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Inhibition of Spoilage Yeasts using Spice Essential Oils and Their Components

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To the Graduate Council:

I am submitting herewith a thesis written by Audra Ann Wallis entitled "Inhibition of Spoilage Yeasts using Spice Essential Oils and Their Components." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

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Inhibition of Spoilage Yeasts using Spice Essential Oils and Their Components

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Audra Ann Wallis
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DEDICATION

I dedicate this work to my family. Rex, Sandy and Alex Wallis

as well as Robert and Peggy Meyer.

Thank you for your limitless and unconditional support.

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First I would like to thank my advisor, Dr. Davidson, for his advice and support while I completed my Masters. I would also like to thank my committee members, Dr. David Golden and Dr. Faith Critzer along with all of the faculty members who have supported my education.

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"You are a child of the universe, no less than the trees and the stars; you have a right to be here. And whether or not it is clear to you, no doubt the universe is unfolding as it should. Therefore be at peace with God, whatever you conceive Him to be, and whatever your labors and aspirations, in the noisy confusion of life keep peace with your soul. With all its sham, drudgery, and broken dreams, it is still a beautiful world. Be cheerful. Strive to be happy."

-Max Ehrmann "Desiderata"

ABSTRACT

Clove bud, cinnamon bark, and thyme oil, along with their components cinnamaldehyde, cinnamic acid, eugenol, carvacrol, and thymol, are widely acknowledged to have antimicrobial properties against bacteria. However, less is know about the inhibitory properties of essential oil components against spoilage yeasts. In this study a minimum inhibitory concentration (MIC) for these essential oils and components was determined using an agar dilution assay for *Torulaspora delbrueckii, Candida krusei, Schizosaccharomyces pombe* and *Zygosaccharomyces bailii*. The efficacy of essential oil components eugenol, carvacrol, cinnamaldehyde and thymol then were evaluated in a model salad dressing. The MIC against all yeasts for cinnamaldehyde and cinnamon bark oil was 50 mg/l. For thymol, thyme oil and carvacrol, the MICs were 200, 400, and 200 mg/l, respectively. *T. delbrueckii* and *C. krusei* required 300 mg/l to achieve an MIC for clove bud oil, and *S. pombe and Z. bailii* had an MIC of 200 mg/l. Eugenol had a MIC of 300 mg/l for *T. delbrueckii*, and *C. krusei* and an MIC of 200 mg/l for *Z. bailii* and *S. pombe.* Cinnamic acid had an MIC of 500 mg/l for *Candida krusei* and *T. delbrueckii*, 400 mg/l for *S. pombe*, and 200 mg/l for *Z. bailii.* To establish efficacy of essential oil components in salad dressing, 4 log CFU/m of yeast cells were added to 39.0±0.5g of model salad dressing with various concentrations of essential oils and incubated at 22˚C. Samples were taken at 0, 6, 12, 24, 48, 72, 96 and 120 h. Cinnamaldehyde at a 500 mg/l concentration was the most effective at inhibiting *S. pombe* and *Z. bailii*. *T. delbrueckii* and *C. krusei* used in this study were unable to grow in the model salad dressing used during the timeframe of the study.

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INTRODUCTION

In 2012, sales of all pourable salad dressings in the US were greater than $1.4 billion. Chief goals when creating a consumable product is that the product be shelf stable and that consumer want to purchase it. However, many of the items used to extend shelf-life of products, such as salad dressings, are not desirable to the modern consumer. With consumers desiring more and more organic and preservative-free products, the search for natural antimicrobials has intensified.

Reduction of shelf-life of food products such as salad dressings can be caused by any number of factors. However, the most common is microbial spoilage. Frequent spoilage contaminants in salad dressings include many strains of yeasts. These microorganisms are unique because of their ability to survive in acidic anaerobic environments with a variety of nutrients. Growth of these microorganisms can cause negative sensory attributes, gas production, changes in color, breaking of emulsions and decreases in pH. Each of these defects can cost manufacturers time and money to resolve.

Contamination from these organisms can come at any step in the manufacturing process. Most organisms can be found as environmental contaminants. Manufacturing facilities with inadequate sanitation and contaminated ingredients are sources for spoilage microorganisms, including yeasts. Detection of these microorganisms is difficult and time consuming, with some analyses taking up to 4 days. While reducing environmental contamination is achievable, complete control of all potential contaminants is nearly impossible. Therefore, use of interventions, such as physical processes to treat the product or chemical antimicrobials incorporated into the product, is important to improve shelf-life.

Antimicrobial food preservatives can be classified as synthetic or natural. While much is known about synthetic antimicrobials, much less is available on the activity of natural antimicrobials, especially against fungi. One group of potential natural antimicrobials is the essential oils (EO) from spices and herbs. EOs oils derived from thyme, clove, cinnamon, oregano and other spices and herbs have repeatedly shown high efficacy as microbial inhibitors. This efficacy can likely be attributed to the phenolic group on the chemical compounds. The chief chemical components of cloves, thyme, oregano and cinnamon are eugenol, thymol, carvacrol, and cinnamaldehyde, respectively, and all are generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA). By incorporating either the active component or the entire oil, a more "natural" product may be created

The objective of this research was to determine the minimum inhibitory concentration of plant EOs and their active chemical components against five common spoilage yeasts known to contaminate salad dressings and other foods and to determine the efficacy of these EO when incorporated into shelf stable ranch salad dressing.

CHAPTER I
REVIEW OF LITERATURE

1) Yeast Characteristics

Yeasts are unicellular eukaryotes classified in the kingdom fungi. Yeasts are common environmental contaminants and are naturally present on a wide variety of foods, such as the skins of grapes, apples and peaches. Yeasts are also frequently associated with insects as a mode of transmission. Most yeast reproduce via asexual budding however some, such as *Schizosaccharomyces pombe,* reproduce by fission.

While the yeast cell wall is considered to be very strong, it can also be very porous. Comprising anywhere from 25-50% of the volume of a yeast cell, the cell wall is composed mostly of mannoprotein, ß1,3 glucan, and chitin (Lipke and Ovaile 1998). Along with these compounds, sterols play a vital role in the permeability of the membrane. In yeast cells ergosterol is the primary sterol present. This counterpart to the cholesterol found in mammalian cells, ergosterol is for cell membrane maintenance and construction as well as regulating the fluidity, permeability and ethanol resistance of the membranes (Deytieux and others 2005).

2) Results of Growth in Foods – From Fermentation to Spoilage

Yeasts have a long history of use in food because of their ability to metabolize sugars in both anaerobic and aerobic conditions, and produce ethanol and carbon dioxide. Their ability to produce ethanol is used in the manufacturing of alcoholic beverages, such as beer, wine and whiskey, while their ability to produce large amounts of carbon dioxide is used in baking. The primary species used in food processing and therefore frequently used as yeast in research *Saccharomyces cerevisiae.*

The ubiquitous nature of yeast makes them likely contaminant of a number of food products, and the effects of the contamination and growth vary with the most noticeable being gas production. Most alterations to the sensory properties of foods contaminated with spoilage

yeasts go unnoticed until cell concentrations reach 5-6 log CFU/g, and no physical effects are seen until numbers reach 7-8 log CFU/g (Fleet 1992). At this level swelling and even explosion of food packages can be seen. Most frequently affected foods include dairy products such as yogurts and cottage cheese (Fleet 1992). However, gas swelling of packaged RTE sliced deli meats, seafood, fruits and vegetables has also been reported (Fleet 1992).

In wine and beers, spoilage often occurs during aging of the products (Loureiro 2003) and the spoilage is more subtle, resulting in nothing more than slight off flavors. In other products such as cheeses and fermented milks, discoloration, texture changes, and gas production can be seen as well as off-flavors (Jakobsen and Narvhus, 1995). When spoilage yeasts contaminate emulsified products, such as salad dressings, breaking of the emulsification can be seen along with gas production (Kurtzman and others 1971). In a study done by Waite, Jones and Yousef, they reported that gas production of fermentation is one of the leading causes of product failure of mayonnaise-based dressings (Waite and others 2009). This gas production can cause damage to the product packaging. Damage to the packaging may compromise the safety of the product by compromising the seal which could introduce more oxygen or other contaminants to the food.

Yeast have very few requirements for survival and have the ability to grow under anaerobic conditions. The ability to thrive in high acid, liquid environments makes spoilage of salad dressing by yeasts common. In one study, of 17 spoiled samples of salad dressing 11 were spoiled by yeasts alone and 2 were contaminated with both yeasts and bacteria. Only 4 were contaminated with bacteria alone (Kurtzman and others 1971).

3) Tolerance to Intrinsic Factors

Most, if not all, yeast strains exhibit some degree of tolerance to high acid environments. Studies have shown a tolerance for most yeast strains to pH as low as 3.0 or as high as 10.0 (Deák 2008). Exporting of protons through the cell membrane allows yeast cells to regulate the pH inside of the cell (Praphailong and Fleet 1997). One theory of pH tolerance is that strains more tolerant to low pH have a more stable and efficient plasma membranes, but this has not been experimentally proven (Praphailong and Fleet 1997).

As many as 800 yeast strains have been isolated from high acid products such as fruit juices and soft drinks (Barnett and others 2000). Because of the low pH (< 4.6), few microorganisms are known to compete with yeasts for growth in these products. A yeast, *Candida davenportii*, identified in a soft drink production facility in the Netherlands caused spoilage at a pH as low as 1.4. The yeast was originally isolated from a wasp found on an external sugar-syrup storage tank tap (Stratford 2002). While active spoilage at a pH of 1.4 is not common, *Zygosaccharomyces bailii* exhibits a particularly strong tolerance to high acid environments. In a study evaluating the pH tolerance of various yeasts, it was found that *Z. bailii* grew better at a pH of 3.0 and actually exhibited no growth at a pH of 7.0 (Praphailong and Fleet 1997). *Z. bailii* also exhibited a higher tolerance to NaCl, glucose, sorbate and benzoate at a pH of 3.0 than at a higher pH (Praphailong and Fleet 1997).

