

ABSTRACT

Title of Document: INTERVENTION STRATEGIES FOR
ESCHERICHIA COLI O157:H7 AND
SALMONELLA IN ORGANIC SOIL AND ON
FRESH PRODUCE

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Philosophy, 2012

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Recently, foodborne diseases caused by *Escherichia coli* O157:H7 and *Salmonella* have been increasingly associated with the consumption of fresh produce. Consumers' demand for safe, natural products has led to research on natural antimicrobials for effective control of foodborne pathogens on fresh produce, which can be inadvertently contaminated by soil. Therefore, there is a need to control microbial loads in soil to minimize contamination. The objectives of this study were to evaluate the antimicrobial activity of cinnamaldehyde, Ecotrol®, eugenol, Sporan® and acetic acid against *E. coli* O157:H7 and *Salmonella* in organic soil, and to evaluate the antimicrobial effects of cinnamaldehyde and Sporan® alone, or in combination with acetic acid against *E. coli* O157:H7, *Salmonella*, and the native microflora of iceberg, romaine and spinach leaves. The quality parameters of the

treated fresh produce were monitored, whereas the modes of action of cinnamaldehyde and Sporan® were investigated.

The results showed that cinnamaldehyde had the highest bactericidal activity against *E. coli* O157:H7 and *Salmonella* in organic soil. Increases in oil concentration resulted in further reduction of both microorganisms. Up to 5 and 6 log CFU/g of *E. coli* O157:H7 and *Salmonella*, respectively, were reduced with 2% Sporan® and acetic acid after 24 h. Sporan® in combination with acetic acid (1000SV) and 800 ppm cinnamaldehyde-Tween reduced significantly *E. coli* O157:H7 (~3 log CFU/g) on iceberg and spinach leaves following treatment at day 0. Likewise, 1000SV treatment reduced *Salmonella* ~ 2.5 log CFU/g at day 0. *E. coli* O157:H7 and *Salmonella* populations in treated iceberg, spinach and romaine leaves were reduced during storage at 4°C.

The native microflora of untreated and treated spinach and lettuce leaves increased during the storage time. The texture and the color of iceberg, romaine and spinach leaves treated with essential oils were not significantly different from the control lettuce after 14 days.

The scanning and transmission electron microscopy of oil-treated bacterial cells indicated possible cell structural damage and leakage of cellular content.

This study shows the potential use of essential oils to effectively reduce *E. coli* O157:H7 and *Salmonella* populations in soil and on fresh produce without adversely affecting leaf color and texture.

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SALMONELLA IN ORGANIC SOIL AND ON FRESH PRODUCE

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2012

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Acknowledgements

First of all, I would like to express my sincere gratitude and appreciation to Dr. Y. Martin Lo, my advisor, for his guidance, encouragement and support throughout this endeavor. He is a great source of inspiration for me. Also, I would like to thank members of my committee, Dr. J. Patel, and Dr. P. Millner, for their helpful insight and comments, Dr. M. Parish, Dr. M. Kantor, Dr. A. Pradhan and Dr. N. Tablante (Dean's Representative) for their help and service on the advisory committee.

Special thanks to my current and former peers Pavan Soma, Eric Thornber, Melody Ge, Michael Wiederoder, Patrick Williams, Nancy Tong, Yuting Zhou, Arpita Mondal, Ansu Cherian, Avani, Sanghvi, Ana Aguado, and Sarah Yachetti for their encouragement and thoughtful questions. I thank specially Eric Thornber, Melody Ge and Pavan Soma for their help and time. Also, I would like to thank all of the family of Food Science faculty for their support and friendship.

My special gratitude goes to the United States Department of Agriculture, Beltsville Agricultural Research Center for financial support. I take this opportunity to thank Dr. Moon Kim, Dr. Conway, Mrs. Lavonda Holler, Euhnee Park, Katherine Darlington, Cheryl Roberts, Paroo Chauchan, Dr. David Ingram, Sean Ferguson and Jay Patel for their encouragement and friendship. Special thanks go to Mr. Gary Bauchan, Mrs Lavonda Holler, Mr. Charlie Murphy and Eunhee Park for technical assistance and Dr. Bryan Vinyard for statistical support.

I thank my family, friends, and siblings for their support, love and encouragement. I thank my husband Telesphore Tchagou, my children Carl Christopher and Kathleen

Telia Tchagou to have bear patience with me and given me the strength to pursue this dream.

Finally, I would like to thank my heavenly Father to have caused me to be at the right place, at the right time with right people to fulfill this work.

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Chapter 1: Introduction

Fruit and vegetables are key components of a healthy diet. They are low fat energy-dense foods, relatively rich in vitamins, minerals, phytonutrients, and other bioactive compounds, as well as a good source of fiber. Despite the nutritional and health benefits of fresh produce, fruit and vegetables are recently recognized as vehicles for foodborne illness in humans. The consumption of fresh produce has now been linked, both epidemiologically and microbiologically, to infectious intestinal disease (CFR FVFCR, 2007). Contamination of fresh produce with enteric pathogens may occur in the field during production, harvesting, and post-harvest processing or at any point from farm to fork. At the field stage many possibilities for contamination (Fig. 1.1.) exist in the environment, which include wildlife intrusion, animal manure, soil amendments, water, and cross-contamination from unsanitized equipment or workers (Beuchat and Ryu, 1997). At present it is unclear to what extent each of the potential sources actually has been involved in known foodborne illness outbreaks as the trace back investigations have not provided a complete resolution of these factors (De Roeve, 1999; Doyle and Erickson, 2008). Environmental factors contributing to contamination of fresh produce have been the subject of considerable research to determine the survival, transport, and fate of major pathogens involved in the recent outbreaks and to very limited extent potential interventions that would reduce or eliminate contamination (Beuchat, 1998).

The major existing interventions involve the use of Good Agricultural Practices (GAPs) at the production level and current Good Manufacturing Practices (cGMPs) at the processing and distribution level (FDA, 1998). Yet, in spite of these interventions, outbreaks still occur. The low infectious dose of some pathogenic bacteria, the limited

effect of many approved sanitizers on produce surface, the resistance to cleaning and disinfection of bacteria in biofilms, and the limitations that outbreak investigators have in tracking a contamination event to a specific location and source remain challenging (Sapers and Doyle, 2009). The interest in the use of essential oils as natural antimicrobials against foodborne pathogens in the food industry has significantly increased (Burt, 2004). However, their uses against *E. coli* O157:H7 and *Salmonella* in organic soil were still unknown. Therefore, effective interventions that can influence improvements in the microbiological safety of fresh produce are needed.

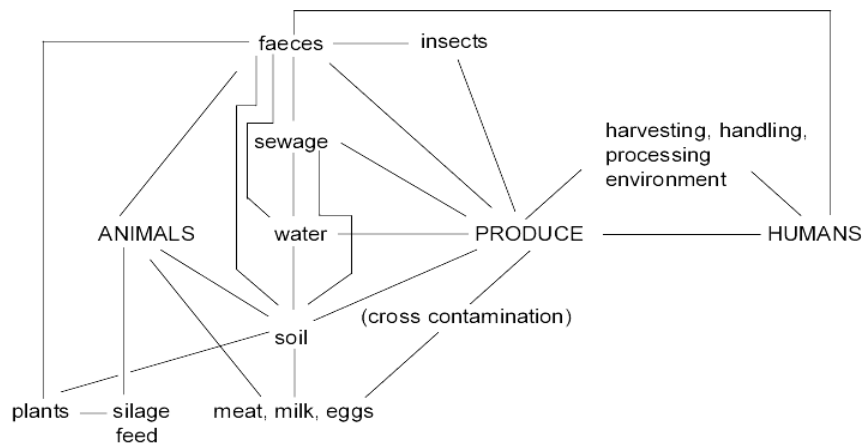


Figure 1.1: Mechanisms by which raw fruits and vegetables may become contaminated with pathogenic microorganisms (Adapted from Beuchat, 1998).

Chapter 2: Literature Review

2.1 *Escherichia coli* O157:H7

Escherichia coli O157:H7 is a Gram-negative, facultative anaerobic rod bacterium representing a significant and widespread environmental health hazard (Brabban et al., 2004). *E. coli* O157:H7 was first isolated from a California woman with bloody diarrhea in 1975 (Doyle et al., 1997), and was first identified as a human pathogen in 1982 associated with outbreaks of bloody diarrhea in Oregon and Michigan, U.S.A (Riley et al., 1983; Wells et al., 1983), and is also linked to sporadic cases of hemolytic uremic syndrome (HUS) in 1983 (Karmali et al., 1983). *E. coli* O157:H7, an enterohemorrhagic (EHEC) strain of *E. coli* are defined as pathogenic *E. coli* strains that produce Shiga toxins (Stxs) and cause hemorrhagic colitis (HC) and the life-threatening HUS in humans (JiYoun et al., 2010). *E. coli* O157:H7 expresses somatic O antigen 157, which is the component of the lipopolysaccharide portion of the cell membrane, and flagella H antigen 7. Most of the virulent factors of *E. coli* O157:H7 are found on the O pathogenicity islands (Spears et al., 2006). Human infection cause by *E. coli* O157:H7 can present a broad clinical spectrum ranging from asymptomatic cases to death. Most cases initiate with non bloody diarrhea and self –resolve without further complication. However, some patients progress to bloody diarrhea or HC in 1-3 days. In 5-10% of HC patients, the disease can progress to the life-threatening HUS or thrombocytopenic purpura (TTP) (Banatvala et al., 2001). The infective dose of *E. coli* O157:H7 could be as low as 10 colony forming units (CFU) needed to cause disease (Chart, 2000; Williams et al., 2006). All age groups are susceptible to infection, but children, elderly and immuno-

compromised people are the most vulnerable to *E. coli* O157:H7 infection and could develop severe illness.

Globally the largest ever reported outbreak of *E. coli* O157:H7 occurred in Japan in 1996 and was linked to the consumption of raw radish sprouts served in school lunches. The total of 6,000 people were affected with three deaths reported (SCF, 2002). In the USA, several *E. coli* O157:H7 infections have been epidemiologically linked to the consumption of lettuce. In 1995, contamination with irrigation water or unsanitary handling of leafy greens were the likely causes of an outbreak associated with lettuce, whereas cross-contamination from meat products was considered the cause of another outbreak involving Iceberg lettuce. Bovine and avian fecal contamination was also considered a potential factor in two outbreaks in 1996 involving mesclun mix lettuce (O'Brien et al., 2000). In September 2006, tainted pre-packaged spinach triggered an *E. coli* O157:H7 outbreak that resulted in five deaths and 205 illnesses. The *E. coli* O157:H7 strain involved in the 2006 spinach outbreak was identified from cattle and wild pig feces (Douglas et al., 2008). In November and December 2006 two additional outbreaks were reported with leafy greens, this time involving iceberg lettuce served in Taco John and Taco Bell lettuce from different suppliers. Both outbreaks were caused by *E. coli* O157:H7 and sickened a combined total of over 150 people (CSPI, 2008). Moreover, *E. coli* O157:H7 inoculated into manure added to planting soil contaminated and survived on lettuce plants grown in that soil. The pathogen was detected within the plant tissues at a soil depth of up to 45 mm (Solomon et al., 2002). Jablasone et al. (2005) reported that *E. coli* O157:H7 was internalized in cress, lettuce, radish and spinach seedling that has been contaminated as seeds, although the cells did not remain internalized in mature

plants. It has been demonstrated that *E. coli* O157:H7 undergoes significant physiological changes during stationary phase, hence capable of adapting to stressful or extreme environmental conditions (Nystrom, 1995; Chung et al., 2006).

2.2 *Salmonella*

Salmonella is a genus name for a group of Gram-negative, non-spore forming facultative rod-shaped bacteria that are members of the family Enterobacteriaceae, trivially known as “enteric” bacteria. *Salmonella* was first isolated by Theobald Smith in 1885 from pigs. The genus name *Salmonella* was derived from the last name of D.E. Salmon, who was Smith’s director (Davis, 2009). In the Approved Lists of Bacterial Names *Salmonella* (S.) includes five species, *S. arizonae*, *S. choleraesuis* (type species of the genus), *S. enteritidis*, *S. typhi* and *S. typhimurium*. *Salmonella typhi* is the cause of typhoid fever, whereas *Salmonella choleraesuis* is primarily a pathogen of swine that occasionally causes systemic infections in humans. *Salmonella enteritidis*, on the other hand, is a common cause of diarrheal infections in humans and animals (Salyers and Whitt, 2001) of which there are a large number (over 2,500) of serotypes of bacteria which are potentially pathogenic and are identified based on its specific protein coating (Poppoff, 2001; Ekdahl et al., 2005).

Salmonella is one of the most important foodborne diseases and causes substantial medical and economic burdens worldwide (De Jong et al., 2006; Voetsch et al., 2004). In the USA, *Salmonella* is responsible of 1.4 million non-typhoidal illnesses annually including 40,000 confirmed cases and 400 deaths (Voetsch et al., 2004). In addition, an estimated 12 to 33 million cases of typhoid fever, a more serious and fatal form of salmonellosis, occurs globally each year and is endemic in many countries of the Indian

subcontinent, South and Central America, and Africa (Klotcho and Wallace, 2009). *Salmonella* is generally associated with consumption of uncooked or undercooked meat, poultry, swine, eggs, or unpasteurized dairy products. It is also found in environmental sources such as water, soil, insects, processing surfaces, and animal feces. However, according to an analysis of food-poisoning outbreaks by the Center for Science in the Public Interest (CSPI, 2009), fresh produce is catching up with chicken as a major culprit of *Salmonella* infections. Produce-related outbreaks tend to be larger than poultry-related outbreaks, and sicken more people, sometimes hundreds at a time. A recent survey from 1996 to 2007 estimated that approximately 33 outbreaks were associated with *Salmonella*-contaminated fruits and vegetables (Callaway, 2008). In recent years, *Salmonella* outbreaks have been traced back to green onions, lettuce, spinach, cantaloupes, tomatoes, cabbage, strawberries, raspberries (Beuchat, 1996), alfalfa, and sprouts, (Shin , 2006), and peanuts (CDC, 2009), as well as salads, melons, and other fruit- and vegetable-containing dishes (CSPI, 2009). In 2008, jalapeno and Serrano pepper with contaminated *Salmonella* St. Paul outbreak was the largest foodborne outbreak which infected 1400 people in 43 states (CDC, 2008). The peppers were received from farms in Mexico, and the FDA investigations traced back the source from one of the farm in Tamaulipas, Mexico (CDC, 2008). Little is known about the survival and growth characteristics of *Salmonella* on these peppers although rapid growth in Jalapeno pepper extract has been reported (Nutt et al., 2003). In February 2009, 235 persons from 14 states were infected with the outbreak strain of *Salmonella* St. Paul linked with the ingestion of contaminated raw alfalfa sprout (Marler, 2010) and in 2005, Ontario was implicated to the most high profile salmonellosis outbreak linked to mung

bean sprout, which resulted in over 600 reported cases (Ye et al., 2010). It is generally recognized that the seed used to prepare sprouts is the primary source of pathogens (Gill et al., 2003; Montville and Shaffner, 2005; Winthrop et al., 2003). Studies have shown that, once the seed is contaminated even at low levels (0.1 log CFU/g) the pathogens can grow rapidly under the warm (20 to 30°C) and humid conditions used in sprout production (Liu et al., 2007; Fu et al., 2008), internalized into developing sprout, and cannot be removed by postharvest washing (Warriner et al., 2003). In 2009, peanut butter and peanut-containing products took the center stage as the largest recall of human food items in the U.S., resulting in over 2,100 products being voluntarily recalled by more than 200 companies. Peanut products contaminated with *Salmonella* Typhimurium caused 714 illnesses and 9 deaths in 46 states. Epidemiologic and laboratory findings indicated that peanut butter and peanut paste produced at on plant were the source of the outbreak (CDC, 2009). This was the second outbreak caused by contaminated peanut butter in the U.S. The first outbreak of contaminated peanut was caused by *Salmonella* Tennessee during 2006-2007 (CDC, 2007). It has been demonstrated that *Salmonella* persist in high fat, low-water-activity foods such as peanut butter (Mattick et al., 2001), and in such foods, *Salmonella* can withstand temperature as high as 90°C for 50 minutes (Shachar and Yaron, 2006). Although contamination of leafy greens with *Salmonella* enterica has not been reported in the U.S. (Barak et al., 2008), over 350 people in England, Northern Ireland, Scotland and the Isle of Man were affected by *Salmonella* Newport in 2004 as a result of the consumption of contaminated Iceberg lettuce (Everis, 2004). Barak et al. (2008) reported that lettuce and tomato have a poor natural attachment compared to other agricultural crops and the *Salmonella* contamination of these crops is not the result of a

pre-harvest contamination via soil. Nevertheless, the way to avoid any foodborne pathogen illnesses is to take effective preventive measures during growing, harvesting, and post-harvest handling.

