Use of plant-derived essential oil compounds, naturally-occurring apple aroma compounds and apple juice flavoring mixtures to control *Escherichia coli* O157:H7

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Abstract

In recent years, there have been a number of studies looking at inhibition of microorganisms by spices, herbs or their extracts. Many of these products have been shown to have antimicrobial activity against foodborne pathogens. The purpose of this research was to evaluate the antimicrobial activity of three essential oil (EO) compounds (thymol, eugenol, and *trans*-cinnamaldehyde) alone and in combination with three naturally-occurring apple aroma (AA) compounds (hexanal, *trans*-2-hexenal and 1-hexanol) to identify the minimum inhibitory concentrations necessary to inhibit *Escherichia coli* O157:H7. Three commercial apple juice flavoring mixtures (natural apple cinnamon, natural apple spice and natural red apple) were additionally tested alone for antimicrobial activity against *E. coli* O157:H7.

The standard agar dilution method (SAD) and checkerboard assay were used to evaluate the efficacy of the nine compounds, alone and in combination against *E. coli* O157:H7. In general, the EO compounds were significantly more effective against *E. coli* O157:H7 than the AA compounds ($P<0.05$). Cinnamaldehye, with an MIC of 0.2 mg/mL, exhibited the highest degree of activity, followed by thymol, eugenol and *trans*-2-hexenal, which each had individual MIC values of 1.6 mg/mL. No synergism was found in the combinations of EO compounds with AA compounds.

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Dedication

I dedicate this work to my grandparents. Thank you for always being so loving, supportive and encouraging. I love you.

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Introduction

Although there have been recent improvements in food production techniques, food safety is still an important public health concern (WHO, 2002b). It has been estimated that there are 9.4 million foodborne illnesses, leading to 55,961 hospitalizations and 1,351 deaths each year in the United States (Scallen, 2011). Therefore, there is still a need for new methods of reducing or eliminating foodborne pathogens. Typically, foods are thermally processed or preserved with chemical compounds such as nitrite, sodium benzoate and sodium metabisulfite that are generally recognized as safe (GRAS) (Davidson and Harrison, 2002). However, incidences of allergic reactions to sulfites have led consumers to become skeptical of "unnatural" food ingredients. In line with this drive for healthier, safer foods, the World Health Organization has called for a reduction in salt intake in order to reduce the incidence of cardiovascular disease (WHO, 2002a). With a reduction in levels of salt used as preservatives in processed foods, it is likely that other antimicrobial compounds will be needed to maintain food safety. Among the alternatives are antimicrobial compounds from natural sources (Aoki et al., 2010).

Antimicrobials are substances that inhibit the growth of microorganisms including bacteria, viruses and fungi (Davidson et al., 2005). Natural antimicrobials are naturally produced and can be isolated from sources such as plants, animals and/or microorganisms (Juneja et al., 2012). These naturally occurring antimicrobials can potentially be applied to foods and beverages to protect quality and extend shelf life of the product. Although some natural antimicrobials are commercially available and

applied in food processing, their efficacy, consumer acceptance and regulation are not well understood (Juneja et al., 2012).

Among the natural antimicrobials being studied are constituents of herbs and spices (Zaika, 1998; Suhr and Nielson, 2003). Effects of spices have been known for centuries, and studies looking at the active components of spices have shown that the essential oils of many spices have antimicrobial effects (Zaika, 1988). Essential oils from cinnamon (cinnamaldehyde), thyme (thymol) and oregano (carvacol) have shown antimicrobial activity against *S*. Typhimurium (Zhou et. al., 2007). Additionally, the essential oil from cloves (eugenol) has been proven to inhibit growth of some strains of *Listeria monocytogenes* and *Escherichia coli* in full fat (4%) milk (Gaysinsky and Taylor, 2007).

In addition to essential oils, other natural antimicrobials that have been studied are aldehydes (Sokovic et al., 2010). Lanciotti et al. showed that hexanal, trans-2-hexenal and hexyl acetate had significant inhibitory effects against *E. coli* O157:H7, *S*almonella Enteriditis and *L. monocytogenes* in a model system, but found conflicting results when applied to packaged fresh sliced apples (Lanciotti et al., 2003).

Marketers recognize that consumers want foods that are convenient, minimally processed, and all natural with no preservatives (Allende et al., 2006). Among consumer trends, one of the most obvious is the increase in consumption of fresh fruits and vegetables and their juices (Zink, 1997). The potential use of natural antimicrobials to combat pathogens such as *E. coli* O157:H7 would likely be appealing to the food industry.

E. coli O157:H7 accounts for approximately 93,000 illnesses, resulting in 20 deaths annually (Scallen et al., 2011). The low infectious dose of *E. coli* O157:H7 coupled with its relative difficulty to eliminate from food, makes this a pathogen of concern in the produce industry (Griffin and Tauxe, 1991). There have been *E. coli* O157:H7 outbreaks in a variety of foods, including ground beef, spinach, and unpasteurized juices and ciders (Beuchat 1996). The use of natural antimicrobials, such as essential oil compounds or apple aroma compounds, shows some potential in combating *E. coli* O157:H7 contamination in fruit juices. This research tested essential oil compounds, apple aroma compounds and apple juice flavoring mixtures, alone and in combination, to determine their efficacy in inhibiting the growth of *E. coli* O157:H7.

