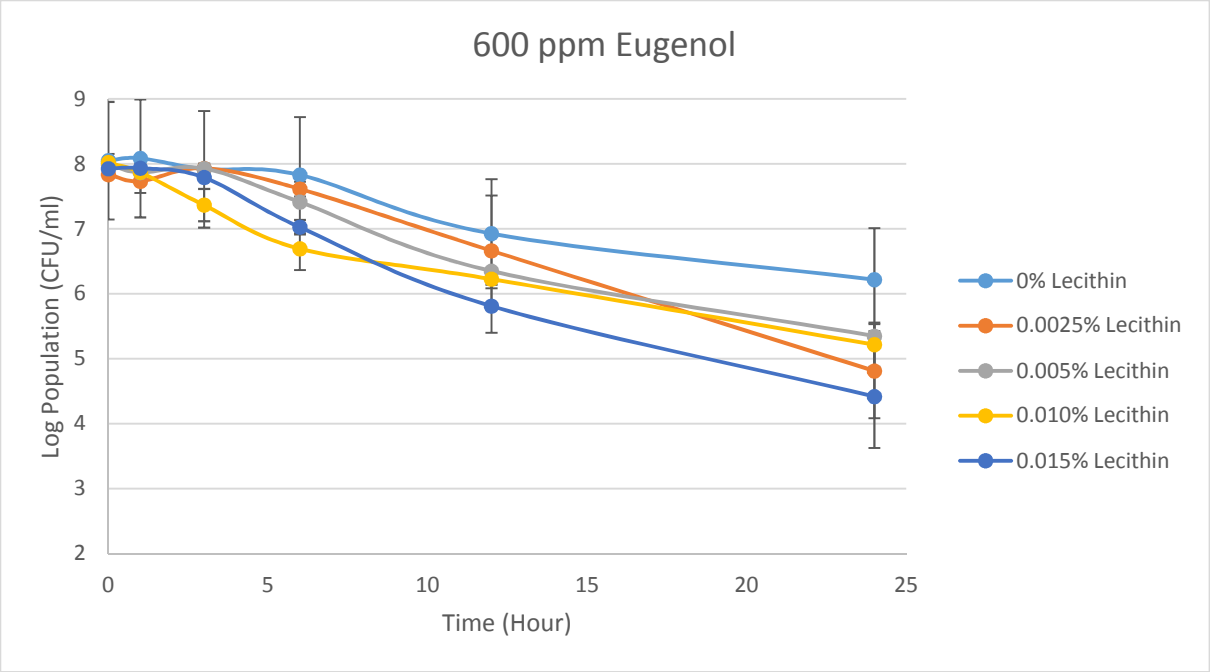

Figure 9 Average log population of Escherichia coli O157:H7 in sodium phosphate buffer (pH 7.2) with varying amounts of soy lecithin, 0.3% w/v media, and 600 ppm eugenol v/v