Yeasts also exhibit a tolerance for reduced water activity. This ability, for most strains can be attributed to trehalose. Trehalose is a non-reducing disaccharide composed of two glucose molecules linked in an α,α-1,1-glycosidic linkage (Elbein and others 2003). Originally considered to act solely as an energy storage for synthesis of cellular components, it is now acknowledged to serve other functions (Elbein and others 2003). In studies with *Saccharomyces*

cerevisiae, trehalose was found to be an important compound for the survival of cells when exposed to severe osmotic stress. Death rates were lower in cells containing more intracellular trehalose (Hounsa and others 1998).

Trehalose may also be related to heat stress resistance. Large amounts of trehalose can also be found in *S. cerevisiae* cells that have been heat shocked, but trehalose supplies quickly disappear when cells are returned to ambient temperature (Hottiger and others 1987). Similar activity was seen in *Schizosaccharomyces pombe* where trehalose was found to protect against both osmotic stress and temperature stresses. Studies show that *S. pombe* cells growing to mid log phase at 27˚C contained minimal amounts of trehalose (Virgilio and others 1990). When cells were transferred to 40˚C, they rapidly accumulated high levels of trehalose which was subsequently lost at lower temperatures. This was only seen when cells were grown in the presence of glucose. Presence of heat shock proteins (hsp) was thought to result in thermal tolerance of yeasts. However, recent studies indicate that production of hsp greatly decreased at 40˚C and higher (Ribeiro and others 1997). This suggests that trehalose is responsible for low water activity and heat tolerance of *S. pombe*.

4) **Yeast Types**

Most authors consider 10 species of yeasts to be the most important in spoilage of food products (Loureiro and Malfeito-Ferreira 2003; Pitt and Hocking 2009a). *Dekkera bruxellensis, Issatchenkia orientalis, Debaryomyces hansenii, Kloeckera apiculata, Pichia membranifaciens, Zygosaccharomyces bailii, Zygosaccharomyces bisporus, Saccharomyces cerevisiae, Zygosaccharomyces rouxii, Schizosaccharomyces cerevisiae* and *Candida holmii* have all been found to cause food spoilage. Over 120 different species of yeast are listed as being associated with food spoilage, however most species show no or poor growth in properly processed foods

(Barnett et al, 1983, Pitt and Hocking, 1985). The yeasts most commonly associated with spoilage of mayonnaise-based salad dressings are *Zygosaccharomyces* species and lactic acid bacteria. These yeast along with *Candida krusei*, *Schizosaccharomyces pombe* and *Torulaspora delbrueckii* were the focus of the present study. Basic growth characteristics for these organisms are listed in table 1.1.

a) *Zygosaccharomyces bailii*

Zygosaccharomyces bailii is a xerophilic spoilage yeast often associated with mayonnaise and salad dressings. Well known for its resistance to weak acid preservatives, *Z. bailii* exhibits resistance to sorbic, benzoic, acetic and propionic acids (Pitt and Hocking 2009a). This tolerance to weak acid preservatives could possibly be attributed to multiple exposures to sub-lethal levels of preservatives such as those found in beverage plant filling machines (Pitt and Hocking 2009a). Like most yeasts, *Z. bailii* ferments glucose to carbon dioxide but it can also grow in the presence of 400 mg/L or more benzoic or sorbic acid. This organism also can grow at pH as low as 2.2. The optimum growth temperature for *Z. bailii* can be influenced by the concentration of glucose in the growth medium. In a medium with 10% w/w glucose, the optimum temperature was found to be 30-32˚C and the minimum growth temperature was 6.6˚C while in a medium containing 60% w/w glucose the optimum and minimum growth temperatures were 34-36˚C and 13˚C, respectively (Pitt and Hocking 2009a).

b) *Candida krusei*

Candida krusei is a spoilage yeast that is capable of growth at temperatures as low as 8˚C and as high as 47˚C (Pitt and Hocking 2009a). It has been reported to be relatively heat resistant for a yeast, surviving 56˚C for 80 min. At 65˚C, 10^4-10^5 of the yeast was killed in 2 min (Pitt and Hocking 2009b; Pitt and Hocking 2009a). While not frequently known to spoil mayonnaise-

based salad dressings, it has been isolated from other high acid products such as citrus fruits, soft drinks and wine (Pitt and Hocking 2009a). *C. krusei* ferments glucose and forms films on the surface of liquid foods it contaminates. It exhibits resistance to a variety of preservatives under anaerobic conditions including sorbic acid, benzoic acid, and methyl paraben (Mollapour and Piper 2001; Piper and others 2001; Warth 1989). Molecular analysis of this yeast linked resistance to acetic acid to the presence of the citrate synthase gene (Pitt and Hocking 2009a).

c) Schizosaccharomyces pombe

Frequently known to contaminate wines and high sugar syrups, *S. pombe* exhibits the ability to grow at low pH and low a_w environments. It grows equally well at 37˚C as it does at 25˚C and may cause spoilage of food products in warmer climates. Some solutes provide protection for *S. pombe* during thermal processing. In the presence of sucrose, the D_{65C} of cells was 1.48 min at a 0.95 a_w while without sugar present, the $D_{65°C}$ was 0.1 min (Duckworth and International Union of Food Science and Technology. 1975). Glucose and fructose were not as protective with $D_{65°C}$ values of 0.41 and 0.27 min. *S. pombe* is unique in that while it can cause spoilage of wine it can also contribute to wine quality. The yeast has the ability to ferment malic acid in wine, which is useful in reducing the "green apple" flavor that high levels of malic acid bring to wine. However, the microorganism is also known to produce off-flavors that make utilizing this ability difficult (Benito and others 2012).

d) Torulaspora delbrueckii

Torulaspora delbrueckii has been associated with the spoilage of dairy products, juices, wines and ready-to-eat (RTE) salad products. In sealed packages of lettuce *T. delbrueckii* was one of the predominant strains identified (King and others 1991). Another study on lightly processed produce (e.g. prepared apple slices, shredded lettuce, trimmed broccoli florets) and

fully processed vegetables and fruits (e.g. fruit/vegetable purees, fruit juices, yogurts with fresh fruits), over a three-year period identified *T. delbrueckii* on both fruits and vegetables (Torok and Jr 1991; Huxsoll and others 1989). However studies have shown that this xerophilic yeast has little tolerance to heat with a $D_{60°C}$ of 0.018 min at a pH of 4.0 (Shearer and others 2002) *T. delbrueckii* can grow at temperatures as low as 4-4.5˚C

Table 1.1 Growth Characteristics of common spoilage yeasts

Yeast	**Temp. Range**	**pH range**	**a_w**	**Commonly associated foods**	**References**
C. krusei	8˚C-47˚C	1.3-	NA	tomato sauce, maize silage, cocoa beans, sour dough, cheese, beverages, olive	(Fleet 1992 ; Pitt and Hocking 2009a)
S. pombe	NA*	0.84	0.81	wine, cocoa, coffee, ketchup raspberry cordial	(Pitt and Hocking 2009a; Tokuoka and Ishitani 1991)
T. delbrueckii	4˚C-	NA*	0.84	juice spoilage, fermented milk beverage, sugar silos, RTE deli salads, kombucha, rice, grapes	(Mu and others 2012; Avila and others 2010; Renault and others 2009)
Z. bailii	4˚C-53˚C	2.2-7.0	.70	spoiled orange juice, spoiled jam, soft drinks, syrup	(Pitt and Hocking 2009a; Pitt and Hocking 2009b)

*NA indicates no value was found in available literature

5) Control of Spoilage Yeasts in Food and Beverages Using Antimicrobials

a) Calcium Disodium Ethylenediaminetetraacetic acid

Calcium disodium ethylenediaminetetraacetic acid (EDTA, molecular weight 410.3 Da) is approved by the US FDA for use as a preservative in non-standardized dressings, French dressing, mayonnaise, salad dressing, sandwich spreads, potato salad and sauces in concentrations not to exceed 75 mg/L for all but sandwich spreads where it can be used in concentrations as high as 100 mg/L (21 CFR 172.120). EDTA is primarily added as a chelation agent to retard auto-oxidation of lipids in foods. Although added to foods to prevent chemical changes, this compound also has antimicrobial properties. EDTA has been studied as an antimicrobial against bacteria, chiefly Gram-negative bacteria, both alone and as an adjunct to other natural antimicrobial compounds. EDTA has be found to enhance the efficacy of lysozyme, a natural antimicrobial found in egg whites as well as human tears, as well as nisin against *Listeria monocytogenes* and *Escherichia coli* (Payne and others 1994; Branen and Davidson 2004). EDTA has been shown to cause *E. coli* to rapidly lose over 50% of its lipopolysaccharide causing significant permeability changes (Leive 1965). This same study also showed that while *E. coli* is naturally impermeable to actinomycin, once treated with EDTA, actinomycin permeated the membrane causing cell death.

Against yeasts, EDTA alone was found to be inhibitory against *S. cerevisiae* as well as *Z. bailii* at a 6.4 g/ml (Kubo and others 2005). In addition, EDTA enhanced the efficacy of polygodial, a strong antifungal compound derived from sprout of *Polygonum hydropiper* (used as a food spice called “tade” in Japan). It has been suggested that EDTA inhibits yeast growth by depriving the cell of the vital zinc ions required to create the cell wall (Brul and Coote 1999;

Brul and others 1997). This interruption caused by EDTA could allow for enhanced antimicrobial activity of any essential oils.

b) Natamycin

Natamycin, *formerly known as pimaricin*, is a highly effective antifungal compound. This polyene macrolide antibiotic is composed of a ring structure with a conjugated double bond located opposite a number of hydroxyl groups, and has a molecular weight of 665.7Da (Figure 1). Produced by *Streptomyces natalensis*, natamycin has a high affinity for ergosterol found in yeast cell membranes. The compound irreversibly binds to the ergosterol preventing it from performing its functions in the membrane. This binding causes significant damage to the cellular structure and leaking of cell contents until cell death (Welscher and others 2008).

