

Antimicrobial Activity of Essential Oils and Their Components Against Lactic Acid Bacteria

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

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December 2013

Acknowledgments

I would first like to thank Dr. Faith Critzer, my major professor, for her willingness to accept me as a student. I could not have asked for a more supportive and knowledgeable advisor, both in and outside the laboratory setting. The encouragement I have received from her to develop and trust my own critical thinking skills has been invaluable, and I am profoundly grateful that she made the decision to mentor me. I would also like to thank Miranda Calderon, who not only gave me the opportunity to discover how much I love food science, but has continued to be a great friend and supporter.

Every member of the Food Science Department has my heartfelt thanks. Nothing could have been done without the aid I received from my instructors, fellow classmates, and friends. Their constant camaraderie, encouragement, and willingness to jump in and help when I've been overwhelmed with work have kept me going for the past year and half.

My family, especially my parents and parents-in-law, has been a constant source of aid, especially this summer. My mom has always been my most enthusiastic cheerleader, and I am thankful for all the times my dad is willing to listen when I am upset. I could not ask for a more loving and supportive family and am continually thankful for the blessing that they are.

My husband, Josh, has my heartfelt thanks as he is the individual who has sacrificed the most by supporting my decision to return to school. Despite the challenges we have had thrown at us over the past 4 years, his positive attitude, kind spirit, and patience have kept us going through it all. I am so thankful I have not had to go through this period of time without him.

Abstract

Efficacy of plant essential oils against spoilage lactic acid bacteria was examined using two different study methods with the goal of determining minimum inhibitory (MIC) and minimum lethal concentrations (MLC) of the essential oils. The initial study included the incorporation of the essential oils, or their major constituents, into agar to allow uniform dispersion of the substance throughout an agar surface. Individual cultures of nine lactic acid bacteria species (*Pediococcus acidilactici*, *Pediococcus damnosus*, *Lactobacillus fermentum*, *Lactobacillus fructivorans*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus plantarum*, *Leuconostoc mesenteroides*, and *Leuconostoc citrovorum*) were spot inoculated onto de Man, Rogosa, and Sharpe (MRS) agar containing the essential oils and incubated under ambient conditions (as determined independently per organism) for 4 days. The plates were examined for the evidence of growth, with the lowest concentration adequate to suppress growth being identified as the MIC. The most antimicrobial compounds were thymol and carvacrol, both of which had MICs of 0.1% w/v or v/v, respectively. Cinnamaldehyde, cinnamon bark oil, eugenol, thyme oil, and clove bud oil had MICs of 0.2% v/v. Cinnamic acid had an MIC of 0.5% w/v, while no MIC was determined for allyl isothiocyanate up to concentrations of 0.75% v/v.

Minimum lethal concentrations were examined using a broth dilution assay for 72 h. Carvacrol, thymol, eugenol, and cinnamaldehyde were dissolved in 95% ethanol to create a 50% stock solution. This solution was then added to MRS broth and mixed thoroughly. Individual cultures of *P. acidilactici*, *L. buchneri*, and *L. citrovorum* were added to the broth at concentrations of 4 log CFU/mL. The broth was spiral plated at 0 (immediately after exposure), 6, 12, 24, 48, and 72 h, and lethality was determined according to log reduction at these time

points. Carvacrol was lethal to against all species at 0.2% (v/v) to the limits of detection (0.95 CFU/mL, while thymol at 0.2% and 0.1% (w/v) prevented recovery of *L. buchneri* and *L. citrovorum*, respectively. Concentrations of cinnamaldehyde at 0.2% were lethal against *L. buchneri* and *L. citrovorum*, and at 0.25% (v/v) against *P. acidilactici*. Eugenol required concentrations in excess of 0.3% (v/v) for universal lethality.

Table of Contents

Introduction	1
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Chapter 1

Review of Literature.....	2
<i>Introduction to Essential Oils</i>	3
<i>Lactic Acid Bacteria</i>	4
<i>Phenolic Mechanisms</i>	5
<i>Isothiocyanates</i>	9
<i>Essential oils in food</i>	11
<i>Additives and combinations that enhance essential oil activity</i>	13
<i>Essential oils on meat, fish and poultry</i>	15
<i>Essential oils and packaging methods</i>	16
<i>Stimulatory effects of essential oils</i>	17
<i>Salad Dressings</i>	18
<i>Use of Essential Oil Combinations</i>	20
<i>List of References</i>	25

Chapter 2

Minimum inhibitory concentrations of essential oils	31
<i>Abstract</i>	32
<i>Introduction</i>	33
<i>Materials and Methods</i>	34
Agar preparation	34
Culture Inoculation	34
<i>Results and Discussion</i>	35
<i>Conclusions</i>	40
<i>List of References</i>	44

Chapter 3

Minimum lethal concentrations of essential oils	47
<i>Abstract</i>	48
<i>Introduction</i>	49
<i>Materials and Methods</i>	50
<i>Results and Discussion</i>	51

<i>Conclusions</i>	56
<i>Figures</i>	59
<i>List of References</i>	64
Conclusion.....	67
<i>List of References</i>	68
Vita	70

List of Tables

Table	Page
1.1 Comparison of individual component MICs and MICs when combined in pairs (adapted from Pei and others 2009).....	25
2.1 Incubation temperatures and atmospheres for ATCC sourced lactic acid bacteria.....	43
2.2 Minimum inhibitory concentrations of essential oils incorporated into MRS agar against lactic acid bacteria.....	44
3.1 Concentrations of carvacrol, cinnamaldehyde, eugenol and thymol examined against <i>P. acidilactici</i> , <i>L. buchneri</i> , and <i>L. citrovorum</i> . Values are v/v except thymol (w/v).....	59

List of Figures

Figure	Page
3.1 Response of a) <i>L. citrovorum</i> , b) <i>P. acidilactici</i> , and c) <i>L. buchneri</i> in MRS broth with varying % (w/v) thymol over 72 h.....	61
3.2 Response of a) <i>L. citrovorum</i> , b) <i>P. acidilactici</i> , and c) <i>L. buchneri</i> in MRS broth with varying % (v/v) cinnamaldehyde over 72 h	62
3.3 Response of a) <i>L. citrovorum</i> , b) <i>P. acidilactici</i> , and c) <i>L. buchneri</i> in MRS broth with varying % (v/v) carvacrol over 72 h	63
3.4 Response of a) <i>L. citrovorum</i> , b) <i>P. acidilactici</i> , and c) <i>L. buchneri</i> in MRS broth with varying % (v/v) eugenol over 72 h.....	64

Introduction

Many herbs and spices have been recognized for their preservative or medicinal properties for millennia. Essential oils present in plant matter have been attributed as principal sources of antimicrobial activity, which has been illustrated against bacteria and fungi (Shelef 1983; Zaika 1988). The understanding of these oil's antimicrobial mechanisms of action has led to increased interest in the specific compounds responsible for this activity, specifically those phenolic in nature (Davidson and others 2005).

Consumers are becoming increasingly aware and concerned about the use of food additives that are synthetically derived or altered. This is driving research to examine essential oils for their use as food preservatives in lieu of traditionally used synthetic compounds. (Fратиanni and others). The aim of this work was to determine what concentrations of several oils or their compounds are effective against lactic acid bacteria, which are responsible for spoilage of acidic foods.

Chapter 1

Review of Literature

Introduction to Essential Oils

Plant derived antimicrobial agents are becoming increasingly popular as food, cosmetic and pharmaceutical preservatives. Essential oils isolated from widely used spices and their plant sources contain compounds known for their antimicrobial activity (Dorman and Deans 2000). Oils containing specific phenols are generally the most effective for antimicrobial application, including those from clove, oregano, and thyme (Deans and others 1995; Holley and Patel 2005; Pyrgotou and others 2010). Many of these oils are most effective against Gram-positive organisms, though some are more antagonistic towards Gram-negative species. (Ouattara and others 1997; Skandamis and others 2002; Holley and Patel 2005). Additional derivatives of these oils may be non-phenolic in nature but still exhibit profound antimicrobial activity, such as allyl isothiocyanate, which may be used against Gram-negative bacteria and fungi (Kyung and Fleming 1997; Lin and others 2000).

Antimicrobials of particular interest in this review include thyme oil, thymol, clove bud oil, carvacrol, cinnamon bark oil, cinnamic acid, cinnamaldehyde, eugenol, and allyl isothiocyanate. Eugenol is the major antimicrobial in clove bud oil, comprising approximately 70-90% of the oil. The remainder of the oil contains compounds such as eugenol acetate (0-15%) and beta caryophyllene (5-15%) (Deans and others 1995; Pino and others 2001; Holley and Patel 2005; Alma and others 2007). Oregano oil is one of the most common sources of carvacrol, which makes up 60-70% of the oil. The antimicrobial activity of thyme oil is most readily attributed to thymol, which is generally present in concentrations around 45% (Bagamboula and others 2004). However, thyme oil may also contain large quantities of carvacrol, which can range from 33% in leaf oils to about 61% in stem oil, depending on

geographic location, time of harvest, or extraction method of the oil (Ultee and others 2002; Belaqziz and others 2013). Cinnamaldehyde is the major antimicrobial component in cinnamon bark oil. Cinnamic acid also participates in the antimicrobial activity of cinnamon oils as well as cloves (Roller and Seedhar 2002; Holley and Patel 2005; Matan and others 2006).

Lactic Acid Bacteria

The term lactic acid bacteria (LAB) encompasses a vast community of Gram-positive, non-spore forming, fermentative microorganisms that are desired in food fermentations but are considered spoilage agents by other constituents of the food industry. End products of their fermentation result in classification into two primary classes: homofermentative and heterofermentative. Under unrestricted ambient growth conditions, homofermentative LAB convert glucose to > 90% lactic acid. Heterofermentative LAB fermentation, on the other hand, results in a blend of end products predominantly comprising lactic acid but that may also include carbon dioxide, ethanol and acetic acid.

Pediococci are homofermentative, acid tolerant bacteria commonly isolated from foods. *P. acidilactici* is commonly required for preservation and flavoring of fermented sausages due to their production of lactic acid. Members of the heterofermentative *Leuconostoc* genus are widely exploited for production of fermented vegetable products such as kimchi and sauerkraut. *Leuconostoc mesenteroides* is also a commonly used culture in dairy fermentations. Lactobacilli are the most abundant and often most acid tolerant of the LAB, and aid in the production of fermented vegetable products, yogurts and cheeses, and breads (Axelsson and others 2004).

As highly as LAB are valued within the food industry, they also account for significant spoilage and monetary loss. Spoilage due to *P. damnosus* has long been the bane of beer producers as its presence results in a buttery off-flavor in finished products (Axelsson and others 2004). *L. fructivorans*, *L. plantarum*, *L. brevis*, and *P. acidilactici* have been implicated in the spoilage of mayonnaise and/or salad dressings (Kurtzman and others 1971; Waite and others 2009). And LAB are the most commonly associated spoilage microorganisms with whole or ground meat, poultry, and fish products (Holley and Patel 2005; Zinoviadou and others 2009; Fratianni and others 2010; Pyrgotou and others 2010).

