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I am submitting herewith a thesis written by Songsong Li entitled "Enhancement of the antimicrobial activity of eugenol and carvacrol against Escherichia coli O157:H7 by lecithin in microbiological media and food." 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.

Federico M. Harte, Major Professor

We have read this thesis and recommend its acceptance:

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(Original signatures are on file with official student records.)

Enhancement of the antimicrobial activity of eugenol and carvacrol against *Escherichia coli* O157:H7 by lecithin in microbiological media and food

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Songsong Li
August 2011

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ABSTRACT

Essential oils (EOs) or their isolated components, such as eugenol and carvacrol, have strong antimicrobial activities against both Gram-positive and Gram-negative bacteria and are generally recognized as safe by the FDA. However their hydrophobic properties limit their dispersion and stabilization in aqueous food systems. This requires higher concentrations, which in turn negatively affect the quality of foods. The objective here was to determine the effect of the natural emulsifier lecithin on the antimicrobial activity of eugenol and carvacrol and possible food applications. *Escherichia coli* K12 and *E. coli* O157:H7 strains 'Cider' and ATCC 43889 were used. Homogenized eugenol and carvacrol, with and without lecithin, were screened for antimicrobial activity. The stability of the samples measured by particle size and zeta potential was not affected by different concentrations of lecithin. For all strains, the antimicrobial activity of carvacrol and eugenol was enhanced significantly ($P<0.05$) by low concentration of lecithin. The D-value (time at a specific concentration of antimicrobial necessary to cause a 90% reduction in viable cells) for *E. coli* K12 exposed to 0.047% v/v eugenol or 0.015 % v/v carvacrol was reduced from 13.3 to 6.3 min and 17.4 to 9.7 min, respectively, with the addition of 0.0025% lecithin (w/v). Similarly 0.0025% w/v lecithin in the presence of 0.058% v/v eugenol or 0.0188% v/v carvacrol, caused the D-value to decrease from 4.0 to 1.2 min and 10.2 to 6.9 min, respectively, for *E. coli* strain 'Cider' and from 6.2 min to 3.6 min and 9.9 to 5.4 min, respectively, for *E. coli* ATCC 43889. Higher lecithin concentrations (> 0.005% w/v) increased D-values compared to lower concentrations. Similar results were found in vegetable juice. The results showed that a small amount of lecithin can enhance the antimicrobial activities of essential oils. Addition of lecithin had

no effect on oil-water emulsion droplet particle size and the stability of the samples was not affected by different concentration of lecithin. We believe that lecithin enhances the antimicrobial activity of eugenol and carvacrol droplets by improving the ionic interactions between the positively charged lecithin-containing essential oil components and negatively charged bacterial cells.

Key words: *Escherichia coli*; lecithin; eugenol; carvacrol; homogenization; vegetable juice

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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Introduction

Essential oils (EOs) (also called volatile or ethereal oils) are aromatic oily liquids obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, wood, fruits and roots) (Burt 2004). They are secondary metabolites, which have functions such as protection against pests, as coloring, scent, or attractants and as the plants' own hormones (Brenes and Roura 2010). EOs are generally a complex combination of components and many have been characterized by gas chromatography and mass spectrometry. Essential oils are generally recognized as safe in the United States of America as flavoring agents, for consumption by human and animals (FDA 2006).

Some EOs also are known to have strong antimicrobial activity against a wide variety of foodborne pathogens. Besides antibacterial properties, EOs or their isolated components exhibit antioxidant (Baratta, Dorman et al. 1998), antifungal (Chao, Young et al. 2000), antiviral (Ramadan and Asker 2009), antiparasitic, and insecticidal properties. Consumers today are interested in "green and natural" food preservatives because of the perceived desire for fewer "synthetic" food additives. Furthermore**,** the World Health Organization has called for lower consumption of salt in order to reduce the incidence of cardiovascular disease (WHO, 2002b). If salt added into the food is reduced, other antimicrobial additives will be necessary to inhibit microorganisms in order to maintain the safety of foods (Burt 2004). While EOs have received considerable attention as

antimicrobial food additives, their hydrophobic properties limit their dispersion and stabilization in aqueous food systems (Brenes and Roura 2010; Sofos and Geornaras 2010). This review is focused on the antimicrobial activity and the methods for, stabilizing EOs and their components in food applications.

History

In ancient times, the Egyptians were the first civilization to extensively make use of aromatherapy and aromatic herbs. These were used in their religion, cosmetics, and for medicinal purposes (Burt 2004). At the same time, the Chinese applied herbs and aromatic plants in their medical system. This practice became an integral part of the Indian Ayurvedic medicinal system.

Spices have been widely used for their perfume, flavor and preservative properties since antiquity (Bauer 2001). The first documented use of EOs was described by Greek and Roman historians for medical treatment functions and aromatherapy massages (Burt 2004). The first authentic written account of distillation of essential oil is ascribed to the Catalan physician, Villanova (ca. 1235–1311). By the 13th century, the pharmacological effect of EOs were described in pharmacopoeias (Bauer 2001), however their use does not appear to have been widespread in Europe until the 16th century. The first antimicrobial research on the vapors of EO was carried out by De la Croix in 1881 (Boyle 1955). In recent times, EOs have been used for flavor and aroma in foods rather than for their medicinal purposes. It has only been recently that the food industry has investigated the antimicrobial activity of EOs.

Extraction

There are many ways to exact EOs from plant materials. In ancient Egypt, most essential oils were produced by means of a type of "enfleurage" extraction method. About 3,500 BC, the Egyptians applied a distillation method to produce the EOs. Distillation as a method of producing EOs was improved in the 9th century by the Persian physician Avicenna (980 - 1,037 AD) (Bauer 2001). Maceration was another extraction method in which oils were used to soak the plant matter, then the oils were heated and the volatile vapors collected. Cold pressing is used to extract the essential oils from citrus rinds such as orange, lemon, grapefruit and bergamot. Solvent exaction is widely used in flavor industry. In this method, a hydrocarbon solvent is added to the plant material to help dissolve the essential oils, and then the solution is filtered and concentrated by distillation. Nowadays, supercritical CO_2 extraction of essential oils is one of the most widely used applications. Supercritical fluid extraction (SFE) can produce superior quality products with no artifacts and results in a better reproduction of the original flavor or fragrance (Reverchon 1997).

In the 16th century, a relatively small number of oils were made, including turpentine, juniper wood, rosemary, spike (lavender), clove, mace, nutmeg, anise and cinnamon (Brenes and Roura 2010). With modern extraction technologies, more and more types of EOs were produced including cilantro, coriander, oregano, rosemary, cinnamon, clove, sage, and thymol.

Composition of EOs

EOs are secondary metabolites of the plants and every plant genus, species and cultivar has a different characteristic EO. Furthermore, the natural origin, environmental and genetic factors may effect the composition of the same EO (Brenes and Roura 2010). The composition of EOs from a particular species of plant can differ between harvesting seasons and between geographical sources (Baranauskiene, Venskutonis et al. 2006). In addition, different extraction methods may result in different composition of EOs. EOs are very complex natural mixtures, which can contain about 20-60 components (Bakkali, Averbeck et al. 2008). They have two main groups: terpenes and aromatic components. According to Burt in Figure 1, geranyl acetate, eugenyl acetate, trans-cinnamaldehyde, menthol, carvacrol, thymol, geraniol, eugenol, p-cymene, limonene, γ-terpinene and carvone are the main components of EOs constituting up to 85% (Burt 2004).

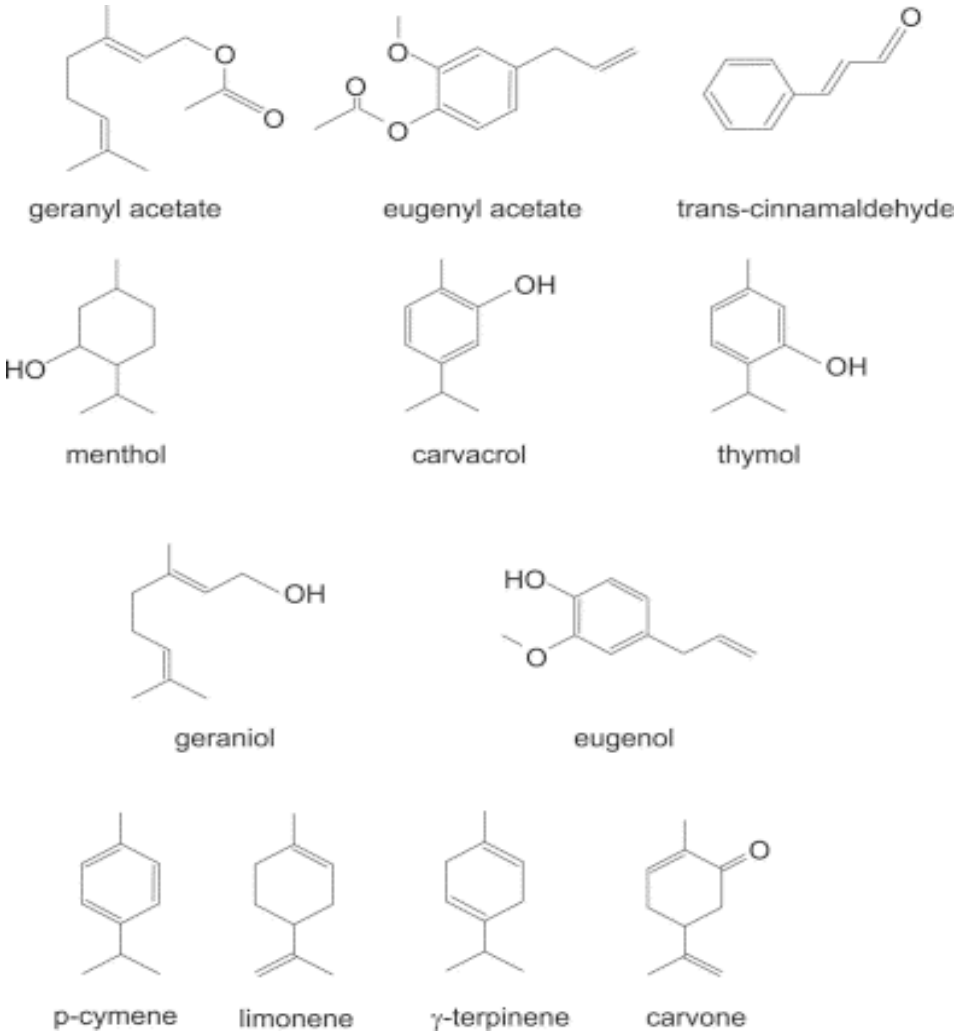

Figure 1. Structural formulae of selected components of EOs (Source: Burt, 2004).