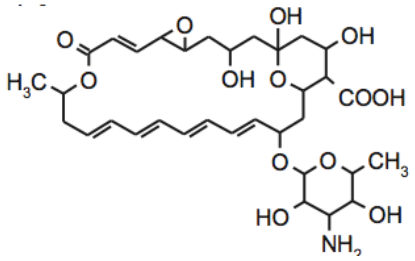

Figure 1.1: Natamycin Chemical Structure

Natamycin is commonly used in manufacturing of cheese to prevent growth of fungi, as it has no effect on the growth of lactic acid bacteria starter culture. Because it is hydrophobic, it also remains at the surface of the cheese where mold is most likely to grow. When tested against Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and other food contaminants, no inhibition was detected (Atta and others 2012). However, when tested against a

number of filamentous and unicellular fungi, concentrations as low as .031 g/ml were inhibitory. Natamycin is GRAS certified by the FDA, has no negative effects on food quality and no resistant yeast strains have been reported (Roller 2003).

In solution, natamycin is susceptible to degradation by light, peroxide or oxygen. A liquid solution with a concentration of .006 g/ml becomes ineffective against microorganisms after 24 h of light exposure (US. Patent 4,536,494). Natamycin is also somewhat vulnerable to low pH. At a pH of 3.5, only 75% of natamycin activity remains (Davidson and Doan 1993). Along with susceptibility of natamycin to low pH, it also has low solubility, with only 0.050 g/ml soluble in water (Davidson and Doan 1993).

c) Nisin

Nisin is a natural antimicrobial peptide produced as a secondary metabolite by strains of *Lactococcus lactis* ssp. *lactis* that is known to inhibit the growth of Gram-positive and Gram-negative bacteria (Hurst and Hoover 1993). Nisin is a large polypeptide with a molecular weight of ca. 3500 Da (Hurst and Hoover 1993). The primary target of nisin in bacteria is the cytoplasmic membrane, where it causes pore formation and increased permeability (Abbe and others 1995). However, some bacteria possess the enzyme nisinase and are thus resistant to nisin exposure (Abbe and others 1995).

Nisin, the active ingredient in the commercial preservative Nisaplin, has been found effective against *Lactobacillus brevis* subsp. *lindneri* isolated from commercial salad dressing in concentrations as low as 200 mg/L for up to 90 days. (Muriana and Kanach 1995). Additionally, this commercially available bacteriocin inhibited 27 of 30 bacterial isolates that were obtained from 6 different brands of salad dressings (Muriana and Kanach 1995). While nisin has been proven effective against lactic acid bacteria, nisin is ineffective against most yeasts and molds

(Davidson and others 2005). This is exemplified in a study where brewer's yeast (*S. cerevisiae*) was exposed to nisin and no inhibition was demonstrated at the same time lactic acid bacteria were eliminated from the contaminated sample (Ogden 1987). The ineffectiveness of nisin against yeast species would render it ineffectual as an antimicrobial.

d) Sorbate, Sorbic Acid

While sorbic acid (molecular weight 112.13 g/mol) is produced synthetically for use as an antimicrobial food preservative, it also occurs naturally in unripen rowanberry oil. Sorbic acid is used in dairy, vegetable and meat products through dry mixing, spraying or immersion (Sofos and Busta 1993). A hurdle in the use of sorbic acid is its limited solubility with 0.16 g solubilizing in100 ml of water at 20˚C. However, the salt of sorbic acid, potassium sorbate, is much more soluble with 58.2 g being soluble in 100 ml of water (Davidson and Taylor 2007).

Sorbates have been applied as antimicrobial food preservatives in a wide range of foods, particularly to inhibit the growth of yeast and molds, at concentrations of 0.02%-0.30% w/v (Sofos and Busta 1993). Activity of sorbates is dependent on a number of factors including pH, a_w, storage temperature, target microorganisms and composition of food (Chipley 1993). The pH of the media used plays a vital role in the antimicrobial activity of sorbates with most inhibition seen below the pKa of 4.75 as the undissociated molecule is 10-600 times more effective than the dissociated form (Davidson and Taylor 2007). There are believed to be multiple mechanisms of inhibition by sorbate, including inhibition of cell wall division in bacterial spores, preventing spore germination in *Clostridium botulinum,* and interfering with amino acid uptake in vegetative cells (Davidson and Taylor 2007). Sorbic acid has been shown inhibitory against a wide range of yeast and fungi, including *C. krusei,* at 0.1% with higher efficacy seen at lower pH (Bell and others 1959)

e) Benzoate and Benzoic Acid

Benzoates and benzoic acid (122.1 Da) were among the first antimicrobials approved by the US FDA for use in food. While they are made synthetically for application as antimicrobial food preservatives, they can be found naturally in cranberries, and other berries (Davidson and Taylor 2007). This compound is GRAS in foods and beverages up to 1 g/l (21CFR184.1021). Benzoic acid is effective against *E. coli, L. monocytogenes, C. krusei* and *P. membranifaciens* (Chipley 1993).As with sorbates and other organic acids, the antimicrobial activity of benzoates occurs only when the molecule is in undissociated state, and it is therefore more effective in foods with a pH below its pKa of 4.19 (Chipley 1993). Benzoic acid and benzoate compounds are thought to inhibit microorganisms by interfering with the cell membrane permeability or interfering with acetic acid metabolism in some yeasts (Chipley 1993).

Yeast tolerance to benzoates is well documented. While most yeasts exhibit some tolerance to benzoates, *Zygosaccharomyces bailii has* been found to exhibit the highest tolerance to benzoic acid, growing at up to 1200 mg/1 at a pH of 3.0 (Praphailong and Fleet 1997). This resistance has been attributed to the *XYbYME2* gene. This gene codes for benzoate-4-hydroxylase activity, which confers the ability to metabolize benzoic acid (Mollapour and Piper 2001; Piper and others 2001). Along with resistance to benzoates, cells grown in the presence of benzoate may exhibit resistance to other low molecular weight, weak lipophilic organic acids. Strains of *C. krusei, S. pombe* and *Z. bailii* grown in the presence of benzoic acid below their MIC were exposed to various concentrations of acetic acid, propanoic acid, benzoic acid and methyl paraben. All strains tested exhibited resistance to the weak organic acids at the levels listed in table 1.2.

Table 1.2: Resistance to weak organic acid preservatives and methyl-paraben by food spoilage yeasts (Warth 1989)

Organism	FRR Number*	Acetic acid (g/L)	Propanoic acid (g/L)	Benzoic acid (g./L)	Methyl paraben (g/L)
C. krusei	1302	13.5	8.0	.44	1.00
S. pombe	2208	12.5	7.7	.50	.50
S. pombe	2535	16.0	9.3	.567	0.72
Z. bailii	1005	24.0	9.0	.65	1.12
Z. bailii	1426	22.0	9.8	1.2	1.11
Z. bailii	1723	22.0	11.0	1.2	1.23
Z. bailii	2227	19.0	10.8	1.25	.99
Z. bailii	1292	21.0	11.0	1.25	1.11
Z. bailii	1278	25.0	13.5	1.3	1.14

* CSIRO FRR Culture Collection

f) DMDC

Dimethyl-dicarbonate (molecular weight 90.08 Da, Fig. 1.2) is an antimicrobial food preservative approved for use in wines, ready to drink teas, noncarbonated juice beverage, and carbonated dilute beverages containing juice and/or fruit flavor (21 CFR 172.133) up to 200 mg/l. Primarily used to control spoilage yeasts in wine, DMDC has an MIC of 150 mg/l against *Z. bailii* in red wine but requires > 300 mg/l to inhibit *S. pombe* (Costa and others 2008).

DMDC's mechanism is most likely related to the inactivation of enzymes (Davidson and Taylor 2007). Use of DMDC in products such as salad dressing has not been approved by the FDA.

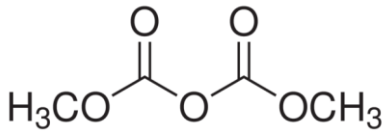

Figure 1.2: DMDC Chemical structure, Molecular weight 90.08 Da

ESSENTIAL OILS AS NATURAL ANTIMICROBIALS

Essential oils (EOs) are complex fragrant compounds derived from plant materials. Typically known for their fragrant and flavoring properties EOs also possess antimicrobial abilities. Both whole plant essential oils and many of the alcohols, ethers, ketones and aldehydes that comprise these oils have well documented antimicrobial properties. Thyme oil, clove bud oil cinnamon bark oil and their components carvacrol, thymol, eugenol, cinnamic acid and cinnamaldehyde have all exhibited antibacterial properties. While the antibacterial effectiveness of EOs has been well documented, little information about their antifungal capabilities is available. Many EOs have been tested against *Saccharomyces cerevisiae* and/or *Candida albicans* but not against other yeasts known to cause spoilage.

1) Types

a) Clove bud oil and Eugenol

Clove bud essential oil and eugenol (2-methoxy-4-[2-propenyl]-phenol, fig 1.3) are extracted from *Eugenia caryophyllata* from the Myrtaceae family. Clove bud oil is comprised of

a number of compounds, many phenolic in nature, with most prevalent constituent being eugenol. Comprising 94.7% of clove bud oil, eugenol is believed to be the most active antimicrobial component (Peter 2001).

Clove bud oil has been shown to inhibit growth of a wide range of bacterial strains. When tested using agar diffusion methods, clove bud oil was shown to inhibit growth of *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and a range of spoilage bacteria (Dorman and Deans, 1999). Using an agar diffusion assay, Conner and Beuchat showed that 10% v/v clove bud oil inhibited growth of 13 different types of spoilage yeasts with an average zone of inhibition of 19 mm (1984).

