Use of plant-derived essential oil compounds and naturally-occurring apple flavor compounds to control foodborne pathogens in apple juice

Takiyah Oniya Abdulmalik

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
in
Food Science and Technology

Renee R. Boyer, Chair
Joshua B. Gurtler
Sean F. O'Keefe
Robert C. Williams

March 30th, 2012
Blacksburg, VA

Keywords: Essential oil compounds, apple aroma compounds, foodborne pathogenic bacteria, apple juice

Use of plant-derived essential oil compounds and naturally-occurring apple flavor compounds to control foodborne pathogens in apple juice

Takiyah Oniya Abdulmalik

ABSTRACT

Recent demands for minimally-processed foods, has led to the exploration of plant-derived essential oil (EO) compounds as an alternative means of preservation. While some of these compounds are effective against foodborne pathogens, their strong aroma and "spicy" flavor are not compatible with the flavor of juice. The purpose of this research was to evaluate the antimicrobial activity of three EO compounds (thymol, eugenol, and *trans*-cinnamaldehyde) alone and in combination with three naturally-occurring apple aroma compounds (hexanal, *trans*-2-hexenal and 1-hexanol) in order to identify combinations that lower the concentrations needed to destroy foodborne pathogens in apple juice.

The standard agar dilution method (SAD) and the Spiral Gradient Endpoint method (SGE) were compared for their abilities to determine minimum inhibitory concentrations (MIC) of the EO compounds. Both methods produced similar patterns of inhibition; however, the MICs produced by the SGE system were significantly higher than those produced by the SAD method of analysis ($P<0.05$). Since the results produced by the SAD method were more comparable with those published in literature, this method was selected for further testing.

In general, the EO compounds were significantly more effective against the test pathogens (*Listeria monocytogenes*, *Salmonella* Typhimurium and *Staphylococcus aurues*) than were the apple aroma compounds ($P<0.05$). Cinnamaldehye exhibited the highest degree of activity, followed by thymol and eugenol. Eugenol was the only compound that acted synergistically with the apple aroma compounds.

The most effective compounds (cinnamaldehyde, eugenol and *trans*-2-hexenal) were then used to inactivate *L. monocytogens* and *S.* Typhimurium in preservative-free apple juice. In most cases, treatment with 0.05% of each compound resulted in a 5 log CFU/ml reduction in bacterial numbers following one day of storage at 4°C or 25°C. Likewise, treatment with antimicrobial combinations (containing 0.025% of *trans*-2-hexenal in combination with 0.025% *trans*-cinnamaldehyde or eugenol) also resulted in a 5 log CFU/ml reduction in bacterial numbers, following one day of storage at 4°C or 25°C. Since these combinations contained half the effective concentration of the essential oil compounds, they may be used to preserve the microbial quality of apple juice, while reducing the likelihood of off flavors in the final juice product.

Dedication

This dissertation is dedicated to my mother, Rosa Abdulmalik;

who taught me how to overcome difficulties, rather than allowing difficulties to overcome me.

Acknowledgements

Above all else, I would like to acknowledge the presence of God in my life. It is by His grace and mercy that I was able to complete this process. Every experience and hardship that I have encountered during my time at Virginia Tech has taught me to recognize and appreciate the many provisions of joy, peace and happiness that He has made for my life.

In addition, I would like to thank my advisor, Dr. Renee Boyer for all of your guidance and support over the past four years. She went above and beyond the call of duty to ensure my success as a student, and for that I am truly grateful. Thank you for always encouraging me to do my best and for taking the time to nurture and mentor me as a student.

I would also like to thank my doctoral committee members (Dr. Joshua Gurtler, Dr. Sean O'Keefe and Dr. Robert Williams,) for all of the assistance and support that they have provided throughout this process. I truly appreciate all of the positive words of encouragement and thought-provoking advice that you have each provided.

In addition, I would like to thank Dr. Hengjian Wang, for all of his assistance with statistical analyses and data interpretation. Likewise, I would like to thank my undergraduate research assistant, Jason Levy, who helped me to complete the laboratory my portion of my research. I truly appreciate all of your hard work and dedication to this project.

I would also like to acknowledge my wonderful parents Sanusi and Rosa Abdulmalik who have supported me throughout my entire academic career. Thanks for always believing in me, even when I didn't believe in myself. To my older brother (Shettima Abdulmalik), thanks for always providing me with a positive perspective on life. Your joyful spirit and positive attitude were just the "pick-me-up" that I needed to get through the difficult days.

I would also like to thank my dear friends (Monica Cohen, Anjeanette Johnson, Shannon Logan, Jennifer Kone, Julie Gillem, Kacie Allen, Brittini R. Brown and Serena L. Parks), for always providing me with a positive outlet for my stress. Thanks for understanding my absence during the busy times and for supporting me during the hard times.

Finally, I would like to thank my wonderful boyfriend, Clarence Sirmons, for all of the comfort and support that he has provided throughout this process. Thanks for always being a shoulder to cry on and a friend to count on.

Attributions

Several individuals contributed to the development and implementation of the research presented in this dissertation. A brief description of their contributions is described below.

Renee R. Boyer – Ph.D. (Assistant Professor, Department of Food Science and Technology, Virginia Tech) is the primary advisor and chair of the author's doctoral committee. She provided assistance with the selection of test compounds and aided in the design of this research project. She also provided funds to support the work presented in this dissertation.

Chapter 3: Comparison of spiral gradient endpoint and standard agar dilution methods to evaluate antimicrobial activity of essential oils against foodborne pathogens

Joshua B. Gurtler –Ph.D. (Research Food Technologist, Food Safety Intervention Technologies, U.S. Department of Agriculture) was a member of the author's committee. He provided the author with initial training on the Spiral Gradient Endpoint method of analysis.

Jason Levy – (Student, Department of Food Science and Technology, Virginia Tech) was the author's undergraduate research assistant. He assisted the author in conducting laboratory experiments.

Sean F. O'Keefe – Ph.D. (Professor, Department of Food Science and Technology, Virginia Tech) was a member of the author's committee. He provided assistance with the selection of test compounds and aided in the design of this research project.

Robert C. Williams – Ph.D. (Associate Professor, Department of Food Science and Technology, Virginia Tech) was a member of the author's committee. His knowledge and expertise in antimicrobial interactions greatly contributed to this work.

Chapter 4: Antimicrobial effects of hexanal, *trans*-2-hexenal and 1-hexanol on foodborne pathogens, alone and in combination with thymol, eugenol and cinnamaldehyde

Joshua B. Gurtler –Ph.D. (Research Food Technologist, Food Safety Intervention Technologies, U.S. Department of Agriculture) was a member of the author's committee. He provided the author with valuable input regarding the activity of these antimicrobial compounds.

Jason Levy – (Student, Department of Food Science and Technology, Virginia Tech) was the author's undergraduate research assistant. He assisted the author in conducting laboratory experiments.

Sean F. O'Keefe – Ph.D. (Professor, Department of Food Science and Technology, Virginia Tech) was a member of the author's committee. He provided assistance with the selection and evaluation of the antimicrobial test compounds.

Robert C. Williams – Ph.D. (Associate Professor, Department of Food Science and Technology, Virginia Tech) was a member of the author's committee. His knowledge and expertise in antimicrobial interactions greatly contributed to this work.

Chapter 5: Inactivation of *Listeria monocytogenes* and *Salmonella* Typhimurium in apple juice using combinations of cinnamaldehyde, eugenol and trans-2-hexenal

Joshua B. Gurtler –Ph.D. (Research Food Technologist, Food Safety Intervention Technologies, U.S. Department of Agriculture) was a member of the author's committee. His knowledge of bacterial inactivation, data interpretation and antimicrobial interactions greatly contributed to this work.

Jason Levy – (Student, Department of Food Science and Technology, Virginia Tech) was the author's undergraduate research assistant. He assisted the author in conducting laboratory experiments.

Sean F. O'Keefe – Ph.D. (Professor, Department of Food Science and Technology, Virginia Tech) was a member of the author's committee. He assisted the author in preliminary experiments concerning the volatility and solubility of the test compounds.

Robert C. Williams – Ph.D. (Associate Professor, Department of Food Science and Technology, Virginia Tech) was a member of the author's committee. His knowledge and expertise in antimicrobial interactions, apple juice preservation, and bacterial inactivation greatly contributed to this work.

Table of Contents

Abstract ii
Dedication iv
Acknowledgements v
Attributions vii
Table of contents ix
List of tables xi
Chapter 1: Introduction 1
Objectives 4
References 5
Chapter 2: Review of the literature 8
Apple juice 8
Apple juice consumption trends 9
Apple juice production 10
Organisms of concern. 18
E. coli O157:H7 18
Listeria monocytogenes 22
Salmonella enterica serovar Typhimurium 24
Apple juice outbreaks 26
Apple juice processing technologies 29
Natural antimicrobials from plants 30
References 52
Chapter 3: Comparison of spiral gradient endpoint and standard agar dilution methods to evaluate antimicrobial activity of essential oils against foodborne pathogens 67
Abstract 68
Introduction 69
Materials and methods 71
Results 76
Discussion 77
References 82
Chapter 4: Antimicrobial effects of hexanal, *trans*-2-hexenal and 1-hexanol on foodborne pathogens, alone and in combination with

thymol, eugenol and cinnamaldehyde 88
Abstract 89
Introduction……..90
Materials and methods 92
Results…….....95
Discussion. 96
References 101
Chapter 5: Inactivation of *Listeria monocytogenes* and *Salmonella* Typhimurium in apple juice using combinations of cinnamaldehyde, eugenol and *trans*-2-hexenal 106
Abstract 107
Introduction……108
Materials and methods 110
Results…….....112
Discussion. 116
References 119
Chapter 6: Conclusions and future directions 127

LIST OF TABLES

CHAPTER 3

Table 1. Individual minimum inhibitory concentrations (MIC) and average gradient minimum inhibitory concentrations (GMIC) for essential oil compounds against three foodborne pathogens 87

Table 2. Antimicrobial interactions for combinations of essential oil compounds against three foodborne pathogens 87

CHAPTER 4

Table 1. Individual minimum inhibitory concentrations (MIC) for antimicrobial test compounds against four foodborne pathogens……...……104

Table 2. Fractional inhibitory concentration (FIC) indices and antimicrobial interactions for combinations containing low concentrations of essential oil (EO) compounds……104

Table 3. Fractional inhibitory concentration (FIC) indices and antimicrobial interactions for combinations containing high concentrations of essential oil (EO) compounds……105

CHAPTER 5

Table 1. Antimicrobial effects of various concentrations of *trans*-cinnamaldehyde on the survival of (a) *S.* Typhimurium and (b) *L. monocytogenes* in pasteurized apple juice (stored at 4˚C AND 25˚C).....123

Table 2. Antimicrobial effects of various concentrations of eugenol on the survival of (a) *S.* Typhimurium and (b) *L. monocytogenes* in pasteurized apple juice (stored at 4˚C AND 25˚C).....124

Table 3. Antimicrobial effects of various concentrations of *trans*-2-hexenal on the survival of (a) *S.* Typhimurium and (b) *L. monocytogenes* in pasteurized apple juice (stored at 4˚C AND 25˚C).....125

Table 4. Antimicrobial effects of essential oil compounds in combination with *trans*-2-hexenal on the survival of (a) *S.* Typhimurium and (b) *L. monocytogenes* in pasteurized apple juice (stored at 4°C and 25°C 126

CHAPTER 1

Introduction

Apple juice and apple cider have traditionally been considered a low risk to food safety (due to their intrinsically low pH and high organic acid content); however, they have caused a number of large-scale outbreaks of foodborne illness (Miller and Kasper 1994; Cody et al., 1999; Sapers et al., 2006; Truong et al., 2010). Outbreaks involving apple juice and cider are most commonly associated with the consumption of raw (unprocessed or preservative-free) products whose ingredient(s) become contaminated by infected fecal matter. Such is the case of the 1974 outbreak (New Jersey, USA), in which 300 people were infected with *Salmonella* Typhimurium, following the consumption of raw apple cider that was produced with contaminated "dropped" apples (Luedtke and Powell 2000). Similarly, in 1993 (Maine, USA), nearly 215 people were infected with *Cryptosporidium* after drinking apple cider that was produced in close proximity to a cow pasture (Luedtke and Powell 2000).

One of the most notable outbreaks was the 1996 Odwalla outbreak, in which 66 people were infected with *E. coli* O157:H7 (12 of which were hospitalized with hemolytic uremic syndrome with 1 child dying from complications from the illness) (Luedtke and Powell 2000). The outbreak spanned four states (and one Canadian province), and lasted over four months (Luedtke and Powell 2000). The source of the outbreak was unpasteurized apple juice, which was produced with decaying fruit under inadequate sanitizing conditions (Cody et al., 1999; Luedtke and Powell 2000). As a direct result of this highly-publicized outbreak, Odwalla recalled all of their apple juice-containing products and began pasteurizing all of their juices (Martinelli and Briggs 1998; Cody et al., 1999). Additionally, this incident prompted the U.S. Food and Drug Administration (FDA) to issue a ruling, requiring a warning label on all

unpasteurized juice products (Burnett 2001; Beerbower et al., 2002; Choi and Nielsen 2004; Vojdani et al., 2008). Furthermore, in 2001 the FDA enacted the Juice HACCP (Hazard Analysis Critical Control Points) rule, which required juice producers to implement a HACCP program for the production of juice and juice-containing products (Beerbower et al., 2002; Vojdani et al., 2008). More importantly, the ruling established a performance standard, which required all juice processors to achieve a 5-log reduction in the pertinent organism of concern (the most resistant microorganism of public health concern that may occur in juice) (Beerbower et al., 2002)n prior to selling juice to the public. In the case of apple juice, these organisms are *E. coli* O157:H7, *Salmonella spp*. and *Cryptosporidium* (Beerbower et al., 2002; Choi and Nielsen 2004; Sapers et al., 2006; Vojdani et al., 2008).

Since 2001, the apple juice industry has relied primarily on thermal pasteurization (>160F for ≥6 seconds) as a means of ensuring product safety; however, due to recent shifts in market trends towards minimally-processed foods, a number of processors have begun to explore the use of non-thermal techniques to safeguard their products arguing that the high pasteurization temperatures alter the natural flavor profiles and nutritional value of fresh juices (Zink 1997; Buchanan et al., 1998; Tajchakavit et al., 1998; Rahman 1999; Beerbower, 2002; Choi and Nielsen 2004; Mosqueda-Melgar et al., 2008). While the production of minimally-processed juice has boosted industry profits, it has also put extreme pressure on regulatory agencies (and food processors) to develop new ways to ensure product safety (Gould 1996; Zink 1997).

Within the past decade, several alternatives to thermal processing have emerged, including UV irradiation (the only FDA-approved alternative to thermal pasteurization of fruit juices), high-pressure processing, microwave heating, ultra filtration, ozone and pulsed electric field (Gould 1996; Buchanan et al., 1998; Tajchakavit et al., 1998; Choi and Nielsen 2004).

While all of these techniques are effective in inactivating target pathogens in foods, their overall acceptance into the food industry is often hampered by negative consumer perceptions regarding their use and labeling (Hunter 2000; DeRuiter and Dwyer 2002; Nayga et al., 2005). Thus, the use of natural preservatives from plant, animal and microbial sources have become increasingly popular (Gould 1996).

Of these compounds, plant-derived antimicrobials represent the largest group of natural preservatives and include substances with antifungal, antimycotic, antiparasitic, antitoxigenic, anti-inflammatory, anticarcogenic and antiviral properties (Sivropoulou et al., 1996; Caccioni et al., 1998; Kalemba and Kunicka 2003; Burt 2004). While these (and other) compounds offer a safe alternative to traditional processing techniques, their use in food products is often hampered by regulatory constraints and their strong sensory profiles (Lambert et al., 2001). In some cases, combinations of multiple antimicrobials may produce synergistic relations which lower the overall effective dose needed to preserve fresh juice products. Such combinations may provide new alternatives to traditional preservation methods, without destroying the natural nutritional and sensory profile of the final juice product.

Objectives

The objectives of this research were to:

1.) Compare the efficacy of the Spiral Gradient Endpoint method of analysis to that of the Standard Agar Dilution method for determining minimum inhibitory concentrations for thymol, eugenol and *trans*-cinnamaldehyde.

2.) Determine the antimicrobial activity of three essential oil compounds (thymol, eugenol and *trans*-cinnamaldehyde) alone and in combination with three apple-flavor compounds (hexanal, *trans*-2-hexenal, and 1-hexanol) against three common foodborne pathogens.

3.) Determine the effectiveness of low levels of *trans*-cinnamaldehyde, eugenol and *trans*-2-hexenal against target pathogens in commercial apple juice.

4.) Evaluate the combined effects of low levels of *trans*-cinnamaldehyde, eugenol and *trans*-2-hexenal against target pathogens in commercial apple juice.

References

Aguilar-Rosas, S.F., Ballinas-Casarrubias, M.L., Nevarez-Moorillon, G.V., Martin-Belloso, O., Ortega-Rivas, E. (2007). "Thermal and pulsed electric fields pasteurization of apple juice: effects on physiochemical properties and flavor compounds." Journal of Food Engineering **83:** 41-46.

Beerbower, B., Botos, M., Bourquin, L., Doughtery, R., Foster, T., Goodrich, R., Harris, L., Grace, K., Howe, Y., King, D., Parish, M., Rushing, J., Sayler, A., Sizer, C., Wang, M., Weddig,L.,Wilhelmsen, E., Williams, D., Wogee, C., and Worobo, R. (2002). Juice HACCP Training Curriculum, First Edition. August 2002, Juice HACCP Alliance.

Buchanan, R. L., Edelson, S.G., Snipes, K. and Boyd, G. (1998). "Inactivation of *Escherichia coli* O157:H7 in apple juice by irradiation." Applied and Environmental Microbiology **64**: 4553-4535.

Burnett, S. L., Beuchat, L.R. (2001). "Food-borne pathogens: human pathogens associated with raw produce and unpasteurized juices, and difficulties in decontamination." Journal of Industrial Microbiology & Biotechnology **27**: 104-110.

Burt, S. (2004). "Essential oils: their antibacterial properties and potential applications in foods-a review." International Journal of Food Microbiology **94**: 223-253.

Caccioni, D. R. L., Guizzardi, M., Biondi, D.M., Renda, A., Ruberto, G. (1998). "Relationship between volatile components of citrus fruit essential oils and antimicrobial action of *Penicillium digitatum* and *Penicillium italicum*." International Journal of Food Microbiology **43**: 73-79.

Choi, L. H., Nielsen, S.S. (2004). "The effects of thermal and non-thermal processing methods on apple cider quality and consumer acceptability." Journal of Food Quality **28**: 13-29.

Cody, S. H., Glynn, K., Farrar, J.A., Cairns, K.L., Griffin, P.M., Kobayashi, J., Fyfe, M., Hoffman, R., King, A.S., Lewis, J.H., Swaminathan, B., Bryant, R.G., and Vugia, D.J. (1999). "An Outbreak of *Escherichia coli* O157:H7 infection from unpasteurized commercial apple juice." Annals of Internal Medicine **130**: 202-209.

DeRuiter, F. E., Dwyer, J. (2002). "Consumer acceptance of irradiated foods: dawn of a new era?" Food Service Technology **2**: 47-58.

Gould, G. W. (1996). "Industry perspectives on the use of natural antimicrobials and inhibitors for food applications." Journal of Food Protection **1996 Supplement**: 82-86.

Hunter, C. (2000). "Changing attitudes to irradiation throughout the food chain." Radiation Physics and Chemistry **57**: 239-243.

Kalemba, D., Kunicka, A. (2003). "Antibacterial and antifungal properties of essential oils " Current Medicinal Chemistry **10**: 813-829.

Lambert, R. J. W., Skandamis, P.N., Coote, P.J., Nychas, G.J.E. (2001). "A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol "Journal of Applied Microbiology **91**: 453-462.

Luedtke, A., Powell, D. (2000). "Fact Sheet: a timeline of fresh juice outbreaks." International Food Safety Network.
Availible at http://www.foodsafety.ksu.edu/en/article-details.php?a=2&c=6&sc=37&id=427 . Accessed 29 March 2012.

Martinelli, K. A., Briggs, W. (1998). "Integrating public relations and legal responses during a crisis: the case of Odwalla, Inc." Public Relations Reviews **24**: 443-460.

Miller, L. G., Kasper, C.W (1994). "*Escherichia coli* O157:H7 acid tolerance and survival in apple cider." Journal of Food Protection **57**: 460-464.

Mosqueda-Melgar, J., Raybaudi-Massilia, R.M., Martin-Belloso, O. (2008). "Non-thermal pasteurization of fruit juices by combining high-intensity pulsed electric fields with natural antimicrobials." Innovative Food Science and Emerging Technologies **9**: 328-340.

Nayga, R. M., Aiew, W., Nichols, J.P. (2005). "Information effects on consumer's willingness to purchase irradiated food products." Review of Agricultural Economics **27**: 37-48.

Rahman, M. S. (1999). Handbook of Food Preservation. New York, NY, Marcel Dekker.
Sapers, G. M., Gorny, J.R., Yousef, A.E., Ed. (2006). Microbiology of Fruits and Vegetables. Boca Raton, FL, Taylor and Francis Group.

Sivropoulou, A., Papanikolaou, E., Nikolaou, C., Kokkini, S., Lanaras, T., Arsenakis, M. (1996). "Antimicrobial and cytotoxic activities of Origanum essential oils." Journal of Agricultural and Food Chemistry **44**: 1202-1205.

Tajchakavit, S., Ramaswamy, H.S., Fustier, P. (1998). "Enhanced destruction of spoilage microorganisms in apple juice during continuous flow microwave heating." Food Research International **31**: 713-722.

Truong, V. Y. T., Boyer, R.R., McKinney, J.M., O'Keefe, S.F. and Williams, R.C. (2010). "Effect of a-cyclodextrin-cinnamic acid inclusion complexes on populations of *Escherichia coli* O157:H7 and *Salmonella enterica* in fruit juices." Journal of Food Protection **73**: 92-96.

Vojdani, J. D., Beuchat, L.R., Tauxe, R.V. (2008). "Juice-associated outbreaks of human illness in the United States, 1995 through 2005 " Journal of Food Protection **71**: 356-364.

Zhao, T., Doyle, M.P., Besser, R.E. (1993). "Fate of enterohemorrhagic *Escherishia coli* O157:H7 in apple cider with and without preservatives." Applied and Environmental Microbiology **59**: 2526-2530.

Zink, D. L. (1997). "The impact of consumer demands and tends on food processing." Emerging

Infectious Disease **3**: 467-469.

CHAPTER 2

Review of the Literature

APPLE JUICE

Juice is defined as the "unfermented but fermentable juice, intended for direct consumption, obtained by the mechanical process from sound, ripe fruits, preserved exclusively by physical means" (Bates et al., 2001). This definition includes reconstituted juices (from concentrate), as well as cloudy, clear or pulpy forms of the products (Bennett 1978; Bates et al., 2001).

While this definition is absolute for most fruit beverages, the terms "apple juice" and "apple cider" are often used interchangeably to describe several forms of apple-based beverages (especially within different regions of the world) (Deng and Cliver 2001; Vojdani et al., 2008). In the United States, the term "apple juice" is used to describe three different types of juices, including "clear" (filtered) juice, apple cider (unfiltered and often unpasteurized juice) and hard cider or apple wine (fermented apple juice containing <7% and >7% alcohol respectively) (Rowles 2000; Deng and Cliver 2001) (Note: In this paper, the term apple cider will be used to describe unpasteurized apple juice). Certain specifications have been adopted within the fruit juice industry, to prevent the sale of illegally adulterated products, which do not qualify as "juice". These provisions provide specific instructions related to the assessment (and adjustment) of the total solids content (denoted by ˚brix), ethanol content, pH, etc. of the final juice product.

In most cases, the acceptable soluble solids content for apple juice is approximately 11.5 ˚brix; however, due to differences in processing techniques (and apple cultivars used for juicing), solids contents as low as 10˚brix are acceptable (Binkley and Wiley 1978; Bates et al., 2001;

FAO/WHO Food Standards 2011). Likewise, the ethanol content of juice is also measured (not to exceed 5 g/kg), as well as the volatile acids content (not to exceed 0.5 g/kg) (FAO/WHO Food Standards 2011). Other factors, such as pH (pH 3.0-4.5) and cloudiness are also monitored to ensure product quality; however, they are not regulated by the U.S. Food and Drug Administration (Jolicoeur).

Apple Juice Consumption Trends

The apple juice industry represents over 80 percent of all processed apple commodities, with domestic production reaching over 148.5 million gallons a year (Fonsah and Muhammad 2008; Geisler 2011). While, the US is one of the leading consumers of processed apple juice, the bulk of this product is imported from China (representing over 46% of global exports), followed by Poland and Germany (Rosa 2001; Horticulture & Tropical Products Division 2003; FAS/ USDA Hotricultural & Tropical Products Division 2006).

Of these products, "clear" apple juice is the second most commonly consumed fruit juice within the United States (orange juice is the first) (Ang et al., 1999). Such products are especially popular among young children (ages 2-5), who derive a large percentage of nutritional value from apple juice products (particularly those fortified with Vitamin C) (Perez et al., 2001; Skinner et al., 2004). In fact, fruit juice accounts for half of the recommended fruit and vegetable intake consumed by children ages 2-18, with apple juice accounting for 50% of this value (Smith and Lifshitz 1994; Dennison 1996). Additionally, the high antioxidant content of apple juice has been proven to decrease lipid oxidation and lower cholesterol in adults (Hyson et al., 2000; Boyer and Liu 2004). However, despite these health benefits, the majority of American adults do not regularly consume commercial apple juice. Rather, cloudy and hard cider are more popular among adults (MacDonald 2000).

Aside from its popularity as a single-component beverage, apple juice is also used as an ingredient in many juice blends and cocktails, including punches and cranberry blends (Dennison et al., 1999). In addition, apple juice has become a popular ingredient in certain vegetable-juice blends, including carrot and tomato juice products (Rowles 2001). While these applications have contributed to the overall popularity of commercial apple juice, there is a growing demand for raw and unprocessed apple juice. Organic apple production increased from 12,772 acres to 17,626 acres between 2005 and 2008 alone; with juice production accounting for 6% total organic apple production (Geisler 2011; Slattery et al., 2011).

Apple Juice Production

Apple juice production is a multi-step process, which utilizes both on the farm and post-harvest processing technologies. While the specific steps of apple juice manufacturing vary depending on the type of juice being produced (cloudy, clear or hard-cider), several steps are conserved within the industry; including harvest, sorting, washing, grinding, juicing (or pressing), filtering (in the case of ultra-clear juice) and bottling. Details related to these processes will be discussed below.

Harvest-

The production of apple juice begins with the harvest of ripe, sound fruit from the tree. In most cases, harvest occurs when apples are close to maturity (as indicated by increased starch content, color intensity and firmness) (Bates et al., 2001). While the majority of apples are processed immediately following harvest, some processors store apples up to several months prior to juicing. In these cases, the fruit must be harvested within 5-20 days of maturity to ensure high fruit-quality during storage and juice extraction (Bates et al., 2001).

Since the growing season of apples (and thus time to maturity) varies, depending on

climate, and cultivar, it is important to measure the degree of ripening of the selected fruit. This can be done using a number of analytical tests, such as the starch/iodine test, pressure test, and soluble solids test. These tests work by measuring the concentration of starch present in the apple (as indicated by the conversion of sugar to starch during ripening) (Bates et al., 2001). Still, other subjective methods exist to help determine the overall level of maturity of apples; including evaluation of ground color (or skin color) change, flesh color change, water core and ease of removal from the spur. However, these methods are greatly affected by time of harvest and nutritional quality and are, therefore, poor indicators of overall fruit maturity (Bates et al., 2001).

Once the apples have reached the appropriate level of maturity, they can be removed from the tree and stored until further processing. In most cases this is done by hand, using harvesting buckets and specially designed poles (to grab hard-to-reach fruit) (Spencer et al., 1993; Earle-Richardson et al., 2005). In this case, it is important to monitor the personal hygiene of workers as they may transmit viral and bacterial pathogens directly to the surface of whole apples. Similarly, it is important to sanitize all harvesting equipment (e.g., boxes, bins, knives and truck beds) prior to harvest, as they can harbor a number of bacterial pathogens.

In addition to manual harvesting techniques, a small number of large-scale producers (those producing >40,000 bushels or 700 metric tons) (Bates et al., 2001) utilize mechanical "shakers" and "catchers" to harvest apples (Peterson et al., 1994). However, it is important to note that the use of mechanical harvesting equipment can cause excessive damage to the fruit (as a result of falling from the tree to the catcher). Such damage can lead to off-flavors in the resulting juice product as well as increase the risk of microbial contamination.

Sorting-

After apples have been harvested from the orchard, they are transported to the processing

house, where they are sorted to remove low-quality (visibly damaged or excessively rotten) fruit from the processing line. This process can be done in conjunction with a washing step or performed separately. In both cases, the resulting batch should be free from bruised and excessively decayed fruit. Similarly, all receiving steps should accept only high-quality apples, which have been produced and harvested under Good Agricultural Practices (GAPs) (Sapers et al., 2006).

Aside from removing low-quality fruit from the processing line, it is also important to limit the use of "windfall" or "drop" apples (i.e., fruit which has fallen from the tree via natural processes) during juice manufacture. The incorporation of such fruit into the juice formulation can significantly increase the risk of contamination in the final product; in fact, it has been demonstrated that aerobic plate counts of freshly produced juice increased from 1.90-3.40 log/ CFU/mL to 4.19 -5.43 log CFU/mL when "drop" apples were included in the final juice formulation (Sapers 2006). Such trends occur because "drop" apples are in contact with the soil for extended periods of time and are, therefore, exposed to microorganisms that may be present in the soil. For this reason, the sorting step often regarded as the first-line of defense against pathogenic contamination of fresh juice products.