Objectives

1) Determine the antimicrobial activity of three essential oil compounds (thymol, eugenol and cinnamaldehyde) against *E. coli* O157:H7
2) Determine the antimicrobial activity of three apple aroma compounds (hexanal, trans-2-hexenal, and 1-Hexanol) against *E. coli* O157:H7
3) Determine the antimicrobial activity of three apple juice flavoring mixtures (natural apple cinnamon, natural apple spice, natural red apple) against *E. coli* O157:H7
4) Determine the antimicrobial activity of three essential oil compounds (thymol, eugenol and cinnamaldehyde) in combination with three apple aroma compounds (hexanal, trans-2-hexenal, and 1-hexanol) against *E. coli* O157:H7

Review of Literature

Escherichia coli O157:H7

E. coli O157:H7 was first recognized as a pathogen in 1982 during an outbreak of hemorrhagic colitis associated with consumption of contaminated hamburgers (Riley et al., 1983). However, it was not until ten years later that *E. coli* O157:H7 became familiar to many consumers when an outbreak linked to the consumption of hamburgers from the Jack in the Box Restaurant chain occurred (Rangel et al., 2005).

There are an estimated 93,094 illnesses due to domestically acquired *E. coli* O157:H7 each year in the United States. Of these illnesses, it is estimated that they result in 2,138 hospitalizations and 20 deaths annually (Scallen et al., 2011). The ability of *E. coli* O157:H7 to cause severe disease in humans is largely associated with the production of Shiga toxin (Stx) (Griffin and Tauxe, 1991; Johannes 2010). Shiga toxin acts by inhibiting protein synthesis in endothelial and other cells (Sandvig, 2002). Shiga toxin producing *E. coli* may cause disease at a low infectious dose, and they have the ability to multiply at temperatures up to 44°C, survive freezing, resist mild heat, resist drying, and can survive exposure to acidic environments (Juneja et al., 1997). Another factor contributing to the danger of *E. coli* O157:H7 as a foodborne pathogen is its prevalence in a wide variety of sources. Although ground beef has traditionally been recognized as the most common source of illness associated with this organism, outbreaks have been linked to many different food products. For example, fresh produce has been the source of a number of outbreak-related *E. coli* O157:H7 infections since 1991. (Rangel, 2005; CDC, 2002). Outbreaks have been linked to alfalfa sprouts, leafy greens such as lettuce and spinach, unpasteurized juices, yogurt,

dried salami, hazelnuts, raw milk and raw cookie dough (Cody et al., 1999; Friedman et al., 1999; Rangel et al., 2005; Breuer et al., 2001).

E. coli O157:H7 in Apple Juice

Although there have been a number of juice-related *E. coli* O157:H7 outbreaks (Table 1), perhaps the most notable is the outbreak in Odwalla brand apple juice. On October 31, 1996, the Food and Drug Administration announced that Odwalla was recalling all of its juice products that contained unpasteurized apple juice (Rowles, 2001). The recall was initiated following 13 reported cases of *E. coli* O157:H7 illness that had been linked to the company's unpasteurized apple juice (Swaminathan et al., 2001). During the outbreak investigation, a strain of *E. coli* O157:H7 identical to the one that had been isolated from case-patients was found in a bottle of unpasteurized Odwalla apple juice (CDC, 1996). Although the investigation never pinpointed the exact source of the *E. coli* at the Odwalla plant, investigators from the FDA found that the plant had used decayed fruit from suppliers to produce their product (Powell, 1998). When the outbreak was over, one child had died and more than 65 individuals were confirmed to have been infected with the bacteria (Martinelli and Briggs, 1998). Following this outbreak, the federal government made it mandatory that warning labels appear on containers of all unpasteurized juices (Hilborn et al., 2000). However, there are still arguments for unpasteurized juices including claims of better taste and nutritional values, therefore alternatives to traditional processing have become a recent topic of research.

Table 1. Outbreaks of *E. coli* O157:H7 in fruit juices in the United States and Canada (Danyluk et al., 2010; Vojdani et al., 2008)

Year	Product	Causative Agent	Location	Venue	Cases (deaths)
1980	Unpasteurized apple cider	*E. coli* O157:H7	Canada (ON)	Local market	14 (1)
1991	Unpasteurized apple cider	*E. coli* O157:H7	Massachusetts	Small cider mill	23 (0)
1996	Unpasteurized apple cider	*E. coli* O157:H7	Canada (BC), USA (CA, CO, WA)	Retail	70 (1)
1996	Unpasteurized apple cider	*E. coli* O157:H7	Connecticut	Small cider mill	14 (0)
1996	Unpasteurized apple cider	*E. coli* O157:H7	Washington	Small cider mill	6 (0)
1997	Unpasteurized apple cider	*E. coli* O157:H7	Indiana	Farm	6
1998	Unpasteurized apple cider	*E. coli* O157:H7	Canada (ON)	Farm/home	14 (0)
1999	Unpasteurized apple cider	*E. coli* O157:H7	Oklahoma	Not reported	25
2004	Unpasteurized apple cider	*E. coli* O111 (and *C. parvum*)	New York	Farm/home	212
2005	Unpasteurized apple cider	*E. coli* O157:H7	Canada (ON)	Not reported	4
2007	Unpasteurized apple cider	*E. coli* O157:H7	Massachusetts	Not reported	9
2008	Unpasteurized apple cider	*E. coli* O157:H7	Iowa	Fair	7
2010	Unpasteurized apple cider	*E. coli* O157:H7	Maryland	Retail	7

