THE EFFICACY OF NATURAL PLANT ANTIMICROBIALS AGAINST *ESCHERICHIA COLI*

by

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DEDICATION

I would like to first thank God for giving me the strength to persevere through these years of graduate school. Looking back I can remember various times where I said a silent prayer before an exam or during an overall rough patch. To my parents Ralston, Zeka, and Linda you have been and will always continue to be an inspiration to me. Your guidance, patience and never ending support have enabled me to become the man that I am today and for that I will always be grateful.

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ABSTRACT

The number of foodborne disease outbreaks related to fresh produce has increased in recent years. This has coincided with a growing public demand for minimally processed fruits and vegetables. Effective produce sanitizers are therefore needed that are at least as effective as chlorine, currently the most commonly used sanitizer. Natural antimicrobials from plant extracts and essential oils are a possible alternative. These are highly effective and may also be used in situations in which chlorine is not advantageous; for instance, in situations in which chlorine has limited efficacy or because of concerns over the production of harmful by-products resulting from chlorine use. Plant derived essential oils have been shown to be antibacterial, antiviral, and antifungal. In this study we examined the use of natural antimicrobials from plant extracts and essential oils as possible alternative sanitizers. We examined these antimicrobials for their efficacy against *Escherichia coli.* In addition, since many of these natural compounds are believed to be membrane active, silver ions were added to some of the tests to assess the potential for synergy between the antimicrobials. Silver ions, although slow-acting on their own, often exhibit a synergistic antimicrobial effect when combined with other membrane active antimicrobials such as oxidizing agents. These studies reveal that plant derived antimicrobials are effective sanitizers with the potential to replace commonly used chlorine.

INTRODUCTION

Problem Definition

Bacteria attributed to foodborne outbreaks associated with produce include *Salmonella enterica*, *Escherichia coli*, *Shigella dysenteriae*, *Campylobacter jejuni*, and *Listeria monocytogenes*. *E. coli* O157:H7 and *S. enterica* are responsible for approximately 61% of all produce-associated illnesses (Olsen et al. 2000).

Bacteria are capable of prolonged survival on produce both pre- and post-harvest. After harvesting, raw fruits and vegetables are usually treated with an aqueous chemical formulation to remove dirt, pesticides, and microorganisms. There are many factors influencing the efficacy of produce sanitizers such as the presence of organic matter (e.g., vegetable tissues, fruit juices, soil), the produce surface properties (e.g., rough/smooth, hydrophilic/hydrophobic, the presence of trichomes/fine hairs), and the type of disinfectant. Organic matter can reduce the sanitizer efficacy by directly interacting with the chemical. Particularly with leafy vegetables, sanitizers are often not as effective as they are on produce with smooth surfaces (e.g., tomatoes) (Beuchat et al. 2001; Koseki et al. 2004; Pirovani et al. 2004). Bacteria adhere to surface irregularities, thus reducing the ability of washing or sanitizing treatments to remove or inactivate attached cells (Ukuku and Sapers 2006).

Chlorine is by far the most commonly used sanitizer ((Suslow, 2000); (Castillo & Rodriguez-Garcia, 2004); Doyle 2005). The concentrations of chlorine used by the industry for surface disinfection of produce range from 50 to 200 mg/L with a contact time of one to two minutes (Beuchat 1998; Yuk et al. 2006); however, this treatment typically results in less than a

2-$\log_{10}$ reduction in the microbial load per gram (Koseki et al. 2003; Koseki et al. 2004; Rodgers et al. 2004; Doyle 2005). Contact of chlorine with open wounds, crevices, or other produce tissues may result in a decrease in its effectiveness. Organic matter rapidly consumes the chlorine via oxidization (Seo and Frank 1999; Suslow, 2000); therefore, when the organic load is high, the chlorine level in the produce wash water needs to be continuously monitored and maintained. Additional drawbacks with chlorine are its corrosive nature to equipment and its ineffectiveness against bacteria that are attached to surfaces (Sapers, 2009). The future of chlorine use is also in question as concerns regarding its carcinogenicity have been raised (Chang et al. 1988; Hidaka et al. 1992).

Literature Review

Foodborne outbreaks related to fresh produce

The ready-to-use (RTU) vegetable industry has expanded at a rapid pace in recent years as a result of continuous consumer demand for fresh, convenient foods (Rico et al., 2007). Fresh fruits and vegetables possess a variety of health benefits. For instance, antioxidants act as free radical receptors. Ascorbic acid and β-carotene are the antioxidants in the highest concentrations in fruits and vegetables (Rico et al., 2007). While the techniques routinely employed in conventional food processing are designed to prolong the shelf-life of fruits and vegetables, the minimal processing to which RTU produce is subjected decreases their shelf-life, rendering them highly perishable (Garcia & Barrett, 2002).

Microorganisms are natural contaminants of fresh produce, with contamination occurring through a variety of mechanisms (Beuchat, 1996). There are five major stages of potential contamination that produce face: pre-harvest, harvest, postharvest, storage, and consumer handling. These include, but are not limited to, the usage of untreated irrigation water or sewage, inappropriate organic fertilizers or inadequately composted manure, pesticides, wild or domestic animals, proximity to urban areas or land used for other types of agriculture or industrial development, or other sources that can occur anywhere from the farm to the fork such as failure during harvesting, transport, storage, processing, packaging, marketing, restaurant services and at home (Mercanoglu Taban & Halkman,).

The range of potential hazards that can be introduced at the pre-harvest stage include bacteria such as enterotoxigenic and enterohemorrhagic *Escherichia coli, Salmonella,*

Campylobacter, *Listeria*, *Shigella*, *Yersinia*, parasites such as *Cryptosproridium*, *Cylospora*, helminths, and viruses such as hepatitis A, and noroviruses. These organisms may be present in water used for irrigation or in soil which the produce is grown (Mercanoglu Taban & Halkman,). In lettuce, cross-contamination can occur through contact with workers' hands (or gloves), knives, automated equipment (conveyor belt), and wash water (Matthews, 2009). Postharvest, numerous opportunities for contamination events occur during transport (open transportation, transport vehicles), processing (immersion in water and cutting or slicing steps), packing (improper packaging, packing equipment), distribution and market or retail (Mercanoglu Taban & Halkman,).

Foodborne outbreaks from contaminated fresh produce have been increasingly recognized in various parts of the world (Lynch et al., 2008). This is due to a combination of increasingly improving outbreak surveillance systems, as well as increased consumption of high risk, minimally treated, ready to eat food items. Ready to eat produce is an ideal vehicle for the transport of foodborne pathogens as they are not subjected to typical processing procedures prior to consumption. In the United States, the proportion of outbreaks linked to fresh produce increased from <1% of all reported outbreaks with known foods in the 1970s to 6% in the 1990s (Sivapalasingam et al., 2004).Outbreak data shows that from 1973 to 2006, roughly 5% of foodborne outbreaks in the U.S. were associated with leafy greens (Smith Dewall & Bhuiya, 2007). Nearly 60% of those outbreaks were caused by norovirus; *Salmonella* and *Escherichia coli* each accounted for about 10% of the outbreaks (Matthews, 2009). In the United States, several major produce outbreaks have been associated with bagged leafy greens. In 2005, bagged salads containing romaine lettuce, red cabbage, and cabbage were linked to an outbreak of *E. coli* 0157:H7 (Smith Dewall & Bhuiya, 2007).

Foodborne outbreaks occur continuously in the United States, usually amongst small groups where the offending pathogen causes self mitigating illness. Nevertheless, large multistate outbreaks cause large numbers of illnesses and even deaths. These large outbreaks often garner excessive media coverage, product recalls, public paranoia, and subsequent economic losses. Listed in Table 1 are the produce associated with multistate outbreaks between the years 2006-2011 as reported by the Centers for Disease Control (CDC) (Anonymous, MMWR 2011). Upon examination, it becomes apparent that *Salmonella* and *Escherichia coli* account most of these large outbreaks.

Table 1. Multistate outbreaks of foodborne illness in the United States from 2006-2011.

Year	Associated Product	Microorganism
2011	Del Monte cantaloupe	*Salmonella* Panama
2011	Papyas	*Salmonella* Agona
2011	Hazelnuts	*Escherichia coli* 0157:H7
2010	Alfalfa Sprouts	*Salmonella*
2010	Alfalfa Sprouts	*Salmonella* Newport
2010	Shredded Romaine Lettuce	*Escherichia coli* 0145
2009	Alfalfa Sprouts	*Salmonella* Saintpaul
2009	Pistachios	*Salmonella* (multiple)

2008	Produce, peppers, tomatoes	*Salmonella* Saintpaul
2006	Tomatoes	*Salmonella* Typhimurium
2006	Fresh Spinach	*Escherichia coli* 0157:H7

Current produce sanitizers

Detergent products reduce microbial populations of produce surfaces by detachment rather than incactivation (Matthews, 2009). Studies have shown that detergents reduce bacterial numbers by 2 to 3 logs while others display reductions with no greater efficacy than normal water (Raiden R.M. et al., 2003). Approved Food and Drug Administration (FDA) surfactants include sodium n-alklybenzene sulfonate, sodium dodecylbenzene, sodium mono- and dimethyle naphthalene sulfonates, and sodium 2-ethylhexyl sulfate (Sapers, 2009) .

Currently, chlorine is the primary postharvest sanitizing agent in use by the fresh produce industry, and canachieve a 1-2 log reduction in microbialnumbers (Doyle, 2005). Chlorine is usually used as sodium or calcium hypochlorite or Cl_2 gas, with working concentrations between 50 to 200 ppm (Sapers, 2009). At a slightly acidic pH, hypochlorite is an extremely effective antimicrobial: however, chlorine is highly reactive with organic species originating in soil and debris or leached from damaged produce into the process water, resulting in rapid chlorine depletion and greater survival of the targeted microflora when the organic load is high (Suslow, 2000). In this case, the chlorine level in wash water needs to be continuously monitored and maintained. Additional drawbacks with chlorine are its corrosive nature, particularly at low pH,

where it shortens the life span of tanks and other stainless steel equipment used in produce packing/processing operations (Sapers, 2009).

Many alternative produce sanitizers have been used. Like chlorine, electrolyzed water sees its efficacy significantly reduced when employed against bacteria attached to produce surfaces, with reductions being generally limited to 1 to 3 logs (Izumi, 1999);(Park et al., 2001). Aqueous chlorine dioxide ClO_2, ,in contrast to hypochlorite, is more effective at neutral pH, less reactive with organics, less corrosive, and forms fewer chlorinated byproducts (Anon., 2001); nevertheless, ClO_2 is unstable, and at partial pressures greater than 120 mm Hg (15.8% by volume at atmospheric pressure), it becomes explosive (Sapers, 2009). Ozone is a highly effective, broad spectrum antimicrobial agent, effective at low concentrations and short contact times (Restaino et al., 1995; Wickramanayake, 1991). Ozone is also highly unstable and rapidly breaks down into nontoxic products. In addition, it is corrosive to equipment and can cause physiological injury to produce and degrade product color and flavor. Ozone is toxic and an irritant to workers at concentrations in air greater than 0.1 ppm and must be adequately vented to avoid worker exposure (Anon., 2001). Peroxyacetic acid (PAA) is highly antimicrobial. It is comprised of equal parts hydrogen peroxide and acetic acid. PAA is approved by both the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) for addition to wash water for the treatment of fruits and vegetables (Sapers, 2009). Unfortunately, due to its strong oxidizing properties, handling of PAA becomes hazardous at high concentrations. Hydrogen peroxide, iodide, and several organic acids have also been approved as produce sanitizers. While all of these sanitizers possess various degrees of effectiveness, none have proven to be more effective than chlorine. Each possesses its own drawbacks. One promising strategy has been that of a combinatorial approach (Beltrán et al., 2005)). Combinations of lactic

acid, chlorinated water, thyme essential oil solution, sodium lactate, citric acid, and hydrogen peroxide, in addition to others have been assessed (Bari et al., 2005).

Produce sanitizers often have limited effectiveness due to a variety of reasons. Inaccessibility is the common theme for pathogen resistance to sanitizers. Irregularities in produce surfaces offer protection from sanitizers. Cracks on the cuticle and trichomes of lettuce confer protection against chlorine inactivation (Gomez-Lopez et al., 2008). Injuries at the surface of produce also protect microorganisms from the effect of sanitizers (Han et al., 2000). Insults to the wax layer, the cuticle, and underlying tissue have been shown to increase bacterial adhesion and resistance to washing and disinfectant treatment (Gomez-Lopez et al., 2008). Injuries (mechanical, bruising, slicing) also offer abundant access to nutrients, which then promotes pathogen survival and multiplication, thus diminishing the effectiveness of sanitizers.

Internalization is another route by which microorganisms avoid sanitizers. Internalization can occur prior to harvesting, during storage, or during process operations. Microorganisms have been shown not only to infiltrate, but also to survive inside fruits and vegetables (www.fda.gov, 2009). Once microorganisms become established in the produce inner tissue they are essentially inaccessible to sanitizers. Leafy greens such as lettuce are particularly susceptible as cutting is a routine step in their processing. These cut surfaces are preferred sites for microbial attachment and internalization.

Bacterial attachment and biofilm formation are additional factors which limit the effectiveness of produce sanitizers. Increasing evidence indicates that microorganisms attached to surfaces are more resistant to sanitizers than their planktonic counterparts (Gomez-Lopez et al., 2008). Biofilms which are often naturally present provide microorganism's protection via reduced diffusion, physiological changes, and the production of enzymes that degrade

antimicrobial substances (Gomez-Lopez et al., 2008). Leafy greens are significantly more susceptible to biofilms as they do not have a peel (unlike oranges, apples, etc).

A final factor influencing sanitizer effectiveness is the initial contamination level. High pathogen loads increase survivability against sanitizers, as they can overwhelm the effectiveness of sanitizers at the levels usually applied.

Natural plant antimicrobial compounds

Bioactive compounds are extra nutritional constituents that typically occur in small quantities in foods. The driving force behind research of these bioactive compounds is the fact that many have shown protective effects with regard to cardiovascular disease (CVD) and cancer (Ullah & Khan, 2008). A great majority of the natural plant compounds and plant extracts exhibit bactericidal activities.Phenolic compounds, commonly referred to as polyphenols, are present in all plants. (Bravo, 1998). To date, more than 8,000 phenolic compounds have been identified, all consisting of variations of the C6 ring structure. Flavonoids are the most common polyphenolic compounds in plants (Kris-Etherton & Keen, 2002). Polyphenols exhibit several biological effects such as anti-inflammatory, anti-microbial, anti-carcinogenic, and anti-HIV, cardioprotective and neuroprotective properties (Ullah & Khan, 2008). For any chemical moiety to exert a biological effect it should be bioavailable, i.e. it must be readily absorbed into the bloodstream and reach concentrations that have the potential to exert effects *in vivo*. Most of the polyphenols are known to be readily absorbed (Ullah & Khan, 2008). Fruits and vegetables such as red, blue, and purple berries, red and purple grapes, red wine, teas (particularly green), apples, pears, raspberries, apples, onions, broccoli, soybeans, legumes, soy foods, Gingko biloba, and

chocolate are common sources of bioavailable polyphenols (Ullah & Khan, 2008).

Polyphenols, in particular flavonoids, have been found to be effective antimicrobials against a wide array of microorganisms. This is probably due to their ability to complex with extracellular and soluble proteins and also with the bacterial cell wall (Tsuchiya et al., 1996). Flavonoids are synthesized by plants in response to microbial infection. Correspondingly, these antimicrobial properties may benefit humans. It is a longstanding belief that people who consume large amounts of fruits and vegetables are healthier than those who do not. Several population studies have reported an inverse association between flavonoid intake and the risk of coronary disease and cancer. In the Zutphen Elderly Study (Kris-Etherton and Keen 2002), a high intake of flavonoind (approximately 30 mg/day) was associated with approximately a 50% reduction in cardiovascular heart disease (CHD) mortality rate compared with individuals who had a low flavonoid intake (<19mg/day). However, there is the "French Paradox"-the observation that mortality from coronary heart disease is relatively low in France despite relatively high levels of dietary saturated fat led to the idea that the regular consumption of red wine (a rich source of polyphenols) might provide additional protection from cardiovascular disease (Criqui & Ringel, 1994). Red wine is a concentrated source of polyphenolic substances and >200 individual phenolic compounds have been identified to date. Studies have shown that red wine inhibits low-density lipoprotein (LDL) *in vitro* and increases the antioxidant capacity of plasmas (Kris-Etherton et al., 2002). One particular polyphenol, resveratrol, found in red wine is responsible for vasorelaxation. Resveratrol provides cardioprotective properties by relaxing endothelium-denuded arteries. Overall, natural polyphenolic compounds possess antioxidant, vasorelaxant and antihypertensive properties what are beneficial to cardiovascular health (Ullah & Khan, 2008).

Epidemiological studies indicate that diets rich in antioxidants can influence the incidence of neurodegenerative disorders. The nervous system is rich in fatty acids and iron. High levels of iron can lead to oxidative stress via the iron-catalyzed formation of reactive oxygen species (ROS) (Bauer & Bauer, 1999). Blueberries, red grapes, and subsequently red wine are excellent sources of antioxidants which interact with ROS. In addition, resveratrol, the phenol antioxidant found in berries and grapes has been reported to possess anticancer properties (Aggarwal et al., 2004) and is able to inhibit the formation of prostate tumors by acting on the regulatory genes such as p53 (Narayanan, 2006). Citrus fruit flavonoids, tangeretin and nobiletin, have also been shown to inhibit human breast cancer cell line MDA-MB-435 and MCF-7 and human colon cancer cell line HT-29 by blocking cell cycle progression at G1 stage of the cell cycle (Morley et al., 2007). Overall, flavonoids are recognized as naturally occurring antioxidants and this property has been implicated for their anticancer activity (Bors et al., 1998).