Table 1. Plant resources of EOs and major components

Plant species	Common name	Source †	Major components
Aniba rosaeodora	Rosewood	W	Linalool (Brenes and Roura 2010)
Citrus aurantifolia	Lime	FR	Geranial Limonene, α-Pinene, γ-Terpinene (Brenes and Roura 2010)
Citrus aurantium	Orange	P	Limonene (Brenes and Roura 2010)
Citrus limon	Lemon	P	
Citrus reticulata var. madurensis	Mandarin	P	Limonene, γ-Terpinen (Brenes and Roura 2010)
Cymbopogon citratus	Lemongrass	L	
Coriandrum sativum	Coriander	S	α–thuyone, β–thuyone (Bakkali, Averbeck et al. 2008)
Cucurbita pepo	Pumpkin	S	
Cupressus sempervirens	Cypress	LT	
Cymbopogon citratus	Lemongrass	L	
Lavandula angustifolia	French lavender	FL	Linalool, Linalyl, Terpinen(Daferera, Ziogas et al. 2000)
Lavandula angustifolia	Tasmanian lavender	FL	
Mentha x piperita	Peppermint	H	Methol, Menthne (Holley and Patel 2005)
Mentha spicata	Spearmint	H	
Ocimum basilicum	Basil	H	Linalool, Methyl chavicol (Holley and Patel 2005)
Rosmarinus officinalis	Rosemary	H	α-Pinene, β-Pinene, 1,8-ocineol(Daferera, Ziogas et al. 2000)
Macadamia integrifolia	Macadamia	NT	
Pimpinella anisum	Aniseed	S	

† B, berry; BD, bud; FL, flower; FR, fruit; H, herb; L, leaf; LT, leaves and twigs; N, needles; NT, nut; P, peel; RH, rhizome; S, seed; W, wood; BK, bulk.

Table 1. Plant resources of EOs and major components. Continued

Plant species	Common name	Source †	Major components
Pinus sylvestris	Pine	N	α-Pinene, β-Pinene, Sabinene , δ -3-carene, Myrcene + α -terpin
Piper nigrum	Black pepper	B	α-Pinene, β-Pinene, Sabinene , δ-3-Carene , Limonene,- β-Caryophyllene
Pogostemon patchouli	Patchouli	L	
Prunus armeniaca	Apricot kernel	S	
Prunus dulcis	Sweet almond	S	
Salvia officinalis	Sage	H	α-thujone, β-thujone, 1,8-ocineol(Daferera, Ziogas et al. 2000; Brenes and Roura 2010)
Salvia sclarea	Clary sage	H	
Cinnamomum zeylanicum	Cinnamon	BK	(E)-Cinnamaldehyde, Benzaldehyde, (E)-Cinnamyl acetate (Unlu, Ergene et al. 2010)
Thymus vulgaris	Thyme	H	1,8-cineol, thymol, β -fenchyl alcohol, nerolidol, terpinolene, α-pinene, myrcene (Bakkali, Averbeck et al. 2008; Asbaghian, Shafaghat et al. 2011)
Origanum majorana	Marjoram	H	Carvacrol, p-cymene(Hammer, Carson et al. 1999)
Origanum vulgare	Oregano	H	
Citrus aurantium var. *bergamia*	Bergamot	EO	α-Pinene, Limonene + β-phellandrener, γ – Terpinene, Linalool , Lynalil acetate(Brenes and Roura 2010)
Syzygium aromaticum	Clove	BD	Eugenol, Eugenyl acetate (Chaieb, Hajlaoui et al. 2007; Bakkali, Averbeck et al. 2008)
Melaleuca alternifolia	Tea tree	LT	Allyl isothiocyanate (Tiwari, Valdramidis et al. 2009)

In Table 1, most of the EOs are characterized by one to three dominant substances. Other than terpenes, aromatic compounds do occur frequently in the EOs. For example, cinnamaldehyde is the major components of the cinnamon oil and eugenol as minor constituents to the aromatic components (Bakkali, Averbeck et al. 2008).

Some studies have shown that some of the major components in EOs are not necessarily the most important to the antimicrobial activity (Chao, Young et al. 2000). Additionally, the unfractionated EOs sometimes have stronger antimicrobial activity than isolated major components (Mourey and Canillac 2002; De Giusti, Aurigemma et al. 2010). This indicates that minor, or even trace elements may be critical to the antimicrobial activity. It is possible that the complexity of an essential oil helps to enhance the antimicrobial activity because bacteria cannot gain tolerance very easily (Burt 2004; Brenes and Roura 2010).

Antimicrobial activity of EOs

Different methods may be used to quantify the antimicrobial activity of EOs. Historically, the disk diffusion method or agar well test was used to screen for antimicrobial activity. However, this method is not accurate because the hydrophobic properties of EOs do not allow them to diffuse in aqueous systems. Thus, to determine antibacterial properties, the agar dilution method or broth dilution method should be used. These can be monitored using visual growth, optical density/turbidity, viable counts, absorbance, conductance, or impedance. To determine the speed and duration of antibacterial activity, time-kill analysis or survival curves can be generated. To observe the physical effects of

antimicrobial compounds on microorganisms, scanning electron microscopy is the most common method used.

Hammer et al. reported on the antimicrobial activity of 52 EOs against 10 different microorganisms (Hammer, Carson et al. 1999) including, *Acinetobacter baumanii*, *Aeromonas veronii* biogroup *sobria*, *Candida albicans, Enterococcus faecalis, Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella enterica* subsp. *enterica* serotype *typhimurium, Serratia marcescens* and *Staphylococcus aureus*. Lemongrass, oregano and bay inhibited all organisms at ≤ 2.0% (v/v) (Table 2). Six oils did not inhibit any organisms at the highest concentration (2.0% (v/v)) including apricot kernel, evening primrose, macadamia, pumpkin, sage and sweet almond. Variable activity was recorded for the remaining oils. Twenty plant EOs and extracts were investigated using a broth microdilution assay against *C. albicans*, *S. aureus* and *E. coli.* The minimum inhibitory concentrations were 0.03% (v/v) thyme oil against *C. albicans* and *E. coli,* and 0.008% (v/v) vetiver oil against *S. aureus*. These results support that plant essential oils and extracts may have a role as preservatives (Hammer, Carson et al. 1999).

Table 2. Minimal Inhibitory Concentration (MIC) of EO composition for different bacteria.

EO composition	Species of bacteria	MIC, approximate range (µl ml^{-1})[a]	References
α-Terpineol	*Escherichia coli*	0.450 – >0.9	(Cosentino, Tuberoso et al. 1999)
	Salmonella typhimurium	0.225	
	Staphylococcus aureus	0.9	
	Listeria monocytogenes	>0.9	
	Bacillus cereus	0.9	
Carvacrol	*E. coli*	0.225 – 5	(Kim, Morr et al. 1996; Helander, Alakomi et al. 1998; Cosentino, Tuberoso et al. 1999; Roller and Seedhar 2002; Bakkali, Averbeck et al. 2008)
	S. typhimurium	0.225 – 0.25	
	Staph. aureus	0.175 – 0.450	
	L. monocytogenes	0.375 – 5	
Eugenol	*E. coli*	1	(Kim, Morr et al. 1996)
	S. typhimurium	0.5	
	L. monocytogenes	>1.0	
Trans-cinnamaldehyte	*E. coli*	3	(Helander, Alakomi et al. 1998)
	S. typhimurium	3	
Thymol	*E. coli*	0.225 – 0.45	(Cosentino, Tuberoso et al. 1999)
	S. typhimurium	3	

[a]**.** In the references MICs have been reported in different units such as ppm, mg ml^{-1}, % (v/v), µl l^{-1} and ug ml^{-1}. For ease of comparison these have been converted to µl ml^{-1}, assuming that EOs have the same density as water.

Eugenol inhibited against *E. coli* with concentration at 1.0 µl ml^{-1} and carvacrol ranging from 0.225 to 5 µl ml^{-1} (Table 2). EOs are effective at preventing the growth and reducing the number of most gram-positive and gram-negative foodborne pathogens at a relatively low concentration.

Mechanisms of antimicrobial activity of Essential Oil Components

The mechanism of action of EOs and their components as antimicrobials has not been fully elucidated. This is complicated by the fact that there are a large number of chemical compounds present in EOs and often they are all needed for antibacterial activity and the EOs does not seem to have a specific cellular target. Thus the antimicrobial mechanism of EOs may not be attributable to one specific mechanism, but rather there may be several targets in the cell. Most of the focus on antimicrobial mechanisms for EOs has been on the cell membrane and targets interconnected with the membrane. For bioactivity, the EOs pass through the cell wall and cytoplasmic membrane (Bakkali, Averbeck et al. 2008), disrupt the structure of different layers of polysaccharides, fatty acids and phospholipids and permeabilize them (Kim, Morr et al. 1996; Helander, Alakomi et al. 1998; Chaieb, Hajlaoui et al. 2007). Several major factors will be discussed individually including cell membrane integrity, leakage of ions and other contents and internal pH.

Cell membrane integrity

Lambert et al. (2001), using fluorescence, showed that *Pseudomonas aeruginosa* and *S. aureus* in the presence of thymol, carvacrol or oregano oils had increased membrane permeability and disruption of the membrane. In another study, when *E. coli* strain rr98089 phage type 34 O157:H7 cells were treated with 625 ul l^{-1} of oregano oil (Mejlholm and Dalgaard 2002), the cell membranes were damaged and loss of cell contents was shown using scanning electron microscopy (Lambert, Skandamis et al. 2001; Chaieb, Hajlaoui et al. 2007). Potential explanations for this mechanism is that the hydrophobic EO components disrupt in the lipids of the bacterial cell membrane thus disturbing the structures of the membrane and rendering them more permeable, It is also associated with loss of ions and reduction of membrane potential, collapse of the proton pump and depletion of the ATP pool (Bakkali, Averbeck et al. 2008).

Leakage of Ions and other Cell Components

Because the cytoplasmic membrane is rendered more permeable by EO components, enzymes such as ATPases, which are known to be located in the cytoplasmic membrane, may be disrupted, and further leakage of ions and other cell contents may occur (Burt 2004). For example, it was shown that oregano essential oil caused both potassium and phosphate leakage from the cytoplasm of *S. aureus* and *P. aeruginosa* (Ultee, Kets et al. 1999; Lambert, Skandamis et al. 2001). Bacteria can tolerate a small amount of leakage from their bacterial cytoplasm without loss of viability, but extensive loss of cell contents or the loss of critical molecules and ions will lead to death (Burt 2004).

Internal and external pH

Changes in the internal pH cells exposed to essential oil components have also been observed (Ultee, Kets et al. 1999; Lambert, Skandamis et al. 2001). Without glucose present, the addition of 0·05% oregano essential oil shortened the time for *S. aureus* to reach an internal pH of 5.05 from an initial external pH of 5.95. With glucose, it took the same amount of time for the control and culture with 0.05% oregano EO to reach a low internal pH of 4.6. This indicated the glucose has the protective role in pH homeostasis.

In conclusion, the chemical structure of the individual EO components affects their precise mode of action and antibacterial activity (Mejlholm and Dalgaard 2002); and the three mechanisms are not occurring alone but interact together to gain the antimicrobial activities.

Application to Food

Although EOs and their components are generally recognized as safe (GRAS) for human and animal consumption under US Federal regulations and they have antimicrobial activity against a wide variety of foodborne pathogens, there are many challenges in applying EOs in food industry. For example, a higher concentration of EOs is required to achieve the same antimicrobial effect in food than in microbiological media. In order to achieve the antimicrobial activities in food, two fold in semi-skimmed milk, 10-fold in pork liver sausage, 50-fold in soup (Corbo, Bevilacqua et al. 2009) and 25- to 100-fold in soft cheese (Burt 2004) were needed comparing to in microbiological media. A high fat content in food appears to markedly reduce the action of EOs in meat products. Mint and

cilantro EOs were not effective in products with a high level of fat, such as pâté (which generally contains 30–45% fat) and a coating for ham containing canola oil (Mead and Griffin 1998; De Giusti, Aurigemma et al. 2010). Because EOs are hydrophobic, they may solubilize in the lipids of a food. Thus amount of EO available to inhibit microorganisms will be relatively less to inhibit bacteria which are present in the aqueous phase (Roller and Seedhar 2002). When applied in food in high concentration, EOs contribute markedly to flavor. Because of the loss of activity of EOs when applied to food products, higher amounts of EOs are needed to achieve the same antimicrobial activities, which may negatively influence the sensory properties of food products.