Washing-

Apples used for juicing, similar to that of whole fruit subject, are subject to a series of washing steps, which help remove dirt and debris from the surface of the fruit. In some cases, the use of a washing step can result in a 1-2 log reduction in microbial numbers (Novak 2003). Nevertheless, if the microbial quality of the water is not properly monitored, this step can increase the overall level of contamination by spreading bacteria from contaminated fruit to non-contaminated apples within the same (or different) batches (Novak 2003). This is particularly

troublesome because pathogens such as *E. coli* O157:H7 are able to survive for extended periods of time in untreated water (Matthews 2006).

Processors, thus, often utilize commercial sanitizers, such as chlorine, ozone, peroxyacetic acid or chlorine dioxide, to control the microbial-load in wash water. Of these compounds, chlorine (200ppm) is the most commonly used sanitizing agent; however, its activity is limited by a number of factors, including: changes in pH (with pH 6.5-7.5 being the optimal range), the presence of organic matter (which can inactivate the chlorine molecule) and limited access to tightly adhering bacteria (such as those growing in biofilms or the stem and calyx areas) (Novak 2003).

Many processors, for this reason, utilize brushing systems to enhance the cleaning process of apples (by removing tightly-adhered bacteria from the surface of the fruit) (Matthews 2006). Most processing facilities utilize one of two types of brushing systems, including flat-bed washers (rotating brushes are arranged perpendicular to the flow of the fruit) and U-shaped washers (brushes are arranged in a U-shape that is perpendicular to the flow of the product) (Novak et al., 2003). In both cases, brushes must be properly sanitized to prevent further contamination (Matthews 2006).

Grinding-

Prior to juicing, apples must undergo a series of grinding steps, which produces a fine "pulp" or "mash". This is typically done using a hammer mill, grating mill or disintegrator, which produces different sized particles (Bates et al., 2001). In order to ensure adequate juicing during later steps, however, careful attention must be paid to the consistency of the resulting mash. In general, the mash should be free from large pieces, which produce low juice yields. On the other hand, pulp should not be too finely mashed, resulting in a product with a high

percentage of suspended solids (Singh 1995).

While grinding is essential to the production of juice, it can contribute to contamination of the final juice product. In nature, apples are exposed to various environmental contaminants, which includes a number of pathogenic and spoilage microorganisms. In most cases, these organisms cannot penetrate beyond the waxy cuticle layer of whole (intact) fruit; however, the grinding process destroys this protective barrier, thus exposing these microorganisms to the fruit tissue. The grinding step is especially troublesome because it can distribute microorganisms throughout the entire batch; contaminating apples from several lots.

Juice Extraction-

Juice extraction can be completed using either continuous or batch systems, depending on the type of extraction equipment used. The most common systems include the hydraulic cider press, the bladder press, continuous screw press and the continuous plate or belt press (Bates et al., 2001). Details related to these systems will be described below.

1.) Hydraulic cider press: The hydraulic batch press system is one of the most common methods of juice extraction. It is comprised of a series of wooden (or metal) racks which are laced with cloth (typically cotton, wool or nylon). These racks are filled with apple pulp and placed one on top of another. When the final rack is in place, juice will begin to flow from the pulp and is called “free-run” juicing, occurring at low pressures of 170-200kPa. A second stage of juicing occurs when pressure (850-900 kPa) is applied to the system resulting in full removal of juice from the macerated apples (Singh 1995). While this system is relatively simple to use, it is very inefficient (taking about 20-30 minutes to generate juice) and is, therefore, unsuitable for producing large amounts of juice (Bates et al., 2001).

2.) Bladder press: The bladder press (commonly called the Willmes Press) is a highly efficient method of juice extraction, operating similar to the hydraulic batch press. It contains a horizontal cylinder lined with cloth, and an inflatable tube, which is situated in the center of the cylinder. Once the pulp is inserted into the cylinder, the tube is inflated; pressing the pulp against the wall of the system. Pressure from the tube (approximately 600 kPa) results in the extraction of juice from the apple mash. The resulting juice is collected in an underlying trough and drained into collection vesicles (Bates et al., 2001).

3.) Continuous screw press: Two types of continuous screw presses are the Jones press (arranged in a vertical manner) and the Reitz press (arranged in a horizontal manner). Both systems contain a large screw-like conveyer, enclosed by a screen cylinder (Bates et al., 2001). When apple pulp is inserted, the screw-like conveyer moves the product through a series of stationary paddles, which squeeze juice from the cells. The resulting juice is allowed to drain from the system into an underlying drip-pan, while the processed pulp is expelled through a separate opening (Singh 1995).

4.) Continuous plate or belt press: This system utilizes a continuously rotating belt to transport apple mash through a set of vertically moving plates that force the juice from the apple mash, which is then collected in an underlying collection-pan. Excess pulp is transported through a set of horizontal rollers and deposited into waste; the belt is then cleaned for subsequent use (Bates et al., 2001).

5.) Screen-type centrifuge: Unlike previously discussed methods, this system utilizes centrifugal force to remove juice from apple pulp and extracted juice is then collected in an underlying trough. While this process is very rapid, it leaves large amounts of juice in the pulp, requiring additional processing (typically using a hydraulic or bladder press

system) to adequately remove juice (Bates et al., 2001).

Juice clarification-

Specific steps involved in apple juice manufacturing vary depending on the type of juice product being produced. “Cloudy” (or natural) apple juice is produced with little to no clarification steps (some producers utilize ascorbic acid to prevent enzymatic browning caused by the enzyme polyphenol oxidase) (Sapers et al., 1969). However, the production of clear and “ultra-clear” juice requires the use of several treatment steps, which remove large amounts of particulate matter. These processes include enzyme treatment, tannin and gelatin treatment, heat clarification, centrifugation, bentonnesite fining and filtration (Bates et al., 2001). The details related to these processes include:

1.) Enzymatic clarification: Prior to filtration, this process to removes excess amounts of colloidal material from the juice suspension, which may clog filters during later clarification steps. These enzymes act by hydrolyzing large amounts of soluble pectin and colloidal material, producing a less-viscous product that easily passes through coarse and fine filters. The two types of enzyme treatments available for commercial use differ in their optimal temperature range. The first set of enzyme treatments, referred to as cold treatment is functional at room temperature are added to juice at 20°C. These enzymes remain active for 6-8 hours (Bates et al., 2001). Conversely, “hot” enzyme treatments are applied to heated juice at 54°C and have relatively short reaction times of 1-2 hours. Both treatments are effective at reducing of the viscosity of the juice product (Bates et al., 2001).

2.) Tannin and gelatin treatment: Tannins are polyphenolic compounds abundant in apple skins (Fournard et al., 2006). When present in the appropriate quantities, they contribute

to the characteristic astringent taste of apple juice; however, when they are present in excess quantities, they can become oxidized and generate off-colors in the final juice product (Vidal et al., 2004). For this reason, it is important to maintain a proper balance of tannins in apple juice. Several systems are available to remove tannins from the juice product, many of which utilize gelatins to precipitate them from solution. In most cases, apple juice is fortified with excess tannin, to prevent complete leeching during later steps, followed by treatment with a complex gel system. The resulting product is a clarified juice that possesses the characteristic "sharp" taste and color of traditional apple juice (Bates et al., 2001).

3.) Heat clarification: This method utilizes "flash" heating (82-85 °C) to coagulate colloidal material, which may be present in the juice matrix. Immediately following heat treatment, juice is rapidly cooled and centrifuged (or filtered) to remove insoluble particulate, resulting in a partially clarified product.

4.) Centrifugation: This method utilizes a continuous or batch centrifuge to reduce the amount of colloidal material present in the juice solution. The resulting product is a juice that is clearer and less viscous than natural or "cloudy" juice, yet not as clear as filtered juice.

5.) Bentonnesite fining: The system utilizes small bentonnesite clay particles, which have a large surface area, to bind to tannins present in apple juice. The resulting product lacks the haze and astringency of "cloudy" or natural apple juice products (Bates et al., 2001).

6.) Filtration: Previously described clarification steps are all used to remove excess colloidal material from freshly pressed apple juice; resulting in a product that is "semi-clear", with little to no haze. However, in order to produce an "ultra-clear" product, apple juice must

pass through a series of filtration steps, which removes the remaining particulate matter. This process includes a number of filtration systems, which include plate and frame filters, continuous filters, and pre-coated filters containing diatomaceous earth (Bates et al., 2001).

Pasteurization and bottling-

Federal law requires that all commercially-processed fruit juices receive a 5-log reduction in the bacterial organism of concern (the organism that is the most resistant to the selected treatment), prior to being sold to the public. While there is no standard procedure for achieving this reduction, most juice processors utilize flash pasteurization (90°C for 20-30 seconds) to destroy target pathogens. This is typically done using thin-tube or plate heat-exchangers, which utilize steam or hot water as the heating agent. In most cases, the juice is processed under frequent agitation, to ensure adequate heating of the juice product (Bates et al., 2001).

Immediately following pasteurization, the juice is aseptically packaged into pre-sterilized containers, which are then sealed and rapidly cooled prior to distribution. The most commonly used packaging materials include enamel-lined cans and glass or plastic bottles; each of which has different effects on post-processing cooling and storage. The majority of apple juice processors utilize plastic bottles, which can be rapidly chilled without damage to the packing material (Bates et al., 2001).

ORGANISMS OF CONCERN

Escherichia coli O157:H7

Illness-

E. coli O157:H7 is estimated to cause nearly 70, 000 cases of foodborne illness each year

(Gaysinsky 2007). In most cases, illness associated with this pathogen is relatively self-limiting (lasting between five and seven days) and includes acute symptoms such as abdominal cramping, fever, nausea, diarrhea and hemorrhagic colitis (bloody diarrhea) (Pai et al., 1988; Chart et al., 1989; Benjamin and Datta 1995; Centers for Disease Control and Prevention 2011). Five to ten percent of all *E. coli* O157:H7 patients develop hemolytic uremic syndrome (HUS), a life-threatening condition characterized by acute renal failure, thrombocytopenia (lowered platelet count), and hemolytic anemia (destruction of red blood cells) (Benjamin and Datta 1995; Wong et al., 2000; Banatvala et al., 2001). HUS is the primary cause of death among *E. coli* O157:H7 victims (especially children < 10 years of age) and produces a number of long-term complications following recovery (Tarr 1995; Centers for Disease Control and Prevention 2011).

Associated outbreaks-

Since the coliform, *E. coli* is found in the digestive tract of most ruminant animals (including cows, deer, and sheep), outbreaks of *E. coli* O157:H7 are most commonly associated with the consumption of raw or undercooked ground beef and milk (Leyer et al., 1995; Berry 2000; Centers for Disease Control and Prevention 2011). Perhaps the most notable outbreak involving *E coli* O157:H7 was the 1993 multistate outbreak involving the consumption of undercooked hamburgers (Food Safety Innovation in the United States). This outbreak resulted in over 700 illnesses and four deaths; and led to new regulation regarding the control and detection of *E. coli* O157:H7 in ground beef (Food Safety Innovation in the United States).

In more recent years, *E. coli* O157:H7 has been associated with a number outbreaks involving high-acid foods, such as apple cider, fermented sausage, yogurt, and mayonnaise (Benjamin and Datta 1995; Berry and Cutter 2000). Such outbreaks occur as a result of the organism's ability to survive under acidic and refrigeration conditions (Vojdani et al., 2008). In

fact, several studies have reported that *E. coli* O157:H7 is capable of surviving for periods up to 21 days in refrigerated (4˚C) apple cider (pH 3.6-4.0) (Miller and Kasper 1994; Vojdani et al., 2008; Ryu 1998). Still other studies have demonstrated the organism's ability to grow in refrigerated (8˚C) apple cider (pH 3.6 to 4.0) (Zhao et al., 1993; Conner and Kotrola 1995). Its ability to survive and possibly grow under these conditions is especially troubling to the apple juice industry, because they are common hurdles used to preserve the microbial quality fresh juice.

Mechanisms of acid survival-

E. coli O157:H7 contains a number of mechanisms that help the cell to remain viable at low pH. The first system (commonly referred to as acid adaptation or acid shock) operates in response to changes in external pH and involves the production of stress-related proteins. The second class of acid-tolerance systems (glutamate decarboxylase and arginine deaminase pathway) acts by degrading amino acid substrates; which in turn leads to a reduction in the level of intercellular protons (Jordan et al., 1999). Both systems allow the cell to maintain an internal pH near neutrality.

Stationary-phase-dependent acid tolerance:

E. coli O157:H7, like most enteropathogenic microorganisms contains a stationary-phase-dependent sigma-factor (called *rpoS*), which regulates the production of a number of stress-related proteins (Cheville et al., 1996). When stationary-growing cells are exposed to mildly acidic conditions (or low concentrations of organic acids, such as benzoic acid or phentylacetic acid), this sigma-factor leads to the production of a nearly 30 proteins, which protect the cell from acid-induced injury (Leyer et al., 1995). These proteins are thought to assist with regulating intercellular pHs through the use of antiporter channels (Jordan et al., 1999).

Additionally, these proteins aid in protecting the cell from environmental stressors, such as heat, starvation and high salt content (Cheville et al., 1996; Bearson et al., 2009).

Glutamate decarboxylase system:

The glutamate decarboxylase system is considered the most effective method for acid survival of *E. coli* O157:H7, operating under extremely low pHs (ca. 2.5), and utilizes the amino acid glutamate as a substrate for enzymatic reactions, which regulate intercellular pH (Bearson et al., 2009). One molecule of glutamate is transported into the cell (via membrane-bound antiporters), where it is decarboxylated into γ -aminobutyric acid (or GABA), via two separate glutamate decarboxylase enzymes. Since each enzyme consumes one proton to complete the reaction, the system leads to a rapid reduction in the concentration of intercellular protons (Bearson et al., 2009). The cell, as a result is able to maintain homeostatic pH conditions near neutrality (Castanie-Cornet et al., 1999; Ma et al., 2003).

Arginine deaminase system:

The arganine deaminase, similar to the action of the glutamate decarboxylae system, pathway acts by degrading the amino acid arginine, into agmatine (releasing one molecule of CO_2 and NH_3) (Davis et al., 1996; Bearson et al., 2009). The resulting molecules are then transported out of the cell via unknown antiporters, which in turn lowers the concentration of intercellular protons. This action helps the cell to maintain internal pH near neutrality (Castanie-Cornet et al., 1999). The resulting ammonia molecules are believed to bind to intercellular proteins, protecting the cell from further acid-induced damage (Davis et al., 1996). Since the system is functional at extremely low pH it is able to protect the cell from internal acid-induced damage, namely denaturation if intercellular proteins.

Listeria monocytogenes

Illness-

Listeria monocytogenes is believed to cause over 1600 cases of foodborne illness and 415 deaths each year; making it one of the most deadly foodborne pathogens (U.S. Department of Health and Human Services 2009). While the organism does not typically produce symptoms in healthy individuals, it does cause mild flu-like symptoms (such as mild fever) as well as a number of gastrointestinal symptoms (such as vomiting, nausea and diarrhea) (Dalton et al., 1997).

In severe cases of listeriosis, the organism causes a number of complications, such as septicemia, meningitis and abortion (Doumith et al., 1991; Farber and Peterkin 1991). These conditions are a result of the organism's ability to invade the intestinal epithelial cells, which releases the pathogen into the blood stream (Gaillard et al., 1987; Garner, et al., 2006). Once the pathogen enters the blood stream, it is engulfed by phagocytic cells where it establishes an infection (Mounier et al., 1990). Once engulfed by the host's phagocytic cells, it is capable of crossing the blood-brain barrier, infecting the meninges, causing meningitis encephalitis (Gaillard et al., 1987; Frye et al., 2002). The organism is also capable of crossing the placental barrier of pregnant women, leading to prenatal infections and abortion (during the second and third trimester) (U.S. Department of Health and Human Services 2009).

Associated Outbreaks-

L. monocytogenes is most commonly associated with the consumption of contaminated dairy products (including both raw and pasteurized milk, cheese, coleslaw and pate) (Gahan et al., 1996). The organism is capable of surviving in a number of high-acid and otherwise acidified foods, including cottage cheese, yogurt, whole-fat cheddar cheese, orange juice, apple cider and

salad dressing (Gahan et al., 1996). The survival of *L. monocytogenes* in high-acid foods is typically a result of improper processing or acidification, which promotes selection for acid-tolerant mutants that are capable of surviving and growing at extremely low pH (O'Driscroll et al., 1996). This is especially true for fermented cheeses, which rely on starter-cultures to control fermentation pH. If starter-cultures fail to adequately reduce the pH of the curd, *L. monocytogens* cells may be pre-sensitized to low-pH, leading to enhanced survival at lower pH (Gahan et al., 1996). Furthermore, gradual reductions in pH (from pH 5.5) trigger a number of virulence factors, contributing to its survival at low pHs (~ pH 3.5) (Gahan et al., 1996).

Mechanisms of Acid Survival-

Acid adaptation in *L. monocytogenes* is believed to occur as a result of the organism's ability to synthesize stress-related proteins (Davis et al., 1996). These proteins are believed to act by repairing acid-damaged DNA as well as stabilizing the cytoplasmic membrane, following exposure to acidic conditions (pH 5.8) (Davis et al., 1996). Furthermore, these proteins provide bacteria with cross-protection against a number of environmental stressors including high osmotic pressure, suboptimal temperatures and surface active agents, such as crystal violet and ethanol (Gahan et al., 1996; O'Driscroll et al., 1996).

It is important to note that *L. monocytogenes* exhibits growth-phase-dependent acid tolerance, confering acid resistance to cells growing in the stationary phase (O'Driscroll, et al., 1996; Phan-Thanh et al., 2000). Stationary-growing cells, unlike exponentially growing cells, display reduced metabolism, decreasing their ability to develop acid tolerance (Davis et al., 1996; DeBiase et al., 1999). For this reason, certain mechanisms must be in place to protect these cells from acid damage.

One such mechanism is the stationary-phase-specific sigma factor, RpoS which initiates

transcription of other stress-related proteins (Davis et al.,1996). While the presence of this gene has not yet been confirmed in *L. monocytogenes*, rpoS it is believed to be identical to genes present in *E. coli* O157:H7 and *Salmonella* Typhimurium, enabling the pathogen to survive in high-acid foods without undergoing severe acid-induced damage (Davis et al., 1996; Koutsoumanis and Sofos 2004).

Salmonella enterica serovar Typhimurium

Illness-

Ingestion of low levels of *Salmonella enterica* serovar Typhimurium (as few as 17 cells) typically produce physiological symptoms within 12-72 hours of consumption (Blaser and Newman 1982; US Department of Health and Human Services 2011). Illness caused by this pathogen is typically relatively self-limiting (lasting between 4-7 days) and is characterized by mild fever, abdominal cramps and diarrhea (Centers for Disease Control and Prevention 2011). However, in severe cases (when infection spreads into the blood stream), the organism may cause death.

Associated Outbreaks-

Outbreaks of salmenollosis are most commonly associated with the consumption of foods of animal origin (e.g. poultry, eggs, milk and beef); however it has recently been implicated as the causative agent in a number of outbreaks, involving the consumption of apple cider and orange juice (Parish 1997; Parish et al., 1997; Poppe et al., 1998; U.S. Food and Drug Administration 2009). While *S. enterica* cannot grow in high-acid products (pH ca.3.6), it contains a number of virulence factors, which allow it to survive at pH as low as 3.0 (Foster 1991; Linton et al., 1999; Uljas and Ingham 1999).

Mechanisms of Acid Survival-

Acid adaptation in *S.* Typhimurium occurs in two successive steps, which are required to protect the cell against severe acid stress (Foster and Hall 1991; Leyer and Johnson 1993). This step-wise progression is believed to occur so that resistance acquired during the first stage offers protection for proteins produced during the second phase (Foster and Hall 1991). These actions provide protection against severe acid damage, as well as a number of other environmental stressors, including heat, osmotic shock and surface-active antimicrobials (Leyer and Johnson 1993).

Preshock:

The first step (commonly referred to as preshock) occurs when exponentially growing cells are exposed to mildly acidic conditions (pH 4-5.8) (Leyer and Johnson 1993). During this stage, the bacterial cell undergoes a number of physiological changes, resulting in the expression of 18 proteins (likely Na^+/H^+ and K^+/H^+ antiporters) (Foster and Hall 1991). These protein are believed to assist in maintaining internal pH around pH 7.6, protecting the cell from protein denaturation (Foster and Hall 1991).

Acid Shock:

The second stage of acid adaptation (commonly referred to as acid shock) occurs when bacterial cells are exposed to severely acidic conditions (pH < 4.5) (Leyer and Johnson 1993). The bacterial cell during this phase, is no longer capable of replicating (an energy-conserving action); rather it produces ca. 52 proteins (likely chaperonins) believed to aid in refolding of denatured proteins (Foster and Hall 1991).

These chaperone molecules (GroEL and DnaK) are identical to those produced during heat-shock and are believed to offer cross resistance to heat damage (O'Driscroll et al., 1996). A

number of studies have also reported that acid-shock proteins protect the cell from stresses caused by salt (or osmotic shock), and certain surface-active antimicrobials, such as crystal violet (Leyer and Johnson 1993). These mechanisms make *Salmonella* spp. especially troublesome to the apple juice industry because it is capable of surviving at extremely low pH (pH 3-4), once thought to inactivate pathogenic microorganisms (Foster and Hall 1991).

APPLE JUICE OUTBREAKS

Apple juice has not traditionally been considered a significant food safety risk due to its intrinsically low pH (3.6-4.0); however, it has recently caused several large-scale outbreaks of foodborne illness (Choi and Nielsen 2004;Vojdani et al., 2008; Truong et al., 2010). Unpasteurized apple juice, was responsible for 10 of 21 CDC-reported juice outbreaks between 1995 and 2005 (Miller and Kasper 1994; Cody et al., 1999; Vojdani et al., 2008; Truong et al., 2010).

To date, *E. coli* O157:H7, *Cryptospodidium* spp. and *S. enterica.* have caused the largest number of apple cider-related outbreaks (Luedtke and Powell 2000; Choi and Nielsen 2004). Additionally, U.S. FDA has also identified *L. monocytogenes* as an emerging threat to food safety, because the bacterium has been isolated from the surface and flesh of apples in several food-safety studies (Roering et al., 1999; Conway et al., 2000; Leverentz et al., 2006). Outbreaks associated with these microorganisms will be discussed below.

Salmonella enterica

The first documented North American outbreak involving consumption of fresh apple cider occurred in 1923, when 24 individuals became ill with typhoid fever (Cornwell 2001; Sapers et al., 2006). It was later reported that apples used to produce the cider were washed in stream water, containing high levels of *S. enterica* Typhi (Luedtke and Powell 2000; Cornwell

2001). Likewise, *S. enterica* was also the causative agent in the 1974 outbreak (New Jersey, USA), whenh 300 people were infected with *S. enterica* Typhimurium after consuming raw apple cider that was produced with contaminated "dropped" apples (Luedtke and Powell 2000).

While outbreaks involving *S. enterica* serovars have caused major problems within the apple juice industry, the organism has not been implicated as the causative agent in a cider-related outbreak since 1974 (*S. enterica* is typically considered a primary organism of concern within the orange juice industry). *E. coli* O157:H7, on the other hand, has caused more than seven major outbreaks involving fresh apple juice within the past twenty years; making it the primary organism of concern to the apple juice industry (Rangel et al., 2005).

E. coli O157:H7

The first documented outbreak involving *E. coli* O157:H7 occurred in 1980; however *E. coli* O157:H7 was not recognized as a pathogen until 1982 when fourteen Canadian children developed HUS following the consumption of fresh apple cider purchased from a local fair (Zhao et al., 1993; Conner and Kotrola 1995; Edlow 2009). The outbreak lasted approximately ten days, resulting in one death (Edlow 2009). Epidemiological studies later revealed that the juice was produced using apples exposed to contaminated fecal matter (Luedtke and Powell 2000).

Twenty-three people, in 1991 also were infected with *E. coli* O157:H7 (16 developing bloody diarrhea and 4 developing HUS), following the consumption of contaminated apple juice sold at a roadside stand. It was later confirmed that the juice was produced using contaminated "dropped" apples washed in poor-quality water (Luedtke and Powell 2000).

The most pivotal juice outbreak, however, was the 1996 Odwalla outbreak, in which 66 people were infected with *E. coli* O157:H7 (12 hospitalized with HUS; 1 child dying from

complications from the illness) (Luedtke and Powell 2000). The outbreak spanned four states (and one Canadian province) spanning over four months (Luedtke and Powell 2000). The source of the outbreak was unpasteurized apple juice produced with decaying fruit, held or processed under inadequate sanitizing conditions (Cody et al., 1999; Luedtke and Powell 2000). As a direct result of this highly-publicized outbreak, Odwalla recalled all of their apple-juice containing products and began pasteurizing all of their juices (Martinelli and Briggs 1998). Aside from these large-scale, commercial outbreaks, a number of small, non-retail outbreaks have also influenced current regulatory policies regarding the production and distribution of fresh apple juice.

The first documented case of *E. coli* O157:H7 poisoning, involving the consumption of non-retail apple juice, occurred in 1996 (Washington State), when 6 people became ill after drinking apple cider prepared at a local church event. It was later documented that the apples used for juicing had been washed in a chlorine rinse, containing improperly monitored chlorine concentrations (Luedtke and Powell 2000). Other incidents, such as the 1998 Ontario, Canada outbreak, also involved the consumption of homemade apple cider. Ten people in this case, were infected with *E. coli* O157:H7 after consuming fresh cider produced on a neighbor's farm. (Luedtke and Powell 2000).

Cryptosporidium spp.

While outbreaks caused by bacterial pathogens are more common in apple cider, a number of outbreaks have also been caused by parasitic microorganisms such as, *Cryptosporidium*, which is the leading cause of illnesses associated with fresh apple cider. The largest *Cryptosporidium* outbreak occurred in 1993 (Maine, USA) causing over 215 cases of cryptosporidiosis (Luedtke and Powell 2000; Sapers et al., 2006). The source of the outbreak was contaminated apple cider sold at a local fair. It was later discovered that the cider was

produced using apples grown in close proximity to a cow pasture (Luedtke and Powell 2000). This particular outbreak was the largest of its kind and therefore extremely well-publicized; however, a number of smaller (less-publicized) outbreaks have also been documented. One example is the 1996 (New York) outbreak, involving thirty-two people that were infected with *Cryptosporidium* after consuming apple juice produced using contaminated well water. Local authorities reported that the water used to wash the apples tested positive for fecal coliforms (Luedtke and Powell 2000).

APPLE JUICE PROCESSING TECHNOLOGIES

Following a series of juice-related outbreaks, the U.S. Food and Drug Administration (FDA) issued a ruling requiring all commercial juice processors to achieve a 5-log reduction in bacterial organisms of concern, prior to selling juice to the public (Beerbower 2002; Choi 2004; Sapers 2006; Vojdani 2008). In most cases, the apple juice industry relies on thermal pasteurization (>160F for ≥6 seconds) to ensure product safety; however, due to recent shifts in market trends, towards minimally-processed foods, many processors have begun to explore use of non-thermal techniques to safe guard their products. (Zhao 1993; Zink 1997; Tajchakavit 1998; Rahman 1999; Beerbower, 2002; Choi 2004; Mosqueda-Melgar 2008).

Non-thermal methods include UV irradiation (the only FDA-approved alternative to thermal pasteurization of fruit juices), high-pressure processing, microwave heating, ultra filtration, ozone and pulsed electric field (Gould 1996; Buchanan 1998; Tajchakavit 1998; Choi 2004). While all of these techniques are effective in inactivating target pathogens in juice, their acceptance by the food industry is often hampered, due to negative consumer perceptions regarding their use and labeling (Hunter 2000; DeRuiter 2002; Nayga 2005). Exploration into the use of natural plant-derived preservatives therefore has become increasingly popular (Gould 1996).

Natural Antimicrobials from Plants

It is well known that plants and some of their extracts exhibit antimicrobial activity, which is hypothesized to have evolved from the plant's natural host defenses against pests and other invasive organisms (Caccioni et al., 1998; Hammer et al., 1999; Burt and Reinders 2003; Holley and Patel 2005). Humans have been aware of these benefits for centuries, with reports of their medicinal use dating to ancient times (Zaika 1988; Suhr and Nielson 2003).

Today, several common flavoring agents, such as garlic, esp. turmeric, black pepper, clove, ginger, cumin, rosemary, etc. are currently being used as therapeutic and antitoxigenic agents to reduce inflammation, prevent cancer, and treat acute respiratory diseases (Arora and Kaur 1999; Dog 2006). While the majority of these compounds are generally recognized as safe (GRAS) by the FDA as flavoring additives their approval as antimicrobial agents is contingent upon research involving their toxicity and compatibility with food matrices (Moleyar and Narasimham 1992; Lambert et al., 2001).

Plant-derived antimicrobials, like their synthetic counterparts are active against a wide array of yeast, molds, and bacteria (Smith-Palmer et al., 1998). Their degree of inhibition is determined by their chemical structure and overall composition with essential oils (EO), organic acids and six-carbon aldehydes (and alcohols) primarily contributing to their antimicrobial nature. Since a large number of organic acids already have GRAS status as food preservatives or acidulants, this paper will focus primarily on the use of essential oil (EO) compounds and volatile six-carbon aldehydes (Gould 1996).

Plant Essential Oils

Essential oils are volatile, aromatic/oily liquids present in the flowers, stems, leaves, roots, wood, bark, etc. of many plant species (Hummelbrunner and Isman 2001; Davidson 2005).