2.3 Current interventions to control contamination in soil during production stage

There are a number of routes by which contamination can occur on fresh produce. The overall goal for field production systems is to implement a series of practices that will contribute to controlling contamination from workers, wildlife, water, soil, amendments, equipment, and other processing chain sources. Once contamination occurs it is difficult to trace back the source, therefore, in 1998, the U.S. Food and Drug Administration (FDA) issued guidelines to minimize microbial contamination of fresh fruits and vegetables (Powell et al., 2008). These voluntary guidelines refer to the control of hazards associated with fruit and vegetable production. In addition, USDA supported development, publication, and education of “Good Agricultural Practices” (GAPs), another set of general guidance practices, that explained in detail relevant practices to reduce microbial contamination hazards with the production and handling environment of fresh fruit and vegetables. After the spinach outbreak with *E. coli* O157:H7 in 2006, the leafy green industry in California and Arizona collectively developed standards with metrics for specific practices and procedures for growers, shippers, packers, and processors involved with various aspects of the production chain, including land history, adjacent land use, water quality, worker hygiene, pesticide and fertilizer use, equipment sanitation, and product transportation (WGA, 2009).

Recently, the USDA-Agricultural Marketing Service (AMS) proposed a federal standard for leafy greens which includes most of the procedures and practices described in the

WGA, 2007 metrics. This proposal is still pending and many comments have been received by the USDA-AMS regarding the proposed rule and its effect on small as well as large producers and processors.

2.3.1 Site selection

Prevention of microbial contamination is preferred to corrective actions after contamination has occurred. Therefore, part of the solution to reducing the risk of contamination can come from avoiding use of cropland that has a history of exposure to microbiological contamination, particularly from intentional recent application of raw manure or close proximity to animal production/housing facilities, or manure, wastewater, and sewage handling and treatment works. Land previously used for animal husbandry may have a high risk of produce contamination because animal manure can introduce disease agents that can survive for months or years in soil. Moreover, to protect fields from inadvertent contamination from animal manure disease agents, cropland should be located away from animal feedlots and definitely avoid slopes that would allow intrusion of runoff water from grazing lands to the fresh produce fields (Rangarajan et al., 2000).

Proximity of high densities of cattle shedding *E. coli* O157:H7 VTEC strains has been highly associated with increased reports of human VTEC cases when humans live in agricultural (rural) regions near the cattle operations or when humans consume contaminated well water or food products produced in regions with the VTEC shedding livestock (Michel et al., 1999; Frank et al., 2008; Strachan et al., 2006). *Salmonella* for example is known to survive more than 968 days in soil (Jones, 1986). Large multi-state outbreaks, such as the *E. coli* O157:H7 outbreak in freshly bagged spinach in September 2006, have occurred (Cooley et al., 2007; Jay et al., 2007) mainly due to river water,

cattle feces, and wild pig feces. In the state of New York in September 1999 the biggest reported outbreak of *E. coli* O157:H7 occurred at a fairground, which included approximately 800 suspected cases. This event was associated with infected well water (CDCa, 2009). A drought followed by an extraordinarily heavy amount of rainfall, were both associated with this large outbreak (Patz et al., 2000). In a 10-year summary of *E. coli* O157:H7 surveillance in Scotland over 60% of the reported cases occurred between May and September (Sharp et al., 1994).

Mukherjee and others (2004) in Minnesota investigated the prevalence of *E. coli*, *Salmonella*, and *E. coli* O157:H7 in a total of 476 and 129 produce samples collected from 32 organic and 8 conventional farms, respectively. The study showed that all samples were virtually free of pathogens. However, *E. coli* was 19 times more prevalent on produce acquired from the organic farms. They implied that this was due to the common use of manure aged for less than a year. The use of cattle manure was found to be of high risk as *E. coli* was found 2.4 times more often on farms using raw manure rather than other animal manures.

2.3.2 Organic and manure amendements for field

Soil amendements are commonly, but not always, incorporated prior to planting into agricultural soils used for lettuce/leafy greens production to add organic and inorganic nutrients to the soil as well as to reduce soil compaction. However, human pathogens may persist in animal manures for weeks or even months (Fukushima et al., 1999; Gagliardi and Karns, 2000).

Livestock manure can be a valuable source of nutrients, but it can also be a source of human pathogens if not managed correctly. USDA National Organic Program certification currently requires only 120 days between incorporation of raw manure into

soil and harvest of leafy greens (USDA-NOP, 2010). Thermophilic composting of manure whereby compost temperatures of 55°C for 3 consecutive days in a static aerated pile, or is turned 5 times during 14 days of exposure to 55°C in a windrow pile, can significantly reduce pathogens, but is not guaranteed to render the compost 'pathogen-free'. Such thermophilically composted manure however may be incorporated (according to the USDA-NOP) into soil prior to planting. There is a recommendation that top-dressing of plants be avoided to reduce the risk of microbial contamination further as plants mature. Manure should be stored as far away as practical from areas where fresh produce is grown and handled the WGA metrics suggest 400 feet.

2.3.3 Rotations

Starvation by deprivation of a suitable host is the key mechanism of plant pathogen control via crop rotation. Judicious crop rotation may be a useful strategy for increasing short term soil organic matter and for establishing healthy, fertile, and productive soils. Rotating cold- and warm-weather crops can suppress weeds by disrupting their life cycles. Alternatively, some crops exude chemicals that suppress weeds (Merfield, 2000). Good crop rotations involve crops that have different planting dates, rooting habits, lengths of production, cultivation requirements, and harvesting requirements (Peet, 2007). All of these factors affect the ability of plants to compete with weeds (Bellows, 2005) and the survival of plant and human/animal disease agents. These systems for plant production that involve balancing production targets for crop yield against weather, biological, physical, economic, and time limitations are highly complex and challenging for producers.

2.3.4 Cover crops

Cover cropping (also called green manuring) is widely recognized as an important tool for soil quality management in organic production systems. Green manuring involves the incorporation into soil of field or forage crops with targeted attributes for soil and crop improvement. Incorporation may occur while the plant is still green or soon after flowering. In addition to providing ground cover and, in the case of a legume, nitrogen fixation, they also help suppress weed growth by competition and smothering and simultaneously contribute to soil organic matter and overall soil tilth (Merfield, 2000). Sometimes insect pests and diseases also are reduced (Sullivan, 2003). Moreover, cover crops help recycle many other nutrients phosphorous, potassium, calcium, magnesium, sulfur, and other elements from the green manure crop in the soil.

2.3.5 Other cultural management practices

It is sometimes possible to create barriers that help protect plants from wild animals. In greenhouses, it is common to use very fine mesh screens that prevent access of insect vectors of fastidious bacteria or viruses. Organic and plastic mulches also can help to protect the “splash zone” parts of plants such as tomatoes and cucurbit fruits from pathogens (Shumann and D’Arcy, 2006). Other organic mulches serve as physical barriers to foliar pathogens.

2.3.6 Solarization

Soil solarization is a nonpesticidal technique that kills a wide range of soil pathogens, nematodes, and weed seeds and seedlings through the high soil temperatures raised by placing plastic sheets on moist soil during periods of high ambient temperature (Shumann and D’Arcy, 2006). Direct thermal inactivation of target organisms was found to be the most important mechanism of solarization biocidal effect, contributed also by a heat-

induced release of toxic volatile compounds and a shift of soil microflora to microorganism antagonist of plant pathogens. Soil temperature and moisture are critical variables in solarization thermal effect, though the role of plastic film is also fundamental for the solarizing process, as it should increase soil temperature by allowing the passage of solar radiation while reducing energetic radiative and convective losses (D'Addabbo et al., 2010). Study done by scientists in Japan reported that soil solarization was able to raise the soil temperature up to 40°C and reduced *E. coli* to < 0.08 CFU/g introduced into the soil in an open upland field (Wu et al., 2009).

2.3.7 Biopesticides- Biofumigation

Biopesticides include naturally occurring substances that control pests (biochemical pesticides), microorganisms that control pests (microbial pesticides), and pesticidal substances produced by plants containing added genetic material (plant-incorporated protectants or PIPs) (U.S. EPA, 2009). Brassica plants are characterized by a high content of glucosinolates and of other sulphur-containing compounds (Walker et al., 1937; Sang et al., 1984, Mayton et al., 1996; Gimsing and Kirkegaard 2006, 2009). Antifungal volatiles such as allylisothiocyanate have been found in leaf extracts of various *Brassica* species (Mayton et al., 1996; Sang et al., 1984). The toxicity of isothiocyanates or other glucosinalate-related compounds to various microorganisms has been well documented (Gamliel, 2000; Bailey and Lazarovits, 2003; Mazzola and Cohen, 2005; PiedraBuena et al., 2006, Mattner et al., 2008; Motisi et al., 2009). Chemicals of this group such as methylisothiocyanate, the active ingredient of metham sodium and dazomet, are widely used as soil fumigants (Lu et al., 2010).

2.3.8 Pesticides

The U.S. government has regulated pesticides since the early 1900s. Pesticide is the name for agricultural chemical that includes herbicides (for the control of weed and other plants), insecticides (for the control of insects), fungicides (for fungi), nematocides (for nematodes, worms), and rodenticides (for rodents). The use of synthetic pesticides in agriculture is the most widespread method for pest control, and it is estimated that farmers spend approximately \$4.1 billion on pesticides annually (US EPA, 2009a). Despite the multiple benefits of pesticides, which include the control of disease organisms, weeds, or insect pest in many circumstances, the direct benefits received by consumers through wider selections and lower prices for food and clothing, and the contribution to enhance human health by preventing disease outbreaks through the control of rodent and insect populations, pesticide compounds are detrimental to human health as well as to the environment. For example, exposure to pesticides can result in death, natural resources can be degraded when pesticide residues in storm water runoff enter streams or leach into groundwater, pesticides that drift from the site of application can harm or kill non-target plants, birds, fish, or other wildlife, and the mishandling of pesticides in storage facilities and in mixing and loading areas can contribute to soil and water contamination (US EPA, 2009b). Moreover, government actions in the U.S., pertaining to the Food Quality Protection Act of 1996, have dramatically restricted the use of many conventional pesticides upon which growers have depended for decades (e.g. organophosphates and carbonates), consequently resulting in the need of alternatives products which are harmless to human and environmentally friendly, and these natural bio pesticides could be useful for organic farmers.

Organic farming is a small, but growing, segment of U.S. agriculture. USDA estimates the value of retail sales of organic foods at \$6 billion in 1999 with about 12,200 organic farmers nationwide, most with small-scale operations (U.S. EPA, 2009c). Organic is a labeling term that denotes products produced under the authority of the U.S. Organic Foods Production Act. Organic producers, based on philosophical preference and conviction or in response to an increasing market opportunity, exclude or prohibit the use of conventional crop inputs common to modern farming. Synthetic pesticides and fertilizers are not allowable in current organic certification program. To achieve optimal quality and economic returns, organic farming systems rely upon crop rotations, crop residues, animal manures, green manures, off-farm organic wastes, mechanical cultivation, mineral bearing rock powders, and biological pest control (Mitchell et al., 2000). Organic agriculture practices do not ensure that products are completely free of residues; however, methods are used to minimize pollution from air, soil and water. Organic food handlers, processors and retailers adhere to standards that maintain the integrity of organic agricultural products (US EPA, 2009c). This includes practices such as minimizing or eliminating the use of herbicides in crop production and antibiotics in animal production.

2.4 Current interventions to control contamination in fresh produce

Public awareness about produce-associated risks reached a tipping-point during the spinach *E. coli* O157:H7 outbreak in the fall of 2006 (Powell, 2008), and in response to the current public health concerns with the safety of fruits and fresh vegetable, researchers have investigated the efficiency of physical, chemical, and biological methods for reducing the populations of microorganisms on whole and fresh cut produce.

Each method has distinct advantages and disadvantages depending upon the type of produce, the type of pathogens, the type of sanitizer, the concentration of the sanitizer, the temperature, the pH, and the exposure time. The best method to eliminate pathogens from produce is to firstly prevent contamination. However, it is not always achieved and the need to wash and sanitize many types of produce remains of great importance to prevent disease outbreaks.

2.4.1 Chlorine and hypochlorite

Chlorine has been used for sanitation purposes in food processing for several decades and is perhaps the most widely used sanitizer in the food industry (Walker and LaGrange, 1991; Cherry, 1999). Chemicals that are chlorine based (as sodium or calcium hypochlorite or Cl_2 gas) are often used to sanitize produce and surfaces within produce processing facilities, and to reduce microbial populations in water used during cleaning and packing operations. The most common forms of free chlorine include liquid chlorine and hypochlorites. Liquid chlorine and hypochlorites are generally used in the 50 to 200 ppm concentration range with a contact surface of 1 to 2 min to sanitize produce surfaces (Beuchat, 1998). The antimicrobial activity of chlorine compounds depends largely on the amount of hypochlorous acid (HOCl) present in the water after the treatment is applied because HOCl transfers across microbial cell walls to kill the microbes. This, in turn, depends on the pH of the water, the amount of organic material in the water, and, to a more limited extent, the temperature of the water. For example, increasing level of organic matter decrease HOCl concentration and also overall antimicrobial activity (Beuchat et al., 2004). Thus, in the management of chlorine, it is important to maximize HOCl concentrations and minimize all other forms of chlorine

In aqueous solutions, the equilibrium between HOCl and the hypochlorite ion (OCl^-) is pH dependent with the concentration of HOCl increasing as pH decreases. It is highly desirable to keep the pH of the water between 6.5 and 7.5 to ensure adequate HOCl activity without the formation of the chlorine gas. Moreover, maximum solubility in water is observed near 4°C; however, it has been suggested that the temperature should be maintained at least 10°C higher than that of produce items in order to reduce the possibility of microbial infiltration caused by a temperature – generated pressure differential (FDA, 2009).