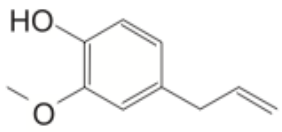

Figure 1.3: Chemical structure of eugenol, molecular weight 164.2 Da (Burt 2004)

The inhibition mechanism of eugenol is likely related to its disruption of cell surfaces. For example, when *Salmonella* Typhi cells were exposed to eugenol, electron micrographs showed severe disruption of the cellular surface (Devi and others 2010). Similar results were found when scanning electron microscopy images were taken of *Saccharomyces cerevisiae* when exposed to eugenol. When *S. cerevisiae* cells were exposed to 3 mM (493 mg/l) eugenol for 1 h, cells were consistently deformed indicating surface damage. This was confirmed by absorbance readings at 260 nm, where release of intracellular components and cellular mortality were seen at a 6 mM (986 mg/l) in 1 h. (Bennis and others 2004)

b) Thyme and Thymol

Thyme can be found growing around the world, with the most common species being *Thymus vulgaris*. Concentrations of thymol (5-methyl-2-[1-methylethyl] phenol, fig 1.4 B) may vary depending on which phase of growth the plant is in when harvested as well as the method used for extraction (steam distillation of supercritical fluid extraction), but can be expected to be between 30-50% v/v of the whole oil (Kunicka-Styzynska 2011; Peter 2001). Thymol can also be found in high concentrations in oregano essential oil.

While thyme oil is an effective antimicrobial in itself, it shows promise as an adjunct to thermal treatments as well. In a study using *Candida lipolytica, Debaryomyces hansenii, Hansenula anomala, Kloeckera apiculata, Lodderomyces elongisporus, Rhodotorula rubra, S. cerevisiae,* and *Torulopsis glabrata,* cells that were heated to 44-54˚C (depending on relative heat sensitivity of each species), showed higher cell mortality when exposed to thyme oil (Conner and Beuchat 1984b). Effectiveness of thyme oil when used alone is showed in a study where thyme oil concentrations as low as 100 µg/ml slowed ethanol production by *Saccharomyces cerevisiae* and *Hansenula anomala* when tested in liquid media (Conner and Beuchat 1984a).

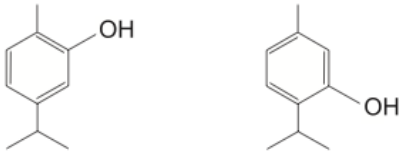

Figure 1.4: Chemical structure of (A) carvacrol, molecular weight 150.217 Da and (B) thymol, molecular weight 150.22 Da (Burt 2004)

Mechanisms of cell inactivation by thymol have been studied on the common baker's yeast, *Saccharomyces cerevisiae*, using both microscopic and molecular methods. Scanning electron microscope images taken of *S. cerevisiae* cells exposed to 270 mg/l concentration of thymol showed severe surface damage. The same study showed that when cells were exposed to 450 mg/l concentrations of thymol, total cell mortality was seen when absorbance was measured at 260 nm (Bennis and others 2004). When microarray studies were done on *S. cerevisiae* exposed to thymol, it was found that thymol affected the expression of a wide variety of genes. Microarray analysis in this study showed that 517 genes were differentially regulated when exposed to thymol. One upregulated gene indicated that exposure to thymol caused the yeast cells to behave as if grown under conditions low in iron (Bi and others 2010).

c) **Carvacrol**

Carvacrol (2-methyl-5-[1-methylethyl] phenol fig 1.4 A) is a phenolic compound that is a major constituent of both thyme and oregano oil. Carvacrol can account for anywhere from 30 to 50% of the whole thyme oil and 40-50% of whole oregano oil, depending on harvest time and extraction methods (Peter 2001) Carvacrol is similar in structure to thymol, with the only variance being the location of a hydroxyl group.

Antimicrobial activity of carvacrol has been widely studied against bacteria. It's efficacy against the foodborne pathogens *Salmonella* Typhimurium, *Staphylococcus aureus, Pseudomonas aeruginosa and E. coli* has been well documented (Davidson and others 2005). However, it has been less extensively studied against spoilage yeasts. In a study completed recently using *Zygosaccharomyces lentus,* it was found that carvacrol was cidal at 225 mg/l in a neutral pH buffer (Roller and Kiskó 2012).

d) Cinnamon bark oil, cinnamaldehyde, and cinnamic acid

Cinnamon is prepared by removing the inner and outer bark from the cinnamon tree, and curled to make a near solid cylinder, or quill. The most common cinnamon tree species are *Cinnamonium zylanicum* and *Cinnamonium aromaticum*. The essential oil makes up 1% (w/w) of the bark. (Peter 2001). As with all essential oils, chemical composition depends on where the plant was grown as well as the time of year it was harvested. Cinnamon bark oil can contain over 30 different volatile compounds (Senanayake and others 1978). Regardless of where cinnamon is grown and harvested, the predominant component is trans- cinnamaldehyde (3-phenyl-2-propenal, fig. 1.5 B), accounting for 60-80% of the oil. Cinnamic acid (trans-3-phenylacrylic acid, fig. 1.5 A) is a lesser component of cinnamon bark EO, accounting for 0.5-3% of the oil.

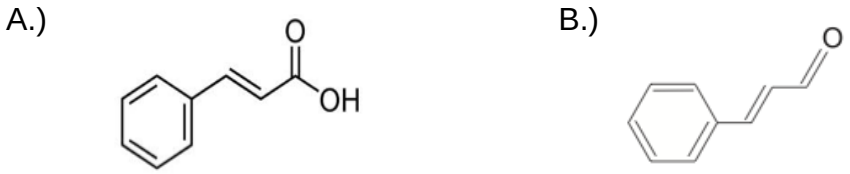

Fig 1.5: A.) Structures of Cinnamic acid (Molecular weight 148.16 Da) and B.) Cinnamaldehyde (Molecular weight 132 Da)

Using an agar diffusion assay, cinnamon bark oil was inhibitory to 13 strains of spoilage yeasts, creating an average zone of inhibition of 18 mm surrounding a concentration of 10% v/v (Conner and Beuchat 1984a). Cinnamaldehyde is also a highly effective antimicrobial, having been found to inhibit growth by interfering with synthesis of ß-(1,3)-glucan as well as synthesis of chitin in *S. cerevisiae* (Bang and others 2000). By interfering with synthesis of both of these compounds, normal yeast cell walls cannot be created, and overall integrity of the cell is

compromised. Trans-cinnamaldehyde has also been proven effective against bacterial pathogens inactivating *Enterobacter* (now *Cronobacter*) *sakazakii* in contaminated infant formula (Amalaradjou and others 2009).

Cinnamic acid (pK_a 4.55) has also been shown to be effective against growth *S. enterica* and *E. coli* in apple cider and orange juice. During a 7-day sampling period, 400 and 1,000 mg/l reduced the amount of pathogen present (Truong and others 2010; Zheng and others 2013). Cinnamic acid has been shown less effective against spoilage bacteria, with no zone of inhibition seen surrounding 13 of 15 produce spoilage bacteria (Zheng et. al. 2013). However, solubility may be an issue when using cinnamic acid, with only 0.496 g dissolving into one liter of distilled water at 25˚C. (Truong 2007). Along with concerns about the solubility of cinnamic acid, very little research has been done utilizing cinnamic acid as an antifungal.

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CHAPTER II
MINIMUM INHIBITORY CONCENTRATION OF ESSENTIAL OILS AND COMPONENTS

Abstract

Clove bud, cinnamon bark, and thyme oil, along with their components cinnamaldehyde, cinnamic acid, eugenol, carvacrol, and thymol, have potential as antimicrobial compounds. In this study the antimicrobial activity against the spoilage yeasts *Torulaspora delbrueckii, Candida krusei, Schizosaccharomyces pombe,* and *Zygosaccharomyces bailii* was evaluated and a minimum inhibitory concentration (MIC) was determined. The MIC against all yeasts for cinnamaldehyde and cinnamon bark oil was 50 mg/l. For thymol, thyme oil and carvacrol, the MICs were 200, 400, and 200 mg/l, respectively. *T. delbrueckii* and *C. krusei* required 300 mg/l to achieve an MIC for clove bud oil, and *S. pombe and Z. bailii* had an MIC of 200 mg/l. Eugenol had a MIC of 300 mg/l for *T. delbrueckii,* and *C. krusei* and an MIC of 200 mg/l for *Z. bailii* and *S. pombe.* Cinnamic acid had an MIC of 500 mg/l for *Candida krusei* and *T. delbrueckii*, 400 mg/l for *S. pombe,* and 200 mg/l for *Z. bailii.* Allyl-isothiocyanate had an MIC greater than 7,500 mg/l for all organisms.

Practical Application: Essential oils provide a natural alternative to control of spoilage yeasts in food products. Essential oils and their components have demonstrated a wide range of antibacterial and antifungal activity, and could be more acceptable to consumers than synthetic preservatives.

Introduction

Yeasts are known to contaminate all types of foods but they are particularly suited to growth in foods with high concentrations of sugar and organic acids or products with lower water activity and lower pH (Fabian and Wethington 1950; Thomas and Davenport 1985; Fleet 1992; Tokuoka 1993). When yeasts grow in foods they can cause spoilage mostly through production of gas (carbon dioxide) and other end-products. Thus, spoilage yeasts frequently leads to the decreased shelf-life that is costly to manufacturers and consumers. Control methods for yeasts may include both physical (heat) and chemical (antimicrobial food preservatives) treatments, but these methods have limitations. For example, the high temperatures required to eliminate bacteria may damage quality of some products, such as emulsified salad dressings. Traditional chemical preservatives, such as benzoic acid, sorbic acid and other frequently used organic acids, have their own limitations. A number of common spoilage yeasts have either an acquired resistance or natural resistance to these weak organic acids (Warth 1989). However, there has been a move by the industry to investigate the use of "naturally occurring" compounds and extracts as antimicrobials including the spice essential oils as a solution to these issues. These compounds are generally recognized as safe (GRAS) when used as flavoring agents. Incorporating EOs, their active components, or related compounds has potential to serve as a secondary barrier to spoilage yeasts.