In addition to their potential as anticancer agents, an important role of plant polyphenols as natural modulators of cancer multidrug resistance (MDR) has been realized recently (Ullah & Khan, 2008). Resistance of recurrent disease to cytotoxic drugs is the principal factor limiting long-term treatment success against cancer. Flavonoids, isoflavones, and green tea polyphenol EGCG have all exhibited anti-MDR activities (Ullah & Khan, 2008). These findings indicate that plant polyphenols will play a significant role in the development of strategies for cancer chemoprevention.

Consumers demand fresh, minimally processed fruits and vegetables throughout the calendar year. While the health benefits of natural, fresh tasting, additive free produce are undeniable, the problem of foodborne pathogens is more prevalent now than at any previous time. Natural plant extracts and oils are more suitable than their synthetic antibiotic counterparts

to address the foodborne pathogen problem. Essential oils in particular are usually formed by several constituents. They may cause deterioration of the cell wall, damage to the cytoplasmic membrane, damage to membrane proteins, leakage of cell contents, coagulation of the cytoplasm, depletion of proton motive force active sites, inactivation of essential enzymes, and disturbance of genetic material functionality (Ayala-Zavala et al., 2008; Burt, 2004; Gutierrez et al., 2008).

The production of natural antimicrobial fruit and vegetable washes/sanitizers could potentially enable consumers to reduce the concentrations of pathogenic bacteria in their homes without the use of potentially harmful chemicals. Additionally, if the agricultural industry can move away from the dependence on chlorine as their primary sanitizer, it would provide both antibacterial efficacies and a 'green' package as many of these natural plant antimicrobials are generally regarded as safe (GRAS) for human consumption.

Natural antimicrobials as produce sanitizers

Natural antimicrobial peptides (AMPs) represent a potentially new category of produce sanitizers. Antimicrobial peptides are usually composed of 12-50 amino acids (Keymanesh et al., 2009). These peptides are vital parts of the innate host immune system and are synthesized by microorganisms as well as multicellular organisms from the plant and animal kingdoms (Brown & Hancock, 2006). Peptide based antimicrobial defense is an evolutionary ancient mechanism, with immediate and non-specific effects against most Gram-negative and Gram-positive bacteria, fungi, viruses and eukaryotic parasites (Vizioli & Salzet, 2002; Wang & Ng, 2005; Wang et al., 2006). AMPs are divided into two categories; non-ribosomally synthesized peptides and

ribosomally synthesized (natural) peptides. Non-ribosomally synthesized peptides are produced mainly by bacteria, while ribosomally (natural) peptides are produced by all organisms including bacteria (Hancock & Chapple, 1999).

Natural antimicrobials are derived from various sources; plants, bacteria, and yeasts are some of the most common producers. Plants produce a wide array of antimicrobial agents which may be separated into several groups. The phenols and polyphenols are a group of bioactive phytochemicals which consist of a single substituted phenolic ring (Cowan, 1999). Catechol and Epicatechin are two forms of simple phenols whose mechanism of action include substrate deprivation and membrane disruption (Peres et al., 1997; Toda et al., 1991). Phenolic acids, quinones, flavonoids, flavones, tannins, and coumarins round out the class of phenolics. Quinone binds to adhesions, causes the formation of complexes in the cell wall, and inactivates enzymes (Duke, 1985; King & Tempesta, 2007). Flavonoids bind to adhesins (Perrett et al., 1995; Rojas et al., 1992), while flavones complex with the cell wall andinactivate enzymes, even inhibiting the reverse transcriptase enzyme of human immunodeficiency virus (HIV) (Brinkworth et al., 1992; Ono et al., 1989; Taniguchi & Kubo, 1993). Tannins (also called proanthocyandins) bind to proteins and adhesins, inhibit enzymes, cause substrate deprivation, complex with the cell wall, disrupt the membrane, and cause metal ion complexation (Brownlee et al., 1990; Butler, 1988; Haslam, 1996; Scalbert, 1991; Schultz, 1988; Serafini et al., 1994; Stern et al., 1996; Stern et al., 1996). Tannins have received a lot of attention as they are found in various green teas and red wines whose consumption have long been credited with therapeutic effects (Serafini et al., 1994).

The fragrance of plants is carried in the so called quinta essential, or essential oil fraction (Cowan, 1999). These oils are secondary metabolites called terpenoids and are highly enriched in compounds based on a isoprene structure (Cowan, 1999). Terpenoids exhibit antimicrobial

activity through membrane disruption (Serafini et al., 1994). Citral, the active component of lemongrass, is a type of terpenoid that causes membrane perturbation; the leakage of specific ions caused by the action on the cell membrane has dramatic effects on proton motive forces, the intracellular ATP content, and the overall cell activity (Somolinos et al., 2010). Eugenol is the primary active component in clove bud, oregano, and allspice essential oils and accounts for their antioxidant properties (Masahiro et al., 2000-10-01). The antioxidant activity may occur via various mechanisms such as the scavenging of radicals and the chelating of metal ions. Eugenol reportedly participates in photochemical reactions (Mihara & Shibamoto, 1982). Grape seed extract (GSE) and green tea are both members of the phenolic family. The high concentrations of epicatechin and catechin in GSE and caffeic acid and epicatechin in green tea extracts account for their high antioxidant activities (Rababah et al., 2004). Carvacrol, the active ingredient in oregano oil, causes depletion of the intracellular ATP pool, changes in the membrane potential, and increases the permeability of the cytoplasmic membrane to proteins and potassium ions (Ultee et al., 2002). Carvacrol partitions the fatty acid chains of the phospholipids in cell membranes, thus forming ion channels which permit ions to escape from the cytoplasm (Cox & Markham, 2007). Cinnamaldehyde, the major active antimicrobial in cinnamon oil, disrupts enzyme activity (Ravishankar et al., 2010).

Oxidation is the primary mechanism of inhibition for a wide range of antimicrobials including several natural antimicrobials. Although biochemical mechanisms of action differ between oxidative biocides, the physiological reactions are largely similar (Denyer & Stewart, 1998). Oxidative biocides are proposed to have multiple targets within a cell. These include peroxidation and disruption of membrane layers, oxidation of oxygen scavengers and thiol groups, enzyme inhibition, oxidation of nucleosides, impaired energy production, disruption of

protein synthesis, and, ultimately, cell death (Finnegan et al., 2010). Oxidation is highly reversible at the cellular level when the antagonist is present at a low level and prokaryotic organisms have evolved various defenses against oxidation (Somolinos et al., 2010). Oxidizing agents are usually low-molecular weight compounds and are considered to pass easily through cell walls/membranes, whereupon they are able to react with internal cellular components, leading to apoptotic and necrotic cell death (Denyer & Stewart, 1998).

Bacteriocins are ribosomally synthesized, extracellularly released, bioactive peptides or peptide complexes which have a bactericidal or bacteriostatic effect on other (usually closed related) species (O'Keeffe & Hill, 1999). Nisin, a bacteriocin produced by certain strains of *Lactococcuc lactis* subsp. *Lactis*, is inhibitiory to a wide range of Gram-positive bacteria, including strains or species of streptococi, staphylococci, lactobacilli, micrococci, *Listeria*, and most spore-forming species of *Clostridium* and *Bacillus* (O'Keeffe & Hill, 1999). Nisin's mode of action is via membrane insertion and pore formation, leading to a rapid and specific efflux of low molecular weight compounds and the depolarization of the membrane (O'Keeffe & Hill, 1999). Nisin has displayed clear synergy with cavacrol and eugenol against both *Bacillus cereus* and *Listeria monocytogenes* (Periago et al., 2001; Pol & Smid, 1999). Currently, nisin is the only bacteriocin to receive FDA approval (O'Keeffe & Hill, 1999).

Silver as an antimicrobial

Silver has no known biological function in living cells and is rarely encountered; thus, there has been little evolutionary pressure to develop a specific mechanism of homeostatic control (Cutting et al., 2007). Silver exhibits low toxicity in the human body, and minimal risk is expected due to

exposure via inhalation, ingestion, dermal application, or through the urological or hematogenous route (Lansdown, 2006). The World Health Organization (WHO) has determined that based on present epidemiological and pharmacokinetic knowledge, a lifetime intake of about 10 g of silver can be considered the human No Observable Adverse Effect Level (NOAEL)(World Health Organization, 1996). Currently, the exposure limits for metallic silver and ionizable silver compounds is <0.10 mg/Ag/L as per EPA standards (Lansdown, 2010; Lansdown, 2010; Lansdown, 2006; Lansdown, 2006). Chronic ingestion or inhalation of silver preparations (especially colloidal silver) can lead to the deposition of silver metal/silver sulfide particles in the skin (argyria), eye (argyrosis), and other organs. These are not life-threatening conditions, but are cosmetically undesirable (Lansdown, 2006).

With the increased usage of silver against bacteria, it is only logical to express concern regarding the emergence of silver resistance. Many bacterial species use efflux pumps to export heavy metals such as silver, copper, nickel, and zinc. These pumps are often membrane adenosine triphosphatases (ATPases), which also act to eliminate antibiotics (Silver & Phung, 2005). The two most likely mechanisms of silver resistance are plasmid acquisition and gene mutation that decrease silver ion uptake or promote its efflux (Chopra, 2007). Silver applications that release low levels of silver ions have the potential to select for resistance, especially if the released silver ion concentration is sub-lethal (Chopra, 2007). Silver ions do not possess a single mode of antimicrobial action. In fact, they interact with a wide range of molecular processes within microorganisms, resulting in a range of effects from inhibition of growth, to loss of infectivity, to cell death. The mechanism depends on both the concentration of silver ions present and the sensitivity of the microbial species to silver. Contact time, temperature, pH, and the presence of free water all impact both the rate and extent of antimicrobial activity (Cooper,

2004). For instance, the antimicrobial activity appears to increase with temperature and pH (Matsumura et al., 2003). Studies have demonstrated that silver ions interact with sulfydryl (-SH) groups of proteins as well as the bases of DNA, leading to either the inhibition of respiratory processes (Bragg & Rannie, 1974) or to DNA unwinding (Batarseh, 2004). Inhibition of cell division and bacterial cell wall damage have also been reported (Richards et al., 1984) and interaction with hydrogen bonding processes has been demonstrated (Russell & Hugo, 1994). Interruption of cell wall synthesis resulting in the loss of essential nutrients has been shown to occur in yeasts (Wells, 1995).

Synergy of silver with other antimicrobials

Silver has been used in combination with a variety of membrane active antimicrobials, producing both synergistic and additive effects. Synergy occurs when two agents working in concert exert a greater antimicrobial effect than when they are administered individually (Privett et al., 2010). It is believed that the activity of the membrane active antimicrobial leads to permeability of the bacterial cell wall, allowing for the rapid penetration of silver ions into the cell.

The combination of silver nanoparticles and ampicillin produced a synergistic effect against both Gram-positive and Gram-negative bacteria. The mode of action of ampicillin is cell wall lysis (Fayaz et al., 2010). CAY-1 is a fungicidal saponin in cayenne pepper fruit (*Capsicum frutescens)* that is active against fungal species such as *Aspergillus* and *Candida*. The antimicrobial properties of saponins are based on their ability to interact with cell walls and membranes, resulting in cell lysis (De Lucca et al., 2009). De Lucca et al. (2009), showed that CAY-1 and Ag significantly reduced the viable counts of *Aspergillus* and *Fusarium* while

drastically reducing the required amounts of both substrates. The combination of nitric oxide (NO) and silver sulfadiazine has been shown to be synergistic against Gram-negative, Gram-positive, and particularly antibiotic- resistant pathogens (Privett et al., 2010). NO is a broad-spectrum antimicrobial, it is a highly reactive radical and frequently combines with locally abundant small molecules such as oxygen (O_2) and superoxide (O_2^-), generating an arsenal of reactive byproducts that include dinitrogen trioxide (N_2O_3) and peroxynitrite ($ONOO^-$). Peroxynitrite is a strong oxidant that can degrade membranes through lipid peroxidation and oxidize nearby proteins, compromising cellular integrity (Privett et al., 2010).

Chitosan, the *N*-deacetylated derivative of chitin, has significant antibacterial activity against a broad spectrum of bacteria. Chitosan's antibacterial activity arises from its polycationic nature. The interaction between the positively charged chitosan and the negatively charged microbial cell leads to the leakage of the intracellular constituents. This happens because positively charged nitrogen atoms displace the divalent cations (Ca^{2+} and Mg^{2+}) that help maintain the integrity of the Gram-negative outer membrane by coordinating lipopolysaccharide (LPS) (Huang et al., 2011). Huang et al. (2011) found synergy between chitosan and silver against Gram-negative species *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in addition to Gram-positive methicillin resistant *Staphylococcus auerus* (MRSA). Independently, synergism has been shown between chitosan and silver nanocomposites against *Staphylococcus aureus* (Monica Potara, 2011) detailed chitosan as supplying the nanoparticles with a positively charged shell that at the same time decreases their aggregation potential, increases the fraction of smaller particles able to penetrate the cell wall, and provides interaction points with negative charges on the cell surface.

Peracetic acid (PAA) (CH_3COOOH) is a compound with a pH value close to 2. It consists

of a quaternary mix of acetic acid, hydrogen peroxide, peracetic acid, and water. It has been used with success in the disinfection of wastewater because of its strong oxidizing properties (Luna-Pabello V. M. et al., 2009). Luna-Pabello et al. (2009) described the synergy between PAA, Ag, and Copper (Cu), as an effective treatment for the removal of fecal coliforms) and helminth eggs in domestic wastewater. The authors speculated that the action of the disinfectant occurs first by weakening the membrane and cellular wall, opening pores, followed by the intervention of Ag and Cu which would affect the cellular material, inducing death. The combination of PAA/Ag/Cu reduced concentrations of both fecal coliforms and helminth ova to below the World Health Organization's standards for wastewater reuse.

Silver and hydrogen peroxide have been examined as a possible alternative to chlorination for disinfection and shelf-life extension of minimally processed iceberg lettuce (Gopal et al., 2010). Washing iceberg lettuce with silver/hydrogen peroxide revealed a stronger disinfection effect on *Pseudomonas, Enterobacteriaceae*, yeasts and molds than chlorine (Gopal et al., 2010). Hydrogen peroxide is a membrane active oxidizer that causes lipid peroxidation (Watt et al., 2004). Overall, numerous independent studies have shown that when combined with a membrane active agent, silver acts in a synergistic manner, increasing the lethal capabilities of the specific combination.

Copper and silver have been used in combination for over a decade to control *Legionella* in water systems due to its low cost and ease of use (Silvestry-Rodriguez et al., 2007). Stout and Yu (Lin et al., 1996)demonstrated that the use of copper and silver in distribution systems controlled *Legionella* in 16 hospitals in the U. S. for 5 years with no evidence for the development of tolerance or resistance. Studies against *Pseudomanas aeruginosa* have shown that the when used in combination, lower amounts of both copper and silver were required to

produce bactericidal results (Fisher et al., 2009). Copper is an essential nutrient that is required for aerobic metabolism; nevertheless, excessive levels of copper can be highly toxic (Rensing & Grass, 2003). This is because accumulation of copper ions or intracellular release of free copper ions from proteins causes cell damage. Copper readily catalyzes reactions that result in the production of hydroxyl radicals through the Fenton and Haber-Weiss reactions (Santo et al., 2011). The highly reactive oxygen intermediates cause lipid peroxidation and oxidation of proteins. Free copper ions are able to oxidize sulfhydryl groups, such as cysteine, in proteins or the cellular redox buffer glutathione. Specifically, copper ions inactivate proteins by damaging Fe-S clusters in cytoplasmic hydratases (Santo et al., 2011). It is logical to assume that silver and copper work well in combination because their antimicrobial efficacies occur via separate mechanisms. Specifically, silver targets enzymes of the bacterial plasma membrane while copper ions inactivate proteins by damaging Fe-S clusters in cytoplasmic hydratases(Santo et al., 2011).

Chlorine, the most common disinfectant, is moderately oxidative and reacts with various components of bacterial cells (White, 1992). One significant inhibition with chlorine is that it reacts with organic matter and subsequently gets depleted. Studies have demonstrated that chlorine and silver can be extremely effective in concert. Specifically, silver has demonstrated greater than 6-$\log_{10}$ reductions against both *P. aeruginosa* and *Aeromonas hydrophila* in municipal tap water (Silvestry-Rodriguez et al., 2007). Additionally, the effectiveness of silver as an antibacterial agent was not interfered with by the presence of free chlorine, even when the chlorine level was significantly elevated (Silvestry-Rodriguez et al., 2007). In this partnership, silver is less of a synergistic component and more of a secondary disinfectant. Silver complements chlorine in that it has no oxidizing capacity but is involved in rendering various enzymes inactive by binding to thiol (-SH) groups in a cell (Lin et al., 1996). Additionally, silver poses no significant risks to human health, and it is relatively in expensive

Dissertation Format

The appendices of this dissertation report the findings of three separate experiments undertaken by the candidate: 1). Synergy of natural plant antimicrobials with silver ions; 2). Natural plant antimicrobials for use as fruit and vegetable sanitizers; 3). Comparison of the efficacy of plant essential oils with their primary active indgredients.