Phenolic Mechanisms

It is commonly believed that the antibacterial activity of phenolic compounds within essential oils, namely carvacrol, thymol and eugenol, is attributed to the hydroxyl group contained on their structures. Lipid solubility of these phenolics allows interaction between the antimicrobial and the fatty acid dense microbial cell membrane (Blaszyk and Holley 1998; Helander and others 1998). While aqueous insolubility is a barrier to overcome in the utilization of oils in food preservation, the lipophilic nature of phenolic compounds and essential oil components is essential for the activity of the antimicrobials.

Theories describing the antimicrobial mechanism of phenolic compounds abound as the pathway of action is unclear and tends to vary by essential oil or microorganism. It is widely held that Gram-positive bacteria experience greater sensitivity to the antimicrobial action of essential oils than Gram-negative species. Gram-positive lactic acid bacteria are a notable exception. Gram-negative bacteria may attribute their increased resistance to the lipopolysaccharide (LPS) rich outer membrane, creating a highly restrictive barrier against large

molecular particles, including toxins and antibiotics (Nikaido 1994). This LPS layer includes several features that aid to the rigid structure of Gram-negative cells. LPS is comprised of saturated fatty acid chains, packing together to form a highly rigid lipid bilayer in contrast to the more fluid, unsaturated fatty acid chains found in membranes of other species. Each LPS molecule also contains a larger quantity of these hydrocarbon chains than glycerophospholipids, further increasing structural rigidity. Gram-positive bacteria lack this extra, more complex membrane, and instead are surrounded by a thick peptidoglycan wall. This peptidoglycan layer strengthens the wall structurally, but is not dense enough to exclude small antimicrobial particles which can permeate the layer and access the cellular membrane (Nikaido 1994; Helander and others 1998; Holley and Patel 2005; Zinoviadou and others 2009; Hyldgaard and others 2012).

Although the cytoplasmic membrane is thought by some to be the site of the vast majority of antimicrobial action, Gram-negative bacteria are not immune to the effects of essential oils, despite their additional outer membrane. One study reported dissolution of cellular membranes of both *Escherichia coli* and *Salmonella* Typhimurium after treatment with either thymol or carvacrol (Helander and others 1998; Holley and Patel 2005). This may be due in part to the presence of porin proteins embedded along the outer membrane of Gram-negative species which aid in the acquisition of nutrients by the cell. These proteins allow the transport of macromolecules and are generally able to exclude toxins and antibiotics from entering the cell. However, some hydrophilic agents and substituted phenolic compounds are able to penetrate the outer membrane via these porin proteins, gaining access to the periplasmic space and cytoplasmic membrane where the targets of their antimicrobial activity

exist. The additional outer membrane does not, therefore, confer absolute immunity to Gram-negative organisms against essential oils (Nikaido 1994).

In both Gram-positive and Gram-negative species, the most readily accepted theory describing cellular death is that it occurs after structural integrity of the cellular membrane is compromised, at which point membrane permeability loses much of its discriminatory nature (Holley and Patel 2005). Carvacrol and thymol share similar structures and are believed to have very similar modes of action. Phenolic terpenoids attribute their antimicrobial activity in part to a hydroxyl group, which in the case of carvacrol and thymol is located on their benzene ring. Carvacrol predominantly acts by incorporating itself into the cellular membrane, increasing fluidity and in turn permeability of the membrane. This results in cellular stress through the leakage of cellular contents, potassium ions, and ATP. Additionally, carvacrol has been described as a proton exchanger, dissociating by relinquishing its proton upon access to the cytoplasm, then taking a cation while exiting the cell (Ultee and others 2002). This action disrupts metabolism and eventually leads to death. Thymol is thought to disrupt the structural and functional integrity of the inner and outer membranes in a similar manner. Both phenolic terpenoids are believed to interfere with proteins that comprise the membrane as well as protein targets within the cell (Ultee and others 2002; Hyldgaard and others 2012).

The most obvious consequence of thymol or carvacrol induced membrane damage is the efflux of cellular components. Loss of cellular content is generally preceded by a loss of potassium ions through the membrane (Holley and Patel 2005). Disruption of the cellular membrane by carvacrol and thymol was demonstrated by their ability to decrease intercellular

levels of ATP in *E. coli* while increasing the levels of ATP found outside of the cell beyond what was normal (Helander and others 1998).

Eugenol and cinnamaldehyde are phenylpropenes synthesized from phenylalanine and contain a six-carbon phenol group. Eugenol is believed to have similar bactericidal activity to thymol and carvacrol, incorporating into the cellular membrane and altering surface and structural proteins. Both compounds are believed to inhibit cellular metabolism and potentially serve as ATPase inhibitors, while cinnamaldehyde may also act through membrane disruption (Gill and Holley 2004; Hyldgaard 2012). Gill and Holley determined that energy generation was significantly reduced in both *Listeria monocytogenes* and *Lactobacillus sake* upon treatment with eugenol and cinnamaldehyde. Further theories describing the potential antibacterial mechanism of essential oils include the inhibition of enzymatic production or activity required for energy generation, disruption in the generation process of ATP, or depletion of ATP already present within the cell (Holley and Patel 2005; Matan and others 2006; Helander and others 1998).

Lactic acid bacteria are understood to be more resistant to the cytotoxic effects of essential oils. Rodríguez and others (2009) suggest one likely reason for LAB resistance to phenolics is because LAB are present and grow on phenol containing plants, and therefore have adapted in order to successfully colonize such antagonistic substrates. Degradation capabilities of phenolic compounds by LAB have also been described, although work on the subject is limited (Rodríguez and others 2009).

Isothiocyanates

Isothiocyanates are natural antimicrobial compounds commonly isolated from plants in the Cruciferae family, which include horseradish and mustard plants. They account for the intense pungent taste of plants within this family. These sulfur containing compounds, the most prevalent being allyl isothiocyanate (AIT), are released upon damage to plant tissues. AIT is thought to interfere with protein structure when present at concentrations that inhibit most microbial growth; free amino groups and disulfide bonds are the predominant protein targets of isothiocyanates (Lin and others 2000; Delaquis and Sholberg 1997; Kawakishi and Kaneko 1987). Luciano and Holley (2009) found that concentrations of 10 to 100 µg/L were sufficient to inhibit the metabolic enzymes present in *E. coli*, including thioredoxin reductase, which when disturbed impairs cellular capacity to maintain an appropriate oxidation state, and acetate kinase, which is responsible in part for the synthesis of acetyl-CoA (Ankri and Mirelman 1999) and crucial for glycolysis. In yeasts also it appears that reduction of enzymatic activity required for metabolism and degradation of disulfide bonds in non-specific enzymatic targets is significant to the antimicrobial action of AIT. Activity against multiple enzymatic targets suggests AIT may use several mechanisms of action to inhibit microbial growth. It has also been suggested that aerobic microorganisms are more sensitive to AIT, indicating that enzymes required for aerobic metabolism are manipulated by isothiocyanate action. However, as some facultative anaerobes also demonstrate suppressed growth or survival upon exposure to AIT, the proposed is unlikely a predominant mechanism (Delaquis and Sholberg 1997). The greatest activity of AIT appears to be against Gram-negative bacteria and some yeasts and molds. Lactic acid bacteria are particularly resistant to AIT-rich essential oils and as of yet, no clear

explanation exists for this tolerance. AIT has been illustrated to effect sensitive bacteria in both stationary and exponential growth phases, which is significant considering most organisms in food matrices are subject to other preservation methods and additives, maintaining suppressed metabolic activity for microbial contaminants. A definitive mechanism of antimicrobial action may need to be understood before an answer to why LAB are so resistant to isothiocyanates is apparent, but reasons are likely similar to those proposed for other essential oil components (Lin and others 2000).

LAB resistance was demonstrated by Ward and others who used a horseradish distillate containing 90% allyl isothiocyanate. Results demonstrated that growth of common foodborne pathogens, including *L. monocytogenes*, *S. Typhimurium*, *E. coli* O157:H7, and *Staphylococcus aureus* was completely inhibited when grown on agar incubated under 4,000 nL horseradish distillate/liter air. By contrast, a 20,000 nL/liter concentration of distillate was required for complete inhibition of *Lactobacillus sake* (Ward and others 1998; Holley and Patel 2005).

AIT and other sulfur containing compounds were evaluated in a liquid medium against a series of Gram-positive and Gram-negative bacteria in addition to several yeast species. All LAB required concentrations of 300-500 ppm AIT for inhibition to occur. MICs for non-LAB species ranged from 50-300 ppm. *E. coli* was the most susceptible Gram-negative bacteria with an MIC of 50 ppm, while *Enterobacter aerogenes* was the least susceptible Gram-negative requiring 300 ppm for inhibition. The yeasts, which included *Saccharomyces* and *Pichia* species, were highly sensitive as inhibition occurred within a range of 1-4 ppm AIT (Kyung and Fleming 1997; Ward and others 1998).

Rapid degradation of AIT is a significant hurdle to overcome prior to application to food preservation. Water, either in vapor or liquid forms, quickly decomposes AIT to produce sulfur, allyl dithiocarbamate, diallyl tetra-sulfide and diallyl penta-sulfide, all with diminished antimicrobial activity. This degradation is further exacerbated by high temperatures and pH (Luciano and Holley 2009; Ward and others 1998; Kawakishi and Kaneko 1987).

Microencapsulation has been explored as a means of adding AIT to suitable food products to reduced degradation prior to target contact while also limiting changes to flavor profiles (Ko and others 2012).

Essential oils in food

It is well accepted that in the majority of instances, greater concentrations of essential oils are required for inhibition of microbial growth in food products than is evident in liquid or agar mediums (Zaika 1988). Intrinsic characteristics of food matrixes, including protein, fat, water activity, or pH all influence the efficiency of antimicrobial additives.

For example, fresh meats, poultry, and fish not only receive minimal processing prior to marketing, but their high lipid and protein content decrease the activity of phenolics. As essential oils are hydrophobic in nature, when applied to the surface of a meat product, the oils often aggregate into the lipid portion of the meat, thus leaving the microbially contaminated surface untreated. They may also be chemically altered, losing antimicrobial capabilities as they come in contact with intrinsic meat components (Quintavalla and Vincini 2002). These products also have pH values near neutral and high water activities, providing an ideal substrate on which microorganisms can flourish (Davidson and others 2013). For these reasons, the use of oil

incorporated films is becoming an increasingly popular area of study (Zivanovic and others 2005; Gómez-Estaca and others 2010; Emiroğlu and others 2010).

Fruit and vegetable products are likely candidates for essential oil application, due in large part to the virtual absence of proteins and lipids in the majority of herbaceous species. However, carbohydrates have demonstrated interference with essential oils within a food system, so further research in this area is still crucial to evaluate their interactions (Davidson and others 2013). Additionally, the surface of most produce is covered with crevices into which microorganisms are able to insert, making uniform application of essential oils to plant surfaces difficult. In juice form, the potential is much more promising as greater homogeneity of the product is achieved, and the majority of fruit and even some vegetable juice cocktails tend to have high acidities. Low pH in juices not only selects for acid tolerant microbial species, but has also been shown to enhance to the antimicrobial properties of many essential oils. For example, Gutierrez and others (2008) found that essential oils applied in a liquid medium at pH 5 increased lag phase and reduced growth of *L. monocytogenes* more effectively than when administered in broth of pH 6 or 7. Additionally, juices are ideal substrates to which essential oils may be incorporated because many plant sources of essential oils are traditionally used with commonly consumed juice beverages, such as cinnamon with apple juice or cider.