The antimicrobial activity of spice essential oils (EOs) and their components have been studied extensively against bacteria and have been demonstrated to be effective at reducing or preventing bacterial growth in a number of products (Peter 2001; Kalemba and Kunicka 2003). EOs from thyme, clove, oregano and cinnamon have been consistently shown to be effective bacterial inhibitors, which is often attributed to their components thymol, eugenol, carvacrol and

cinnamaldehyde, respectively. However, effects of EOs on spoilage yeasts have not been as thoroughly studied.

The objective of this research was to determine the minimum inhibitory concentration of EOs and their active chemical components, along with cinnamic acid and allyl-isothiocyanate, against four spoilage yeasts known to contaminate high acid food.

Materials and Methods

Culture

Zygosaccharomyces bailii, Candida krusei, and *Schizosaccharomyces pombe* were obtained from commercial ketchup samples and *Torulaspora delbrueckii* obtained from laboratory stock. *T. delbrueckii,* and *C. krusei* were incubated for 24 h in 10ml of Yeast Extract Peptone Dextrose broth (YEPD, Difco, Sparks, MD). *S. pombe* was incubated for 24 h in Yeast Extract Glucose broth (YEG) and *Z. bailii* was incubated for 48 h in YEPD broth. All cultures were transferred 3 times prior to use and incubated at 32˚C on a shake incubator. Cells were serially diluted in 0.1% peptone (Difco, Sparks, MD) to a 10^4 concentration prior to use in antimicrobial assay.

Antimicrobials

Cinnamon bark oil, clove bud oil, thyme oil, carvacrol, cinnamic acid, trans-cinnamaldehyde, eugenol, thymol and allyl-isothiocyanate were obtained from Sigma-Aldrich (St. Louis, MO). All oils and components were greater than or equal to 98% purity, with the exception of allyl-isothiocyanate which was no less than 93% purity. Essential oils and components were added to tubes in 50-500 mg/l using pure oil for all but thymol and cinnamic acid, which was added using 10% w/v stock solutions dissolved in EtOH. A 500 mg/l EtOH control was used to ensure that it did not influence inhibition.

Antimicrobial Assay

The antimicrobial activity of the compounds was evaluated using a modified agar dilution assay (Mann and Markham 1998). Yeast Extract Glucose (YEG) Agar was prepared by combining 30 g Glucose with 5 g Difco Yeast Extract (Sparks, MD) and sterilized by autoclaving at 121˚C for 15 min. 50 ml was then dispensed into sterile dilution bottles and essential oil concentrations added. Essential oils and agar were combined by mechanical agitation, allowed to cool and then poured into sterile petri plates. Plates were allowed to stand in the dark overnight to allow oils to evenly disperse through agar and then refrigerated to until use.

Essential oil plates were spot inoculated with 10µl of 10^4 concentrations of cells in triplicate. Experiments were performed in triplicate. Plates were incubated at 32˚C and observed over 7 days to determine the minimum inhibitory concentration (MIC), which was defined as the lowest essential oil concentration in which no growth was seen.

Results and Discussion

Of the three phenolic based essential oils (cinnamon, clove, thyme), five essential oil components (thymol, carvacrol, eugenol, trans-cinnamaldehyde, cinnamic acid) and one sulfur based isothiocyanate from mustard (allyl-isothiocyanate) evaluated, cinnamon bark oil and trans-cinnamaldehyde were the most effective with inhibition of all yeast strains at 50 mg/l (Table 2.1). Clove bud oil and its primary component eugenol were equally effective, inhibiting growth of *T. delbrueckii* and *C. krusei* at 300 mg/l, and *S. pombe* and *Z. bailii* at 200 mg/l. Carvacrol and thymol were twice as effective on a concentration basis as the complete essential oil, thyme oil. They inhibited growth of all species tested at 200 mg/l, while thyme oil required 400 mg/l. The phenolic acid cinnamic acid was slightly less effective on a concentration basis against *T. delbrueckii*, *C. krusei*, and *S. pombe* than the essential oils or EO components inhibiting growth

at 500 mg/l, 500 mg/l and 400 mg/l, respectively. However, it was equally as effective as clove bud oil, eugenol, carvacrol, and thymol against *Z. bailii*. The mustard component, allyl-isothiocyanate was the least effective compound with no inhibition of any of the tested yeast strains by concentrations > 7,500 mg/l. Finally, no inhibition was seen in the ethanol control.

The efficacy of cinnamon bark oil and clove bud oil components over the efficacy of the whole oil can be attributed to the components comprising the majority of the oil. This is best exhibited with the cinnamon bark oil. Trans-cinnamaldehyde can be anywhere from 60-80% of the total composition of cinnamon bark oil and was as effective as the whole oil on a concentration basis, while cinnamic acid is a lesser component and was less effective on a concentration basis as the whole oil, 0.5-4% of the whole oil (Senanayake and others 1978; Ooi and others 2006). The same is true for clove bud oil and eugenol with eugenol making up 94% of clove bud oil and was equally effective as the whole oil. The exception to this is thyme oil and its components carvacrol and thymol. Thymol makes up 44.6% of thyme oil while carvacrol is present at 4.6% (Kunicka-Styzynska, 2011). Therefore, when used at the same concentrations, the individual components were present at 2 to 20 times higher than what was in the oil and they were in fact twice as effective as the whole oils.

Thyme oil, clove oil and cinnamon oil have been shown to be active against *Staphylococcus aureus, E. coli, Pseudomonas aeruginosa, Klebsiella pneumonia, Enterococcus faecalis, Listeria monocytogenes* and a number of other pathogenic and spoilage bacteria using broth dilution and agar diffusion assays (Kalemba and Kunicka 2003; Peter 2001; Zaika 1988). However, in depth research on fungal inhibition has not extended beyond the common baker's yeast, *Saccharomyces cerevisiae*. When *S. cerev*isiae cells were exposed to either 493 mg/l of eugenol, 270 mg/l of thymol or 2000 mg/l v/v of clove bud oil for 1 h, damage to and

deformation of the outer surface of the yeast cell was seen (F. Chami 2005; Bennis and others 2004). Cell surfaces were severely impacted with misshapen and cracked cell surfaces observed using scanning electron microscopy. Further experiments in these studies used 260nm spectrophotometry to observe leakage of intracellular contents, which is believed to be the cause of cell mortality. Conner and Beuchat tested 10% v/v cinnamon, thyme, clove and oregano oil against 13 genera of yeast using an agar diffusion assay and found the most effective were cinnamon (18 mm zone), clove (19 mm zone), and thyme (23 mm zone (1984). Hammer et al. tested thyme and clove oil against *Candida albicans* and found MICs of 0.03% and 0.12% v/v, respectively using an agar dilution method similar to that used in the present study (1999). MICs of 330 mg/l for clove bud oil, 295 mg/l concentrations of eugenol and 270 mg/l concentrations of thymol were found for *S. cerevisiae* in a 0.2% w/v agar-broth medium (Bennis and others 2004; F. Chami 2005)

Allyl-isothiocyanate has been found to be an effective bactericide in gaseous form against *E. coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium, *Pseudomonas corrugata*, *Penicillium expansum*, *Aspergillus flavus* and *Botrytis cinerea* at 100-1,000 µg/l (Delaquis and Sholberg 1997). However, the highly volatile nature of allyl-isothiocyanate makes incorporation into an agar medium like the one used in our study difficult. Also, studies have also shown degradation of the compound occurs in liquid medium. This degradation renders allyl-isothiocyanate ineffective as an antimicrobial (Luciano and Holley 2009).

In the present study, cinnamic acid was found to inhibited growth of *T. delbrueckii* and *C. krusei* at 500 mg/l, *S. pombe* at 400 mg/l and *Z. bailii* at 200 mg/l. Very few studies have been completed using cinnamic acid as an antifungal, however studies have shown it to be effective against the pathogens *S. enterica* and *E. coli* at 400-1000 mg/l (Truong and others 2010; Zheng

and others 2013). While the results of our study show potential for cinnamic acid as an antifungal, the relatively low solubility of cinnamic acid, 0.496 g/l, could be problematic for inhibiting organisms with MIC values above that concentration (Truong 2007).

The yeast strains in this study are all frequently known to contaminate high acid as well as high sugar containing food products. These species have been found in products ranging from ketchup, beer, wine salad dressing, fruit juices and soft drinks (Loureiro 2003; Fleet 1992). Studies have shown that intrinsic factors such as pH, food components (protein, lipid, and cations), water activity and other components with in the food matrix could interfere with the antimicrobial activity of essential oils (Zaika 1988). While this study shows that essential oils have the ability to inhibit growth of spoilage yeasts, further studies need to be done to prove their efficacy in a food matrix.

Conclusions

Cinnamon bark oil, clove oil, and thyme oil, as well their components carvacrol, eugenol, thymol, and cinnamaldehyde, show potential for antimicrobial application in foods. The low MIC values of these compounds are promising, as there is likely to be less issue with flavor compatibility in food products. Since the oil components were capable of inhibiting growth at lower concentrations, they show more promise for use in foods than whole oils. Allyl-isothiocyanate has very little potential for use in liquid form.