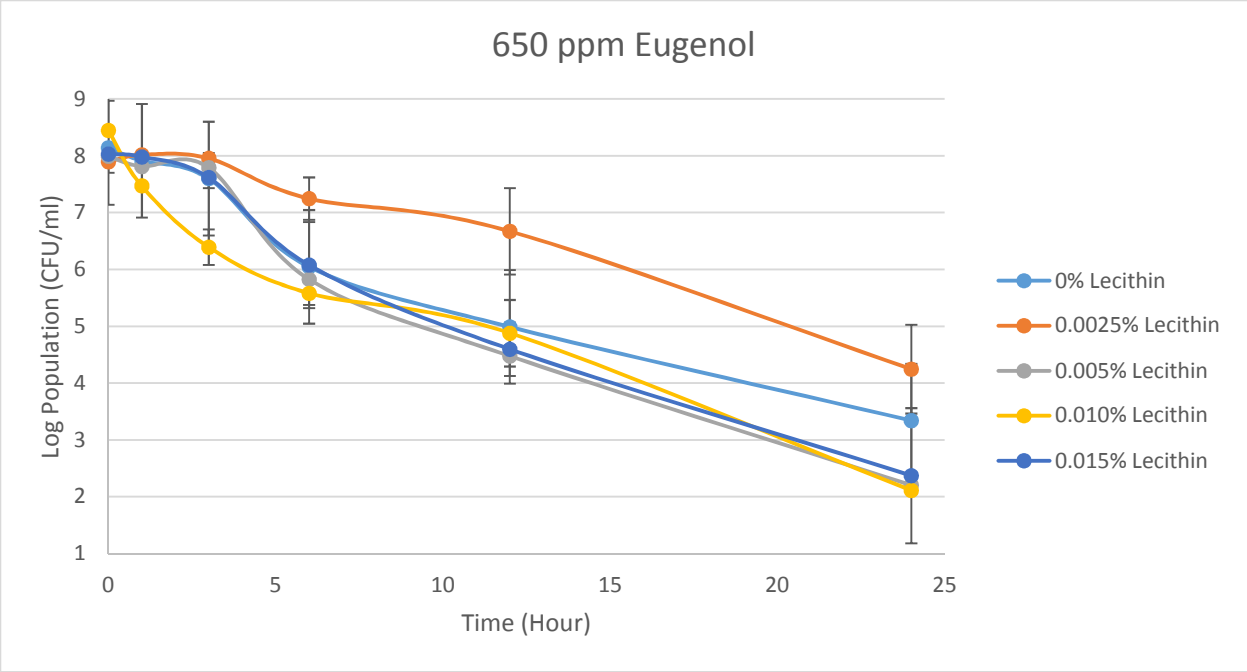

Figure 10 Average log population of Escherichia coli O157:H7 in sodium phosphate buffer (pH 7.2) with varying amounts of soy lecithin, 0.3% w/v media, and 650 ppm eugenol v/v

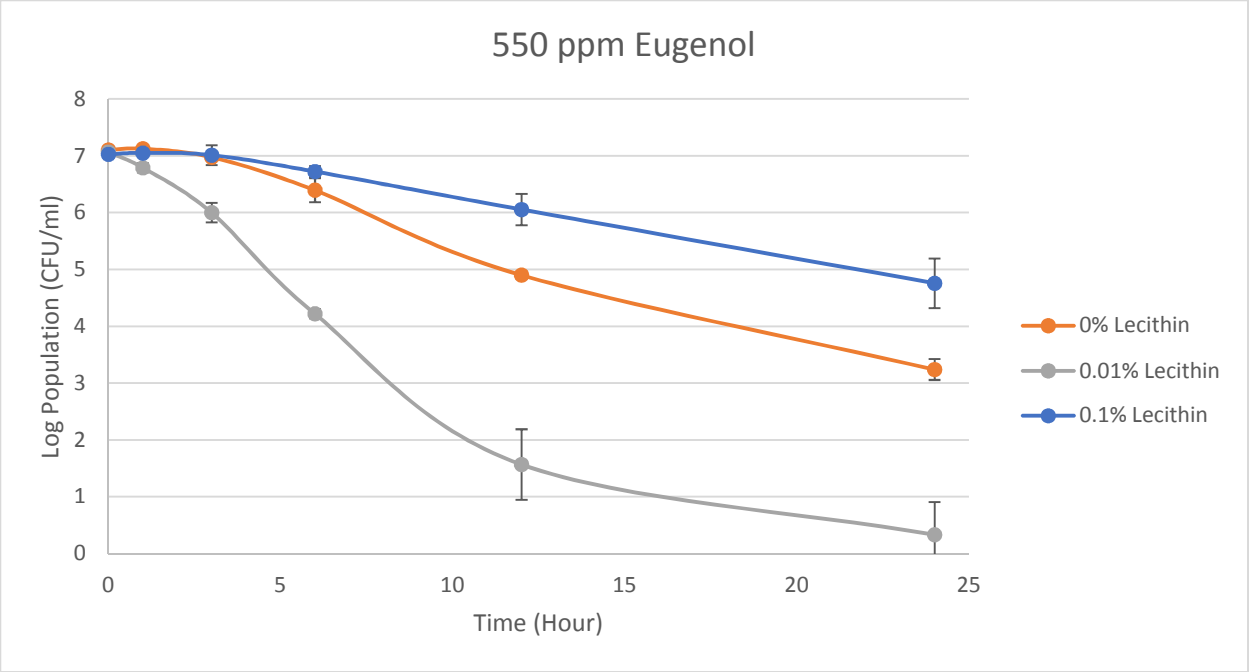

Figure 11 Average log population of Escherichia coli O157:H7 in sodium phosphate buffer (pH 7.2) with varying amounts of soy lecithin, 0.03% w/v media, and 550 ppm eugenol v/v