The extrinsic factors, pH, storage temperature and package atmosphere, all have effects on the antimicrobial activity of EOs (Mead and Griffin 1998; Skandamis and Nychas 2001; Gharsallaoui, Roudaut et al. 2007). Usually the lower the pH, the better effect to inhibit the bacteria (Tassou, Drosinos et al. 1995). At pH 7 the synergistic action of nisin and carvacrol was significantly greater at 30°C than at 8°C, which would indicate temperature-induced changes in the permeability of the cytoplasmic membrane (Burt 2004). Available oxygen influences the antibacterial activity of EOs. One possible explanation for this, is that when oxygen is present at low concentrations, fewer oxidative changes occur to EOs or cells generating energy via anaerobic metabolism are more sensitive to the toxic action of EOs (Burt 2004; De Giusti, Aurigemma et al. 2010).

EOs have antimicrobial activities against gram-negative and gram-positive organisms (Canillac and Mourey 2001; Burt and Reinders 2003; Fitzgerald, Stratford et al. 2004; Friedman, Henika et al. 2004; Burt, Vlielander et al. 2005; Gaysinsky, Davidson et al.

2005). Generally, EOs are slightly more active against gram-positive than gram-negative bacteria (Canillac and Mourey 2001; Mejlholm and Dalgaard 2002; Burt 2004; Brenes and Roura 2010). The reason is that the outer membrane of the gram-negative organisms containing lipopolysaccharide protects the bacteria from EOs' disruption. However, *P. aeruginosa* (gram-negative) in particular, appear to be least sensitive to the action of EOs (Burt 2004). In addition, the geographical origin and harvesting period affect the EOs in composition between batches, which further cause variability in the degree of susceptibility of gram-negative and gram-positive bacteria (Burt 2004).

There have been many studies on the effectiveness of EOs and their components against foodborne microorganisms. Eugenol, coriander, clove, oregano and thyme oils were found to be effective at levels of 5–20 µl g^{-1} inhibiting *L. monocytogenes, A. hydrophila* and autochthonous spoilage flora (Chaieb, Hajlaoui et al. 2007; Gharsallaoui, Roudaut et al. 2007) in microbial media. Oregano oil is more effective in/on fish than mint oil on *L. monocytogenes* (Roller and Seedhar 2002). In dairy products, cinnamon, cardamom and clove oils were more effective than mint oil on *S. Enteritidis*. (Mead and Griffin 1998). For vegetables, all EOs and their components were effective at 0.1–10 µl g^{-1} in washing water on *E. coli* and six *Salmonella* serotypes with a decrease in storage temperature and/or a decrease in the pH of the food (Skandamis and Nychas 2001). Carvacrol and cinnamaldehyde in Kiwifruit 0.15 µl mL^{-1} in dipping solution (Roller and Seedhar, 2002) can be effective, but less effective on honeydew melon. It is possible that this difference has to do with the difference in pH between the fruits; the pH of kiwifruit was 3.2–3.6 and of the melon 5.4–5.5 (Tassou, Drosinos et al. 1995). Burt (2004) suggested the

following antimicrobial activity ranking for EOs (in order of decreasing antibacterial activity): oregano/clove/coriander/cinnamon > thyme > mint > rosemary > mustard > cilantro/sage.

Another approximate general ranking of the isolated EO components is shown below (in order of decreasing antibacterial activity): eugenol > carvacrol/cinnamic acid > basil methyl chavicol > cinnamaldehyde > citral/geraniol (Burt 2004).

Application Technologies

In order to improve the antimicrobial effectiveness of EO components applied to foods and thus reduce the overall effect on flavor, researchers have attempted a number of strategies. These include combinations with physical processes and addition of other compounds to evaluate potential synergies.

Physical

EOs can be suspended in phosphate saline buffer by employing vigorous shaking. More efficient methods involve mechanical methods involving homogenization, either rotary homogenization or high pressure homogenization (HPH). Other physical technologies have also been studied. For example, pulsed electric fields (PEF) were also studied but this process did not improve the inactivation of vegetative *B. cereus* cells with carvacrol (Pol and Smid, 1999). High Hydrostatic Pressure (HHP) was shown to enhance the antimicrobial activity of 3 mmol l^{-1} thymol or carvacrol at 300 MPa HHP (Karatzas et al., 2001). Modified atmosphere packaging (40% CO_2, 30% N_2 and 30% O_2) along with

oregano oil was found to delay microbial growth and suppress spoilage microorganisms in minced beef) (Skandamis and Nychas 2001).

Chemical

Surfactants such as Tween-20 (Polyoxyethylene (20) sorbitan monolaurate), Tween-acetone in combination with Tween-80 (Polyoxyethylene (80) sorbitan monolaurate), polyethylene glycol, propylene glycol, and dimethyl sulfoxide, can be applied to stabilize the oil-in-water EO emulsion to encapsulate or enhance the antimicrobial activities. It has been shown that the MICs of oregano and clove oils were significantly higher with Tween-80 or ethanol than with agar. It has been reported that the liposome-encapsulated nisin with EDTA nearly-completely inhibited *E. coli* O157:H7 with lower concentration of antimicrobial needed (Taylor, Bruce et al. 2008). However, Baskaran et al. concluded solvents and detergents could decrease the antibacterial effect of eugenol and carvacrol (Baskaran, Kazmer et al. 2009). Other chemicals such as ethanol, agar and methanol are also used in research. For example, the use of agar (0.2%) could produce a better homogenous emulsion than in absolute ethanol (Baskaran, Kazmer et al. 2009).

Yet another approach is encapsulation of EOs in beta-cyclodextrin to control the odor and reactivity of active compounds throughout the release of natural antimicrobial compounds such as oregano oil and thymol (Varona, Kareth et al. 2010). Chitosan enriched with EOs such as oregano, coriander, basil and anise showed similar antimicrobial activities when applied alone or incorporated in the film (Zivanovic, Chi et al. 2005). Other encapsulation methods have been studied. Nisin or thymol, which were spray-dried and encapsulated by zein, were also more effective than the free antimicrobials in inhibiting the growth of *L. monocytogenes* in 2% reduced fat milk in

growth media (Xiao 2010; Xiao 2011). However after encapsulation, the antimicrobial activities were similar or even lower.

An emulsifier is a surface-active substance, which has a strong tendency to adsorb at oil-water interfaces, thereby promoting the formation of oil-in-water (O/W) emulsion, and the rapid stabilization of nano-size emulsion droplets by interfacial action. Lecithin, which consists of a mixture of various phospholipids, is the only natural small-molecule emulsifier available in food industry (Dickinson 1993).

The objective of this research was to evaluate the effect of lecithin on the antimicrobial activity of the essential oil components, eugenol and carvacrol, against *E. coli* O157:H7 in microbiological media and foods.

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ENHANCEMENT OF THE ANTIMICROBIAL ACTIVITY OF EUGENOL AND CARVACROL AGAINST *ESCHERICHIA COLI* O157:H7 BY LECITHIN IN MICROBIOLOGICAL MEDIA AND FOOD

This chapter is a lightly revised version of a paper by the same name that will be submitted to the International Journal of Food Protection in July 2011 by Songsong Li, P. Michael Davidson and Federico M. Harte.

My use of "our" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) the experimental work, (2) most of the collection and analysis of data, (3) most of the gathering and interpretation of literature, and (4) most of the writing.

Abstract

Novel approaches for controlling pathogens in foods include the use of antimicrobial essential oil (EOs) components, such as eugenol and carvacrol. While EOs have antimicrobial activity against a wide variety of pathogens, their hydrophobic properties limit their dispersion and stabilization in aqueous food systems. Thus high concentrations of EOs are required to inhibit microorganisms which, in turn, may negatively affect the sensory properties of certain foods. The objective of this research was to determine the effect of the natural emulsifier, lecithin, on the antimicrobial activity of eugenol and carvacrol against *Escherichia coli* K12, and *E. coli* O157:H7 strains 'Cider' and ATCC 43889 in microbiological media and vegetable juice. Homogenized eugenol and carvacrol with and without lecithin were screened for antimicrobial activity. The stability of the samples, as measured by particle size and zeta potential, was not affected by lecithin. For all strains of *E. coli*, the antimicrobial activity of carvacrol and eugenol was enhanced significantly ($P<0.05$) by low concentration of lecithin. The D-value (time at a specific concentration of antimicrobial necessary to cause a 90% reduction in viable cells) for *E. coli* K12 with 0.047% v/v eugenol or 0.015 % v/v carvacrol was reduced from 13.3 to 6.3 min and from 17.4 to 9.7 min in the presence of 0.0025% lecithin (w/v). Similarly, the D-value for 0.058% v/v eugenol and 0.0188% v/v carvacrol with 0.0025% w/v lecithin decreased from 4.0 to 1.2 min and 10.2 to 6.9 min for *E. coli* strain 'Cider', respectively. For *E. coli* ATCC 43889 under the same conditions, the D-value decreased from 6.2 min to 3.6 min and 9.9 to 5.4 min, respectively. Higher lecithin concentration (> 0.005% w/v) resulted in increased D-values compared to lower concentrations. Similar results were found in vegetable juice. The addition of lecithin had no effect on oil-water emulsion

droplet particle size. A hypothesis for the mechanism of lecithin enhancement of the antimicrobial activity of eugenol and carvacrol is that small quantities of lecithin promote ionic interactions between the EOs and negatively charged bacterial cells.

Key words: *Escherichia coli*; lecithin; eugenol; carvacrol; homogenization; vegetable juice

Introduction

Essential oils (EOs), also called volatile or ethereal oils, are aromatic oily liquids obtained from plant materials (flowers, buds, seeds, leaves, twigs, bark, wood, fruits and roots) and are classified as secondary metabolites. The essential oil components, eugenol and carvacrol, are generally recognized as safe as flavoring agents in the US (FDA 2006) and have been repeatedly shown to have strong antimicrobial activity against a wide variety of spoilage and pathogenic bacteria. The antimicrobial activity of EO components has been attributed to the presence of terpenoid and phenolic groups. Geranyl acetate, eugenyl acetate, trans-cinnamaldehyde, menthol, carvacrol, thymol, geraniol, eugenol, p-cymene, limonene, γ-terpinene and carvone constitute up to 85% active components in EOs (Burt 2004).Most components in EOs are described as defined "fast-acting", i.e. effective within one hour (Friedman, Henika et al. 2004). Eugenol has antimicrobial activity against foodborne pathogens at concentrations ranging from 0.4 to 2.5 $\mu l\ ml^{-1}$ (Burt and Reinders 2003), while, for carvacrol, the range is 0.15 to 0.75 $mg\ g^{-1}$ (Ultee, Kets et al. 1999).

The hydrophobic properties of EO components limit their dispersion and stabilization in aqueous food systems, leading to an increase in the concentration required for antimicrobial functions, which can lead to phase separation and negatively affect the quality of foods. Additionally, EO components have effects on the sensory properties of foods and thus it is often desirable to use them at the lowest possible concentrations. In order to optimize the use of EOs in food applications, a number of methods have been

studied including emulsification, encapsulation and incorporation into packaging (Taylor, Bruce et al. 2008), (Skandamis and Nychas 2001), (Zivanovic, Chi et al. 2005), (Varona, Kareth et al. 2010), (Xiao 2010; Xiao 2011), and (Varona, Kareth et al. 2010). Most or all of these processes are relatively costly. The focus of this research was to evaluate the direct use of an emulsifier, lecithin, to enhance the antimicrobial activity of eugenol and carvacrol and thus reduce the effective concentrations needed.