The use of spices and spice blends in many food products that already contain high levels of similar seasonings has also been examined. Ideally, reengineering of food formulations that contain high levels of spices such as oregano or thyme seasonings could take advantage of the already present sources of essential oils. Unfortunately, some work has shown that spices stimulate the growth and acid production of LAB (Shelef 1983). In a study by Nes and Skjelkvale

(1982), seasoned dry sausages were inoculated with three strains of *Lactobacillus plantarum*.

Acid production and glucose consumption within sausages was augmented in those utilizing a natural spice blend. However, seasoning with oleoresins derived from the same spices resulted in no change in acid production, indicating that oleoresins had no effect on cellular growth

Spices themselves are not likely suitable replacements for concentrated volatile oils. The quantity of seasoning used is typically far too small to contain sufficient quantities of essential oil to confer antimicrobial activity. Effective dosages are likely only realistic for highly seasoned foods like fermented meat or vegetable products, which are coincidentally foods in which viable cultures are desirable. Bacterial contamination of spices and seasonings is a common occurrence as well; as the majority of essential oils are contained within the plant matter and not on the surface, contaminants may be present on the surface of leaves or stems in spice blends and serve as an inadvertent source of contamination (Dorman and Deans 2000). Utilizing oil components instead of spice blends would circumvent these issues.

Additives and combinations that enhance essential oil activity

The insoluble nature of essential oils makes delivery of phenolic compounds to the liquid fraction of food products difficult (Blaszyk and Holley 1998). When essential oils are added to a food matrix, they typically partition off into the lipid portion of the food, leaving the aqueous segment devoid of phenolics. While viable microorganisms can be present in lipid dense zones, growth occurs within the aqueous regions; in a food product, it is feasible that microbial spoilage occurs unchecked despite a high essential oil concentration as the microorganisms are unlikely to come into contact with the lipid-bound phenolic compounds. Adding essential oils to high fat foods generally results in high phenol concentrations in the lipid

portion with little present in the aqueous segment of a food. Attempts to solubilize essential oils have been generally unsuccessful, often with the solubilizing agent interfering with the antimicrobial activity of the oils (Blaszyk and Holley 1998).

Inclusion of other additives or processes, however, has been highly successful in improving the activity and efficiency of essential oils within food products. The addition of humectants, such as salt and sugar, has been shown to improve the antimicrobial action of cinnamon or clove against mold (Shelef 1983). Moleyar and Narasimham (1992) found that 500 µg/mL cinnamaldehyde was required for inhibition of the foodborne organisms at 30°C, but at 20°C and 25°C the MIC dropped to 400 µg/mL (Blaszyk and Holley 1998).

In a study by Blaszyk and Holley, eugenol was combined with monolaurin, a GRAS status additive, and sodium citrate for testing against two *Lactobacillus* species and *Leuconostoc mesenteroides* for four days in MRS broth. Monolaurin also exhibits antimicrobial activity against Gram-positive bacteria as well as fungi. When evaluated alone, *L. mesenteroides* proved to be more sensitive than the lactobacilli to the antimicrobial activity of eugenol, especially at a lower temperature of 7°C. Both *Lactobacillus* species were relatively unhindered by the eugenol, although inhibition did appear to increase for one of the species at 7°C. The authors concluded that the most effective inhibition came with the use of all three additives simultaneously; a combination of eugenol at 1000 ppm in conjunction with 0.4% sodium citrate and 250 ppm monolaurin completely inhibited growth of *L. curvatus*. Additionally, sodium citrate at 0.2 or 0.4% significantly inhibited *L. sake* growth with 250 ppm monolaurin at either 500 or 1000 ppm eugenol, while *L. mesenteroides* growth was greatly hindered with 500 ppm eugenol and 100 ppm monolaurin (Blaszyk and Holley 1998). By understanding and

manipulating additive or synergistic relationships among essential oils and other food additives, it is possible that reduced concentrations may be necessary for food preservation, resulting in fewer negative sensory changes occurring in food products.

Pyrgotou and others (2010) examined the effect of oregano oil (carvacrol 57.7% and thymol 2.8%) on salted rainbow trout under modified atmosphere conditions. Oil was added to salted fish at 0.2% and 0.4% (w/v), then placed under modified atmosphere conditions (45% CO₂/5% O₂/50% N₂) and stored for 21 days under refrigerated conditions. The study found that LAB were significantly inhibited by the essential oil/salt/MAP conditions with a log reduction of approximately 2.6 CFU/g at both concentrations. Unfortunately, a trained sensory panel scored the odor of the treated, cooked samples as unacceptable because of off-odors.

Essential oils on meat, fish and poultry

The chemical composition and physical characteristics of meat makes it a suitable environment for bacterial growth, which includes species such as LAB, *Pseudomonas*, and a host of foodborne pathogens. LAB spoilage in meats is a relevant problem as they are facultative anaerobes that can grow and continue to spoil foods under chilled conditions (Fратиanni and others 2010; Pyrgotou and others 2010). It has been shown that the more complex a food matrix is, especially in items like meats that are high in fat and protein, the less effective essential oil activity is against resident bacteria (Shelef 1983; Zinoviadou and others 2009; Gill and Holley 2005).

Fратиanni and others (2010) treated fresh strips of chicken breast meat with an agar slurry solution containing 0.5% thyme and balm essential oils for 15 min. Samples were stored for 21 days at 4°C. Thyme was incredibly effective at controlling LAB growth for the duration of

the study; 21 day counts were only 0.8×10^3 CFU/mL, which was consistent throughout the entire 3 weeks. The antibacterial effect of balm oil was much less evident until the day 21, with balm oil closely matching the untreated control up until that point. *Salmonella* on the treated chicken was very sensitive to the activity of balm oil, while thyme oil very effectively reduced growth of *E. coli*.

Essential oils and packaging methods

The use of plant essential oils is predominantly restricted by sensory acceptability as essential oils have 'Generally Recognized as Safe' (GRAS) status (Lambert and others 2001; Roller and others 2002; Zinoviadou and others 2009; Rodríguez and others 2009). To circumvent this issue, which may include changes in flavor, astringency or appearance, packaging materials and films may be utilized to aid in the impartation of essential oil antimicrobial benefits without negative flavor profile modification (Zinoviadou and others 2009). A study by Zinoviadou and others (2009) put varied levels of oregano oil (which is naturally high in carvacrol) into whey protein films prior to covering beef cuts. In a 12 day trial, lactic acid bacteria on product covered by films containing no oregano oil increased nearly 5 log CFU/cm², while organisms on beef covered by films containing 1.5% oregano oil increased less than 1 log CFU/cm² (Zinoviadou and others 2009).

Essential oils have also been considered for potential application in modified atmosphere conditions. Modified atmosphere packaged (MAP) and vacuum-packaged (VP) products are often selective for *Lactobacillus* or *Leuconostoc* species, making these organisms the primary source of spoilage in such conditions (Holley and Patel 2005). Matan and others incubated a broad spectrum of yeast and bacteria under the volatilized gas phases of cinnamon

and clove oils. The oils, at a 1:1 ratio, were effective against all microorganisms, especially *Pediococcus halophilus* (recently reclassified as *Tetragenococcus halophilus*). The inhibitory period increased when oxygen was reduced, and further increased when CO₂ levels rose. The most significant inhibition was evident when low oxygen was used in conjunction with high CO₂ levels. In fact, under <0.05% oxygen and 40% CO₂, *P. halophilus* growth was delayed for 38 days on MRS agar (Matan and others 2006; Axelsson and others 2004).

Work with oregano oil in modified atmosphere packaged and vacuum-packaged food showed that organism selectivity as well as essential oil inhibition occurred (Skandamis and others 2002). Oregano oil was found to have less inhibitory potential in aerobically packaged products, as well as in MAP or VP foods with highly oxygen permeable films. However, lactic acid bacteria along with *Salmonella* Typhimurium were found to be highly susceptible to an oil concentration of 0.8% w/v regardless of atmosphere or film permeability (Skandamis and others 2002).

Stimulatory effects of essential oils

A team of researchers at Danisco studied the possible substitution of essential oils or their components for avilamycin, an antimicrobial growth promoter the use of which, like all such promoters, is prohibited by the European Union. Use of this and other antimicrobial growth promoters have been used in poultry and meat animals for decades to accelerate body mass accumulation (Lin and others 2013). The intent of essential oil use in this study was to find an alternative to antibiotic growth promoters in effort to limit the increase of antibiotic resistant bacteria species that may be due in part to the increased use of these antibiotics.

Ideally, essential oils would target pathogenic bacteria without harming beneficial bacteria and could potentially slow the spread antibiotic resistance (Ouwehand and others 2010).

Five LAB species with reported high sensitivity to avilamycin were tested against 13 essential oils or extracts thereof, including carvacrol, cinnamaldehyde, eugenol, thymol, and thyme oil. *Bifidobacterium breve* was found not only to be resistant to the majority of the essential oils, but exhibited pronounced stimulatory effects when exposed to eugenol. Similarly, *Lactobacillus fermentum* growth was stimulated by carvacrol, cinnamaldehyde, thymol, and thyme oil, although it was found to be sensitive to several of the essential oils. *Lactobacillus reuteri* exhibited sensitivity only when exposed to the highest concentration of carvacrol and thymol, while *Bifidobacterium longum* was sensitive only to the highest concentrations of the oils examined (500 mg/l) Only *Bifidobacterium animalis* ssp. *lactis* was sensitive to the essential oils at all concentrations (5, 50, and 500 mg/l) (Ouwehand and others 2010).

Salad Dressings

Mayonnaise and salad dressings are exempt from acidified food regulations in United States due in large part to their extensive history as safe food products and intrinsic characteristics that prevent them from being environments conducive to microbial growth. The acidity of these products, which must remain below pH of 4.6 to prevent growth of *Clostridium botulinum*, sufficiently prevents survival of other foodborne pathogens that may be introduced during manufacture or post-production of these products (Smith and Stratton 2006). Outbreaks do occur in parts of the world where homemade products are commonly made with raw eggs and lower acidities, but in commercially available salad dressings in the US, this problem is virtually non-existent. The water portion of commercial dressing products is predominantly

acetic acid, with lactic or citric acids also being present in some instances. Large quantities of additional ingredients also have high acidities, including lemon juice and fermented dairy products (Smittle 2000; Radford and others 1991).

Microbial spoilage of these products, however, is of commercial interest as lactic acid bacteria are capable of surviving and growing under low pH conditions. *Lactobacilli* are the most acid tolerant of the LAB, the majority of which are able to ferment sugars at pH of 4.4 and below (Axelsson and others 2004). Kurtzman and others (1971) reported the possibility of utilizing sweeteners that are not readily fermented by LAB to reduce the likelihood of spoilage due to these organisms. Many LAB, including *L. fructivorans* ferment glucose readily but can also utilize sucrose, albeit at suppressed rates. While sucrose monoesters were found to have no effect on the growth or lag time of *L. fructivorans* in salad dressing, a significant reduction was evident in the final colony counts (Yang and others 2003). While sucrose esters do not appear to have promise in terms of LAB inhibition, the utilization of non-carbohydrate containing artificial sweeteners has potential as a means of limiting the spoilage capacity of these acid tolerant bacteria.