These compounds are responsible for the flavor and aroma characteristics of herbs and spices, and are therefore added to foods as flavoring agents

To date, over 3,000 different essential oils have been identified, with 300 available for commercial use (Burt 2004). Of this group, over 1,340 species have been reported to exhibit antimicrobial effects, while the broadest range of antimicrobial activity is associated with oils of oregano, garlic, clove, lemongrass, rosemary, sage and vanillin exhibiting (Hammer et al., 1999; Suhr and Nielson 2003; Davidson 2005; Holley and Patel 2005). Furthermore, several studies have reported that thyme, bay, cinnamon and nutmeg oils are also active against a wide array of microorganisms, including *Staphylococcus epidermidis, S. aureus*, *S.* Enteritidis *S.* Typhi, *E. coli*, *Bacillus cereus* and *L. monocytogeness* (Rees et al., 1993; Smith-Palmer et al., 1998; Arora and Kaur 1999; Benkeblia 2004; Holley and Patel 2005). In most cases, however the activity of these compounds changes depending on the structure and overall composition of the target food product.

It is generally understood that EOs (due to their hydrophobic nature) are more likely to coalesce with the lipid phase of foods; thus limiting their activity in high-fat food products (Tassou 1995; Burt et al., 2005). High-acid foods are also capable of suppressing the activity of EOs, making it extremely difficult for processors to predict their activity within actual food systems. Despite this phenomenon, several studies have reported on the enhanced killing effects of EOs against pathogens within a fruit juice matrix.

Freidman et al. (2004) reported that oregano oil, geraniol, cinnamon leaf, lemon oil, cinnamon bark oil, and lemon oil were all effective antimicrobials against *E. coli* and *S. enterica* serovars in an apple juice matrix. Raybaudi-Massilia et al. (2006) also identified lemongrass, cinnamon and geranoil as being effective against *S. enterica* Enteritidis, *E. coli* and *L. inocua* in

apple and pear juice. Although the inhibitory nature of these oils is not fully understood, it has been suggested that only certain chemical constituents contribute to their overall antimicrobial properties.

EOs are comprised of over 60 different classes of compounds, including terpenes, phenolic compounds, aliphatatic alcohols, aldehydes, ketones, and isoflavonoids (Nychas 1995; Davidson 2005). These constituents are divided into two groups based on their relative concentrations, with major constituents present in high concentrations (up to 85% of total EO content) and minor constituents present in lower concentrations (Burt 2004). It is believed that major constituents primarily contribute to the antimicrobial activity, while minor constituents produce synergistic effects that can enhance inhibition (Hummelbrunner and Isman 2001).

Plants within the same species typically produce similar essential oil profiles; however small changes in composition (i.e. chemical structure, or presence/absence of a functional group), can lead to variation in overall activity, including cell wall degradation, cell membrane disruption, inactivation of metabolic enzymes, disruption of protein synthesis and interruption of genetic systems (Holley and Patel 2005). Their composition and activity can vary, depending on geographic location, environmental conditions, location within the plant, method of extraction, maturity of the plant, etc. (Cosentino et al., 1999; Cimanga et al., 2002) .

Phenolic compounds represent the largest group of plant-derived antimicrobials, and include substances such as eugenol, cinnamic acid, gallic acid, thymol, citral, oleuropein, etc. (Gould 1996; Holley and Patel 2005). These compounds are hydrophobic in nature and, therefore, capable of inserting themselves into the lipid portion of the cytoplasmic membrane leading to the formation of pores, leakage of intercellular fluid, dissipation of the proton motive force, and in some cases, cell death (Davidson 2005).

These compounds are active against a wide array of foodborne pathogens, including *S. enterica* Typhimurium, *S. aureus*, *E. coli*, *Vibrio parahaemolyticus*, *Clostridium perfringens* and *Pseudomonas flourescens* (Davidson 2005). In general, however, Gram-positive microorganisms are more susceptible to the action of these compounds with Gram-negative microorganisms displaying increased levels of resistance, due to the presence of a highly selective outer-membrane (Davidson 2005; Holley and Patel 2005). While the activity of these compounds varies between studies, it is agreed that certain compounds are consistently effective against important foodborne pathogens.

Freidman, et al. (2002) screened the activity of 23 different EO constituents, finding that carvacrol, cinnamaldehyde, thymol, salicylaldehyde, geraniol, isoeugenol, citral, perillaldehyde, estragole and eugenol were effective against a number of foodborne pathogens, including *C. jejuni*, *E. coli*, *L. monocytogenes* and *S.enterica*. Kim et al. (1995) further confirmed the activity of citral, perillaldehyde, carvacrol, and geraniol, against *E. coli*, *S.* Typhimurium, *L. monocytogenes*, and *V. vulnificus*. While these and other studies document the activity of numerous EO components, only a handful of compounds show promise as potential antimicrobial agents in foods; these compounds will be further discussed in this paper.

Thymol

Thymol (5-methyl-2-(1-methylethyl)-phenol) is a potent antimicrobial compound that's abundant in the leaves, stem, roots, etc. of thyme and origanum species (Karapinar and Aktug 1987; Bagamboula et al., 2004) and is currently approved for use as a synthetic flavoring agent (up to 50mg/kg) (Bogadanov et al., 1998). Thymol has been demonstrated to inhibit the growth of several spoilage and pathogenic microorganisms, including *E. coli* O157:H7, *L. monocytogenes*, *S. enterica* serovars, *Shigella* spp., etc (Bagamboula et al., 2000; Ettayebi et al.,

2004).

Thyol, like most hydrophobic compounds, is assumed to disrupt the cytoplasmic membrane, which in turn leads to leakage of intercellular fluids and cell death (Helander, et al., 1998). However, unlike other phenolics, thymol possesses outer-membrane-disintegrating activity, making it extremely active against a number of Gram-negative microorganisms (Olasupo, et al., 2003). Such broad-spectrum activity makes thymol amongst the most effective and commonly studied EO compounds to date (Nazer, et al., 2005).

Thymol's antimicrobial properties were first reported in the mid 1980s when Karpinair, et al. (1987), used the standard agar dilution method to evaluate its activity against several foodborne pathogens. This study revealed that concentrations as low as 25 µg/ml and 75µg/mL could inhibit the growth of *S. aureus* and *V. parahemolyticus,* respectively (within a general growth medium) (Karapinar and Aktug 1987). Helander et al. (1998) later evaluated its activity against *E. coli* O157:H7, and *S.* Typhimurium, using the bioluminescense assay and reported that thymol inhibited the growth of both organisms at concentrations of 1-3mM. Olasupo et al. (2003) further confirmed these findings, when they reported that concentrations of 1.0 and 1.2 mmol prevented the growth of *S. enterica* Typhimurium and *E. coli*, respectively.

While these and other reports prove that thymol is extremely effective against pathogens within a laboratory setting only a few studies have evaluated its activity within a food matrix. Thymol, in one study, was evaluated alone and in combination with cymine to determine its antimicrobial effects in carrot juice, chicken soup, and pumpkin cream (Delgado et al., 2004). It was reported that low concentrations of thymol (up to 2.0 mmol/L) reduced *B. cereus* counts by one log cycle within 24 hours, although cymene wasn't as effective, requiring concentrations up to 4 mmol/L to inhibit growth. Furthermore, it was also established that low concentrations of

thymol and cymine produced synergistic effects within the tested food products, lowering the concentration of thymol needed to inhibit bacterial growth (Delgado et al., 2004).

Such synergistic relationships are important to the food industry because, despite its enhanced antimicrobial properties, thymol has an extremely pungent flavor, which can produce strong phenolic, medicinal, woody and spicy flavor profiles at very low concentrations (thymol has an aroma detection threshold between 86-790 ppm). Such descriptors can lead to off flavors in juices, which are commonly perceived as spoilage by consumers. Combining thymol with other naturally occurring antimicrobial compounds can, however, lower the concentration of antimicrobials needed to inhibit bacterial growth, without compromising product safety and quality.

One compound that has received a great deal of attention in recent years is nisin (a bacteriocin produced by *Streptococcus lactis*), believed to enhance the killing effects of thymol and other EO compounds. While nisin alone has very strong antimicrobial properties, against Gram-positive microorganism, its activity when used in combination with other treatments is not always as effective. Such is the case for Olasupo et al. (2003), who concluded that thymol has no noticeable effect on the activity of nisin against similar organisms. In this case, low concentrations of nisin and thymol were applied to *Listeria monocytogenes* in a general growth medium, and monitored after a short period of time to determine its killing effects. Conversely, Ettayebi et al. (2000), reported that thymol-nisin combinations produce synergistic effects against common foodborne pathogens. Despite inconsistencies in reported data, thymol continues to be the subject of testing for antimicrobial properties.

Aside from interactions with nisin, thymol has also been proven to produce synergistic effects when used in combination with other EO compounds. Nazer et al. (2005), provided a

comprehensive review of combinational treatments, and concluded that thymol acted synergistically with citral, geranoil, eugenol and carvacrol. In addition, Pei et al. (2009) also evaluated the activity of thymol alone and in combination with several EO compounds, reporting that thymol enhanced efficacy when paired with eugenol and carvacrol (with MIC values decreasing from 400 to 100 mg/L when used in combination). These and other reports suggest that thymol has great potential as a preservative agent, although a great deal of sensory analysis is still needed before it can be effectively adopted by the food industry.

Eugenol

Eugenol (4-allyl-2-methoxyphenol) is a phenolic compound abundant in the oils of clove (representing 93-95% of the total oil content), allspice (up to 9.33%), and cinnamon (up to 5.38%) (Karapinar and Aktug 1987; Ouattara et al., 1997; Dusan et al., 2006). Eugenol is currently approved for use within the food and cosmetic industries as a flavoring and perfume agent, respectively; and has widespread application within the dental industry, as an ingredient in toothpastes, mouthwashes, dental sealants and local anesthetics (Schwarts et al., 1998; Dusan et al., 2006; Amiri et al., 2008). Aside from commercial applications, eugenol also possess strong antimicrobial, anti-inflammatory and antioxidant properties, which makes it a suitable alternative to traditional food preservatives (Ali et al., 2005).

To date, eugenol has been proven to inhibit the growth of several of foodborne pathogens, including *E. coli, L. monocytogenes, S. aureus, Campylobacter jejuni, S. enterica,* etc. (Ali et al., 2005; Dusan et al., 2006; Hemaiswarya and Doble 2009). Eugenol is also active against a number of food-spoilage organisms, including *Lactobacillus curvatus, Lactobacillus sake, Leuconostoc mesenteroide,* etc. (Bartels 1947; Blaszyk and Holley 1998).

While eugenol's exact mode of action is not fully understood, it is generally assumed that

it acts by disrupting the cytoplasmic membrane of bacterial cells(due to its hydrophobic nature) which, in turn, causes leakage of intercellular ions (such as K+), as well as loss of membrane-bound lipids and proteins then leads to cell death, due to rapid depletion of the proton motive force and leakage of intercellular ATP. (Ouattara et al., 1997; Walsh et al., 2003; Gill and Holley 2006;Gaysinsky et al., 2007; Galluci et al., 2009; Hemaiswarya and Doble 2009; Oyedemi et al., 2009).

It is important to note, however, that these actions are highly dose-responsive, requiring higher concentrations to produce bactericidal effects (rather than bacteriostatic inhibition) (Galluci et al., 2009). These actions were proven by Gill et al. (2006), who reported an 80% increase in membrane permeability of *E. coli* O157:H7 following treatment with 5 mM and 10 mM of eugenol, respectively.

Aside from its membrane-dissipating activity, eugenol is believed to inhibit the activity of membrane-bound ATPase (the enzyme responsible for the generation of ATP and pH regulation) which, in turn, causes secondary antagonism in bacterial cells (Gill and Holley 2006; Ahmad et al., 2010). This mechanism was confirmed by, Ahmad et al. (2010), who reported that low concentrations (500µg/ml) of eugenol greatly reduced the intercellular pH of various *Candida spp.;* an action likely caused by increased H+ efflux from the cell. Gill et al. (2006) also demonstrated that low concentrations of eugenol (5 mM) inhibited flagellar motility in *E. coli* and *L. monocytogenes*, likely caused by a combination of membrane disruption (as evidenced by depletion of the proton motive force) and inactivation of membrane-bound proteins (Gill and Holley 2006). The broad-spectrum activity of eugenol makes it an ideal candidate for antimicrobial combination testing.

Since eugenol is effective against both Gram-negative and Gram-positive bacteria, it is

commonly used to enhance the activity of non-specific antimicrobials (and antibiotics), which have limited activity against gram-negative bacteria (Holetz et al., 2002; Gill and Holley 2004; Hemaiswarya and Doble 2009). A study involving the antibiotic resistance of *E. coli, E. aerogenes, P. aeruginosa, S.enterica* Typhimurium *and P. vulgaris*, assessed the ability of eugenol to enhance the uptake vancomycin and β-lactam into the outer membrane of target cells. This action reduced the concentration (of antibiotics) needed to inhibit microbial growth thus lowering the likelihood of antibiotic resistance in tested pathogens (Hemaiswarya and Doble 2009).

Similar patterns of synergism were also reported by Blaszyk et al. (1998), who evaluated the activity of eugenol (500 and 1000ppm) in combination with monolaurin (100-250 ppm) and sodium citrate (0.2 and 0.4%) against *E. coli* and *L. monocytogenes*. It was hypothesized that sodium citrate behaved as a chelator, increasing permeability of the outer membrane of *E. coli* and rendering the cell susceptible to the action of eugenol. It was later noted that monolaurin (an emulsifier) increased the overall solubility of eugenol, causing increased damage to the cytpolasmic membrane. This synergism was expected because the compounds attacked different target sites within the cell

Conversely, a number of studies have proven that eugenol is often incapable of enhancing the activity of other membrane-active compounds. This is especially true for nisin (a bacteriocin produced by *L. lactis*) which, despite its membrane-dissipating activity has limited activity against Gram-negative bacteria (Abee et al., 1994). Olasupo et al. (2003) reported that the membrane-damaging activity of eugenol was not sufficient to allow nisin to enter the cells of *S. enterica* Typhimurium and *E. coli.* Thus resulting in a combined MIC similar to that of the individual compound (Olasupo et al., 2003).

A number of membrane-active essential oil compounds have also been proven to suppress the activity of eugenol, as demonstrated by Galluci et al. (2009), who evaluated the activity the thymol, geraniol, carvacrol and methanol, in combination with eugenol (against *S. aureus*, *E. coli* and *B. cereus*). Eugenol-containing combinations produced antagonistic (or indifferent) effects, resulting in a MIC greater than that of the individual compound.

Certain high-acid and fatty foods have also been proven to inhibit the activity of eugenol. Because eugenol is relatively hydrophobic in nature, it typically associates with the lipid phase of foods, reducing its ability to interact with target bacteria, which exist in the aqueous phase (Blaszyk and Holley 1998; Gaysinsky et al., 2007). For this reason, a number of studies have utilized food-grade surfactants to enhance dispersion of eugenol into the aqueous phase (Li 2011). Low concentrations of lecithin (0.0025 and 0.005%), in one study were proven to enhance the antimicrobial activity of eugenol against *E. coli* present in vegetable juice (pH 4.16-4.21) (Li 2011). It is suggested that lecithin (due to its zwitterionic properties) formed a stable emulsion in solution, which enhanced the attachment of eugenol to the surface of the bacteria, allowing eugenol to be used in food environments that are, otherwise, unaffected by its antimicrobial activity.

It is important to note, however that the solubility-enhancing properties of emulsifiers are only effective at relatively low concentrations, due to their inherent nature to form micelles in solution (Li 2011). When surfactants are applied at concentrations above their "critical micelle concentration", the antimicrobial activity of eugenol is suppressed to a level equal to (or below) its individual level of activity. This was the case for Gaysinsky et al. (2007) who reported that high concentrations of Surfynol 485W (a common surfactant) did not improve the activity of eugenol against *L. monocytogenes* and *E. coli* O157:H7 in reduced-fat milk as eugenol (when

incorporated into the micelar complex) becomes localized in the lipid portion of the milk, lowering the concentration available to interact with the bacterial membrane. Despite its limitations in high-fat foods, eugenol, however, remains the focus of several studies, which aim to preserve minimally-processed foods.

Cinnamaldehyde

Cinnamaldehyde or cinnamic aldehyde (3-phenyl-2-propenal) is an aromatic aldehyde abundant in cinnamon-bark oil, representing ca. 70%-90% of the total composition of cinnamon oil (Ali et al., 2005; Chen et al., 2009). It is largely responsible for the characteristic flavor and aroma of cinnamon (U.S. Environmental Protection Agency 2011). Cinnamaldehyde is currently approved for use within the food industry at concentrations of 9-4900 ppm, to enhance the cinnamon-flavor of certain candies, ice creams, non-alcoholic beverages, and chewing gums (U.S. Environmental Protection Agency 2011). Cinnamaldehyde is also used as a fungicide to control the spread (and growth) of corn rootworm, *Verticillium* spot, *Rhizoctonia*, Pythium and Pitch canker disease on a number of field crops (Niu et al., 2006). Aside from its commercial applications, cinnamaldehyde has been studied for its enhanced ability to inhibit the growth of harmful bacteria (Davidson 2005).

Like most EO compounds, cinnamaldehyde is effective against a number of pathogenic microorganisms (including *S.* Typhimurium*, K. pnuemoniae, H. pylori, E. coli, C. botulinum, V. parahaemolyticus, S. aureus* and *B. cerus*) (Ali et al., 2005; Gill and Holley 2006; Ooi 2006). Unlike other phenolic EO compounds, cinnamaldehyde does not act by disrupting the cytoplasmic membrane of bacterial cells and is therefore incapable of releasing membrane-bound lipopolysaccharides and proteins from the cell (Helander et al., 1998; Gill and Holley 2006). Moreover, cinnamaldehyde does not possess strong ATPase-inhibiting activity, which affects its

overall ability to inhibit microbial growth (Gill and Holley 2006).

While its specific mode of action is unknown, several studies suggest that cinnamaldehyde is capable of disrupting bacterial quorum sensing, by inhibiting the binding of certain signal molecules (particularly 3-hydroxy-C4- and 3-oxo-C12-HSL) (Niu et al., 2006). Furthermore, the compound has also been proven to block the synthesis of certain autoinducer molecules such as those involved with the AI-2 synthetic pathway (Niu et al., 2006). Such actions make cinnamaldehyde extremely effective at inhibiting the growth of harmful foodborne pathogens.

Several studies have in fact reported that the activity of cinnamaldehyde is comparable to or greater than the activity of other membrane-active essential oil compounds. One study evaluating the activity of several EO compounds (including carvacrol, (+) carvone, thymol and *trans*-cinnamaldehyde) against *E. coli* O157:H7 and *S. enterica* Typhimurium in a general growth medium reported that cinnamaldehyde was among the most active EO compounds (with an MIC of 3 mM for both organisms), followed by (+) carvone (MIC=10 mM) (Helander et al., 1998). Similar patterns of activity were reported by Zhou et al. (2007), who reported that cinnamaldehye was significantly more effective than thymol and carvacrol (which produced MICs that were twice that of cinnamaldehyde) at inhibiting the growth of *S.* Typhimurium.

Such activity has led a number of researchers to evaluate the antimicrobial efficacy of cinnamaldehyde in model food systems, particularly fresh fruit juices. In recent years, low concentrations of cinnamaldehyde have been used to inhibit the growth of common foodborne pathogens, including *E. coli* O157:H7 and *S. enterica* in fresh and commercially processed apple juices (Friedman et al., 2004; Baskaran et al., 2010). In addition, cinnamaldehyde (in combination with eugenol) was used to prevent the outgrowth of *Alicyclobacillus acidoterrestis*

spores in commercial apple juice (Bevilacqua et al., 2010). Cinnamaldehyde has also been used to preserve a number of other food products, including carrot broth and cooked ground turkey, making it an ideal candidate for use as a food preservative (Valero and Frances 2006; Juneja and Friedman 2007).

Six-carbon Aldehydes ($C_6H_{12}O$)

Aside from the inhibitory effects of plant-derived essential oil compounds, a number of six-carbon aldehydes (and their corresponding alcohols) also exhibit strong antimicrobial activity against a number of postharvest spoilage microorganisms and foodborne pathogens (including: *Botrytis cinerea, Alternaria alternate*, *Colectotrichum gleosporioides, L. monocytogenes, E. coli, S. enterica* Enteritidis*, P. aeruginosa, E. aerogenes* and*, P. vulgari*) (Muroi et al., 1993; Gardini et al., 1997; Bate and Rothstein 1998; Gardini et al., 2001; Song et al., 2007; Patrigani et al., 2008).

These compounds are naturally present in plants at varying concentrations; and are likely a byproduct of the lipoxygenase pathway (a set of enzymatic reactions, which are induced during "wounding" in damaged plant tissue) (Archbold et al., 1997; Bate and Rothstein 1998; Gardini et al., 2001; Neri et al., 2006). They are thought to possess strong inhibitory properties, to protect the plant from secondary infection by opportunistic pathogens, as well as reduce insect feeding rates, preventing further tissue damage (Gardini et al., 1997; Bate and Rothstein 1998; Patrigani et al., 2008).

Aside from their antimicrobial properties, these volatile aldehydes serve as precursor molecules to the formation of secondary alcohols and esters (such as hexanol, hexylacetate, hexylbutanoate, and hexylhexanoate), which are responsible for the characteristic "green" notes of many fruit and vegetable products(Flath et al., 1967; Gardini et al., 2001; Fan et al., 2006).

For this reason, these compounds are commonly used as flavoring additives to enhance the "fruity" aroma of commercial products.

A number of these compounds (including ethyl 2-methylbutyrate, ethyl butyrate, hexyl acetate, 2-Methylbutyl acetate, butyl acetate, hexanal, *(E)*-2-hexenol, *(E)*-2-hexenal, hexanol and 2-Methylbutanol) are used to restore the natural apple aroma to concentrated apple juice (Da Costa and Cannon 2010). Of these compounds, several aldehydes (namely *trans*-2-hexenal, hexanal, and 1-hexanol) have been proven to exhibit strong antimicrobial effects against a number of foodborne pathogens. The activity of these compounds will be discussed in this section, with the exception of hexanol (the alcohol derivative of hexanal), which will be discussed in a later section.

Hexanal and trans-2-hexenal

Hexanal ($C_6H_{12}O$) and *trans*-2-hexenal ($C_6H_{10}O$, commonly referred to as leaf aldehyde) are both volatile six-carbon aldehydes that are formed during the oxidation of linoleic acid (a reaction which occurs during the lipoxygenase pathway) (Gardini et al., 2001; Neri et al., 2006; Sholberg and Randall 2007). They are currently approved for use within the food industry (as flavoring and fragrance additives) to enhance the "green" and "fruity" flavors of various fruit-based products (Neri et al., 2006; Da Costa and Cannon 2010).

Aside from their commercial applications, both compounds are known to possess antifungal (and antibacterial) properties, allowing them to be used as postharvest fumigants and sanitizers to control the growth of several postharvest spoilage organisms and foodborne pathogens, including *Botrytis cinerea, Alternaria alternate*, *Colectotrichum gleosporioides, L. monocytogens, E. coli S. enterica* Enteritidis*, P. aeruginosa, E. aerogenes* and *P. vulgari* (Muroi, et al., 1993; Gardini et al., 1997; Bate and Rothstein 1998; Gardini et al., 2001; Song et al., 2007;

Patrigani et al., 2008)

Hexanal (in its vapor phase) has been used to suppress the development of blue and gray mold (caused by *Penicillum expansium* and *Botryis cinera*, respectively) on whole and sliced apples as well as to slow the rate of decay (caused by *B. cinerea* and *M. fructicola*) on stored raspberries and peaches (Fan et al., 2006; Sholberg and Randall 2007; Song et al., 2007). Likewise, *trans*-2-hexenal has been evaluated for its ability to inhibit the growth of certain disease-causing fungi (e.g. *Aspergillus flavus* and *Penicillium expansum*) as well as to preserve the microbial quality of certain teas, sliced apples and grapes (Gardini et al., 2001; Neri et al., 2006).

Aside from the previous applications, both compounds have been evaluated for their effectiveness as preservatives in modified atmosphere packaging (MAP). Hexanal (when applied at a constant rate of 40-70μL L^{-1}) was found to inhibit the growth of *Botrytis cinerea* on whole tomatoes as well as increasing the rate of respiration of unripe fruit, thus delaying the reddening process (Utto et al., 2008). *Trans*-2-hexenal (when applied to ordinary and modified atmosphere packages) decreased the requisite concentration of *E. coli* by 3.7 and 1.3 log CFU/g, respectively on fresh sliced apples (Lanciotti et al., 2003). These properties make them ideal alternatives to traditional processing technologies.

Although both compounds are effective against a wide array of microorganisms, including both Gram-negative and positive bacteria, the activity of *trans*-2-hexanal appears to be far more effective than hexanal and similar aldehydes (Lanciotti et al., 2003; Patrigani et al., 2008). Several studies, have reported that the MIC of hexanal was up to 10 times greater than *trans*-2-hexenal when tested against *E. coli*, *S. enterica* Enteritidis and *L. monocytogenes* (Lanciotti et al., 2003). The inhibitory effects of *trans*-2-hexenal exceeded that of hexanal and a

number of essential oil compounds when evaluated against *Monillinia laxa* (causative agent of brown rot on stone fruit) (Neri et al., 2007).

Both compounds, are believed to behave as surface-active detergents, attaching to the sulfhydryl group of membrane-bound proteins via nucleophillic interactions involving their polar carbonyl groups (Kubo et al., 1995; Lanciotti et al., 2003; Neri et al., 2006; Neri et al., 2007). *Trans*-2-hexenal (an α, β-unsaturated aldehyde) however, contains a carbonyl group that is in close proximity to its double bond. This particular configuration makes *trans*-2-hexenal especially reactive with the sulfhydryl and amino groups of membrane-bound proteins, thus amplifying its effects on cell viability (Deng et al., 1993; Muroi et al., 1993; Archbold et al., 1997; Lanciotti et al., 2003; Neri et al., 2006; Neri et al., 2007; Patrigani et al., 2008). Conversely, hexanal, is a saturated aldehyde (lacking double bonds) making it significantly less reactive with membrane-bound proteins; thus, it is far less active against test microorganisms (Zhou and Decker 1999; Neri et al., 2006). Other differences in antimicrobial activity is the fact that the two compounds react differently to changes in environmental stimuli (including temperature, water activity (a_w) and solute content), with temperature having the greatest impact on overall activity (Gardini et al., 2001).

The antimicrobial activity of hexanal (and *trans*-2-hexenal, to a lesser extent) is highly dependent upon its vapor pressure (i.e., tendency to enter into the vapor phase) (Corbo et al., 2000). Because temperature has a direct effect on vapor pressure (with vapor pressure increasing proportional to changes in temperature), antimicrobial activity of such compounds should increase with an increase in temperature (Gardini et al., 2001; Lanciotti et al., 2003).

Several studies have reported that the antimicrobial activity of *trans*-2-hexenal decreases, following an increase in temperature (Corbo et al., 2000; Gardini et al., 2001). These differences

may be attributed to the fact that *trans*-2-hexenal has a relatively low vapor pressure (6.6 mmHG) compared to that of hexanal (11.26 mmHg), therefore they have different degrees of volatility (Gardini et al., 2001). Several studies have also reported that the activity of *trans*-2-hexenal is dependent upon antimicrobial concentration, with higher concentrations being more effective against test pathogens (Deng et al., 1993; Gardini et al., 2001).

While both compounds exhibit dose-dependent interactions, several studies have reported these concentrations lead to a degradation of quality in fresh fruit products (Deng et al., 1993; Corbo et al., 2000; Song et al., 2007; Neri et al., 2007). This is especially true for hexanal, which has been proven to cause black, scald-like discolorations on the surface of some pome fruits (e.g., Anjou pears and apples) (Sholberg and Randall 2007). High concentrations of *trans*-2-hexenal (20µl/L) have also been proven to generate dark-yellow and brown spots on peaches, nectarines, pears and apricots (Neri et al., 2006; Neri et al., 2007). These phytotoxic effects are likely a result of the compounds' innate ability to stimulate plant-defense reactions (e.g., allene oxide synthase and lipoxygenase), which causes a build-up of defense-related proteins, often associated with lingification of leaves (Neri et al., 2007).

Aside from these effects, high concentrations of these aldehydes have been proven to generate off-flavors in certain fruit products, particularly in drupe or stone fruits. High concentrations of *trans*-2-hexenal (used to control the growth of *Monilinia laxa* on stone fruit) have been associated with the production of "green" (leafy) flavors in nectarine and peaches; "fermented-acetic" or "cheesy" flavors in plums, as well as "green-bitter" flavors in nectarine (Neri et al., 2007).

These effects are likely a result of the plant reducing carbon-carbon double bonds in the target molecule, which leads to a production of *trans*-2-hexen-1-ol (alcohol derivative of *trans*-2-

hexenal) and *trans*-2-hexenyl acetate (ester derivative of *trans*-2-hexenal) (Hamilton-Kemp et al., 1996). Hexanal is also vulnerable to this process, because it is readily converted into its alcohol-derivative (hexanol), which has a slightly different flavor profile from hexanal (Song et al., 1996; Patrigani et al., 2008). While these responses do not affect the overall fruit-quality of most products (as indicated by pH, firmness, ˚brix and titratable acidity), they are often perceived as spoilage by consumers and are, therefore, undesirable within the food industry (Sholberg and Randall 2007).

For these reasons, these compounds are often evaluated in combination with a number of pre- and postharvest preservatives, which help to lower the effective concentration needed to inhibit microbial growth. Such synergism reduces the occurrence of deleterious sensory effects caused by high concentrations of the test aldehydes (Spotts et al., 2007).

One compound that has received a great deal of attention in recent years is 1-methylcyclopropene (a synthetic olefin used to block the activity of ethylene gas). When used in combination with hexanal gas, the treatment effectively inhibited the development of "bull's–eye rot" on cold-stored d' Anjou pears, while maintaining the fruit's natural firmness (Spotts et al., 2007). Combinations of hexanal, *trans*-2-hexenal and hexyl acetate also produced marked killing effects against *E. coli, S. enterica* Enteritidis and *L. monocytogenes* (Ayala-Zavala et al., 2009). While the above combinations offer new alternatives for food preservation, several additives have been proven to reduce the activity of these compounds.