An emulsifier is a surface-active substance, which has a strong tendency to adsorb at oil-water interfaces, thereby promoting the formation of oil-in-water (O/W) emulsion, and the rapid stabilization of nano-size emulsion droplets by interfacial action. Lecithin, which consists of a mixture of various phospholipids, is the only natural small-molecule emulsifier available in food industry (Dickinson 1993). The specific objectives of this research were to evaluate the effect of lecithin on the antimicrobial activity of the essential oil components, eugenol and carvacrol, against *E. coli* K12 and *E. coli* O157:H7 strains 'Cider' and ATCC 43889.

Material and methods

Culture preparation

Escherichia coli K12, *E. coli* O157:H7 strains 'Cider' and ATCC 43889 were stock cultures obtained from the Department of Food Science and Technology collection at the University of Tennessee, Knoxville. All cultures were grown statically under aerobic conditions in tryptic soy broth (TSB; Difco, Sparks, MD) for 24 h and stocks were made

using glycerol and stored at -20°C. Working cultures were prepared by inoculating glycerol stocks into TSB and incubating at 37°C overnight.

Time kill assays

Laboratory grade vegetable lecithin (Fisher Scientific, Fair Lawn, NJ) solutions were prepared by dissolving in phosphate buffer (ca. pH = 7.20) and then heating to boiling with stirring for at least 5 min. Sterilized deionized water was added to the cooled mixture to replace the water lost during boiling.

In preliminary experiments, 0.047 %, 0.058 %and 0.058 % v/v eugenol showed 3 to 6 log CFU/ml reduction after 30 min for *E. coli* K12, *E. coli* O157:H7 strains 'Cider' and ATCC 43889, respectively. For carvacrol, 2-3 log CFU/ml reductions for the same respective strains was obtained with 0.015%, 0.0188%, and 0.0188% v/v. Eugenol, 99% or carvacrol, ≥98% (Acros Organics, Fair Lawn, NJ) was suspended in 47 ml phosphate buffer with or without the addition lecithin to a final concentration 0.025%, 0.05% 0.10%, 0.15% w/v. All samples and controls (no lecithin) were subjected to rotary homogenization using a Polytron PT 10/35 GT (Kinematica, Inc., Bohemia, New York) at about 10,000 rpm (rotary head diameter 12 mm) for 3 min. The droplet size was measured (2 ml of sample) using a Delsa Nano^{TM} C particle size analyzer (Beckman Coulter, Fullerton, CA). Zeta potential was measured only for 0.094% v/v eugenol and 0.030% v/v carvacrol; each sample was measured at least 3 times. All samples were then mixed with 45 ml of phosphate buffer (pH = 7.2) and 10 ml of overnight culture of *E. coli* to achieve an initial count of ca. 8.6 log CFU/ml.

Vegetable juice (VJ; V8, Campbell Soup Company, Camden, NJ) was purchased from a local grocery store. The pH of the VJ ranged from 4.16 to 4.21. Ninety ml of VJ were mixed with or without sterile? water containing lecithin at (0.1 % w/v and 0.080% v/v eugenol. Samples were then subjected to rotary homogenization as described above. Homogenized emulsions were then mixed with 10 ml of *E. coli* to ca. 8 log CFU/ml. Every 10 min, a sample was taken, serially diluted and plated in tryptic soy agar (TSA; Fisher Scientific, USA), and survivors enumerated after 24 h aerobic incubation at 37°C. A D-value (time at a specific concentration of eugenol or carvacrol to achieve a 90% reduction in *E. coli* cells) was calculated in the log linear region of the inactivation curve. All experiments were done in triplicate and average values were reported.

Statistical analysis

Data were analyzed as a complete randomized design with at least 3 replicates by analysis of variance (ANOVA) using the general linear model (SAS 9.2, SAS Institute, Cary, NC). Least significant differences (LSD) were used to compare treatment mean values when significant differences were found ($p < 0.05$). Error bars represent half of the LSD in all figures centered by the mean. The correlation between particle size / D-value and zeta potential / D-value were evaluated (SAS 9.2, SAS Institute, Cary, NC).

Results and Discussion

Prior to homogenization, rapid phase separation of free eugenol was observed in the phosphate buffer (pH = 7.2). Treatment with a rotary homogenization eliminated the phase separation for the duration of experiments. Emulsions made with lecithin were light yellowish in color and exhibited a thin foam layer on top. Emulsions without lecithin appeared transparent following rotary homogenization. Carvacrol emulsion showed similar appearance. No phase separation was observed in any of the homogenized samples throughout the experiments.

A strong enhancement of the activity of both eugenol and carvacrol was demonstrated with all three strains of *E. coli* (**Figure 2 - 7**) at low concentrations of lecithin. The lowest concentration of lecithin exhibited the greatest antimicrobial enhancement against all *E. coli* strains. The same response was observed with lecithin at 0.0025% or 0.005% w/v in vegetable juice against *E. coli* O157:H7 Cider (**Figure 8)**.

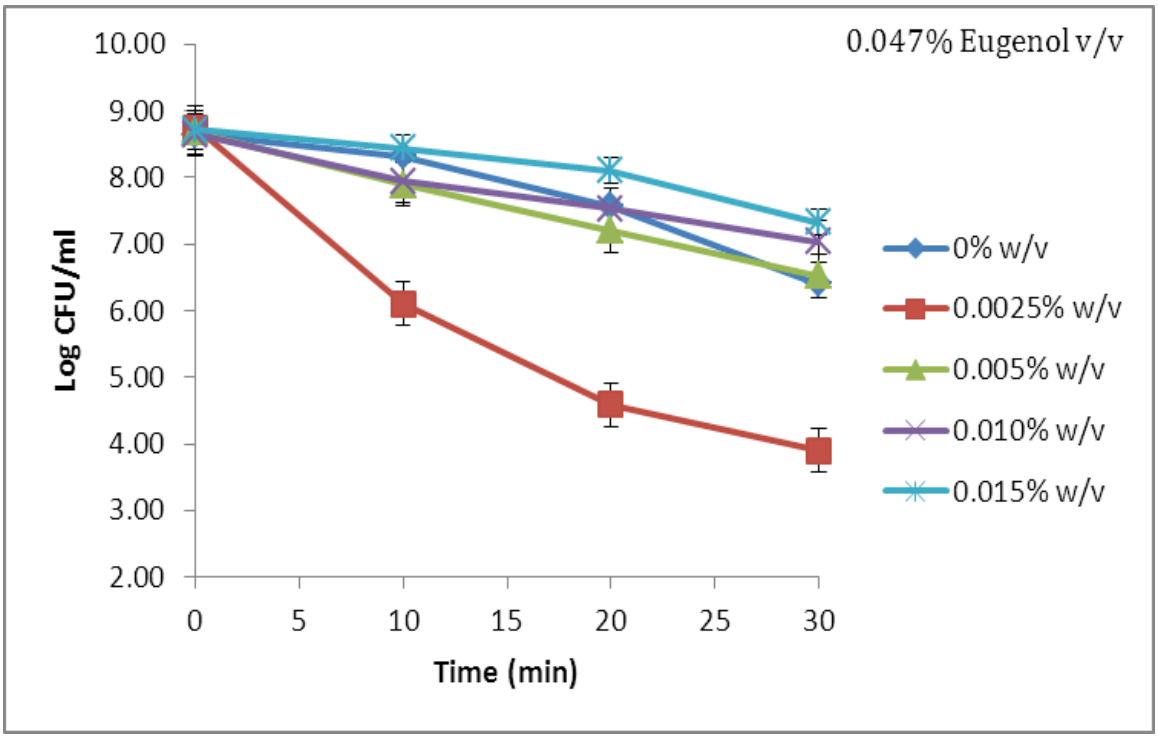

Figure 2. Inactivation of *E. coli* K12 in vitro at 37°C with varying concentrations of lecithin, 0.047% v/v eugenol and an initial count of ca. 8.6 logs CFU/ml. Error bars are LSD for the mean.

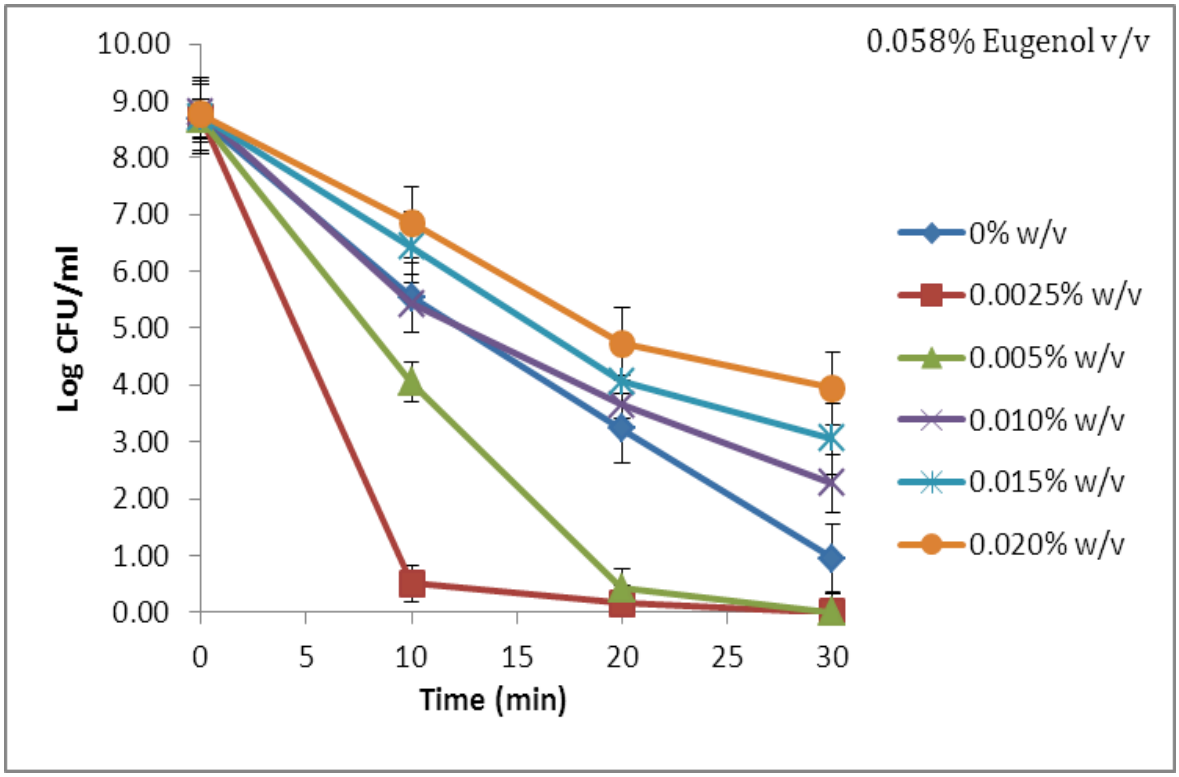

Figure 3. Inactivation of *E. coli* strain 'Cider' in vitro at 37°C with varying concentrations of lecithin, 0.058% v/v eugenol and an initial count of ca. 8.6 logs CFU/ml. Error bars are LSD for the mean.