Plant oils in homemade mayonnaise were examined for the influence they may contribute to the elimination of *Salmonella* Enteritidis. The study found that a recipe containing extra virgin olive oil resulted in faster *Salmonella* death rates when compared to blended olive or sunflower oils. They determined that this increased bactericidal activity was due not only to the lower acidity of the oil (0.5% oleic acid), but also to the more complex phenolic profile of the olive oils, particularly that originating from Greece. The Greek oils contained a wider range of phenols including tyrosol, oleuropein, caffeic acid, and vanillic acid (Radford and others

1991). Manufacturers interested in incorporating essential oils into their dressing and mayonnaise products should first explore the possibility of using ingredients that may contain high levels of phenolics. Using phenols that are present in already incorporated ingredients may produce a product that is more organoleptically acceptable than would occur with the addition of extraneous essential oils.

Use of Essential Oil Combinations

Increasingly apparent is the fact that individual essential oils or their compounds will be not be sufficient to satisfactorily inhibit the growth of spoilage bacteria while still producing an acceptable food product. MICs for oils range from 1-5%, which due to the strong scent and flavor characteristics of the majority of essential oils, makes these preservatives difficult to disguise. Combinations of essential oils or seasonings with high essential oil fractions may be the most effective oil based method for a myriad of food products, allowing for concentrations less than individual oil MICs. This method results in more subtle flavor and odor profiles as opposed to highly concentrated, more objectionable flavors (Moleyar and Narasimham 1992). Resistance to sub-lethal concentrations of whole plant organ oils (such as clove bud oil) is less likely to occur than when single essential oil components (for example, eugenol) are used because of the diversity of antimicrobial agents within essential oils (Shelef 1983; Pei and others 2009; Angienda and Hill 2012). Antimicrobial agents other than the phenolics do exist in whole essential oils, but are often not worth the difficulty to extract due to their inferior activity and quantity within the plant material. However, when allowed to remain in the oils alongside highly active phenolic compounds, their activity may be indispensable to the antibacterial activity of the essential oils as a whole (Holley and Patel 2005).

One such example of such cooperation is p-cymene, a precursor to carvacrol that is believed to work synergistically with carvacrol as an antimicrobial agent. While exhibiting insufficient antimicrobial activity independently, it has been shown to cause greater expansion and swelling of the bacterial membrane than what carvacrol alone is capable. This expansion results in leakage of ions from the cell; while incapable of cellular destruction single-handedly, this destabilization may increase cellular exposure and susceptibility to the cation exchanger carvacrol, leading to ATP depletion and cellular death (Ultee and others 2002; Hyldegard and others 2012).

As aldehydes and phenols are credited as conferring the majority of the antimicrobial properties of essential oils, synergistic mechanisms have been observed when combining these highly active compounds. Pei and others (2009) found the MICs of cinnamaldehyde, eugenol, carvacrol, and thymol to be significantly reduced when pairs of these agents were administered against *E. coli* (see table 1.1).

Specifically, the MICs for eugenol and cinnamaldehyde were reduced from 1600 and 400 mg/L, respectively, to 400 and 100 mg/L (Pei and others 2009). This synergism is potentially attributable to the differing protein target sites of eugenol and cinnamaldehyde; eugenol inhibits enzymatic activity while cinnamaldehyde interferes with the action of amino acid decarboxylases (Wendakoon and Sakaguchi 1993). Eugenol and thymol are believed to work synergistically with thymol disrupting and disintegrating the outer membrane of Gram-negative species and allowing eugenol access to the cytoplasm to disrupt enzymes. The MIC for thymol decreases from 400 to 100 mg/L when combined with 400 mg/L of eugenol. Independently, eugenol required 1600 mg/L for inhibition (Pei and others 2009). Supporting work by Moleyar

and Narasimham (1992) found that combining cinnamaldehyde at 250 µg/mL with eugenol at 500 µg/mL was effective at inhibiting bacterial growth for thirty days while individually at similar concentrations the compounds were ineffective.

The interest in using individual components of essential oils for use as preservatives is due to several distinct advantages. One such benefit is the standardization of commercial oil constituents by uniform distillation (Holley and Patel 2005). Composition of natural oils varies widely based on a multitude of factors, including plant organ of origin, geographical location and time of harvest. The eugenol content of clove bud oil, for example, is approximately 70%, while clove leaf oil contains 78.1% eugenol. Carvacrol has also been found to range from virtually non-existent to comprising 4/5 of some oregano oils (Pino and others 2001; Gómez-Estaca and others 2010).

Essential oils and their components may provide a solution for the growing demand of natural preservative methods that require minimal processing. Even more exciting is the fact that these are already approved for use in foods, meaning that once the issues of application and concentration are resolved, food producers can almost immediately begin using essential oils in their food formulations. Future work must comprise studies that determine which essential oils are most appropriate for preservation, what concentrations and delivery methods are most appropriate and effective, and what foods or packaging methods are most ideal for reformulation or reengineering to take advantage of the antimicrobial activity of essential oils.

Tables

Table 1.1. Comparison of individual component MICs and MICs when combined with one other essential oil compound (adapted from Pei and others 2009).

	Essential Oil Component			
	Carvacrol	Cinnamaldehyde	Eugenol	Thymol
Individual MIC (mg/L)	400	400	1600	400
Combined MIC (mg/L)	100 (w/eugenol)	100 (w/eugenol)	400 (w/ thymol or cinnamaldehyde)	100 (w/eugenol or cinnamaldehyde)

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Chapter 2

Minimum inhibitory concentrations of essential oils

Abstract

The essential oils of clove bud, cinnamon bark and thyme, as well as some of the individual compounds that comprise these essential oils, including allyl isothiocyanate, carvacrol, cinnamaldehyde, cinnamic acid, eugenol, and thymol were assessed for antimicrobial activity against nine lactic acid bacteria species. Carvacrol and thymol were the most inhibitory with MICs of 0.1% (v/v and w/v, respectively). Cinnamaldehyde, cinnamon bark oil, clove bud oil, eugenol, and thyme oil were moderately inhibitive (MICs = 0.2% v/v), while cinnamic acid (w/v) required a concentration of 0.5% to inhibit microbial growth. The MIC for allyl isothiocyanate was not determined as the effective dose exceeded the concentration tested within this study (0.75% v/v). Results indicate that individual essential oil components and whole essential oils may be suitable for their antimicrobial properties in food. However, the concentrations required for inhibition will need to be evaluated for incorporation as food preservatives because of their organoleptic impact.

Keywords: *Essential oils, minimum inhibitory concentration, lactic acid bacteria*

Practical Application: Essential oils are plant derived compounds that consumers may view as more natural and safer alternatives to synthetic preservatives. These oils have been demonstrated to have antibacterial and antifungal activity within food systems, and may be ideal additives to food formulations in order to meet the growing consumer demand for organic and natural food products.

Introduction

The antimicrobial activity of plants and their essential oils has been recognized and exploited for thousands of years. Plants commonly incorporated as flavorings into cuisines worldwide, such as cinnamon, sage, and thyme have been and continue to be explored as potential food preservatives. Efficacy has been observed against Gram-positive and negative bacteria as well as fungi (Shelef 1983; Zaika 1988; Hammer and others 1999). More recently, emphasis has been directed towards the extraction of essential oils from plant matter and even further to concentrated antimicrobial components, namely phenolic compounds, for use as antimicrobial food preservatives (Davidson and others 2005).

Despite a long history of safe use in food products compounded along with assurance from regulatory authorities regarding the safety of commercially employed synthetic preservatives, increasing consumer trend towards perceived natural additives is encouraging food companies to consider alternative preservative methods and components. Plant sources are commonly examined as many species utilize antimicrobial agents and toxins to combat microbial invasion or consumption by invertebrate, avian, and mammalian predators (Ames and others 1990; Sofos and others 1998).

Applications of plant essential oils have been studied extensively against foodborne pathogens, but a significant void exists in research with spoilage microorganisms. General consensus suggests that Gram-positive organisms are typically more sensitive than Gram-negative organisms to essential oils, with some exceptions. Lactic acid bacteria (LAB), for example, seem less susceptible to the inhibitory action of essential oils than most bacteria, and

in fact have been shown to have stimulated growth in the presence of some oils (Shelef 1983; Zaika and others 1983; Kivanç and others 1991; Holley and Patel 2005; Angienda and Hill 2012).

The object of this study was to determine the minimum inhibitory concentrations (MIC) of the selected essential oils or essential oil components against a collection of lactic acid bacteria that are commonly associated with spoilage of acid foods at ambient temperature and atmospheric growth conditions.

Materials and Methods

Agar preparation

De Man, Rogosa, Sharpe agar (MRS; Oxoid, Basingstoke, UK or Difco, Sparks, MD) was sterilized (121°C for 15 min) and allowed to cool to approximately 60°C. Following cooling, allyl isothiocyanate, carvacrol, cinnamon bark oil, trans-cinnamaldehyde, clove bud oil, eugenol, trans-cinnamic acid, thymol, and thyme oil (Sigma, St. Louis, MO) were incorporated into the medium. The mixture was stirred rapidly and poured into petri plates before separation of the oil could occur.

Culture Inoculation

Cultures of the following lactic acid bacteria were grown in 10 mL tubes of MRS broth to stationary phase (approximately 10^9 or 10^8 CFU/mL): *Pediococcus acidilactici* ATCC 8042, *Pediococcus damnosus* ATCC 11308, *Leuconostoc citrovorum* ATCC 23065, *Leuconostoc mesenteroides* ATCC 8293, *Lactobacillus buchneri* ATCC 9460, *Lactobacillus brevis* ATCC 367, *Lactobacillus fermentum* ATCC 8289, *Lactobacillus fructivorans* ATCC 8288, and *Lactobacillus plantarum* ATCC 4008. Cultures were serially diluted to 10^4 CFU/mL in 0.1% peptone water (Acros

Organics, Geel, Belgium). MRS plates with antimicrobial incorporated were divided into 3 segments, upon which 10 µL of diluted culture was spot inoculated. Plates were incubated according to temperature and oxygen specifications required for each species according to ATCC recommendations (Table 2.1). Anaerobic conditions were achieved using anaerobe sachets (Becton, Dickinson and Company, Sparks, Maryland). Inhibition was determined by lack of colony formation on agar surface after 96 h, while growth indicated an insufficient antimicrobial concentration. Three samples were evaluated per replicate and each experiment was replicated three times (n=9).