Such is the case for *trans*-2-hexenal, whose activity was inhibited by the presence of common food additives. Combinations containing vitamin C (used to prevent oxidative damage to its enal group), anacardic acid (potent antimicrobial naturally present in cashew apples) and indole (green-tea flavor compound used to enhance the activity of similar aldehydes) all

produced additive (rather than synergistic) when evaluated against *P. ovale, S. mutant, S. aureus*, *P. aeruginos,* and *E. coli*, respectively (Muroi et al., 1993). Such relationships lead to an overall increase in the effective dose needed to inhibit microbial growth; an action which may have adverse effects on fruit quality.

For this reason, a great deal of research is still needed to identify effective concentrations and combinations which preserve foods without altering the overall quality of the product. Furthermore, it is important to understand the cytotoxic effects of these compounds when evaluated at their effective concentrations.

1-hexanol

1-hexanol (or 1-hexyl alcohol) is a volatile six-carbon alcohol that is produced during the metabolic break down of hexanal (an intermediate 6-C aldehyde that is produced during the lipoxygenase pathway) (Hamilton-Kemp et al., 1996). It is characterized as having a strong "green" aroma; and is therefore used to enhance the overall "fruity" flavor of many fruit-based products (particularly apple juice) (Neri et al., 2007; Da Costa and Cannon 2010). Unlike the activity of its corresponding aldehydes (hexanal and *trans*-2-hexenal), 1-hexanol displays limited antimicrobial activity against certain types of microorganisms (Archbold et al., 1997). For example, the MIC value of *trans*-2-hexenal, is up to sixteen times lower than that of 1-hexanol (for *P. ovale, P. chrysogenum,* and *T. mentagrophytes*) (Kubo et al., 1995). Despite this fact, several studies have demonstrated its ability to inhibit the growth of several postharvest pathogens, including *Botytis cinera*, *Rhizopus stolonifer* and *Penicillium* spp. (Archbold et al., 1997; Li et al., 2009).

Like most alcohols, 1-hexanol acts by disrupting the cytoplasmic membrane of bacterial cells, which causes an increase in membrane fluidity, and leakage of intercellular components

(Ingram and Vreeland 1980). However, 1-hexanol has additional inhibitory features, which enhance its effects within the target cell. The compound has been proven to decrease the concentration of unsaturated fatty acids present in the cytoplasmic membrane as well as decrease membrane polarization, an action which leads to cell death (Ingram and Vreeland 1980). These properties make 1-hexanol up to ten times more effective at increasing membrane fluidity, compared to other short-chain alcohols (Ingram and Vreeland 1980).

Despite its inhibitory effects within the cell, the activity of 1-hexanol is highly dependent upon environmental stimuli, with vapor pressures having a significant impact on its overall activity (Kim and Shin 2004). Like most volatile compounds, the activity of 1-hexanol increases proportional to changes in temperature, with higher temperatures having a positive effect on activity. High concentrations (3M) of salt, sodium sulfate and potassium chloride have also been proven to increase the activity of 1-hexanol, by increasing the number of volatile molecules present in a system (Ingram and Vreeland 1980). Unlike other volatile alcohols (and aldehydes), the activity of 1-hexanol is relatively unaffected by changes in pH, making it suitable for the preservation of certain high-acid fruit-based products (Kim and Shin 2004).

For this reason, 1-hexanol has been used to preserve the quality of a number of fresh fruits, such as raspberries and strawberries (Vaughn et al., 1993). One study reported that low concentrations of 1-hexanol (0.1μL/mL) inhibited the growth of three postharvest pathogens (including *Alternaria alternate*, *Botrytis cinera*, and *Colletotrichum goleosporioides*) (Vaughn et al., 1993). Furthermore, the concentrations used in this study did not produce deleterious effects on the overall quality of both strawberry and raspberry fruit when compared to several aldehydes, which caused significant damage to fruit tissue (Vaughn et al., 1993).

Aside from its inhibitory effects against postharvest fungi, 1-hexanol has been used to

control the growth of several foodborne pathogens, including *S. aureus*, *E. coli*, and *S. enterica* Enteritidis, (Morris et al., 1979; Kubo et al., 1993; Nakamura and Hutanaka 2002). While the inhibitory effects of 1-hexanol are typically less potent than that of hexanal (its corresponding aldehyde), certain studies have reported that the two compounds produced comparable inhibitory effects. One study reported that the two compounds resulted in uniform MICs of 12.5 μg/mL for *E. coli* O157:H7, *S. aureus*, and *S. enterica* Enteritidis (Nakamura and Hutanaka 2002). Both compounds produced similar levels of inhibition, when evaluated for their bactericidal effects (Nakamura and Hutanaka 2002).

Similar patterns were also reported by Kubo et al. (1995), who evaluated the antimicrobial activity of eleven olive oil flavor compounds. In their study, the MICs (and minimum bactericidal concentrations MBCs) of 1-hexanol were almost identical to that of hexanal against a number of microorganisms, including *B. subtilis, B. ammoniagenes, S. aureus, S. mutans, P. acnes, P. aeruginosa, E. aerogenes, E. coli, P. vulgaris, S. cervisiae, C. utilis, P. ovale, P., chryogenum, T. metagrophytes* and *A. niger*.

In addition to its antimicrobial properties, 1-hexanol is essential to the development of certain aroma compounds in ripe fruits, such as apples and strawberries (Knee and Hatfield 1981; Hamilton-Kemp et al., 1996). These aromatic compounds are produced as a result of the plant's ability to metabolizes alcohols into their corresponding acetate esters (Hamilton-Kemp et al., 1996). The compound, 1-hexanol is readily esterified into hexyl acetate as well as a number of butyrates (compounds responsible for the strong "fruity" aroma of many fruit products) (Ahmed et al., 1978; Knee and Hatfield 1981; Hamilton-Kemp et al., 1996). These flavor-developments can be used to mask the flavor (and aroma) of stronger antimicrobial compounds, which lack compatibility with certain fruit-based products. This feature makes 1-hexanol an ideal

candidate for the preservation of fruit-based products.

References

Abee, T., F. M. Rombouts, et al., (1994). "Mode of action of nisin Z against *Listeria monocytogenes* Scott A grown at high and low temperatures." Applied and Environmental Microbiology **60**(6): 1962-1968.

Ahmad, A., A. Kahn, et al., (2010). "Proton translocating ATPase mediated fungicidal activity of eugenol and thymol " Fitoterapia **81**: 1157-1162.

Ahmed, E. M., R. A. Dennison, et al., (1978). "Flavor and odor thresholds in water of selected orange juice components." Journal of Agricultural and Food Chemistry **26**(1): 187-191.

Ali, S. M., A. A. Khan, et al., (2005). "Antimicrobial activities of eugenol and cinnamaldehyde against the human gastric pathogen *Helicobacter pylori*." Annals of Clinical Microbiology and Antimicrobials **4** (20).

Amiri, A., R. Dugas, et al., (2008). "In vitro and in vitro activity of eugenol oil (*Eugenia caryophylata*) against four important postharvest apple pathogens." International Journal of Food Microbiology **126**: 13-19.

Ang, C. Y. W., K. Liu, et al., Eds. (1999). Asian foods science and technology. Fruit Products. Lancaster, PA, Techomic Publishing Company, Inc.

Archbold, D. D., T. R. Hamilton-Kemp, et al., (1997). "Identifying natural volatile compounds that control gray mold (*Botrytis cinera*) during postharvest storage of strawberry, blackberry and grape." Journal of Agricultural and Food Chemistry **45**: 4032-4037.

Arora, D. S. and J. Kaur (1999). "Antimicrobial activity of spices." International Journal of Antimicrobial Agents **12**: 257-262.

Ayala-Zavala, J. F., G. A. Gonzalez-Aguilar, et al., (2009). "Enhancing safety and aroma appealing of fresh-cut fruits and vegetables using the antimicrobial and aromatic power of essential oils." Journal of Food Science **74**(7): R84-R91.

Bagamboula, C. F., M. Uyttendaele, et al., (2004). "Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards *Shigella sonni* and *S. flexneri*." Food Microbiology **21**: 33-42.

Banatvala, N., P. M. Griffin, et al., (2001). "The United States national prospective hemolytic uremic syndrome study: microbiologic, serologic, clinical, and epidemiologic findings." Journal of Infectious Diseases **183**(7): 1063-1070.

Bartels, H. A. (1947). "The effect of eugenol and oil of cloves on the growth of microorganims." American Journal of Orthodontics and Oral Surgery **33**(6): B458-B465.

Baskaran, S. A., M. A. R. Amalaradjou, et al., (2010). "Inactivation of *Escherichia coli* O157:H7

in apple juice and apple cider by trans-cinnamaldehyde." International Journal of Food Microbiology **141**: 126-129.

Bate, N. J. and S. J. Rothstein (1998). "C6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes." The Plant Journal **16**(5): 561-569.

Bates, R. P., J. R. Morris, et al., (2001). Principles and practices of small- and medium-scale fruit juice processing. Food Science and Human Nutrition Department University of Florida, Food and Agriculture Organization-United Nations Agricultural Services Bulletin. 146. Available at http://www.fao.org/DOCREP/005/Y2515E/y2515e00.htm . Accessed 29 March 2012.

Bearson, B. L., I. S. Lee, et al., (2009). "*Escherichia coli* O157:H7 glutamate- and arginine-dependent acid-resistance systems protect against oxidative stress during extreme acid challenge." Microbiology **155**: 805-812.

Beerbower, B., M. Botos, et al., (2002). Juice HACCP training curriculum, First Edition August 2002, Juice HACCP Alliance.

Benjamin, M. M. and A. R. Datta (1995). "Acid tolerance of enterohemorrhagic *Escherichia coli*." Applied and Environmental Microbiology **61**(4): 1669-1672.

Benkeblia, N. (2004). "Antimicrobial activity of essential oil extracts of various onions (*Allium cepa*) and garlic (*Allium satvum*)." Lebensm.-Wiss. u-Technol. **37**: 263-268.

Bennett, J. E., and A.E. , Jennings (1978). "Factors influencing susceptibility of nocardia species to trimethoprim-sulfamethoxazole " Antimicrobial Agents and Chemotherapy **13**(4): 624-627.

Berry, E. D. and C. Cutter (2000). "Effects of acid adaptation of *Escherichia coli* O157:H7 on efficacy of acetic acid spray washes to decontaminate beef carcass tissue. " Applied and Environmental Microbiology **66**(4): 1493-1498.

Bevilacqua, A., M. R. Corbo, et al., (2010). "Combining eugenol and cinnamaldehyde to control the growth of Allicyclobacillus acidoterrestris." Food Control **21**: 172-177.

Binkley, C. R. and R. C. Wiley (1978). "Continuous diffusion-extraction method to product apple juice." Journal of Food Science **43**(3): 1019-1023.

Blaser, M. J. and L. S. Newman (1982). "A review of human salmonellosis: I. infective dose." Reviews of Infectious Diseases **4**(6): 1096-1106.

Blaszyk, M. and R. A. Holley (1998). "Interaction of monolaurin, eugenol and sodium citrate on growth of common meat spoilage and pathogenic organisms." International Journal of Food Microbiology **39**: 175-183.

Bogadanov, S., A. Imdorf, et al., (1998) "Thymol residues in wax and honey after apilife VAR treatment." 1-9. Swiss Bee Research Centere. Dairy Research Station, Liebefeld, CH-3003 Bern.

Boyer, J. and R. H. Liu (2004). "Apple phytochemicals and their health benefits." Nutrition Journal **3**(5).

Burt, S. (2004). "Essential oils: their antibacterial properties and potential applications in foods-a review." International Journal of Food Microbiology **94**: 223-253.

Burt, S. A. and R. D. Reinders (2003). "Antibacterial activity of selected plant essential oils against Escherichia coli O157:H7." Letters in Applied Microbiology **36**: 162-167.

Burt, S. A., R. Vilielander, et al., (2005). "Increase in activity of essential oil components carvavrol and thymol against *Escherichia coli* O157:H7 by addition of food stabilizers." Journal of Food Protection **68**(5): 919-926.

Caccioni, D. R. L., M. Guizzardi, et al., (1998). "Relationship between volatile components of citrus fruit essential oils and antimicrobial action of *Penicillium digitatum* and *Penicillium italicum*." International Journal of Food Microbiology **43**: 73-79.

Castanie-Cornet, M. P., T. A. Penfound, et al., (1999). "Control of acid resistance in *Escherichia coli*." Journal of Bacteriology **181**(11): 3525-3535.

Centers for Disease Control and Prevention. (2011). "*Escherichia coli* O157:H7 and other shiga toxin-producing *Escherichia coli* (STEC)." Available at http://www.cdc.gov/nczved/divisions/dfbmd/diseases/ecoli_o157h7/ . Accessed 29 March 2012.

Centers for Disease Control and Prevention (2011). Investigation announcement: multistate outbreak of human Salmonella Typhimurium infections associated with exposure to clinical and teaching microbiology laboratories. W. Division of Foodborne, and Environmental Diseases. Availible at http://www.cdc.gov/salmonella/typhimurium-laboratory/042711/index.html. Accessed 12 March 2012.

Chart, H., S. M. Scotland, et al., (1989). "Serum antibodies to *Escherichia coli* serotype O157:H7 in patients with hemolytic uremic syndrome." Journal of Clinical Microbiology **27**(2): 285-290.

Chen, F., Z. Shi, et al., (2009). "Antioxidant and antibacterial activities of eugenol and carvacrol-grafted chitosan nanoparticles." Biotechnology and bioengineering **104**(1): 30-39.

Cheville, A. M., K. W. Arnold, et al., (1996). "rpoS regulation of acid, heat, and salt tolerance in Escherichia coli O157:H7." Applied and Environmental Microbiology **62**(5): 1822-1824.

Choi, L. H. and S. S. Nielsen (2004). "The effects of thermal and nonthermal processing methods on apple cider quality and consumer acceptability." Journal of Food Quality **28**(1): 13-29.

Cimanga, K., K. Kambu, et al., (2002). "Correlation between chemical composition and antibacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo." Journal of Ethnopharmacology **79**: 213-220.

Cody, S. H., K. Glynn, et al., (1999). "An outbreak of *Escherichia coli* O157:H7 infection from unpasteurized commercial apple juice." Annals of Internal Medicine **130**(3): 202-209.

Conner, D. E. and J. S. Kotrola (1995). "Growth and survival of *Escherishia coli* O157:H7 under acidic conditions." Applied and Environmental Microbiology **61**(1): 382-385.

Corbo, M. R., R. Lanciotti, et al., (2000). "Effects of hexenal, *trans*-2-hexenal, and storage temperature on shelf life of fresh sliced apples." Journal of Agricultural and Food Chemistry **48**: 2401-2408.

Cornwell, C. M. M. (2001). Fate of *Salmonella Montevideo*, *Salmonella Gaminara*, and *Salmonella Poona* in homemade unpasteurized fruit and vegetable juices. Master of Science, The University of Georgia. Available at http://athenaeum.libs.uga.edu/handle/10724/5697. Accessed 09 March 2012.

Cosentino, S., C. I. G. Tuberoso, et al., (1999). "In-vitro antimicrobial activity and chemical composition of Sardinian Thymus essential oils." Letters in Applied Microbiology **29**: 130-135.

Da Costa, N. C. and R.J., Cannon Eds. (2010). Flavors in noncarbonated beverages. Washington, DC, Oxford University Press.

Dalton, C. B., C. C. Austin, et al., (1997). "An outbreak of gastroenteritis and fever due to *Listeria monocytogenes* in milk." New England Journal of Medicine **336**: 100-106.

Davidson, P. M. (2005). Antimicrobials in food 3rd edition. New York, Taylor & Francis.

Davis, M. J., P. J. Coote, et al., (1996). "Acid tolerance in *Listeria monocytogenes*: the adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance." Microbiology **142**(10): 2975-2982.

DeBiase, D., A. Tramonti, et al., (1999). "The response to stationary-phase stress conditions in Escherichia coli: role and regulation of the glutamic acid decarboxylase system." Molecular Microbiology **32**(6): 1198-1211.

Delgado, B., A. Palop, et al., (2004). "Combined effect of thymol and cymene to control the growth of *Bacillus cereus* vegetative cells." European Food Research and Technology **218**: 188-193.

Deng, M. and D. O. Cliver (2001). "Inactivation of *Cryptosporidium parvum* oocysts in cider by flash pasteurization." Journal of Food Protection **64**(4): 523-527.

Deng, W., T. R. Hamilton-Kemp, et al., (1993). "Effects of six-carbon aldehydes and alcohols on

bacterial proliferation." Journal of Agricultural and Food Chemistry **41**: 506-510.

Dennison, B. A. (1996). "Fruit juice consumption by infants and children: a review." Journal of the American College of Nutrition **15**(5): 4s-11s.

Dennison, B. A., H. L. Rockwell, et al., (1999). "Children's growth parameters vary by type of fruit juice consumed." Journal of the American College of Nutrition **18**(4): 346-352.

Dog, T. L. (2006). "A reason to season: the therapeutic benefits of spices and culinary herbs." The Journal of Science and Healing **2**(5): 446-449.

Doumith, M., C. Cazalet, et al., (1991). "New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays." Microbiology Reviews **55**(3): 476-511.

Dusan, F., S. Marian, et al., (2006). "Essential oils-their antimicrobial activity against *Escherichia coli* and effect on intestinal cell viability." Toxicology in Vitro **20**: 1435-1445.

Earle-Richardson, G., P. Jenkins, et al., (2005). "An ergonomic intervention to reduce back strain among apple harvest workers in New York state." Applied Economics **36**: 327-334.

Edlow, J. A. (2009). The deadly dinner party and other medical detective stories.

Ettayebi, K., J. E. Yamani, et al., (2000). "Synergistic effects of nisin and thymol on antimicrobial activities in *Listeria monocytogenes* and *Bacillus subtilis*." FEMS Microbiology Letters **183**: 191-195.

Fan, L., J. Song, et al., (2006). "Effect of hexanal vapor on spore viability of *Penicillium expansum*, lesion development on whole apples and fruit volatile biosynthesis." Journal of Food Science 71(3): M105-M109.

FAO/WHO Food Standards (2011). "Codex standard for apple juice reserved exclusively by physical means." CODEX STAN 48.

Farber, J. M. and P. I. Peterkin (1991). "*Listeria monocytogenes*, a food-borne pathogen." Microbiology Reviews **55**(3): 476-511.

FAS/ USDA Horticultural & Tropical Products Division (2006). "World apple juice situation: global apple juice production recedes as China's apple crop declines, trade to continue strong in MY 2005/2006." Availible at: http://www.fas.usda.gov/htp/Hort_Circular/2006/05-06/Apple%20Juice%20Feature%20May%202006.pdf. Accessed 01 March 2012.

Flath, R. A., D. R. Black, et al., (1967). "Identification and organoleptic evaluation of compounds in delicious apple essence." Journal of Agricultural and Food Chemistry **15**(1): 29-35.

Fonsah, E. G. and A. Muhammad (2008). "The demand for imported apple juice in the United States." Journal of Food Distribution Research **39**(1): 57-61.

Food Safety Innovation in the United States empirical investigation: a closer look at food safety drivers in the meat industry. Economic Research Service/USDA. Available at: http://www.ers.usda.gov/Publications/aer831/aer831fm.pdf. Accessed 01 March 2012.

Foster, J. W. (1991). "*Salmonella* acid shock proteins are required for the adaptive acid tolerance response." Journal of Bacteriology **173**(21): 6896-6902.

Foster, J. W. and H. K. Hall (1991). "Inducible pH homeostasis and the acid tolerance response of *Salmonella* Typhimurium." Journal of Bacteriology **173**(16): 5129-5135.

Fournard, D., A. Vicens, et al., (2006). "Accumulation and extractability of grape skin tannins and anthocyanins at different advanced physiological stages." Journal of Agriculture and Food Chemistry **54**(19): 7331-7338.

Friedman, M., P. R. Henika, et al., (2002). "Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*." Journal of Food Protection **65**(10): 1545-1560.

Friedman, M., P. R. Henika, et al., (2004). "Antibacterial activities of plant essential oils and their components against *Escherichia coli* O157:H7 and *Salmonella enterica* in apple juice." Journal of Agricultural and Food Chemistry **52**: 6042-6048.

Frye, D. M., R. Zweig, et al., (2002). "An outbreak of febrile gastroenteritis associated with delicatessen meat contaminated with *Listeria monocytogenes*." Clinical Infectious Diseases **35**(8): 943-949.

Gahan, C. G., B. O'Driscoll, et al., (1996). "Acid adaptation of *Listeria monocytogenes* can enhance survival in acidic foods and during milk fermentation." Applied and Environmental Microbiology **62**(9): 3128-3132.

Gaillard, J. L., P. Berche, et al., (1987). "In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line caco-2." Infection and Immunity **55**(11): 2822-2829.

Galluci, M. N., M. Oliva, et al., (2009). "Antimicrobial combined action of terpenes against the food-borne microorganims *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*." Flavour and Fragrance Journal **24**: 348-354.

Gardini, F., R. Lanciotti, et al., (1997). "Antifungal activity of hexanal as dependent on its vapor pressure." Journal of Agricultural and Food Chemistry **45**: 4297-4302.

Gardini, F., R. Lanciotti, et al., (2001). "Effect of *trans*-2-hexenal on the growth of *Aspergillus flavus* in relation to its concentration, temperature and water activity." Letters in Applied

Microbiology **33**: 50-55.

Garner, M. R., B. L. Njaa, et al., (2006). "Sigma B contributes to *Listeria monocytogenes* gastrointestinal infection but not to systemic spread in the guinea pig infection model." Infection and Immunity **74**(2): 876-886.

Gaysinsky, S., T. M. Taylor, et al., (2007). "Antimicrobial efficacy of eugenol microemulsions in milk against *Listeria monocytogenes* and *Escherichia coli* O157:H7." Journal of Food Protection **70**(11): 2631-2637.

Geisler, M. (2011). "Commodity apple profile." Agricultureal Marketing Resource Center. Availible at: http://www.agmrc.org/commodities__products/fruits/apples/commodity_apple_profile.cfm. Accessed 24 February, 2012.

Gill, A. O. and R. A. Holley (2006). "Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics." International Journal of Food Microbiology **108**: 1-9.

Gill, A. O. and R. A. Holley (2006). "Inhibition of membrane bound ATPase of *Escherichia coli* and *Listeria monocytogens* by plant oil aromatics." International Journal of Food Microbiology **111**: 170-174.

Gill, A. O. and R. A. Holley (2004). "Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*." Applied and Environmental Microbiology **70**(10): 5750-5755.

Gould, G. W. (1996). "Industry perspectives on the use of natural antimicrobials and inhibitors for food applications." Journal of Food Protection **1996** Supplement: 82-86.

Hamilton-Kemp, T. R., D. D. Archbold, et al., (1996). "Metabolism of natural volatile compounds by strawberry fruit." Journal of Agricultural and Food Chemistry **44**: 2802-2805.

Hammer, K. A., C. F. Carson, et al., (1999). "Antimicrobial activity of essential oils and other plant extracts." Journal of Applied Microbiology **86**: 985-990.

Helander, I. M., H. L. Alakomi, et al., (1998). "Characterization of the action of selected essential oil components on Gram-negative bacteria." Journal of Agricultural and Food Chemistry **46**: 3590-3595.

Hemaiswarya, S. and M. Doble (2009). "Synergistic interaction of eugenol with antibiotics against Gram negative bacteria." Phytomedicine **16**(11): 997-1005.

Holetz, F. B., G. L. Pessini, et al., (2002). "Screening of some plants used in the Brazilian folk medicine for the treatment of infectious disease." Memorias do Instituto Oswaldo Cruz, Rio de Janeiro **97**(7): 1027-1031.

Holley, R. A. and D. Patel (2005). "Improvement in shelf-life and safety of perishable foods of plant essential oils and smoke antimicrobials." Food Microbiology **22**: 273-292.

Horticulture & Tropical Products Division (2003). "World apple juice situation: global apple juice production and trade to remain strong in 2003/2004." Availible at: http://fruitjuiceaustralia.org/publications/db/Apple%20Juice%20Feature.pdf Accessed 15 January 2012.

Hummelbrunner, L. A. and M. B. Isman (2001). "Acute, sub-lethal, anti-feedant, and synergistic effects of monoterpenoid essential oil compounds on the tobacco cutworm, *Spodoptera litura* (*Lep., Noctuidae*)." Journal of Agricultural and Food Chemistry **49**(2): 715-720.

Hyson, D., D. Studebaker-Hallman, et al., (2000). "Apple juice consumption reduces plasma low-density lipotrotein oxidation in healthy men and women. "Journal of Medicinal Food **3**: 159-166.

Ingram, L. O. and N. S. Vreeland (1980). "Differential effects of ethanol and hexanol on the *Escherichia coli* cell envelope." Journal of Bacteriology **144**(2): 481-488.

Jolicoeur, C. "Acidity and pH of apple juice." Availible at: http://cjoliprsf.awardspace.biz/Documents/Acidity-pH.pdf Accessed on 12 January 2012.

Jordan, K. N., L. Oxford, et al., (1999). "Survival of low-pH stress by *Escherichia coli* O157:H7: correlation between alterations in the cell envelope and increased acid tolerance " Applied and Environmental Microbiology **65**(7): 3048-3055.

Juneja, V. K. and M. Friedman (2007). " Carvacrol, cinnamaldehyde, organo oil and thymol inhibit *Clostridium perfringens* spores germination and outgrowth in ground turkey during chilling " Journal of Food Protection **70**(1): 218-222.

Karapinar, M. and S. E. Aktug (1987). "Inhibition of foodborne pathogens by thymol, eugenol, menthol and anethole." International Journal of Food Microbiology **4**: 161-166.

Kim, Y. and D. Shin (2004). "Volatile constituents from the leaves of *Callicarpa japonica* Thunb. and their antibacterial activities." Journal of Agricultural and Food Chemistry **52**: 781-787.

Knee, M. and S. G. S. Hatfield (1981). "The metabolism of alcohols by apple fruit tissue." Journal of the Science of Food and Agriculture **32**: 593-600.

Koutsoumanis, K. P. and J. N. Sofos (2004). "Comparative acid stress response of *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella* Typhimurium after habituation at different pH conditions." Letters in Applied Microbiology **38**: 321-326.

Kubo, A., C. S. Lunde, et al., (1995). "Antimicrobial activity of the olive oil flavor compounds."

Journal of Agricultural and Food Chemistry **43**: 1629-1633.

Kubo, I., H. Muroi, et al., (1993). "Antibacterial activity of long-chain alcohols: the role of hydrophobic alkyl groups." Bioorganic and Medicinal Chemistry Letters **3**(6): 1305-1308.

Lambert, R. J. W., P. N. Skandamis, et al., (2001). "A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol." Journal of Applied Microbiology **91**: 453-462.

Lanciotti, R., N. Belletti, et al., (2003). "Application of hexanal, (E)-2-hexenal, and hexyl acetate to improve safety of fresh-sliced apples." Journal of Agricultural and Food Chemistry **51**: 2958-2963.

Leyer, G. J., L. Wang, et al., (1995). "Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods." Applied and Environmental Microbiology **61**(10): 3752-3755.

Leyer, G. J. and E. A. Johnson (1993). "Acid adaptation induces cross-protection against environmental stresses in *Salmonella* Typhimurium." Applied and Environmental Microbiology **59**(6): 1842-1847.

Li, S. (2011). Enhancement of the antimicrobial actiivty of eugenol and carvacrol against *Escherichia coli* O157:H7 by lecithin in microbiological media and food. Master of Science, University of Tennessee. Available at: http://trace.tennessee.edu/cgi/viewcontent.cgi?article=2156&context=utk_gradthes Accessed 01 January 2012

Li, Y., H. Yuah, et al., (2009). "Identification of pathogenic fungi from the post-harvest strawberry and effect of hexanol treatment on them." Journal of Xinjiang Agricultural University.

Linton, M., J. M. J. McClements, et al., (1999). "Survival of *Escherichia coli* O157:H7 during storage in pressure-treated orange juice " Journal of Food Protection **62**(9): 1038-1040.

Luedtke, A. and D. Powell. (2000). "Fact sheet: a timeline of fresh juice outbreaks." International Food Safety Network

Ma, Z., S. Gong, et al., (2003). "GadE (YhiE) activates glutamate decarboxylase-dependent acid resistance in Escherichia coli K-12." Molecular Microbiology **49**(5): 1309-1320.

MacDonald, J. M. (2000). "Demand, information, and competition: why do food prices fall at seasonal demand peaks?" Journal of Industrial Economics **48**(1): 27-45.

Martinelli, K. A. and W. Briggs (1998). "Integrating public relations and legal responses during a crisis: the case of Odwalla, Inc." Public Relations Reviews **24**(4): 443-460.

Matthews, K. R. (2006). Microbiology of fresh produce. Washington, DC, ASM Press.

Miller, L. G. and C. W. Kasper (1994). "*Eschericiha coli* O157:H7 acid tolerance and survival in apple cider." Journal of Food Protection **57**(6): 460-464.

Moleyar, V. and P. Narasimham (1992). "Antibacterial activity of essential oil components." International Journal of Food Microbiology **16**: 337-342.

Morris, J. A., A. Khettry, et al., (1979). "Antimicrobial activity of aroma chemicals and essential oils." Journal of the American Oil Chemist's Society **56**: 595-603.

Mounier, J., A. Ryter, et al., (1990). "Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocytelike cell line caco-2." Infection and Immunity **58**(4): 1048-1058.