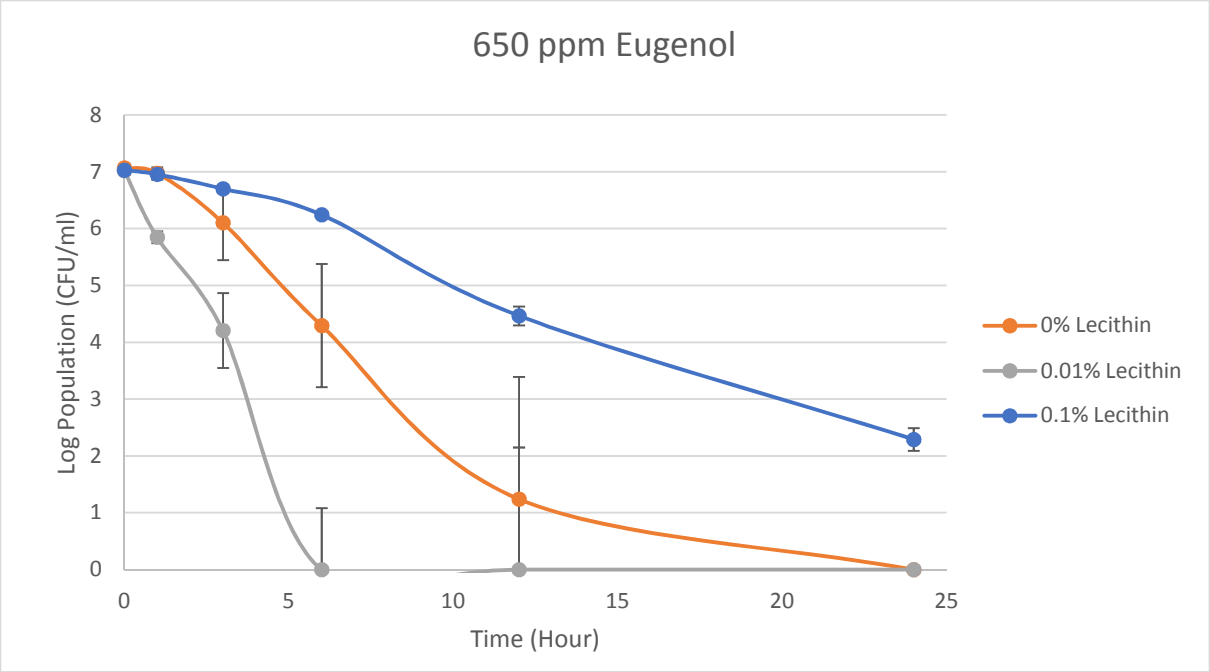

Figure 12 Average log population of Escherichia coli O157:H7 in sodium phosphate buffer (pH 7.2) with varying amounts of soy lecithin, 0.03% w/v media, and 650 ppm eugenol v/v