Below pH 6.0, noxious chlorine gas (Cl_2) is formed and does not serve as an effective water disinfectant. Above pH 7.5 very little (<50%) chlorine can exist as active HOCl while most becomes inactive hypochlorite. With very long contact time, OCl^- does have some antimicrobial activity but would not be expected to result in beneficial control in typical postharvest handling systems (Suslow, 1997).

Effects of chlorine on bacterial pathogens inoculated onto produce have been investigated with mixed results. Studies indicate those chlorine concentrations traditionally used with produce (<200 ppm) are not particularly effective at reducing microbial populations on lettuce. Survival of *E. coli* O157:H7 on cut lettuce pieces after submersion for 90 s in a solution of 20 ppm chlorine at 20 or 50°C was not significantly different from the non chlorine treatment (Li et al., 2001). Spray treatment of lettuce with 200 ppm chlorine was no more effective at removing *E. coli* O157:H7 than treatment with deionized water (Beuchat, 1999). Increasing the exposure time from 1 to 5 min did not result in an increased kill. Likewise, Adams et al. (1989) indicated that a standardized washing procedure for lettuce leaves was only slightly improved with inclusion of 100 ppm

chlorine over tap water alone. Although a reduction of pH of the chlorine solution to between 4.5 and 5.0 increased lethality up to 4-fold, longer wash times (from 5 to 30 min) did not result in increased removal of microorganisms.

Since chlorine reacts with organic matter, components leaching from tissues of cut produce surfaces may neutralize some of the chlorine before it reaches microbial cells, thereby reducing its effectiveness. Additionally, crevices, cracks, and small fissures in produce, along with the hydrophobic nature of the waxy cuticle on the surface of many fruit and vegetables, may prevent chlorine and other sanitizers from reaching the microorganisms (Adams et al., 1989; Zhang and Farber, 1996). In addition, chlorine is known to interact with organic matter present in water to generate a spectrum of by-products including trihalomethanes (chloroform, bromodichloromethane, chlorodibromomethane and bromoform), haloacetic acids, haloacetonitriles, haloketones, chloral hydrate and chloropicrin (CFRFVFC, 2007), many of which are of concern as potential residuals on foods.

The use of chlorine washes or sprays must comply with the legal definition of a processing; they should not perform a function in the final product and should leave no residues that present a health risk. The toxicological profiles of chlorination by-products are incomplete. Concerns have been expressed regarding their carcinogenic and reproductive toxicity potentials. However, the data so far remains inconclusive and is certainly not robust enough on which to base any potential changes to current processing/disinfection practices (Fawell, 2000). U.S. Environmental Protection Agency (EPA) study on the carcinogenic activity of, and potential interactions between, different trihalomethanes in drinking water was also inconclusive (Pereira, 2000). The

occupational exposure limit (ceiling) is 1 ppm in the U.S. (instantaneous up to 15 minutes) (OSHA).

2.4.2 Chlorine dioxide

The major advantages of chlorine dioxide (ClO_2) over HOCl include reduced reactivity with organic matter and greater activity at neutral pH; however, stability of chlorine dioxide may be a problem because it must be generated on site and can be explosive when concentrated. ClO_2 forms fewer organohalogens than HOCl , although its oxidizing power is reported as 2.5 times that of chlorine (Benarde et al., 1967). Its mechanism of action involves disruption of cell protein synthesis and membrane permeability control. A maximum of 200 ppm ClO_2 is allowed for sanitizing of processing equipment and 3 ppm maximum is allowable for contact with whole produce. Only 1 ppm maximum is permitted for peeled potatoes. Treatment of produce with chlorine dioxide must be followed by a potable water rinse or blanching, cooking, or canning (CFR, 2000a).

ClO_2 gas reduced the numbers of *E. coli* O157:H7 on injured green pepper surfaces (Han et al., 2000). Treatment of surface-injured green peppers with 0.6 and 1.2 ppm ClO_2 gas reduced populations of *E. coli* O157:H7 by 3.0 and 6.4 log, respectively. These researchers noted that no significant growth of *E. coli* O157:H7 was observed on uninjured pepper surfaces, but significant growth occurred on injured pepper surfaces within 24 h at 37 °C.

2.4.3 Acidified sodium chlorite

Acidified sodium chlorite has been approved for use on certain meats, seafood, poultry, and raw fruits and vegetables as either a spray or dip in the range of 500 to 1200 ppm (CFR, 2000b). Reactive intermediates of this compound are highly oxidative with broad spectrum germicidal activity. Applications of 500 ppm acidified ClO_2 significantly

reduced populations of *E. coli* O157:H7 (>1 log) on germinated alfalfa seeds, but did not control the growth of the pathogen during the sprouting process (Taormina and Beuchat, 1999a).

2.4.4 Use of non-chlorine compounds for disinfection

2.4.4.1 Ozone

Ozone is another strong oxidizing agent used in process water, drinking water, and swimming pools. In clean water free of organic debris and soil particulates, ozone is a highly effective sanitizer at concentrations of 0.5 to 2 ppm. Ozone is almost insoluble in water (0.00003 g/100 ml at 20°C); its disinfectants activity is unaffected in water with a pH from 6 to 8. Ozone is highly corrosive to equipment and lethal to humans with prolonged exposure at concentrations above 4 ppm. Ozone is readily detectable by human smell at 0.01 to 0.04 ppm. At 1 ppm ozone has a pungent, disagreeable odor and is irritating to eyes and throat (Suslow, 1997).

Ozone is highly unstable in water and decomposes to oxygen in water with suspended soil and organic matter, the half-life of ozone activity may be less than 1 minute. The use of ozone as an antimicrobial agent in food processing was reviewed by Kim et al. (1999) and Xu (1999); however, little has been reported about the inactivation of pathogens on produce. Kim et al. (1999) reported a 2 log CFU /g reduction in total counts for shredded lettuce suspended in water ozonated with 1.3 mM ozone at a flow rate of 0.5 L/min.

2.4.4.2 Organic acids

Organic acids are naturally-occurring compounds in fruit and vegetables or they may be accumulated as a result of fermentation, which sometimes is relied upon to retard the growth of some microorganisms and prevent the growth of others. Foodborne bacteria

capable of causing human illness cannot grow at pH values less than about 4.0 (Beuchat, 1998). The mode of action of organic acids (Figure 1.2.) is attributed to direct pH reduction, depression of the internal pH of microbial cells by ionization of the undissociated acid molecule, or disruption of substrate transport by alteration of cell membrane permeability (Davidson, 2001).

The use of acetic acid to inactivate pathogenic bacteria on fresh parsley was studied by Karapinar and Gonul (1992). Populations of *Y. enterocolitica* inoculated onto parsley leaves were reduced >7 logCFU after washing for 15 min in solutions of 2% acetic acid or 40% vinegar. Treatment in 5% acetic acid for 30 min did not result in any recovery of aerobic bacteria, while treatment with vinegar gave a 3 to 6 log decrease in aerobic counts, depending upon acetic acid concentration and exposure time. Treatment of whole parsley leaves for 5 min at 21 °C with 7.6 % acetic acid reduced populations of *S. sonnei* more than 7 log CFU/g(Wu et al., 2000).

Fresh Express™, a leading producer of bagged salad greens in the USA, has announced that it is abandoning the standard industry practice of washing salad greens and leafy vegetables with chlorine and substituting an acid mix, Fresh Rinse™ , containing lactic acid and peracetic acid (Anonymous, 2010).

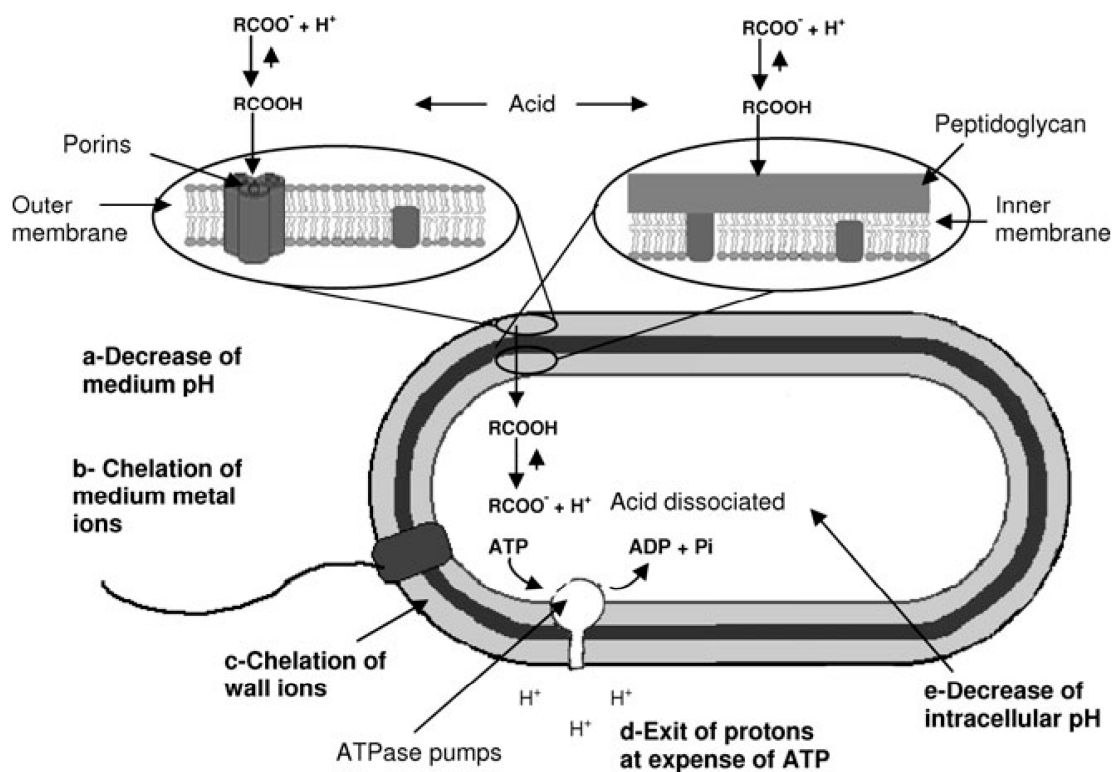


Figure 2.2: Mechanisms of action of organic acids in a bacterial cell from a-e.

The left amplification illustrates how the organic acids can pass through the outer membrane in Gram-negative bacteria, whereas the right amplification shows how they can pass through the inner membrane in Gram-positive (adapted from Davidson 2001).

2.4.4.3 Peroxyacetic acid

Peroxyacetic acid has recently been approved for use on produce in California Department of Pesticide Regulation. For the treatment of fruit and vegetable surfaces, current formulations combine 11% hydrogen peroxide and 15% acetic acid. The labeled rate for surface contact on produce is 80 ppm. After application of peroxyacetic acid for disinfection, produce must be rinsed with potable water. Peroxyacetic acid is a colorless liquid with an acid odor; as a concentrate it is considered a hazardous substance and a severe irritant if breathed (Suslow, 1997). However, recent studies have shown that food containing residues of acetic acid and octanoic acid arising from the use of peroxyacid

antimicrobial solutions has previously been considered as safe for human consumption (EFSA, 2005b; WHO, 2005).

Masson (1990) reported in a study that 100-fold reductions in total counts and fecal coliforms on cut-salad mixtures were observed after treatment with 90 ppm peroxyacetic (peracetic) acid or with 100 ppm chlorine. The subsequent inhibition of microbial growth during storage of salads was attributed to residual peracetic activity. When used at 40 and 80 ppm, a sanitizer containing peracetic acid (TsunamiTM, Ecolab, Mendota Heights, MN) significantly ($P<0.05$) reduced *Salmonella* and *E. coli* O157:H7 populations on cantaloupe and honeydew melon surfaces (Park and Beuchat, 1999).

2.4.4.4 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) possesses bactericidal and inhibitory activity due to its properties as an oxidant. In addition, a report published by the Joint FAO/WHO Expert Committee on FOOD Additives (JECFA) considered that due to the high reactivity of hydrogen peroxide towards organic matter they would break down into acetic acid, octanoic acid, and water and therefore does not pose a risk (EFSA, 2005a). The antimicrobial activity of hydrogen peroxide depends on temperature, pH, and other environmental factors (EFSA, 2005b).

The antimicrobial activity of H_2O_2 has been investigated and well documented by Juven and Pierson's (1996). *Salmonella* populations on alfalfa sprouts were reduced approximately 2 log CFU/g after treatment for 2 min with 2% H_2O_2 or 200 ppm chlorine (Beuchat and Ryu, 1997). Use of a 1% H_2O_2 spray on alfalfa seeds and sprouts did not control growth of *E. coli* O157:H7 (Taormina and Beuchat, 1999b). In the same study, *Shigella* inoculated onto lettuce was reduced by approximately 4 log CFU/g after dipping

in H₂O₂ combined with either 2 or 5% acetic acid; however, obvious visual defects were noted on the treated lettuce. The same treatment gave similar results for *E. coli* O157:H7 inoculated onto broccoli florets or tomatoes with minimal visual defects. Shredded lettuce was severely browned upon dipping into a solution of H₂O₂.

2.4.4.5 Essential oils

In the last 15 years, interest in alternative postharvest disease management practices other than chemical pesticides has increased due to the interest and consumer pressure to eliminate chemical residues on fruit. Numerous plant-derived compounds with antimicrobial properties have been studied for use in food systems (Cherry, 1999). Although their usefulness may be limited due to undesirable sensory effects, naturally-derived food compounds and essences have shown antimicrobial activity against human pathogens in laboratory studies. Compounds such as cinnamaldehyde, diacetyl, benzaldehyde, pyruvic aldehyde, piperonal, basil methyl chavicol, carvacrol, vanillin, psoralens, jasmonates, allylisothiocyanate, hop resins, and essences of garlic, clove, cinnamon, coriander, and mint have been studied for antimicrobial activity in various food systems (Bowles et al., 1995; Bowles and Juneja, 1998; Buta and Moline, 1998; Cerrutti et al., 1997; ; Chantaysakorn and Richter, 2000; Delaquis and Mazza, 1995; Isshiki et al., 1992; Lis-Balchin et al., 1996; Ulate-Rodriguez et al., 1997; Tokuoka and Isshiki, 1994; Wan et al., 1998).