Muroi, H., A. Kubo, et al., (1993). "Antimicrobial activity of cashew apple flavor compounds." Journal of Agricultural and Food Chemistry **41**:1106-1109.

Nakamura, S. and A. Hutanaka (2002). "Green-lead derived C6-aroma compounds with potent antibacterial action the act on both Gram-negative and Gram-positive bacteria." Journal of Agricultural and Food Chemistry **50:** 7639-7644.

Nazer, A. I., A. Kobilinsky, et al., (2005). "Combinations of food antimicrobials at low levels to inhibit the growth of Salmonella sv. Typhimurium: a synergistic effect?" Food Microbiology **22**: 391-398.

Neri, F., M. Mari, et al., (2006). "Control of *Penicillium expansum* by plant volatile compounds." Plant Pathology **55**: 100-105.

Neri, F., M. Mari, et al., (2007). "Fungicidal activity of plant volatile compounds for controlling *Monilinia laxa* in stone fruit." Plant Disease **91**(1): 30-35.

Niu, C., S. Afre, et al., (2006). "Subinhibitory concentrations of cinnamaldehyde interfere with quorum sensing. " Letters in Applied Microbiology **43**: 489-494.

Novak, J. S., G. M. Sapers, et al., Eds. (2003). Microbial safety of minimally processed foods. Boca Raton, FL, CRC Press.

Nychas, G. J. E. (1995). Natural antimicrobials from plants. New Methods of Food Preservation. G. W. Gould. London, Blackie Academic Professional.

O'Driscroll, B., C. G. Gahan, et al., (1996). "Adaptive acid tolerance response in *Listeria monocytogenes*: isolation of an acid-tolerant mutant which demonstrates increased virulence." Applied and Environmental Microbiology **62**(5): 1693-1698.

Olasupo, N. A., D. J. Fitzgerald, et al., (2003). "Activity of natural antimicrobial compounds against *Escherichia coli* and *Salmonella enterica* serovar Typhimurium." Letters in Applied Microbiology **36**: 448-451.

Ooi, L. S. M. (2006). "Antimicrobial activities of cinnamon oil and cinnamaldehyde from the Chinese medicinal herb Cinnamomum cassia blume." The American Journal of Chinese Medicine **34**(3): 511-522.

Ouattara, B., R. E. Simard, et al., (1997). "Antibacterial activity of selected fatty acids and essential oils against six meat spoilage organisms." International Journal of Food Microbiology **37**: 155-162.

Oyedemi, S. O., A. I. Okoh, et al., (2009). "The proposed mechanism of bactericidal action of eugenol, alpha-terpineol and gamma-terpinene against *Listeria monocytogenes*, *Streptococcus pyogenes*, *Proteus vulgaris* and *Escherichia coli*." African Journal of Biotechnology **8**(7): 1280-1286.

Pai, C. H., N. Ahmed, et al., (1988). "Epidemiology of sporadic diarrhea due to verocytotoxin-producing *Escherichia coli*: a two-year prospective study." Journal of Infectious Diseases **157**(5): 1054-1057.

Parish, M. E. (1997). "Public health and non-pasteurized fruit juices." Critical Reviews in Microbiology **23**(2): 109-119.

Parish, M. E., J. A. Narciso, et al., (1997). "Survival of *Salmonella* in orange juice." Journal of Food Safety **17**(4): 273-281.

Patrigani, F., L. Uucci, et al., (2008). "Effects of sub-lethal concentrations of hexanal and 2-(E)-hexenal on membrane fatty acid composition and volatile compounds of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* Eneteritidis and *Escherichia coli*." International Journal of Food Microbiology **123**: 1-8.

Pei, R., F. Zhou, et al., (2009). "Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. coli* with an improved method." Journal of Food Science **74**(7): M379-M383.

Perez, A., B. Lin, et al., (2001) "Demographics profile of apple consumption in the United States." Fruit and Tree Nuts S&O/FTS-292.

Peterson, D. L., S. S. Miller, et al., (1994). "Harvesting semidwarf freestanding apple trees with an over-the-row mechanical harvester." Journal of the American Society of Horticultural Science **119**(6): 1114-1120.

Phan-Thanh, L., F. Mahouin, et al., (2000). "Acid responses of *Listeria monocytogenes*." International Journal of Food Microbiology **55**: 121-126.

Poppe, C., N. Smart, et al., (1998). "*Salmonella* Typhimurium DT104: a virulent and drug-resistant pathogen." The Canadian Veterinary Journal **39**(9): 559-565.

Rangel, J. M., P. H. Sparling, et al., (2005). "Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States 1982-2002." Emerging Infectious Disease **11**(4): 603-609.

Raybaudi-Massilia, R. M., J. Mosqueda-Melgar, et al., (2006). "Antimicrobial activity of essential oils on *Salmonella Enteritidis*, *Escherichia coli*, and *Listeria innocua* in fruit juices." Journal of Food Protection **69**(7): 1579-1586.

Rees, L. P., S. F. Minney, et al., (1993). "A quantitative assessment of the antimicrobial activity of garlic (*Allium sativum*)." World Journal of Microbiology and Biotechnology **9**: 303-307.

Rosa, S. (2001, May 8, 2001). "Outlook for concentrated apple juice production and trade in selected countries." Accessed at: http://www.fas.usda.gov/htp/Hort_Circular/2001/01-06/Statistics/AJFeature.PDF. Accessed 29 March 2012.

Rowles, K. (2000) "Hard Cider and Apple Wine." Marketing analyst, Department of Agricultural Resource and Managerial Economics.

Rowles, K. (2001). "Processed apple product marketing analysis: apple juice and cider."

Sapers, G.M., K.B. Hicks, et al., (1969) "Control of enzymatic browning in apple with ascorbic acid derivatives, polyphenol oxidase inhibitors, and complexing agents. " Journal of Food Science **54** (4): 997-1002.

Sapers, G.M., J.R. Gorny, et al., Eds. (2006). Microbiology of fruits and vegetables. Boca Raton, FL, Taylor and Francis Group.

Schwartz, R.S., D.F. Murchison, et al., (1998). "Effects of eugenol and noneugenol endodontic sealer cements on post retention." Journal of Endodontics **24**(8): 564-567.

Sholberg, P.L. and P. Randall (2007). "Fumigation of stored pome fruit with hexanal reduces blue and gray mold decay." HortScience **42**(3): 611-616.

Singh, R.K., Ed. (1995). Food process design and evaluation. Lancaster, PA, Technomic Publishing Company, Inc.

Skinner, J. D., P. Ziegler, et al., (2004). "Transitions in infants' and toddlers' beverage patterns." Journal of the American Dietetic Association **104**(Supplement 1): 45-50.

Slattery, E., M. Livingston, et al., (2011). Characteristics of conventional and organic apple production in the United States. United States Department of Agriculture-Economic Research Service. Accessed at: http://www.ers.usda.gov/Publications/FTS/2011/07Jul/FTS34701/fts34701.pdf Accessed 29 March 2012.

Smith, M. M. and F. Lifshitz (1994). "Excess fruit juice consumption as a contributing factor in nonorganic failure to thrive." Pediatrics **93**(3): 438-443.

Smith-Palmer, A., J. Stewart, et al., (1998). "Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens." Letters in Applied Microbiology **26**: 118-122.

Song, J., P. D. Hildebrand, et al., (2007). "Effect of hexanal vapor on the growth of postharvest pathogens and fruit decay." Journal of Food Science **72**(4): M108-M112.

Song, J., R. Leepipattanawit, et al., (1996). "Hexanal vapor is a natural, metabolizable fungicide: inhibition of fungal activity and enhancement of aroma biosynthesis in apple slices." Journal of the American Society for Horticultural Science **121**(5): 937-942.

Spencer, J. R., B. Z. Hernandez, et al., (1993). Dermal and urinary monitoring of peach and apple harvesters exposed to organophosphate in Sutter, Stanislaus and Madera counties, 1989 and 1990. California Environmental Protection Agency Department of Pesticide Regulation Worker Health and Safety Branch. Sacramento, CA. Available at: http://www.cdpr.ca.gov/docs/whs/pdf/hs1577.pdf. Accessed 14 January 2012.

Spotts, R. A., P. L. Sholberg, et al., (2007). "Effects of 1-MCP and hexanal on decay of d' Anjou pear fruit in long-term cold storage." Postharvest Biology and Technology **44**: 101-106.

Suhr, K. I. and P. V. Nielson (2003). "Antifungal activity of essential oils evaluated by two different techniques against rye bread spoilage fungi." Journal of Applied Microbiology **94**: 665-674.

Tarr, P. I. (1995). "*Escherichia coli* O157:H7: clinical, diagnostic, and epidemiological aspects of human infection." Clinical Infectious Diseases **20**(1): 1-10.

Tassou, C. C., E.H., Drosinos, et al. (1995). "Effects of essential oil from mint (*Mentha piperita*) on *Salmonella enteritidis* and *Listeria monocytogenes* in model food systems at 4°C and 10°C." Journal of Applied Microbiology **78**(6): 593-600.

Truong, V. Y. T., R. R. Boyer, et al., (2010). "Effect of a-cyclodextrin-cinnamic acid inclusion complexes on populations of Escherichia coli O157:H7 and *Salmonella enterica* in fruit juices." Journal of Food Protection **73**(1): 92-96.

Uljas, H.E. and S. C. Ingham (1999). "Combinations of intervention treatments resulting in 5-Log10-unit reductions in numbers of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT104 organisms in apple cider." Applied and Environmental Microbiology **65**(5): 1924-1929.

U.S. Environmental Protection Agency (2011). Cinnamaldehyde (040506) fact sheet. Office of Pesticide Programs. Washington, D.C., Ombudsman, Biopesticides and Pollution Prevention Division. Accessed at: http://www.epa.gov/oppbppd1/biopesticides/ingredients/factsheets/factsheet_040506.htm. Accessed 12 March 2012.

U.S. Department of Health and Human Services. (2009). "Bad bug book: foodborne pathogenic microorganisms and natural toxins handbook *Listeria monocytogenes*." Available at: http://www.fda.gov/food/foodsafety/foodborneillness/foodborneillnessfoodbornepathogensnaturaltoxins/badbugbook/ucm070064.htm. Accessed 21March 2012.

U.S. Department of Health and Human Services (2011). Bad Bug Book: foodborne pathogenic microorganisms and natural toxins handbook onset, duration, and symptoms of foodborne illness. United States Food and Drug Administration. Available at: http://www.fda.gov/food/foodsafety/foodborneillness/foodborneillnessfoodbornepathogensnaturaltoxins/badbugbook/ucm071342.htm. Accessed 21March 2012.

U.S. Food and Drug Administration (2009). Bad bug book: foodborne pathogenic microorganisms and natural toxins handbook *Salmonella* spp. Silver Spring, MD. Available at: http://www.fda.gov/food/foodsafety/foodborneillness/foodborneillnessfoodbornepathogensnaturaltoxins/badbugbook/ucm069966.htm. Accessed 29 March 2012.

Utto, W., A. J. Mawson, et al., (2008). "Hexanal reduces infection of tomatoes by *Botrytis cinera* whilst maintaining quality." Postharvest Biology and Technology **47**: 434-437.

Valero, M. and E. Frances (2006). "Synergsitic bactericidal effect of carvacrol, cinnamaldehyde or thymol and refrigeration to inhibit *Bacillus cereus* in carrot broth." Food Microbiology 23: 68-73.

Vaughn, S. F., G. F. Spencer, et al., (1993). "Volatile compounds from raspberry and strawberry fruit inhibit postharvest decay fungi." Journal of Food Science **58**(4): 793-796.

Vidal, S., L. Francis, et al., (2004). "Taste and mouth-feel properties of different types of tannin-like polyphenolic compounds and anthocyanins in wine." Analytica Chimica Acta **153**(1): 57-65.

Vojdani, J. D., L. R. Beuchat, et al., (2008). "Juice-associated outbreaks of human illness in the United States, 1995 through 2005 " Journal of Food Protection **71**(2): 356-364.

Walsh, S., J. Maillard, et al., (2003). "Activity and mechanisms of action of selected biocidal agents on Gram-positive and -negative bacteria." Journal of Applied Microbiology **94**: 240-247.

Wong, C. S., S. Jelacic, et al., (2000). "The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections." The New England Journal of Medicine **342**(26): 1930-1936.

Zaika, L. L. (1988). "Spices and Herbs: their antimicrobial activity and its determination." Journal of Food Safety **9**: 97-118.

Zhao, T., M. P. Doyle, et al., (1993). "Fate of enterohemorrhagic *Escherishia coli* O157:H7 in apple cider with and without preservatives." Applied and Environmental Microbiology **59**(8): 2526-2530.

Zhou, F., B. Ji, et al., (2007). "The antibacterial effect of cinnamaldehyde, thymol, carvacrol and their combinations against the foodborne pathogen *Salmonella* Typhimurium." Journal of Food Safety **27**(2): 124-133.

Zhou, S. and E. Decker (1999). "Ability of amino acids, dipeptides, polyamines, and sulfhydryls to quench hexanal, a saturated aldehydic lipid oxidation product." Journal of Agricultural and Food Chemistry **47**: 1932-1936.

CHAPTER 3

Comparison of spiral gradient endpoint and standard agar dilution methods to evaluate antimicrobial activity of essential oils against foodborne pathogens

Abdulmalik, T.O.[1]*, R.R.Boyer[1], J.B.Gurtler[2], J. Levy[1], S.F. O'Keefe[1] , R.C. Williams[1]

[1]Virginia Polytechnic Institute and State University, Department of Food Science and Technology. 22 Duck Pond Drive, Blacksburg VA 24061 USA
2 Food Safety Intervention Technologies, U.S. Department of Agriculture, ARS, Eastern Regional Research Center. 600 E. Mermaid Ln. Wyndmoor, PA 19038-8598

Key Words: Foodborne pathogens, antimicrobials, essential oils, spiral gradient endpoint method, standard agar dilution method

ABSTRACT

There are several methods currently available to evaluate the inhibitory effects of plant-derived antimicrobials; however, they differ in their abilities to establish minimum inhibitory concentrations (MIC). These inconsistencies may delay the adoption of new antimicrobial compounds by the food industry. Therefore, it is important to establish uniform testing protocols which produce consistent MIC values. In this study, two common methods of analysis (the standard agar dilution (SAD) method and the Spiral Gradient Endpoint (SGE) method) were compared for their abilities to determine MIC values for thymol, eugenol and cinnamaldehyde against three foodborne pathogens (*L. monocytogenes*, *S.* Typhimurium and *S. aureus*). The effects of combining these compounds were also evaluated with both methods. MIC values obtained from the SGE software were significantly higher than those produced by SAD; however, both methods produced comparable trends in overall antimicrobial activity. In general, cinnamaldehyde was the most active essential oil compound, followed by thymol and eugenol, with overall MIC values of 0.2, 0.4, and 1.6 mg/mL, respectively, for all three pathogens by SAD. SGE produced similar trends with cinnamaldehyde having the highest activity against *L. monocytogenes, S.* Typhimurium, and *S. aureus* (GMIC = 6.0, 26.24, and 4.8 mg/mL, respectively), followed by thymol (GMIC = 42.76, 24.96, 28.16 mg/mL, respectively) and eugenol (GMIC = 133.12, 102.91, and 61.44 mg/mL, respectively). SGE software classified all antimicrobial combinations as being antagonistic in nature; whereas SAD classified the majority of combinations as being indifferent in nature. Results generated by the SAD method of analysis were comparable with those reported in literature; therefore, it can be concluded that the SAD method more accurately evaluates the antimicrobial activity of EO compounds.

INTRODUCTION

Thymol, eugenol and cinnamaldehyde are antimicrobial compounds abundant in the oils of thyme, clove and cinnamon, respectively (Karapinar and Aktug 1987; Bagamboula et al., 2004; Kwan et al., 2006). These compounds are active against a wide variety of pathogenic microorganisms, including *Escherichia coli* O157:H7, *Shigella* spp., *Listeria monocytogenes*, *Salmonella enterica* serovars, *Vibrio vulnificus*, *Staphylococcus aureus*, and *Bacillus cereus* (Ettayebi et al., 2000; Walsh et al., 2003; Bagamboula et al., 2004; Gill and Holley 2004; Holley and Patel 2005; Si et al., 2006; Hemaiswarya and Doble 2009). These compounds are generally recognized as safe by the U.S. Food and Drug Administration as flavoring additives;however, their use as natural preservatives is often limited by their strong organoleptic characteristics and limited solubility (Aeschbach et al., 1994; Bogadanov et al., 1998; Burt 2004; Valero and Giner 2006; Hemaiswarya and Doble 2009). Research has been conducted to identify effective combinations, which lower the minimum concentration needed to inhibit microbial growth in foods, without contributing to off-flavors in certain products (Valero and Frances 2006; Zhou et al., 2007; Yen and Chang 2008; Pei et al., 2009).

The antimicrobial activities of thymol, eugenol and cinnamaldehyde have been well documented; however, there are discrepancies regarding the reported minimum inhibitory concentration (MIC) for each compound as well as their combinations (Pauli and Kubeczka 1997; Delgado et al., 2004; Zhou et al., 2007; Pei et al., 2009). These variations are likely a result of a lack of standardization among test methods (Pati and Kurade; Hill 1991). To date, several methods have been developed to evaluate the antimicrobial activity of essential oil compounds, each of which differs in terms of overall sensitivity and reproducibility. Most test methods fall into one of three main categories, including diffusion, dilution and bioautographic

methods; however, they each have inherent differences, which contribute to the overall variation of reported MIC values (Hammer et al., 1999; Burt 2004; Holley and Patel 2005; Valgas et al., 2007). Therefore, it has become increasingly important to develop uniform testing protocols, allowing for more accurate evaluation and application of test compounds (Hammer et al., 1999; Holley and Patel 2005).

The method approved by the National Committee for Clinical Laboratory Standards is the standard agar dilution (SAD) method (Hammer et al., 1999; Holley and Patel 2005). Although this method is reliable and commonly cited in literature, it is labor-intensive and costly; making it difficult to inexpensively evaluate large numbers of antimicrobial compounds and combinations (Baker 1991; Hill 1991; Karlsson et al., 2002; Luangtongkum et al., 2007). Therefore, a number of automated methods have been developed to assist in rapid screening of test compounds including the Spiral Gradient Endpoint (SGE) method of analysis.

SGE is a relatively new method of analysis, which rapidly computes MIC values, while minimizing the need for serial dilutions and incremental test plates (Hill 1991). The SGE software calculates an MIC based on agar depth and molecular weight of the test compound (Spiral Biotech 2009). SGE also extrapolates a gradient minimum inhibitory concentration (GMIC), which is a value considered to be comparable to other two-fold dilution methods (Spiral Biotech 2009). The GMIC value allows researchers to compare their test results with those MIC values already published in literature, establishing uniformity between methods.

Although SGE has been in use for several years, it has only been verified using a small number of antimicrobial compounds, including certain types of antibiotics, sanitizers and bacteriocins (De Martinis et al., 1997). Furthermore, SGE has only been tested against a select

number of microorganisms, including anaerobic bacteria, Gram-positive bacteria and a few spoilage yeasts (and unidentified bacteria) (Wexler et al., 1991; Schmalreck et al., 1995; Wexler et al., 1996; Winniczuk and Parish 1997). Several studies have confirmed that SGE is up to 96% compatible with the traditional SAD method; however, there is still a large void in the literature regarding its effectiveness in determining synergistic relationships between multiple compounds (particularly hydrophobic substances) (Hill 1991; Wexler et al., 1991; Wexler et al., 1996).

The purpose of this study was to evaluate the efficacy of the SGE system for identifying individual and synergistic relationships between certain classes of antimicrobial compounds. By comparing the results produced by SGE with those of the traditional SAD method, SGE may be used to rapidly screen antimicrobial compounds in future studies in our lab; with the ultimate goal of identifying combinations of antimicrobials that inhibit microbial growth without contributing to off-flavors in foods. Since the individual and combined activities of thymol, eugenol and cinnamaldehyde have been widely studied in the literature, it will be easy to compare their effectiveness between both methods. Furthermore, the evaluation of their combined activity against common foodborne pathogens is an important step towards their adoption within the food industry.

MATERIALS AND METHODS

Microorganisms: *Listeria monocytogenes* (Scott A), *Staphylococcus aureus* (ATCC 25923), and *Salmonella enterica* serovar Typhimurium (ATCC 14028) were all obtained from the frozen bacterial culture collection at Virginia Tech (Blacksburg, VA). Prior to each experiment, stored cultures were activated by two successive transfers in tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) at 35˚C for 24 hours. Samples were then streaked onto differential

media (xylose lysine desoxycholate (XLD) agar, moxified Oxford (MOX) agar and mannitol salt (MAS) agar), for *S.* Typhimurium, *L. monocytogenes* and *S. aureus*, respectively; and a single representative colony (for each organism) was selected for further confirmation. *S.* Typhimurium, *L. monocytogenes* and *S. aureus* cultures were confirmed using a *Salmonella* latex agglutination test (Oxoid Ltd.; Basingstoke, Hants, UK), API *Listeria* strips (Biomerieux, Inc., Durham, NC), and the BBLTM StaphylosideTM Latex agglutination test (Becton Dickson Microbiology Systems, Franklin Lakes,NJ), respectively.

Upon confirmation, a single representative colony of each organism was inoculated into individual test tubes containing 10mL of sterile TSB. Samples were then incubated for 24 hours at 35ºC, yielding a final concentration of 10^8-10^9 CFU/mL. This culture was transferred into fresh broth every 18-24 hours, in order to ensure a uniform culture for the duration of the experiment.

Preparation of Chemicals and Antimicrobial Agents: Thymol (99%), eugenol (>89%) and cinnamaldehyde (89%) were all were purchased from SAFC Supply Solutions, St. Louis, MO, USA. Prior to experimentation, all test compounds were weighed into individual glass beakers, containing 0.5% Tween-20 (Fisher Scientific, Fairlawn, NJ) (Tween-20 was included to enhance solubility of the test compounds). These solutions were then emulsified for 30 seconds using a micro-emulsifying needle (Cadence Science, Staunton, VA) and filter sterilized, using a 45 µL disposable syringe filters (Fisher Scientific).

Determination of Minimum Inhibitory Concentrations

Standard Agar Dilution Method: MICs were determined using the standard agar dilution (SAD) method, according to guidelines established by the National Committee for Clinical

Laboratory standards (Hecth et al., 2007). Briefly, antimicrobial compounds at concentrations of 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 mg/mL were dissolved into separate containers (one concentration per container) of molten tryptic soy agar (TSA; Becton, Dickinson and Company), containing 0.5% Tween-20.

Molten agar solutions were mixed vigorously by hand for one minute to evenly distribute the compound throughout the agar, poured into individual petri dishes and allowed to solidify. Individual plates were then spot inoculated with a 2.5 µL culture of each test organism (diluted to log 10^6 CFU/mL). Plates were incubated at 35˚C and evaluated after 24 hours (*L. monocytogenes* was evaluated after 48 hours) for the presence or absence of growth. The MIC for each compound was considered the lowest concentration that completely inhibited growth. Control plates (0.5% Tween-20 with no added antimicrobial) were included to ensure that Tween-20 had no inhibitory effects on test organisms. All samples were plated in duplicate and the experiment was replicated three times.

Synergistic effects of two compounds were determined using the "checkerboard" assay, as described by Rosenblatt and Rand (1995). Briefly, each essential oil was dissolved into an individual container of molten TSA, containing 0.5% tween-20. However, the concentration of these compounds did not exceed their previously determined MIC value. The secondary test compound (ranging from 0.2- 3.2mg/mL) was then added to each container; resulting in a series of mixed-ratio combinations. For example, cinnamaldehyde at 0.2 mg/mL and 0.4 mg/mL (individual MIC = 0.4 mg/mL) was tested in combination with eugenol at 0.2, 0.4, 0.8, 1.6 and 3.2 mg/mL, respectively. All combinations were poured into individual petri plates and allowed to solidify. Plates were then separated into four quadrants and spot inoculated with a 2.5 µL culture of each test organism (diluted to 6 log CFU/mL). Samples were incubated at 35˚C for 48

hours and evaluated after 24 hours for the presence or absence of growth. MICs were determined as the lowest concentration that completely inhibited growth.

The Fractional Inhibitory Concentration (FIC) index was used to describe the nature of each interaction. FIC values were calculated according to the procedure outlined by Botelho (2000). Briefly, ΣFIC = FIC A + FIC B, where FIC A is the MIC of the combination / MIC of compound A alone, and FIC B is the MIC of the combination / MIC of compound B alone.

However, since the checkerboard method of analysis generates numerous MIC values; antimicrobial interactions were determined using combinations that contained the lowest effective concentration of each test compound (i.e., combinations with two low EO concentrations (0.2 or 0.4 mg/mL) were preferred over combinations which contained high concentrations (0.8 or 1.6 mg/mL of one or both compounds)). Combination relationships were defined as follows: synergistic when $\Sigma FIC \leq 0.5$, antagonistic when $\Sigma FIC > 4$ and indifferent when $\Sigma FIC > 0.5$ to < 4 (Botelho 2000).

Spiral Gradient Endpoint: Antimicrobial compounds were dissolved in 2% Tween-20 and dispensed onto a 15 cm agar plate (containing 25 mL of solidified TSA), using an Autoplate 4000® spiral plater (Spiral Biotech, Norwood, MA). Plates were dried for one hour to allow test compounds to diffuse into the agar, thus establishing a concentration gradient within the test plate. Overnight cultures of each test organism were diluted to 7 log CFU/ml (according to SGE protocol) and streaked radially across the concentration gradient. Plates were then incubated at 35°C for 24 hours. In all cases, a control plate (2% Tween-20 with no added antimicrobial) was included to ensure that Tween-20 had no inhibitory effects on test organisms. All samples were plated in duplicate and the experiment was replicated three times.

The endpoints of growth were then measured and entered into the SGE system for determination of a MIC value. In this case, the MIC value was categorized as biostatic or biocidal, depending on whether the observed streak produced a gradual reduction in colony size or a distinct line of demarcation, respectively. Similarly, SGE also extrapolated a GMIC value, which was used to compare results with other two-fold dilution methods.

Synergistic effects were determined according to the procedure outlined by SGE user's manual (Spiral Biotech 2009). Briefly, stock solutions for each set of binary combinations were prepared, such that their ending radii were equal. Individual compounds were then dispensed exponentially onto 15 cm agar plates, using the spiral plater. Plates were allowed to dry for one hour, followed by deposition of the second test compound. Once both compounds had diffused into the media, microorganisms (7 log CFU/mL) were streaked radially across the plates and incubated at 35˚C; plates were evaluated at 24 hrs (*L. monocytogenes* was evaluated after 48 hours), to determine end points for growth. Synergistic relationships were indicated by an observable shift outward, whereas inhibitory effects were determined by an observable shift towards the center of the plate. All samples were plated in duplicate and the experiment was replicated three times.

Statistical Analysis: Data were analyzed using the General Linear Model (GLM) procedure of SAS (V. 9.1.3, Statistical Analysis Systems Institute, Inc. 2006). The factorial complete randomized design was utilized to test (F tests) the effect of the compound treatments on the pathogen growth and the interaction between the compound treatments with pathogens. If the tests were significant ($P < 0.05$), the levels (types) of the main effects (compounds and pathogens) were separated using Tukey's HSD tests.

RESULTS

The antimicrobial activities of thymol, cinnamaldehyde and eugenol were evaluated against several foodborne pathogens (including *L. monocytogenes*, *S. aureus* and *S.* Typhimurium), using the SGE system, as well as the SAD method of analysis. In all cases, GMIC indexes (extrapolated two-fold dilution values) generated by the SGE system were significantly higher than those produced by SAD (Table 1). Despite this variation, both methods of analysis generated similar patterns of inhibition among the three test compounds.

According to the SAD method, cinnamaldehyde was the most active EO compound, with reported MIC values of 0.2, 0.2 and 0.4 mg/mL for *L. monocytogenes*, *S. aureus* and *S.* Typhimurium, respectively. Thymol produced similar levels of inhibition, and eugenol was the least inhibitory of the three EO compounds (Table 1). SGE produced similar patterns of inhibition, with cinnamaldehyde displaying the highest degree of antimicrobial activity (GMIC = 6.00, 26.24 and 4.80 mg /mL for *L. monocytogenes*, *S.* Typhimurium and *S. aureus*, respectively), followed by thymol and eugenol. In both cases, cinnamaldehyde was the only compound that exhibited organism-specific interactions, producing significantly lower MIC values for *L. monocytogenes* and *S. aureus* (both Gram-positive bacteria) (Table 1).

The combined activities of thymol, eugenol and cinnimaldehyde were also determined, using the checkerboard method of analysis (for SAD) and the SGE system. In general, the two methods differed in their ability to identify synergistic and antagonistic relationships (Table 2). The checkerboard method of analysis classified most interactions as indifferent. Thymol/cinnamaldehyde and thymol/eugenol combinations were the only exceptions, with each producing antagonistic effects for *L. monocytogenes* (Table 2). However, it is important to note

that unlike the individual compounds, antimicrobial combinations did not produce organism-specific interactions; rather all combinations produced similar levels of inhibition against test organisms.

SGE identified antagonistic relationships for most combinations; however, it is interesting to note that while the majority of test compounds did not produce synergistic effects, they did display enhanced inhibition when used in certain combinations. This is particularly true for eugenol, which generated significantly lower MIC values when used in combination with thymol (compared to eugenol/cinnamaldehyde) for *L. monocytogenes,* and *S.* Typhimurium ($P<0.05$). Similarly, thymol/eugenol combinations produced significantly lower MIC values than thymol / cinnamaldehyde combinations for *S.* Typhimurium ($P<0.05$). Cinnamaldehyde also produced similar effects when used in combination with thymol (compared to cinnamaldehyde/eugenol combinations), producing enhanced effects against *L. monocytogenes* and *S. aureus*, respectively ($P<0.05$).