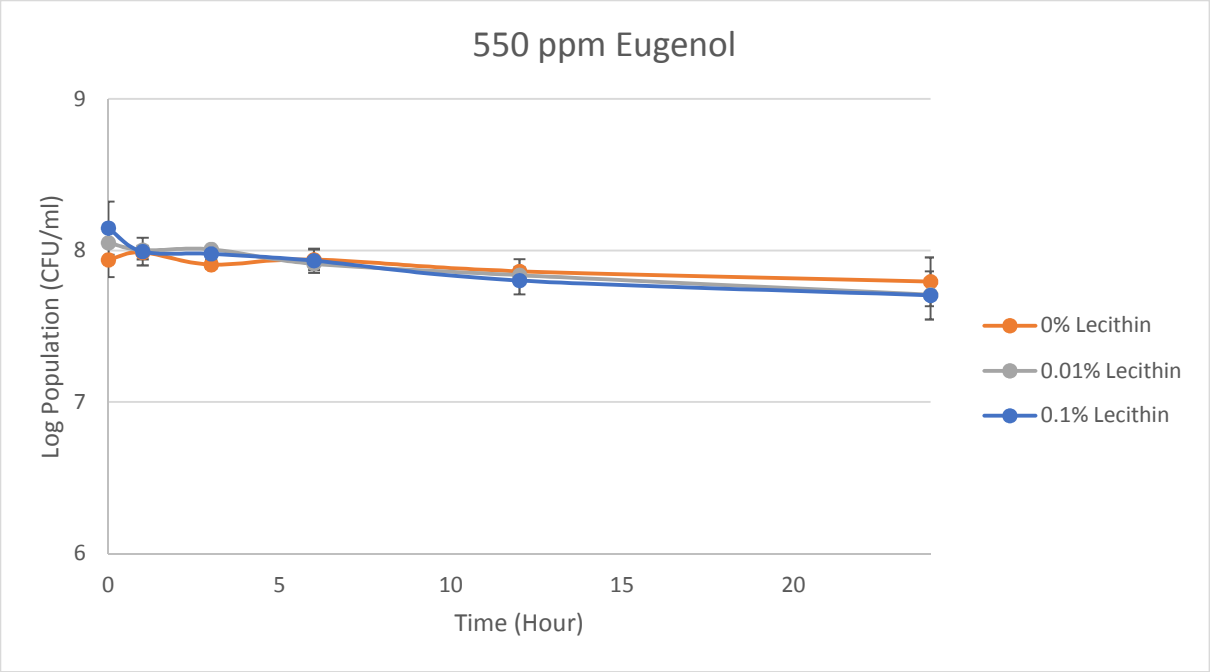

Figure 13 Average log population of Escherichia coli O157:H7 in sodium phosphate buffer (pH 7.2) with varying amounts of soy lecithin, 0.9% w/v media, and 550 ppm eugenol v/v

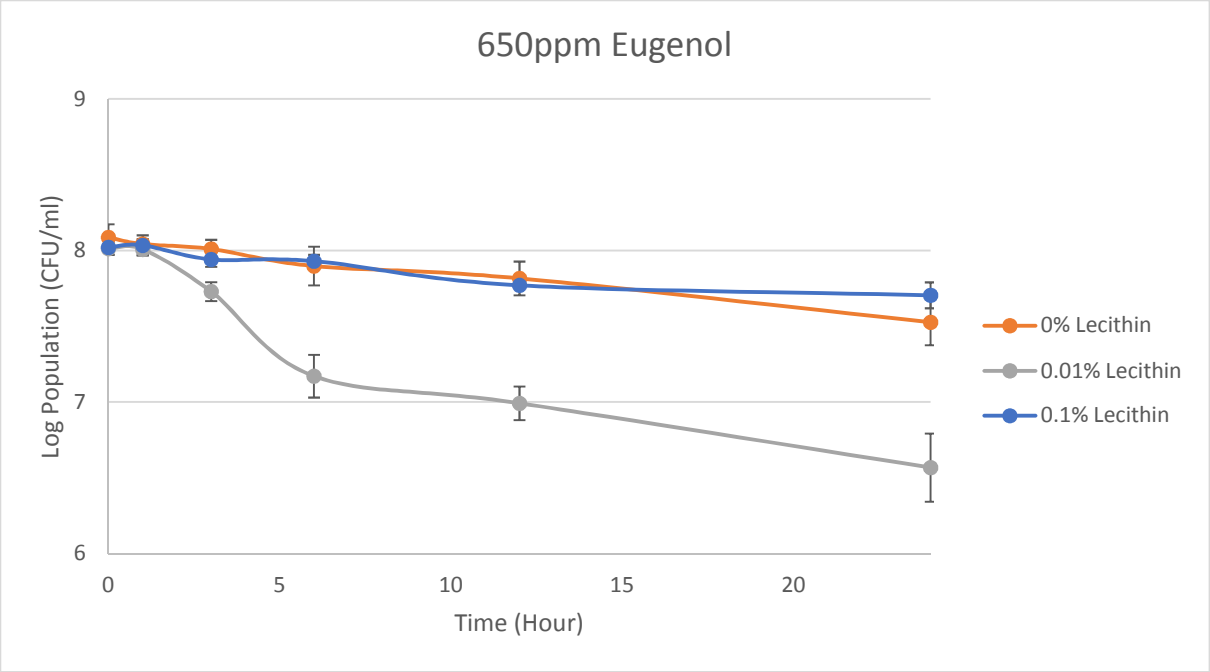

Figure 14 Average log population of Escherichia coli O157:H7 in sodium phosphate buffer (pH 7.2) with varying amounts of soy lecithin, 0.9% media, and 650 ppm eugenol v/v

Vita

Sasha Wilkinson was born in Orange, California to parents Christina Delaney and Ronald Wilkinson. She moved to Tennessee in 2001 and attended Heritage High School in Maryville, Tennessee where she graduated with honors. After high school, she continued her education at the University of Tennessee where she graduated *cum laude* with a Bachelor's degree in Food Science and Technology after completing a product development internship at Mars Petcare.