Although the antimicrobial properties of essential oils (EOs) and their components have been reviewed in the past, their mechanisms of action have not been studied in great detail. Considering the large number of different groups of chemical compounds present in EOs, it is most likely that their antibacterial activity is not attributable to one specific

mechanism, but rather to interaction with several specific targets in the cell (Burt, 2004). Nychas et al. (2003) and Burt (2004) have reported the location and mechanisms of action in the bacterial cell of EOs, for instance: degradation of the cell wall, damage to cytoplasmic membrane and membrane proteins, leakage of cell contents, coagulation of cytoplasm, and depletion of the proton motive force (Figure 2.3.). Nychas et al. (2003) indicated that the mode of action of EOs is concentration dependent, indicating that low concentrations inhibit enzymes associated with energy production, while higher amounts may precipitate proteins. Further information is needed regarding the effects of specific plant derivatives, and other naturally occurring compounds, on human pathogens and produce.

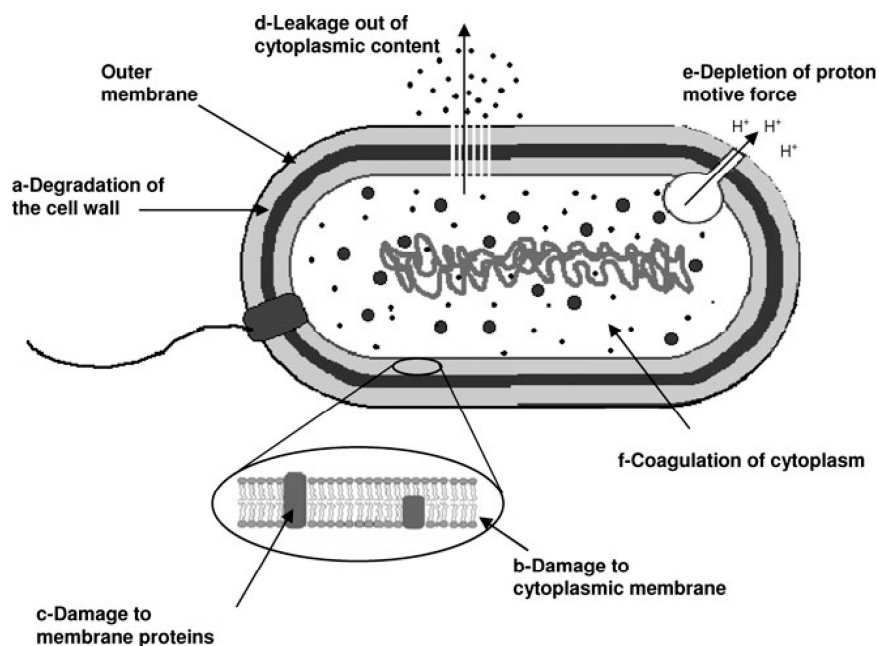


Figure 2.3: Mechanisms of action of essential oils and their components in a bacterial cell (a - f). The amplification illustrates the mode of action at the inner membrane (adapted from Burt 2004).

2.5 Summary

Foodborne outbreaks associated with consumption of leafy greens contaminated with *Salmonella* and *E. coli* O157:H7 have been increasing in the U.S. and worldwide. Food products must be free of these pathogens to protect public health. However, despite adherence to Good Agricultural Practices and other measures taken during production, harvesting, processing, transportation, and distribution, food-associated outbreaks still occur. Results of trace-back investigations associated with recent foodborne outbreaks with lettuce and tomatoes have strongly implicated contamination at the field production stage of the farm-to-fork continuum. Wildlife, surface water, proximity to animal and compost production operations, dust, and insects in the outdoor environment are primary suspect sources for contamination of soil and produce. Organic growers usually incorporate organic soil amendments, such as animal manures, green manures, cover crops, compost, and mixed organic fertilizers, to improve soil quality. However, despite the benefits, raw manure or improperly prepared compost are known reservoirs for pathogens like *E. coli* O157:H7 and *Salmonella* spp. Current Good Manufacturing Practices and Good Agricultural Practices guidelines have a strong emphasis on measures to reduce food safety risks at preharvest and postharvest stages of the fresh produce supply chain. However, additional technologies or development of methods are needed to strengthen current methods used by growers throughout the food production, harvesting and processing stages of the fresh produce supply chain. The studies reported here were conducted to evaluate strategies for reducing or eliminating *Salmonella* and *E. coli* O157:H7 on leafy greens from the preharvest stage via contaminated soil to processing in the leafy green washing stage.

Chapter 3: Research Objectives

The ultimate goal of this project is to develop an effective intervention strategy for reducing *E. coli* O157:H7 and *Salmonella* with essential oils on produce (iceberg and romaine lettuce and spinach). In order to achieve this goal, the effect of essential oils will be examined during the pre harvest and the post harvest stage of the production. Therefore, this study covers three specific objectives:

- 1) To evaluate the inhibitory effect of essential oils such as cinnamaldehyde, Ecotrol[®], eugenol, Sporan[®] and acetic acid against *E. coli* O157: H7 and *Salmonella* in organic soil.
- 2) To evaluate the effect of cinnamaldehyde, and Sporan[®] alone or in combination with acetic acid on the microbiological of spinach leaves, iceberg and romaine lettuce. Further, the physical properties such as color, and texture as well as sensory quality of treated leaves will be evaluated.
- 3) To study the inhibitory activities and modes of action of cinnamaldehyde, Sporan[®], and Sporan[®]-acetic acid against the five mixed strains of *E. coli* O157:H7 and *Salmonella*. Furthermore, visualize the effects of cinnamaldehyde, Sporan[®], and Sporan[®]-acetic acid on *E. coli* O157:H7 and *Salmonella* cells through scanning electron microscopy and transmission electron microscopy.

Chapter 4: Antimicrobial Activity of *Essential* Oils against *E. coli* O157:H7 and *Salmonella* in Organic Soil

(Published in Yossa et al., Food Control 21 (2010) 1458-1465)

4.1 Overview

Soil can be a significant source of preharvest contamination of produce by pathogens. Demand for natural pesticides such as essential oils for organic farming continues to increase. Antimicrobial activity of essential oils *in vitro* has been well documented, but there is no information about their efficacy in soil. In this study, we examined the antimicrobial activity of several essential oils against *E. coli* O157:H7 and *Salmonella* in soil.

Two essential oils (cinnamaldehyde and eugenol), two bio-pesticides (Ecotrol® and Sporan®) containing essential oils, and an organic acid (acetic acid) at 0.5%, 1.0%, 1.5% and 2.0%, were mixed with organic sandy soil and inoculated with five different strains of *E. coli* O157:H7 separately. Soils were incubated at room temperature and samples obtained at 1, 7 and 28 days were enumerated to determine survival. The bactericidal effect of 0.5% cinnamaldehyde was evidenced by a 10-fold reduction in *E. coli* O157:H7 as compared to other treatments. *E. coli* populations in soil were reduced by up to 5 log CFU/g after 24h incubation with 2% cinnamaldehyde, Ecotrol®, Sporan® or acetic acid. In contrast, the antimicrobial effect of eugenol was not evident either at 0.5% or 2%. Four logs of reduction in *E. coli* concentrations were obtained after 7 days of incubation with 1% cinnamaldehyde, 1.5%, acetic acid or 2% Ecotrol® or Sporan® at. Overall, *E. coli*

O157:H7 strain 4406 was the most sensitive of all the five strains tested to essential oils at 2% as evidenced by significant reduction (detection limit <1 bacterial count) from 0 to 4 weeks. In general, increases in essential oil concentrations corresponded to reduced survival of *E. coli* with all oils used in this study.

Results show the potential for oils to effectively reduce *E. coli* populations in soil. Interventions that significantly reduce survival of *E. coli* in soil prior to or during crop growth while simultaneously contributing to crop pest control could offer producers promising options to reduce potential contamination of fresh organic produce inadvertently contaminated by soil.

4.2 Introduction

Foodborne diseases continue to be a serious threat to public health all over the world. The incidence of illnesses appears to be increasing on a global basis including developed and industrialized countries. Foodborne illness outbreaks associated with *E. coli* O157:H7 on meat and fresh produce products have occurred in the US since 1982 despite awareness and diligence by industry. With 76 million estimated illnesses, more than 300,000 hospitalizations, and 5,000 deaths annually in the US attributed to food-borne illness (Seto et al., 2007), the associated annual estimated economic loss ranges from \$5-6 billion (Murphy et al., 2003). Consumption of refrigerated ready-to-eat (RTE), fresh-cut fruits and vegetables, often eaten with minimal processing, are a potential source of foodborne infection.

In 2006 consumption of contaminated raw spinach killed three, brought devastating kidney failure to 23, hospitalized more than 75, and sickened 205 people in the U.S. The spinach was traced back to product grown, processed, and packaged in California by the

largest producer of organically certified lettuce and spinach in the United States (CDC, 2006). As was subsequently reported in a study of 15 Minnesota farms, organic produce was six times more likely to be contaminated with *E. coli* (non-pathogenic), than conventional (Mukherjee et al., 2004).

Workers, visitors, animal feces, equipments, improper composting and farm runoff have been suggested as sources of contamination of field grown fresh produce. Due to the limited options for treatment of O157:H7 illnesses and lack of human vaccines avoiding exposure is currently the most viable option (Karmali, 1998; Li et al., 2000). Therefore the prevention of infection requires control measures at all stages of the food chain, from agricultural production on the farm to the table of consumers. Recent studies have linked *E. coli*, a traditional indicator of fecal contamination, to unexpected (non-fecal) habitats including a variety of soils across different climatic regions. Persistence of *E. coli* in non-host environments, has led to the suggestion that *E. coli* may no longer be useful as a fecal indicator organism (Power et al., 2005). Although *E. coli* O157:H7 are associated with feces from livestock and wildlife, it is clear that they are transported in surface runoff, and accumulate in sediments and soils contaminated by these animals (Guber et al., 2006; Meals and Braun, 2006; Millner, 2009,). These bacteria may also migrate into groundwater (Brabban et al., 2004). In addition, *E. coli* O157:H7 survives, replicates, and moves within soil, and the presence of manure enhances this survival (Gagliardi and Karns, 2000). Evidence shows it survives in coastal subtropical soils even after drying (Solo-Gabriele et al., 2000) and in agricultural soils well after manure application (Topp et al., 2003). Once *E. coli* populations are established in soil, a portion can become naturalized or autochthonous and even survive freeze/thaw cycles (Ishii, 2006).

Nematodes can vector *E. coli* and contribute to its spread and persistence in soil (Anderson et al., 2006). Soil reservoirs pose a serious risk to public health primarily through the fresh food chain.

Only a few ways have been suggested for eliminating *E. coli* O157: H7 from manure. Composting and anaerobic digestion, along with some advanced manure management technologies, and the addition of various chemicals, such as lime, have been used successfully, to reduce pathogen levels (Millner, 2009). Eliminating pathogens from livestock would aid in reducing soil and water contamination with various fecal pathogens. Researchers are currently investigating several approaches to eliminating *E. coli* O157:H7 from livestock, including antibiotics, antimicrobials, probiotics, vaccines and bacteriophage (Brabban et al., 2004).

Widespread use of pesticides has significant drawbacks including increased cost, handling hazards, concerns about pesticide residues on food, and threats to human health and environment (Paster and Bullerman, 1988). Public demand for safe produce has increased interest in investigating alternative soil and crop management practices that do not rely on use of synthetic chemical pesticides and fertilizers. Essential oils with pesticidal activity are increasingly used in organic production systems because they tend to have low mammalian toxicity, few non-target environmental effects, and wide public acceptance (Paster et al., 1995; Paranagana, 2003). Essential oils are volatile compounds produced by plants as secondary metabolites in particular cells or formed as glandular hairs (Hili et al., 1997). Among these natural antimicrobials are eugenol (85%) from clove oil (Farag et al., 1989), thymol and oregano, carvacrol from oregano and thyme oils, vanillin from vanilla, allicin from garlic, cinnamic-aldehyde from cinnamon, and

allyl isothiocyanate from mustard (Tzortzakis, 2008). Ecotrol®, a concentrated, commercial blend of rosemary and peppermint oils (10% and 2% respectively), is a broad spectrum contact insecticide/miticide effective against many insects (Belanger, 2006). It has minimal environmental impact in a formulation suitable for both conventional and organic applications (Anonymous, 2009). It can be applied to agricultural crops including vegetables and cole, herbs and spices, citrus, pome and stone fruits, nuts, berries, fruits, and grapes (Anonymous, 2005). Sporan® is a curative and preventive contact fungicide useful against a broad range of diseases, including but not limited to blights, molds, scabs, and mildews (Anonymous, 2009). It is composed of rosemary (18%), clove (10%) and thyme (10%) oils and can be applied to a wide variety of agricultural crops. Sporan® and Ecotrol® EC are listed by the Organic Material Review Institute (www.omri.org) for use in organically certified production systems.

Other types of antimicrobials used by the food industry are organic acids such as (acetic, benzoic, lactic, sorbic, propionic) fatty acids, parabens, bacteriocins (nisin), sulfites, sucrose esters, and other antimicrobials including natamycin and lysozyme (Krotcha, 2002). Furthermore, organic acids and their salts are promising agents because of their acceptance in food products and low cost (Miller et al., 1996). Organic acids have been tested for disinfecting meat, fish and minimally processed fruits and vegetables. The antimicrobial activity of organic acids is due to the pH reduction, depression of internal pH of microbial cell and disruption of substrate transport by altering cell membrane permeability (Beuchat, 1998). Acetic acid at 10-20% concentration has been used as a burn down, non-selective, organic herbicide (Dayan et al., 2009). No data are currently available on efficacy of essential oils or commercial products containing such oils in soil.

Hence quantitative data are needed on the antimicrobial activities of essential oils in soil to determine their efficacy in reducing survival of *E. coli* O157:H7 in a main component of an organic production environment, the soil. In this study, we evaluated the inhibitory effect of cinnamaldehyde, Ecotrol®, eugenol, Sporan® and acetic acid against *E. coli* O157:H7 in organic soil.

4.3 Materials and methods

4.3.1 Preparation of bacterial strains

Five nalidixic acid resistant strains of *E. coli* O157:H7 were used in the study. The strains RM 4406, RM 4688, and RM 1918 (clinical isolates from lettuce outbreaks), RM 4407 (clinical isolate from spinach outbreak), and RM 5279 (clinical isolate, bagged vegetable isolate) were kindly provided by Robert Mandrell (U.S. Department of Agriculture, Agricultural Research Service, Albany, CA). All cultures were maintained at -80°C in 20% glycerol. Each strain was aseptically sub-cultured in Tryptic Soy Broth (TSB, pH 7.2 Acumedia, Lansing, MI) supplemented with 50 ppm nalidixic acid (TSBN, Sigma-Aldrich, St. Louis, MO) for 24h at 37°C and stored at 4°C for long term use. Prior to the experiment, cultures were grown in TSBN and incubated for 24 h at 37°C. Cells were centrifuged (7500g, 10min, 10°C), and cell pellets were suspended in 0.1% sterile peptone water (Acumedia). The cell density of individual strains was adjusted to obtain final concentration to 8 log CFU/ml. The populations of individual strains were verified on tryptic soy agar containing 50 ppm nalidixic acid (TSAN) by spot plate technique.