DISCUSSION

The minimum inhibitory concentrations (MIC) of thymol, cinnamaldehyde and eugenol were determined for *L. monocytogenes*, *S. aureus* and *S.* Typhimurium, using the Spiral Gradient Endpoint (SGE) system, as well as the Standard Agar Dilution (SAD) method of analysis. Both methods produced similar patterns of inhibition (with cinnamaldehyde exhibiting the highest degree of antimicrobial activity, followed by thymol and eugenol); however, the MICs produced by SGE were significantly higher than those produced by SAD.

There was a high degree of variation between the two test methods, which may be attributed to the fact that SGE and SAD each represent different classes of antimicrobial tests

(diffusion and dilution methods, respectively), which differ in their ability to establish a concentration gradient (Hill and Schalkowsky 1990; Davidson 2005). While both tests measure the minimum concentration needed to inhibit microbial growth (as determined by an absence of growth along incremental concentration gradients), SGE also relies on the ability of test compounds to diffuse laterally into the test medium, thus establishing a gradient within one agar plate (Hill and Schalkowsky 1990; Spiral Biotech 2009). However, if these compounds do not adequately diffuse into the agar matrix, SGE loses its ability to accurately measure antimicrobial activity and inhibition (Hill and Schalkowsky 1990). The overall rate of diffusion is influenced by the polarity of the test compounds (with polar compounds diffusing quicker than non-polar compounds), as well as the depth of agar and amount of time allotted to establish a gradient (Pati and Kurade ; Hammer et al., 1999). While SGE testing protocols require that plates dry for a minimum of one hour prior to inoculation, it cannot be proven that volatile hydrophobic test compounds (such as the ones used in this study) fully diffuse into the agar (which is relatively hydrophilic), rather than adhering to or evaporating from the surface of the agar plate (Duckworth and Yaphe 1971; Laas 1972; Holley and Patel 2005; Spiral Biotech 2009). Such factors limit the ability of SGE to accurately measure antimicrobial activity and generate high degrees of variation between test compounds.

Most EO compounds are relatively insoluble in water thus they do not remain in solution long enough to interact with test microorganims (Mann and Markham 1998). This is extremely problematic, because most EO compounds act by disrupting the cytoplasmic membrane of bacterial cells, which in turn causes dissipation of cellular proton motive force and leakage of intercellular ions (Cox 2000; Gardini 2001; Nazer 2005; Hemaiswarya 2009). If test compounds do not come into contact with the bacterial membrane, they cannot confer antimicrobial activity

(Mann and Markham 1998; Di Pasua et al., 2006). For this reason, Tween 20 (a common emulsifier) was used to enhance the overall solubility of test compounds (organic solvents were not used because they produced inhibitory effects against test microorganisms) (data not shown) (Mann and Markham 1998; Nino and Patino 1998; Fisher and Phillips 2006). Tween 20 acts by forming a micelle around hydrophobic compounds, thus suspending them in water (Mann and Markham 1998). While this action does not typically affect antimicrobial activity, high concentrations (i.e., >3%) of Tween 20 have been shown to produce adverse effects during antimicrobial screening (Baker 1983; Kim et al., 1995; Kim et al., 1995; Blaszyk and Holley 1998; Mann and Markham 1998)

It is possible that for SGE testing, Tween 20 (2%), produced a large number of micellear complexes, which reduced the number of compounds available to interact with test organisms (Mann and Markham 1998). This is especially true for the antimicrobial combination testing, because SGE protocols requires that two layers of antimicrobials be deposited onto the surface of the agar plate. In this case, excessive amounts of Tween 20 were deposited onto the agar plate, further contributing to antimicrobial inhibition. As a result, the overall concentration of antimicrobial needed to inhibit microbial growth increased.

The SAD method, unlike SGE is capable of suspending test compounds in molten agar prior to experimentation; thus requiring significantly lower concentrations of Tween 20 (0.5%) to solubilize test compounds. Such differences explain why MIC values produced by SAD were highly comparable with those published in the literature (Zhou et al., 2007; Pei et al., 2009). In addition to these factors, SAD and SGE each differ in their abilities to identify and interpret antimicrobial interactions. While both methods are capable of detecting synergistic, indifferent

and additive relationships, the criteria used for categorizing such interactions vary between the methods.

SAD, as stated earlier utilizes a "checkerboard" design to analyze large numbers of antimicrobial combinations often resulting in numerous MIC values for each drug combinations. For this reason the FIC index (the numeric scale used for categorizing antimicrobial interactions) was calculated using combinations containing the lowest effective concentration of each test compound (i.e., combinations with two low EO concentrations (0.2 or 0.4 mg/mL) were preferred over combinations containing high concentrations (0.8 or 1.6mg/mL) of one or both test compounds). While the above specifications made it feasible to analyze large numbers of antimicrobial combinations, the FIC index used for this particular study differed from those published in literature (Bennett 1978; Yan and Hancock 2001; Perea et al., 2002).

Because there is no set procedure for interpreting antimicrobial interactions, it is common for researchers to manipulate the FIC index to reflect varying degrees of synergism (Bonapace 2002; Dorsthorst et al., 2002). In our case, antimicrobial interactions were determined according to the following scale: synergistic (FIC<0.5); indifferent (FIC 0.5-4); antagonistic (FIC>4) (Botelho 2000) .While this scale is recommended by the American Society for Microbiology it covers a narrower range of FIC values (compared to traditional FIC indexes), thus decreasing the likelihood of identifying synergistic relationships. Such variations make it difficult to compare our results with those published in literature (Bonapace 2002). Furthermore, the high degree of subjectivity associated with the checkerboard method (for SAD) makes it difficult to compare our results with those from multi-comparison methods, such as SGE.

The SGE system, as stated earlier categorizes antimicrobial interactions based on differences in microbial inhibition (specifically linear endpoints of growth). Synergistic effects are determined as visible a shift outward from a reference endpoint, corresponding to the individual compound (Spiral Biotech 2009). While the results produced by SGE are easier to interpret than those produced by SAD, they do not account for differences in hydrophobicity (and solubility) of test compounds; therefore SGE is not currently recognized as an official method of antimicrobial testing (Clinical and Laboratory Standards Institute 2007).

Since both procedures vary in their ability to interpret antimicrobial combinations, it is difficult to draw conclusions about the overall efficacy of either test method. At best, it can be concluded that SAD is better suited for the evaluation of hydrophobic test compounds (compared to SGE), because it allows for more adequate dispersal of test compounds into the agar matrix; however, due to the subjective nature of the checkerboard design, results produced by SAD (for antimicrobial combinations) are not comparable with those published in literature. More research is therefore needed to identify testing methods which yield consistent results.

References

Aeschbach, R., J. Loliger, et al., (1994). "Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol." Food Chemistry and Toxicology **32**(1): 31-36.

Bagamboula, C. F., M. Uyttendaele, et al., (2004). "Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards *Shigella sonni* and *S. flexneri*." Food Microbiology **21**: 33-42.

Baker, C. N., S.A., Stocker, et al. (1991). "Comparison of the E Test to agar dilution, broth microdilution, and agar diffusion susceptibility testing techniques by using a special challenge set of bacteria." Journal of Clinical Microbiology **29**(3): 533-538.

Baker, R. C., D.V., Vadehra, et al. (1983). "Neutralization of antimicrobial properties of lauricidin by tweens." Journal of Food Science **6**(1): 1-12.

Bennett, J. E., and A.E. Jennings (1978). "Factors influencing susceptibility of nocardia species to trimethoprim-sulfamethoxazole " Antimicrobial Agents and Chemotherapy **13**(4): 624-627.

Blaszyk, M. and R. A. Holley (1998). "Interaction of monolaurin, eugenol and sodium citrate on growth of common meat spoilage and pathogenic organisms." International Journal of Food Microbiology **39**: 175-183.

Bogadanov, S., A. Imdorf, et al., (1998) "Thymol residues in wax and honey after apilife VAR treatment." Swiss Bee Research Centere. Dairy Research Station, Liebefeld, CH-3003 Bern.

Bonapace, C. R., J.A., Bosso, et al. (2002). "Comparison of methods of interpretation of checkerboard synergy testing." Diagnostic Microbiology and infectious Disease **44**: 363-366.

Botelho, M. G. (2000). "Fractional inhibitory concentration index of combinations of antibacterial agents against cariogenic organisms." Journal of Dentistry **28**(8): 565-570.

Burt, S. (2004). "Essential oils: their antibacterial properties and potential application in foods-a review." International Journal of Food Microbiology **94**: 223-253.

Clinical and Laboratory Standards Institute (2007). Performance standards for antimicrobial susceptibility testing: Seventeenth Informational Supplement. Clinical and Laboratory Standards Institute. Wayne, PA.

Davidson, P. M. (2005). Antimicrobials in food. New York, Taylor & Francis.

De Martinis, E. C. P., A.D., Crandall, et al. (1997). "Influence of pH, salt and temperature on nisin resistance in *Listeria monocytogenes*." Journal of Food Protection **60**(4): 420-423.

Delgado, B., A. Palop, et al., (2004). "Combined effect of thymol and cymene to control the growth of Bacillus cereus vegetative cells." European Food Research and Technology **218**: 188-193.

Di Pasua, R., N., Hoskins, et al. (2006). "Changes in membrane fatty acids composition of microbial cells induced by addiction of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing Media." Journal of Agricultural and Food Chemistry **54**: 2745- 2749.

Dorsthorst, D. T. A., P.E., Verweij, et al. (2002). "Comparison of the fractional inhibitory concentration index with response surface modeling for characterization of in vitro interaction of antifungals against intraconazole- susceptible and -resistant *Aspergillus fumigatus* isolates." Antimicrobial Agents and Chemotherapy **46**(3): 702-707.

Duckworth, M. and W. Yaphe (1971). "The structure of agar: Part I fractionation of a complex mixture of polysaccharides." Carbohydrate Research **16**(1): 189-197.

Ettayebi, K., J. E. Yamani, et al., (2000). "Synergistic effects of nisin and thymol on antimicrobial activities in *Listeria monocytogenes* and *Bacillus subtilis*." FEMS Microbiology Letters **183**: 191-195.

Fisher, K. and C.A. Phillips (2006). "The effect of lemon, orange and bergamot essential oils and their components on the survival of *Campylobacter jejuni*, *Escherichia coli* O157, *Listeria monocytogenes*, *Bacillus cereus* and *Staphylococcus aureus* in vitro and in food systems." Journal of Applied Microbiology **101**: 1232-1240.

Gill, A. O. and R.A., Holley, (2004). "Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*." Applied and Environmental Microbiology **70**(10): 5750-5755.

Hammer, K. A., C.F., Carson, et al. (1999). "Antimicrobial activity of essential oils and other plant extracts." Journal of Applied Microbiology **86**: 985-990.

Hecth, D. W., D.M., Cirton, et al. (2007). Methods for antimicrobial susceptibility testing of anaerobic bacteria, approved standard-seventh edition Clinical and Laboratory Standards Institute. Wayne, PA. 27.

Hemaiswarya, S. and M. Doble (2009). "Synergistic interaction of eugenol with antibiotics against Gram negative bacteria." Phytomedicine **16**(11): 997-1005.

Hill, G. B. (1991). "Spiral gradient endpoint method compared to standard agar dilution for susceptibility testing of anaerobic Gram-negative Bacilli." Journal of Clinical Microbiology **29**(5): 975-979.

Hill, G. B. and S. Schalkowsky (1990). "Development and evaluation of the spiral gradient endpoint method for susceptibility testing of anaerobic Gram-negative bacilli." Reviews of Infectious Diseases **12**(2): S200-S209.

Holley, R. A. and D. Patel (2005). "Improvement in shelf-life and safety of perishable foods of plant essential oils and smoke antimicrobials." Food Microbiology **22**: 273-292.

Karapinar, M. and S.E. Aktug (1987). "Inhibition of foodborne pathogens by thymol, eugenol, menthol and anethole." International Journal of Food Microbiology **4**: 161-166.

Karlsson, M., S.L., Oxberry, et al. (2002). "Antimicrobial susceptibility testing of Australian isolates of *Brachyspira hyodysenteriae* using a new broth dilution method." Veterinary Microbiology **84**: 123-133.

Kim, J., M.R., Marshall, et al. (1995). "Antibacterial activity of some essential oil components against five foodborne pathogens." Journal of Agricultural and Food Chemistry **43**: 2839-2845.

Kim, J. M., M.R.,Marshall, at al. (1995). "Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella typhimurium* in culture medium and fish cubes." Journal of Food Science **60**(6): 1364-1368.

Kwan, K. Y., A.J.,Allchorne, et al. (2006). "TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction." Neuron **50**: 277-289.

Laas, T. (1972). "Agar derivatives for chromatography, electrophoresis and gel-bound enzymes." Journal of Chromatography **66**(2): 347-355.

Luangtongkum, T., T.Y., Morishita, et al. (2007). "Comparison of antimicrobial susceptibility testing of *Campylobacter spp*. by the agar dilution and the agar disk diffusion methods." Journal of Clinical Microbiology **45**(2): 590-594.

Mann, C. M. and J.L. Markham (1998). "A new method for determining the minimum inhibitory concentration of essential oils." Journal of Applied Microbiology **84**: 538-544.

Nino, M. R. and J.M.R. Patino (1998). "Surface tension of bovine serum albumin and tween 20 at the air-aqueous interface." Journal of the American Oil Chemist's Society **75**(10): 1241-1248.

Pati, U. S. and N.P. Kurade "Antibacterial screening methods for evaluation of natural products." Regional Station Indian Veterinary Research Institute.

Pauli, A. and K. Kubeczka (1997). Evaluation of inhibitory data of essential oil constituents obtained with different microbiological testing methods. Essential Oils: Basic and Applied Research Carol Stream, IL, Allured.

Pei, R., F., Zhou, et al. (2009). "Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. coli* with an improved method." Journal of Food Science **74**(7): M379-M383.

Perea, S., G. Gonzalez, et al. (2002). "In Vitro interaction of caspofungin acetate with voriconazole against clinical isolates of Aspergillus spp." Antimicrobial Agents and Chemotherapy **46**(9): 3039-3041.

Schmalreck, A. F., I., Kottman, et al. (1995). "An evaluation of seven methods of testing in vitro susceptibility of clinical yeast isolates to fluconazole." Mycoses **38**(9-19): 359-368.

Si, W., J., Gong, et al. (2006). "In vitro assessment of antibacterial activity of carvacrol, thymol and cinnamaldehyde towards *Salmonella* serotype Typhimurium DT104: effects of pig diets and emulsification in hydrocolloids." Journal of Applied Microbiology **101**(6): 1282-1291.

Spiral Biotech, I. (2009). Spiral Gradient Endpoint User Guide. Norwood, Massachusetts.

Valero, M.and E Frances (2006). "Synergsitic bactericidal effect of carvacrol, cinnamaldehyde or thymol and refrigeration to inhibit Bacillus cereus in carrot broth." Food Microbiology **23**: 68-73.

Valero, M. and M.J. Giner (2006). "Effects of antimicrobial components of essential oils on growth of Bacillus cereus INRA L2104 in and the sensory qualities of carrot broth." International Journal of Food Microbiology **106**: 90-94.

Valgas, C., S., Machado de Souza, et al. (2007). "Screening methods to determine antibacterial activity of natural products." Brazilian Journal of Microbiology **38**: 369-380.

Walsh, S., J., Maillard et al. (2003). "Activity and mechanisms of action of selected biocidal agents on Gram-positive and -negative bacteria." Journal of Applied Microbiology **94**: 240-247.

Wexler, H. M., E., Molitoris, et al. (1996). "Comparison of spiral gradient endpoint and agar dilution methods for susceptibility testing of anaerobic bacteria: a multilaboratory collaborative evaluation." Journal of Clinical Microbiology **34**(1): 170-174.

Wexler, H. M., E., Morlitoris, et al. (1991). "Comparison of spiral gradient and conventional agar dilution for susceptibility testing of anaerobic bacteria." Antimicrobial Agents and Chemotherapy **35**(6): 1196-1202.

Winniczuk, P. P. and M.E. Parish (1997). "Minimum inhibitory concentration of antimicrobials against micro-organims related to citrus juice." Food Microbiology **14**: 373-381.

Yan, H. and R.E.W. Hancock (2001). "Synergistic Interactions between mammalian antimicrobial defense peptides." Antimicrobial Agents and Chemotherapy **45**(5): 1558-1560.

Yen, T. and S.T. Chang (2008). "Synergistic effects of cinnamaldehyde in combination with eugenol against wood decay fungi." Bioresource Technology **99**(1): 232-236.

Zhou, F., B. Ji, et al. (2007). "The antibacterial effect of cinnamaldehyde, thymol, carvacrol and their combinations against the foodborne pathogen *Salmonella* Typhimurium." Journal of Food Safety **27**(2): 124-133.

Tables

Table 1. Individual Minimum Inhibitory Concentrations (MIC) and Average Gradient Minimum Inhibitory Concentrations (GMIC) for Essential Oil Compounds against Three Foodborne Pathogens

	MIC in mg/mL					
	L. monocytogenes		*S.* Typhimurium		*S. aureus*	
Antimicrobial	SGE	SAD	SGE	SAD	SGE	SAD
Cin	6.00[b]	0.2[a]	26.24[b]	0.4[b]	4.80[b]	0.2[b]
Eug	133.12[a]	1.6[a]	102.91[a]	1.6 [a,b]	61.44[a]	1.6[a]
Thy	42.76[b]	0.4[a]	24.96[b]	0.4[b]	28.16[b]	0.4[b]

Thy (thymol); Cin (cinnamaldehyde); Eug (eugenol); SGE (spiral gradient endpoint); SAD (standard agar dilution)
[a-c] Means with the same letter are not significantly different

Table 2. Antimicrobial Interactions for Combinations of Essential Oil Compounds against Three Foodborne Pathogens

Interaction Type						
	L. monocytogenes		*S.* Typhimurium		*S. aureus*	
Combination	SGE	SAD	SGE	SAD	SGE	SAD
Thy+Cin	Ant	Ind (3)	Ant	Ind (2)	Ant	Ind (1.5)
Thy+Eug	Ant	Ant (5)	Ant	Ind (2.5)	Ant	Ind (2.5)
Eug+ Cin	Ant	Ind (1.13)	Ant	Ind (1.25)	Ant	Ind (1.13)

Thy (thymol); Cin (cinnamaldehyde); Eug (eugenol); SGE (spiral gradient endpoint); SAD (standard agar dilution)
Ant (Antagonism); Ind (Indifferent interactions); Syn (synergism)
Numbers in parenthesis are FIC values generated by the checkerboard method of analysis.

CHAPTER 4

Antimicrobial effects of hexanal, *trans*-2-hexenal and 1-hexanol on foodborne pathogens, alone and in combination with thymol, eugenol and cinnamaldehyde

Abdulmalik, T.O.[1]*, R. R. Boyer[1], J. B. Gurtler[2], J. Levy[1], J., S. F. O'Keefe[1], R. C. Williams[1]

[1]Virginia Polytechnic Institute and State University, Department of Food Science and Technology. 22 Duck Pond Drive, Blacksburg VA 24061 USA
[2]Food Safety Intervention Technologies, U.S. Department of Agriculture, ARS, Eastern Regional Research Center. 600 E. Mermaid Ln. Wyndmoor, PA 19038-8598

Key words: thymol, eugenol, cinnamaldehyde, hexanal, hexenal, *trans*-2-hexenal, hexanol, 1-hexanol, *L. monocytogenes, S. aureus*, *S*. Typhimurium.

ABSTRACT

The antibacterial activity of plant-derived essential oils has been recognized for many years. Of these compounds, thymol, eugenol and cinnamaldehyde are effective against a number of foodborne pathogens; however their use within the food industry is often limited by their negative organoleptic effects and limited solubility. They are, therefore, not compatible within fruit-based foods, which often do not effectively mask the flavor of these compounds. Combining these compounds with naturally-occurring fruit flavorants may results in a lower effective concentration, to minimize off-flavors in the final product. In this study, the antimicrobial activity of three apple aroma compounds (hexanal, *trans*-2-hexenal and 1-hexanol) was evaluated individually and in combination with thymol, eugenol and cinnamaldehyde, to determine their combined effects against three foodborne pathogens (*Listeria monocytogenes*, *Salmonella enterica* Typhimurium and *Staphylococcus aureus*). The antimicrobial effects of the individual compounds, as well as the combined effects of both compounds, were determined using the standard agar dilution method of analysis. Cinnamaldehyde was the most inhibitory essential oil compound, followed by thymol and eugenol, with overall MIC values of 0.2, 0.4, and 1.6 mg/mL, respectively. *Trans*-2-hexenal was the most effective apple aroma compound , with an MIC of 1.6 mg/mL, followed by hexanol (MIC =12.8 mg/ml) and hexanal (MIC =25.6 mg/ml). Synergistic relationships were identified for combinations containing high concentrations of eugenol (0.8 or 1.6mg/mL; however, all other combinations had a negligible effect on antimicrobial activity, with MIC values similar to their original concentration.

INTODUCTION

The antimicrobial properties of plant essential oils (EOs) and their extracts have been recognized for years, with reports of their medicinal use dating back to historic times (Zaika 1988; Suhr and Nielson 2003). These compounds have been used primarily as flavoring and perfume additives; however, in response to the recent demand for natural and fresh-like food products, their use as natural food preservatives has been evaluated (Kim et al., 1995; Burt 2004). Thymol, eugenol and cinnamaldehyde are three EO compounds that are effective against a number of foodborne pathogens (Friedman et al., 2002; Davidson 2005).

Thymol is a phenolic compound abundant in the leaves, stems, roots, etc. of thyme and *Origanum* species of plant (Karapinar and Aktug 1987; Bagamboula et al., 2004; Sokmen et al., 2004). Thymol is currently approved for use as a synthetic flavoring agent (at a maximum concentration of 50 mg/kg) and can inhibit the growth of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* serovars, *Staphylococcus aureus* and *Shigella* spp. (Bogadanov et al., 1998; Ettayebi et al., 2000; Walsh et al., 2003; Bagamboula et al., 2004). Thymol's antimicrobial activity has been evaluated within a number of food matrices; including carrot broth, chicken soup and pumpkin cream; however, its use within the food industry is often limited due to its strong sensory profile and poor solubility in water (Aeschbach et al., 1994; Delgado et al., 2004; Valero and Frances 2006; Valero and Giner 2006).

Eugenol and cinnamaldehyde are antimicrobial compounds found in the oils of clove and cinnamon, respectively (Kwan et al., 2006). These compounds are inhibitory to a number of foodborne pathogens, including *E. coli*, *L. monocytogenes*, *S. enterica* Typhimurium, *Campylobacter jejuni*, *Vibrio vulnificus* and *Bacillus cereus* (Gill and Holley 2004; Si, Gong et

al., 2006; Hemaiswarya and Doble 2009). While both compounds are generally recognized as safe (GRAS) by the United States Food and Drug Administration (FDA), their use within the food industry is limited, due to negative organoleptic properties (Burt 2004; Valero and Giner 2006; Hemaiswarya and Doble 2009). Consequently, the exploration of effective natural antimicrobials has shifted to explore compounds with less impact on flavor profiles.

Hexanal ($C_6H_{12}O$, commonly referred to as hexanaldehyde), *trans*-2-hexenal ($C_6H_{10}O$) and 1-Hexanol (CH3 (CH2)5OH) are naturally occurring fruit flavorants used within the food industry to enhance the fruity and woody nuances of concentrated apple juices (Burdock 2005; Da Costa 2010). While these compounds have been proven to exhibit varying degrees of antimicrobial activity (e.g., against *Pichia subpelliculosa*, *E. coli*, *S.* Enteritidis, and *L. monocytogenes*), their effective dosages often exceed consumer-acceptable limits (Corbo 2000; Lanciotti 2003; Burdock 2005). Therefore, they are commonly evaluated in combination with other fruit extracts, to enhance their antimicrobial effects against common foodborne pathogens (Lanciotti 2003).

The goal of this study was to combine the antimicrobial activity of three essential oil compounds with three naturally-occurring apple flavorants (which exhibit limited degrees of antimicrobial activity) in order to identify synergistic relationships to lower the effective concentration of one or both compounds needed to inhibit the growth of common foodborne pathogens.

MATERIALS AND METHODS

Bacterial Cultures: The following bacterial strains were used for this study: *L. monocytogenes* (Scott A), *S. aureus* (ATCC 25923), and *S. enterica* Typhimurium (ATCC 14028). All test cultures were obtained from the frozen (-80°C) culture collection at Virginia Tech (Blacksburg, VA).

Preparation of Bacterial Cultures: Stored cultures were activated by two successive transfers in tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) at 35˚C for 24 hours. Samples were then streaked onto differential media (xylose lysine desoxycholate (XLD) agar, moxified Oxford (MOX) agar and mannitol salt (MAS) agar for *S.* Typhimurium, *L. monocytogenes* and *S. aureus*, respectively); and a single representative colony (for each organism) was selected for further confirmation. *S.* Typhimurium, *L. monocytogenes* and *S. aureus* cultures were confirmed using the *Salmonella* latex agglutination test (Oxoid Ltd., Basingstoke,Hants, UK), API *Listeria* strips (Biomerieux, Inc., Durham, NC), and the BBL™ Staphyloside™ Latex test (Becton Dickson Microbiology Systems, Franklin Lakes, NJ), respectively.

Upon confirmation, a single representative colony of each organism was inoculated into individual test tubes, containing 10 mL of sterile tryptic soy broth. Samples were then incubated for 24 hours at 35ºC, yielding a final concentration of 10^8-10^9 CFU/mL. This culture was transferred into fresh broth every 18-24 hours, in order to ensure a uniform culture for the duration of the experiment.

Preparation of Chemicals and Antimicrobial Agents: Prior to experimentation, all apple flavor compounds**,** hexanal (96%), 1-hexanol (99%) and *trans*-2-hexenal (99%) (Acros Organics,

Fair Lawn, NJ) and all essential oils, thymol, (99%), eugenol (≥89%); and cinnamaldehyde (89%) (SAFC Supply Solutions, St. Louis, MO) were weighed into individual glass beakers, containing 0.5% Tween-20 (Fisher Scientific, Fairlawn, NJ). Tween-20 was included to enhance solubility of the test compounds. This solution was then emulsified for 30 seconds, using a micro-emulsifying needle (Cadence Science, Staunton, VA),filter sterilized, using a 45μL disposable syringe filter (Fisher Scientific) and dispensed into individual containers of molten tryptic soy agar (TSA; Becton, Dickinson and Company).

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

Determination of Synergistic Effects: Antimicrobial interactions between test compounds were evaluated using the checkerboard assay, as previously described (Rosenblatt 1974; Rand 1995).

Briefly, each essential oil compound (at 0.2, 0.4, 0.8 or 1.6 mg/mL) was dissolved into an individual container of molten TSA, containing 0.5% tween-20 with the exception of cinnamaldehyde, whose individual MIC did not exceed 0.4 mg/mL.

One of the three apple aroma (AA) compounds (0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 and 25.6 mg/mL) were then separately added to each container, resulting in a series of eight mixed ratio combinations. For example, cinnamaldehyde at 0.2 mg/mL and 0.4 mg/mL (previously determined MIC values) were tested in combination with hexanal at 0.2, 0.4, 0.8, 1.6, 3.2, 6.5 12.8 and 25.6 mg/mL, respectively. All combinations were poured into individual petri plates and allowed to solidify. Plates were then separated into four quadrants and spot inoculated and incubated as previously described. MICs were determined as the lowest concentration that completely inhibited microbial growth.

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

Because the checkerboard method of analysis generates numerous MIC values, antimicrobial interactions (and FIC indexes) were determined using combinations that contained the lowest effective concentration of each test compound (typically 0.2 or 0.4 mg/mL of each compound). In addition, FIC indexes were also determined for combinations containing high

concentrations of each compound (0.8 or 1.6 mg/mL), in order to understand the effects of EO concentrations on antimicrobial activity.

RESULTS

The antimicrobial activities of eugenol, cinnamaldehyde and thymol were evaluated alone and in combination with three apple aroma compounds (1-hexanol, *trans*-2-hexenal and hexanal) to determine their individual and combined effects against *L. monocytogenes*, *S. aureus* and *S. enterica* Typhimurium. Of the EO compounds, cinnamaldehyde exhibited the highest degree of antimicrobial activity, with reported MIC values of 0.4 mg/mL for *S. enterica* Typhimurium; and 0.2 mg/mL for *L. monocytogenes* and *S. aureus* (Table 1). Thymol generated similar levels of inhibition for all test microorganisms. Eugenol was the least inhibitory of the three EO compounds (although there was no statistical difference between the treatments) (Table 1).

Of the apple aroma compounds, *trans*-2-hexenal exhibited the highest degree of antimicrobial activity (MIC = 1.6, for *L. monocytogenes* and *S. aureus*; MIC = 3.2 mg/mL for *S. enterica* Typhimurium) (Table 1). Hexanal and 1-hexanol were significantly less active against *E. coli* O157:H7 and *L. monocytogenes*, (Table 1). Since the AA compounds displayed limited degrees of activity against test organisms, they were evaluated in combination with the EO compounds, in an effort to identify synergistic relationships, which enhance their effects against the test pathogens.

Combination testing revealed that low concentrations (0.2 or 0.4 mg/mL) of thymol or eugenol in combination with hexanal and *trans*-2-hexenel resulted in antagonistic effects; whereas, cinnamaldehyde-containing combinations produced indifferent interactions

(0.5>FIC<4) (Table 2). Likewise, the majority of high-concentration combinations (0.8 or 1.6mg/mL) resulted in indifferent relationships (with FIC indexes ranging between 0.51 and 2.06) (Table 3). Eugenol was the only exception, with all combinations producing synergistic effects (FIC< 0.5). Interestingly, all antimicrobial combinations generated organism-specific interactions, in which the Gram-negative bacteria required higher concentrations to completely inhibit microbial growth than the Gram-positive microorganisms (data not shown).