The dissertation author was responsible for all of the research presented in the manuscripts appended to this dissertation. Dr. Kelly Bright was consulted on the experimental design discussed in Appendix A, B, C, and also consulted on the statistical analysis utilized in Appendix A, B, C.

PRESENT STUDY

The objective of the current study was to determine the efficacy of natural antimicrobials from plant extracts and essential oils as possible alternative sanitizers.

The methods, results, and conclusions of this study are presented in the manuscripts appended to this dissertation. The following is a summary of the most important findings in this paper:

The manuscript "Synergy of natural plant antimicrobials with silver ions" is in Appendix A. Several separate experiments were conducted in which the effectiveness of citral, cinnamaldehyde, carvacrol, allspice oil, and olive extracts were determined against *Escherichia coli.* In addition, since many of these natural compounds are believed to be membrane active, silver ions were added to some of the tests to assess the potential for synergy between the antimicrobials. All of the natural plant antimicrobials evaluated in the current study produced significant reductions in bacterial populations within 1 to 30 minutes of exposure. Nevertheless, a synergistic effect was observed with all of the antimicrobials when they were combined with silver ions with the exception of olive extract (which exhibited an antagonistic effect). The combined treatments produced more rapid and significant reductions in *E. coli* populations than the individual antimicrobials alone.

The manuscript "Natural plant antimicrobial for use as fruit and vegetable sanitizers" is in Appendix B. Five natural plant derived antimicrobials were examined for their efficacy against *Escherichia coli:* olive extract, clove bud oil, allspice oil, green tea extract, and grape seed extract. The two essential oils, clove bud and allspice, were highly effective, producing

>5.65 $\log_{10}$ reductions in bacterial populations within one minute of exposure. Olive extract also was demonstrated to be a promising antimicrobial, producing statistically significant reductions within 15 minutes of exposure. The other extracts of green tea and grape seed only produced significant reductions after 24 hours of exposure. Nevertheless, these extracts could still be useful in combination with a rapidly acting antimicrobial to provide a long-lasting antimicrobial residual on fresh produce, possibly improving the shelf life of perishable foods.

The manuscript "Comparison of the antimicrobial efficacy of plant essential oils with their primary active ingredients" is in Appendix C. We compared the antimicrobial efficacy of several plant derived essential oils with that of their primary active ingredients against *Escherichia coli.* In one experiment the antimicrobial efficacies of lemongrass oil and it active ingredient citral were assessed. We found that 0.1% citral was equally as effective as the 0.3% lemongrass oil. A second experiment compared cinnamon oil and its active ingredient cinnamaldehyde, with both producing similar results. The final experiment paired oregano oil and its active ingredient carvacrol a concentration of 0.02%. A significant reduction was observed within five minutes with carvacrol whereas 20 minutes was required for the oregano oil. In general, for this study the active ingredient produced statistically significant reductions in *E. coli* populations more rapidly and at lower concentrations than its corresponding essential oil. Nevertheless, the essential oils also were quite effective, producing >5.0-$\log_{10}$ reduction within one to 10 minutes of exposure.

REFERENCES

1. Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y. 2004. Role of resveratrol in prevention and therapy of cancer: Preclinical and clinical studies. *Anticancer Res* **24:** 2783-2840.

2. Anonymous. 2001. Chapter V. Methods to reduce/eliminate pathogens from fresh and fresh-cut produce. *In* Analysis and evaluation of preventive control measures for the control and reduction/elimination of microbial hazards on fresh and freshcut produce. U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition. Accessed August 9, 2011. http://www.fda.gov/Food/ScienceResearch/ResearchAreas/SafePracticesforFoodProcesses/ucm091363.htm

3. Ayala-Zavala JF, Del-Toro-Sánchez L, Alvarez-Parrilla E, González-Aguilar GA. 2008. High relative humidity in-package of fresh-cut fruits and vegetables: Advantage or disadvantage considering microbiological problems and antimicrobial delivering systems? *J Food Sci* **73:** R41-R47.

4. Bari ML, Ukuku DO, Kawasaki T, Inatsu Y, Isshiki K, Kawamoto S. 2005. Combined efficacy of nisin and pediocin with sodium lactate, citric acid, phytic acid, and potassium sorbate and EDTA in reducing the *Listeria monocytogenes* population of inoculated fresh-cut produce. *J Food Protect* **68:** 1381-1387.

5. Batarseh KI. 2004. Anomaly and correlation of killing in the therapeutic properties of silver (I) chelation with glutamic and tartaric acids. *J Antimicrob Chemother* **54:** 546-548.

6. Bauer V, Bauer F. 1999. Reactive oxygen species as mediators of tissue protection and injury. *Gen Physiol Biophys* **18:** 7-14.

7. Beltrán D, Selma MV, Tudela JA, Gil MI. 2005. Effect of different sanitizers on microbial and sensory quality of fresh-cut potato strips stored under modified atmosphere or vacuum packaging. *Postharvest Biol Technol* **37:** 37-46.

8. Beuchat LR. 1996. Pathogenic microorganisms associated with fresh produce. *J Food Protect* **59:** 204-216.

9. Bors W, Heller W, Michel C, Saran M. 1990. Flavonoids as antioxidants: Determination of radical scavenging efficiencies. In Methods in Enzymology. 186: 343-355.

10. Bragg PD, Rannie DJ. 1974. The effect of silver ions on the respiratory chain of *Escherichia coli*. *Can J Microbiol* **20:** 883-889.

11. Bravo L. 1998. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutr Rev* **56:** 317-333.

12. Brinkworth RI, Stoermer MJ, Fairlie DP. 1992. Flavones are inhibitors of HIV-1 proteinase. *Biochem Biophys Res Commun* **188:** 631-637.

13. Brown KL, Hancock RE. 2006. Cationic host defense (antimicrobial) peptides. *Curr Opin Immunol* **18:** 24-30.

14. Brownlee HE, McEuen AR, Hedger J, Scott IM. 1990. Anti-fungal effects of cocoa tannin on the witches' broom pathogen *Crinipellis perniciosa*. *Physiol Mol Plant Pathol* **36:** 39-48.

15. Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods - A review. *Int J Food Microbiol* **94:** 223-253.

16. Butler LG. 1988. Effects of condensed tannins on animal nutrition. *In* Chemistry and significance of condensed tannins. Hemingway RW, Karchesy JJ (Eds.). Plenum Press, NY, pp. 391-402.

17. Chopra I. 2007. The increasing use of silver-based products as antimicrobial agents: a useful development or a cause for concern? *J Antimicrob Chemother* **59:** 587-590.

18. Cooper R. 2004. A review of the evidence for the use of topical antimicrobial agents in wound care. Accessed August 9, 2011.
http://www.worldwidewounds.com/2004/february/Cooper/Topical-Antimicrobial-Agents.html

19. Cowan MM. 1999. Plant products as antimicrobial agents. *Clin Microbiol Rev* **12:** 564-582.

20. Cox SD, Markham JL. 2007. Susceptibility and intrinsic tolerance of *Pseudomonas aeruginosa* to selected plant volatile compounds. *J Appl Microbiol* **103:** 930-936.

21. Criqui MH, Ringel BL. 1994. Does diet or alcohol explain the French paradox? *Lancet* **344:** 1719-1723.

23. Cutting K, White R, Edmonds M. 2007. The safety and efficacy of dressings with silver: Addressing clinical concerns. *Int Wound J* **4:** 177-184.

24. De Lucca AJ, Boue S, Sien T, Cleveland TE, Walsh TJ. 2009. Silver enhances the in vitro antifungal activity of the saponin, CAY-1. *Mycoses* **54:** e1-9.

25. Denyer SP, Stewart GSAB. 1998. Mechanisms of action of disinfectants. *Int Biodeterior Biodegrad* **41:** 261-268.

26. Doyle MP. 2005. Food antimicrobials, cleaners, and sanitizers. Food Research Institutes, UW-Madison, FRI Briefings. **9:** 1.

27. Duke JA. 2002. CRC Handbook of medicinal herbs, 2nd Ed. Duke JA, Bogenschutz-Godwin MJ, du Cellier J (Eds.). CRC Press, New York, New York.
29. Fayaz AM, Balaji K, Girilal M, Yadav R, Kalaichelvan PT, Venketesan R. 2010. Biogenic

synthesis of silver nanoparticles and their synergistic effect with antibiotics: a study against Gram-positive and Gram-negative bacteria. *Nanomedicine* **6:** 103-109.

30. Finnegan M, Linley E, Denyer SP, McDonnell G, Simons C, Maillard J. 2010. Mode of action of hydrogen peroxide and other oxidizing agents: Differences between liquid and gas forms. *J Antimicrob Chemother* **65:** 2108-2115.

31. Fisher K, Pope M, Phillips C. 2009. Combined effect of copper and silver against *Pseudomonas aeruginosa*. *J Hosp Infect* **73:** 180-182.

32. Gomez-Lopez VM, Ragaert P, Debevere J, Devlieghere F. 2008. Decontamination methods to prolong the shelf-life of minimally processed vegetables, state-of-the-art. *Crit Rev Food Sci Nutr* **48:** 487-495.

33. Garcia E, Barrett DM. 2002. Preservative treatments for fresh cut fruits and vegetables. Accessed August 9, 2011. http://ucanr.org/sites/Zann_test/files/28711.pdf

34. Gopal A, Coventry J, Wan J, Roginski H, Ajlouni S. 2010. Alternative disinfection techniques to extend the shelf life of minimally processed iceberg lettuce. *Food Microbiol* **27:** 210-219.

35. Gutierrez J, Barry-Ryan C, Bourke P. 2008. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *Int J Food Microbiol* **124:** 91-97.

36. Han Y, Sherman DM, Linton RH, Nielsen SS, Nelson PE. 2000. The effects of washing and chlorine dioxide gas on survival and attachment of *Escherichia coli* O157: H7 to green pepper surfaces. *Food Microbiol* **17:** 521-533.

37. Hancock RE, Chapple DS. 1999. Peptide Antibiotics. *Antimicrob Agents Chemother* **43:** 1317-1323.

38. Haslam E. 1996. Natural polyphenols (vegetable tannins) as drugs: Possible modes of action. *J Nat Prod* **59:** 205-215.

39. Huang L, Dai T, Xuan Y, Tegos GP, Hamblin MR. 2011. Synergistic combination of chitosan acetate with nanoparticle silver as a topical antimicrobial: efficacy against bacterial burn infections. *Antimicrob Agents Chemother* **55:** 3432-3438.

40. Izumi H. 1999. Electrolyzed water as a disinfectant for fresh-cut vegetables. *J Food Sci* **64:** 536-539.

42. Keymanesh K, Soltani S, Sardari S. 2009. Application of antimicrobial peptides in agriculture and food industry. *World J Microbiol Biotechnol* **25:** 933-944.

43. King SR, Tempesta MS. 2007. From shaman to human clinical trials: The role of industry in ethnobotany, conservation and community reciprocity. *Ciba Found Symp* **185:** 197-213.

44. Kris-Etherton P, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD. 2002. Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *Am J Med* **113:** 71-88.

45. Kris-Etherton P, Keen CL. 2002. Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health. *Curr Opin Lipidol* **13:** 41-49.

47. Lansdown AB. 2006. Silver in health care: antimicrobial effects and safety in use. *Curr Probl Dermatol* **33:** 17-34.

46. Lansdown AB. 2010. A pharmacological and toxicological profile of silver as an antimicrobial agent in medical devices. *Adv Pharmacol Sci* **2010:** 910686.

48. Lin YE, Vidic RD, Stout JE, Yu VL. 1996. Individual and combined effects of copper and silver ions on inactivation of *Legionella pneumophila*. *Water Res* **30:** 1905-1913.

49. Luna-Pabello VM, Ríos Miranda M, Jiménez B, orta de Velasquez MT. 2009. Effectiveness of the use of Ag, Cu and PAA to disinfect municipal wastewater. *Environ Technol* **30:** 129-139.

50. Lynch FM, Tauxe RV, Hedberg WC. 2008. The growing burden of foodborne outbreaks due to contaminated fresh produce: Risks and oppurtunities. *Epidemiol Infect* **137:** 307-315.

51. Masahiro O, Midori H, Shiro U, Toyoshige E. 2000. Antioxidant activity of eugenol and related monomeric and dimeric compounds. *Chem Pharm Bull* **48:** 1467-1469.

52. Matsumura Y, Yoshikata K, Kunisaki S, Tsuchido T. 2003. Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate. *Appl Environ Microbiol* **69:** 4278-4281.

53. Matthews KR. 2009. Chapter 8. Leafy Vegetables. *In* The produce contamination problem: Causes and Solutions. Sapers, Solomon, Matthews (Eds.), pp. 165-188. Academic Press, Maryland Heights, MO.

54. Mercanoglu Taban B, Halkman AK. 2011. Do leafy green vegetables and their ready-to-eat [RTE] salads carry a risk of foodborne pathogens? *Anaerobe*. **Epub ahead of print.**

55. Mihara S, Shibamoto T. 1982. Photochemical reactions of eugenol and related compounds: Synthesis of new flavor chemicals. *J Agric Food Chem* **30:** 1215-1218.

56. Potara M, Jakab E, Damert A, Popescu O, Canpean V, Astilean S. 2011. Synergistic antibacterial activity of chitosan-silver nanocomposites on *Staphylococcus aureus*. *Nanotechnology* **22:** 135101.

58. Morley KL, Ferguson PJ, Koropatnick J. 2007. Tangeretin and nobiletin induce G1 cell cycle arrest but not apoptosis in human breast and colon cancer cells. *Cancer Lett* **251:** 168-178.

59. Narayanan BA. 2006. Chemopreventive agents alter global gene expression pattern:

Predicting their mode of action and targets. *Curr Cancer Drug Targets* **6:** 711-727.

61. O'Keeffe T, Hill C. 1999. Bacteriocins: potential in food preservation. *In* Encyclopedia of food microbiology, Robinson RK, Batt CA, Patel PD (Eds.), pp. 183-191. Academic Press, Maryland Heights, MO.

62. Ono K, Nakane H, Fukushima M, Chermann J, Barré-Sinoussi F. 1989. Inhibition of reverse transcriptase activity by a flavonoid compound, 5,6,7-trihydroxyflavone. *Biochem Biophys Res Commun* **160:** 982-987.

63. Park C, Hung Y, Doyle MP, Ezeike GOI, Kim C. 2001. Pathogen reduction and quality of lettuce treated with electrolyzed oxidizing and acidified chlorinated water. *J Food Sci* **66:** 1368-1372.

64. Peres MT, Delle Monache F, Cruz AB, Pizzolatti MG, Yunes RA. 1997. Chemical composition and antimicrobial activity of *Croton urucurana Baillon* (*Euphorbiaceae*). *J Ethnopharmacol* **56:** 223-226.

65. Periago PM, Palop A, Fernandez PS. 2001. Combined effect of nisin, carvacrol and thymol on the viability of *Bacillus cereus* heat-treated vegetative cells. *Food Sci Technol Int* **7:** 487-492.

66. Perrett S, Whitfield PJ, Sanderson L, Bartlett A. 1995. The plant molluscicide *Millettia thonningii* (*Leguminosae*) as a topical antischistosomal agent. *J Ethnopharmacol* **47:** 49-54.

67. Pol IE, Smid EJ. 1999. Combined action of nisin and carvacrol on *Bacillus cereus* and *Listeria monocytogenes*. *Lett Appl Microbiol* **29:** 166-170.

68. Privett BJ, Deupree SM, Backlund CJ, Rao KS, Johnson CB, Coneski PN, Schoenfisch MH. 2010. Synergy of nitric oxide and silver sulfadiazine against Gram-negative, Gram-positive, and antibiotic-resistant pathogens. *Mol Pharm* **7:** 2289-2296.

69. Rababah TM, Hettiarachchy NS, Horax R. 2004. Total phenolics and antioxidant activities of fenugreek, green tea, black tea, grape seed, ginger, rosemary, gotu kola, and ginkgo extracts, vitamin E, and tert-butylhydroquinone. *J Agric Food Chem* **52:** 5183-5186.

70. Raiden RM, Sumner SS, Eifert JD, Pierson MD. 2003. Efficacy of detergents in removing *Salmonella* and *Shigella* spp. from the surface of fresh produce. *J Food Protect* **66:** 2210-2215.

71. Ravishankar S, Zhu L, Reyna-Granados J, Law B, Joens L, Friedman M. 2010. Carvacrol and cinnamaldehyde inactivate antibiotic-resistant *Salmonella enterica* in buffer and on celery and oysters. *J Food Protect* **73:** 234-240.

72. Rensing C, Grass G. 2003. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. *FEMS Microbiol Rev* **27:** 197-213.

73. Restaino L, Frampton E, Hemphill J, Palnikar P. 1995. Efficacy of ozonated water against

various food-related microorganisms. *Appl Environ Microbiol* **61:** 3471-3475.

74. Richards RM, Odelola HA, Anderson B. 1984. Effect of silver on whole cells and speroplasts of a silver resistant *Pseudomonas aeruginosa*. *Microbios* **39:** 151-157.

75. Rico D, Martín-Diana AB, Barat JM, Barry-Ryan C. 2007. Extending and measuring the quality of fresh-cut fruit and vegetables: A review. *Trends Food Sci Technol* **18:** 373-386.