Table 2.1 Minimum inhibitory concentrations in mg/l of essential oils and components for spoilage yeasts

Essential Oil or Oil Component	*T. delbrueckii*	*C. krusei*	*S. pombe*	*Z. bailii*
Spice Essential Oils				
Cinnamon Bark Oil	50	50	50	50
Clove Bud Oil	300	300	200	200
Thyme Oil	400	400	400	400
Spice Essential Oil Components				
Cinnamaldehyde	50	50	50	50
Eugenol	300	300	200	200
Carvacrol	200	200	200	200
Thymol	200	200	200	200
Cinnamic Acid	500	500	400	200
Mustard Component				
Allyl-isothiocyanate	>7500	>7500	>7500	>7500

[a]Defined as lowest concentration of oil used in which no growth was seen

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CHAPTER III
EFFICACY OF ESSENTIAL OIL COMPONENTS AS ANTIMICROBIALS IN SALAD DRESSING MODEL

Abstract

Essential oil components are widely acknowledged to have antimicrobial properties against bacteria. However, less is known about the inhibitory properties of essential oil components against spoilage yeasts in a food matrix. The efficiency of the essential oil components carvacrol, cinnamaldehyde, eugenol and thymol was evaluated against four common spoilage yeasts *Torulaspora delbrueckii, Candida krusei, Schizosaccharomyces pombe* and *Zygosaccharomyces bailii.* In a model salad dressing, 4 log CFU/ml of yeast cells were added to 39.0±0.5g of salad dressing with various concentrations of essential oils and the products were incubated at 22˚C. Samples were taken at 0, 6, 12, 24, 48, 72, 96 and 120 h. Cinnamaldehyde at a 500 mg/l concentration was the most effective at inhibiting *S. pombe* and *Z. bailii*. *T. delbrueckii* and *C. krusei* used in this study were unable to grow in the model salad dressing used during the timeframe of the study.

Practical Application: Spoilage yeast cost the food industry a substantial amount of money every year. Because of their resistance to traditional food antimicrobials, alternatives to these are being sought out. Along with the increasing microbial resistance, consumers also have a negative idea of traditional antimicrobials. Essential oils could provide a consumer friendly, effective alternative to control of spoilage organisms.

Introduction

The role of yeasts in the production of commercial cheese, bread, beer, wine and other products is very well known. However, the same species of yeast used intentionally in the production of many foods, can be a nuisance when they contaminate other products. The ability of yeasts to produce ethanol and carbon dioxide in anaerobic conditions can have adverse effects on quality of food products and the integrity of food packaging. The ubiquitous nature of yeast can make contamination prevention very difficult, thus the food manufacturing industry relies on other control methods such as heat and chemical food preservatives.

The trend in healthy eating has boosted the popularity of fresh produce and thus pourable salad dressings; with cumulative dressing sales in 2012 exceeding $1.5 billion and 80% of households consuming a mayonnaise-based salad dressing (Anonymous, 2012). Increased consumption of these products has made maintaining a high product quality more important than ever. Unfortunately, salad dressings are among the many foods which yeast can contaminate and grow (Fabian and Wethington 1950; Kurtzman and others 1971). Typically the high acid ($pH<4.5$) of salad dressings is relied on to prevent the growth of spoilage and pathogenic bacteria (Smith and Stratton). However, high acid environments are of no consequence to many strains of yeast with some species surviving pH as low as 3.0 (Deák 2008). Using heat as a method to control microbial populations also presents complications, as temperatures required to eliminate fungal and bacterial contamination can degrade the emulsions present in many salad dressing. Thus, manufacturers must rely on other methods to prevent growth of spoilage organisms.

Some of the most common antimicrobials used in salad dressing are benzoates, sorbates, sorbic acid and acetates. Activity of these preservatives is dependent on a range of factors including the pH, water activity, storage temperature and composition of the food. These

antimicrobials are more effective at a low pH, making them popular for use in salad dressings. However, many yeast possess genetic or acquired resistance mechanisms to these weak organic acids including the ability to metabolize these compounds as well as the ability to pump out dissociated anions (Dawidowicz and Rado 2010 ; Mollapour and Piper 2001; Piper and others 2001). With this in mind, research has turned to alternative antimicrobials.

One potential solution to this resistance is the use of spice essential oils and their components, which have long been known to have antimicrobial properties (Peter 2001; Zaika 1988). A variety of essential oils have been proven effective against both Gram-positive and Gram-negative spoilage and pathogenic bacteria (Peter 2001; Zaika 1988; Hammer and others 1999; Burt 2004). However, their efficacy against yeasts is not as well documented. *In vitro* studies have shown 493 mg/l eugenol, carvacrol and clove bud oil to be effective at eliminating *Saccharomyces cerevisiae* (Bennis and others 2004; F. Chami 2005). Thymol has also been shown as an effective inhibitor of the spoilage yeast *Debaryomyces hansenii*, with an MIC of 100-125mg/l when tested in a broth dilution assay (Curtis and others 1996). Studies have shown that the main constituent of cinnamon bark oil, trans-cinnamaldehyde, interferes with the synthesis of the *Saccharomyces cerevisiae* cell wall leading to cell death (Bang and others 2000). While there are some studies showing efficacy of essential oil components in agar and broth mediums, very little is known about the efficacy of these essential oil components against spoilage yeasts when incorporated into a food matrix.

The objective of this study was to evaluate the efficacy of carvacrol, cinnamaldehyde, eugenol and thymol against the spoilage yeasts *Torulaspora delbrueckii, Candida krusei, Schizosaccharomyces pombe* and *Zygosaccharomyces bailii* in a model salad dressing.

Materials and Methods

Yeast

Torulaspora delbrueckii, Candida krusei, and *Zygosaccharomyces bailii* were revived from frozen stock in Yeast Extract Peptone Dextrose (YEPD, Difco, Sparks, MD) agar. *Schizosaccharomyces pombe* was revived in yeast extract glucose agar (YEG) created by combining 30 g glucose with 5 g yeast extract (Difco, Sparks, MD). *T. delbrueckii, C. krusei* and *S. pombe* were incubated for 24 h, and *Z. bailii* for 48 h. All cultures were incubated at 32˚C in a shaking incubator and transferred three times prior to use. *C. krusei, Z. bailii,* and *S. pombe* were obtained from spoiled commercial ketchup while *T. delbrueckii* was a laboratory stock strain. Cultures were incubated to a stationary phase, washed and re-suspended in peptone water. Then 40µls of washed cells were added to 39.0 ± 0.5g of salad dressing. Samples were then thoroughly mixed, shaking by hand for 60s.

Model Salad Dressing

The salad dressing model was based on that used by Yang et al. (Yang and others 2003). All ingredients used were purchased at a local grocery store. Dressing was prepared by aseptically combining 281 g sterile DI water, 160 g corn syrup, 76 g distilled white vinegar, 46 g cornstarch, 22 g sucrose, and 15 g salt over heat until starch paste was formed. Then, 54 g egg yolk, 312 g soybean oil, 30 g sterile DI water and 76 g white vinegar were whipped together using a sterile mixer to form a mayonnaise base. The starch paste was allowed to cool to room temperature and then whipped together with the mayonnaise base to form dressing with a final volume of 1.2 L. Final pH of salad dressing was 3.3 ± 0.2 and was adjusted to pH of 4.2 ± 0.2 using 5N NaOH.

Antimicrobials

Carvacrol, trans-cinnamaldehyde, eugenol, and thymol were obtained from Sigma-Aldrich (St. Louis, MO) and were greater than or equal to 98% purity. The concentrations of essential oils component to use was determined by increasing the previously determined minimum inhibitory concentrations (MIC) by a factor of 10 to attempt to overcome interference from the food matrix. Carvacrol, eugenol and cinnamaldehyde were solubilized in 95% ethanol to create a 10% v/v stock solution and thymol was dissolved in 95% ethanol to create a 10% w/v solution. The component volumes listed in Table 3.1 were added to 39.0±0.5g of salad dressing and combined by hand shaking for 30 seconds. Sorbic and benzoic acid were obtained from Sigma-Aldrich (St. Louis, MO). These compounds were dissolved in the vinegar and water used to create starch paste prior to addition of other ingredients. Starch paste was then combined with mayonnaise base using sterile mixer. Sorbic acid was added at 0.1% and 0.3% w/v while benzoic acid was added at 0.1% and 0.05% w/v.

Sampling and Controls

Samples were removed and the population of yeast determined by spread-plating at 0, 6, 12, 24, 48 and 72 h for *S. pombe* and *Z .bailii* with two additional time points at 96 and 120 h for the latter. Samples were plated on YEG agar and incubated at 32˚C. Uninoculated controls were used to insure that salad dressing was free of microbial contaminants. Also, ethanol was added at 2,000 mg/l concentration to evaluate interference with microbial growth caused by ethanol. All experiments were done in triplicate (n=3). Results were analyzed using SAS (9.3, Cary, NC) Mixed Model Analysis of Variance with the GLIMMEX procedure ($p<.05$) and LSD.

Results and Discussion

S. pombe and *Z. bailii* were the only two yeast that grew in the salad dressing under the experimental conditions, increasing from 4.6 and 4.4 log CFU/ml to 6.8 and 6.7 log CFU/ml after 72 and 96 h, respectively (Table 3.2, 3.3). Ethanol had a significant ($p<0.05$) inhibitory effect on both yeast but the difference was ≤ 1 log CFU/ml at all test times. For *S. pombe,* 2,000 mg/l carvacrol or thymol caused significant ($p<0.05$) inhibition after 24 h incubation but the difference between the controls and treatments was ≤ 1 log CFU/ml. Eugenol at 2,000 mg/l extended the lag phase of *S. pombe* to 48 h but there was no difference between the eugenol and the ethanol control by 72 h. At 48 and 72 h, cinnamaldehyde was significantly more inhibitory than the other components tested with final cell count at 72 h decreased to 3.85 log CFU/ml from 4.71 log at time 0. In comparison to the ethanol controls, there was no significant impact on the growth of *Z. bailii* by carvacrol, eugenol and thymol at 2,000 mg/l. However, cinnamaldehyde at 500 mg/l was significantly inhibitory ($p<0.05$), with a 1-1.2 log difference seen between it and the ethanol control at 48-96 h.

Benzoic acid at both 0.05% and 0.1% were both equally effective at inhibiting growth of *S. pombe,* and the same was true for 0.1% and 0.3% sorbic acid. Comparing sorbic acid at 0.01% and 0.03% and cinnamaldehyde at 500 mg/l, equal inhibition of *S. pombe* was seen, total cell counts at 72 h decreasing approximately 0.5 log CFU/ml from time 0. Cinnamaldehyde at 500mg/l also performed as well as 0.05% benzoic acid at controlling the growth of *Z. bailii.* However, 0.01% benzoic acid and 0.1% sorbic acid were more effective at controlling *Z. bailii* growth than the thymol, carvacrol and eugenol at 2,000 mg/l. Of all the compounds tested, sorbic acid at 0.3% caused the most *Z. bailii* inhibition with total cell counts decreasing slightly from 4.54 to 4.41 log CFU/ml over the course of incubation.