DISCUSSION

Our results indicate that cinnamaldehyde was the most active EO compound, followed by thymol and eugenol. These results were similar to the patterns found by Zhou et al. (2007), who evaluated the activity of cinnamaldehyde and thymol against *S. enterica* Typhimurium in a general growth medium. In this case, cinnamaldehyde produced MIC values of 200 mg/L, and thymol required significantly higher concentrations (400 mg/L) to achieve the same level of inhibition. Similarly, Pei et al. (2009) evaluated the activity of eugenol, cinnamaldehyde and thymol against generic strains of *E. coli*, and reported MIC values of 1600, 400 and 4000 mg/L, respectively.

This variation in activity is likely caused by small differences in the structure and relative hydrophobicity of each test compound (Gardini 2001). While most EO compounds act by disrupting the cytoplasmic membrane of bacterial cells(which in turn induces a dissipation of the proton motive force and leakage of intercellular ions), certain EO compounds target additional sites within the cell, making them more active against test organisms (Cox 2000; Gardini 2001; Nazer 2005; Hemaiswarya 2009).

Such is the case for eugenol, which contains a free hydroxyl group that is believed to interact with the sulfhydryl group of surface-active proteins (Cowan 1999; Pei 2009). These interactions suppress the activity of key enzymes involved in the metabolic break-down of glucose (namely ATPase) leading to a depletion of intercellular ATP and cell death (Gill 2004; Hemaiswarya 2009). For this reason, eugenol is typically active against both Gram-positive and Gram negative microorganisms (Arora 1999). Thymol also triggers a depletion of intercellular ATP (although it acts by disrupting the outer membrane of target cells), explaining why thymol and eugenol both produced similar MIC values for all test organisms (Helander 1998).

Cinnamaldehyde, however, displayed organism-specific interactions, requiring slightly higher concentrations to completely inhibit the growth of *S. enterica* Typhimurium, when compared to MICs for *L. monocytogenes* and *S. aureus*. This variation is likely caused by the fact that cinnamaldehyde possesses a highly active carbonyl group, which inhibits the action of amino acid decarboxylase (Pei 2009). While this action is generally effective in preventing bacterial proliferation, it is often less effective against Gram-negative microorganisms, because Gram-negative bacteria contain a highly selective outer membrane, which inhibits the entry of large hydrophobic EO compounds (Ouattara 1997; Delaquis 2002).

In the case of the apple aroma compounds, *trans*-2- hexenal displayed the highest degree of antimicrobial activity, followed by 1-hexanol and hexanal. Similar patterns of inhibition have been observed in a number of studies, which suggests that the structure and stability of test aldehydes greatly influences their level of inhibition against test pathogens (Kubo et al., 1995; Lanciotti et al., 2003). Alkyl aldehydes behave as surface-active detergents, which attach to the sulfydryl group of membrane-bound proteins via nucleophilic interactions involving their polar carbonyl groups (Kubo et al., 1995; Lanciotti et al., 2003; Neri et al., 2006; Neri et al., 2007).

These interactions compromise the cytoplasmic membrane, leading to cell death (Kubo et al., 1995).

While the majority of alkyl aldehydes produce similar levels of inhibition, some compounds, such as *trans*-2-hexanal, exhibit exceptional killing ability (Lanciotti et al., 2003). It is assumed that *trans*-2- hexenal passively diffuses into the cell, where the highly active enal group attaches to membrane-bound proteins (Muroi et al., 1993; Lanciotti et al., 2003). Since hexanal and other test aldehydes do not contain the enal group, they can be expected to display limited activity against test microorganisms.

Interestingly, the other two AA aldehydes produced similar MICs for both Gram-positive and Gram-negative microorganisms. This pattern is likely due to the fact that these compounds are all small hydrophobic substances, which easily penetrate the outer membrane of gram negative microorganisms via porins (Lanciotti et al., 2003). Once inside the cell, their hydrophobic carbon tails interact with integral proteins, such as transport and ion channel proteins, causing membrane disruption eventually leading to cell death (Patrigani et al., 2008).

In this experiment, combinations containing high concentrations of eugenol with the AA aldehydes produced a number of synergistic relationships, while thymol and cinnamaldehyde resulted in indifferent interactions. Such patterns are contrary to the results reported by Nazer et al. (2005) who evaluated the activity of thymol and eugenol in combination with several aromatic and acidic compounds. Their results indicate that thymol produced the highest main effects, while eugenol had the smallest influence on overall antimicrobial activity. These patterns were thought to occur because of the concentration-dependent activity of EO compounds (Nazer et al., 2005).

However, at the concentrations used for our study, the enhanced effects of eugenol are likely to exist because of the compound's ability to penetrate the bacterial membrane, allowing other compounds with different modes of action to target sites within the cell (Hemaiswarya and Doble 2009; Pei et al., 2009). More specifically, it is assumed that eugenol pre-sensitizes bacterial cell membranes to the action of secondary compounds, enhancing their overall effects against test organisms (Hemaiswarya and Doble 2009).

While eugenol-containing combinations produced a number of synergistic effects, the majority of EO combinations resulted in antagonistic effects. Such relationships are likely a result of the antioxidant properties of the EO compounds. Several studies have reported that EO compounds, such as thymol and eugenol, interact with hexanal (a secondary byproduct of lipid oxidation, involving linoleic acid) and 1-hexanol to prevent their oxidative conversion into hexanoic acid, a compound that leads to off-flavors and decreased shelf life of foods (Marcuse 1973; Zhou 1999; Brink et al., 2000; Lee and Shibamotto 2002; Lee et al., 2005). In fact, thymol (and eugenol, to a lesser extent) has been proven to inhibit the oxidative conversion of hexanal to hexanoic acid in foods for periods up to 30 days (Lee 2005). Such interactions may have suppressed the antimicrobial activity of EO compounds.

While it is customary to limit the amount of essential oil components in foods (due to their volatility and decreased solubility in water), the use of high EO concentrations may prove beneficial in foods where the odor or aroma of EO compounds are effectively masked. The use of eugenol, in combination with hexanal, 1-hexanol and *trans*-2-hexenal offers new possibilities for preserving fresh food products. This synergistic relationship may lower the effective concentrations needed to preserve foods, without altering the natural profile of the food product.

Nevertheless, further research is required to understand the activity of these compounds in model food systems, as well as their overall sensorial acceptability in foods.

References

Aeschbach, R., J. Loliger, et al. (1994). "Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol." Food Chemistry and Toxicology **32**(1): 31-36.

Bagamboula, C. F., M. Uyttendaele, et al. (2004). "Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards *Shigella sonni* and *S. flexneri*." Food Microbiology **21**: 33-42.

Bogadanov, S., A. Imdorf, et al. (1998) "Thymol residues in wax and honey after apilife VAR treatment." 1-9. Swiss Bee Research Centre, Dairy Research Station, Liebefeld, CH-3003 Bern.

Brink, G., I. Arends, et al., (2000). "Green, catalytic oxidation of alcohols in water." Science **287**: 1636-1639.

Burt, S. (2004). "Essential oils: their antibacterial properties and potential applications in foods-a review." International Journal of Food Microbiology **94**: 223-253.

Davidson, P. M. (2005). Antimicrobials in food. New York, Taylor & Francis.

Delgado, B., A. Palop, et al. (2004). "Combined effect of thymol and cymene to control the growth of *Bacillus cereus* vegetative cells." European Food Research and Technology **218**: 188-193.

Ettayebi, K., J. E. Yamani, et al. (2000). "Synergistic effects of nisin and thymol on antimicrobial activities in *Listeria monocytogenes* and *Bacillus subtilis*." FEMS Microbiology Letters **183**: 191-195.

Friedman, M., P. R. Henika, et al. (2002). "Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*." Journal of Food Protection **65**(10): 1545-1560.

Gill, A. O. and R. A. Holley (2004). "Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*." Applied and Environmental Microbiology **70**(10): 5750-5755.

Hecth, D. W., D. M. Cirton, et al. (2007). Methods for antimicrobial susceptibility testing of anaerobic bacteria, approved standard-seventh edition Clinical and Laboratory Standards Institute. Wayne, PA. 27.

Hemaiswarya, S. and M. Doble (2009). "Synergistic interaction of eugenol with antibiotics against Gram negative bacteria." Phytomedicine **16**(11): 997-1005.

Karapinar, M. and S. E. Aktug (1987). "Inhibition of foodborne pathogens by thymol, eugenol, menthol and anethole." International Journal of Food Microbiology **4**: 161-166.

Kim, J. M., M. R. Marshall, et al. (1995). "Antibacterial activity of carvacrol, citral, and geraniol against *Salmonella* Typhimurium in culture medium and fish cubes." Journal of Food Science **60**(6): 1364-1368.

Kubo, A., C. S. Lunde, et al. (1995). "Antimicrobial activity of the olive oil flavor compounds." Journal of Agricultural and Food Chemistry **43**: 1629-1633.

Kwan, K. Y., A. J. Allchorne, et al. (2006). "TRPA1 contributes to cold, mechanical, and chemical nociception but is not essential for hair-cell transduction." Neuron **50**: 277-289.

Lanciotti, R., N. Belletti, et al. (2003). "Application of hexanal, (E)-2-hexenal, and hexyl acetate to improve safety of fresh-sliced apples." Journal of Agricultural and Food Chemistry **51**: 2958-2963.

Lee, K. and T. Shibamotto (2002). "Determination of antioxidant potential of volatile extracts isolated from various herbs and spices." Journal of Agricultural and Food Chemistry **50**: 4947-4952.

Lee, S., K. Umano, et al. (2005). "Identification of volatile components in basil (*Ocimum basilicum L.*) and thyme leaves (*Thymus vulgaris L.*) and their antioxidant properties." Food Chemistry **91**: 131-137.

Muroi, H., A. Kubo, et al. (1993). "Antimicrobial activity of cashew apple flavor compounds." Journal of Agricultural and Food Chemistry **41**:1106-1109.

Nazer, A. I., A. Kobilinsky, et al. (2005). "Combinations of food antimicrobials at low levels to inhibit the growth of *Salmonella* sv. Typhimurium: a synergistic effect?" Food Microbiology 22: 391-398.

Neri, F., M. Mari, et al. (2006). "Control of *Penicillium expansum* by plant volatile compounds." Plant Pathology **55**: 100-105.

Neri, F., M. Mari, et al. (2007). "Fungicidal activity of plant volatile compounds for controlling *Monilinia laxa* in stone fruit." Plant Disease **91**(1): 30-35.

Patrigani, F., L. Uucci, et al. (2008). "Effects of sub-lethal concentrations of hexanal and 2-(E)-hexenal on membrane fatty acid composition and volatile compounds of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* Eneteritidis and *Escherichia coli*." International Journal of Food Microbiology **123**: 1-8.

Pei, R., F. Zhou, et al. (2009). "Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. coli* with an improved method." Journal of Food Science **74**(7): M379-M383.

Si, W., J. Gong, et al. (2006). "In vitro assessment of antibacterial activity of carvacrol, thymol and cinnamaldehyde towards *Salmonella* serotype Typhimurium DT104: effects of pig diets and emulsification in hydrocolloids." Journal of Applied Microbiology **101**(6): 1282-1291.

Sokmen, M., J. Serkedjieva, et al. (2004). "In vitro antioxidant, antimicrobial, and antiviral activities of the essential oil and various extracts from herbal parts and callus cultures of *Origanum acutidens*." Journal of Agricultural and Food Chemistry **52**(11): 3309-3312.

Suhr, K. I. and P. V. Nielson (2003). "Antifungal activity of essential oils evaluated by two different techniques against rye bread spoilage fungi." Journal of Applied Microbiology **94**: 665-674.

Valero, M. and M. J. Giner (2006). "Effects of antimicrobial components of essential oils on growth of *Bacillus cereus* INRA L2104 in and the sensory qualities of carrot broth." International Journal of Food Microbiology **106**: 90-94.

Walsh, S., J. Maillard, et al. (2003). "Activity and mechanisms of action of selected biocidal agents on Gram-positive and -negative bacteria." Journal of Applied Microbiology **94**: 240-247.

Zaika, L. L. (1988). "Spices and Herbs: Their antimicrobial activity and its determination." Journal of Food Safety **9**: 97-118.

Tables

Table 1. Individual Minimum Inhibitory Concentrations (MIC) for antimicrobial test compounds against four foodborne pathogens.

Antimicrobial	MIC in mg/mL		
	L. monocytogenes	*S. aureus*	*S.* Typhimurium
EO Compounds			
Eugenol	1.6 [b]	1.6 [b,c]	1.6 [b,c]
Thymol	0.4 [b]	0.4 [c]	0.4 [c]
Cinnamaldehyde	0.2 [b]	0.2 [c]	0.4 [c]
AA Compounds			
Hexanal	12.8 [a]	25.6 [a]	25.6 [a]
trans-2-hexenal	1.6 [b]	1.6 [b,c]	3.2 [b,c]
1-hexanol	12.8 [a]	12.8 [a]	12.8 [a]

EO Essential oil; AA apple aroma
[a-c] Means with the same letter are not significantly different

Table 2. Fractional inhibitory concentration (FIC) indices and antimicrobial interactions for combinations containing low concentrations of essential oil (EO) compounds

Combination	FIC Indexes					
	L. monocytogenes		*S.* Typhimurium		*S. aureus*	
	FIC	Interpretation	FIC	Interpretation	FIC	Interpretation
Thy + Hexanal	33.00	ANT[+]	65.00	ANT[+]	32.50	ANT[++]
Thy + Hexanol	8.25	ANT[++]	8.25	ANT[++]	8.25	ANT[++]
Thy + Hexenal	0.63	IND[+]	2.25	IND[++]	2.50	IND[+]
Eug + Hexanal	4.50	ANT[+]	17.00	ANT[+]	8.50	ANT[++]
Eug + Hexanol	4.50	ANT[++]	4.50	ANT[++]	9.00	ANT[+]
Eug + Hexenal	1.00	IND[+]	1.50	IND[+]	1.00	IND[+]
Cin + Hexanal	1.02	IND[+]	1.02	IND[++]	1.01	IND[++]
Cin + Hexanol	1.02	IND[+]	2.06	IND[+]	1.02	IND[+]
Cin + Hexenal	1.13	IND[+]	1.13	IND[+]	1.02	IND[+]

Abbreviations: FIC, Fractional Inhibitory Concentration; SYN, synergy (FIC≤0.5); IND, indifference (FIC >0.5<4); ANT, antagonism (FIC≥4)

[+]These combinations contain 0.2 mg/mL of essential oil compounds (thymol, cinnamaldehyde or eugenol)
[++]These combinations contain 0.4 mg/mL of essential oil compounds (thymol, cinnamaldehyde or eugenol)

Table 3. Fractional inhibitory concentration (FIC) indices and antimicrobial interactions for combinations containing high concentrations of essential oil (EO) compounds

Combination	FIC Indexes					
	L. monocytogenes		*S.* Typhimurium		*S. aureus*	
	FIC	Interpretation	FIC	Interpretation	FIC	Interpretation
Thy + Hexanal	0.52	IND[+]	0.51	IND[++]	0.51	IND[+]
Thy + Hexanol	0.52	IND[+]	0.52	IND[++]	0.52	IND[+]
Thy + Hexenal	0.63	IND[+]	1.13	IND[+]	0.63	IND[+]
Eug + Hexanal	0.14	SYN[++]	0.13	SYN[++]	0.13	IND[++]
Eug +Hexanol	0.14	SYN[++]	0.14	SYN[++]	0.14	IND[++]
Eug + Hexenal	0.25	SYN[+]	0.19	SYN[++]	0.50	IND[+]

Abbreviations: FIC, Fractional Inhibitory Concentration; SYN, synergy (FIC≤0.5); IND, indifference (FIC >0.5<4); ANT,antagonism (FIC≥4)

[+]These combinations contain 0.8 mg/mL of essential oil compounds (thymol, cinnamaldehyde or eugenol)
[++]These combinations contain 1.6mg/mL of essential oil compounds (thymol, cinnamaldehyde or eugenol)

CHAPTER 5

Inactivation of *Listeria monocytogenes* and *Salmonella* Typhimurium in apple juice using combinations of cinnamaldehyde, eugenol and *trans*-2-hexenal

Abdulmalik, T.O.[1]*, R.R.Boyer[1], J.B.Gurtler[2], J. Levy[1], S.F. O'Keefe[1] , R.C. Williams[1]

[1]Virginia Polytechnic Institute and State University, Department of Food Science and Technology. 22 Duck Pond Drive, Blacksburg VA 24061 USA
2 Food Safety Intervention Technologies, U.S. Department of Agriculture, ARS, Eastern Regional Research Center. 600 E. Mermaid Ln. Wyndmoor, PA 19038-8598

Key Words: Foodborne pathogens, eugenol, *trans*-cinnamaldehyde, essential oils, *trans*-2-hexenal, apple juice

ABSTRACT

The antimicrobial activity of two plant essential oil compounds (*trans*-cinnamaldehyde and eugenol) were evaluated alone and in combination with *trans*-2-hexenal (a naturally-occurring apple flavorant) to determine their ability to inactivate *Salmonella* Typhimurium and *Listeria monocytogenes* in commercial apple juice. Apple juice samples containing 0.025%, 0.05%, 0.075% and 0.125% of each individual compound or dual-combinations containing 0.025% of each test compound were inoculated with 7-8 log CFU/mL of *S.*Typhimurium or *L. monocytogens* and stored at 4°C and 25°C. Bacterial counts were determined after 0, 1, 3 and 7 days of storage, using tryptic soy agar. Treatments with 0.05% of *trans*-cinnamaldehyde, eugenol and *trans*-2-hexenal resulted in a 5 log CFU/ml reduction in bacterial numbers within one day of storage at both 4°C and 25°C. The only exception was *trans*-2-hexenal, which resulted in a 4 log CFU/ml reduction in *L. monocytogense* counts, after one day of storage at 4°C. Antimicrobial combinations containing 0.025% *trans*-cinnalamdehyde or 0.025% eugenol in combination with 0.025% *trans*-2-hexenal resulted in 5 and >7 log CFU/ml reductions in *S.* Typhimurium counts following one day of storage at 4°C and 25°C, respectively. Likewise, both treatments resulted in 4 and 8 log CFU/ml reductions in *L. monocytogenes* counts, following one day of storage at 4°C and 25°C, respectively.

INTRODUCTION

Apple juice has traditionally been considered a low risk to food safety, due to its intrinsically low pH and organic acid content (Sapers et al., 2006; Truong et al., 2010); however, it has recently become the cause of several large-scale outbreaks of foodborne illness involving *E. coli* O157:H7, *Salmonella* Typhurimum and *Cryptosporidium parvum* (Miller and Kasper 1994; Cody et al., 1999; Truong et al., 2010). The United States Food and Drug Administration (U.S. FDA) has also identified *Listeria monocytogenes* as an emerging threat to food safety, because it has been isolated from the flesh of apples in several food-safety studies (Roering et al., 1999; Conway et al., 2000; Leverentz et al., 2006).

As a result of these highly publicized outbreaks, the U.S. FDA now requires that fruit juice processors achieve a 5-log reduction in the "pertinent organisms of concern", prior to selling juice to the public (Uljas and Ingham 1999; Williams et al., 2005). In most cases, the apple juice industry utilizes thermal pasteurization (>160F for ≥6 seconds) as a means of ensuring product safety (Zhao et al., 1993; Tajchakavit et al., 1998; Beerbower, 2002). However, due to recent demands for minimally-processed foods, many processors have explored the use of non-thermal techniques (such as UV irradiation, high-pressure processing, microwave heating, ultra filtration, ozone, pulsed electric field, etc.) as an alternative means of ensuring product safety (Gould 1996; Zink 1997; Buchanan et al., 1998; Tajchakavit et al., 1998; Rahman 1999; Choi and Nielsen 2004; Mosqueda-Melgar et al., 2008).

While these technologies are all capable of producing a 5 log CFU/ml reduction in bcteria, their acceptance into the fresh juice industry is often hampered by negative consumer perceptions as well as high implementation cost (Hunter 2000; DeRuiter and Dwyer 2002; Nayga et al., 2005). For this reason, research has been conducted to identify natural

antimicrobials (particularly plant-derived compounds), which inhibit the survival and growth of pathogenic microorganisms (Gould 1996).

Of these compounds, cinnamaldehyde and eugenol, major constituents of cinnamon and clove oil, respectively, are effective against a number of pathogenic microorganisms, including *E. coli, E. sakazakii, L. monocytogenes, Bacillus cerus, S.* Typhimurium and *V. vulnificus* (Gill and Holley 2004; Hemaiswarya and Doble 2009). While both compounds are Generally Recognized as Safe (GRAS) by the U.S. FDA, their use within the apple juice industry is limited because of negative organoleptic effects and limited solubility (Burt 2004; Valero and Giner 2006; Hemaiswarya and Doble 2009). There has been interest in identifying antimicrobial combinations, with other natural preservatives which lower the concentration needed to inhibit pathogenic growth in foods (Zhou et al., 2007; Pei et al., 2009).

Trans-2-hexenal ($C_6H_{10}O$) is a common leaf-aldehyde that is naturally present in apples, strawberries, apricot, potatoes, etc. (Visser et al., 1979; Lanciotti et al., 2003; Burdock 2005; Da Costa and Cannon, 2010). *Trans*-2-hexenal is characterized by its "fruity" and "green" aroma and is commonly used to restore the natural apple-aroma of fresh and concentrated apple juice (Burdock 2005; Da Costa and Cannon 2010). *trans*-2-hexenal is active against a number of foodborne pathogens (e.g., *S*. Enteritidis, *E. coli* and *L. monocytogenes*), however its effective dose often exceeds acceptable sensory limits of 10 ppm; therefore, it is commonly used in combination with other naturally-occurring antimicrobials (particularly other apple-aldehydes) to inhibit the growth of pathogens on apple-based products (Corbo et al., 2000; Lanciotti et al., 2003; Burdock 2005).

The objective of this study was to evaluate the antimicrobial activities of cinnamaldehyde and eugenol alone and in combination with *trans*-2-hexenal in order to identify synergistic relationships to lower the effective concentration of EO compounds needed to inactivate pathogens in apple juice. This research may offer alternatives for the preservation of fresh apple cider, without contributing to off flavor in the final product.

MATERIALS AND METHODS

Microorganims: *Salmonella* Typhimurium ATCC 14028 and *Listeria monocytogenes* (Scott A) were both obtained from the frozen (-80°C) bacterial culture collection at Virginia Tech (Blacksburg, VA).

Preparation of Antimicrobial Agents: *Trans*-2-hexenal (99% purity) was purchased from Acros Organics, New Jersey, USA; eugenol (≥89% purity) and cinnamaldehyde (89% purity) were both purchased from SAFC Supply Solutions (St. Louis, MO). Prior to experimentation, all test compounds were filter sterilized, using a 45µL disposable syringe filters (Fisher Scientific, Fairlawn, NJ) and stored in separate sterile glass beakers.

Sample Preparation: Commercially pasteurized apple juice (Mott's® Natural apple juice, with no added preservatives) was purchased from a local grocery store. The initial microbial quality of the juice was evaluated by spread-plating onto tryptic soy agar (TSA, for enumeration of aerobic microorganims), saboraud dextrose agar (SAD, for enumeration of yeast), xylose lysine desoxycholate agar (XLD, for enumeration of *Salmonella* Typhimurium) and moxified Oxford agar (MOX, for enumeration of *L. monocytogenes*).

Test samples of juice were prepared according to the procedure outlined by Baskaran, et al. (2010). Briefly, 90 mL aliquots of apple juice were transferred to individual glass containers

containing various concentrations of test compounds (0.025%, 0.05%, 0.075% and 0.125%). Juice solutions were shaken vigorously by hand for 30 sec. to ensure adequate dispersal of test compounds.

Dual-combinations, containing eugenol in combination with *trans*-2-hexenal; or cinnamaldehyde in combination with *trans*-2-hexenal, were prepared in a similar fashion, except that total antimicrobial concentration did not exceed 0.050% (0.025% of each test compound).

Microbiological Analysis: Prior to experimentation, stored cultures of *Salmonella* Typhimurium and *L. monocytogenes* were streaked onto differential media and a single representative colony (one for each organism) was selected for further confirmation. *S.* Typhimurium was confirmed using the *Salmonella* latex agglutnation test (Oxoid Ltd., Basingstoke, Hants, UK) and *L. monocytogenes* was confirmed using API *Listeria* strips (Biomerieux, Inc., Durham, NC).

Upon confirmation, a single representative colony from each organism was inoculated into individual test tubes containing 10mL of sterile tryptic soy broth (TSB) and incubated for 24 hours at 35ºC. Following incubation, bacterial cultures were then inoculated into 100 mL of TSB and incubated with shaking (200 rpm) at 37˚C 24 for h. Overnight cultures consistently yielded a concentration of log 8-9 log CFU/mL, as confirmed by the standard plate count technique).

The 100mL culture was then pelleted by centrifugation (5,000 RPM for 10 minutes at 4˚C). The supernatant was removed, and cells were re-suspended in 100mL of 0.85% sterile bacterial saline (This procedure was repeated twice). Upon final centrifugation, bacterial cells were re-suspended in 100mL of Mott's ®Natural apple juice (samples taken from this step consistently yielded microbial counts of 8-9 log CFU/mL).

Immediately following this step, 10mL samples of the inoculated juice were added to 90mL of the previously-prepared antimicrobial mixtures (1:10 dilution). Samples were then inverted by hand (15 sec) to ensure adequate dispersal of test compounds. The resulting sample was then distributed into four 25-mL test tubes, each containing 20 mL of the prepared juice samples and stored at either 4˚C or 25˚C (two tubes per temperature). Microbial counts were taken at 0, 1, 3, and 7 days of storage.

All samples were diluted with 0.1% peptone water when necessary and spiral plated (Autoplate 4000® spiral plater ; Spiral Biotech, Norwood, MA) onto 10mm TSA plates. All plates were then incubated for 24 hr (37˚C) and enumerated using a ProtoCOL® automated colony counter (Microbiology International, Frederick, MD). All samples were plated in duplicate and the experiment was replicated four times.

pH measurement: The pH of each sample with and without added antimicrobials were determined using an Accument Excel XL 20 pH and conductivity meter (Fisher Scientific, Pittsburgh, PA) on days 0,1, 3 and 7.

Statistical Analysis: Bacterial counts were analyzed using the General Linear Model (GLM) procedure of SAS (V. 9.1.3, Statistical Analysis Systems Institute, Inc. 2006). The factorial complete randomized design (F tests) was utilized to analyze the individual and combined effects of the test compounds on pathogens in the juice. If tests were significant ($P < 0.05$), the levels (types) of main effects (compounds and pathogens) were separated using Fisher's (LSD) tests.

Results

The uninoculated apple juice was free from bacterial and yeast growth, as indicated by an absence of growth on TSA and SDA plates, respectively. Juice was also free of *S.* Typhimurium,

L. monocytogenes, as indicated by an absence of growth on XLD and MOX agars, respectively. The addition of test antimicrobials did not significantly affect the pH of the stored juice (pH=3.54±0.05).

S. Typhimurium and *L. monocytoegens* were inoculated into apple juice at a concentration of 7.0-8.0 log CFU/ml and plated using the Autoplate 4000® spiral plater, which has a minimum limit of detection of 2.0 log CFU/ml. Bacterial counts that were below this limit of detection were assumed to have undergone a 5 log CFU/ ml reduction.

When inoculated into the control samples (commercially pasteurized apple juice, containing no added antimicrobial), *S.* Typhimurium counts decreased by 6 and 3 log CFU/ml following seven days of storage at 4ºC and 25ºC, respectively (Table 1a). Likewise, *L. monocytogenes* counts decreased by 5 and 2 log CFU/ml following 7 days of storage at 4ºC and 25ºC, respectively (Table 1b). While the control samples produced significant reductions in bacterial numbers within the seven day storage period, samples treated with low concentrations of eugenol, *trans*-cinnamaldehyde and *trans*-2-hexenal typically resulted in significantly ($P<0.05$) greater inactivation of the two test pathogens on day 1 or day 3 of storage (compared to bacterial levels in the control samples) (Tables 1-3).

Eugenol (0.05%) treated juice, inoculated with *S.* Typhimurium, resulted in a 5 log CFU/ml reduction in bacterial counts following one day of storage at 4˚C and 25˚C. These counts were both significantly lower than those obtained from the control samples ($P<0.05$) (Table 2a). Likewise, treatment with 0.05% *trans*-2-hexenal, resulted in 5 and ≥7log CFU/ml reductions in bacterial numbers, following one day of storage at 4˚C and 25˚C, respectively (with both treatments being significantly lower than the control sample ($P<0.05$) (Table 3a). While,

treatment with 0.05% *trans*-cinnamaldhyde produced similar patterns of inactivation (≥7 log CFU/ml reduction in bacterial counts, following one day of storage at 4˚C and 25˚C), treatment with 0.025% *trans*-cinnamaldehyde also resulted in a 5 log CFU/ml reduction in bacterial counts, following one day of storage at 25˚C (both concentrations were significantly different from the control sample ($P<0.05$)) (Table 1a).

Eugenol (0.05%) treated juice inoculated with *L. monocytogenes*, resulted in a 6 log CFU/ml reduction in bacterial counts, following one day of storage at 4˚C and 25˚C (both treatments were statistically lower than the control group ($P<0.05$)) (Table 2b). Likewise, treatment with 0.05% *trans*-cinnamaldehyde also resulted in a 6 log CFU/ml reduction in bacterial numbers, following one day of storage at 4˚C and 25˚C (both treatments were statically lower than the control sample ($P<0.05$)) (Table 1b). Similarly, treatment with 0.05% *trans*-2-hexenal, resulted in 4 and ≥7 log CFU/ml reductions in bacterial counts, following one day of storage at 4˚C and 25˚C, respectively (with the later treatment being significantly different from the control sample ($P<0.05$) (Table 3b).