Antimicrobials

The quality of a food product diminishes from the time of harvest until it is consumed. This loss of quality can be attributed to microbiological, enzymatic, chemical or physical changes (Gould, 2000). However, food preservation technologies protect food from these changes. Historically, heating, cooling, drying or fermentation have been popular methods to achieve safe, quality food, but recent attention has been on

the use of antimicrobial compounds (Varoquaux and Wiley, 1994). Antimicrobials are defined as “substances used to preserve food by preventing growth of microorganisms and subsequent spoilage, including fungistats, mold and rope inhibitors” in the Code of Federal Regulations (FDA, 2006). Consumer demand for high quality, “safe” food products has led producers to looking at natural sources of antimicrobials to inhibit food-spoilage organisms or foodborne pathogens (Rai and Chikindas, 2011). Many foods naturally contain compounds such as herbs and spices which are known to have antimicrobial activity and have potential in extending the shelf life of a food product (Tiwari et al., 2009). Some of these naturally occurring compounds have been studied for their use as direct food additives. However, most of these are not present at high enough concentrations in foods to be antimicrobials without some purification. Challenges lie in using purified constituents of the natural compounds, due to the potential of altering the sensory characteristics of the food (Davidson et al., 2005). Additionally, in order to be useful in the food industry, antimicrobial compounds must be applied without causing a significant increase in the costs for formulation, processing or marketing (Beuchat and Golden, 1989).

Essential Oils

Wilkins and Board (1989) reported that more than 1,340 plants are known to be potential sources of antimicrobial compounds. As early as 5,000 years ago, ancient Chinese, Indian and Middle Eastern civilizations documented uses of plants as medicinal remedies (Burt, 2004). Despite years of use of plants in medicinal ways, most antimicrobial research has been performed recently. Antimicrobial components of herbs and spices have been characterized as phenolics, terpenoids, alkaloids, lectins,

and polypeptides (Cowan, 1999). Antimicrobial substances vary in content from the allicin in garlic (0.3-0.5%) to eugenol in cloves (16-18%) (Davidson et al., 2005). Clove (eugenol), cinnamon (cinnamaldehyde), thyme (thymol), oregano (carvacol) and vanilla (vanillin) have all been noted as having a broad spectrum of antimicrobial activity against numerous bacteria and fungi (Cowan 1999; Naidu 2000). Growth and aflatoxin production by *Aspergillis parasiticus* in broth was inhibited by 200-300 ppm of cinnamon and clove oils (Bullerman et al., 1977). Friedman et al. (2004) found that carvacrol, oregano oil, geraniol, eugenol, cinnamon leaf oil, citral, clove bud oil, lemongrass oil, cinnamon bark oil, and lemon oil were effective in reducing *E. coli* O157:H7 populations by 50% in apple juice. Baskarin et al. (2012) reported that trans-cinnamaldehyde concentrations of 0.125%v/v and 0.075%v/v completely inactivated *E. coli* O157:H7 after one and three days of storage in apple juice and apple cider, respectively, while a concentration of 0.025%v/v reduced *E. coli* O157:H7 populations to undetectable levels after five days. However, in many cases, the concentration of the compound in its natural form is too low to be successfully used as an antimicrobial (Davidson et al., 2005).

Natural Aldehydes

In addition to essential oil compounds, naturally occurring six-carbon aldehydes (aroma compounds) have also been shown to have antimicrobial activity against a number of foodborne pathogens. Studies have shown that naturally occurring aldehydes are effective in inhibiting *L. monocytogenes, E. coli, S.* Enteritidis*, P. aeruginosa,* and *E. aerogenes* growth (Muroi et al., 1993; Gardini et al., 1997; Gardini et al., 2001; Song et al., 2007; Patrigani et al., 2008). For example, Lanciotti et al. (2003),

has shown that hexanal, hexyl acetate, and trans-2-hexenal used at 150, 150, and 20 ppm, respectively, displayed bactericidal effects on *L. monocytogenes* and were effective in extending the lag phase of *E. coli* and *S. enteritidis* in vitro.

Aroma compounds are naturally present in plants, and believed to possess inhibitory properties against a number of bacterial species as a defense mechanism (Patrigani et al., 2008). Their aroma properties are thought to be due to the natural formation of alcohols and esters by the aldehydes, which often impart a "green" characteristic to a number of fruits (Gardini et al., 2001). For this research, hexanal, trans-2-hexenal and 1-hexanol were studied for antimicrobial properties against *E. coli* O157:H7.

Essential oils mode of action

The degree of sensitivity of a microorganism to an antimicrobial varies based on a number of factors, including the strain tested and environmental conditions (Paster et al., 1990). Additionally, it has been observed that Gram positive bacteria exhibit a higher degree of sensitivity than Gram negative bacteria to antimicrobial compounds in spices (Shelef, 1983). Farag et al. (1989) observed that *E. coli* was less resistant than *P. fluorescens* when tested with essential oils from sage, rosemary, cumin, clove and thyme. Paster et al. (1990) found that *S.* Typhimurium was more sensitive than *P. aeruginosa* to carvacol (oregano) and thymol (thyme). Reasons for these variations have been studied through possible modes of action (Davidson et al., 2005). However, the exact mode of action has not been defined.

In some cases, the effect of phenolic compounds is concentration dependent. At low concentrations, phenols affect enzyme activity, particularly those associated with

energy production, while at high concentrations, they cause protein denaturation (Viuda-Martos et al., 2007). Carvacrol, thymol and trans-cinnamaldehyde are reported to alter intra- and extracellular ATP content of *E. coli* O157:H7 cells, thereby achieving some form of disruption to the plasma membrane of the cell (Helander et al., 1998). Rojas-Grau et al. (2006) has studied the effects of oregano, cinnamon and lemon grass oils and their active components in apple puree and against *E. coli* O157:H7. It was found that the essential oils potentially work by targeting several areas in the cell including damage to the cell wall, cytoplasmic membrane or membrane proteins, depletion of proton motive active sites or inactivation of essential enzymes.