Results and Discussion

Great variation in efficacy existed among the antimicrobials tested (Table 2.2). Thymol (w/v) and carvacrol (v/v) were the most inhibitory oils with MICs of 0.05% against all LAB except *L. plantarum* (carvacrol) and *L. buchneri* (carvacrol and thymol), where the MIC was 0.1% w/v or v/v in both instances. Cinnamaldehyde and cinnamon bark oil were the next most effective with MICs of 0.2% v/v, although the majority of species were inhibited by 0.1% v/v of each antimicrobial. *P. acidilactici* was the only species not inhibited by 0.1% v/v cinnamaldehyde. *P. acidilactici* and *L. mesenteroides* also were the not inhibited by cinnamon bark oil concentrations < 0.2% v/v. Clove bud oil, eugenol and thyme oil also had MICs of 0.2% v/v. The results of our study are consistent with the available literature reporting that LAB are inherently more resistant to essential oils than most Gram-negative and Gram-positive species. One group reported that concentrations of 0.12% (v/v) clove oil and 0.03% thyme oil were sufficient to inhibit *S. aureus* and *E. coli* (Hammer and others 1999). The LAB we examined, however, necessitated MICs of 0.2% for both thyme and clove oils. Cinnamic acid required higher

concentrations of 0.5% w/v to inhibit six LAB and 0.2% to inhibit the remaining three. By far the least effective antimicrobial was allyl isothiocyanate (AIT), which demonstrated no or minimal inhibition against seven LAB at the concentrations tested. *L. buchneri* and *L. fructivorans* were the only two organisms inhibited each requiring doses of 0.5% v/v AIT.

It is widely held that the antimicrobial properties of herbs stem from the essential oil fraction of the plants (Zaika and others 1983; Paster and others 1990; Hyldgaard and others 2012). Whether individual components (i.e. thymol) are more effective than whole oils (i.e. thyme oil) is much debated. For example, p-cymene, a precursor to carvacrol, is thought to play a supporting role in the antimicrobial efficacy of carvacrol in oregano oils. While insufficient to initiate a noticeable antimicrobial response independently, p-cymene is thought to mediate to the destructive action of carvacrol by inserting into the membrane, which also serves as the antimicrobial target of carvacrol, causing greater membrane expansion than carvacrol alone could. This may allow more complete penetration into the membrane and as a result, enhanced bactericidal action (Ultee and others 2002; Hyldgaard and others 2012). All volatile oils contain multiple antimicrobial compounds which may exert different but complimentary mechanisms of action against bacterial cells.

Despite the multitude of essential oils and compounds scrutinized for potential food preservative application, those derived from cloves, cinnamon, thyme and oregano dominate available literature as they frequently appear to be the most efficacious against the realm of food related bacteria. Carvacrol, cinnamaldehyde and thymol, compounds from oregano, cinnamon and thyme, respectively, were determined to be the most effective against a host of pathogenic and beneficial Gram-positive and Gram-negative bacteria when compared to

compounds such as citral, benzaldehyde, cresol, limonene or rosemary oil (Ouweland and others 2010). Barbosa and colleagues found clove to be the most effective oil against foodborne pathogens and spoilage microbiota alike, with thyme also exhibiting strong antimicrobial activity (Barbosa and others 2009). When tested on fish muscle extract, clove and thyme oils were the most inhibitory compared to fennel, Cyprus, lavender, herb-of-the-cross, pine and rosemary (Gómez-Estaca and others 2010). Our results indicated that clove bud and thyme oils have comparable antimicrobial efficacy, both having MICs of 0.2% v/v. However, the major component of thyme oil, thymol, worked more effectively as an isolated compound than eugenol, the primary antimicrobial compound in clove bud oil. Eugenol may contribute most of its activity while in an essential oil to synergism with other components within the clove bud oil, which might explain why it was less effective than thymol as an isolated antimicrobial. This interaction should be further investigated in future studies.

Structural differences in chemical compounds result in variances in mechanisms of action against bacterial species. Synergism has been speculated to occur among compounds when they are added to a formulation simultaneously, such as occurs with whole oils or blends of compounds (Bassolé and Juliani 2012). However, our results did not indicate any such advantage due to natural synergism, as the independent compounds thymol and carvacrol were more efficacious antimicrobials than the whole essential oils. It has been mentioned, however, that combinations or whole oils may be beneficial as a means of preventing the development of microbial resistance (Bassolé and Juliani 2012). The previously mentioned variances in action may be crucial in circumventing the development of microbial resistance. Application of more than one compound would facilitate inhibition through multiple pathways,

making target organisms less likely to adapt to any one individual agent. While our work indicated that whole essential oils required higher concentrations for inhibition, they should still be considered for application in foods. Man-made blends of oil compounds also have potential for preservative application. Future work examining these particular compounds and understanding their modes of bactericidal action may lead to essential oil cocktails that require even lower concentrations within a food formulation, resulting in diminished cost and lessened impact on flavor.

While studies involving herbaceous matter against pathogens abound, less work evaluating the effects of herbs and essential oils against lactic acid bacteria exists. Some of the earliest studies utilized herbs and spices in heavily seasoned foods that require LAB for production, such as sausages. Zaika and others (1983) found that acid production from LAB in liquid medium and at concentrations used to produce a Lebanon style bologna (Zaika and others, 1979) was stimulated in the presence of spice blends once resistance to initial bacteriostatic activity developed. Nes and Skjelkvåle (1982) demonstrated the effects of both natural spices and oleoresins against starter cultures of LAB commonly used in dry sausages. They concluded that while the oleoresins have no inhibitory effect on the LAB, a natural spice blend at 0.0025%, 0.005%, and 0.01% stimulated acid production and cell proliferation within the sausage.

Stimulated growth is not only a result of whole spices but also essential oils. *L. fermentum* exhibited sensitivity to several of the oils evaluated in a liquid medium, but had increased growth when exposed to carvacrol, cinnamaldehyde, oregano oil, thymol, and thyme oil. In addition, *Lactobacillus reuteri* also was unaffected by essential oil exposure up to 500

mg/l carvacrol or thymol (Ouwehand and others 2010). Addition of the MICs of spices or oils is essential so inhibition is achieved against spoilage LAB as lower concentrations appear to enhance the fermentative capabilities of some of these bacteria.

The poor performance of allyl isothiocyanate in this report is consistent with previous studies. The activity of AIT appears to be greatest when in a gaseous form, although there have been successes with the use of AIT as a liquid additive. However, exposure to an aqueous matrix tends to cause severe degradation to AIT, such that its antimicrobial activity is significantly diminished requiring high concentrations for inhibition (Kawakishi and Kaneko 1987; Luciano and Holley 2009). Use of AIT is inhibited by its strong organoleptic characteristics and water insolubility, further encouraging use of the volatile compound in its gaseous state (Lin and others 2000; Delaquis and Sholberg 1997; Ko and others 2012).

The majority of species examined in this study were heterofermentative, with the exception of the two *Pediococci*. There appears to be little difference in behavior between the homo- and heterofermentative species when exposed to the essential oils. *P. damnosus* had similar sensitivity to several of the *Lactobacilli* in terms of inhibition. *P. acidilactici* was the most resistant species examined, but the next most resistant organism, the heterofermentative *L. plantarum*, did not have substantially higher MICs. While it appears no metabolic pathway provides a distinct advantage, a larger pool of organisms including more homofermentative species needs to be analyzed before a conclusion should be made.

Our study examined the antimicrobial efficacy of essential oils when dispersed throughout an agar model. This serves as part of a foundation working towards application within food products, but results should not be directly extrapolated into a food model without

additional examination. Ingredients and other factors within food systems, including lipids, proteins, pH, and water activity frequently interfere with the antimicrobial action of essential oils, necessitating larger concentrations for inhibition (Zaika 1988; Nychas and Tassou 2000). Also, as mentioned previously, MICs do not indicate lethality; however, they do provide a starting point from which to better understand microbial resistance to essential oils. They direct us towards determining what essential oil concentrations will be sufficient for food preservation, and if it is determined that these cannot work independently, then it may provide framework to how essential oils may fit within a preservative hurdle technique. Future work will need to determine bactericidal concentrations of these oils against LAB before turning attention towards application within a food model.

Conclusions

The most promising information gleaned from this study is the efficacy of the essential oil components carvacrol and thymol. These agents have by far best potential for application within a food system as they have lowest MICs of 0.1% (v/v and w/v, respectively) against all nine LAB examined. Lower MIC values of these strongly scented and flavored compounds increase the likelihood of preservative application without detriment to organoleptic properties of a food. However, cinnamaldehyde, cinnamon bark oil, clove bud oil, thyme oil, and eugenol are also promising prospects with MICs of 0.2% v/v and should not yet be discounted as ineffectual by the food industry.

Tables

Table 2.1. Incubation temperatures and atmospheres for lactic acid bacteria.

Organism	ATCC #	Atmosphere	Temp (°C)
<i>Pediococcus acidilactici</i>	8042	anaerobic	37
<i>Pediococcus damnosus</i>	11308	anaerobic	30
<i>Leuconostoc citrovorum</i>	23065	aerobic	30
<i>Leuconostoc mesenteroides</i>	8293	aerobic	26
<i>Lactobacillus buchneri</i>	9460	anaerobic	37
<i>Lactobacillus brevis</i>	367	aerobic	30
<i>Lactobacillus fermentum</i>	8289	aerobic	37
<i>Lactobacillus fructivorans</i>	8288	aerobic	30
<i>Lactobacillus plantarum</i>	4008	aerobic	37

*Percent w/v thymol and cinnamic acid or percent v/v allyl isothiocyanate, carvacrol, cinnamaldehyde, cinnamon bark oil, clove bud oil, eugenol, and thyme oil

Table 2.2. Minimum inhibitory concentrations of essential oils incorporated into MRS agar against lactic acid bacteria.

Antimicrobial	MIC (%)*								
	<i>P. acidilactici</i> ATCC 8042	<i>P. damnosus</i> ATCC 11308	<i>L. plantarum</i> ATCC 4008	<i>L. buchneri</i> ATCC 9460	<i>L. fermentum</i> ATCC 8289	<i>L. brevis</i> ATCC 367	<i>L. fructivorans</i> ATCC 8288	<i>L. mesenteroides</i> ATCC 8293	<i>L. citrovorum</i> ATC 23065
Allyl Isothiocyanate	>0.75	>0.75	>0.75	0.5	>0.75	>0.75	0.5	>0.75	>0.75
Carvacrol	0.05	0.05	0.1	0.1	0.05	0.05	0.05	0.05	0.05
Cinnamaldehyde	0.2	0.05	0.1	0.05	0.05	0.1	0.05	0.1	0.1
Cinnamic acid	0.5	0.5	0.2	0.5	0.5	0.5	0.5	0.2	0.2
Cinnamon bark oil	0.2	0.05	0.1	0.05	0.1	0.1	0.05	0.2	0.1
Clove bud oil	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1	0.2
Eugenol	0.2	0.2	0.2	0.2	0.1	0.1	0.2	0.1	0.1
Thyme oil	0.2	0.2	0.2	0.2	0.1	0.2	0.1	0.1	0.1
Thymol	0.05	0.05	0.05	0.1	0.05	0.05	0.05	0.05	0.05

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Chapter 3

Minimum lethal concentrations of essential oils

Abstract

The bactericidal capability of the essential oil components carvacrol, cinnamaldehyde, eugenol, and thymol was evaluated against three lactic acid bacteria species: *Pediococcus acidilactici*, *Lactobacillus buchneri*, and *Leuconostoc citrovorum*. Thymol at 0.1% (w/v) was bactericidal against *L. citrovorum* (> 4-log reduction), but resulted in a > 2-log CFU/mL reduction against *L. buchneri* and 2-log reduction in *P. acidilactici*. Cinnamaldehyde at 0.2% to 0.25% (v/v) was effective against *L. citrovorum*, *L. buchneri*, and *P. acidilactici*, after 48 h resulting in a > 2-log reduction. All three organisms were susceptible to 0.2% carvacrol with > 3-log reduction observed after exposure for 6 h. Eugenol was the least effective of the antimicrobials. Concentrations of 0.2 and 0.25% (v/v) were needed to achieve an initial reduction in population, > 3-log CFU/mL after 6 h exposure. However, at 0.2%, *P. acidilactici* and *L. buchneri* recovered to initial populations in 48-72 h. Increasing the concentration to 0.3% caused a > 3-log reduction of *P. acidilactici* during the course of the study, but *L. buchneri* re-grew to initial populations.