Overall antimicrobial activity of essential oil components was reduced compared to that in microbiological media most likely because of interference by the lipids present, as the model salad dressing was approximately 35% soybean oil. Interference with lipophilic antimicrobials by oil is well documented in literature. Gutierrez and others showed that high concentrations of sunflower oil have a negative impact on the antimicrobial activity when thyme oil was tested against *Listeria monocytogenes* (Gutierrez and others 2008). Glass and Johnson executed a study observing the effect of fat on antibotulinal activity of several lipophilic food preservatives, including nisin and lysozyme, using 0%, 10% and 20% milk-fat and liquid soybean oil. They found that the effect of nisin was diminished, with optical density (OD_{640}) increasing to from 0.3 in the presence of nisin only to 0.7 and 0.75 in the presence of 20% milk fat and 20% soybean oil respectively (Glass and Johnson 2004). Similar studies showed decreased efficacy of clove and cinnamon bark oil against *Listeria monocytogenes* in whole milk (3.5% milk fat) over semi-skimmed milk (1.5% milk fat), where a 500 mg/l concentration of cinnamon bark oil kept microbial growth stationary in whole milk, and reduced microbial population by 1 log in skim milk, (Cava and others 2007). This study also shows, clove bud oil was also more effective at inhibiting growth of *L. monocytogenes* in skim milk than whole milk. At 2,000 mg/l, approximately one log reduction in skim milk was seen in 4 days, while a nearly one log increase was seen in whole milk during the same time period.

The presence of starch in the model salad dressing may also have interfered with the efficacy of the essential oil components used. Starch accounted for approximately 4.6% of the overall makeup of the salad dressing used in the study. There are conflicting studies as to the ability of starch to interfere with essential oil efficacy. Gutierrez and others studied the effects of thyme and oregano oil in the presence of potato starch and found that the lag phase of *L.*

monocytogenes decreased when grown in the presence of either 5% or 10% (w/v) starch and both oregano and thyme oil, but efficacy of the oils was enhanced in the presence of 1% starch increasing the length of the lag phase (Gutierrez and others 2008). Starch was also found to interfere with the efficacy of chitosan against the spoilage yeast *Candida lambica*. At 1% w/v water-soluble starch was found to have no impact on the efficacy of chitosan inhibiting yeast growth, however at 30% w/v, activity of chitosan was significantly decreased, with a shorter lag phase and increased growth rate of the yeast in a broth medium. (Devlieghere and others 2004)

The results of this study show that essential oil components could effectively inhibit growth of spoilage yeasts in salad dressing if added at higher concentrations. However, sensory properties should be taken into consideration. Little research has been done to evaluate the palatability of essential oil components in foods similar to the salad dressing used in this study. When applied to fresh cut fruits (apples pears, grapes, peaches and melons) carvacrol, and cinnamic acid had no effect on sensory attributes at concentrations ≤ 0.015% (v/w) and whole cinnamon oil did not affect sensory attributes below 0.7% (v/w) (Raybaudi-Massilla and others 2009). Palatability of essential oils has been much more thoroughly researched in fish and meat products. Thyme oil applied as a coating for cooked shrimps was not found unfavorable when applied in a 0.9% (v/w), but was found unacceptable at 1.8% (v/w) concentration (Burt 2004). Oregano oil at 0.8% (v/v) was found acceptable on surface treated beef fillets after storage at 5˚C and cooking (Tsigarida and others 2000). A study performed by Ifesan et al. (2009) examined the palatability of *Eleutherine americana* crude extract, commonly used in Thai cuisine, when incorporated into salad dressing. Nineteen trained panelists in this study found that incorporation of this crude oil had no significant impact on taste or overall acceptability. This study shows that

depending on the composition of the salad dressing, components may be highly compatible to the overall flavor of the dressing.

Conclusion

This study indicates that essential oil components have the potential to be effective inhibitors of spoilage yeasts in salad dressings. These components have the potential to be as effective as traditional antimicrobials currently used in in food manufacturing.

Table 3.1: Maximum concentrations of essential oils added to salad dressings.

EO Component	Concentration (in mg/l)	Volume 10% Solution added
Thymol	2,000	800 µl
Eugenol	2,000	800 µl
Carvacrol	2,000	800 µl
Cinnamaldehyde	500	200 µl

Table 3.2: Growth of *Schizosaccharomyces pombe* in model salad dressing with essential oil components and traditional antimicrobials over time

	Hour					
	0	6	12	24	48	72
Control	4.61± 0.09[a] A[b]	4.45 ± 0.04 AB	4.54 ± 0.17 A	5.07 ± 0.19 A	5.88 ± 0.24 A	6.76 ± 0.25 A
0.05% Benzoic Acid	4.54 ± 0.22 A	4.51 ± 0.25 AB	4.40 ± 0.02 ABC	4.41 ± 0.12 BC	4.51 ± 0.16 CD	4.54 ± 0.12 C
0.1% Benzoic Acid	4.49 ± 0.18 A	4.39 ± 0.17 AB	4.47 ± 0.23 ABC	4.38 ± 0.32 BC	4.21 ± 0.10 DE	4.41 ± 0.04 C
0.03% Sorbic Acid	4.59 ± 0.15 A	4.41 ± 0.04 AB	4.43 ± 0.41 ABC	4.42 ± 0.12 BC	4.15 ± 0.19 E	3.77 ± 0.07 D
0.1% Sorbic Acid	4.57 ± 0.14 A	4.54 ± 0.10 A	4.20 ± 0.02 C	4.20 ± 0.19 C	3.74 ±0.34 F	3.72 ± 0.22 D
Ethanol Control	4.57 ± 0.17 A	4.5 ± 0.14 A	4.53 ± 0.12 AB	4.42 ± 0.00 BC	5.83 ± 0.46 A	6.05 ± 0.02 B
Carvacrol 2,000 mg/l	4.64 ± 0.14 A	4.49 ± 0.21 AB	4.54 ± 0.14 A	4.63 ± 0.07 B	5.43 ± 0.06 B	6.18 ± 0.05 B
Cinnamaldehyde 500 mg/l	4.71 ± 0.11 A	4.22 ± 0.20 B	4.22 ± 0.21 BC	4.17 ± 0.02 C	4.03 ± 0.12 EF	3.85 ± 0.13 D
Eugenol 2,000 mg/l	4.78 ± 0.11 A	4.4 ± 0.16 AB	4.18 ± 0.22 C	4.43 ± 0.06 BC	4.54 ± 0.70 C	5.96 ± 0.24 B
Thymol 2,000 mg/l	4.61 ± 0.00 A	4.43 ± 0.13 AB	4.37 ± 0.22 ABC	4.52 ± 0.04 B	5.35 ± 0.08 B	6.16 ± 0.09 B

[a]Standard deviations are indicated in log CFU/ml

[b]Different letters within columns are significantly different ($p \leq 0.05$)

Table3.3: Growth of *Zygosaccharomyces bailii* in model salad dressing with components and traditional antimicrobials over time

	Hour						
	0	6	12	24	48	72	96
Control	4.44 ± 0.11[a] A[b]	4.46 ± 0.08 AB	4.44 ± 0.47 BCD	5.45 ± 0.48 A	6.47 ± 0.11 A	6.67 ± 0.12 A	6.67 ± 0.42 A
0.05% Benzoic Acid	4.43 ± 0.26 A	4.50 ± 0.30 A	5.21 ± 0.12 A	5.42 ± 0.25 A	5.57 ± 0.32 D	5.54 ± 0.16 C	5.83 ± 0.08 C
0.1% Benzoic Acid	4.52 ± 0.33 A	4.54 ± 0.24 A	4.79 ± 0.21 B	4.3 ± 0.19 BCD	4.97 ± 0.48 D	5.56 ± 0.36 C	5.71 ± 0.07 CD
0.3% Sorbic Acid	4.54 ± 0.29 A	4.39 ± 0.31 AB	4.33 ± 0.37 CD	4.08 ± 0.40 D	4.09 ± 0.13 E	3.96 ± 0.15 E	4.14 ± 0.04 F
0.1% Sorbic Acid	4.65 ± 0.49 A	4.27 ± 0.23 AB	4.12 ± 0.31 D	4.24 ± 0.42 CD	4.24 ± 0.42 E	4.63 ± 0.09 D	4.87 ± 0.05 E
Ethanol Control	4.26 ± 0.32 A	4.10 ± 0.20 B	4.56 ± 0.11 BC	4.61 ± 0.60 BC	5.97 ± 0.26 B	6.24 ± 0.25 B	6.45 ± 0.13 AB
Carvacrol 2,000 mg/l	4.43 ± 0.05 A	4.5 ± 0.09 A	4.43 ± 0.09 BCD	4.56 ± 0.16 BC	5.5 ± 0.12 BC	6.21 ± 0.20 B	6.34 ± 0.08 AB
Cinnamaldehyde 500 mg/l	4.45 ± 0.06 A	4.50 ± 0.06 A	4.37 ± 0.08 CD	4.53 ± 0.04 BC	4.76 ± 0.51 D	5.28 ± 0.44 C	5.44 ± 0.35 D
Eugenol 2,000 mg/l	4.46 ± 0.08 A	4.38 ± 0.09 AB	4.46 ± 0.02 BCD	4.35 ± 0.17 BCD	5.51 ± 0.31 BC	5.96 ± 0.19 B	6.29 ± 0.19 B
Thymol 2,000 mg/l	4.50 ± 0.03 A	4.49 ± 0.10 A	4.39 ± 0.08 CD	4.65 ± 0.34 B	5.46 ± 0.34 C	6.31 ± 0.26 AB	6.40 ± 0.03 AB

[a]Standard deviations are indicated in log CFU/ml

[b]Different letters within columns are significantly different ($p \leq 0.05$)

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CONCLUSION

The low MIC values for cinnamon bark oil, clove oil, and thyme oil as well as their carvacrol, eugenol, thymol and cinnamaldehyde, show potential for antimicrobial application in foods. Because of the low MIC values, there is likely to be less issue with flavor compatibility in food products. Since the oil components inhibited growth at lower concentrations, they show more promise for use in foods than whole oils. Allyl-isothiocyanate, in liquid form, has very little potential for use in foods as the MIC values are high.