Limitations

Most studies related to antimicrobial efficacy of EOs have been conducted in vitro using microbiological media (Ting and Diebel 1992; Remmal et al 1993; Hammer et al 1999; Griffin et al 2000; Elgayyar et al 2001; Gomez-Estaca et al 2010). Because of this, there is limited understanding related to their efficacy when applied to food matrices. For example, plant EOs of thyme, clove, and pimento were tested against *L. monocytogenes* and were found to be highly effective in peptone water. However, when the EOs were applied in a food system, their efficacy was reduced due to the interaction with food components (Rai and Chikindas, 2011). In general, higher concentrations of EOs are required in foods than in laboratory media (Shelef, 1983; Smid and Gorris, 1999). Similarly, Shekarforoush et al. (2007) found that oregano and nutmeg were effective against *E. coli* O157:H7 in a broth system, but had no effect in ready-to-cook chicken. In many food products, activity of the hydrophobic EO constituents are impaired by interactions with food matrix components such as fat,

starch, and proteins (Hyldgaard et al., 2012). Additionally, the antimicrobial potential of EOs also depends on pH, temperature and level of microbial contamination. In general, spices are less effective in food than in culture media and Gram positive bacteria are more sensitive than Gram negative (Davidson et al., 2005).

Synergy

Additions of high concentrations of natural antimicrobials are often limited in food products due to negative sensory changes. Because of this, the use of lower concentrations of two or more antimicrobials in combination have been explored (Lvetal 2011). There have been a number of studies looking at this potential for synergy between essential oils and conventional antibiotics. Results have shown variations based on the test organism and essential oil used. Synergism has been observed between cinnamaldehyde and clindamycin against *Clostridium difficile* and between oregano oil and sarafloxacin, levofloxacin, maquindox, florfenicol and doxycycline against *E. coli* (Thormar, 2011). A combination of antagonistic, synergistic and indifferent interactions was seen between ciprofloxacin and tea-tree, thyme, peppermint and rosemary oils against *Klebsiella pneumoniae*, based on the concentration of each compound (van Vuuren et al., 2009). A combination of cinnamaldehyde with thymol or carvacol has proven to display synergism against *S*. Typhimurium. It has also been shown that nerolidol was not synergistic with eugenol or thymol against *E. coli* or *P. aeruginosa* (Thormar, 2011). Overall, there appears to be no clear trends for the efficacy of essential oils when used in combination with other compounds. Whether the combination of two antimicrobial agents results in an antagonistic, indifferent or synergistic effect depends on the mechanisms exerted by each agent against the test

organism, the characteristics of the test organism and any chemical interactions between the two antimicrobial agents (Bassole and Juliani, 2012). This is supported by two studies investigating terpene combinations, which found that interactions could be synergistic, indifferent or antagonistic, depending on concentrations and ratios of components (Thormar, 2011)

Method for antimicrobial testing

There are a number of different methods that are used to analyze antimicrobial effects. These test methods have been described in literature (Barry, 1976; Davidson and Parish, 1989). In this experiment, the agar dilution method was used. Agar dilution assays are typically used when quantitative data are desired to determine whether an antimicrobial is lethal to a test microorganism (Davidson et al., 2005). This method offers the researcher flexibility in determining the activity of a novel antimicrobial and/or the effect of environmental conditions on the growth of the microorganism in the presence of a known antimicrobial (Griffin et al., 2000). With this method, the minimum inhibitory concentration (MIC) is determined, with the MIC being defined as the lowest concentration of an antimicrobial that prevents growth of a microorganism after a specified incubation period (Davidson et al., 2005). There are several advantages of the agar dilution method, including the ability to test a large number of strains at once, contamination is easily detected, and the medium can contain opaque compounds (Davidson et al., 2005).

Materials and Methods

Bacterial Cultures

The following bacterial strains were used for this study: *E. coli* O157:H7 (cider), *E. coli* O157:H7 (beef), *E. coli* ATCC 25922. All test cultures were obtained from the frozen (-80°C) culture collection at Virginia Tech (Blacksburg, VA).

Preparation of Bacterial Cultures

Stored cultures were activated by two successive transfers in tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) at 35^{o}C for 24 hours. Samples were then streaked onto differential media (Sorbitol Maconkey, SMAC) and a single representative colony was selected for further confirmation. Both *E. coli* O157:H7 strains were confirmed using *E. coli* O157:H7 latex agglutination test (Remel Lenexa, KS). Upon confirmation, a single representative colony of each organism was inoculated into individual test tubes, containing 10 mL of sterile tryptic soy broth. Samples were then incubated for 24 hours at 35^{o}C, yielding a final concentration of 10^{7}-10^{8} CFU/mL. This culture was transferred into fresh tryptic soy broth every 18-24 hours, in order to ensure a uniform culture for the duration of the experiment.

Preparation of Chemicals and Antimicrobial Agents

Prior to experimentation, all apple aroma compounds**:** hexanal (96%), 1-hexanol (99%) and *trans*-2-hexenal (99%) (Acros Organics, Fair Lawn, NJ), all essential oils: thymol, (99%), eugenol (≥89%), cinnamaldehyde (89%) (SAFC Supply Solutions, St. Louis, MO) and all apple juice flavoring mixtures: natural apple cinnamon, natural apple spice, and natural red apple (Gold Coast Ingredients, Inc, Commerce, CA) were

weighed into individual glass beakers, containing 0.5% Tween-20 (Fisher Scientific, Fairlawn, NJ). Tween-20 was included to enhance solubility of the test compounds. This solution was then emulsified for 30 seconds, using a micro-emulsifying needle (Cadence Science, Staunton, VA), filter sterilized using a 0.45µm disposable syringe filter (Fisher Scientific) and dispensed into individual containers of molten tryptic soy agar (TSA; Becton, Dickinson and Company).