Keywords: *Essential oils, lactic acid bacteria, minimum lethal concentrations*

Practical application: Food manufacturers are subject to increased consumer pressure to remove synthetic preservatives and additives from food labels and replace them with natural additives. These demands are increasing the interest of application of essential oils to any potential facet of the food industry. However, despite the Generally Recognized as Safe (GRAS) status these essential oils have been designated, research is still necessary to overcome several

issues that utilization of these oils present, including achievement of uniform dispersion throughout a food matrix, prevention of negative flavor or odor qualities, and determination of effective concentrations for microbial inhibition or inactivation.

Introduction

Essential, or volatile, oils are rapidly gaining popularity as food preservatives as consumer enthusiasm for organic and minimally processed foods continues to escalate (Baratta and others 1998). These plant-derived oils, which play an integral in plant defense, are demonstrated antimicrobial activity against Gram-positive and negative bacteria, as well as fungal species (Shelef 1983; Ames and others 1990; Sofos and others 1998).

Producers of salad dressings and mayonnaise products are frequently plagued by spoilage of their finished product, which typically can receive no heat treatment to eliminate bacterial and fungal spoilage species due temperature induced alterations to the dressing's consistency. Product pH is often sufficient to inhibit the majority of microbial growth (Smith and Stratton 2006), but growth and spoilage of dressings due to acid tolerant species is still an all too common occurrence. The utilization of natural processing hurdles, especially the incorporation of essential oils into product formulations or packaging materials, is an exciting prospect in the realm of food preservation, an area that is receiving increased consumer criticism for its use of synthetic preservative agents.

Phenols appear to be predominantly responsible for the antimicrobial activity of essential oils (Bassolé and Juliani 2012). Cinnamaldehyde confers the majority of activity to cinnamon oils. Thyme and oregano are bactericidal due to high concentrations of thymol and carvacrol, and eugenol is attributed with the antimicrobial activity in clove oils (Martínez-Tomé

and others 2001; Alma and others 2007). The utilization of individual volatile oil components has the distinct advantage of uniformity over the whole essential oils. Oil composition varies according to a myriad of factors, including harvest season, geographic location and extraction method. For instance, clove bud oil harvested in Cuba contained 69.8% eugenol, while eugenyl acetate and β -caryophyllene comprised 16.1% and 13.0% of the essential oil, respectively (Pino and others 2001). Turkish clove bud oil contained similar quantities of eugenyl acetate and β -caryophyllene with 15% and 5-12%, respectively, but anywhere from 80-90% eugenol (Alma and others 2007). Incorporating essential oil components as a supplementary hurdle within a series of food preservative techniques allows for greater control and uniformity in antimicrobial efficacy when juxtaposed to whole oils.

The objective of this work was to determine the Minimum Lethal Concentrations (MLCs) for select lactic acid bacteria when exposed to carvacrol, cinnamaldehyde, eugenol, and thymol in a broth system over 72 h. This information will help food processors assess what concentrations may be needed to inactivate these organisms in a food system.

Materials and Methods

In vitro examination of the bactericidal effects of carvacrol, cinnamaldehyde, eugenol and thymol (Sigma, St. Louis, MO) occurred against three genus of lactic acid bacteria (LAB); *Pediococcus acidilactici* ATCC 8042, *Lactobacillus buchneri* ATCC 9460 and *Leuconostoc citrovorum* ATCC 23065. Antimicrobials were dissolved into 95% ethanol to create stock solutions at 50% initial concentration (v/v; w/v thymol). de Man, Rogosa, Sharpe (MRS) broth (Oxoid, Basingstoke, UK) was combined with stock solution to desired essential oil concentration; 500 μ L of 6 log CFU/mL culture was inoculated into 49.5 mL MRS + essential oil

solution to achieve a final inoculum of 4 log CFU/mL. Concentrations of each antimicrobial examined in this study were determined based on minimum inhibitory concentrations from previous work and are shown in Table 3.1.

Broth was incubated at 37°C (*P. acidilactici* and *L. buchneri*) or 30°C (*L. citrovorum*) according to recommended culture conditions (ATCC 2012). At 0 (immediately after exposure), 6, 12, 24, 48, and 72 h populations of each LAB were determined. Ten-fold dilutions occurred in buffered peptone water (Oxoid, Basingstoke, UK) and spiral plated (WASP; Don Whitley Scientific, West Yorkshire, UK) on MRS agar in duplicate. Anaerobic incubation was achieved using anaerobe sachets (Becton, Dickinson and Company, Sparks, Maryland) for *P. acidilactici* and *L. buchneri*. *L. citrovorum* was incubated aerobically. Plates were incubated for 24 h for *P. acidilactici* and *L. citrovorum* and while *L. buchneri* required a 48 h incubation.

Analysis of variance was used to determine statistical differences among species within each examined antimicrobial concentration. Statistical analysis was accomplished with the mixed models procedure (PROC MIXED) using SAS 9.3 (SAS Institute Inc; Cary, NC) with significance set at $p < 0.05$. Three replicates of each species against each antimicrobial concentration were analyzed with sampling occurring in triplicate ($n=9$).

Results and Discussion

As would be expected, the three LAB species examined varied in their sensitivity to essential oil exposure. *L. citrovorum* and *P. acidilactici* were initially inhibited by 0.5% (w/v) thymol, but had recovered to approximately 5 and 6 log CFU/mL by 72 hours, respectively (Figure 3.1a and b) All three species behaved statistically differently at a concentration of 0.1% thymol after exposure for 72 h ($p < 0.05$). As can be seen in Figure 3.1, *L. citrovorum* populations

were below the limit of detection (0.95 log CFU/mL) after exposure for 72 h, while *P. acidilactici* initially experienced a 3 log CFU/mL reduction starting at hour 6 but had recovered by hour 72 to the initial inoculum level of 4 log CFU/mL. A 2 log CFU/mL reduction was evident for *L. buchneri* after 24 h and continued through 72 h sampling at 0.1% thymol (Figure 3.1c). *L. buchneri* was additionally exposed to 0.2% (w/v) thymol, which resulted in almost instantaneous lethality as no viable colonies were recovered immediately after inoculation and throughout the remainder of the study (Figure 3.1c).

At 0.2% (v/v) cinnamaldehyde, *L. citrovorum* (Figure 3.2a) and *L. buchneri* (Figure 3.2c) were statistically similar with a > 3-log reduction after 72 h of exposure ($p>0.05$). This concentration resulted in a >2-log CFU/mL reduction of *P. acidilactici* after 72 h (Figure 3.2b), but the organism required a concentration of 0.25% to be reduced to a non-detectable level after exposure for 48 h.

Carvacrol at 0.2% (v/v) rapidly inactivated all three organisms below the level of detection (> 3-log CFU/mL reduction) after 6 h of exposure (Figures 3.3). When the carvacrol concentration was reduced to 0.1%, *P. acidilactici* and *L. citrovorum* had statistically similar reductions of approximately 3 log CFU/mL from 24-72 h (Figure 3.3a and b). At 48 h, neither species had recoverable colonies, but by 72 h, both had similarly recovered to approximately 1.5 log CFU/mL ($p>0.05$). Meanwhile, at 0.1% carvacrol *L. buchneri* only decreased 1 log CFU/mL after 24 h and experienced a 2 log reduction after 72 h of exposure (Figure 3.3c).

All three organisms were inhibited by eugenol at 0.25% (v/v) for some time. *L. citrovorum* and *L. buchneri* recovered statistically similarly to around 2 log CFU/mL by 72 h after little to no recoverable growth through much of the study ($p>0.05$; Figure 3.4a and c). After

having no recoverable colonies (> 3 log-reduction) at 6 and 12 h, *P. acidilactici* recovered to 3 log CFU/mL by 24 h and 4 log CFU/mL by 72 h (Figure 3.4b). *L. buchneri* was finally inhibited at a concentration of 0.3% (Figure 3.4c), but *P. acidilactici* had statistically similar recovery at 0.3% to what it had at 0.25%, recovering to 4 log CFU/mL after 72 h of exposure ($p>0.05$; Figure 3.4b).

The appreciation for the antimicrobial, preservative, and medicinal properties of plant products is by no means a novel sentiment (Hammer and others 1999; Prabuseenivasan and others 2006). However, they are decreasingly being viewed as simply “traditional” remedies as their application in mainstream medicine is becoming increasingly practical. While a cinnamon oil product has been shown to be effective as a treatment for oral candidiasis in patients with HIV (Quale and others 1996), eugenol and cinnamaldehyde have been shown to inhibit growth of the stomach ulcer causing agent *Helicobacter pylori*, without the development of microbial resistance to these agents (Ali and others 2005; Prabuseenivasan and others 2006). As popular as these components are becoming within the medical community, exploitation of essential oils is perhaps even more applicable to the food industry, where consumers are clamoring for what they perceive as “natural” preservatives and additives. Fortunately for these consumers, essential oils fit the bill as both naturally derived and already stamped with the FDA’s Generally Recognized as Safe (GRAS) status.

Due to the fact that essential oils intrinsically exhibit strong flavor and odor characteristics, strong antimicrobial potency of these oils and their compounds is imperative so only minimal concentrations are required. Essential oils that contain phenols and terpenes generally have the highest antimicrobial activity, while oils containing fewer phenols but

greater quantities of other compounds such as ketones, esters, acids, and others are typically less efficacious as antimicrobials. For example, thymol and carvacrol are the major antimicrobial components of the essential oil of oregano, and have been shown to have an additive effect when combined. Against *Pseudomonas aeruginosa*, these two components were identified as responsible for 96% of the inhibitory action exerted by oregano oil (Lambert and others 2001). Utilizing these individual compounds rather than the entire essential oil allows more concise and concentrated application of antimicrobial agents in order to exert the greatest amount of activity possible.

Using individual components of essential oils instead of whole oils provides other distinct advantages. Oil uniformity varies greatly depending on time of harvest, climate, oil extraction method, and a multitude of other factors. Disruption in supply chain or a poor harvest year could impact the oil composition and potentially distort the preservative capacity within a food formulation. Difficulty also arises in comparing results obtained from essential oil testing when not only variation exists among oils from study to study, but common names of oils also can encompass a number of different species and subspecies of plants (Hammer and others 1999; Chang and others 2001; Gómez-Estaca and others 2010). Using an oil constituent eliminates the likelihood of variability associated with whole oils. This study focused on essential oil components rather than the whole oils as an attempt to determine if using constituents provided the hypothesized benefit of concentrated antimicrobial action.