76. Rojas A, Hernandez L, Pereda-Miranda R, Mata R. 1992. Screening for antimicrobial activity of crude drug extracts and pure natural products from Mexican medicinal plants. *J Ethnopharmacol* **35:** 275-283.

78. Russell AD, Hugo WB. 1994. Antimicrobial activity and action of silver. *Prog Med Chem* **31:** 351-370.

79. Santo CE, Lam EW, Elowsky CG, Quaranta D, Domaille DW, Chang CJ, Grass G. 2011. Bacterial killing by dry metallic copper surfaces. *Appl Environ Microbiol* **77:** 794-802.

80. Sapers MG. 2009. Chapter 16. Disinfection of contaminated produce with conventional washing and sanitizing technology. *In* The Produce Contamination Problem: Causes and Solutions. Sapers, Solomon, Matthews (Eds.), pp. 393-424. Academic Press, Maryland Heights, MO.

81. Scalbert A. 1991. Antimicrobial properties of tannins. *Phytochemistry* **30:** 3875-3883.

82. Schultz JC. 1989. Tannin-insect interactions. *In* Chemistry and significance of condensed tannins. Hemingway RW, Karchesy JJ (Eds.). Plenum Press, NY, pp. 417-433

83. Serafini M, Ghiselli A, Ferro-Luzzi A, Melville CAS. 1994. Red wine, tea, and antioxidants. *Lancet* **344:** 626-626.

85. Silver S, Phung L. 2005. A bacterial view of the periodic table: Genes and proteins for toxic inorganic ions. *J Ind Microbiol Biotechnol* **32:** 587-605.

86. Silvestry-Rodriguez N, Bright KR, Uhlmann DR, Slack DC, Gerba CP. 2007. Inactivation of *Pseudomonas aeruginosa* and *Aeromonas hydrophila* by silver in tap water. *J Environ Sci Health A Tox Hazard Subst Environ Eng* **42:** 1579-1584.

87. Sivapalasingam S, Friedman CR, Cohen L, Tauxe RV. 2004. Fresh produce: A growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *J Food Protect* **67:** 2342-2353.

88. Smith Dewall C, Bhuiya F. 2007. Outbreak Alert 2007. Center for Science in the Public Interest. Ninth Edition. www.cspinet.org

89. Somolinos M, García D, Condón S, Mackey B, Pagán R. 2010. Inactivation of *Escherichia*

coli by citral. *J Appl Microbiol* **108:** 1928-1939.

90. Stern JL, Hagerman AE, Steinberg PD, Mason PK. 1996. Phlorotannin-protein interactions. *J Chem Ecol* **22:** 1877-1899.

91. Suslow T. 2000. Chapter 6. Chlorination in the production and postharvest handling of fresh fruits and vegetables. *In* Fruit and vegetable processing. McLaren D (Ed.). Accessed August 9, 2011. http://www.sdhdidaho.org/eh/pdf/Chlorination_of_fruits_and_veggies.PDF

92. Taniguchi M, Kubo I. 1993. Ethnobotanical drug discovery based on medicine men's trials in the African Savanna: Screening of East African plants for antimicrobial activity II. *J Nat Prod* **56:** 1539-1546.

93. Toda M, Okubo S, Ikigai H, Suzuki T, Suzuki Y, Shimamura T. 1991. The protective activity of tea against infection by *Vibrio cholerae* O1. *J Appl Microbiol* **70:** 109-112.

94. Tsuchiya H, Sato M, Miyazaki T, Fujiwara S, Tanigaki S, Ohyama M, Tanaka T, Iinuma M. 1996. Comparative study on the antibacterial activity of phytochemical flavanones against methicillin-resistant *Staphylococcus aureus*. *J Ethnopharmacol* **50:** 27-34.

95. Ullah FM, Khan WM. 2008. Food as medicine: Potential therapeutic tendencies of plant derived polyphenolic compounds. *Asian Pacific J Cancer Prev* **9:** 187-195.

96. Ultee A, Bennik MHJ, Moezelaar R. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Appl Environ Microbiol* **68:** 1561-1568.

97. Vizioli J, Salzet M. 2002. Antimicrobial peptides versus parasitic infections? *Trends Parasitol* **18:** 475-476.

98. Wang HX, Ng TB. 2005. An antifungal peptide from the coconut. *Peptides* **26:** 2392-2396.

99. Wang J, Zhao X, Liang Y, Li L, Zhang W, Ren Q, Wang L, Wang L. 2006. Molecular characterization and expression of the antimicrobial peptide defensin from the housefly (*Musca domestica*). *Cell Mol Life Sci* **63:** 3072-3082.

100. Watt BE, Proudfoot AT, Vale JA. 2004. Hydrogen peroxide poisoning. *Toxicological Reviews* **23:** 51-57.

101. Wells TN, Scully P, Paravicini G, Proudfoot AE, Payton MA. 1995. Mechanism of irreversible inactivation of phosphomannose isomerases by silver ions and flamazine. *Biochemistry* **34: 7**896-7903.

102. White GC. 1992. The handbook of chlorination and alternative disinfectants, 3rd Ed. John Wiley & Sons, Inc., Hoboken, New Jersey.

103. Wickramanayake GB. 1991. Chapter 10. Disinfection and sterilization by ozone. *In*

Disinfection, Sterilization, and Preservation, 4th ed. Block SS (Ed.). Lea &Febiger, Philadelphia.

104. World Health Organization. 1996. Silver in drinking water: Background document for the development of WHO Guidelines for Drinking Water Quality. WHO, Geneva, Switzerland. Accessed August 9, 2011. http://www.who.int/water_sanitation_health/dwq/chemicals/silver.pdf

105. Food and Drug Administration. 2009. Potential for infiltration, survival and growth of human pathogens within fruits and vegetables. Accessed August 9, 2011. http://www.fda.gov/Food/FoodSafety/HazardAnalysisCriticalControlPointsHACCP/JuiceHACCP/ucm082063.htm

APPENDIX A:

SYNERGY OF NATURAL PLANT ANTIMICROBIALS WITH SILVER IONS

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Running Title: Synergy of silver and plant antimicrobials

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Abstract

Antimicrobials from plant sources are a possible alternative to currently used sanitizers and disinfectants. Plant antimicrobials are highly effective and may also be used in situations in which the use of other antimicrobials is not advantageous; for instance, in situations in which chemical antimicrobials have limited efficacy or because of concerns over the production of harmful by-products resulting from their use. The effectiveness of citral, cinnamaldehyde, carvacrol, and allspice oil were determined against *Escherichia coli in vitro*. In addition, since many of these plant compounds are believed to be membrane active, silver ions were included in some of the tests to assess the potential for synergy between the antimicrobials and silver ions. Silver ions, although slow-acting on their own, often exhibit a synergistic antimicrobial effect when combined with other membrane active antimicrobials such as oxidizing agents. Citral, cinnamaldehyde, carvacrol, and allspice oil produced significant reductions ($P \leq 0.05$) in bacterial populations within one to 30 minutes of exposure. These treatments combined with silver produced more rapid and statistically significant ($P \leq 0.05$) reductions in *E. coli* populations than the silver or plant antimicrobials individually. The use of these plant antimicrobials combined with silver ions could therefore result in highly effective and inexpensive antimicrobial treatments that could be used in a wide variety of applications, are safe for human consumption, and are active in the presence of organic matter.

Introduction

There will always be a need for new effective alternatives to existing sanitizers and disinfectants as new pathogens emerge and microorganisms develop resistances to existing antimicrobials. In addition, in certain situations, only "green" or non-toxic antimicrobials may be used: for instance, to sanitize food or food contact surfaces, to treat water, or to disinfect environmental surfaces in areas with sensitive populations (e.g., day care centers, hospital intensive care units) that may not tolerate exposure to harsher chemicals. Also, in some current applications, existing antimicrobials have limited efficacy.

Natural antimicrobials from plants are one such possible alternative. Plants produce natural antimicrobials in various areas such as in the roots, leaves, bark, and stem, coinciding with the various assaults that the plant might encounter in the environment (Burt 2004). Numerous plant extracts, essential oils, and their components have significant antimicrobial properties (Didry et al. 1994; Friedman et al. 2002; Olasupo et al. 2003; Singh et al. 2003; Gupta and Ravishankar 2005; Knowles et al. 2005; Peñalver et al. 2005; Uhart et al. 2006; Callaway et al. 2008; Nannapaneni et al. 2008; Ravishankar et al. 2008; Ravishankar et al. 2009; Ravishankar et al. 2010). Many plant antimicrobials are routinely used and are often found in the average kitchen cabinet. This common and longstanding usage has earned these antimicrobials the label of Generally Regarded As Safe (GRAS) compounds (Dillon 1999; Ress et al. 2003; Adams et al. 2004; Knowles et al. 2005). In addition, these compounds are likely not affected by the presence of organics and therefore may be easier to maintain at the appropriate effective concentrations than antimicrobials such as chlorine (Dillon 1999).

Silver (Ag) ion has been used as a disinfectant since ancient times. It is known to bind to

disulfide (S–S) and sulfhydryl groups (–SH) on proteins in microbial cell walls, interfering in critical membrane transport and biochemical pathways (Silvestry-Rodriguez et al. 2007b). Silver has been shown to be effective against yeasts, viruses, and a wide variety of bacteria (Silvestry-Rodriguez et al. 2007b). Unlike numerous other antimicrobials, silver is not considered a hazardous substance (Ibarluzea et al. 1998; Kim et al. 2002; World Health Organization 1996). Both the Environmental Protection Agency (EPA) and the World Health Organization (WHO) regard silver as safe for human consumption (Environmental Protection Agency 2002; Silvestry-Rodriguez et al. 2007b; World Health Organization 1996). The only known side effect of silver consumption in humans is argyria (irreversible skin discoloration) and argyrosis (discoloration of the eye), which only occur with the ingestion of regular gram quantities of silver over several years or by the administration of high concentrations to ill individuals. These conditions are not dangerous, but are cosmetically undesirable (Lansdown 2006). The amount of silver needed to achieve the desired antimicrobial effect is usually on the order of 10 to 100 parts per billion (µg/L). Based on our previous work, for a 1000 µg/L silver treatment on 10 grams of lettuce, the amount remaining on the lettuce after treatment is approximately 0.06 µg/gram of lettuce (unpublished data). Assuming that a person consumes 200 grams of lettuce 365 days a year, after 76 years, they will have consumed 0.333 grams of silver. Based on epidemiological and pharmacokinetic data, a lifetime limit of 10 grams of silver is considered a No Observable Adverse Effect Level (NOAEL) for humans (World Health Organization 1996). It would therefore require more than 2,280 years before enough silver would be consumed to cause any adverse effects.

As such, silver has been used in numerous applications in which it is either ingested or in contact with exposed tissues. For instance, silver has been used in Mexico for more than fifty

years in commercially available consumer produce washes such as Microdyn®, BacDyn® plus, Biopur®, Bacterin®, and Gadicin® Argentum, among others.

Silver has been used in combination with a variety of membrane active antimicrobials such as oxidizers (e.g., chlorine), producing additive and sometimes synergistic antimicrobial effects (Yahya et al. 1992; Armon et al. 2000; Butkus et al. 2004, Silvestry-Rodriguez et al. 2007b). Synergy occurs when two agents working in concert exert a greater antimicrobial effect than merely the sum of their individual effects. Such synergy can result in the use of lower concentrations of each antimicrobial, potentially lowering costs and reducing the risks of toxicity (Privett et al. 2010). It has been postulated that the oxidizer disrupts the cell wall and effects the rapid penetration of silver into the cell, where irreversible precipitation of the DNA occurs (Armon et al. 2000; Yahya et al. 1992).

The goal of this study was to identify natural plant antimicrobials with enhanced or synergistic antimicrobial efficacy when combined with silver ions against *Escherichia coli*. No such previous studies have been conducted. It is believed that membrane active natural antimicrobials will have synergistic activities when used in combination with silver, similar to membrane active oxidizing agents such as chlorine.

Materials and Methods

Maintenance and preparation of bacterial isolates

Escherichia coli strain 25922 was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and was maintained on Tryptic Soy Agar (TSA; Difco, Sparks, MD) with incubation for 18 to 24 hours at 37°C. Prior to the start of each experiment, an Erlenmeyer flask containing 100 ml of Tryptic Soy Broth (TSB; Difco, Sparks, MD) was inoculated with the organism and incubated on an orbital shaker (Model G33; New Brunswick Scientific, Edison, NJ) at 300 rpm at 37°C overnight. After incubation, the *E. coli* were pelleted via centrifugation (9,820 × *g*, 15 min, 20°C, JA-14 rotor, Beckman J2-21 centrifuge; Beckman Coulter, Inc., Fullerton, CA). The pelleted cells were washed by resuspension in 100 ml of physiological saline (0.85% NaCl) followed by centrifugation as described previously. This step was repeated one additional time. The final pellet was resuspended in 10 ml of sterile phosphate buffered saline (PBS; pH7.4; Sigma-Aldrich, St. Louis, MO). The test suspensions were then prepared by adding small volumes of the bacterial suspension to 10 ml of sterile PBS, resulting in an optical turbidity (measured using a BIOLOG turbidimeter, Hayward, CA) equivalent to a McFarland number 0.5 optical density standard [= 1.5×10^8 colony-forming units (CFU)/ml]. This solution was then diluted further in sterile PBS to achieve the desired final test concentration (approximately 1.0×10^7 CFU/ml).

Antimicrobials preparation

A stock solution of silver (Ag) ions was prepared immediately prior to the start of each experiment by adding $AgNO_3$ (J.T. Baker, Phillipsburg, NY) to distilled water to obtain a Ag ion concentration of 100 mg/L (100 ppm). A 10 µl volume of this stock was then added to test flasks containing 10 ml of solution, resulting in a final silver concentration of 100 µg/L (100 ppb).

Allspice essential oil was obtained from Lhasa Karnak Herbal Co. (Berkley, CA). Citral (mixture of *cis* and *trans*, >96%), carvacrol (>98%), and cinnamaldehyde (93%) were purchased from Sigma-Aldrich (St. Louis, MO). The natural antimicrobials were diluted (volume to volume) using PBS.

Experimental protocol

The carvacrol, cinnamaldehyde, citral, and allspice were evaluated in separate experiments. The disinfection treatments for each experiment included the following: 1) 100 µg/L silver, 2) the diluted plant antimicrobial, and 3) 100 µg/L silver and the diluted natural plant antimicrobial. A control with *E. coli* in PBS but no added antimicrobial was also included. Purified stocks of the bacteria were added separately to the antimicrobial solutions (final concentration of ~1.0×10^7 cfu/ml) and the flasks were placed on an orbital shaker (300 rpm) for the duration of the experiment. Experiments were performed in triplicate at room temperature (24°C) in 50 ml polypropylene conical tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). At predetermined time intervals, 100 µl samples were collected and neutralized with Dey Engley (D/E) neutralizing broth (Difco, Sparks, MD) at a ratio of 1:10. The D/E is necessary to

neutralize the antimicrobial effect of silver whereas the plant antimicrobials are neutralized via dilution. Samples were assayed immediately.

Assay for bacteria

Bacterial samples were serially diluted in physiological saline and the surviving bacteria were enumerated using the spread plate method on duplicate plates of Levine Eosin Methylene Blue Agar plates (EMB; Becton, Dickinson and Company, Franklin Lakes, NJ). The plates were incubated at 37°C for 24 hours and the surviving bacterial colonies were counted.

Statistical analysis

Data were reported as logarithmic reduction using the formula $\log_{10} (N_0 / N_t)$, where N_0 was the concentration of *E. coli* at time zero and N_t was the surviving concentration at time *t*. A Student's t-test was used to determine if there were significant differences between the control and the antimicrobial treatments (the reduction at each time exposure was compared to the control after 30 minutes) or between the various treatments (for each exposure time). Differences were considered statistically significant if the resultant *P* value was ≤ 0.05.

Results

The results for each of the plant antimicrobials alone or in combination with silver are shown in Tables 1 through 4. No significant reductions were observed with the Ag treatment within 30 minutes of exposure for any of the experiments.

Statistically significant ($P \leq 0.05$) reductions (compared to the control) were observed within 5 minutes of exposure (and each time interval thereafter) with both the citral alone and the citral combined with Ag treatments (Table 1). The reduction in *E. coli* observed for the combined citral/Ag treatment was greater than that observed with the citral treatment at each time interval. This difference between the two treatments was statistically significant after 10 and 20 minutes of exposure. There was no significant difference between the two treatments after 30 minutes of exposure; however, the reduction with the citral/Ag treatment had reached the limit of detection of the assay (50 CFU/ml or >5.28 $\log_{10}$ reduction) and thus could potentially have been greater.

Likewise, significant reductions were observed within 5 minutes of exposure (and each time interval thereafter) with both the carvacrol alone and the carvacrol combined with Ag treatments (Table 2). The carvacrol/Ag combined treatment also yielded greater reductions than carvacrol alone at both 10 and 30 minutes of exposure. Similarly, the reductions observed with cinnamaldehyde (Table 3) were significant within 20 minutes in comparison to the control. The reduction was more rapid when cinnamaldehyde was combined with silver, with a significant reduction observed within 10 minutes of exposure. In addition, the cinnamaldehyde/Ag combination had greater reductions ($P \leq 0.05$) at both the 20- and 30-minute exposure intervals.