Determination of Minimum Inhibitory Concentrations

Minimum Inhibitory Concentrations (MIC) were determined using the standard agar dilution (SAD) method according to guidelines established by the National Committee for Clinical Laboratory Standards (Hecth et al., 2007). Briefly, antimicrobial compounds at concentrations of 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 mg/mL (and up to 51.2 mg/mL for the apple juice flavoring mixtures) were dissolved into separate containers of molten TSA, containing 0.5% Tween-20. Molten agar solutions were mixed vigorously by hand for one minute to evenly distribute the compound throughout the agar, then poured into individual petri dishes and allowed to solidify. Individual plates were then divided into thirds and spot inoculated with 2.5 µL of each *E. coli* culture (diluted to log 10^6 CFU/mL). Plates were incubated at 35^{o}C and evaluated after 24 hours and 48 hours for presence or absence of growth. MICs were considered the lowest concentration completely inhibiting growth. Control plates (0.5% Tween-20 with no added antimicrobial) were also included to ensure that Tween-20 had no inhibitory effects on *E. coli* O157:H7. All samples were plated in duplicate and the experiment was replicated three times.

Determination of Synergistic Effects

Antimicrobial interactions between test compounds were evaluated using the checkerboard assay, as described by Rosenblatt 1974 and Rand 1995. Briefly, each essential oil compound (at 0.2, 0.4, 0.8 or 1.6 mg/mL) was dissolved into an individual container of molten TSA, containing 0.5% Tween-20 with the exception of cinnamaldehyde, which was only tested in combination at 0.2 and 0.4 mg/mL (due to its individual MIC of 0.2 mg/mL). One of the three apple aroma (AA) compounds (at 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 and 25.6 mg/mL) was then separately added to each container, resulting in a series of eight mixed ratio combinations. For example, cinnamaldehyde at 0.2 mg/mL and 0.4 mg/mL were each tested in combination with hexanal at 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 12.8 and 25.6 mg/mL. All combinations were poured into individual petri plates and allowed to solidify. Plates were then divided into thirds and spot inoculated with a 2.5 µL culture of each of the three *E. coli* cultures (diluted to log 10^6 CFU/mL) and incubated at 35^{o}C. Plates were read at 24 and 48 hours. MICs were determined as the lowest concentration that completely inhibited microbial growth.

The Fractional Inhibitory Concentration (FIC) index was used to describe the nature of each interaction. FIC values were calculated according to the procedure outlined by Botelho (2000). Briefly, ΣFIC = FIC A + FIC B, where FIC A is the MIC of the combination/MIC of compound A alone, and FIC B is the MIC of the combination/MIC of compound B alone. Combination relationships are defined as follows: synergistic when $\Sigma FIC \leq 0.5$, antagonistic when $\Sigma FIC > 4$ and indifferent when $0.5 < \Sigma FIC < 4$.

Results

The minimum inhibitory concentration (MIC) of three EO compounds (eugenol, cinnamaledyhe, thymol), three AA compounds (1-hexanol, *trans*-2-hexenal and hexanal), and three apple juice flavoring mixtures (natural apple cinnamon, natural apple spice, and natural red apple) against two strains of *E. coli* O157:H7 and a non-pathogenic surrogate (*E. coli* ATCC 25922) were determined.

When the EO compounds were tested alone, cinnamaldehyde exhibited the highest degree of antimicrobial activity against all three strains of *E. coli* tested, with reported MIC values of 0.2 mg/mL (Table 1). Thymol and eugenol generated the same level of inhibition against *E. coli* O157:H7, each with an MIC value of 1.6 mg/mL. (Table 1).

When the AA compounds were tested alone, *trans*-2-hexenal exhibited the highest degree of antimicrobial activity (MIC = 1.6 mg/mL against each strain) (Table 1). Hexanal and 1-hexanol were significantly less active, with MICs of 12.8 and 6.4 mg/mL respectively (Table 1). Since the AA compounds displayed limited degrees of activity against test organisms, they were evaluated in combination with the EO compounds in an effort to identify synergistic relationships which may enhance their effects against *E. coli* O157:H7.

Of the apple juice flavoring mixtures, apple cinnamon exhibited the highest degree of antimicrobial activity (MIC = 12.8 mg/mL), followed by apple spice with an MIC of 51.2 mg/mL. Red apple flavoring did not exhibit any antimicrobial activity against any strain at the levels tested (Table 1)

Combination testing

Combination testing revealed that both low and high concentrations of essential oil compounds in combination with apple aroma compounds resulted in an indifferent effect against both *E. coli* O157:H7 stains and *E. coli* ATCC 25922 (Tables 2.1, 2.2, 2.3, 3.1, 3.2, 3.3, 4.1, 4.2, 4.3, 5.1, 5.2, 5.3).

Discussion

The results indicate that cinnamaldehyde was the most active EO compound, followed by thymol and eugenol. These results are similar to those shown by Pei et al. (2009) who evaluated the activity of cinnamaldehyde, eugenol and thymol against strains of *E. coli* in vitro and reported MIC values of 400, 1600 and 4000 mg/L, respectively. Blaszyk and Hilley reported that with greater than 1000 μg/mL of eugenol, the growth of *E. coli* O157:H7 and *L. monocytogenes* was inhibited at 18^{o}C in microbiological media. Bullerman et al. (1977) showed that cinnamaldehyde at 150 ppm and eugenol at 125 ppm were inhibitory against aflatoxin production by *Aspergillus parasiticus*.