Of particular concern are the concentrations at which, after initial inhibition and what appeared to be bactericidal activity, bacteria recovered and rebounded to initial inoculum levels. This is apparent with *P. acidilactici* at concentrations of 0.3% eugenol and 0.1% thymol,

as well as *L. citrovorum* at 0.25% eugenol. The same occurrence has been reported with eugenol and carvacrol against *E. coli* O157:H7 and with cumin essential oil against *L. mesenteroides* at concentrations of 0.015%, 0.03%, and 0.06% (Kivanç and others 1991; Kim and others 1995). This tendency of bacteria to overcome inhibition due to essential oils is disconcerting, as preservative effects of essential oils may be overcome prior to the expiration of a product's shelf-life, resulting in premature spoilage of a food item. Because of this, it is crucial that accurate MLC's are determined and proven effective in media and food matrixes or utilized with other hurdles if this pattern is observed.

Our previous studies revealed that thymol and carvacrol were the most inhibitory components analyzed against LAB. The current study indicated that at 0.1% (v/v) carvacrol, all species were inhibited, and at 0.2% all species were inactivated > 3-log CFU/mL after 6h. While 0.1% (w/v) thymol was initially lethal to all species (> 2-log CFU/mL reduction), *P. acidilactici* recovered after 48 h and re-grew to initial populations by 72 h. The results of this study also indicate that cinnamaldehyde is comparably effective to thymol and carvacrol as a bactericidal agent. The compound was lethal to *L. citrovorum* and *L. buchneri* with a > 3-log reduction after 48 and 72 h, respectively. *P. acidilactici* suffered a 2 log reduction at 0.2% (v/v) after 72 h, but 0.25% resulted in undetectable populations. Cinnamon or cinnamon compounds are often used as the predominant flavoring agents in many food products, and with bactericidal capabilities evidenced from the cinnamon derived component cinnamaldehyde, application to food formulations can serve a two-fold purpose.

Eugenol required the largest bactericidal concentration. At 0.25% (v/v), the population of *P. acidilactici* after 72 h was statistically similar to the initial inoculum level, while *L. buchneri*

and *L. citrovorum* only experienced 2 log reductions. Even at 0.3%, viable *P. acidilactici* colonies re-grew to initial populations, making the compound an unlikely candidate for application within a food model as the only antimicrobial agent or control. It has been noted that much larger quantities of these components are required in a food matrix to exert the same lethality evident in a broth dilution (Zaika 1988). The already large quantities of eugenol required for lethality in broth medium indicate the necessity of bactericidal concentrations may make it difficult to apply to foods at levels required for inhibition without significantly altering the flavor profiles of these products.

Conclusions

The MLC for carvacrol was 0.2% (v/v), and 0.2% thymol (w/v) was sufficient to inactivate two of the examined LAB species below the limit of detection. A 3-log reduction was evident against two LAB species at 0.2% cinnamaldehyde (v/v), while one required 0.25% before the MLC was attained over 72 h. Up to 24 h, *P. acidilactici* was not recovered by the highest concentration of eugenol examined, 0.3% (v/v), but recovered to initial populations by 72 h. Carvacrol, thymol, and cinnamaldehyde are likely candidates for use as food preservative agents; eugenol may require concentrations too high to provide sufficient protection against spoilage unless utilized in a system where it can also serve as a compatible flavoring compound.

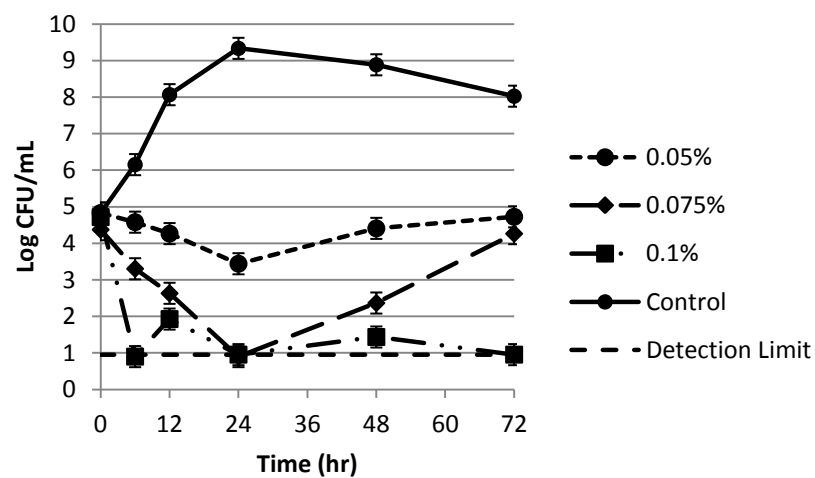
Tables

Table 3.1 Concentrations of carvacrol, cinnamaldehyde, eugenol and thymol examined against *P. acidilactici*, *L. buchneri*, and *L. citrovorum*. Values are v/v except thymol (w/v).

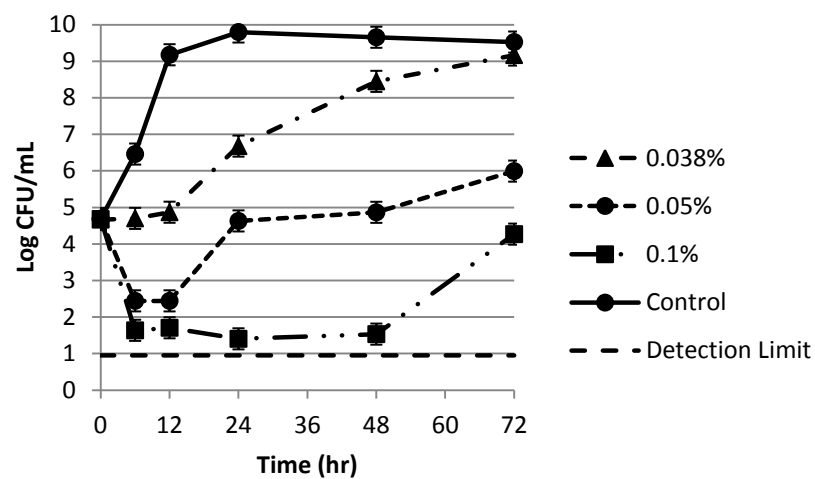
Antimicrobial	Concentration	Species		
		<i>P. acidilactici</i>	<i>L. buchneri</i>	<i>L. citrovorum</i>
Carvacrol	0.025%			x
	0.038%			x
	0.05%	x	x	x
	0.075%		x	
	0.1%	x	x	x
	0.2%	x	x	x
Cinnamaldehyde	0.025%		x	
	0.05%			x
	0.075%		x	x
	0.1%		x	x
	0.175%	x		
	0.2%	x	x	x
	0.25%	x		
Eugenol	0.075%			x
	0.1%			x
	0.175%	x	x	
	0.2%	x	x	x
	0.25%	x	x	x
	0.3%	x	x	
Thymol	0.038%	x		
	0.05%	x	x	x
	0.075%		x	x
	0.1%	x	x	x
	0.2%		x	

Figures

a)



b)



c)

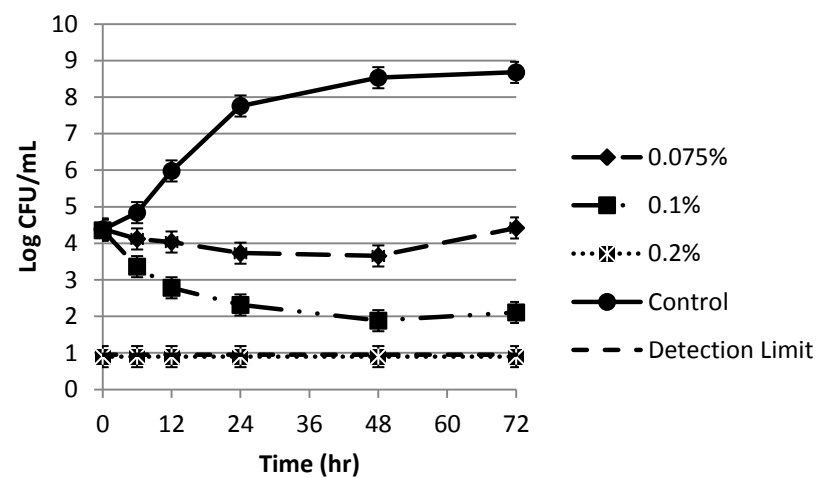
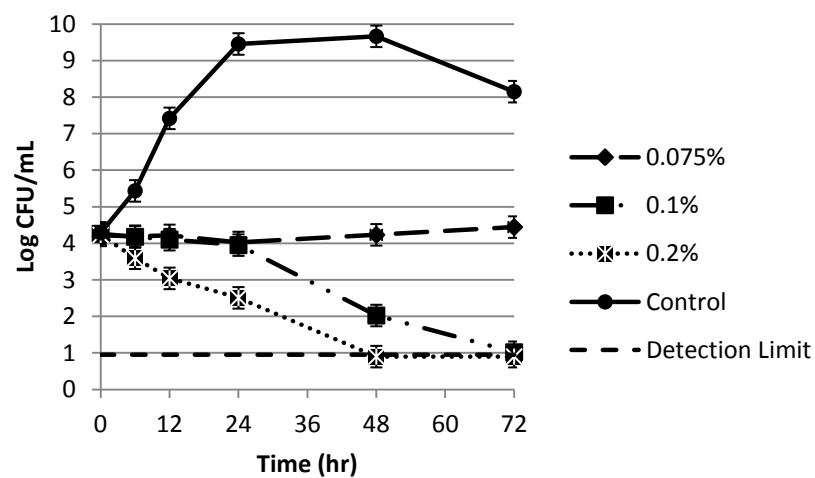
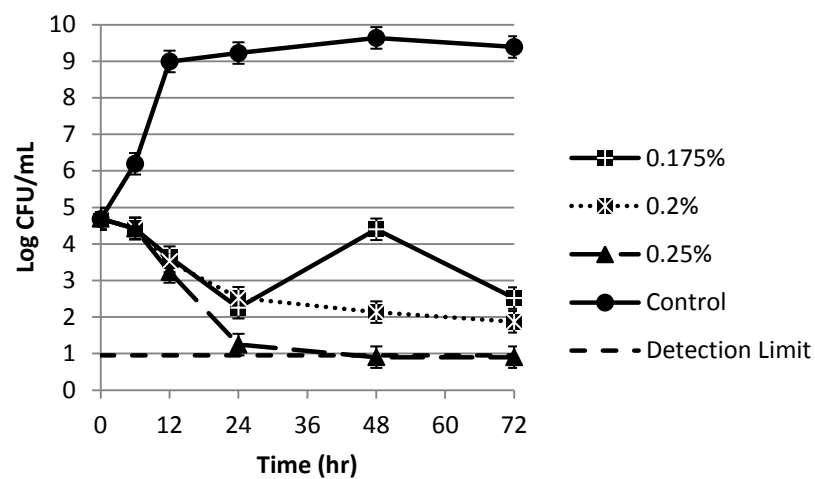