No significant reductions were observed with the 0.5% allspice treatment (Table 4). In contrast, a significant reduction of 2.68-$\log_{10}$ was observed after 30 minutes when the antimicrobial was combined with Ag.

Discussion

New strategies for combating the ever growing problem of microbial pathogens will require creativity. Plant antimicrobials and silver have been around for millennia; however, their combined efficacies have never been explored. Many plant antimicrobials are known to be membrane active and thus have the potential for synergy with silver ions. For instance, carvacrol, found in oregano, partitions the fatty acid chains of cell membrane phospholipids, thus forming ion channels which permit ions to escape the cytoplasm (Cox and Markham 2007). Citral, the active component of lemongrass, is a type of terpenoid that causes the leakage of specific ions because its action on the cell membrane has dramatic effects on proton motive forces, the intracellular ATP content, and the overall cell activity (Somolinos et al. 2010). Allspice attributes its antimicrobial capabilities to its primary active component eugenol that has a variety of antioxidant properties (Masahiro et al. 2000) such as the scavenging of radicals and the chelating of metal ions. Eugenol also reportedly participates in photochemical reactions (Mihara and Shibamoto 1982). Cinnamaldehyde, the major active antimicrobial in cinnamon oil, disrupts enzyme activity (Ravishankar et al. 2010).

Silver is an effective antimicrobial but requires hours to produce a significant reduction in bacterial populations. No such reductions were observed within the 30-minute time scale of the experiments included in the current study. Therefore, any enhanced reduction when combined with a plant antimicrobial may be attributed to synergy. Several plant antimicrobials were found to be synergistic with silver ions when combined in solution. The combined treatments produced more rapid and significant reductions in *E. coli* populations than the individual antimicrobials alone. These plant antimicrobials were highly effective at concentrations of 0.04% carvacrol,

0.1% citral, 0.2% cinnamaldehyde, and 1.0% allspice, eliciting >5.0-$\log_{10}$ reductions within 1 to 10 minutes of exposure (unpublished data); however, the concentrations were halved (0.02% carvacrol, 0.05% citral, 0.1% cinnamaldehyde, 0.5% allspice) so that they would only yield partial reductions of the bacterial population. Thus, any enhanced antimicrobial effect due to the addition of silver could then be observed and quantified.

Unlike other produce sanitizers such as ozone, chlorine and hydrogen peroxide, plant antimicrobials and silver are not as sensitive to the presence of organic matter (Silvestry-Rodriguez et al. 2007a; Matthews 2009). As a result, they should retain their antimicrobial efficacy for longer periods and should not require the frequency of application or as much monitoring of antimicrobial solutions as many traditionally used antimicrobials. In addition, since both the plant antimicrobials and silver are considered safe for human consumption at these levels, these antimicrobials may be used in applications in which other antimicrobials are considered unsafe. Finally, plant antimicrobials can be fairly expensive to produce due to the costs associated with their extraction and purification. Although silver is considered a precious metal, it is quite inexpensive (costing a fraction of a U.S. cent) at the concentrations (100 ppb) required to produce such a synergistic effect. Therefore, combining plant antimicrobials with silver could produce a more cost-effective product.

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References

Adams MR, Hartley AD, Cox LJ. 1989. Factors affecting the efficacy of washing procedures used in the production of prepared salads. *Food Microbiol* **6:** 69-77.

Adams TB, Cohen SM, Doull J, Feron VJ, Goodman JI, Marnett LJ, Munro IC, Portoghese PS, Smith RL, Waddell WJ, Wagner BM. 2004. The FEMA GRAS assessment of cinnamyl derivatives used as flavor ingredients. *Food Chem Toxicol* **42:** 157–185.

Armon R, Laot N, Lev O, Shuval H, Fattal B. 2000. Controlling biofilm formation by hydrogen peroxide and silver combined disinfectant. *Water Sci Technol* **42:** 187-192.

Beuchat LR. 1998. Surface decontamination of fruits and vegetables eaten raw: a review. *Food Saf Issues*. WHO/FSF/FOS/ 98.2. World Health Organization, Geneva.

Beuchat LR, Harris LJ, Ward TE, Kajs TM. 2001. Development of a proposed standard method for assessing the efficacy of fresh produce sanitizers. *J Food Protect* **64:** 1103-1109.

Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods - A review. *Int J Food Microbiol* **94:** 223-253.

Butkus MA, Labare MP, Starke JA, Moon K, Talbot M. 2004. Use of aqueous silver to enhance inactivation of coliphage MS-2 by UV disinfection. *Appl Environ Microbiol* **70:** 2848-2853.

Callaway TR, Carroll JA, Arthington JD, Pratt C, Edrington TS, Anderson RC, Galyean ML, Ricke SC, Crandall P, Nisbet DJ. 2008. Citrus products decrease growth of *E. coli* O157:H7 and *Salmonella* Typhimurium in pure culture and in fermentation with mixed ruminal microorganisms in vitro. *Foodborne Pathog Dis* **5:** 621-627.

Castillo C, Rodriguez-Garcia MO. 2004. Bacterial hazards in fresh and fresh-cut produce: sources and control. In: Preharvest and Postharvest Food Safety. Beier RC, Pillai SD, Phillips DT, Riprin RL (Eds.) pp. 43-57. Black Publishing Ames, IA.

Chang TL, Streicher R, Zimmer H. 1988. The interaction of aqueous solutions of chlorine with malic acid, tartaric acid, and various fruit juices, a source of mutagens. *Anal Lett* **21:** 2049.

Cox SD, Markham JL. 2007. Susceptibility and intrinsic tolerance of *Pseudomonas aeruginosa* to selected plant volatile compounds. *J Appl Microbiol* **103:** 930-936.

Didry N, Dubreuil LB, Pinkas MA. 1994. Activity of thymol, carvacrol, cinnamaldehyde and eugenol on oral bacteria. *Pharm Acta Helv* **69:** 25-28.

Dillon VM. 1999. Natural Anti-Microbial Systems (a) Preservative effects during storage. *In* Encyclopedia of food microbiology, Robinson RK, Batt CA, Patel P (Eds.). Academic Press, Boston, Massachussetts

Doyle MP. 2005. Food antimicrobials, cleaners, and sanitizers. Food Research Institutes, UW-Madison, FRI Briefings. **9:** 1.

Environmental Protection Agency. 2002. National Secondary Drinking Water Regulations. http://www.epa.gov/safewater/mcl.html. Retrieved July 20, 2011.

Friedman M, Henika PR, Mandrell RE. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J Food Protect* **65:** 1545-1560.

Gupta S, Ravishankar S. 2005. A comparison of the antimicrobial activity of garlic, ginger, carrot, and turmeric pastes against *Escherichia coli* O157:H7 in laboratory buffer and ground beef. *Foodborne Pathog Dis* **2:** 330-340.

Hidaka T, Kirigaya T, Kamijo M. 1992. Disappearance of residual chlorine and formation of chloroform in vegetables treated with sodium hyphochlorite. *J Food Hyg Soc Japan*. **33:** 267.

Ibarluzea J, Moreno B, Zigorraga C, Castilla T, Martinez M, Santamaria J. 1998. Determinants of the microbiological water quality of indoor swimming-pools in relation to disinfection. *Water Res* **32:** 865–871.

Kim H, Shim J, Lee S. 2002. Formation of disinfection by-products in chlorinated swimming pool water. *Chemosphere* **46:** 123–130.

Knowles JR, Roller S, Murray DB, Naidu AS. 2005. Antimicrobial action of carvacrol at different stages of dual-species biofilm development by *Staphylococcus aureus* and *Salmonella enterica* serovar Typhimurium. *Appl Environ Microbiol* **71:** 797-803.

Koseki S, Yoshida K, Kamitani Y, Itoh K. 2003. Influence of inoculation method, spot inoculation site, and inoculation size on the efficacy of acidic electrolyzed water against pathogens on lettuce. *J Food Protect*.**66:** 2010-2016.

Koseki S, Yoshida K, Isobe S, Itoh K. 2004. Efficacy of acidic electrolyzed water for microbial decontamination of cucumbers and strawberries. *J Food Protect* **67:** 1247-1251.

Lansdown A. 2006. Silver in health care: antimicrobial effects and safety in use. *Curr Probl Dermatol* **33:** 17-34.

Masahiro O, Midori H, Shiro U, Toyoshige E. 2000. Antioxidant activity of eugenol and related monomeric and dimeric compounds. *Chem Pharm Bull* **48:** 1467-1469.

Mihara S, Shibamoto T. 1982. Photochemical reactions of eugenol and related compounds:

synthesis of new flavor chemicals. *J Agric Food Chem* **30:** 1215-1218.

Nannapaneni R, Muthaiyan A, Crandall PG, Johnson MG, O'Bryan CA, Chalova VI, Callaway TR, Carroll JA, Arthington JD, Nisbet DJ, Ricke SC. 2008. Antimicrobial activity of commercial citrus-based natural extracts against *Escherichia coli* O157:H7 isolates and mutant strains. *Foodborne Pathog Dis* **5:** 695-699.

Olasupo NA, Fitzgerald DJ, Gasson MJ, Narbad A. 2003. Activity of natural antimicrobial compounds against *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. *Lett Appl Microbiol* **37:** 448-451.

Peñalver P, Huerta B, Borge C, Astorga R, Romero R, Perea A. 2005. Antimicrobial activity of five essential oils against origin strains of the *Enterobacteriaceae* family. *APMIS* **113:** 1-6.

Pirovani M, Piagentini A, Guemes D, Silvana A. 2004. Reduction of chlorine concentration and microbial load during washing-disinfection of shredded lettuce. *Int J Food Sci Tech* **39:** 341-347.

Privett BJ, Deupree SM, Backlund CJ, Rao KS, Johnson CB, Coneski PN, Schoenfisch MH. 2010. Synergy of nitric oxide and silver sulfadiazine against Gram-negative, Gram-positive, and antibiotic-resistant pathogens. *Mol Pharm* **7:** 2289-2296.

Ravishankar S, Zhu L, Joens L, Friedman M. 2008. Plant-derived compounds inactivate antibiotic resistant *Campylobacter jejuni* strains. *J Food Protect* **71:** 1145-1149.

Ravishankar S, Zhu L, Olsen CW, McHugh TH, Friedman M. 2009. Edible apple film wraps containing plant antimicrobials inactivate foodborne pathogens in meat and poultry products. *J Food Sci* **74:** M440-M445.

Ravishankar S, Zhu L, Reyna-Granados J, Law B, Joens L, Friedman M. 2010. Carvacrol and cinnamaldehyde inactivate antibiotic-resistant *Salmonella enterica* in buffer and on celery and oysters. *J Food Protect* **73:** 234-240.

Ress NB, Hailey JR, Maronpot RR, Bucher JR, Travlos GS, Haseman JK, Orzech DP, Johnson JD, Hejtmancik MR. 2003. Toxicology and Carcinogenesis studies of microencapsulated citral in rats and mice. *Toxicol Sci* **71:** 198–206.

Rodgers SL, Cash JN, Siddiq M, Ryser ET. 2004. A comparison of different chemical sanitizers for inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes* in solution and on apples, lettuce, strawberries, and cantaloupe. *J Food Prot*ect **67:** 721-731.

Sapers MG. 2009. Disinfection of contaminated produce with conventional washing and sanitizing technology. *In* The produce contamination problem: Causes and solution. Sapers GM, Solomon EB, Matthews KR (eds.), pp. 393-424. Academic Press, Boston, Massachussetts.

Seo KH, Frank JF. 1999. Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and

bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. *J Food Protect* **62:** 3-9.

Silver S. 2003. Bacterial silver resistance: Molecular biology and uses and misuses of silver compounds. *FEMS Microbiol Rev* **27:** 341-353.

Silvestry-Rodriguez N, Bright KR, Uhlmann DR, Slack DC, Gerba CP. 2007a. Inactivation of *Pseudomonas aeruginosa* and *Aeromonas hydrophila* by silver in tap water. *J Environ Sci Health, Part A Tox Hazard Subst Environ Eng* **42:** 1579-1584.

Silvestry-Rodriguez N, Sicairos-Ruelas EE, Gerba CP, Bright KR. 2007b. Silver as a disinfectant. *Rev Environ Contam Toxicol* **191:** 23-45.

Singh N, Singh RK, Bhunia AK. 2003. Sequential disinfection of *Escherichia coli* O157:H7 inoculated alfalfa seeds before and during sprouting using aqueous chlorine dioxide, ozonated water, and thyme essential oil. *Lebensm Wiss U.-Technol* **36:** 235-243.

Somolinos M, García D, Condón S, Mackey B, Pagán R. 2010. Inactivation of *Escherichia coli* by citral. *J Appl Microbiol* **108:** 1928-1939.

Suslow T. 2000. Chapter 6. Chlorination in the production and postharvest handling of fresh fruits and vegetables. *In* Fruit and vegetable processing. McLaren D (Ed.). Accessed August 9, 2011. http://www.sdhdidaho.org/eh/pdf/Chlorination_of_fruits_and_veggies.PDF

Uhart M, Maks N, Ravishankar S. 2006. Effect of spices on growth and survival of *Salmonella* Typhimurium DT 104 in ground beef stored at 4 and 8°C. *J Food Saf* **26:** 115-125.

Ukuku DO, Sapers GM. 2006. Microbiological safety issues of fresh melons, p. 29-30. *In:* Microbiology of fruits and vegetables. Sapers GM, Gorny JR, Yousef AE (eds.), CRC Pres, Boca Raton, FL.

Yahya MT, Straub TM, Gerba CP. 1992. Inactivation of coliphage MS-2 and poliovirus by copper, silver and chlorine. *Can J Microbiol* **38:** 430-435.

Yuk HG, Bartz JA, Schneider KR. 2006. The effectiveness of sanitizer treatments inactivation of *Salmonella spp.* from bell pepper, cucumber, and strawberry. *J Food Sci* **71:** 95-99.

Table 1. Reduction (Log_{10} ± standard deviation)[§] in the population of *Escherichia coli* after exposure to 0.05% citral (v/v) with and without silver (100 ppb) in PBS.

Time (min)	Control	Treatment		
		Citral	Citral + Silver	Silver
1	ND	0.06 ± 0.03	0.06 ± 0.05	ND
5	ND	1.52* ± 0.19	2.25* ± 0.61	ND
10	ND	2.63* ± 0.76	4.30*[†] ± 0.47	ND
20	ND	3.57* ± 0.35	> 5.28*[†] ± 0.40	ND
30	0.05 ± 0.03	4.35* ± 0.61	> 5.28* ± 0.34	0.01 ± 0.04

ND Not determined

§ Inoculated with 1.7 x 10^7 CFU/ml

* Reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobials)

† Reductions were significantly different between citral with and without silver

Table 2. Reduction (Log_{10} ± standard deviation)[§] in the population of *Escherichia coli* after exposure to 0.02% carvacrol (v/v) with and without silver (100 ppb) in PBS.

Time (min)	Control	Treatment		
		Carvacrol	Carvacrol + Silver	Silver
5	ND	0.35* ± 0.07	0.45* ± 0.16	0.08 ± 0.12
10	ND	0.50* ± 0.05	1.03*[†] ± 0.07	0.00 ± 0.06
30	0.09 ± 0.04	1.44* ± 0.06	2.31*[†] ± 0.04	0.00 ± 0.04

ND Not determined

§ Inoculated with 2.9 x 10^7 CFU/ml

* Reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobials)

† Reductions were significantly different between carvacrol with and without silver

Table 3. Reduction (Log_{10} ± standard deviation)[§] in the population of *Escherichia coli* after exposure to 0.1% cinnamaldehyde (v/v) with and without silver (100 ppb) in PBS.

Time (min)	Control	Treatment		
		Cinnamaldehyde	Cinnamaldehyde + Silver	Silver
1	ND	0.05 ± 0.04	0.10 ± 0.08	ND
5	ND	0.18 ± 0.04	0.15 ± 0.05	ND
10	ND	0.17 ± 0.07	0.54* ± 0.24	ND
20	ND	0.69* ± 0.12	1.72*[†] ± 0.48	ND
30	0.08 ± 0.04	1.79* ± 0.36	4.46*[†] ± 0.94	0.12 ± 0.10

ND Not determined

§ Inoculated with 2.1 x 10^7 CFU/ml

* Reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobials)

† Reductions were significantly different between cinnamaldehyde with and without silver

Table 4. Reduction (Log_{10} ± standard deviation)[§] in the population of *Escherichia coli* after exposure to 0.5% allspice oil (v/v) with and without silver (100 ppb) in PBS.