When incorporated into a salad dressing food model, efficacy of the essential oil components thymol, carvacrol and eugenol are decreased at the concentrations tested. However, cinnamaldehyde remains an effective inhibitor of yeast growth in the food model. Essential oil components have the potential to be as, or more effective than the traditional antimicrobials sorbic and benzoic acid when tested in a salad dressing model.

APPENDIX

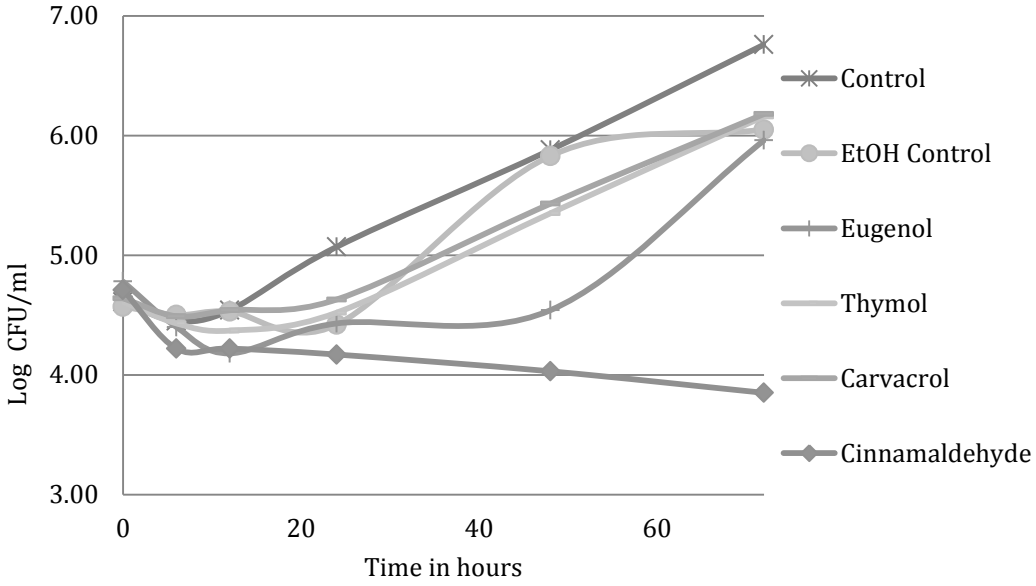

Figure A1 :Effect of Essential Oil Components in Salad Dressing Model- *Schizosaccharomyces pombe*

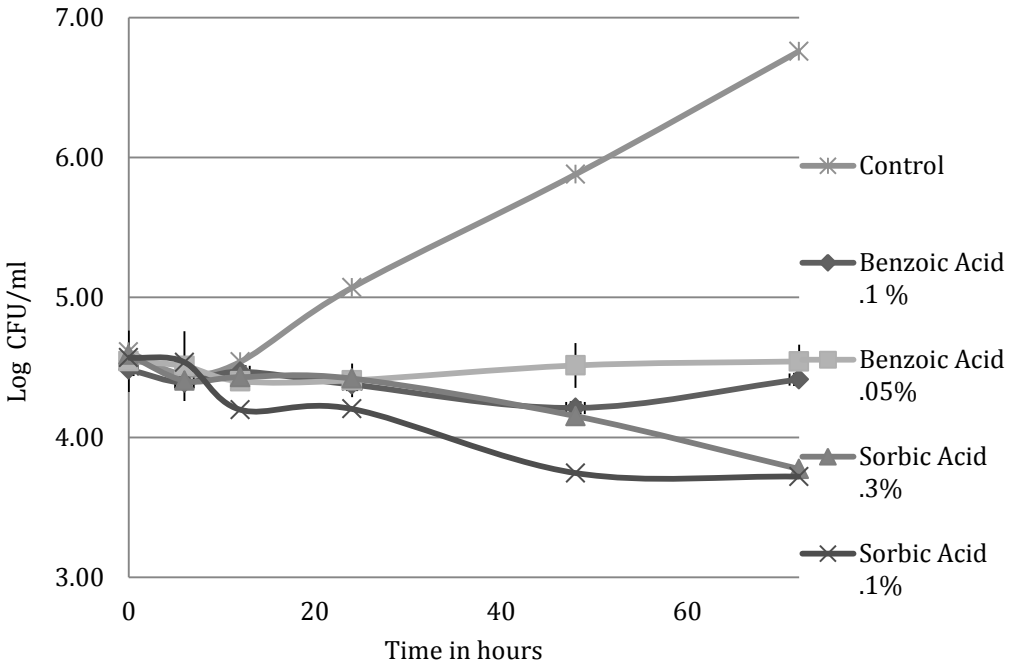

Figure A2: Effect of Traditional Antimicrobials in a Salad Dressing Model- *Schizosaccharomyces pombe*

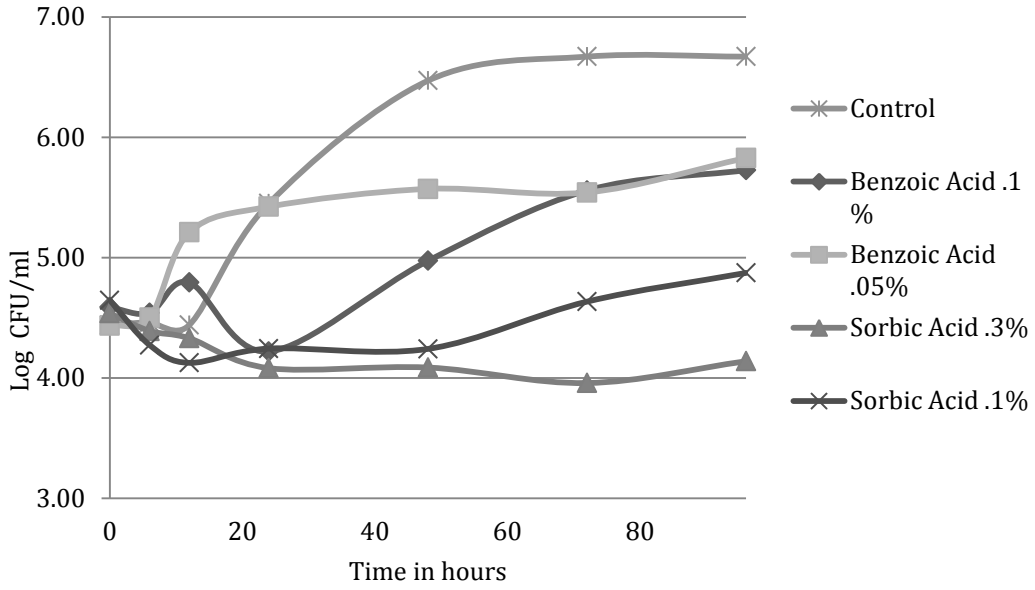

Figure A3: Effect of Traditional Antimicrobials in Model Salad Dressing -*Zygosaccharomyces bailii*

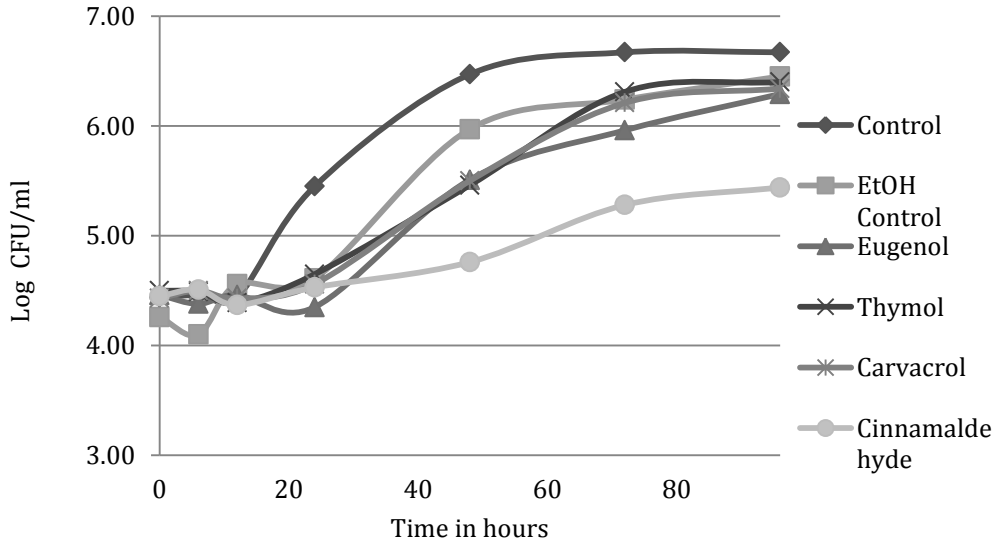

Figure A4: Effect of Essential Oil Components in a Salad Dressing Model
-Zyogosaccharomyces bailii

VITA

Audra A. Wallis was born in 1990 in Cleveland, TN, and is the daughter of Rex and Sandy Wallis. In 2008 Audra followed her older brother Alex to the University of Tennessee where she majored in Food Science and Technology. Audra completed her undergraduate degree in 2012 and immediately began to pursue a masters. Upon completion of her masters in Food Science and Technology with an emphasis in Microbiology, she plans to move to Logan, Utah where she will be a production team advisor for Schreiber Foods.