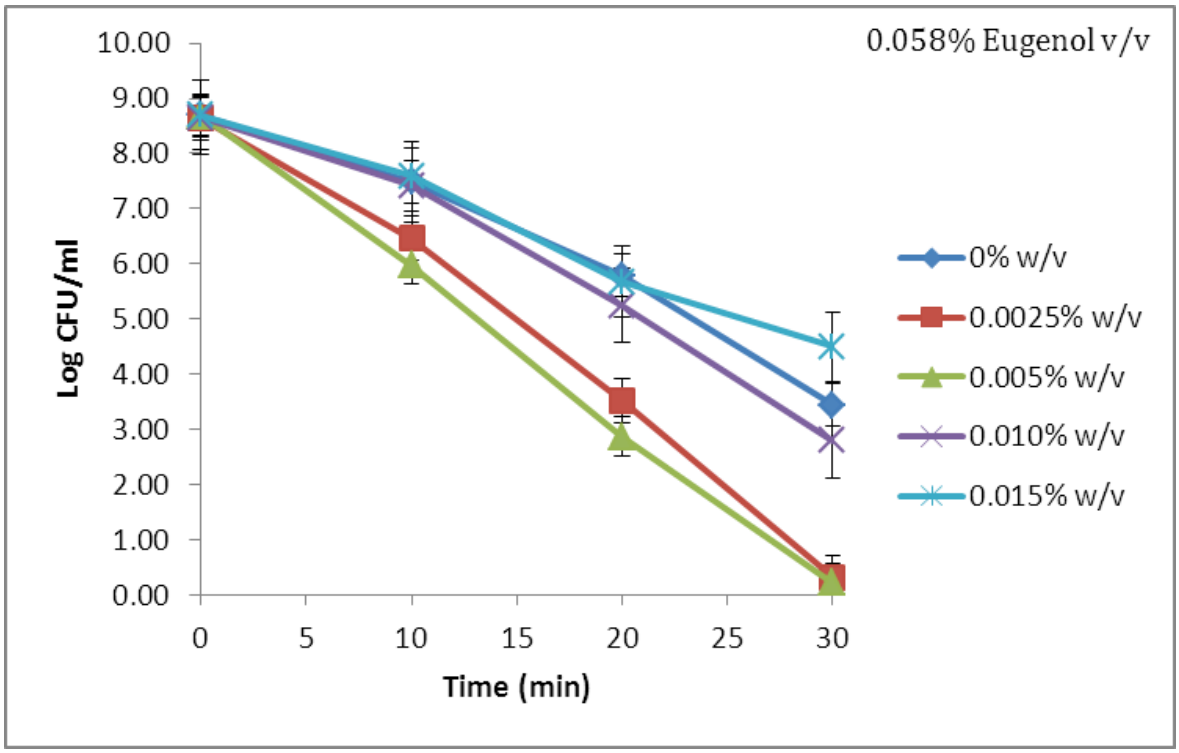

Figure 4. Inactivation of *E. coli* O157:H7 ATCC strain 43889 in vitro at 37°C with varying concentrations of lecithin, 0.058% v/v eugenol and an initial count of ca. 8.6 logs CFU/ml. Error bars are LSD for the mean.

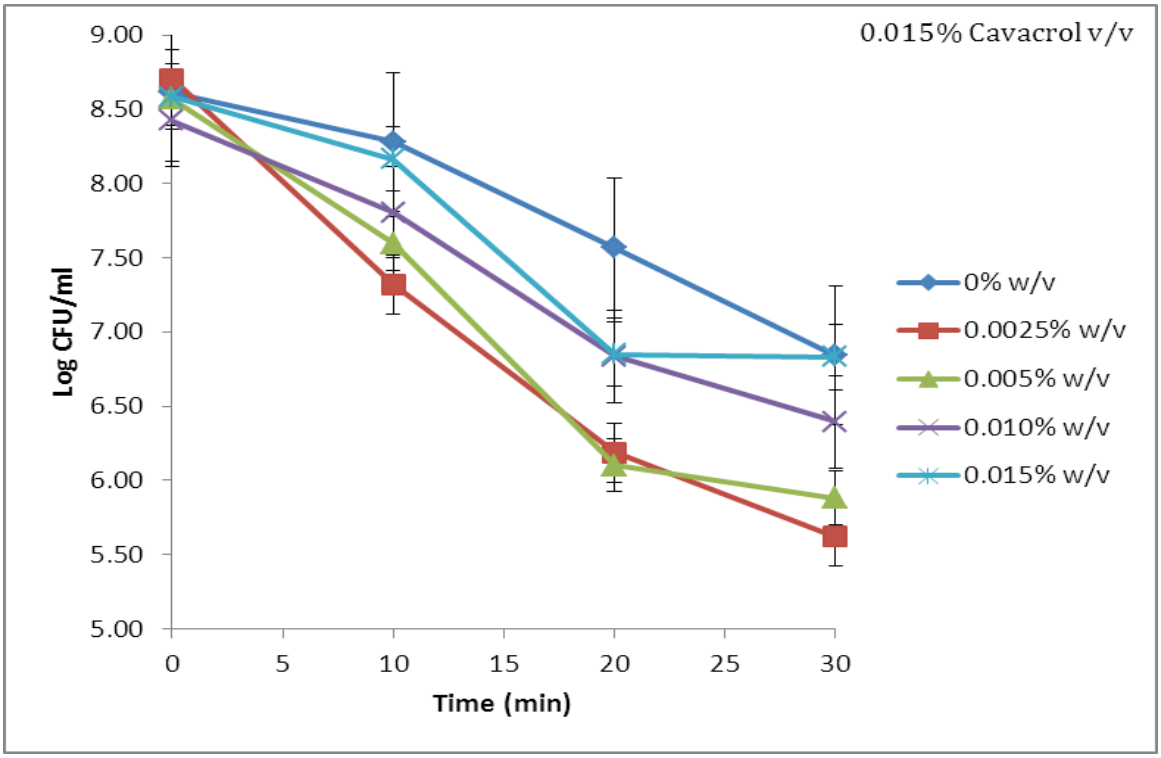

Figure 5. Inactivation of *E. coli* K12 in vitro at 37°C with varying concentrations of lecithin, 0.0150% v/v carvacrol and an initial count of ca. 8.6 logs CFU/ml. Error bars are LSD for the mean.

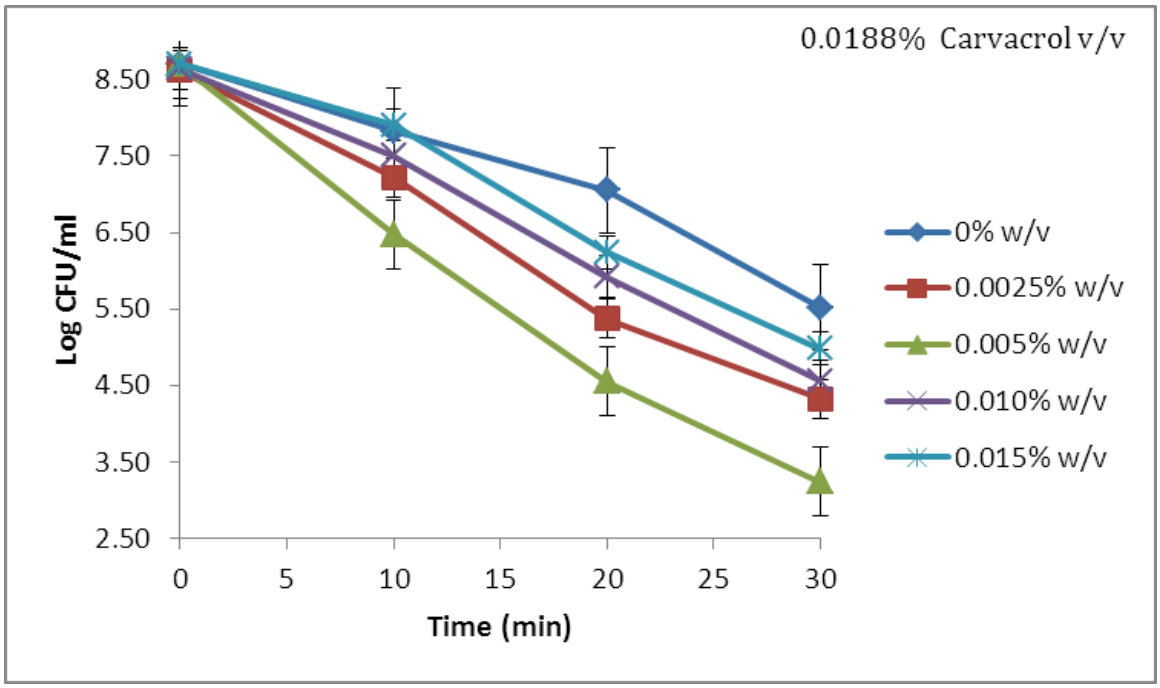

Figure 6. Inactivation of *E. coli* O157:H7 strain 'Cider' in vitro at 37°C with varying concentrations of lecithin, 0.0188% v/v carvacrol and an initial count of ca. 8.6 log CFU/ml. Error bars are LSD for the mean.

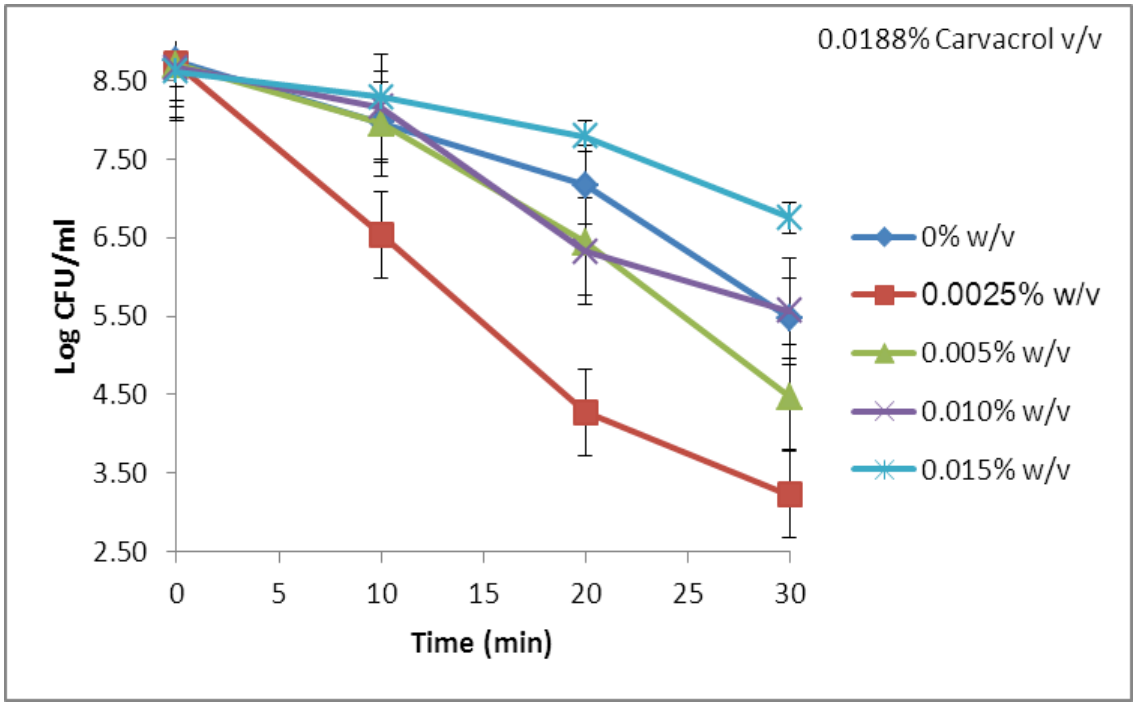

Figure 7. Inactivation of *E. coli* O157:H7 ATCC strain 43889 in vitro at 37°C with varying concentrations of lecithin, 0.0188% v/v carvacrol and an initial count of ca. 8.6 log CFU/ml. Error bars are LSD for the mean.