Since the mode of action of EO compounds is not fully understood, differences in antimicrobial activity can only be hypothesized. Eugenol is a hydrophobic compound that associates with lipid fractions (Gaysinky et al., 2007). Its antimicrobial action against Gram negative and Gram positive organisms is thought to be contributed to interactions with the cell membrane (Lee et al., 2007). It is believed that the mode of action for the antimicrobial activity of cinnamaledyde varies based on the concentration level. At low concentrations, cinnamaldehyde inhibits enzymes involved in cytokinesis. At higher but still sub lethal concentrations, it acts as an ATPase inhibitor and at lethal concentrations it perturbs the cell membrane (Hyldgaard et al., 2012). Finally, the mode of action for thymol is believed to be similar to eugenol in that it involves outer and inner membrane disruption and interaction with membrane proteins.

In the case of the AA compounds, *trans*-2-hexenal exhibited the highest degree of antimicrobial activity, followed by 1-hexanol and hexanal. Similar results have been

observed in other studies. Research by Lanciotti et al. (2003) showed that *E. coli* and *L. monocytogenes* were inhibited by 20 and 50 ppm, respectively, of *trans*-2-hexenal, while 200 and 500 ppm, respectively, of hexanal was required to achieve the same level of inhibition. Like EO compounds, the precise mode of action is not clear for AA compounds. However, it is believed that hexanal acts as a surfactant, attaching to sulfydryl groups of membrane-bound proteins, disrupting the cytoplasmic membrane and ultimately leading to cell death. Unlike hexanal, *trans*-2-hexenal most likely acts by permeating across the plasma membrane by passive diffusion (Muroi et al., 1993). Similar to hexanal, it is believed that the small hydrophobic cells of 1-hexanol penetrate the outer membrane of the cell via porin proteins (Beuchat, 2002). Once inside the cell, the hydrophobic carbon tails of 1-hexanol interact with proteins causing membrane disruption eventually leading to cell death (Patrigani et al., 2008).

In this research, testing combinations of EO compounds with AA compounds against *E. coli* O157:H7 and nonpathogenic *E. coli* ATCC 25922 over a varying range of concentrations resulted in indifferent relationships for all combinations. Such varying results from studies involving interactions of antimicrobials, antibiotics and/or aromatic compounds, points to the fact that there is a current lack of knowledge in area. Conflicting data on the mode of action of the individual essential oil constituents is one of the main reasons researchers have only a vague understanding about synergistic and antagonistic relationships (Hylgaard et al., 2012).

Conclusions and future research

The idea of a potential for synergistic or antagonistic interactions between antimicrobials and antibiotics is based on data that are over a decade old (Davidson and Parish, 1989). However, with a renewed interest in the subject and a development of new techniques, it is likely that significant advances can be made in understanding how antimicrobial compounds interact in combination. Future research should explore the mode of action of individual EO constituents in depth, as well as investigate the mechanisms of possible synergistic interactions between different constituents.

Although the use of EO compounds are often limited due to the sensory alterations they impart, their potential as antimicrobial agents could be beneficial if the flavor could be masked. This is where the potential for use of aroma compounds can be beneficial. Seeing as aroma compounds showed activity at relatively low levels as well as their natural occurrence in fruits makes the AA compounds potential candidates for antimicrobial use in minimally processed fruit juices. Nevertheless, further research is required to understand the activity of these compounds in model food systems, as well as their overall sensorial acceptability in foods.

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Tables

Table 1. Individual Minimum Inhibitory Concentrations (MIC) for antimicrobial test compounds against *E. coli* O157:H7 (cider), *E. coli* O157:H7 (beef) and *E. coli* ATCC 25922

Antimicrobial	MIC in mg/mL		
EO compounds	*E. coli* O157:H7 (cider)	*E. coli* O157:H7 (beef)	*E. coli* ATCC 25922
Eugenol	1.6[c]	1.6[c]	1.6[c]
Thymol	1.6[c]	1.6[c]	1.6[c]
Cinnamaldehyde	0.2[d]	0.2[d]	0.2[d]
AA compounds			
Hexanal	12.8[a]	12.8[a]	12.8[a]
1-Hexanol	6.4[b]	6.4[b]	6.4[b]
Trans-2-hexenal	1.6[c]	1.6[c]	1.6[c]
Apple juice flavoring			
Apple cinnamon	12.8	12.8	12.8
Apple spice	51.2	51.2	51.2
Red apple	N/A	N/A	N/A

EO = Essential Oil, AA= Apple Aroma

[a-d]Means with the same letter are not significantly different

Table 2.1: MICs, FIC values and antimicrobial interactions for combinations containing 0.2 mg/mL of Essential Oil compounds against *E. coli* O157:H7 (cider)

Combination		FIC Indicies			
	MIC	FIC A	FIC B	ΣFIC	Interpretation
Eug + Hexanal	12.8	0.125	1	1.125	Indifferent
Eug + 1-Hexanol	12.8	0.125	2	2.125	Indifferent
Eug + Trans-2-hexenal	0.8	0.125	0.5	0.625	Indifferent
Thy + Hexanal	25.6	0.125	2	2.125	Indifferent
Thy + 1-Hexanol	6.4	0.125	1	1.125	Indifferent
Thy + Trans-2-hexenal	1.6	0.125	1	1.125	Indifferent
Cinn + Hexanal	0.4	1	0.031	1.031	Indifferent
Cinn + 1-Hexanol	3.2	1	0.5	1.5	Indifferent
Cinn + Trans-2-hexenal	0.2	1	0.125	1.125	Indifferent
*Eug + Thymol	0.8	0.125	0.5	0.625	Indifferent