Time (min)	Control	Treatment		
		Allspice Oil	Allspice Oil + Silver	Silver
1	ND	0.00 ± 0.16	0.04 ± 0.23	0.00 ± 0.03
5	ND	0.00 ± 0.05	0.00 ± 0.09	0.00 ± 0.08
10	ND	0.00 ± 0.17	0.12 ± 0.26	0.00 ± 0.12
20	ND	0.00 ± 0.08	0.03 ± 0.19	0.00 ± 0.23
30	0.58 ± 0.34	0.55 ± 0.04	2.68*† ± 0.16	0.29 ± 0.32

ND Not determined

§ Inoculated with 6.6 x 10^6 CFU/ml

* Reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobials)

† Reductions were significantly different between allspice oil with and without silver

APPENDIX B:

EFFICACY OF PLANT ANTIMICROBIALS AGAINST *ESCHERICHIA COLI*

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Abstract

In the current study, we examined five natural plant derived antimicrobials for their efficacy against *Escherichia coli*: olive extract, clove bud oil, allspice oil, green tea extract, and grape seed extract. The two essential oils, clove bud and allspice, were highly effective, producing >5.52-$\log_{10}$ reductions in bacterial populations within one minute of exposure at concentrations of 0.5% and 1.0% (vol/vol), respectively. Olive extract also was demonstrated to be a promising antimicrobial, producing statistically significant ($P \leq 0.05$) reductions (>1.0-$\log_{10}$) within 15 minutes of exposure and achieving a 4.81-$\log_{10}$ reduction after 30 minutes of exposure. The extracts of green tea and grape seed only produced significant reductions after 24 hours of exposure; nevertheless, these extracts could still be useful in combination with a rapidly acting antimicrobial to provide a long-lasting antimicrobial residual. All of the antimicrobials included in this study are generally regarded as safe (GRAS) for human consumption and could therefore be used in numerous “green” applications such as for sanitizing foods and as a non-toxic surface decontaminant.

Introduction

Volatile oils or essential oils are aromatic viscous liquids obtained from plant materials (e.g., flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruit, and roots) (Burt 2004). They may be separated from plants by distillation, extraction, and cold pressing (Burt 2004; Rasooli 2007); steam distillation is the most commonly employed commercial method (van de Braak and Leijten 1999). Plant extracts are obtained from many of these same sources but are in a powdered form. Plant extracts and essential oils have been used in many applications such as to provide flavoring to foods and fragrances in perfumes, and have been added to shampoos, toothpastes, ointments, and cosmetics (Burt 2004). They are primarily of interest due to many having natural antimicrobial properties (Burt 2004).

Essential oils may have as many as 60 individual components (Senatore, 1996; Russo et al., 1998) and therefore often have multiple effects on the bacterial cell (Burt 2004). They may cause deterioration of the cell wall (Thoroski et al. 1989; Burt 2004), damage to the cell membrane (Ultee et al. 2002) and to membrane proteins (Ultee et al. 1999), increased membrane permeability and the leakage of cell contents (Ultee et al. 2002; Burt 2004), coagulation of the cytoplasm (Gustafson et al. 1998), reduction of the proton motive force (Ultee et al. 1999), inactivation of essential enzymes (Wendakoon and Sakaguchi 1995; Cowan 1999; Ayala-Zavala et al. 2008), and disturbance of genetic material functionality (Ayala-Zavala et al. 2008).

Essential oils and extracts often have a dominant antimicrobial component or active ingredient (Burt 2004). For example, eugenol is the primary active component in clove bud oil (up to 85%) (Farag et al. 1989; Bauer et al. 2001) and accounts for its antioxidant properties (Ogata et al. 2000). Eugenol is also the active ingredient and primary component in Allspice (Takemasa et al. 2009).

Eugenol is a phenolic. The phenols and polyphenols are a group of bioactive phytochemicals which consist of a single substituted phenolic ring (Cowan 1999). In general, phenols or polyphenols exhibit the greatest antimicrobial efficacy of the various active ingredients found in plants (Burt 2004). The antioxidant activity may occur via various mechanisms such as the scavenging of radicals and the chelating of metal ions. Eugenol reportedly participates in photochemical reactions (Mihara and Shibamoto 1982) and inhibits the production (Farag et al. 1989) and activity of enzymes (Wendakoon and Sakaguchi 1995). It may also cause changes in membrane permeability as a consequence of the exit of potassium ions (Walsh et al. 2003). Cell wall deterioration and lysis have also been observed (Thoroski et al. 1989)

Extracts of olive pulp, grape seeds, and green tea also contain members of the phenolic family. Catechol and Epicatechin are two forms of simple phenols whose mechanism of action include substrate deprivation and membrane disruption (Toda et al. 1991; Peres et al. 1997). Grape seed extract contains proanthocyanidins and olive extract contains oleuropein; green tea contains tannin, caffeine, and catechins. The high concentrations of epicatechin and catechin in grape seed extract and caffeic acid and

epicatechin in green tea extracts account for their high antioxidant activities (Rababah et al. 2004).

In this study, we examined five plant antimicrobial essential oils and extracts (clove flower bud oil, allspice oil, olive extract, green tea extract, and grape seed extract) for their antimicrobial efficacy against *Escherichia coli in vitro*.

Materials and Methods

Preparation of Escherichia coli and natural antimicrobials

Escherichia coli strain 25922 was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Long-term stocks of the *E. coli* strain were stored at -80°C in fetal bovine serum. The bacterium was maintained on tryptic soy agar (TSA; Difco, Sparks, MD). Prior to the start of each experiment, a 250 ml capacity Erlenmeyer flask containing 100 ml of tryptic soy broth (TSB; Difco, Sparks, MD) was inoculated with the *E. coli* strain and incubated on an orbital shaker (250 rpm) at 37°C for 18 hours. After incubation, the *E. coli* cells were pelleted via centrifugation (9,820 × *g*, 15 min, 20°C). The pelleted cells were washed twice to remove organics by resuspension in 100 ml of physiological saline (0.85% NaCl) followed by centrifugation as before. The final pellet was resuspended in 10 ml of sterile phosphate buffered saline (PBS; pH7.4; Sigma-Aldrich, St. Louis, MO). The test bacterial suspensions were then prepared by adding small volumes of the bacterial suspension to 10 ml of sterile PBS, resulting in an optical turbidity (measured using a BIOLOG turbidimeter, Hayward, CA) equal to a McFarland number 0.5 optical density standard. This is equivalent to approximately 1.5×10^8 colony-forming units (CFU)/ml.

Allspice and clove bud essential oils were obtained from Lhasa Karnak Herbal

Co. (Berkley, CA), green tea polyphenols extract was obtained from LKT Laboratories, Inc. (St. Paul, MN), grape seed extract was obtained from Swanson Health Products (Fargo, ND), and olive extract was obtained from CreAgri, Inc. (Hayward, CA). The plant antimicrobials were dissolved and diluted (volume to volume or weight to volume) using PBS.

Experimental protocol

Allspice oil, clove bud oil, olive extract, green tea extract, and grape seed extract were evaluated in separate experiments. Screening trials consisted of systematically examining each plant antimicrobial for efficacy against *E. coli*. The method consisted of identifying appropriate working concentrations for the antimicrobials to produce significant reductions. Initially, the essential oils were evaluated at 0.1% and 1.0% (vol/vol) concentrations and the extracts at 1.0% and 2.0% (wt/vol). These concentrations were then adjusted up or down as needed based on the experimental results. A control with *E. coli* in PBS but no added antimicrobial was also included in each experiment. Purified stocks of the bacteria were added separately to the antimicrobial solutions (to a final concentration of ~1.0 x 10^7 cfu/ml) and the tubes were placed on an orbital shaker (Model G33; New Brunswick Scientific, Edison, NJ) at 300 rpm. Experiments were performed in triplicate at room temperature (24°C) in 50 ml polypropylene conical tubes (Becton Dickinson and Company, Franklin Lakes, NJ). At predetermined time intervals

(usually between 1 and 30 minutes of exposure), 100 µl samples were collected and placed in 900 µl of Dey Engley (D/E) neutralizing broth (Difco, Sparks, MD). The D/E was included since simultaneous studies were being conducted with other antimicrobials that required this neutralizer. D/E has been used in similar studies in the past with plant antimicrobials (Mattson et al. 2011). The plant antimicrobials are also neutralized via dilution using D/E. The samples were assayed immediately.

Assay for bacteria

The surviving bacteria were enumerated by performing ten-fold serial dilutions in physiological saline and spread plating on duplicate plates of Levine eosin methylene blue agar plates (EMB; Becton, Dickinson and Company, Sparks, MD). The plates were incubated for 18 to 24 hours at 37°C and the bacterial colonies counted.

Statistical analysis

Data were reported as logarithmic reduction using the formula $\log_{10} (N_0 / N_t)$, where N_0 was the concentration of *E. coli* at time zero and N_t was the surviving concentration at time *t*. A Student's t-test was used to determine if there were significant differences

between the control and the antimicrobial treatments (the reduction at each time exposure was compared to the control at the end of the experiment). Differences were considered statistically significant if the resultant *P* value was ≤ 0.05. Differences between the reductions observed between different concentrations for each antimicrobial were also evaluated for statistical significance using a Student's t-test.

Results

The results for the experiments conducted with olive extract are shown in Table 1. The 1.0% and the 2.5% concentrations were both effective, yielding similar statistically significant ($P \leq 0.05$ in comparison to the control) $\log_{10}$ reductions in *E. coli* populations at the earlier exposure time intervals (i.e., 15 and 20 minutes). However, the 2.5% treatment exhibited significantly greater reductions than the 1.0% treatment with longer exposure times (i.e., 25 and 30 minutes). For example, a 4.81-$\log_{10}$ reduction was observed with the 2.5% concentration after 30 minutes of exposure versus only a 3.08-$\log_{10}$ reduction with the 1.0% concentration.

The higher concentration of clove bud oil (0.5% vol/vol) was rapidly effective, causing a highly significant reduction within one minute of exposure (Table 2). In fact, the number of surviving *E. coli* were below the detection limit of the assay (i.e., < 50 CFU/ml and therefore a >5.52-$\log_{10}$ reduction). A nearly 10-fold lower concentration of 0.06% was also fairly effective, resulting in a significant reduction of 0.32-$\log_{10}$ in *E. coli* within five minutes; nonetheless, this concentration required 30 minutes of exposure to reduce the population to a level (> 4.34-$\log_{10}$) near that observed with the 0.5% treatment. The differences between the reductions observed with the two concentrations were statistically significant ($P \leq 0.05$) at all of the time exposures with the exception of 30 minutes (in which both were below the detection limit of the assay and thus could not be directly compared).

In contrast, a two-fold increase in allspice oil from 0.5% to 1.0% resulted in a vast difference in antimicrobial efficacy (Table 3). No significant reductions were observed with the lower concentration within 30 minutes of exposure, whereas a highly significant reduction (in comparison to both the control and the 0.5% treatment) of >5.52-$\log_{10}$ was found with the 1.0% treatment after one minute of exposure and every point thereafter.

The green tea extract (Table 4) and the grape seed extract (Table 5) were only tested at higher concentrations (6.0% and 5.0% wt/vol, respectively) and for longer exposure times (up to 24 hours) based on the results of preliminary studies (unpublished). Concentrations higher than this are difficult to dissolve in the PBS test solution. These two extracts were not nearly as effective as the other antimicrobials included in this study. Nonetheless, they did produce significant reductions (1.93-$\log_{10}$ and >5.05-$\log_{10}$, respectively) in the *E. coli* population after 24 hours of exposure.

Discussion

In the current study, we tested several plant based compounds with antimicrobial activities that make them promising candidates for use as antimicrobials. Clove bud and allspice oils and their active ingredient, eugenol, have previously been shown to be effective against several foodborne pathogens (Friedman et al. 2002; Friedman et al. 2004; Du et al. 2009; Mattson et al. 2011; Pérez-Conesa et al. 2011). These oils were similarly effective in the current study, achieving complete reductions (> 5.52-$\log_{10}$) in *E. coli* within one minute of exposure at low concentrations (0.5% and 1.0%, respectively). Little data is available regarding the antimicrobial efficacy of olive pulp extracts; nonetheless, with nearly a 5-log reduction observed within 30 minutes of exposure, this extract is also highly effective and could be useful as an antimicrobial in various applications.

Previous studies with grape seed extract have demonstrated poor antibacterial efficacy (Mayer et al. 2008); however, in one study, grape seed extract was shown to be effective against Gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) strains (Al-Habib et al. 2010). Multiple studies with plant antimicrobials have indicated that many are more effective against Gram-positive than they are against Gram-negative bacteria (Farag et al. 1989; Delaquis et al. 2002; Burt 2004). This might explain why both grape seed extract and green tea extract had limited efficacy against the Gram-negative *E. coli* in the current study, requiring 24 hours to achieve a significant reduction.

Various studies have been conducted in the past that indicate that plant antimicrobials are effective in real world applications (Du et al. 2009; Ravishankar et al. 2009; Mattson et al. 2011; Mild et al. 2011; Pérez-Conesa et al. 2011). We believe that clove bud oil, allspice, and olive extract can have similar practical uses, particularly in the form of a spray which could be used in various applications. They could be used to treat foods such as meats and fresh produce throughout the various stages of production from the processing plant, to the grocery store, to the consumer household. Another possible use is as a surface disinfectant to kill pathogenic microorganisms on fomites (inanimate surfaces). Even the green tea extract and the grape seed extract are effective with long exposure times; therefore, these may be used in situations in which a rapid kill is not required or used in combination with a rapidly effective antimicrobial to provide a long-lasting residual effect.

An advantage that natural antimicrobials might have over many traditional antimicrobials such as chlorine is that they likely have a longer lasting residual efficacy (Sapers 2009). In addition, they are generally regarded as safe (GRAS) for human consumption (Burt 2004; Soni et al. 2006; Baskaran et al. 2010; Kumar et al. 2010; Hsu et al. 2011) and may therefore be used in situations in which toxic antimicrobials could not.

One great challenge when employing plant essential oils/extracts as food sanitizers is the problem of compatibility with respect to odor and taste. Whether they are derived from fruits or vegetables, all have specific aromatic notes. It is important to take

advantage of these aroma notes and pair oils/extracts with compatible food items, much like how wine and cheeses are paired. For example, green tea extract could be used with apples, lemons, and other fruits traditionally combined with hot and cold tea drinks. Grape seed extract could potentially be applied to grapes, strawberries, and other vine-based fruits. While further testing is needed to elucidate the best olfactory pairings, the potential combinations are abundant. Many essential oil source products such as garlic and oregano are staples of many cuisines worldwide. Additionally, spices such as cinnamon and allspice are commonly added to foods as flavor additives. Creating a compatibility chart would therefore not be difficult. An effort should also be made to use the lowest effective concentrations, which should then minimize the aromatic and sensory effects and lower costs.

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References

Al-Habib A, Al-Saleh E, Safer AM, Afzal M. 2010. Bactericidal effect of grape seed extract on methicillin-resistant *Staphylococcus aureus* (MRSA). *J Toxicol Sci* **35:** 357-364.

Ayala-Zavala JF, Del-Toro-Sánchez L, Alvarez-Parrilla E, González-Aguilar GA. 2008. High relative humidity in-package of fresh-cut fruits and vegetables: Advantage or disadvantage considering microbiological problems and antimicrobial delivering systems? *J Food Sci* **73:** R41-R47.

Baskaran Y, Periyasamy V, Venkatraman AC. 2010. Investigation of antioxidant, anti-inflammatory and DNA-protective properties of eugenol in thioacetamide-induced liver injury in rats. *Toxicol* **268:** 204–212.

Bauer K, Garbe D, Surburg H. 2001. Common fragrance and flavor materials: Preparation, properties and uses. Wiley-VCH, Weinheim, p. 293.

Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods - a review. *Int J Food Microbiol* 94: 223-253.

Cowan MM. 1999. Plant products as antimicrobial agents. *Clin Microbiol Rev* **12:** 564-582.

Delaquis PJ, Stanich K, Girard B, Mazza G. 2002. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *Int J Food Microbiol* **74:** 101–109.

Du WX, Olsen CW, Avena-Bustillos RJ, McHugh TH, Levin CE, Mandrell R, Friedman M. 2009. Antibacterial effects of allspice, garlic, and oregano essential oils in tomato films determined by overlay and vapor-film methods. *J Food Sci* **74:** M390-M397.

Farag RS, Daw ZY, Hewedi FM, El-Baroty GSA. 1989. Antimicrobial activity of some Egyptian spice essential oils. *J Food Protect* **52:** 665–667.

Friedman M, Henika PR, Mandrell RE. 2002. Bactericidal activities of plant essential oils and some of the isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J Food Protect* **65:** 1545-1560.

Friedman M, Henika PR, Levin CE, Mandrell RE. 2004. Antibacterial activities of plant enssential oils and their components against *Escherichia coli* O157:H7 and *Salmonella enterica* in apple juice. *J Agric Food Chem* **52:** 6042-6048.

Gustafson JE, Liew YC, Chew S, Markham JL, Bell HC, Wyllie SG, Warmington JR. 1998. Effects of tea tree oil on *Escherichia coli*. *Lett Appl Microbiol* **26:** 194-198.

Hsu YW, Tsai CF, Chen WK, Huang CF, Yen CC. 2011. A subacute toxicity evaluation of green tea (Camellia sinensis) extract in mice. *Food Chem Toxicol* **49:** 2624-2630.

Kumar A, Shukla R, Singh P, Dubey NK. 2010. Chemical composition, antifungal and antiaflatoxigenic activities of Ocimum sanctum L. essential oil and its safety assessment as plant based antimicrobial. *Food Chem Toxicol* **48:** 539-543.

Mattson TE, Johny AK, Amalaradjou MAR, More K, Schreiber DT, Patel J, Venkitanarayanan K. 2011. Inactivation of *Salmonella* spp. on tomatoes by plant molecules. *Int J Food Microbiol* **144:** 464-468.

Mayer R, Stecher G, Wuerzner R, Silva RC, Sultana T, Trojer L, Feuerstein I, Krieg C, Abel G, Popp M, Bobleter O, Bonn GK. 2008. Proanthocyandins: target compounds as antibacterial agents. *J Agric Food Chem* **56:** 6959-6966.