4.3.2 Essential oil treatments

Cinnamaldehyde (Sigma-Aldrich), Ecotrol® (EcoSMART Tech., Alpharetta, GA) Eugenol (Fisher Scientific, Pittsburgs, PA), Sporan® (EcoSMART Tech.), and acetic

acid (20%, Knouse Foods, Biglerville, PA). The desired concentrations (0.5%, 1.0%, 1.5% and 2.0%) of these treatments were freshly prepared before each use by dispersing them in a sterile distilled water containing 0.5% (w/v) of Tween 20 (Fisher Scientific, Pittsburg, PA). Most of the studies about spices or their essential oils are conducted in vitro conditions and an emulsifier or solvent such as ethanol, methanol or Tween are used to dissolve essential oils (Burt, 2004). The suspension was vortexed before using in soil.

4.3.3 Inoculation of soil

Soils (Downer-Ingleside loamy sand, Coarse-loamy, siliceous, semiactive, mesic Typic Hapludults) were obtained from the USDA Beltsville Agricultural Research Center North Farm high tunnels managed for four years according to USDA-National Organic Program guidelines. Soil (10 g) was placed into a sterile whirl-pak filter bag (Nasco, Fort Atkinson, WI) and inoculated with 100 μ L of designated inocula to obtain 6 log CFU/g soil. Samples were mixed vigorously to distribute the inoculum, and each desired concentration (0.5%, 1%, 1.5%, and 2.0%) was added in soil and mixed again. The bags were closed, and incubated at room temperature (22°C) for 28 days. Soil sample inoculated with *E. coli* O157:H7 served as control.

4.3.4 Enumeration of *E. coli* O157:H7

On each sampling date (1, 7, 28 days), 10ml (w/v) of sterile peptone water (0.1%) was added to each soil bag and the bag was pummeled for 2 min (Bagmixer, Interscience, St. Nom, France). Appropriately diluted suspensions were spiral plated (Whitley automatic spiral plater, Whitley Scientific, West Yorkshire, England) on Sorbitol MacConkey media (Acumedia) supplemented with 0.05mg/l of cefixime, 2.5mg/l of potassium tellurite (Invitrogen, Carlsbad, CA, CtSMAC.) and 50 ppm nalidixic acid (Sigma-Aldrich,

CtSMAC-N), and incubated at 37°C for 24 h. Presumptive colonies of *E. coli* O157:H7 were confirmed using Dry Spot latex agglutination assay (Remel, Lenexa KS).

4.3.5 Statistical analysis

E. coli O157: H 7 populations obtained at each sampling period treated with different oils were converted to log CFU/g. The experiment was performed in triplicate. The data were analyzed by quadratic response surface model for each oil concentration applied to each strain using “Proc RSReg” statement (SAS 8.2, Cary, NC). Contour graphs were produced for each of the 25 (strain x oil concentration). The effects of oils, strains, sampling time and interaction effects were determined. In all cases, the level of statistical significance was $P < 0.05$.

4.4 Results

4.4.1 Inactivation of *E. coli* O157:H7 in soil by cinnamaldehyde, Ecotrol®, eugenol, Sporan® and acetic acid after 24h

E. coli O157:H7 were not detected in uninoculated soil used in this study. The effect of cinnamaldehyde, Ecotrol®, eugenol, Sporan® and acetic acid on survival of *E. coli* inoculated in soil are presented in Tables 4.1- 3. The populations of *E. coli* O157:H7 varied from 5.10 to 6.55 log CFU/g in inoculated control.

In general, the antimicrobial effect of oils varied with *E. coli* O157:H7 strains. The strain 4406 was the most sensitive to these oils followed by strain 4407, 4688, 5279, and 1918. Cinnamaldehyde significantly reduced *E. coli* O157:H7 in soil compared to other oils used in this study. At 0.5% level, cinnamaldehyde was the most effective antimicrobial in reducing *E. coli* O157:H7 in soil (Table 4.1). Populations of *E. coli* O157:H7 strain 4407 recovered from soil treated with 0.5% cinnamaldehyde (4.57 log CFU/g) were

significantly lower than those recovered from control soil (6.55 log CFU/g). Acetic acid at 0.5% reduced *E. coli* O157:H7 strain 4406 by 1.33 log CFU/g. The effect of Ecotrol®, eugenol and Sporan® at 0.5% was not evident in reducing *E. coli* O157:H7. The population of *E. coli* O157:H7 were reduced with an increase in concentration of cinnamaldehyde, Sporan®, and acetic acid. However, increasing concentration of these treatments from 0.5% to 1% did not yield significant reduction of *E. coli* O157:H7 in soil. *E. coli* O157:H7 populations recovered from soil treated with 1% cinnamaldehyde were significantly lower than the *E. coli* O157:H7 population recovered from control soil. The *E. coli* O157:H7 populations in soils treated with 1.5% cinnamaldehyde were significantly lower than the *E. coli* O157:H7 populations recovered from soil treated with 0.5%, or 1% cinnamaldehyde. At least 5 log reductions in *E. coli* O157:H7 strains 4688 and 5279 were observed with 1.5% cinnamaldehyde. *E. coli* O157:H7 recovered from soils treated with 1.5% Ecotrol® or acetic acid was not significantly different from those treated with 0.5% Ecotrol® or acetic acid. Strain 4407 was the most vulnerable to 1.5% Sporan® with 4 log reduction followed by strains 4406, 1918, and 4488 with 3 log CFU/g reductions. Populations of *E. coli* O157:H7 strains 4407, 5279 and 4688 were non-detectable (detection limit 1.39 log CFU) in 2% cinnamaldehyde treated-soil after 24 h. Likewise, complete inhibition of strain 4406 and 4407 was observed with 2% concentration of Sporan® or Ecotrol®.

Table 4.1: Impact of essential oils and acetic acid on *E. coli* O157:H7 in soil after 24 h

Treatment	Conc. (%)	Populations of <i>E. coli</i> O157:H7 strains (log CFU/g) ^{a,b}				
		4406	4407	1918	5279	4688
Control	0	5.10±0.92	6.55±0.91	6.06±0.98	5.50±0.97	6.02±0.75
Cinnamaldehyde	0.5	4.07±0.40A	4.57±0.46A	4.69±0.72A	4.30±0.53A	4.69±0.27A
	1	2.59±0.26A	3.60±0.17A	3.64±0.34A	3.65±0.14A	3.77±0.21A
	1.5	0.00±0.00B	0.73±0.26B	0.57 ±0.48B	0.00±0.00B	0.00±0.00B
	2	0.77±0.33B	0.00±0.00B	1.09±0.88B	0.00±0.00B	0.00±0.00B
Ecotrol®	0.5	3.95±0.27A	5.38±0.60A	5.65±0.43A	5.51±0.53A	4.69±0.42A
	1	4.33±1.13A	4.91±0.18A	5.11±0.28A	5.17±0.43A	4.58±0.50A
	1.5	3.08±0.64A	4.00±1.15A	3.95±0.53A	4.13±0.71A	3.95±0.61A
	2	0.00±0.00B	0.00±0.00B	1.12±0.593B	1.31±0.27B	1.09±0.88B
Eugenol	0.5	4.34±1.30A	5.79±0.65A	5.99±0.10A	6.01±0.26A	6.32±0.37A
	1	3.94±0.17A	5.37±1.28A	5.70±0.42A	5.37 ±0.9AB	3.70±0.45A
	1.5	4.59±1.38A	4.76±0.91AB	3.29±0.23B	3.67±1.16BC	3.67±0.45B
	2	1.66±0.87B	3.60±0.19B	3.32±0.29B	2.80±0.38C	1.98±0.43B
Sporan®	0.5	4.60±1.11A	5.65±0.54A	5.66±0.71A	5.78±0.20A	5.77±0.62A
	1	3.49±0.65AB	4.43±0.09A	5.08±0.29AB	4.57±0.53AB	5.01±0.19AB
	1.5	2.87±0.47B	2.42±0.30B	3.82±0.99B	3.71±0.70B	3.31±0.66B
	2	0.00±0.00C	0.00±0.00C	1.39±0.40C	0.87±0.50C	0.82±0.43C
Acetic acid	0.5	3.77±0.27A	5.55±0.15A	5.77±0.24A	5.37±0.28A	5.53±0.17A
	1	3.91±0.15A	5.54±0.20A	5.33±0.57A	5.64±0.28A	4.57±1.63A
	1.5	3.00±0.50A	3.86±0.52A	4.30±1.48A	4.37±0.67A	3.96±0.70A
	2	0.00±0.00B	0.90±0.56B	1.51±0.62B	1.66±0.87B	0.77±0.33B

^a Mean ± standard deviation^b Means in the same column within the treatment with different letters are significantly different (P < 0.05).

4.4.2 Inactivation of *E. coli* O157:H7 in soil by cinnamaldehyde, Ecotrol®, eugenol, Sporan® and acetic acid after 7 days

After 7 days, *E. coli* O157:H7 populations remained either identical or increased in most treated soil samples with exception of 0.5% and 1% cinnamaldehyde, 1.5% Sporan®, and 1.5% acetic acid treatment. Recovery of *E. coli* O157:H7 strains 4407, 1918, and 4688 after 7 days in soil treated with 1 % cinnamaldehyde (1.72, 1.37, and 0.87 log CFU/g), were significantly lower than those recovered after 24hr (3.60, 3.64, and 3.77 log CFU/g), respectively. Likewise, significant reduction of *E. coli* O157:H7 strains 4407, 1918, 4688, and 5279 were observed in soils treated with 1.5% acetic acid after 7 days in comparison to *E. coli* O157:H7 populations recovered after 24 h. In contrast, Sporan® and eugenol at 0.5% level in soil increased *E. coli* O157:H7 by ca. 1 log after 7 days. Soil treatment with 2% eugenol also resulted in increase of up to 3 log CFU /g *E. coli* O157:H7 strains 4407 and 1918 after 7 days. After 7 days of incubation, all *E. coli* O157:H7 strains were non-detectable in soil treated with 2% cinnamaldehyde, or with 2% acetic acid except strain 5279. *E. coli* O157:H7 strains 4407 and 4688 were not recovered in soil treated with 2% Sporan®.

Table 4.2: Impact of essential oils and acetic acid on *E. coli* O157:H7 in soil after 7 days

Treatment	Conc. (%)	Populations of <i>E. coli</i> O157:H7 strains (log CFU/g) ^{a,b}				
		4406	4407	1918	5279	4688
Control	0	4.18±1.61	5.76±0.63	5.76±1.35	5.22±0.93	5.82±0.64
Cinnamaldehyde	0.5	0.67±0.15A	2.86±0.25A	3.74±0.64A	3.33±0.35A	3.62±0.22A
	1	1.61±0.40A	1.72±0.51A	1.37±0.23B	2.50±0.75A	0.87±0.50BC
	1.5	1.23±0.58A	0.83±0.43B	0.93±0.60B	2.86±1.01A	2.15±0.41AB
	2	0.00±0.00A	0.00±0.00B	0.00±0.00B	0.00±0.00B	0.00±0.00C
Ecotrol®	0.5	4.84±0.79A	5.76±1.00AB	6.92±0.42A	6.05±0.56A	6.51±0.56A
	1	4.94±1.25A	5.81±0.97A	6.33±0.57A	5.34±1.08A	5.81±0.25A
	1.5	2.34±0.41B	4.04±0.33B	4.14±0.40B	2.84±0.57B	3.09±1.21B
	2	1.91±1.13B	1.97±0.41C	0.57±0.48C	1.70±0.99B	0.00±0.00C
Eugenol	0.5	4.30±0.52A	6.45±0.64A	6.79±0.36A	6.45±0.33AB	5.80±1.98AB
	1	5.02±0.41A	6.68±0.75A	6.93±0.46A	6.80±1.15A	7.14±0.50A
	1.5	3.94±0.95A	5.98±0.10A	5.70±0.33A	4.99±0.70B	4.38±1.06BC
	2	5.07±0.56A	6.05±0.11A	6.05±0.04A	3.25±0.56C	3.79±0.81C
Sporan®	0.5	4.64±0.46A	6.32±0.98A	6.94±0.23A	6.72±0.36A	6.86±0.43A
	1	5.33±1.06A	6.25±1.56A	6.54±0.94A	5.83±1.49A	5.66±0.96A
	1.5	0.67±0.55B	1.33±1.15B	2.89±0.66B	1.96±0.24B	3.12±0.24B
	2	0.57±0.48B	0.00±0.00B	1.36±0.36B	1.87±0.62B	0.00±0.00C
Acetic acid	0.5	2.23±1.96A	4.65±0.24A	5.80±0.07A	5.28±0.61A	5.44±0.36A
	1	3.28±0.17AB	4.33±0.64A	4.98±0.64A	4.53±0.44A	4.24±0.27A
	1.5	0.77±0.33BC	2.19±0.85B	2.39±0.82B	1.81±0.87B	1.63±0.59B
	2	0.00±0.00C	0.00±0.00C	0.00±0.00C	0.73±0.26B	0.00±0.00B

^a Mean ± standard deviation; ^b Means in the same column within the treatment with different letters are significantly different (P < 0.05).

4.4.3 Inactivation of *E. coli* O157:H7 in soil by cinnamaldehyde, Ecotrol®, eugenol, Sporan® and acetic acid after 28 days

E. coli O157:H7 populations in treated soil were reduced further with most treatments during 28 days of incubation at room temperature (22 °C). However, the difference in recovery of *E. coli* O157:H7 between 7 and 28 days were not significant at 0.5% level of these treatments except Sporan® with strain 4407. Further, significant reductions were observed mainly with strain 5279 and 4407 at 1% and 1.5% levels. In general, the increased concentration of test compounds in the soil was associated with increased bacterial inhibition after 28 days. Cinnamaldehyde and Ecotrol® at 2% levels reduced the populations of *E. coli* O157:H7 strains 4407 and 1918 to undetectable level. Similarly, acetic acid at 1.5% and 2% levels reduced all *E. coli* O157:H7 strains to non-detectable levels. When compared at 28 days, *E. coli* O157:H7 4406 and 4407 were the most sensitive strains at 0.5% or 1% levels of Ecotrol®, eugenol and Sporan®. Acetic acid, cinnamaldehyde, and Ecotrol® were the most effective treatment when compared at 28 days. Overall, eugenol was the least effective in reducing *E. coli* O157:H7 in soil.