FIC A = Fractional inhibitory concentration for EO compound
FIC B = Fractional inhibitory concentration for AA compound
ΣFIC = FIC A + FIC B, Indifference=(0.5<ΣFIC<4)
*Eugenol was added at 0.2 mg/mL

Table 2.2: MICs, FIC values and antimicrobial interactions for combinations containing 0.2 mg/mL of Essential Oil compounds against *E. coli* O157:H7 (beef)

Combination		FIC Indicies			
	MIC	FIC A	FIC B	ΣFIC	Interpretation
Eug + Hexanal	12.8	0.125	1	1.125	Indifferent
Eug + 1-Hexanol	6.4	0.125	1	1.125	Indifferent
Eug + Trans-2-hexenal	0.8	0.125	0.5	0.625	Indifferent
Thy + Hexanal	25.6	0.125	2	2.125	Indifferent
Thy + 1-Hexanol	6.4	0.125	1	1.125	Indifferent
Thy + Trans-2-hexenal	1.6	0.125	1	1.125	Indifferent
Cinn + Hexanal	0.4	1	0.031	1.031	Indifferent
Cinn + 1-Hexanol	3.2	1	0.5	1.5	Indifferent
Cinn + Trans-2-hexenal	0.2	1	0.125	1.125	Indifferent
*Eug + Thymol	0.8	0.125	0.5	0.625	Indifferent

*Eugenol added at 0.2 mg/mL

Table 2.3: MICs, FIC values and antimicrobial interactions for combinations containing 0.2 mg/mL of Essential Oil compounds against *E. coli* ATCC 25922

Combination		FIC Indicies			
	MIC	FIC A	FIC B	ΣFIC	Interpretation
Eug + Hexanal	12.8	0.125	1	1.125	Indifferent
Eug + 1-Hexanol	12.8	0.125	2	2.125	Indifferent
Eug + Trans-2-hexenal	0.8	0.125	0.5	0.625	Indifferent
Thy + Hexanal	25.6	0.125	2	2.125	Indifferent
Thy + 1-Hexanol	6.4	0.125	1	1.125	Indifferent
Thy + Trans-2-hexenal	0.8	0.125	0.5	0.625	Indifferent
Cinn + Hexanal	0.4	1	0.031	1.031	Indifferent
Cinn + 1-Hexanol	3.2	1	0.5	1.5	Indifferent
Cinn + Trans-2-hexenal	0.2	1	0.125	1.125	Indifferent
*Eug + Thymol	0.8	0.125	0.5	0.625	Indifferent

*Eugenol added at 0.2 mg/mL

Table 3.1: MICs, FIC values and antimicrobial interactions for combinations containing 0.4 mg/mL of Essential Oil compounds against *E. coli* O157:H7 (cider)

Combination		FIC Indicies			
	MIC	FIC A	FIC B	ΣFIC	Interpretation
Eug + Hexanal	12.8	0.25	1	1.25	Indifferent
Eug + 1-Hexanol	6.4	0.25	1	1.25	Indifferent
Eug + Trans-2-hexenal	0.8	0.25	0.5	0.75	Indifferent
Thy + Hexanal	12.8	0.25	1	1.25	Indifferent
Thy + 1-Hexanol	6.4	0.25	1	1.25	Indifferent
Thy + Trans-2-hexenal	0.8	0.25	0.5	0.75	Indifferent
Cinn + Hexanal	0.2	2	0.016	2.016	Indifferent
Cinn + 1-Hexanol	0.2	2	0.031	2.031	Indifferent
Cinn + Trans-2-hexenal	0.2	2	0.125	2.125	Indifferent
*Eug + Thymol	0.8	0.25	0.5	0.75	Indifferent

*Eugenol added at 0.4 mg/mL

Table 3.2: MICs, FIC values and antimicrobial interactions for combinations containing 0.4 mg/mL of Essential Oil compounds against *E. coli* O157:H7 (beef)

Combination		FIC Indicies			
	MIC	FIC A	FIC B	ΣFIC	Interpretation
Eug + Hexanal	12.8	0.25	1	1.25	Indifferent
Eug + 1-Hexanol	3.2	0.25	0.5	0.75	Indifferent
Eug + Trans-2-hexenal	0.8	0.25	0.5	0.75	Indifferent
Thy + Hexanal	12.8	0.25	1	1.25	Indifferent
Thy + 1-Hexanol	6.4	0.25	1	1.25	Indifferent
Thy + Trans-2-hexenal	0.8	0.25	0.5	0.75	Indifferent
Cinn + Hexanal	0.2	2	0.016	2.016	Indifferent
Cinn + 1-Hexanol	0.2	2	0.031	2.031	Indifferent
Cinn + Trans-2-hexenal	0.2	2	0.125	2.125	Indifferent
*Eug + Thymol	0.8	0.25	0.5	0.75	Indifferent

*Eugenol added at 0.4 mg/mL

Table 3.3: MICs, FIC values and antimicrobial interactions for combinations containing 0.4 mg/mL of Essential Oil compounds against *E. coli* ATCC 25922