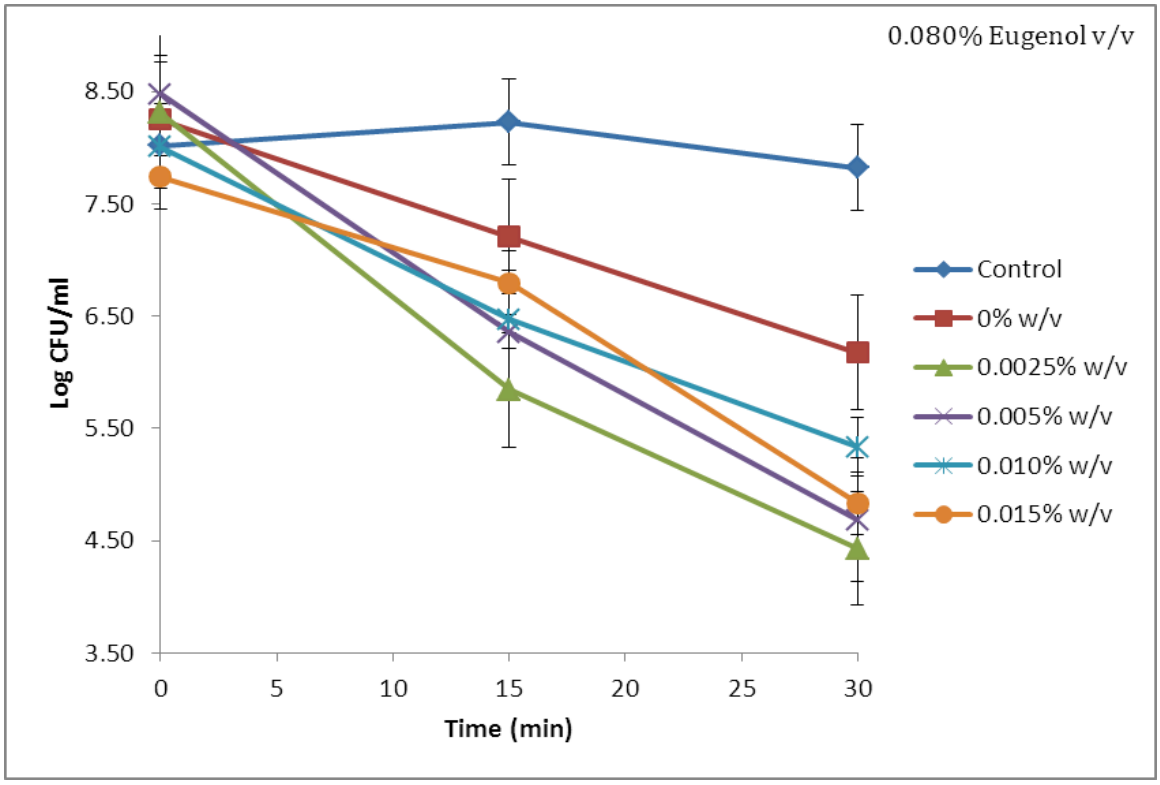

Figure 8. Inactivation of *E. coli* O157:H7 strain 'Cider' in vitro at 37°C with varying concentrations of lecithin, 0.080% v/v eugenol and an initial count of ca. 8.0 log CFU/ml. Error bars are LSD for the mean.

Eugenol

For *E. coli* K12 in the presence of eugenol, the D-value was decreased from 13.32 ± 1.23 for the control to 6.31 ± 1.42 min with 0.0025% w/v lecithin ($p < 0.05$, **Figure 9**). As the lecithin concentration increased, the D-value also increased. At 0.010, 0.015, 0.02 % w/v lecithin, D-values were not significantly different than samples without lecithin ($p > 0.05$). At 0.015% w/v lecithin, the D-value (23.52 ± 1.42 min) was greater than the control ($p < 0.05$). For *E. coli* O157:H7 'Cider', the D-value decreased from 4.01 ± 0.47 to 1.23 ± 0.47 min in the presence of 0.025% w/v lecithin ($p < 0.05$, **Figure 10**). However when the lecithin concentration increased, the D-value also increased. For *E. coli* O157:H7 ATCC 43889, the D-value decreased significantly ($p<0.05$) from 6.27 ± 0.34 for the control to 3.60 ± 0.34 and 3.51 ± 0.31 min with 0.0025% and 0.005% w/v lecithin, respectively (**Figure 11**). Increasing the lecithin concentration resulted in an increased D-value up to 6.97 ± 0.34 min at 0.015% lecithin ($p < 0.05$). In vegetable juice, the D-value was 15.31 ± 4.10 min in the control (0% lecithin). At 0.0025% and 0.005% lecithin, the D-value was significantly lower at 7.77 ± 0.65 min and 8.11 ± 1.49 min, respectively ($p<0.05$, **Figure 15**).

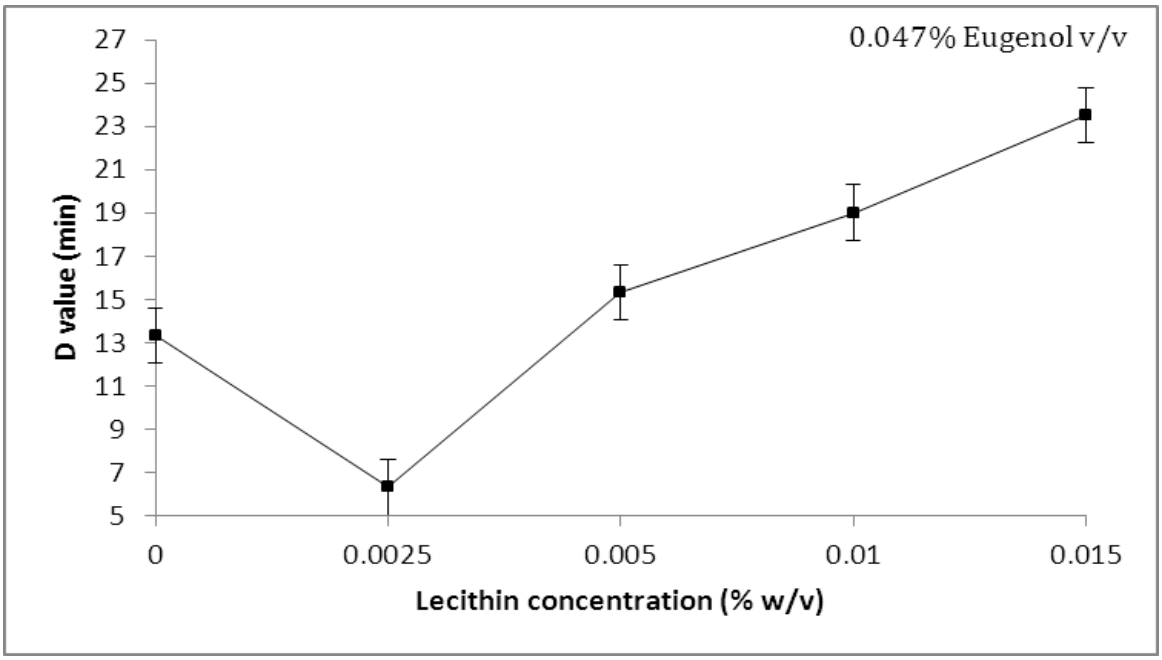

Figure 9. D-value of *E. coli* K12 in vitro at 37°C with varying concentrations of lecithin, 0.047% v/v eugenol and an initial count of ca. 8.6 log CFU/ml. Error bars are LSD for the mean.

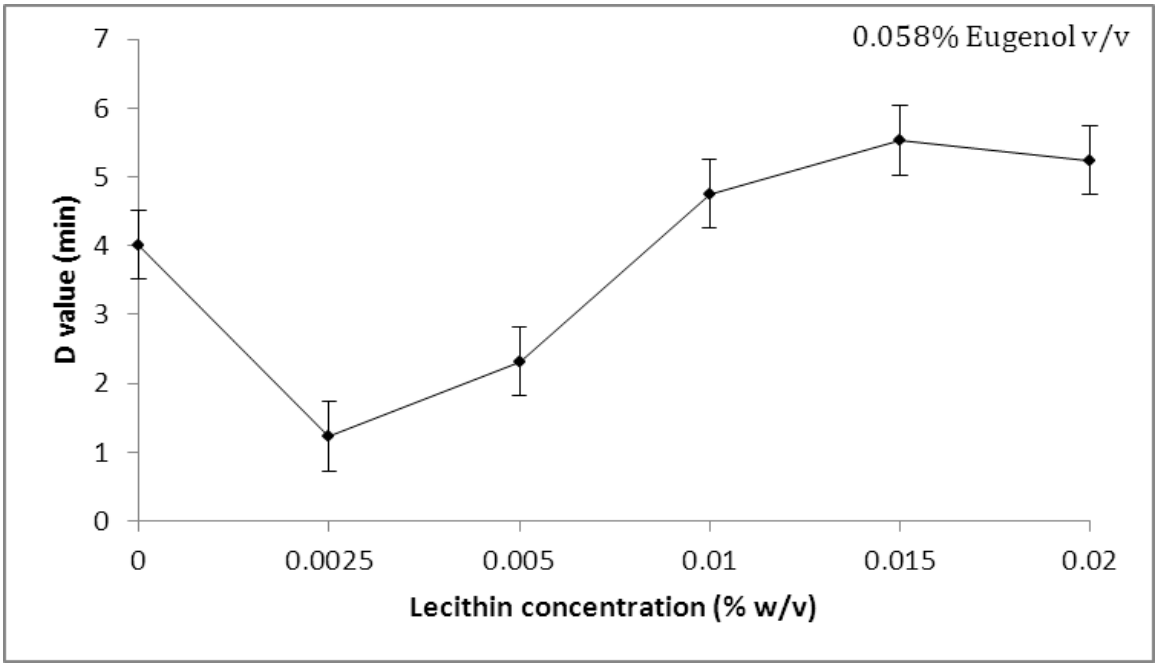

Figure 10. D-value of *E. coli* O157:H7 strain 'Cider' in vitro at 37°C with varying concentrations of lecithin, 0.058% v/v eugenol and an initial count of ca. 8.6 log CFU/ml. Error bars are LSD for the mean.