Mihara S, Shibamoto T. 1982. Photochemical reactions of eugenol and related compounds: Synthesis of new flavor chemicals. *J Agric Food Chem* **30:** 1215-1218.

Mild RM, Joens LA, Friedman M, Olsen CW, McHugh TH, Law B, Ravishankar S. 2011. Antimicrobial edible apple films inactivate antibiotic resistant and susceptible *Campylobacter jejuni* strains on chicken breast. *J Food Sci* **76:** M163-M168.

Ogata M, Hoshi M, Urano S, Endo T. 2000. Antioxidant activity of eugenol and related monomeric and dimeric compounds. *Chem Pharm Bull* **48:** 1467-1469.

Peres MT, Delle Monache F, Cruz AB, Pizzolatti MG, Yunes RA. 1997. Chemical composition and antimicrobial activity of *Croton urucurana Baillon* (*Euphorbiaceae*). *J Ethnopharmacol* **56:** 223-226.

Pérez-Conesa D, Cao J, Chen L, McLandsborough L, Weiss J. 2011. Inactivation of *Listeria monocytogenes* and *Escherichia coli* O157:H7 biofilms by micelle-encapsulated eugenol and carvacrol. *J Food Protect* **74:** 55-62.

Rababah TM, Hettiarachchy NS, Horax R. 2004. Total phenolics and antioxidant activities of fenugreek, green tea, black tea, grape seed, ginger, rosemary, gotu kola, and ginkgo extracts, vitamin E, and tert-butylhydroquinone. *J Agric Food Chem* **52:** 5183-5186.

Rasooli I. 2007. Food preservation – A biopreservative approach. *Food* **1:** 111-136.

Ravishankar S, Zhu L, Olsen CW, McHugh TH, Friedman M. 2009. Edible apple film wraps containing plant antimicrobials inactivate foodborne pathogens in meat and poultry products. *J Food Sci* **74:** M440-M445.

Russo M, Galletti GC, Bocchini P, Carnacini A. 1998. Essential oil chemical composition of wild populations of Italian oregano spice (*Origanum vulgare* ssp. hirtum (Link) Ietswaart): A preliminary evaluation of their use in chemotaxonomy by cluster analysis: 1. Inflorescences. *J Agric Food Chem* **46:** 3741– 3746.

Sapers MG. 2009. Chapter 16. Disinfection of contaminated produce with conventional washing and sanitizing technology. *In* The Produce Contamination Problem: Causes and Solutions. Sapers, Solomon, Matthews (Eds.), pp. 393-424. Academic Press, Maryland Heights, MO.

Senatore F. 1996. Influence of harvesting time on yield and composition of the essential oil of a thyme (*Thymus pulegioides* L.) growing wild in Campania (Southern Italy). *J Agric Food Chem* **44:** 1327– 1332.

Soni MG, Burdock GA, Christian MS, Bitler CM, Crea R. 2006. Safety assessment of aqueous olive pulp extract as an antioxidant or antimicrobial agent in foods. *Food Chem Toxicol* **44:** 903-915.

Takemasa N, Ohnishi S, Tsuji M, Shikata T, Yokoigawa K. 2009. Screening and analysis of spices with ability to suppress verocytotoxin production by *Escherichia coli* O157. *J Food Sci* **74:** M461-M466.

Thoroski J, Blank G, Biliaderis C. 1989. Eugenol induced inhibition of extracellular enzyme production by *Bacillus cereus. J Food Protect* **52:** 399-403.

Toda M, Okubo S, Ikigai H, Suzuki T, Suzuki Y, Shimamura T. 1991. The protective activity of tea against infection by *Vibrio cholerae* O1. *J Appl Microbiol* **70:** 109-112.

Ultee A, Kets EPW, Smid EJ. 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Appl Environ Microbiol* **65:** 4606-4610.

Ultee A, Bennink MHJ, Moezelaar R. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Appl Environ Microbiol* **68:** 1561-1568.

van de Braak SAAJ, Leijten GCJJ. 1999. Essential Oils and Oleoresins: A Survey in the Netherlands and other Major Markets in the European Union. CBI, Centre for the Promotion of Imports from Developing Countries, Rotterdam, p. 116.

Walsh SE, Maillard JY, Russell AD, Catrenich CE, Charbonneaus DL, Bartolo RG. 2003. Activity and mechanisms of action of selected biocidal agents on Gram positive and negative bacteria. *J Appl Microbiol* **94:** 240–247.

Wendakoon CN, Sakaguchi M. 1995. Inhibition of amino acid decarboxylase activity of *Enterobacter aerogenes* by active components in spices. *J Food Protect* **58:** 280–283.

Table 1. Reduction (Log_{10} ± standard deviation)[§] in the population of *Escherichia coli* after exposure to olive extract in PBS.

Time (min)	Olive Extract (wt/vol)	
	1.0 %	**2.5 %**
15	1.02* ± 0.15	1.22* ± 0.25
20	2.29* ± 0.11	2.24* ± 0.12
25	2.73* ± 0.11	3.39*[†] ± 0.15
30	3.08* ± 0.01	4.81*[†] ± 0.23

§ Inoculated with 1.5 x 10^7 CFU/ml and 1.2 x 10^7 CFU/ml for the experiment with 1.0% and 2.5% olive extract, respectively. The experiments were conducted in triplicate.

* Reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobial).

† Reductions were significantly different ($P \leq 0.05$) between the two different olive extract concentrations.

Table 2. Reduction (Log_{10} ± standard deviation)[§] in the population of *Escherichia coli* after exposure to clove bud oil in PBS.

Time (min)	Clove Bud Oil (vol/vol)	
	0.06 %	**0.5 %**
1	0.00 ± 0.08	> 5.52*[†] ± 0.00
5	0.32* ± 0.10	> 5.52*[†] ± 0.00
10	1.20* ± 0.26	> 5.52*[†] ± 0.00
20	2.85* ± 0.68	> 5.52*[†] ± 0.00
30	> 4.34* ± 0.98	> 5.52* ± 0.00

[§] Inoculated with 1.3 x 10^7 CFU/ml and 2.3 x 10^7 CFU/ml for the experiment with 0.06% and 0.5% clove bud oil, respectively. The experiments were conducted in triplicate.

* Reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobial).

† Reductions were significantly different ($P \leq 0.05$) between the two different clove bud oil concentrations.

Table 3. Reduction (Log_{10} ± standard deviation)[§] in the population of *Escherichia coli* after exposure to allspice oil in PBS.

Time (min)	Allspice Oil (vol/vol)	
	0.5 %	**1.0 %**
1	0.00 ± 0.16	>5.52*[†] ± 0.00
5	0.00 ± 0.05	>5.52*[†] ± 0.00
10	0.00 ± 0.17	>5.52*[†] ± 0.00
20	0.00 ± 0.08	>5.52*[†] ± 0.00
30	0.00 ± 0.04	>5.52*[†] ± 0.00

§ Inoculated with 6.6 x 10^6 CFU/ml and 2.3 x 10^7 CFU/ml for the experiment with 0.5% and 1.0% allspice oil, respectively. The experiments were conducted in triplicate.

* Reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobial).

† Reductions were significantly different ($P \leq 0.05$) between the two different allspice oil concentrations.

Table 4. Reduction (Log_{10} ± standard deviation)[§] in the population of *Escherichia coli* after exposure to green tea extract in PBS.

Time (hours)	6.0% Green Tea Extract (wt/vol)	
4	0.00	± 0.08
6	0.00	± 0.00
24	1.93*	± 0.03

§ Inoculated with 4.0 x 10^7 CFU/ml. The experiment was conducted in duplicate.

* Reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobial).

Table 5. Reduction (Log_{10} ± standard deviation)[§] in the population of *Escherichia coli* after exposure to grape seed extract in PBS.

Time (hours)	5.0% Grape Seed Extract (wt/vol)	
1	0.00	± 0.01
4	0.00	± 0.05
6	0.00	± 0.09
24	> 5.05*	± 0.00

§ Inoculated with 1.8 x 10^7 CFU/ml. The experiment was conducted in duplicate.

* Reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobial).

APPENDIX C:

COMPARISON OF THE ANTIMICROBIAL EFFICACY OF PLANT ESSENTIAL OILS WITH THEIR PRIMARY ACTIVE INGREDIENTS AGAINST *ESCHERICHIA COLI*

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Running Title: Efficacy of plant antimicrobials and their active ingredients

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Abstract

Plant derived essential oils have been shown to have significant antimicrobial properties. In the current study, we compared the antimicrobial efficacy of several plant derived essential oils with that of their primary active ingredients against *Escherichia coli*. The antimicrobial efficacy of all of the antimicrobials studied was concentration dependent, with higher concentrations yielding greater bacterial reductions. The active ingredient carvacrol produced statistically significant reductions in *E. coli* populations more rapidly than its corresponding essential oil. For instance, 0.02% carvacrol produced a 2.27-$\log_{10}$ reduction in *E. coli* after thirty minutes of exposure, whereas the same concentration of oregano oil yielded a 1.0-$\log_{10}$ reduction in the same period of time. A concentration of 0.2% cinnamaldehyde produced a similar reduction (5.11-$\log_{10}$) after 10 minutes of exposure to that produced by 1.25% cinnamon oil after one minute (>5.53-$\log_{10}$). A 0.05% concentration of citral yielded significant reductions (in comparison to the control) more rapidly than lemongrass oil; however, with longer exposure times, the reductions observed with the 0.05% lemongrass oil were greater (e.g., >5.62-$\log_{10}$ after 30 minutes versus 4.30-$\log_{10}$ with citral). All of the essential oils and their active ingredients were quite effective, producing >5.0-$\log_{10}$ reductions within one to 10 minutes of exposure. The essential oils are typically less costly to produce and less pungent than their active ingredients; therefore, essential oils may be a better option for use as antimicrobials in many applications.

Introduction

The fragrance of plants is carried in the quinta essential, or essential oil fraction (Cowan 1999). Essential oils are of particular interest since such oils have been shown to possess antibacterial, antifungal, antiviral, insecticidal, and antioxidant properties (Kordali et al. 2005). Essential oils obtained from plant material (e.g., flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits, and roots) are typically a mixture of many compounds. The active ingredient is often the dominant component, accounting for greater than 50% of its chemical composition (Burt 2004).

Many of these compounds are generally regarded as safe (GRAS) for human consumption (Ress et al. 2003; Adams et al. 2004; Knowles et al. 2005). Oregano oil has been used to add flavor to foods including salad dressings, tomato sauces, and pizzas (Ravishankar et al. 2009). Lemongrass is widely used for food flavoring, as a fragrance component in perfumes, and for its analgesic and anti-inflammatory characteristics (Ress et al. 2003; Katsukawa et al. 2010). Cinnamon oil is also used as a flavoring agent in various foods (Friedman et al. 2000).

The antimicrobial efficacy of essential oils may be due to a variety of effects. They may cause deterioration of the cell wall (Thoroski et al. 1989; Burt 2004), damage to the cell membrane (Ultee et al. 2002) and to membrane proteins (Ultee et al. 1999), increased membrane permeability and the leakage of cell contents (Ultee et al. 2002; Burt 2004), coagulation of the cytoplasm (Gustafson et al. 1998), reduction of the proton motive force (Ultee et al. 1999), inactivation of essential enzymes (Wendakoon and

Sakaguchi 1995; Cowan 1999; Ayala-Zavala et al. 2008), and disturbance of genetic material functionality (Ayala-Zavala et al. 2008).

Terpenoids are secondary metabolites and are highly enriched in compounds based on an isoprene structure (Cowan 1999). Terpenoids exhibit antimicrobial activity through membrane disruption (Serafini et al. 1994). Citral is a type of terpenoid that causes membrane perturbation and the leakage of specific ions. Its action on the cell membrane also has dramatic effects on proton motive forces, the intracellular ATP content, and the overall cell activity (Somolinos et al. 2010). Carvacrol is hydrophobic and partitions the fatty acid chains of membrane phospholipids, thus forming ion channels which permit ions to escape the cytoplasm (Cox and Markham 2007; García-García et al. 2011). This causes depletion of the intracellular ATP pool, changes in the membrane potential, and increases in the permeability of the cytoplasmic membrane to proteins and potassium ions (Ultee et al. 2002). Cinnamaldehyde reacts chemically with the cell membrane (Friedman et al. 2002; Friedman et al. 2003) and is known to disrupt bacterial enzyme activity (Ravishankar et al. 2010). Cinnamon oil is thought to bind proteins (Wendakoon and Sakuguchi 1995), thereby inhibiting their function.

In the current study, we compared the antimicrobial efficacy of three essential oils, oregano oil, lemongrass oil, and cinnamon oil, with their primary active ingredients (carvacrol, citral, and cinnamaldehyde, respectively) against *Escherichia coli*.

Materials and Methods

Preparation of Escherichia coli *and natural antimicrobials*

Escherichia coli strain 25922 was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Long-term stocks of the *E. coli* strain were stored at -80°C in fetal bovine serum. The bacterium was maintained on tryptic soy agar (TSA; Difco, Sparks, MD). Prior to the start of each experiment, an Erlenmeyer flask containing 100 ml of Tryptic Soy Broth (TSB; Difco, Sparks, MD) was inoculated with the organism and incubated on an orbital shaker (Model G33; New Brunswick Scientific, Edison, NJ) at 300 rpm at 37°C overnight. After incubation, the *E. coli* were pelleted via centrifugation (9,820 × *g*, 15 min, 20°C). The pelleted cells were washed twice to remove organics by resuspension in 100 ml of physiological saline (0.85% NaCl) followed by centrifugation as described previously. The final pellet was resuspended in 10 ml of sterile phosphate buffered saline (PBS; pH7.4; Sigma-Aldrich, St. Louis, MO). The test bacterial suspensions were then prepared by adding small volumes of the bacterial suspension to 10 ml of sterile PBS, resulting in an optical turbidity (measured using a BIOLOG turbidimeter, Hayward, CA) equivalent to a McFarland number 0.5 optical density standard. This is equivalent to approximately 1.5×10^8 colony-forming units (CFU)/ml. This solution was then diluted further in sterile PBS to achieve the desired final test concentration (approximately 1.0×10^7 CFU/ml).

Oregano, cinnamon, and lemongrass essential oils were obtained from Lhasa

Karnak Herbal Co. (Berkley, CA). Citral (mixture of *cis* and *trans*, >96%), carvacrol (>98%), and cinnamaldehyde (93%) were purchased from Sigma-Aldrich (St. Louis, MO). The antimicrobials were diluted (volume to volume) using PBS.

Experimental protocol

The plant essential oils and their active components were evaluated in separate experiments. Initially, these antimicrobials were evaluated at 0.1% and 1.0% (vol/vol) concentrations. These concentrations were then adjusted up or down as needed based on the experimental results. A control with *E. coli* in PBS but no added antimicrobial was also included. Purified stocks of the bacteria were added separately to the antimicrobial solutions (to a final concentration of ~1.0 x 10^7 cfu/ml) and the flasks were placed on an orbital shaker (300 rpm) for the duration of the experiment. Experiments were performed in triplicate at room temperature (24°C) in 10 ml volumes of PBS (pH 7.4) in 50 ml polypropylene conical tubes (Becton Dickinson and Company, Franklin Lakes, NJ). At predetermined time intervals (between 1 and 30 minutes of exposure), 100 µl samples were collected and placed in Dey Engley (D/E) neutralizing broth (Difco, Sparks, MD) at a ratio of 1:10. The D/E was included since simultaneous studies were being conducted with other antimicrobials that required this neutralizer. D/E has been used in similar studies in the past with plant antimicrobials (Mattson et al. 2011). The plant antimicrobials are also neutralized via dilution using D/E. The samples were assayed immediately.

Assay for bacteria

The surviving bacteria were enumerated by performing ten-fold serial dilutions in saline buffer solution (0.85% NaCl) and spread plating on duplicate plates of Levine Eosin Methylene Blue Agar plates (EMB; Becton, Dickinson and Company, Sparks, MD). The plates were incubated for 24 hours at 37°C and the bacterial colonies counted.

Statistical analysis

Data were reported as logarithmic reduction using the formula $\log_{10} (N_0 / N_t)$, where N_0 was the concentration of *E. coli* at time zero and N_t was the surviving concentration at time *t*. A Student's t-test was used to determine if there were significant differences between the control and the antimicrobial treatments (the reduction at each time exposure was compared to the control after 30 minutes). Differences were considered statistically significant if the resultant *P* value was ≤ 0.05. Differences between the reductions observed between separate experiments with comparable concentrations of the essential oil and its active ingredient were also evaluated for statistical significance using a Student's t-test.

Results

The reductions observed in the controls (no added antimicrobials) after thirty minutes ranged from no reduction to 0.16-$\log_{10}$ (Average = 0.03-$\log_{10}$). These were subtracted from the reductions reported for each treated sample (Tables 1-3) to allow for direct statistical comparisons between experiments.

In the first set of experiments, the antimicrobial efficacies of lemongrass oil and its active ingredient citral were assessed (Table 1). Lemongrass oil concentrations of 0.05% and 0.3% were both highly effective, with >4-$\log_{10}$ reductions in *E. coli* populations within 10 and 5 minutes of exposure, respectively. The higher concentration yielded statistically significant reductions ($P \leq 0.05$ in comparison to the control) more rapidly than the lower concentration. In addition, the reduction observed at five minutes of exposure with the 0.3% lemongrass oil (4.37-$\log_{10}$) was significantly greater (P = 0.00055) than that observed with the 0.05% concentration (0.55-$\log_{10}$). The detection limit of the assay (< 50 CFU/ml) was reached by 5 and 10 minutes of exposure for the high and low concentrations, respectively (indicated by >5.94 and >5.62 $\log_{10}$ reductions).