Table 4.3: Impact of essential oils and acetic acid on *E. coli* O157:H7 in soil after 28 days

Treatment	Conc. (%)	Populations of <i>E. coli</i> O157:H7 strains (log CFU/g) ^{a,b}				
		4406	4407	1918	5279	4688
Control	0	4.83±2.08	4.33±0.44	5.52±0.60	5.17±1.96	5.25±1.67
Cinnamaldehyde	0.5	4.40±0.66A	4.33±1.24A	2.47±1.51A	4.35±0.54A	4.27±1.09A
	1	1.76±0.66B	0.67±0.15B	0.00±0.00B	0.00±0.00B	0.57±0.38B
	1.5	1.19±0.56B	0.00±1.26B	0.00±0.00B	1.54±0.66B	1.27±0.20B
	2	0.57±0.48B	1.19±0.55B	0.00±0.00B	0.00±0.00B	0.77±0.33B
Ecotrol®	0.5	3.82±0.31A	4.16±0.15A	5.92±0.05A	5.43±0.56A	5.29±0.05A
	1	2.51±0.82A	3.67±0.35A	5.02±0.21A	3.46±1.03B	4.21±0.57A
	1.5	2.26±0.49A	0.67±0.55B	3.16±0.55B	2.88±0.20B	1.67±1.46B
	2	0.00±0.00B	0.00±0.00B	0.00±0.00C	1.01±0.74C	1.49±1.29B
Eugenol	0.5	4.32±0.62A	5.28±0.17A	5.44±0.31A	4.91±0.64A	4.77±0.70A
	1	3.59±0.53A	5.03±0.91A	5.43±0.79A	5.51±0.94A	5.47±0.11A
	1.5	3.21±1.07A	4.38±0.31A	4.67±0.81A	1.80±0.56B	4.10±0.95A
	2	4.26±1.13A	5.33±0.59A	5.50±0.31A	4.46±0.62A	5.05±0.86A
Sporan®	0.5	4.69±0.75A	3.33±1.61AB	6.43±0.19A	5.75±0.78A	5.30±0.33A
	1	3.28±0.52AB	4.27±0.24A	5.35±0.51A	5.31±0.92A	4.21±0.62A
	1.5	2.14±0.42B	2.18±0.43B	2.00±0.30B	1.57±0.37B	2.12±0.10B
	2	1.64±0.85B	0.00±0.00C	1.38±0.40B	1.81±0.57B	0.00±0.00C
Acetic acid	0.5	4.21±1.45A	4.20±0.62A	5.83±0.35A	5.79±0.94A	5.91±0.26A
	1	1.48±0.38B	0.00±0.00B	3.82±0.45B	4.40±1.22A	5.46±1.22A
	1.5	0.00±0.00B	0.00±0.00B	0.00±0.00C	0.00±0.00B	0.00±0.00B
	2	0.00±0.00B	0.00±0.00B	1.06±0.84C	0.00±0.00B	0.00±0.00 B

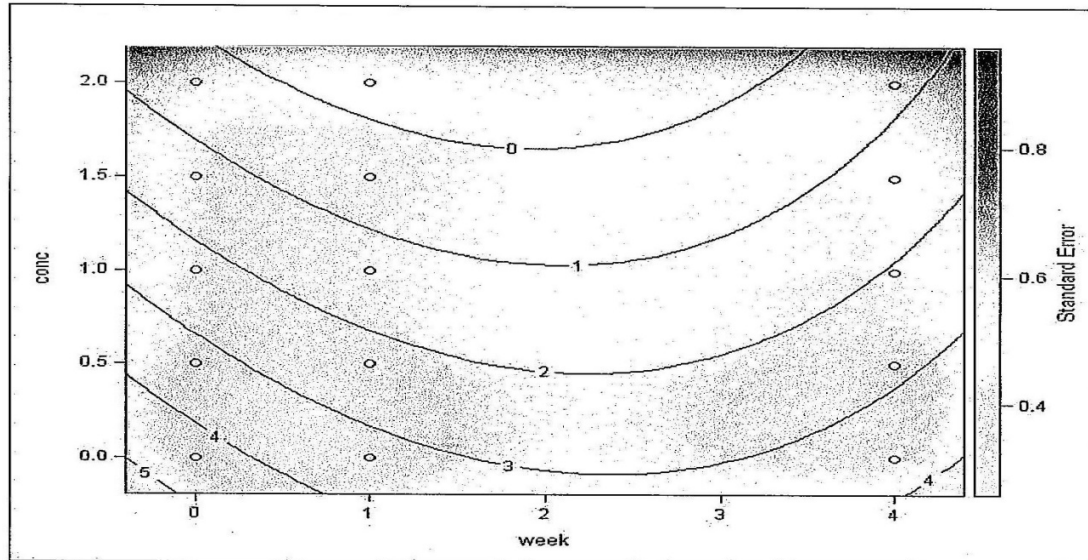
^a Mean ± standard deviation; ^b Means in the same column within the treatment with different letters are significantly different (P < 0.05).

4.4.4 Range of oil concentration (%) / weeks for which quadratic response surface model predict bacterial count to be < 1 log

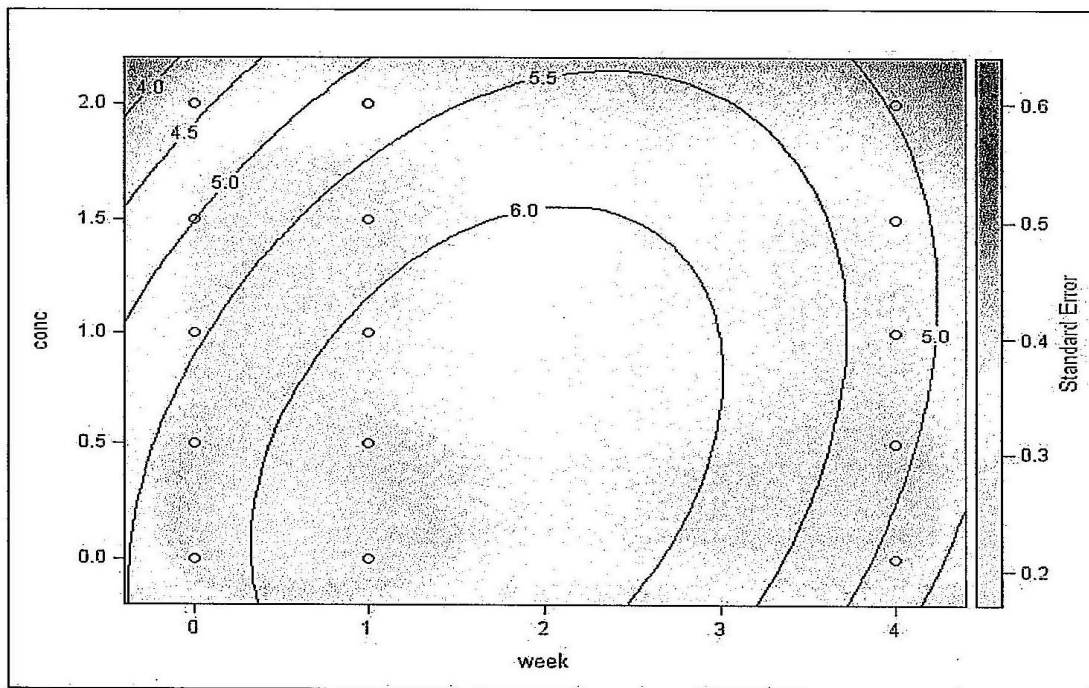
Quadratic response surface modelling analysis for predicting *E. coli* O157:H7 populations in soil are shown in Table 4.4. Selected response contour graphs are shown in Figure 4.1. The model predicted that populations the individual strains of *E. coli* O157:H7 would be less than 1 log CFU/g when 2% cinnamaldehyde, Ecotrol®, Sporan®, and acetic acid were applied to soil. Eugenol at any concentration from 0.5-2.0% would not be effective in achieving cell concentrations of less than 1 log at any time. *E. coli* O157:H7 populations would be at least 2 log CFU/g if eugenol was used in soil. Representative contour charts (Fig. 4.1) indicated how survival of *E. coli* O157:H7 will be affected by different treatments. With increase in concentration, *E. coli* O157:H7 populations changed as indicated in line patterns (each line represents specific log CFU).

Figure 4.1: Representative contour charts for predicting the effect of treatments on *E. coli* O157:H7 in soil. The charts show changes in *E. coli* O157:H7 populations with an increase in concentration of antimicrobial treatment during storage. The curved lines in the chart represent *E. coli* O157:H7 populations (log CFU/g). (a) Effect of cinnamaldehyde on *E. coli* O157:H7 strain 4406 (at least 1.5% cinnamaldehyde required to reduce *E. coli* O157:H7 populations below 1 log CFU/g in soil). (b) Effect of Ecotrol® on *E. coli* O157:H7 strain 1918 in soil (up to 1% Ecotrol® in soil does not reduce *E. coli* O157:H7 in soil). (c) Effect of eugenol on *E. coli* O157:H7 strain 4407 in soil (*E. coli* O157:H7 populations were always more than 4 log CFU/g in soil). (d) Effect of cinnamaldehyde on *E. coli* O157:H7 strain 5279 in soil (*E. coli* O157:H7 were reduced to <1 log CFU/g within 24 h when more than 1.5% cinnamaldehyde were used). (e) Effect of acetic acid on *E. coli* O157:H7 strain 4406 in soil (*E. coli* O157:H7 were reduced to below 1 log CFU/g after 1 week when acetic acid was used at 1.5% levels).

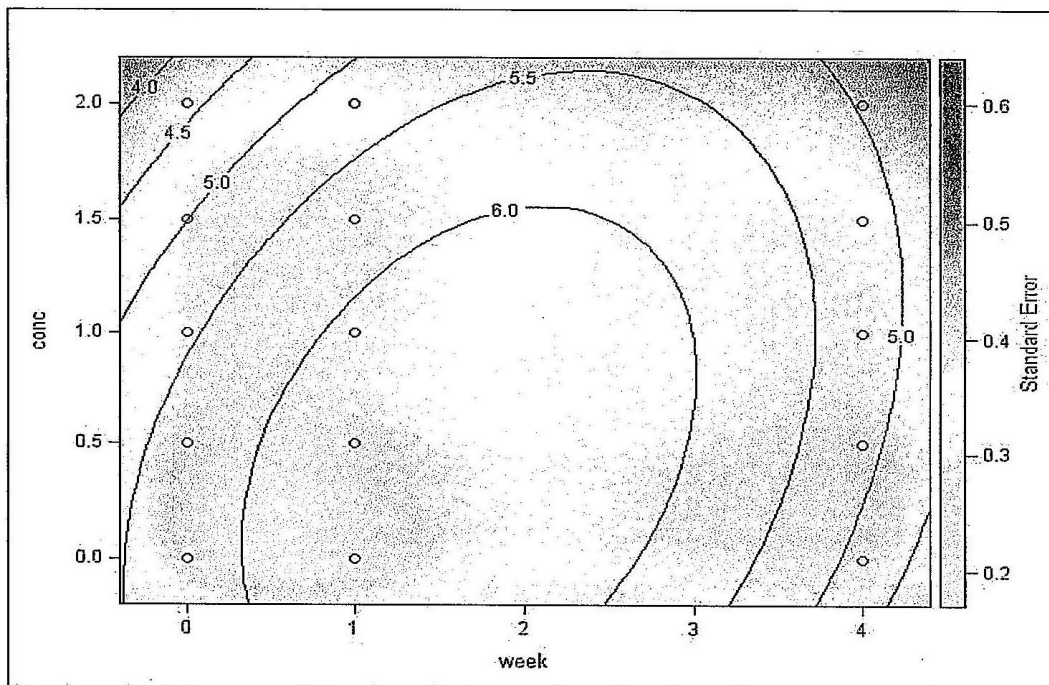
(a)



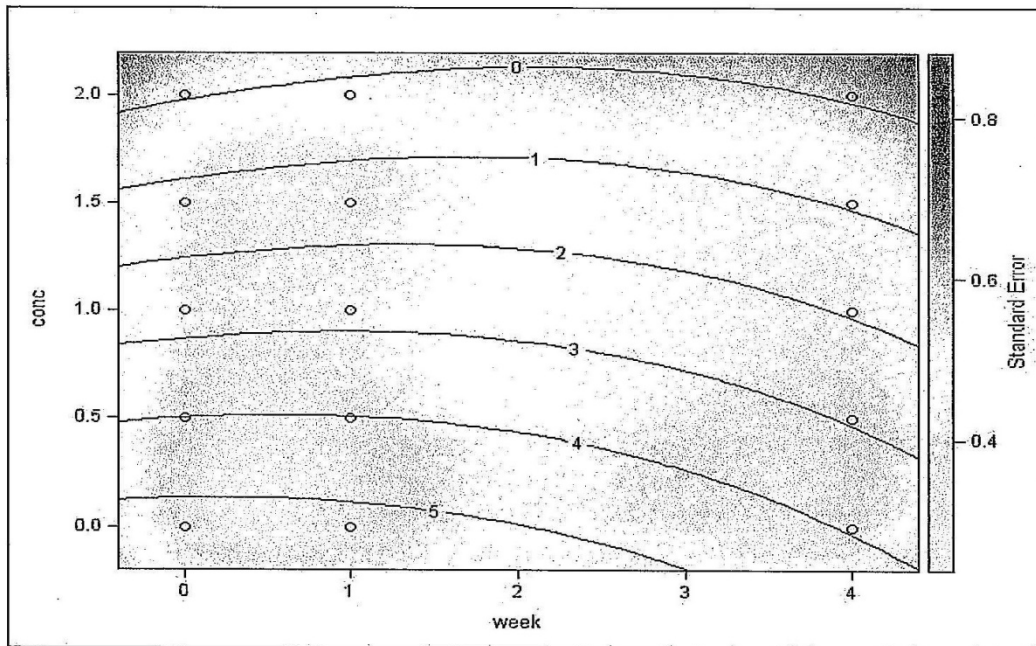
(b)



(c)



(d)



(e)

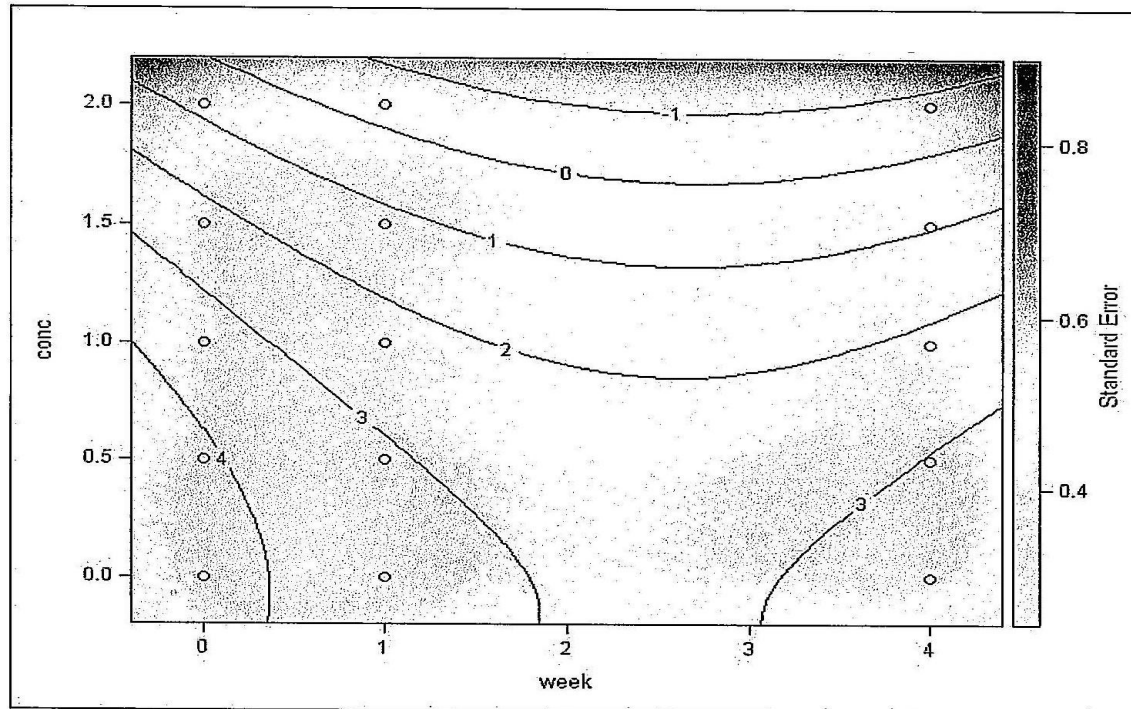


Table 4.4: Quadratic response surface model prediction for *E. coli* O157:H7 (< 1 log CFU/g) in soil treated with oils and acetic acid