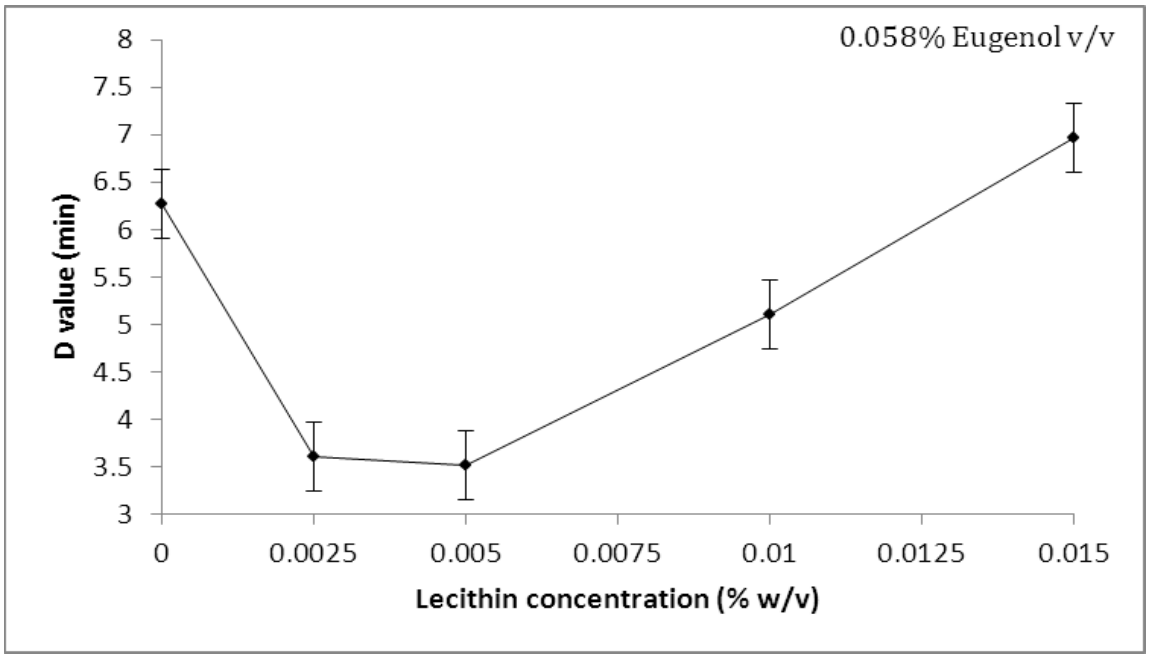

Figure 11. D-value of *E. coli* O157:H7 ATCC strain 43889 in vitro at 37°C with varying concentrations of lecithin, 0.058% v/v eugenol and an initial count of ca. 8.6 log CFU/ml. Error bars are LSD for the mean.

Carvacrol

The D-value with carvacrol emulsion showed similar effect with the addition of lecithin. For *E. coli* K12, the D-value decreased from 17.36 ± 4.25 for the control to 9.73 ± 1.12 min and 10.49 ± 0.73 with 0.0025% and 0.005% w/v lecithin ($p<0.05$, **Figure 12**). For *E. coli* O157:H7 'Cider', the D-value dramatically dropped from 10.24 ± 3.17 without lecithin to 6.30 ± 0.48 min, 4.88 ± 0.48 min by adding 0.0025% and 0.005% w/v lecithin respectively ($p < 0.05$, **Figure 13**). For *E. coli* O157:H7 ATCC 43889, the D-value started at 12.08 ± 1.20 min without lecithin. With 0.0025% w/v lecithin, the D-value significantly decreased to 4.92 ± 0.88 min ($p<0.05$, **Figure 14**). Extensive lecithin hindered the interaction of carvacrol.

The particle sizes of eugenol droplets were between 100 to 700 nm and zeta potentials were between – 40 to – 110 mV (Table 3) in all experiments. There were no statistically significant correlations between the particle size and D-value, or zeta potential and D-value for *E. coli* strains ($p > 0.05$ and $r < 0.7$).

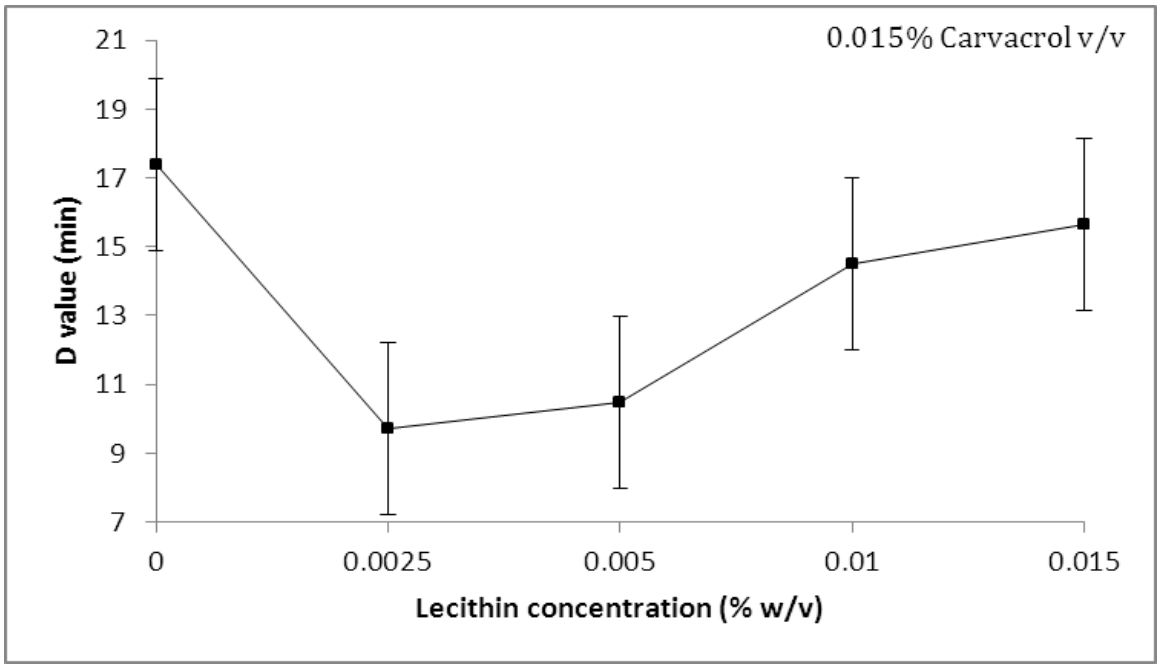

Figure 12. D-value of *E. coli* K12 in vitro at 37°C with varying concentrations of lecithin, 0.0150% v/v carvacrol and an initial count of ca. 8.6 log CFU/ml. Error bars are LSD for the mean.

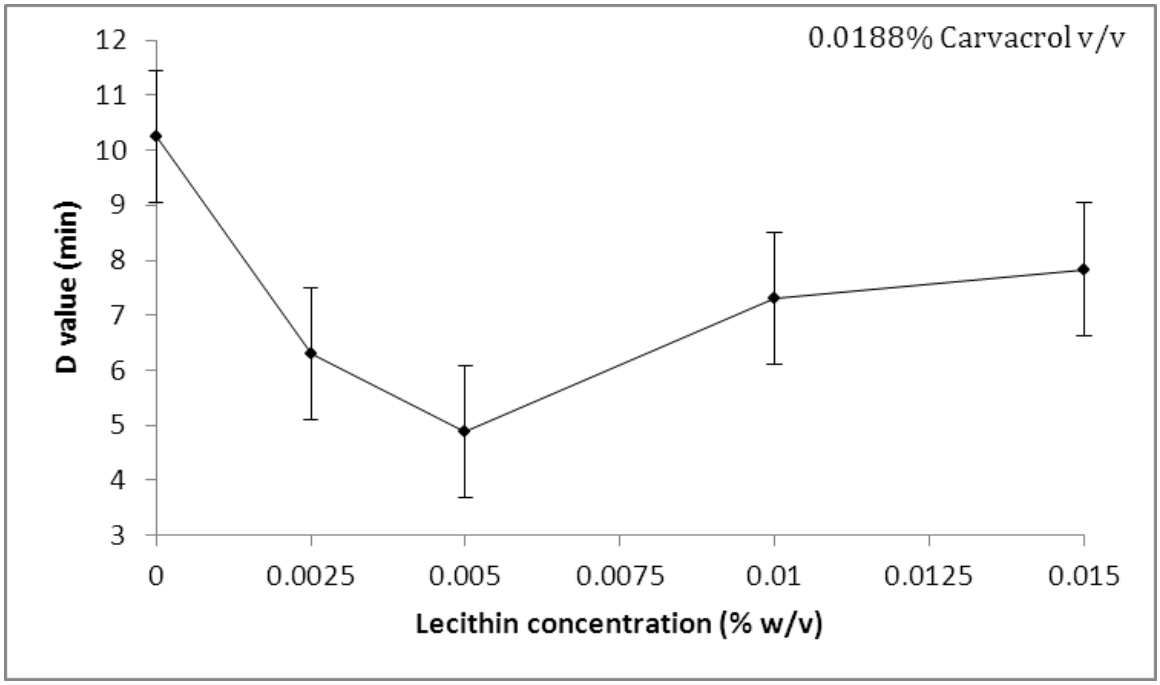

Figure 13. D-value of *E. coli* O157:H7 strain 'Cider' in vitro at 37°C with varying concentrations of lecithin, 0.0188% v/v carvacrol and an initial count of ca. 8.6 log CFU/ml. Error bars are LSD for the mean.

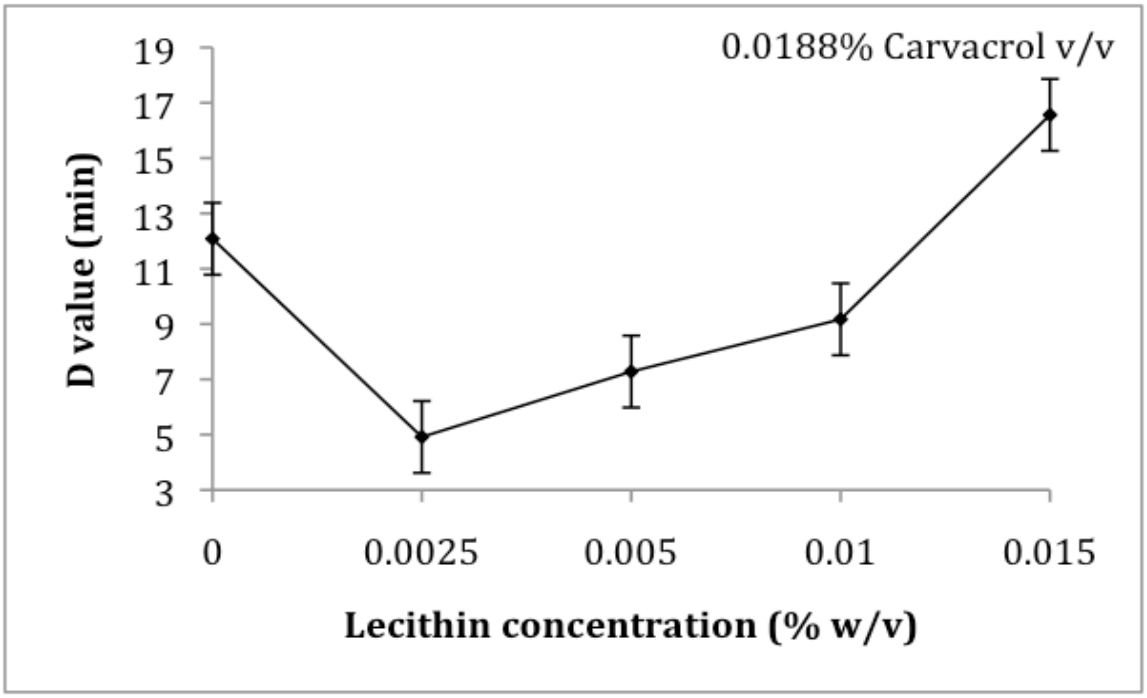

Figure 14. D-value of *E. coli* O157:H7 strain 'Cider' in vitro at 37°C with varying concentrations of lecithin, 0.0188% v/v carvacrol and an initial count of ca. 8.0 log CFU/ml. Error bars are LSD for the mean.