Figure 3.1 Response of a) *L. citrovorum*, b) *P. acidilactici*, and c) *L. buchneri* in MRS broth with varying % (w/v) thymol over 72 h

a)



b)



c)

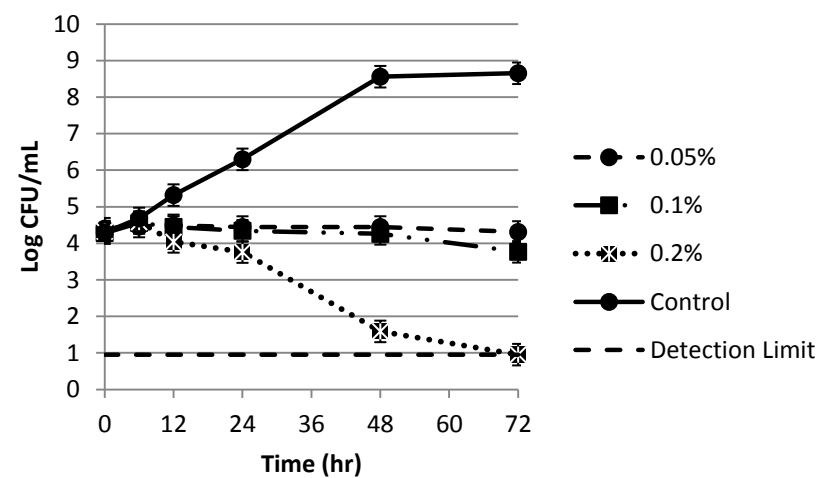
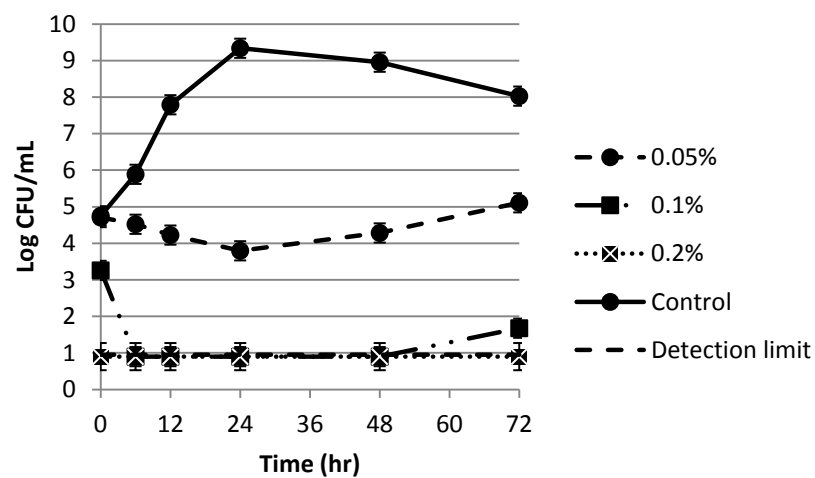
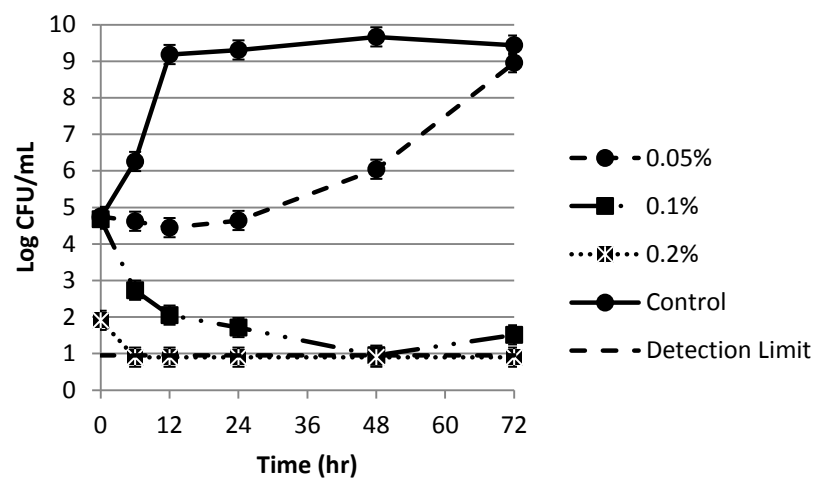


Figure 3.2 Response of a) *L. citrovorum*, b) *P. acidilactici*, and c) *L. buchneri* in MRS broth with varying % (v/v) cinnamaldehyde over 72 h

a)



b)



c)

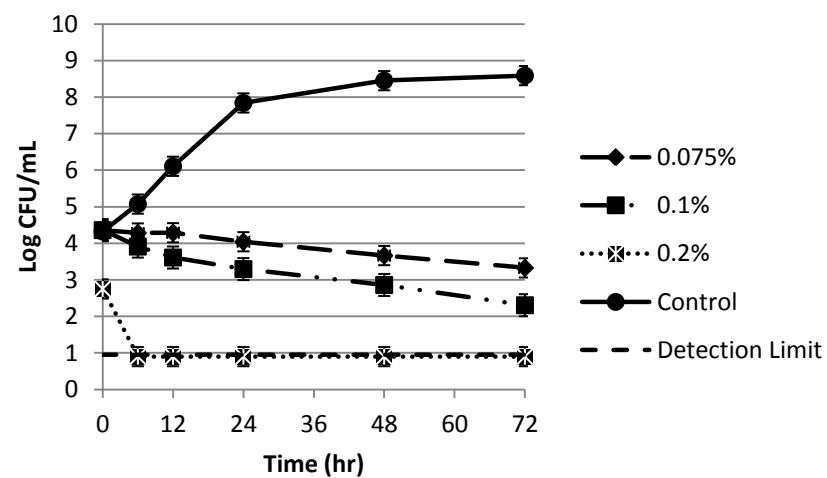
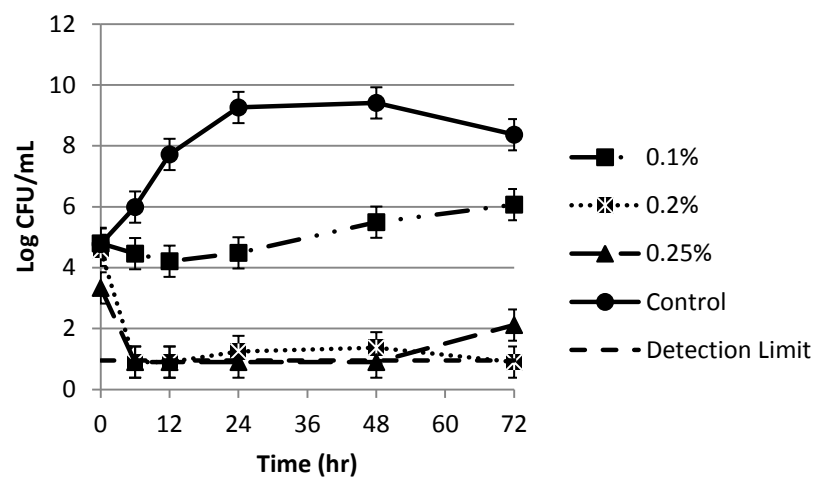
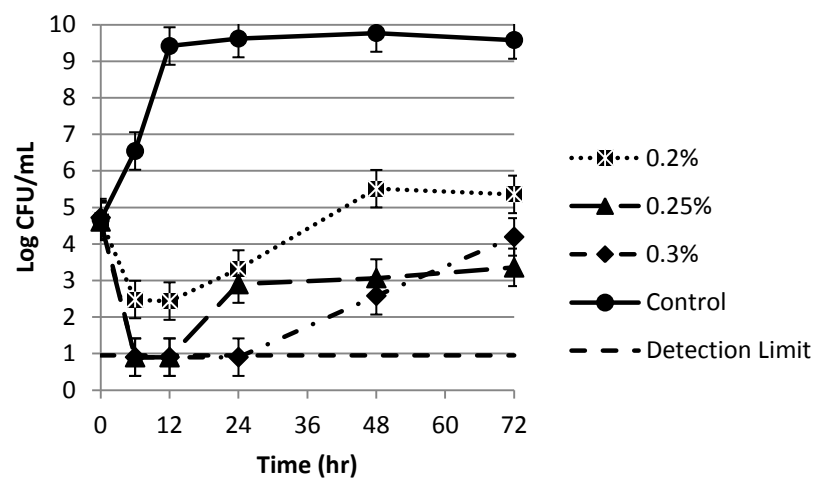


Figure 3.3 Response of a) *L. citrovorum*, b) *P. acidilactici*, and c) *L. buchneri* in MRS broth with varying % (v/v) carvacrol over 72 h

a)



b)



c)

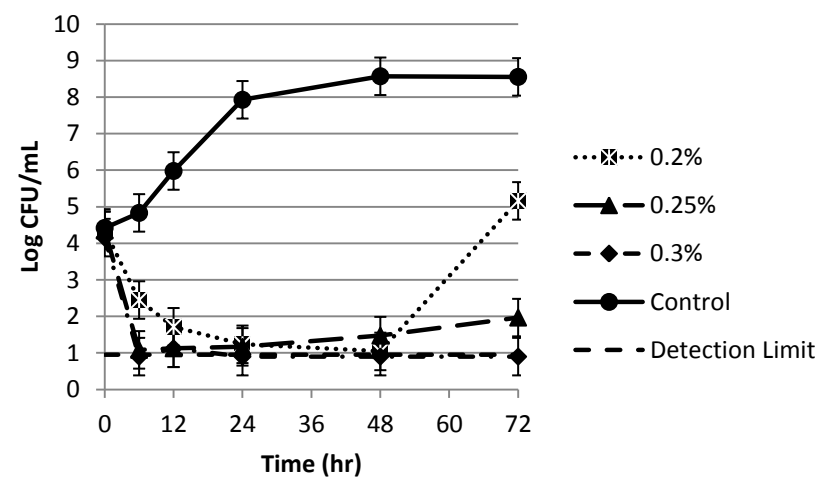


Figure 3.4 Response of a) *L. citrovorum*, b) *P. acidilactici*, and c) *L. buchneri* in MRS broth with varying % (v/v) eugenol over 72 h

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Conclusion

The substitution of essential oils for traditional food preservatives is promising. Given the abundant literature regarding essential oils against foodborne pathogens, it is evident that enthusiasm exists for essential oils as effective food additives. However, as research exploring their potential against spoilage species is limited, much work is still to be done before these can be applied effectively to foods.

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Vita

Laurel (Dunn) Gann, the daughter of Lewis and Jean Dunn, was born on November 3, 1987 in Gretna, Louisiana. After a series of cross-country moves, her family finally settled in Cleveland, TN, where Laurel spent the majority of her school-aged years. Upon graduation from Cleveland High School in 2006, Laurel followed her two older brothers, Matthew and Gregory, to study at the University of Tennessee, where she graduated with a Bachelor's degree in Food Science and Technology in 2009. Laurel later returned to UT and completed her Master's degree in Food Science with a focus in Food Microbiology. She plans to pursue her doctorate in Food Microbiology at the University of Tennessee.