<i>E. coli</i> O157:H7 strain	Prediction of <i>E. coli</i> O157:H7 populations to be less than 1 CFU/g in soil with treatment				
	Cinnamaldehyde	Ecotrol®	Eugenol	Sporan®	Acetic acid
1918	1.5 - 2% / 2-4 weeks	2% / 0 - 4 weeks	Counts always > 3 log CFU/g	2% / 0 - 4 weeks	2% / 2 - 4 weeks
4406	1.5 - 2% / 0-4 weeks	2% / 0 - 4 weeks	Counts always > 3 log CFU/g	2% / 0 - 4 weeks	2% / 0 - 4 weeks, 1.5% / 1 - 4 weeks
4407	>1.5 % / 0-4 weeks	2% / 3 - 4 weeks	Counts always > 4 log CFU/g	> 1.75% / 3 - 4 weeks	2% / 1 - 4 weeks, 1.5% / 3 - 4 weeks
4688	> 1.5% / 0-4 weeks	2% / 0 - 4 weeks	Counts always > 2 log CFU/g	2% / 0 - 4 weeks	2% / 0 week, 1.75% / 2 -4 weeks
5279	> 1.75% / 0-4 weeks	counts always > 1 log CFU/g	Counts always > 3 log CFU/g	2% / 0 - 4 weeks	2% / 1 - 4 weeks

4.5 Discussion

Among fresh fruits and vegetables, lettuce appears to be more susceptible to bacterial contamination. Not only have a number of outbreaks caused by *E. coli* O157:H7 (Tauxe et al., 1997) been linked to the consumption of lettuce, but recent evidence suggests that foodborne pathogens can be internalized into lettuce leaves (Solomon et al., 2002). That report provided evidence that O157:H7 could be transmitted from contaminated manure and irrigation water applied to soil into the subsurface tissues of lettuce leaves. Moreover, Wachtel and others (2002) found in their study the predominance of O157:H7 attached to the roots both singly and in small aggregates. Therefore, this study showed for the first time ever the impact of essential oils in organic soil.

Ecotrol® and Sporan® at the lower concentrations (0.5 and 1) reduced *E. coli* O157:H7 by 2 log CFU within 24 h. However, the populations of *E. coli* O157:H7 were increased following 7 days. The effects of these oils at higher levels were more inhibitory and *E. coli* O157:H7 populations reduced further with storage time. These compounds initially may have killed or injured the bacteria by affecting the cellular structures or biochemical pathways and processes of the growing cells. However, after 24 h surviving bacteria multiplied rapidly after recovering from the initial inhibitory effect. Shelef (1980) and Zaika (1984) reported that Food systems due to their complex structures require higher amounts of essential oils or their components than laboratory media. Protein and fat components of foods bind essential oil compounds, reduce their availability, and protect microorganisms from their

antimicrobial action (Shelef, 1984; Raccach, 1984). The same the greater availability of nutrients in organic soils may have enable bacteria to repair cells faster. Not only can the intrinsic but extrinsic factors influence bacterial sensitivity. At a concentration level of 1, 1.5, and 2%, eugenol reduced the bacteria, within the 24hr, but failed to kill them after. Kim (1995) reported similar results with eugenol and showed an inhibitory effect against *E. coli* O157:H7 in liquid media at 1000 ppm but incomplete lethality of the bacteria at that concentration. Moreover, evaporation and homogenization of eugenol may affect the results. Although Tween 20 was used to increase the solubility of this hydrophobic compound, homogenization was still difficult.

Cinnamaldehyde, Ecotrol®, and Sporan®, at 1.5% and 2.0% show large reductions in bacteria. Organic soil due to its complexity and nutrient composition may require large concentrations of essential oils. Zaika and Rios (1988) and Burt (2004) reported that the extent of microbial inhibition by spices and herbs depends on the combination of natural substance (oil), microorganism, and other storage/environmental factors (temperature, humidity, preservatives, etc.). Furthermore, many researchers found that foods due to their complex structures require greater amounts of essential oils or their components than do laboratory media to achieve comparable amounts of bacterial inhibition (Shelef, 1980 and 1984; Zaika 1988).

Cinnamaldehyde had the most potent inhibitory/bactericidal activity against the five strains of *E. coli* O157:H7 followed by acetic acid, Sporan®, Ecotrol® and eugenol. Cinnamaldehyde at 1.5% was highly bactericidal against all five strains of *E. coli*

O157:H7 as evidenced by the 5 log reduction compared with the control. *E. coli* O157:H7 populations in soil were reduced by ca. 5 log CFU/g when acetic acid, Sporan® or Ecotrol® were used at 2.0 % concentration. The inhibitory effect of Eugenol was evident during the initial 24 h only. Strains of *E. coli* O157:H7 re grew (Fig. 4.1).

This study showed that *E. coli* can survive more than 28 days in organic soil. Paul and Clark (1996) reported that soils provide a wealth of nutrients that can be utilized by a variety of microorganisms. The dissolved organic matter in soil is a cocktail of aromatic organic derived from lignin, some oligomeric sugar derivatives derived from cellulose and hemicelluloses, and fatty acids between C₁₄ and C₅₄, believed to derive from both plant wall material and dead bacteria (Huang et al., 1998; Kalbitz et al., 2000). Proteomic analysis has revealed that *Bacillus cereus* cells growing in soil utilize soil-associated carbohydrates, fatty acids and perhaps amino acids (Luo et al., 2007). Therefore, it is not surprising that enteric bacteria are capable of surviving in soil. Some types of soils may act as a reservoir for enteric bacteria. Often, bacteria in soil persist in a stressed state because of their exposure to fluctuations in a wide range of environmental parameters. Some of these stressed cells are occasionally resuscitated by passive internalization in plant structural openings (e.g., stomata, wounds, stem scars), by earthworms, or by ingestion by a mammalian host (Williams et al., 2006). Therefore, development of interventions that can significantly reduce survival of *E. coli* in soil prior to or during crop growth while simultaneously contributing to crop pest control could provide crop producers a useful aid in

reducing potential contamination of fresh organic produce inadvertently contaminated by soil.

Persistence of essential oil constituents in natural environments appears to be limited. Murray (2000) reported that eugenol and other essential oil constituents were not persistent in freshwater laboratory tests. These compounds are also non persistent in soils (Misra and Pavlostathis, 1997). Eugenol is completely degraded to common organic acids by soilborne *Pseudomonas* species (Rabenhorst, 1996). Concerns about essential oil residues on food crops should be mitigated by the growing body of evidence that some essential oil constituents acquired through the diet are actually beneficial to human health (Huang et al., 1994).

Although the antimicrobial properties of essential oils and their components have been reviewed in the past (Koedam, 1977a, b; Shelef, 1983; Nychas, 1995), the mechanism of action has not been studied in great detail (Lambert et al., 2001). It is suggested that the antimicrobial activity of essential oils is attributed to more than one mode of action (Burt, 2004). The mechanism of action of all antimicrobials can be as follow: cell membrane damage, inactivation of essential enzymes and destruction of genetic material (Kim et al., 1995a; Juven et al., 1994; Farag et al., 1989b; Davidson and Branen 1981). Sub- lethal concentrations of eugenol have been found to inhibit production of amylase and proteases by *B. cereus*. Cell wall deterioration and high degree of cell lysis were also noted (Thoroski et al., 1989). The hydroxyl group on eugenol is thought to bind to proteins, preventing enzyme action in *Enterobacter. aerogenes* (Wendakoon and Sakaguchi, 1995). Wendakoon and Sakaguchi (1995) reported that cinnamaldehyde inhibits amino acid decarboxylase enzyme activity in

E. aerogenes. Gill and Holley 2006 reported that plant aromatic oils such as eugenol, carvacrol and cinnamaldehyde inhibited the membrane-bound ATPase activity of *E. coli* and *Listeria monocytogenes*. Previous studies have demonstrated that leaf essential oils from cinnamaldehyde type of *Cinnamomum osmophloeum* had excellent antitermite, antibacterial, antimite, antimildew, antimosquito and antipathogenic activities (Chang et al., 2001; Cheng et al., 2004; Lee et al., 2005).

4.6 Conclusion

The results of this study show the efficacy of essential oils in controlling important foodborne pathogen in soil, and the possibility of extending the application of Ecotrol® and Sporan® to control *E. coli* O157:H7. The significant reduction of *E. coli* could greatly reduce potential contamination of fresh organic produce inadvertently contaminated by soil. Moreover, growers of inorganic produce could apply essential oils to soil in order to avoid pesticide residues in food and thereby reduce exposure to pesticide.

Chapter 5: Inactivation of *Salmonella* in Organic Soil by Cinnamaldehyde, Eugenol, Ecotrol®, and Sporan®

(Published in Yossa et al., Foodborne Pathogens and Disease 8(2) (2011) 311-317)

5.1 Overview

Salmonella can survive in soil for months to years; consequently, soil can be a preharvest source of contamination of produce. Elimination of *Salmonella* with natural products and processes such as essential oils is important to prevent infection among consumers. Essential oils (distilled extract from plants) have been mainly evaluated in liquid medium and foods in which minimum inhibitory concentration (MIC) is determined. However, there are no reports describing the impact of essential oils in soil, especially organic soil. We evaluated essential oils for controlling *Salmonella enterica* serovars in organic soil.

Two essential oils (cinnamaldehyde and eugenol), two bio-pesticides (Ecotrol® and Sporan®) and an organic acid (20% acetic acid) at 0.5%, 1.0%, 1.5% and 2.0%, were mixed with organic sandy soil and inoculated with six different serovars of *Salmonella enterica* separately. Soils were incubated at room temperature and samples obtained at 1, 7 and 28 days were enumerated to determine survival.

The bactericidal effect of cinnamaldehyde was evident at 0.5%, 1.0%, 1.5%, and 2% and during all times of incubation. Overall, *S. Negev* was the most sensitive strain to oils resulting in significant reductions compared with other strains. Increases in oil concentration resulted in further reduction of *Salmonella* with all oils used in the study. Up to six log reductions in *Salmonella* serovars Typhimurium, Negev and

Newport were found after one day when cinnamaldehyde, Ecotrol®, eugenol, Sporan®, or acetic acid was used at 2% level. This study shows the potential use of essential oils to effectively reduce *Salmonella* populations in soil. The significant reduction of *Salmonella* could greatly reduce potential contamination of fresh organic produce inadvertently contaminated by soil.

5.2 Introduction

Approximately 1.4 million cases of *Salmonella* infections occur every year in the U.S., resulting in 15,000 hospitalizations and 580 deaths (Shin, 2006). Cost estimates per case of human salmonellosis range from \$4.6 to \$40 million (WHO, 2005). Salmonellosis in humans is generally contracted through the consumption of contaminated foods of animal origin mainly meat, poultry, eggs and milk, although other food including fresh produce such as lettuce (Gillespie, 2004), tomatoes, cantaloupe, and alfalfa sprouts (Shin, 2006) contaminated with manure or irrigation water have been implicated in its transmission (WHO, 2005). Compared to other bacteria, *Salmonella* has high survival rates in aquatic environments (Chao et al., 1987; Winfield and Groisman, 2003). *Salmonella* can be widely disseminated in soil and sediment, even in the absence of active fertilization, as a result of water currents, underground spring, and rain runoff carrying contaminated material (Abdel-Monem and Dowidar, 1990; Chao et al., 1987).

Produce can become contaminated with pathogenic microorganisms at any point during farm to fork continuum. Potential sources of *Salmonella* contamination on the field could be: field fertilized with untreated manure (Beuchat, 2002) or sewage as a soil amendment, field irrigated with water contaminated with animal and human

waste, water used to apply fungicides and insecticides, wildlife and domestic animal grazing on or near the fields, dust, equipment exposed to contaminated mud or water, transport vehicles, processing equipment, and workers (Western Growers Association, 2010). Non-composted manure or improperly composted manure used on the farm, or manure that enters surface waters, may contain these pathogens and subsequently contaminate produce (Millner, 2009). Eliminating pathogens from livestock, for example by vaccination, may help reduce shedding in manure and consequently reduce the potential for soil and water contamination. However, this method may run into major problems related to adverse immunological reactions in cattle and regulatory issues (Brabban et al., 2004). While such approaches are still being investigated and developed, other researchers are seeking a variety of conventional, novel, and natural alternatives to reduce the risk of produce contamination.

Although a great deal is already known about *Salmonella* spp., these organisms continue to provide new challenges to food safety, particularly because of the evolution of new strains resulting from the acquisition of genes conferring characteristics such as multiple antibiotic resistance (Blackburn and McClure, 2002). Hence, there is a continued need for research and information concerning the reduction of these bacteria. The use of natural products as antibacterial compounds appeals to growers for controlling pathogenic bacteria in organic systems without resorting to traditional agrichemical fumigants, and pesticides (Conner, 1993; Dorman and Deans, 2000). Public concern about the long-term health and environmental effects of synthetic pesticides has increased interest in use of natural

pesticides of both microbial and plant origin in the global market place. Natural insecticides based on essential oils are used by farmers for pest and disease management (Isman, 2006). It has been reported that those plant essential oils not only repel insects, but also have contact and fumigant insecticidal actions against specific pests, and fungicidal actions against plant pathogens (Isman, 2006). However, no study has been done on the impact of those natural pesticides against *Salmonella* in soil.