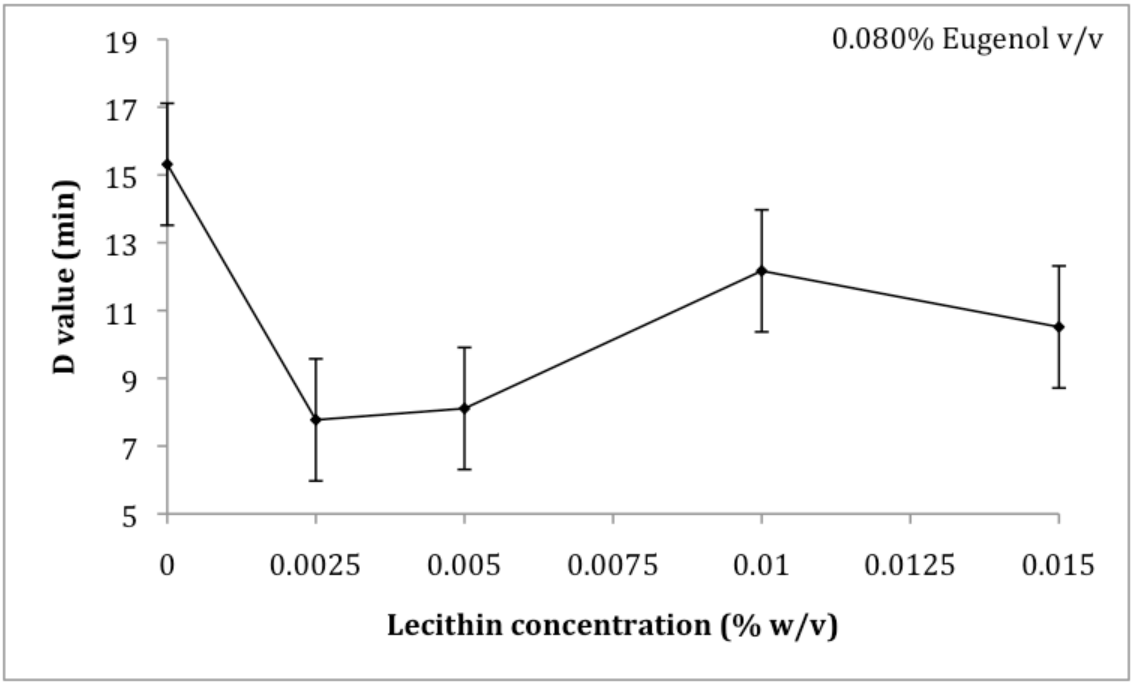

Figure 15. D-value of *E. coli* O157:H7 strain 'Cider' in vitro at 37°C with varying concentrations of lecithin, 0.080% v/v eugenol and an initial count of ca. 8.0 log CFU/ml. Error bars are LSD for the mean.

Table 3. Average and standard deviation for particle size (nm) and zeta potential (mV) of the eugenol and carvacrol droplets dispersed in phosphate buffer containing different concentration of lecithin.

***E. coli* strain -**	K12	'Cider'	ATCC 43889	_	K12	'Cider'	ATCC 43889	_
Eugenol Concentration (% v/v)	**0.094**	**0.116**	**0.116**	**0.094**	**0.0030**	**0.0376**	**0.0376**	**0.030**
Lecithin Concentration (% w/v)	**Average Particle Size ± std (nm)**			**Zeta Potential ± std (mV)**	**Average Particle Size ± std (nm)**			**Zeta Potential ± std (mV)**
0	636.9±53.9	313.83±48.69	381.02±15.74	-49.3±5.5	638.83±374	410.03±90.69	212.60±26.20	-70.0±4.7
0.0025	389.31±101.43	327.80±73.41	362.12±36.87	-67.8±1.5	229.9±26.09	239.77±25.61	280.1±88.96	-53.6±13.4
0.005	407.19±263.90	435.61±60.41	416.74±27.76	-69.0±13.8	207.6±2035	239.22±24.16	240.83±9.66	-58.3±9.4
0.01	596.90±144.16	435.61±60.11	486.08±108.17	-70.8±11.2	263.53±22.49	265.831±41.68	314.10±14.19	53.76±1.03
0.015	514.27±153.99	496.50±70.28	558.49±136.45	-107±15.2	247.93±17.45	222.8±77.78	252.23±3.97	-62.7±6.1
0.020		483.37±20.50						

Results indicate that lecithin-induced surface modifications of the nano-size EO droplet are beneficial, not only to promote a better stability of essential oils in aqueous systems, but also to improve their antimicrobial properties. The critical micelle concentration (CMC) of phosphatidycholine, the major components in lecithin, is ca. 0.1 wt% (McKee, Layman et al. 2006). Because the concentrations of lecithin used in the study were lower than the CMC, micelle formation of lecithin did not occur and was not playing a role in enhancement of the antimicrobial activity of the EO components. The findings indicated that, at low concentrations lecithin enhanced the antimicrobial activity of eugenol and carvacrol against *E. coli*. However, above some critical concentration of lecithin, the antimicrobial activity returned to the level found in control samples without lecithin. Our results with high concentration of lecithin are in agreement with Friedman et al. who observed that 0.25% soy lecithin reduced the antimicrobial activity of oregano and thyme essential oils (Friedman, Henika et al. 2004).

Lecithin is a natural zwitterionic surfactant. It is hypothesized that lecithin promotes antimicrobial activity by improving interaction between the charged EO droplets and the generally negatively charged bacterial surface (Schwegmann, Feitz et al. 2010). In this way, lecithin not only helps stabilize the emulsion by forming a surfactant layer against aggregation, but improves the antimicrobial properties by promoting better EO droplet-bacteria contact. According to literature, the proposed mechanisms of bacterial inhibition by EOs or their components involves charged EO component droplets contacting the bacterium, the EO components disrupting into the lipid cell membrane, the EO components disrupting membrane structures, resulting in increased permeability

(Lambert, Skandamis et al. 2001; Estevez and Cava 2006) and leakage (Burt 2004). Furthermore enzymes such as ATPases, known to be located in the cytoplasmic membrane, are disturbed which causes leakage of ions and other cell contents (Burt 2004). Changes in the internal pH of the cells has also been observed (Ultee, Kets et al. 1999; Lambert, Skandamis et al. 2001; Skandamis and Nychas 2001).

At high concentrations of lecithin, it is hypothesized that lecithin phospholipids form bi-layers or multilayers, which physically hinder contact between EO component droplets and bacterial surfaces (Burt and Reinders 2003). Phosphatidylcholine, a major phospholipid in lecithin, can partially neutralize the antimicrobial activity of EO components against bacterial cells. These results also explain the negative effect of lecithin used in studies to improve antimicrobial activity of EOs (Friedman, Henika et al. 2004). Further studies are needed to elucidate specific mechanisms of action and to implement further applications in fluid foods.

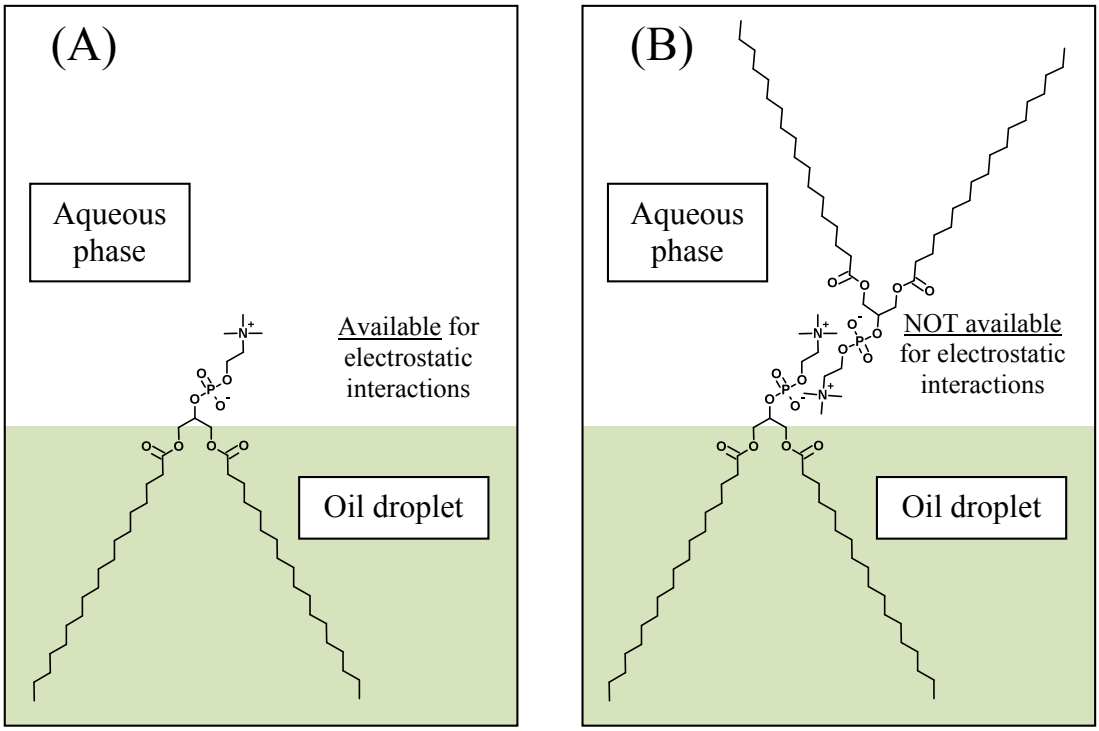

Figure 16. Schematic representation of the effect of low (A) and high (B) concentration of phosphatidylcholine, a major component in lecithin, on the ability of eugenol oil droplets to exhibit electrostatic interaction with bacterial surfaces.

Conclusion

Both in microbiological media and food, low concentration of lecithin can effectively enhance the antimicrobial activities of eugenol / carvacrol to prevent the growth of *E. coli* O157:H7. In contrast, in most cases at high concentrations of lecithin, the antimicrobial activities of the isolated EO component were hindered. The hypothesis is that lecithin promotes antimicrobial activity by improving interaction between the charged EO droplets and the generally negatively charged bacterial surface. Further studies are needed to elucidate specific mechanisms of action and to implement further applications in fluid foods as a food additive to prevent the growth of *E. coli* O157:H7.

Acknowledgments

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Vita

Songsong Li was born in a beautiful village, named Yanghua, Hunan Province, China. He enjoyed the countryside life for fourteen years with his grandparents. After that, he moved to a small city to continue his primary school, Middle School. After High School, he was luckily enrolled in Northeast Forestry University to pursue degrees in Food Science and Technology and English. It is the double majors that helped him improve his English writing, reading and speaking, introduce him an occidental worldview. After obtaining his Bachelor of engineering in Food Science and Technology and Bachelor of Arts in English from NEFU with excellent grades, he had the opportunity to continue his graduate study in the University of Tennessee, Knoxville. During his stay in UT, Knoxville, he majored in Food Science and Technology, minored in Statistics and worked as a graduate research assistant under the guidance of Dr. Federico Harte. His research is focusing on enhancement of the antimicrobial activity of eugenol and carvacrol against *Escherichia coli* O157:H7 by lecithin in microbiological media and food. Also he has developed a dragon fruit sports drink to compete in the 2011 Danisco Knowledge Award New Product Competition. As a graduate student he was awarded the Hazelwood Graduate Student Scholarship from the College of Agricultural Sciences and Natural Resources (2009), and Graduate Student Travel Award (2011). He also volunteered in various activities: FSC cheese sale fundraising (2009, 2010), Relay for Life (2010, 2011), Bearden Middle School Science Olympia (2010. 2011), vice president of UTK Chinese Students and Scholars Association (2010) etc. In the future, he would like to start his challenge in the Food Industry to develop healthy and high quality food products.