Combination		FIC Indicies			
	MIC	FIC A	FIC B	ΣFIC	Interpretation
Eug + Hexanal	12.8	0.25	1	1.25	Indifferent
Eug + 1-Hexanol	6.4	0.25	1	1.25	Indifferent
Eug + Trans-2-hexenal	0.8	0.25	0.5	0.75	Indifferent
Thy + Hexanal	12.8	0.25	1	1.25	Indifferent
Thy + 1-Hexanol	6.4	0.25	1	1.25	Indifferent
Thy + Trans-2-hexenal	0.8	0.25	0.5	0.75	Indifferent
Cinn + Hexanal	0.2	2	0.016	2.016	Indifferent
Cinn + 1-Hexanol	0.2	2	0.031	2.031	Indifferent
Cinn + Trans-2-hexenal	0.2	2	0.125	2.125	Indifferent
*Eug + Thymol	0.8	0.25	0.5	0.75	Indifferent

*Eugenol added at 0.4 mg/mL

Table 4.1: MICs, FIC values and antimicrobial interactions for combinations containing 0.8 mg/mL of Essential Oil compounds against *E. coli* O157:H7 (cider)

Combination		FIC Indicies			
	MIC	FIC A	FIC B	ΣFIC	Interpretation
Eug + Hexanal	3.2	0.5	0.25	0.75	Indifferent
Eug + 1-Hexanol	1.6	0.5	0.25	0.75	Indifferent
Eug + Trans-2-hexenal	0.4	0.5	0.5	1.00	Indifferent
Thy + Hexanal	1.6	0.5	0.125	0.625	Indifferent
Thy + 1-Hexanol	1.6	0.5	0.25	0.75	Indifferent
Thy + Trans-2-hexenal	0.2	0.5	0.125	0.625	Indifferent
*Eug + Thymol	0.2	0.5	0.125	0.625	Indifferent

*Eugenol added at 0.8 mg/mL

Table 4.2: MICs, FIC values and antimicrobial interactions for combinations containing 0.8 mg/mL of Essential Oil compounds against *E. coli* O157:H7 (beef)

Combination		FIC Indicies			
	MIC	FIC A	FIC B	ΣFIC	Interpretation
Eug + Hexanal	3.2	0.5	0.25	0.75	Indifferent
Eug + 1-Hexanol	0.8	0.5	0.125	0.625	Indifferent
Eug + Trans-2-hexenal	0.4	0.5	0.125	0.625	Indifferent
Thy + Hexanal	1.6	0.5	0.125	0.625	Indifferent
Thy + 1-Hexanol	1.6	0.5	0.25	0.75	Indifferent
Thy + Trans-2-hexenal	0.4	0.5	0.25	0.75	Indifferent
*Eug + Thymol	0.2	0.5	0.125	0.625	Indifferent

*Eugenol added at 0.8 mg/mL

Table 4.2: MICs, FIC values and antimicrobial interactions for combinations containing 0.8 mg/mL of Essential Oil compounds against *E. coli* ATCC 25922

Combination		FIC Indicies			
	MIC	FIC A	FIC B	ΣFIC	Interpretation
Eug + Hexanal	3.2	0.5	0.25	0.75	Indifferent
Eug + 1-Hexanol	0.8	0.5	0.125	0.625	Indifferent
Eug + Trans-2-hexenal	0.4	0.5	0.125	0.625	Indifferent
Thy + Hexanal	0.8	0.5	0.063	0.563	Indifferent
Thy + 1-Hexanol	0.2	0.5	0.031	0.531	Indifferent
Thy + Trans-2-hexenal	0.2	0.5	0.125	0.625	Indifferent
*Eug + Thymol	0.2	0.5	0.125	0.625	Indifferent

*Eugenol added at 0.8 mg/mL

Table 5.1: MICs, FIC values and antimicrobial interactions for combinations containing 1.6 mg/mL of Essential Oil compounds against *E. coli* O157:H7 (cider)

Combination		FIC Indicies			
	MIC	FIC A	FIC B	ΣFIC	Interpretation
Eug + Hexanal	0.2	1	0.016	1.016	Indifferent
Eug + 1-Hexanol	0.2	1	0.031	1.031	Indifferent
Eug + Trans-2-hexenal	0.2	1	0.125	1.125	Indifferent
Thy + Hexanal	0.2	1	0.016	1.016	Indifferent
Thy + 1-Hexanol	0.4	1	0.063	1.063	Indifferent
Thy + Trans-2-hexenal	0.4	1	0.25	1.25	Indifferent

Table 5.2: MICs, FIC values and antimicrobial interactions for combinations containing 1.6 mg/mL of Essential Oil compounds against *E. coli* O157:H7 (beef)

Combination		FIC Indicies			
	MIC	FIC A	FIC B	ΣFIC	Interpretation
Eug + Hexanal	0.2	1	0.016	1.016	Indifferent
Eug + 1-Hexanol	0.2	1	0.031	1.031	Indifferent
Eug + Trans-2-hexenal	0.2	1	0.125	1.125	Indifferent
Thy + Hexanal	0.2	1	0.016	1.016	Indifferent
Thy + 1-Hexanol	0.4	1	0.063	1.063	Indifferent
Thy + Trans-2-hexenal	0.4	1	0.25	1.25	Indifferent

Table 5.3: MICs, FIC values and antimicrobial interactions for combinations containing 1.6 mg/mL of Essential Oil compounds against *E. coli* ATCC 25922

Combination		FIC Indicies			
	MIC	FIC A	FIC B	ΣFIC	Interpretation
Eug + Hexanal	0.2	1	0.016	1.016	Indifferent
Eug + 1-Hexanol	0.2	1	0.031	1.031	Indifferent
Eug + Trans-2-hexenal	0.2	1	0.125	1.125	Indifferent
Thy + Hexanal	0.2	1	0.016	1.016	Indifferent
Thy + 1-Hexanol	0.2	1	0.031	1.031	Indifferent
Thy + Trans-2-hexenal	0.2	1	0.125	1.125	Indifferent