The reductions observed with 0.05% and 0.1% citral were also rapid, with statistical significance reached within 5 minutes of exposure for both. The 0.1% citral treatment yielded a significantly greater reduction in *E. coli* than the 0.05% at every time interval. Interestingly, the 0.05% citral, although more rapidly effective than the 0.05% lemongrass oil (with a significantly greater reduction after five minutes), did not yield

reductions as great at later time exposures (e.g., 4.30-$\log_{10}$ versus >5.62-$\log_{10}$ after 30 minutes of exposure). In fact, the reductions observed for the 0.05% lemongrass oil were significantly higher than the 0.05% citral for the 20 and 30 minute exposure times. In contrast, the 0.1% citral was equally as effective ($P = 0.52$) as the 0.3% lemongrass oil, yielding 4.13-$\log_{10}$ and 4.37-$\log_{10}$ reductions, respectively after five minutes of exposure, and reductions to below the detection limit thereafter (>5.53 and >5.94 $\log_{10}$ reductions).

The results for cinnamon oil and its active ingredient cinnamaldehyde are shown in Table 2. Cinnamon oil was tested at concentrations of 0.8% and 1.25%. The 0.8% trial produced a significant reduction ($P \leq 0.05$) of 2.20-$\log_{10}$ within one minute of exposure. The numbers of surviving bacteria at all subsequent time intervals were below the detection limit of the assay (>6.15-$\log_{10}$ reduction). The number recovered following exposure to the 1.25% concentration was below the detection limit (>5.53-$\log_{10}$) within one minute of exposure.

Cinnamaldehyde concentrations of 0.1% and 0.2% were evaluated. The 0.1% trial produced a significant reduction of 2.01-$\log_{10}$ after 15 minutes (in comparison to the control); the 0.2% trial produced a significant result of 0.69-$\log_{10}$ within one minute of exposure. Although much lower concentrations of cinnamaldehyde were tested in comparison to the cinnamon oil, similar reductions were observed, albeit with longer exposure times. For instance, 0.2% cinnamaldehyde resulted in a 5.11-$\log_{10}$ reduction after 10 minutes, whereas 1.25% cinnamon oil produced a >5.53-$\log_{10}$ reduction after 1 minute.

The results for oregano oil and its primary active ingredient carvacrol are shown in Table 3. Both were tested at a concentration of 0.02%. A significant reduction (in comparison to the control) of 0.48-$\log_{10}$ was observed within five minutes with the 0.02% carvacrol, whereas 20 minutes was required for the oregano oil (0.72-$\log_{10}$ reduction). Likewise, greater reductions ($P \leq 0.05$) were observed with the carvacrol at each common time exposure. Exposure to 0.02% carvacrol resulted in 0.48-$\log_{10}$ to 2.27-$\log_{10}$ reductions between five and 30 minutes for *E. coli*, whereas exposure to 0.02% oregano oil resulted in 0.10-$\log_{10}$ to 1.00-$\log_{10}$ reductions in the same time frame. At higher concentrations of 0.05% and 0.04% for oregano oil and carvacrol, respectively, the *E. coli* populations were reduced to below the detection limit within five minutes of exposure.

Discussion

Previously published research has demonstrated the efficacy of essential oils against a variety of pathogenic bacteria (Friedman et al. 2002; Ultee et al. 2002; Friedman et al. 2003; Knowles et al. 2005; Ravishankar et al. 2008; Ravishankar et al. 2009; Espina et al. 2010; Mild et al. 2011). However, these studies often only focus on either a specific essential oil or a particular active ingredient. Little information regarding the efficacy of essential oils in comparison to their active ingredients is available. In a few studies, crude essential oils have been found to be more effective than their individual components (Lachowicz et al. 1998; Vardar-Unlu et al. 2003), suggesting additive or possibly synergistic effects between the components. In the current study, we examined the efficacy of three essential oils and their corresponding active ingredients against a laboratory strain of *E. coli*. Our results demonstrated that two of the active ingredients, cinnamaldehyde and carvacrol, were more effective, often at lower concentrations, than their corresponding essential oils. Delaquis et al. (2002) observed a similar result with individual fractions of cilantro and dill essential oils. In the current study, citral was more rapidly effective, yet did not produce reductions as great as lemongrass oil with longer exposure periods. These differences may be at least partially explained by the relative proportion of the active ingredient found in the essential oil. For instance, the carvacrol content of oregano oil may be as high as 85% and the cinnamaldehyde content of cinnamon oil as high as 86%, depending on their geographical origin (Ravishankar et al. 2009). Lemongrass oil contains multiple components including citral (57.5%), citral

diethylacetal (24.7%), limonene (6.4%), citral acetate (2.1%), myrcene (1.2%), and methyl heptenone (1.2%) (Katsukawa et al. 2010).

Comparing the efficacies of essential oils to that of their respective active ingredients allows for the establishment of a catalog of effective options. In addition, the antimicrobial efficacy of the essential oils and their active ingredients appears to be concentration dependent. In other words, with increasing concentrations, greater antimicrobial efficacy was observed. Therefore concentration is a key factor that should be considered prior to their use. Other factors such as cost, availability, and organoleptic properties will all affect the usage of essential oils or their active ingredients against pathogens in various applications. Essential oils, although usually not quite as effective as their active ingredients, exhibited significant antimicrobial activity. They are also likely far less costly to produce since there are fewer extraction steps and have less pungent organoleptic properties. Essential oils may therefore be a more viable option for use as an antimicrobial product. Future strategies could include combining essential oils or their active ingredients with other plant antimicrobials or with chemical agents such as chlorine in a combinatorial approach (Beltrán et al. 2005).

Probably the most obvious use for plant antimicrobials is as food sanitizers for meats and fresh fruits and vegetables. Unlike many other antimicrobials such as chlorine, plant antimicrobials are not sensitive to organic matter (Sapers 2009). Therefore, they could potentially promote longer shelf life for food products because of their longer lasting residual effect (Sapers 2009). As mentioned previously, these compounds are

GRAS; therefore, any food sanitizer based upon these antimicrobials would need to undergo only minimal regulatory testing.

These plant antimicrobials could be used in a wide variety of other applications as well. For instance, they could potentially be used as preservatives in cosmetics and lotions, they could be used to coat textiles (e.g., antimicrobial grocery bags), they could be added to laundry or dishwashing detergents, and they could be used in antimicrobial surface coatings or in liquid or spray applications to disinfect fomites (inanimate surfaces). They could also be used to treat food contact surfaces such as in the domestic kitchen, in restaurants, or in food processing facilities.

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References

Adams TB, Cohen SM, Doull J, Feron VJ, Goodman JI, Marnett LJ, Munro IC, Portoghese PS, Smith RL, Waddell WJ, Wagner BM. 2004. The FEMA GRAS assessment of cinnamyl derivatives used as flavor ingredients. *Food Chem Toxicol* **42:** 157–185.

Ayala-Zavala JF, Del-Toro-Sánchez L, Alvarez-Parrilla E, González-Aguilar GA. 2008. High relative humidity in-package of fresh-cut fruits and vegetables: Advantage or disadvantage considering microbiological problems and antimicrobial delivering systems? *J Food Sci* **73:** R41-R47.

Beltrán D, Selma MV, Tudela JA, Gil ML. 2005. Effect of different sanitizers on microbial and sensory quality of fresh-cut potato strips stored under modified atmosphere or vacuum packaging. *Postharvest Biol Technol* **37:** 37-46.

Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods - a review. *Int J Food Microbiol* **94:** 223-253.

Cowan MM. 1999. Plant products as antimicrobial agents. *Clin Microbiol Rev* **12:** 564-582.

Cox SD, Markham JL. 2007. Susceptibility and intrinsic tolerance of *Pseudomonas aeruginosa* to selected plant volatile compounds. *J Appl Microbiol* **103:** 930-936.

Delaquis PJ, Stanich K, Girard B, Mazza G. 2002. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *Int J Food Microbiol* **74:** 101–109.

Espina L, Somolinos M, Pagán R, García-Gonzalo D. 2010. Effect of citral on the thermal inactivation of *Escherichia coli* O157:H7 in citrate phosphate buffer and apple juice. *J Food Protect* **73:** 2189-2196.

Friedman M, Kozukue N, Harden LA. 2000. Cinnamaldehyde content in foods determined by gas chromatography-mass spectrometry. *J Agric Food Chem* **48:** 5702–5709.

Friedman M, Henika PR, Mandrell RE. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J Food Prot*ect **65:** 1545–1560.

Friedman M, Henika PR, Mandrell RE. 2003. Antibacterial activities of phenolic benzaldehydes and benzoic acids against *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes*, and *Salmonella enterica*. *J Food Protect* **66:** 1811–1821.

García-García R, López-Malo A, Palou E. 2011. Bactericidal action of binary and ternary mixtures of carvacrol, thymol, and eugenol against *Listeria innocua. J Food Sci* **76:** M95-100.

Gustafson JE, Liew YC, Chew S, Markham JL, Bell HC, Wyllie SG, Warmington JR. 1998. Effects of tea tree oil on *Escherichia coli*. *Lett Appl Microbiol* **26:** 194-198.

Katsukawa M, Nakata R, Takizawa Y, Hori K, Takahashi S, Inoue H. 2010. Citral, a component of lemongrass oil, activates PPARα and γ and suppresses COX-2 expression. *Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids.* **1801:** 1214-1220.

Knowles JR, Roller S, Murray DB, Naidu AS. 2005. Antimicrobial action of carvacrol at different stages of dual-species biofilm development by *Staphylococcus aureus* and *Salmonella enterica* serovar Typhimurium. *Appl Environ Microbiol* **71:** 797–803.

Kordali S, Kotan R, Mavi A, Cakir A, Ala A, Yildirim A. 2005. Determination of the chemical composition and antioxidant activity of the essential oil of *Artemisia dracunculus* and of the antifungal and antibacterial activities of Turkish *Artemisia absinthium, A. dracunculus, Artemisia santonicum,* and *Artemisia spicigera* essential oils. *J Agric Food Chem* **53:** 9452-9458.

Lachowicz KJ, Jones GP, Briggs DR, Bienvenu FE, Wan J, Wilcock A, Coventry MJ. 1998. The synergistic preservative effects of the essential oils of sweet basil (*Ocimum basilicum* L.) against acid-tolerant food microflora. *Lett Appl Microbiol* **26:** 209-214.

Mattson TE, Johny AK, Amalaradjou MAR, More K, Schreiber DT, Patel J, Venkitanarayanan K. 2011. Inactivation of *Salmonella* spp. on tomatoes by plant molecules. *Int J Food Microbiol* **144:** 464-468.

Mild RM, Joens LA, Friedman M, Olsen CW, McHugh TH, Law B, Ravishankar S. 2011. Antimicrobial edible apple films inactivate antibiotic resistant and susceptible *Campylobacter jejuni* strains on chicken breast. *J Food Sci* **76:** M163-168.

Ravishankar S, Zhu L, Law B, Joens L, Friedman M. 2008. Plant-derived compounds inactivate antibiotic-resistant *Campylobacter jejuni* strains. *J Food Protect* **71:** 1145–1149.

Ravishankar S, Zhu L, Olsen CW, McHugh TH, Friedman M. 2009. Edible apple film wraps containing plant antimicrobials inactivate foodborne pathogens on meat and poultry products. *J Food Sci* **74:** M440-M445.

Ravishankar S, Zhu L, Reyna-Granados J, Law B, Joens L, Friedman M. 2010. Carvacrol and cinnamaldehyde inactivate antibiotic-resistant *Salmonella enterica* in buffer and on celery and oysters. *J Food Protect* **73:** 234-240.

Ress NB, Hailey JR, Maronpot RR, Bucher JR, Travlos GS, Haseman JK, Orzech DP, Johnson JD, Hejtmancik MR. 2003. Toxicology and Carcinogenesis studies of microencapsulated citral in rats and mice. *Toxicol Sci* **71:** 198–206.

Sapers MG. 2009. Chapter 16. Disinfection of contaminated produce with conventional washing and sanitizing technology. *In* The Produce Contamination Problem: Causes and Solutions. Sapers, Solomon, Matthews (Eds.), pp. 393-424. Academic Press, Maryland Heights, MO.

Serafini M, Ghiselli A, Ferro-Luzzi A. 1994. Red wine, tea, and antioxidants. *Lancet* **344:** 626.

Somolinos M, García D, Condón S, Mackey B, Pagán R. 2010. Inactivation of *Escherichia coli* by citral. *J Appl Microbiol* **108:** 1928-1939.

Thoroski J, Blank G, Biliaderis C. 1989. Eugenol induced inhibition of extracellular enzyme production by *Bacillus cereus. J Food Protect* **52:** 399-403.

Ultee A, Kets EPW, Smid EJ. 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Appl Environ Microbiol* **65:** 4606-4610.

Ultee A, Bennik MHJ, Moezelaar R. 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Appl Environ Microbiol* **68:** 1561-1568.

Vardar-Unlu G, Candan F, Sokmen A, Daferera D, Polissiou M, Sokmen M, Donmez E, Tepe B. 2003. Antimicrobial and antitoxic activity of the essential oil and methanol extracts of *Thymus pectinatus* Fisch. et Mey. var. *pectinatus* (Lamiaceae), *J Agric Food Chem* **51:** 63-67.

Wendakoon CN, Sakaguchi M. 1995. Inhibition of amino acid decarboxylase activity of *Enterobacter aerogenes* by active components in spices. *J Food Protect* **58:** 280-283.

Table 1. Reduction (Log_{10} ± standard deviation)[§] in the population of *Escherichia coli* after exposure to various concentrations of lemongrass oil or its active ingredient, citral in PBS.

Time (min)	Lemongrass Oil (vol/vol)		Citral (vol/vol)	
	0.05 %	**0.3 %**	**0.05 %**	**0.1 %**
1	0.02 ± 0.08	ND	0.01 ± 0.03	0.23[†] ± 0.11
5	0.55 ± 0.32	4.37*[†] ± 0.01	1.47* ± 0.19	4.13*[†] ± 0.54
10	4.79* ± 1.44	> 5.94* ± 0.00	2.58* ± 0.76	> 5.53*[†] ± 0.00
20	> 5.62* ± 0.00	> 5.94* ± 0.00	3.52* ± 0.35	> 5.53*[†] ± 0.00
30	> 5.62* ± 0.00	> 5.94* ± 0.00	4.30* ± 0.62	> 5.53*[†] ± 0.00

ND Not determined.

§ The initial bacterial inocula ranged from 1.7 x 10^7 to 4.3 x 10^7 CFU/ml. All experiments were conducted in triplicate.

* The reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobial).

† Reductions were significantly different ($P \leq 0.05$) between the two concentrations of each antimicrobial.

Table 2. Reduction (Log_{10} ± standard deviation)[§] in the population of *Escherichia coli* after exposure to various concentrations of cinnamon oil or its active ingredient, cinnamaldehyde in PBS.

Time (min)	Cinnamon Oil (vol/vol)		Cinnamaldehyde (vol/vol)	
	0.8 %	**1.25 %**	**0.1 %**	**0.2 %**
1	2.20* ± 0.07	> 5.53*[†] ± 0.00	ND	0.69* ± 0.04
5	> 6.15* ± 0.00	> 5.53* ± 0.00	0.01 ± 0.07	3.01*[†] ± 0.40
10	> 6.15* ± 0.00	> 5.53* ± 0.00	ND	5.11* ± 0.91
15	> 6.15* ± 0.00	> 5.53* ± 0.00	2.01* ± 0.58	ND
30	> 6.15* ± 0.00	> 5.53* ± 0.00	4.92* ± 0.62	> 5.64* ± 0.00

ND Not determined.

§ The initial bacterial inocula ranged from 1.7 x 10^7 to 8.9 x 10^7 CFU/ml. All experiments were conducted in triplicate.

* The reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobial).

† Reductions were significantly different ($P \leq 0.05$) between the two concentrations of each antimicrobial.

Table 3. Reduction (Log_{10} ± standard deviation)[§] in the populations of *Escherichia coli* after exposure to various concentrations of oregano oil or its active ingredient, carvacrol in PBS.

Time (min)	Oregano Oil (vol/vol)		Carvacrol (vol/vol)	
	0.02 %	**0.05 %**	**0.02 %**	**0.04 %**
1	0.00 ± 0.05	0.32 ± 0.53	ND	ND
5	0.10 ± 0.09	> 5.35*† ± 0.00	0.48* ± 0.10	> 5.67*† ± 0.00
10	0.36 ± 0.28	> 5.35*† ± 0.00	0.98* ± 0.05	> 5.67*† ± 0.00
20	0.72* ± 0.38	> 5.35*† ± 0.00	ND	ND
30	1.00* ± 0.49	> 5.35*† ± 0.00	2.27* ± 0.22	> 5.67*† ± 0.00

ND Not determined.

§ The initial bacterial inocula ranged from 1.1 x 10^7 to 2.4 x 10^7 CFU/ml. All experiments were conducted in triplicate.

* The reduction was statistically significant ($P \leq 0.05$) in comparison to the control (with no antimicrobial).

† Reductions were significantly different ($P \leq 0.05$) between the two concentrations of each antimicrobial.