It is important to note, however that treatment with higher concentrations of *trans*-2-hexenal (up to 0.125%) produced slightly antagonistic effects on the level of inactivation of *L. monocytogenes* in the refrigerated samples. Treatment with 0.075% and 0.125% *trans*-2-hexenal resulted in 5 and 4 log CFU/ml reductions in bacterial numbers following 3 days of storage, respectively, which was less than occurred with 0.05%. While these effects were not statistically different than those elicited by 0.05% ($p>0.05$) they do suggest that concentrations of 0.05% *trans*-2-hexenal are potentially more effective at inactivating *L. monocytogenes* in the refrigerated apple juice.

Likewise, 0.05% was the minimum effective concentration for eugenol and *trans*-cinnamaldehyde, which produced 5-log CFU/ml reductions in bacterial numbers, following one day of storage at either temperature. For this reason, antimicrobial combinations (containing *trans*-cinnamaldehyde in combination with *trans*-2-hexenal or eugenol in combination with *trans*-2-hexenal) were evaluated at a total concentration of 0.05% (containing 0.025% of each test compound). Table 4 summarizes the effects of these combinations on the survival of test pathogens in the stored apple juice.

At 4˚C, treatment with 0.025% eugenol + 0.025% *trans*-2-hexenal resulted in a 5 log CFU/ml reduction in *L. monocytogenes* counts following one day of storage; however these counts were not statistically lower than those obtained from the control group ($p>0.05$). When these samples were stored at 25˚C, this treatment produced an 8 log CFU/ml reduction in bacterial counts, which was statistically lower than the control samples ($p<0.05$). Similar patterns of inactivation were seen in samples that were treated with 0.025% *trans*-cinnamaldehyde + 0.025% *trans*-2-hexenal. In this case, storage at 4˚C resulted in a 4 log CFU/ml reduction in bacterial numbers, while storage at 25˚C produced an 8 log CFU/ml reduction in bacterial numbers, with the 25 ˚C treatment being statistically different from the control group ($P<0.05$) (Table 4b).

In the case of *S.* Typhimurium, treatment with 0.025% *trans*-cinnamaldehyde + 0.025% *trans*-2-hexenal resulted in 5 and ≥7 log CFU/ml reductions in bacterial numbers following one day of storage at 4˚C and 25˚C, respectively. Both treatments were statistically different from the control group ($P<0.05$). Similarly, treatment with 0.025% eugenol+ 0.025% *trans*-2-hexenal resulted in 4 and 7 log CFU/ml reductions in bacterial counts following one day of storage at 4˚C

and 25˚C, respectively. Both treatments were statistically different from the control sample ($P<0.05$) (Table 4a).

Discussion

Treatment with various concentrations *trans*-2-hexenal, eugenol and *trans*-cinnamaldehyde and their combinations resulted in significant reductions in *S.* Typhimurium and *L. monocytogenes* populations in the stored apple juice. The magnitude of these reductions was influenced by changes in storage temperature and antimicrobial concentration. The antimicrobials evaluated in this study were typically more effective when applied at higher concentrations and these dose-dependent interactions were consistent with patterns described in the literature. One study, which evaluated the antimicrobial activity of various concentrations of *trans*-cinnamaldehyde against *E. coli* O157:H7 in apple juice, reported that treatment with 0.025%, 0.075% and 0.125% at 23˚C resulted in complete inactivation of the test pathogen, following 5, 3 and 1day of storage respectively. However, it is important to note that this study reported adverse effects on antimicrobial activity for all concentrations, when stored at refrigeration temperatures (with inactivation occurring at 3 and 5 days of storage following treatment with 0.l25% and 0.075%, respectively) (Baskaran et al., 2010).

Likewise, the compounds evaluated in our study also exhibited temperature-dependent interactions; in most cases, samples stored at 25˚C resulted in significant reductions in bacterial numbers (compared to control samples), whereas treatment with similar concentrations did not always result in significant reductions in bacterial numbers when stored at 4˚C. Similar patterns of inhibition have also been reported for apple juice treated with 0.3% cinnamon, where storage at 20˚C resulted in significantly greater levels of in activation of *S.* Typhimurium than samples

stored 5˚C (Yuste and Fung 2003). Similarly, treatment with *trans*-cinnamaldehyde at a concentrations of 0.67% was three times more effective against *Salmonella* enterica, when stored at 37˚C than at 4˚C (Friedman et al., 2004).

These patterns of inactivation are thought to be a result of the organism's decreased metabolism and growth rates during storage at refrigeration temperatures, which decreases its susceptibility to the test compound (Yuste and Fung 2003; Yuste and Fung 2004; Baskaran et al., 2010). In addition, *S.* Typhimurium and *L. monocytogenes* are both capable of decreasing membrane fluidity by an altering the proportion and composition of fatty acids present in the plasma membrane, following exposure to sub-lethal temperatures (Yuste et al., 2002). These mechanisms limit the migration of antimicrobials into the plasma membrane and reduce their effects within the bacterial cell (Yuste and Fung 2003). This phenomenon particularly true for the EO test compounds (eugenol and cinnamaldehyde), which act by disrupting the cytoplasmic membrane of bacterial cells through association with the lipid portion of the cytoplasmic membrane (Gaysinsky et al., 2007; Hemaiswarya and Doble 2009).

While *trans*-2-hexenal also acts by disrupting the cytoplasmic membrane of bacterial cells; it specifically targets the activity of membrane-bound proteins (Kubo et al., 1995; Lanciotti et al., 2003; Neri et al., 2006; Neri et al., 2007). Therefore, its antimicrobial activity is not significantly affected by changes in membrane fluidity. Instead it is assumed that the antimicrobial activity of *trans*-2-hexenal is directly related to its vapor pressure which increases proportional to an increase in temperature (Gardini et al., 2001; Lanciotti et al., 2003).

When the two classes of compounds tested in this study were elevated in combination with one another, similar patterns of inhibition were produced (all treatments were slightly more

effective at 25˚C, than at 4˚C). However, it is assumed that the natural antioxidant properties of the EO compounds further suppressed the activity of the test compounds at refrigeration temperatures (Brink et al., 2000; Lee et al., 2005). This reaction is especially true for eugenol, which has been proven to interact with 6-carbon aldehydes, to prevent their oxidative conversion into hexanoic acid (a compound that leads to off-flavors and decreased shelf life of foods) (Brink et al., 2000; Lee and Shibamotto 2002; Lee et al., 2005). Such interactions explain why eugenol-containing combinations resulted in slightly lower levels of inactivation of the test pathogens when compared to cinnamaldehyde-containing combinations.

Despite their temperature-dependent activity, all combinations containing *trans*-2-hexenal in combination with eugenol or *trans*-cinnamaldehyde resulted in significant reductions in bacterial numbers following one day of storage at 4˚C or 25˚C. These effects were similar to patterns produced by the individual compound. Therefore, these combinations at half the concentration of the individual compound may be used to preserve the microbial quality of fresh apple juice, without altering its sensory properties.

References

Baskaran, S. A., M. A. R. Amalaradjou, et al. (2010). "Inactivation of *Escherichia coli* O157:H7 in apple juice and apple cider by *trans*-cinnamaldehyde." International Journal of Food Microbiology **141**: 126-129.

Brink, G., I. Arends, et al. (2000). "Green, catalytic oxidation of alcohols in water." Science **287**: 1636-1639.

Buchanan, R. L., S. G. Edelson, et al. (1998). "Inactivation of *Escherichia coli* O157:H7 in apple juice by irradiation." Applied and Environmental Microbiology **64**(11): 4553-4535.

Burdock, G. A. (2005). Fenaroli's handbook of flavor ingredients-fifth edition. Boca Raton, FL, CRC Press.

Burt, S. (2004). "Essential oils: their antibacterial properties and potential application in foods-a review." International Journal of Food Microbiology **94**: 223-253.

Choi, L. H. and S. S. Nielsen (2004). "The effects of thermal and nonthermal processing methods on apple cider quality and consumer acceptability." Journal of Food Quality **28**(1): 13-29.

Cody, S. H., K. Glynn, et al. (1999). "An outbreak of *Escherichia coli* O157:H7 infection from unpasteurized commercial apple juice." Annals of Internal Medicine **130**(3): 202-209.

Conway, W. S., B. Leverentzx, et al. (2000). "Survival and growth of *Listeria monocytogenes* on fresh-cut apple slices and its interaction with *Glomerella cingulata* and *Penicillium expansum*." Plant Disease **84**: 177-181.

Corbo, M. R., R. Lanciotti, et al. (2000). "Effects of hexenal, *trans*-2-hexenal, and storage temperature on shelf life of fresh sliced apples." Journal of Agricultural and Food Chemistry **48**: 2401-2408.

Da Costa, N. C. and R.J., Cannon Eds. (2010). Flavors in noncarbonated beverages. Washington, DC, Oxford University Press.

DeRuiter, F. E. and J. Dwyer (2002). "Consumer acceptance of irradiated foods: dawn of a new era?" Food Service Technology **2**: 47-58.

Gardini, F., R. Lanciotti, et al. (2001). "Effect of *trans*-2-hexenal on the growth of *Aspergillus flavus* in relation to its concentration, temperature and water activity." Letters in Applied Microbiology **33**: 50-55.

Gaysinsky, S., T. M. Taylor, et al. (2007). "Antimicrobial efficacy of eugenol microemulsions in milk against *Listeria monocytogenes* and *Escherichia coli* O157:H7." Journal of Food Protection **70**(11): 2631-2637.

Gill, A. O. and R. A. Holley (2004). "Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*." Applied and Environmental Microbiology **70**(10): 5750-5755.

Gould, G. W. (1996). "Industry perspectives on the use of natural antimicrobials and inhibitors for food applications." Journal of Food Protection 1996 Supplement: 82-86.

Hemaiswarya, S. and M. Doble (2009). "Synergistic interaction of eugenol with antibiotics against Gram negative bacteria." Phytomedicine **16**(11): 997-1005.

Hunter, C. (2000). "Changing attitudes to irradiation throughout the food chain." Radiation Physics and Chemistry **57**: 239-243.

Kubo, A., C. S. Lunde, et al. (1995). "Antimicrobial activity of the olive oil flavor compounds." Journal of Agricultural and Food Chemistry **43**: 1629-1633.

Lanciotti, R., N. Belletti, et al. (2003). "Application of hexanal, (E)-2-hexenal, and hexyl acetate to improve safety of fresh-sliced apples." Journal of Agricultural and Food Chemistry **51**: 2958-2963.

Lee, K. and T. Shibamotto (2002). "Determination of antioxidant potential of volatile extracts isolated from various herbs and spices." Journal of Agricultural and Food Chemistry **50**: 4947-4952.

Lee, S., K. Umano, et al. (2005). "Identification of volatile components in basil (*Ocimum basilicum L.*) and thyme leaves (*Thymus vulgaris L.*) and their antioxidant properties." Food Chemistry **91**: 131-137.

Leverentz, B., W. C. Conway, et al. (2006). "Biocontrol of the foodborne pathogens *Listeria monocytogenes* and *Salmonella enterica* Serovar Poona on fresh-cut apples with naturally occurring bacterial and yeast antagonists." Applied and Environmental Microbiology **72**(2): 1135-1140.

Miller, L. G. and C. W. Kasper (1994). "*Escherichia coli* O157:H7 acid tolerance and survival in apple cider." Journal of Food Protection **57**(6): 460-464.

Mosqueda-Melgar, J., R. M. Raybaudi-Massilia, et al. (2008). "Non-thermal pasteurization of fruit juices by combining high-intensity pulsed electric fields with natural antimicrobials." Innovative Food Science and Emerging Technologies **9**: 328-340.

Nayga, R. M., W. Aiew, et al. (2005). "Information effects on consumer's willingness to purchase irradiated food products." Review of Agricultural Economics **27**(1): 37-48.

Neri, F., M. Mari, et al. (2006). "Control of *Penicillium expansum* by plant volatile compounds." Plant Pathology **55**: 100-105.

Neri, F., M. Mari, et al. (2007). "Fungicidal activity of plant volatile compounds for controlling *Monilinia laxa* in stone fruit." Plant Disease **91**(1): 30-35.

Pei, R., F. Zhou, et al. (2009). "Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. coli* with an improved method." Journal of Food Science **74**(7): M379-M383.

Rahman, M. S. (1999). Handbook of food preservation. New York, NY, Marcel Dekker.

Roering, A. M., J. B. Luchansky, et al. (1999). "Comparative survival of *Salmonella* Typhimurium DT104, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in preservative-free apple cider and simulated gastric fluid." International Journal of Food Microbiology **46**(263-269): 263.

Sapers, G. M., J. R. Gorny, et al. Eds. (2006). Microbiology of fruits and vegetables. Boca Raton, FL, Taylor and Francis Group.

Tajchakavit, S., H. S. Ramaswamy, et al. (1998). "Enhanced destruction of spoilage microorganisms in apple juice during continuous flow microwave heating." Food Research International **31**(10): 713-722.

Truong, V. Y. T., R. R. Boyer, et al. (2010). "Effect of a-cyclodextrin-cinnamic acid inclusion complexes on populations of *Escherichia coli* O157:H7 and *Salmonella enterica* in fruit juices." Journal of Food Protection **73**(1): 92-96.

Uljas, H., E. and S. C. Ingham (1999). "Combinations of intervention treatments resulting in 5-log10-unit reductions in numbers of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium DT104 organisms in apple cider." Applied and Environmental Microbiology **65**(5): 1924-1929.

Valero, M. and M. J. Giner (2006). "Effects of antimicrobial components of essential oils on growth of *Bacillus cereus* INRA L2104 in and the sensory qualities of carrot broth." International Journal of Food Microbiology **106**: 90-94.

Visser, J. H., S. V. Straten, et al. (1979). "Isolation and identification of volatiles in the foliage of potato, *Solannum tuberosum*, a host plant of the Colorado beetle." Journal of Chemical Ecology **5**(1): 13-25.

Williams, R. C., S. S. Sumner, et al. (2005). "Inactivation of *Escherichia coli* O157:H7 and *Salmonella* in apple cider and orange juice treated with combinations of ozone, dimethyl dicarbonate, and hydrogen peroxide." Journal of Food Science **70**(4): M197-M201.

Yuste, J. and D. Y. C. Fung (2003). "Evaluation of *Salmonella* Typhimurium, *Yersinia enterocolitica* and *Staphylococcus aureus* counts in apple juice with cinnamon, by conventional media and thin agar layer method." Food Microbiology **20**: 365-370.

Yuste, J. and D. Y. C. Fung (2004). "Inactivation of *Salmonella* Typhimurium and *Escherichia coli* O157:H7 in apple juice by a combination of nisin and cinnamon." Journal of Food Protection **67**(2): 371-377.

Yuste, J., D. Y. C. Fung, et al. (2002). "Combination of carbon dioxide and cinnamon to inactivate Escherichia coli O157:H7 in apple juice." Journal of Food Science **67**(8): 3087-3090.

Zhao, T., M. P. Doyle, et al. (1993). "Fate of enterohemorrhagic *Escherishia coli* O157:H7 in apple cider with and without preservatives." Applied and Environmental Microbiology **59**(8): 2526-2530.

Zhou, F., B. Ji, et al. (2007). "The antibacterial effect of cinnamaldehyde, thymol, carvacrol and their combinations against the foodborne pathogen *Salmonella* Typhimurium." Journal of Food Safety **27**(2): 124-133.

Zink, D. L. (1997). "The impact of consumer demands and trends on food processing." Emerging Infectious Disease **3**(4): 467-469.

Tables

Table 1. Antimicrobial effects of various concentrations of *trans*-cinnamaldehyde on the survival of (a) *S.* Typhumirium and (b) L. monocytogenes in pasteurized apple juice (stored at 4˚C AND 25˚C).

a.)

	Initial Count (log CFU/mL)	Day 1	Day 3	Day 7
4˚C				
Control	7.35±0.26^{a}	5.04±0.52^{a}	4.24±0.65^{a}	1.53±0.63^{a}
0.025%	7.40±0.20^{a}	4.25±0.23^{b}	1.75±0.61^{b}	ND^{b}
0.050%	7.63±0.06^{a}	ND^{c}	0.71±0.27^{b,c}	ND^{b}
0.075%	6.90±0.40^{a}	0.12±0.12^{c}	ND^{c}	ND^{b}
0.125%	5.92±0.31^{b}	ND^{c}	ND^{c}	ND^{b}
25˚C				
Control	7.35±0.26^{x}	4.55±0.67^{x}	5.42±0.29^{x}	5.41±0.03^{x}
0.025%	7.40±0.20^{x}	1.87±0.96^{y}	1.12±0.91^{y}	ND^{y}
0.050%	7.63±0.60^{x}	ND^{z}	0.81±0.28^{y}	ND^{y}
0.075%	6.90±0.40^{x}	0.33±0.33^{y,z}	ND^{y}	ND^{y}
0.125%	5.92±0.31^{y}	0.29±0.29^{y,z}	ND^{y}	ND^{y}

b.)

	Initial Count (log CFU/mL)	Day 1	Day 3	Day 7
4˚C				
Control	7.34±0.77^{a}	4.61±0.58^{a}	3.71±0.36^{a}	2.65±0.26^{a}
0.025%	6.79±0.81^{a}	4.25±0.73^{a}	3.26±0.64^{a,b}	2.20±0.53^{a}
0.050%	7.94±0.04^{a}	2.39±0.23^{b}	0.63±0.23^{d}	ND^{b}
0.075%	6.54±0.75^{a}	2.82±0.33^{b}	2.22±0.01^{b,c}	2.62±0.18^{a}
0.125%	6.52±0.67^{a}	ND^{c}	1.81±0.74^{c,d}	ND^{b}
25°C				
Control	7.34±0.77^{x}	3.94±0.29^{x}	5.35±0.30^{x}	5.36±0.10^{x}
0.025%	6.79±0.81^{x}	3.26±0.33^{x,y}	2.55±0.27^{y}	0.65±0.65^{y}
0.050%	7.94±0.04^{x}	2.33±0.25^{y}	0.70±0.10^{y}	ND^{y}
0.075%	6.54±0.75^{x}	0.65±0.53^{z}	1.13±1.13^{y}	1.15±0.15^{y}
0.125%	6.52±0.67^{x}	ND^{z}	1.30±1.30^{y}	ND^{y}

ND; Not Detectable

$^{a-d}$ Means with the same letter are not significantly different (P<0.05)

$^{x-z}$ Means with the same letter are not significantly different (P<0.05)

Table 2. Antimicrobial effects of various concentrations of eugenol on the survival of (a) *S.* Typhumirium and (b) *L. monocytogenes* in pasteurized apple juice (stored at 4˚C AND 25˚C)

a.)

	Initial Count (log CFU/mL)	Day 1	Day 3	Day 7
4˚C				
Control	7.35±0.26^{a}	5.04±0.52^{a}	4.24±0.65^{a}	1.53±0.63^{a}
0.025%	7.00±0.30^{a}	4.42±0.45^{a}	2.79±1.02^{a}	0.48±0.28^{b}
0.05%	6.63±0.46^{a}	1.78±0. 22^{b}	0.87±0.27^{b}	ND^{b}
0.075%	4.18±0.30^{b}	0.12±0.12^{c}	ND^{b}	ND^{b}
0.125%	4.21±0.59^{b}	ND^{c}	ND^{b}	ND^{b}
25˚C				
Control	7.35±0.26^{x}	4.55±0.67^{x}	5.42±0.29^{x}	5.41±0.03^{x}
0.025%	7.00±0.30^{x}	4.04±0.60^{x}	2.40±1.33^{y}	ND^{y}
0.05%	6.63±0.46^{x}	1.97±1.00^{y}	1.02±0.17^{y,z}	ND^{y}
0.075%	4.18±0.30^{y}	ND^{y}	ND^{z}	ND^{y}
0.125%	4.21±0.59^{y}	ND^{y}	ND^{z}	ND^{y}

b.)

	Initial Count (log CFU/mL)	Day 1	Day 3	Day 7
4˚C				
Control	7.34±0.77^{a}	4.61±0.58^{a}	3.71±0.36^{a}	2.65±0.26^{a}
0.025%	7.23±0.64^{a}	4.86±0.43^{a}	3.05±0.49^{a}	1.62±0.81^{b}
0.05%	8.09±0.04^{a}	1.83±0.62^{b}	0.97±0.13^{b,c}	ND^{c}
0.075%	6.61±0.78^{a}	ND^{c}	1.59±0.86^{b}	ND^{c}
0.125%	7.520.05^{a}	ND^{c}	ND^{c}	ND^{c}
25°C				
Control	7.34±0.77^{x}	3.94±0.29^{x}	5.35±0.30^{x}	5.36±0.10^{x}
0.025%	7.23±0.64^{x}	3.70±0.62^{x}	2.55±0.28^{y}	ND^{y}
0.05%	8.09±0.04^{x}	2.07±0.32^{y}	0.24±0.18^{z}	ND^{y}
0.075%	6.61±0.78^{x}	ND^{z}	ND^{z}	ND^{y}
0.125%	7.52±0.05^{x}	ND^{z}	ND^{z}	ND^{y}

ND, Not Detectable

$^{a-c}$ Means with the same letter are not significantly different (P<0.05)

$^{x-z}$ Means with the same letter are not significantly different (P<0.05)

Table 3. Antimicrobial effects of various concentrations of *trans*-2-hexenal on the survival of (a) *S.* Typhumirium and (b) *L. monocytogenes* in pasteurized apple juice (stored at 4˚C and 25˚C)

a.)

	Initial Count (log CFU/mL)	Day 1	Day 3	Day 7
4˚C				
Control	7.35±0.26^{a}	5.04±0.52^{a}	4.24±0.65^{a}	1.53±0.63^{a}
0.025%	6.93±0.36^{a}	3.95±0.67^{a,b}	2.28±0.76^{b}	0.15±0.15^{b}
0.05%	7.53±0.14^{a}	2.40±0.48^{b,c}	1.19±0.27^{b,c}	ND^{b}
0.075%	7.59±0.09^{a}	1.68±0.97^{c,d}	ND^{c}	0.15±0.15^{b}
0.125%	7.220.34^{a}	ND^{d}	ND^{c}	ND^{b}
25˚C				
Control	7.35±0.26^{x}	4.55±0.67^{x}	5.42±0.29^{x}	5.41±0.03^{x}
0.025%	6.93±0.36^{x}	2.36±1.18^{y}	0.94±0.94^{y}	1.26±0.96^{y}
0.05%	7.53±0.14^{x}	ND^{z}	0.72±0.44^{y}	ND^{y}
0.075%	7.59±0.09^{x}	0.37±0.20^{z}	0.57±0.47^{y}	1.51±0.09^{y}
0.125%	7.220.34^{x}	ND^{z}	ND^{y}	ND^{y}

b.)

	Initial Count (log CFU/mL)	Day 1	Day 3	Day 7
4˚C				
Control	7.34±0.77^{a}	4.61±0.58^{a}	3.71±0.36^{a}	2.65±0.26^{a}
0.025%	7.10±0.74^{a}	5.08±0.59^{a}	2.70±0.41^{a,b}	0.96±0.63^{b}
0.05%	8.19±0.12^{a}	4.80±0.40^{a}	1.71±0.53^{b}	ND^{b}
0.075%	6.54±0.78^{a}	3.89±0.74^{a,b}	1.95±0.55^{b}	ND^{b}
0.125%	6.82±0.74^{a}	2.27±0.59^{b}	2.75±0.28^{a,b}	ND^{b}
25˚C				
Control	7.34±0.77^{x}	3.94±0.29^{x}	5.35±0.30^{x}	5.36±0.10^{x}
0.025%	7.10±0.74^{x}	3.33±0.29^{x}	2.59±0.29^{y}	ND^{y}
0.05%	8.19±0.12^{x}	ND^{z}	1.01±0.20^{z}	ND^{y}
0.075%	6.54±0.78^{x}	2.33±0.12^{y}	0.53±0.87^{z}	ND^{y}
0.125%	6.82±0.74^{x}	ND^{z}	0.55±0.64^{z}	ND^{y}

ND, Not Detectable

$^{a-d}$ Means with the same letter are not significantly different (P<0.05)

$^{x-z}$ Means with the same letter are not significantly different (P<0.05)

Table 4. Antimicrobial effects of essential oil compounds in combination with *trans*-2-hexenal on the survival of (a) *S.* Typhimurium and (b) *L. monocytogenes* in pasteurized apple juice (stored at 4°C and 25°C)

a.)

	Initial Count (log CFU/mL)	Day 1	Day 3	Day 7
4°C				
Control	7.35±0.26^{a}	5.04±0.52^{a}	4.24±0.65^{a}	1.53±0.63^{a}
Eug+Hex (0.025%)	7.72±0.05^{a}	3.34±0.47^{b}	1.42±0.56^{b}	0.82±0.71^{a}
Cin+Hex (0.025%)	7.76±0.05^{a}	2.13±0.82^{b}	0.87±0.38^{b}	0.80±0.69^{a}
25°C				
Control	7.35±0.26^{x}	4.55±0.67^{x}	5.42±0.29^{x}	5.41±0.33^{x}
Eug+Hex (0.025%)	7.72±0.05^{x}	0.46±0.46^{y}	ND^{y}	ND^{y}
Cin+Hex (0.025%)	7.76±0.05^{x}	ND^{y}	ND^{y}	ND^{y}

b.)

	Initial Count (log CFU/mL)	Day 1	Day 3	Day 7
4°C				
Control	7.34±0.77^{a}	4.61±0.58^{a}	3.71±0.36^{a}	2.65±0.26^{a}
Eug+Hex (0.025%)	8.02±0.09^{a}	3.30±0.24^{a}	1.39±0.64^{b}	2.45±0.08^{a}
Cin+Hex (0.025%)	8.13±0.03^{a}	4.23±0.34^{a}	0.82±0.52^{b}	2.48±0.16^{a}
25°C				
Control	7.34±0.77^{x}	3.94±0.29^{x}	5.35±0.30^{x}	5.36±0.10^{x}
Eug+Hex (0.025%)	8.02±0.09^{x}	0.72±0.43^{y}	ND^{y}	ND^{y}
Cin+Hex (0.025%)	8.13±0.03^{x}	0.40±0.40^{y}	ND^{y}	ND^{y}

ND(Not Detectable); Eug (Eugenol), Cin (*trans*-cinnamaldehyde), Hex (*trans*-2-hexenal)

$^{a-b}$ Means with the same letter are not significantly different (P<0.05)

$^{x-z}$ Means with the same letter are not significantly different (P<0.05)

CHAPTER 6

Conclusions and Future Directions

The antimicrobial properties of essential oil compounds have been recognized for years. While several studies have demonstrated the effectiveness of these compounds against foodborne pathogens in apple juice, the concentrations used in these studies often exceeds consumer acceptable limits. Therefore, the purpose of this research was to evaluate the antimicrobial activity of three essential oil compounds (thymol, eugenol and *trans*-cinnamaldehyde) alone and in combination with three naturally-occurring apple flavorants (hexanal, *trans*-2-hexenal and 1-hexanol) in order to identify synergistic relationships that lower the effective concentrations needed to inactivate foodborne pathogens in apple juice.

This research confirmed that the essential oil compounds were more effective at inhibiting the growth of *L. monocytogenes*, *S. aureus* and *Salmonella* Typhimurium than the apple aroma compounds. It was also revealed that hexanal (a compound previously proven to exhibit strong antimicrobial properties) exhibited little to no inhibitory effects against these three pathogens. The most interesting finding, however, was that eugenol (at concentrations of 0.8 and 1.6 mg/mL) was the only essential oil compound that produced synergistic relationships in combination with the apple aroma compounds.

When compounds were applied to an apple juice matrix, 0.05% of each compound (or combinations containing 0.025% of *trans*-2-hexenal in combination with eugenol or *trans*-cinnamaldehyde) were capable of reducing *L. monocytogenes* and *S.* Typhimurium populations by 5 log CFU/ml within one day of storage at 4°C or 25°C. These results suggest that *trans*-2-hexenal can be used to lower the concentration of essential oils compounds needed to inactivate

pathogens in apple juice. This research provides new insight into the use of essential oil compounds for the preservation of unprocessed apple juice; however, several factors must be addressed before these treatments can be adopted by the fruit juice industry.

First, it is important to determine the effectiveness of these treatments against *E. coli* O157:H7 in an apple juice matrix. Since this microorganism has caused a number of large-scale apple juice-related outbreaks, it is typically regarded as the primary organism of concern to the apple juice industry. Therefore, alternative juice processing technologies must demonstrate a 5 log CFU/ml reduction in *E. coli* O157:H7 populations prior to gaining acceptance within the apple juice industry and approval by the U.S. FDA.

Likewise, this research can also be expanded to include a number of spoilage and pathogenic fungi such as *Zygosaccharomyces bailii*, *Saccharomyces cerevisiae* , etc. These organisms are capable of growing in apple juice, especially when stored at room temperature, and elicit a number of adverse organoleptic effects, which significantly reduce shelf-life. For this reason, it may be of interest to identify concentrations that extend the lag phase of these organisms, thus producing a shelf-stable product.

When an effective concentration is established (for both pathogenic bacteria and spoilage fungi), it is important to determine the consumer acceptability of these products. Because cinnamaldehyde and eugenol are both extremely pungent flavor compounds, they may impart off-flavors in juices, even when applied at low concentrations. Therefore, it is important to determine if these treatments are detectable and/or acceptable within an apple juice matrix. In this case, it is important to note that cinnamon (from which cinnamaldehyde is derived) and clove (from which eugenol is derived) are commonly used to season spiced apple cider.

Therefore, some consumers may find these compounds to be acceptable within a typical apple juice matrix.