Essential oils, also called volatiles, are aromatic oily liquids obtained from plant materials (flowers, buds, seeds, leaves, twig bark, herbs, woods, fruits, and roots), which can be obtained by fermentation, extraction, or distillation (Hili et al., 1997; Burt, 2004). Among these natural antimicrobials are eugenol from cloves, thymol from thyme, carvacrol from oregano, allicin from garlic, cinnamic aldehyde from cinnamon, and allyl isothiocyanate from mustard (Tzortzakis, 2009). Previous studies have demonstrated that leaf essential oils of the cinnamaldehyde had excellent antitermite, antibacterial, antimite, antimildew, antimosquito and antipathogenic activities (Chang et al., 2001; Cheng et al., 2004; Lee et al., 2005). Cinnamaldehyde also inhibited the growth of *Clostridium botulinum* (Bowles et al., 1997), *Escherichia coli* O157:H7, and *Salmonella enterica* serovar Typhimurium (Helander et al., 1998) in liquid media. *E. coli* O157:H7 and *Listeria monocytogens* were inhibited when 1000 ppm eugenol was added in Tryptic soy broth (TSB) (Blaszyk and Holley, 1998). Ecotrol®, based on a concentrated blend of 10% rosemary and 2% peppermint oils, is effective against many insects. It can be applied to agricultural crops including vegetables and cole, herbs and spices, citrus, pomes and stone fruits, nuts, berries,

fruits, and grapes (Anonymous, 2005). Sporan® is a fungicide against a broad range of diseases, including blights, molds, scabs, and mildews (Anonymous, 2008). It is composed of rosemary, clove and thyme oils and is suitable for use on agricultural crops. It disrupts the cell membrane of fungal hyphae and spores resulting in cell death. Sporan® and Ecotrol® have been approved by Organic Material Review Institute (OMRI) for application on foliar tissues (Anonymous, 2008).

Organic acids and their salts are promising as antimicrobial agents due to their acceptance in food products and low cost (Miller et al., 1996). Organic acids have been used for controlling pathogens in ready-to-eat meats (Patel et al., 2009) and minimally-processed fruits and vegetables. The antimicrobial activity of organic acids is due to the pH reduction, depression of internal pH of microbial cells, and disruption of substrate transport by altering cell membrane permeability (Beuchat, 1998). This is the first report on the impact of essential oils in organic soil on survival of *Salmonella*. This project was performed to determine the effect of essential oils: cinnamaldehyde, eugenol; natural pesticides: Ecotrol® and Sporan®; and acetic acid on organic soil experimentally contaminated with *Salmonella*.

5.3 Materials and methods

5.3.1 Preparation of bacterial strains

Six *S. enterica* serovars were used in the study. *S. Thompson* 2051H, *S. Tennessee* 2053N, and *S. Negev* 26 H (Thyme isolates) were provided by Tom Hammack (Food and Drug, College Park, MD). *S. Braenderup* (CDC clinical isolate # 95-682-997). *S. Typhimurium*, and *S. Newport* (CDC clinical isolate #9113) were used from our

Environmental Microbial and Food Safety Laboratory culture collection. Bacteria strains were prepared as indicated above.

5.3.2 Essential oil treatments

Cinnamaldehyde (Sigma-Aldrich, St Louis, MO), Ecotrol® (EcoSMART Tech., Alpharetta, GA), eugenol (Fisher Scientific, Pittsburg, PA), Sporan® (EcoSMART Tech), and acetic acid (Fleischmann's, Baltimore, MD) were used in the study. Four individual suspensions with the desired concentrations (0.5%, 1.0%, 1.5% and 2.0%, v/v) were freshly prepared by dispersing them in sterile distilled water containing 0.5% (w/v) Tween 20 (Fisher Scientific) to dissolve essential oil as reported by Burt (2004). A 20% concentration of acetic acid was prepared using sterile distilled water. Unamended soils were also inoculated to serve as controls.

5.3.3 Inoculation of soil

Organic soil (Downer-Ingleside loamy sand, coarse-loamy, siliceous, semiactive, mesic Typic Hapludults) was obtained from the USDA Beltsville Agricultural Research Center North Farm. Soil was mixed, screened to remove stones and debris, and stored in a sterile plastic bag prior to treatment. Soil (10 g) was placed into sterile filter bags (Nasco Whirl-Pak, Fort Atkinson, WI) for each treatment-strain combination, inoculated with 100 μ L of 8 log CFU/ml of designated inocula, and vigorously shaken/massaged to distribute the inoculum thoroughly. Essential oil preparations (0.5%, 1.0%, 1.5%, and 2.0%) were added individually to these filter bags, mixed thoroughly before closure, and incubated at room temperature (22°C) for 28 days.

5.3.4 Enumeration of *Salmonella*

On days 1, 7, and 28, 10ml sterile peptone water (0.1%) was added to each soil bag and the bag was pummeled for 2 min (Bagmixer, Interscience, St. Nom, France). Serially diluted soil suspensions were spiral plated on selective agar (XLT4, Acumedia) and incubated at 37°C for 24 h. Typical *Salmonella* colonies were counted after incubation of 24 h at 37°C. Randomly selected colonies were confirmed by latex agglutination assay (Remel Inc., Lenexa, KS).

5.3.5 Statistical analysis

Colony counts of presumptive *Salmonella* for each sampling period were converted to log CFU/g. The experiment was performed in triplicate. Data were analyzed by a three-way ANOVA using the “Proc Mixed” procedure (SAS 8.2, Cary, NC) for effects of oils, oil concentrations, strains, sampling time, and their interactions. In all cases, the level of statistical significance was $P < 0.05$.

5.4 Results

5.4.1 Inactivation of *Salmonella* in soil after 24 h

Salmonella was not detected in uninoculated soil used in this study. The impacts of treatment with cinnamaldehyde, Ecotrol®, eugenol, Sporan® and acetic acid on *Salmonella* inoculated in soil are presented in Tables 1- 3. Recovery of *Salmonella* after 24 h of antimicrobial treatment varied with the serovar. *Salmonella* populations in untreated soil ranged from 5.92 to 6.36 log CFU/g (Table 1). After 24 h, *Salmonella* populations in soil treated with cinnamaldehyde at 0.5%, 1%, 1.5%, and 2% were undetectable (< 1 log CFU/g), except for Thompson and Tennessee. *Salmonella* serovars recovered in soil treated with Ecotrol® at 0.5% were not significantly different from those populations recovered in untreated soil. Eugenol at

0.5% concentrations reduced Typhimurium and Tennessee serovars in soil by 4 and 2 log CFU/g, respectively. Only *S. Braedenrup* was reduced significantly when 0.5% Sporan® was used in soil. Overall, *Salmonella* populations were reduced with increased concentrations of Sporan®, acetic acid, eugenol, and Ecotrol®. *S. Negev*, Newport, and Thompson serovars were significantly reduced when 1% Ecotrol® was used, whereas, only Braedenrup was significantly reduced in soil treated with 1% eugenol. Likewise, treatment with 1.0% acetic acid reduced ($P < 0.05$) all serovars used in the study; up to 4.8 log reductions in Negev and Newport serovars were observed with 1% acetic acid. *Salmonella* serovar populations in soil treated with 1.5% essential oils were significantly lower than corresponding serovars recovered from soil treated with 0.5% oils with the exception of eugenol. *S. Negev* strain was the most sensitive when treated with 1.5% Ecotrol® or eugenol resulting in ca. 4.5 log reductions. About 5 log reductions in all but Typhimurium serovars were observed with 1.5% Sporan® in soil. Populations of all *salmonella* strains were undetectable (< 1 log CFU/g) in 2% cinnamaldehyde- and acetic acid-treated soil after 24 h. Likewise, complete inhibition of serovars Braedenrup and Typhimurium was observed with 2% Ecotrol®, serovar Negev with 2% eugenol, and serovars Typhimurium, Negev and Newport with 2% Sporan®.

Table 5.1: Impact of essential oils and acetic acid on *Salmonella* in soil after 24 h

		Populations of <i>Salmonella</i> serovars in soil treated with oils*					
Treatment	Conc. (%)	Braedenrup	Typhimurium	Negev	Newport	Thompson	Tennessee
control	0	6.36±0.29 ^{ax}	6.09±0.24 ^{ax}	5.92±0.38 ^{ax}	6.10±0.26 ^{ax}	6.10±0.30 ^{ax}	6.07±0.30 ^{ax}
cinnamaldehyde	0.5	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{bx}	1.05±1.05 ^{bx}	0.67±0.67 ^{cx}
	1	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}	0.00±0.00 ^{dx}	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}
	1.5	0.00±0.00 ^{dx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{dx}	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}
	2	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{dx}	0.00±0.00 ^{cx}
Ecotrol®	0.5	5.73±0.30 ^{abx}	5.94±0.04 ^{ax}	4.71±0.38 ^{ax}	4.97±0.30 ^{ax}	5.37±0.44 ^{ax}	5.93±0.72 ^{ax}
	1	5.06±0.37 ^{ax}	5.09±0.33 ^{ax}	3.85±0.21 ^{bx}	4.40±0.32 ^{bx}	4.34±0.36 ^{bx}	4.72±0.65 ^{ax}
	1.5	3.63±0.47 ^{bx}	2.47±1.37 ^{bxy}	1.62±0.83 ^{by}	3.28±0.64 ^{bxy}	3.13±0.50 ^{bxy}	3.38±0.48 ^{bx}
	2	1.00±0.00 ^{bxy}	0.00±0.00 ^{cy}	0.00±0.00 ^{by}	1.29±1.29 ^{bctx}	2.30±0.30 ^{bctx}	1.06±1.06 ^{bctx}
eugenol	0.5	5.01±0.11 ^{abxy}	2.22±2.22 ^{bz}	5.14±0.28 ^{axy}	5.43±0.75 ^{axy}	5.83±0.37 ^{ax}	4.02±2.09 ^{by}
	1	0.00±0.00 ^{cz}	5.29±0.51 ^{ax}	4.02±1.42 ^{aby}	5.93±0.20 ^{abx}	4.79±0.31 ^{abx}	5.04±0.04 ^{ax}
	1.5	2.49±1.25 ^{bctx}	2.66±1.33 ^{bxy}	1.55±1.55 ^{by}	3.25±0.78 ^{bx}	3.39±1.79 ^{bx}	3.18±1.68 ^{bxy}
	2	0.83±0.83 ^{by}	2.77±0.50 ^{bx}	0.00±0.00 ^{by}	2.90±1.48 ^{bx}	2.96±1.52 ^{bx}	2.59±1.30 ^{bx}
Sporan®	0.5	4.52±0.18 ^{bx}	5.06±0.33 ^{ax}	4.46±0.88 ^{ax}	4.71±0.46 ^{ax}	4.85±0.62 ^{ax}	5.21±0.20 ^{abx}
	1	2.43±1.36 ^{bxy}	3.20±0.20 ^{bx}	0.66±0.66 ^{cz}	2.35±1.19 ^{ctxyz}	1.47±1.47 ^{cyz}	1.56±1.56 ^{bctxyz}
	1.5	0.90±0.90 ^{cdx}	2.08±1.32 ^{bx}	1.00±1.00 ^{bx}	0.90±1.55 ^{cx}	1.27±0.23 ^{bx}	0.90±0.90 ^{cx}
	2	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.88±0.88 ^{cdx}	0.56±0.56 ^{cx}
acetic acid	0.5	5.04±0.46 ^{abx}	5.30±0.27 ^{ax}	5.04±0.11 ^{ax}	4.96±0.52 ^{ax}	5.33±0.09 ^{ax}	4.87±0.75 ^{abx}
	1	3.00±0.68 ^{bxy}	2.44±0.16 ^{bxyz}	1.01±1.01 ^{cz}	1.46±0.80 ^{cdyz}	3.75±0.39 ^{bx}	2.62±0.26 ^{bxyz}
	1.5	0.66±0.66 ^{dxy}	0.00±0.00 ^{cy}	0.56±0.56 ^{bxy}	0.00±0.00 ^{cy}	2.23±0.40 ^{bx}	0.56±0.56 ^{cxy}
	2	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{dx}	0.00±0.00 ^{cx}

* Counts (log CFU/g) ± standard deviation; ^{abc} Means with different letter in the column within the treatment are significantly different (P < 0.05); ^{xyz} Means with different letter in the row are significantly different (P < 0.05).

5.4.2 Inactivation of *Salmonella* in soil after 7 days

After 7 days, *Salmonella* populations remained the same or decreased in most treated samples. *Salmonella* Negev recovered after 7 days from soil treated with 0.5% eugenol, Sporan® or acetic acid were significantly lower than their corresponding populations recovered after 24 h. Conversely, the occasional significant increase was observed in *Salmonella* populations after 7 days, such as increase in *S. Typhimurium* with eugenol at 0.5%; and Braedenrup and Newport with 1.5% Sporan® treatment. *Salmonella* strains in soil treated with cinnamaldehyde were still undetectable at 7 days with concentrations of 0.5%, 1.0%, 1.5%, and 2.0%. *S. Negev* recovered after 7 days in soil treated with 0.5% eugenol, Sporan®, and acetic acid (2.93, < 1, and 2.45 log CFU/g) were significantly lower than those recovered after 24h (5.44, 4.71 and 4.96 log CFU/g), respectively. Similarly, *S. Thompson* strain recovered in soil treated with 0.5% Sporan® after 7 days (2.9 log CFU/g) were lower ($P < 0.05$) than the Thompson strain recovered at 24 h (4.85 log CFU/g). After 7 days incubation, all *Salmonella* serovars were undetectable in soil treated with 1.5 or 2% acetic acid, or with 2.0% Ecotrol® except *S. Braedenrup*. Likewise, *S. Braedenrup*, *S. Typhimurium*, *S. Negev*, and *S. Tennessee* were undetectable in soil treated with 2.0% Sporan®; *S. Negev* and *S. Thompson* were not detectable in soil treated with 2% eugenol.

Table 5.2 : Impact of essential oils and acetic acid on *Salmonella* in soil after 7 days

Treatment	Conc. (%)	Populations of <i>Salmonella</i> serovars in soil treated with oils*					
		Braedenrup	Typhimurium	Negev	Newport	Thompson	Tennessee
Control	0	5.76±0.29 ^{ax}	5.58±0.23 ^{ax}	5.16±0.41 ^{ax}	5.65±0.22 ^{ax}	5.64±0.10 ^{ax}	5.82±0.16 ^{ax}
cinnamaldehyde	0.5	0.00±0.00 ^{bx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}
	1	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}
	1.5	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}	0.00±0.00 ^{dx}
	2	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}
Ecotrol®	0.5	5.57±0.30 ^{ax}	5.09±0.26 ^{ax}	4.90±0.56 ^{ax}	4.88±0.23 ^{abx}	5.31±0.35 ^{ax}	5.63±0.20 ^{ax}
	1	5.43±0.14 ^{ax}	5.52±0.45 ^{ax}	5.02±0.64 ^{ax}	4.92±0.14 ^{ax}	5.22±0.26 ^{ax}	5.71±0.15 ^{ax}
	1.5	4.21±0.66 ^{abx}	4.12±0.31 ^{abx}	0.00±0.00 ^{bz}	0.00±0.00 ^{cz}	2.88±1.45 ^{bxy}	2.10±1.24 ^{cy}
	2	1.00±1.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}
Eugenol	0.5	5.30±0.24 ^{ax}	5.19±0.16 ^{ax}	2.93±1.52 ^{by}	6.22±0.76 ^{ax}	5.27±0.47 ^{ax}	5.79±0.14 ^{ax}
	1	1.49±1.49 ^{bcy}	4.65±0.41 ^{ax}	1.31±1.31 ^{by}	5.41±0.45 ^{ax}	4.89±0.25 ^{ax}	5.36±0.19 ^{ax}
	1.5	3.88±0.38 ^{bxy}	2.63±1.35 ^{byz}	1.31±2.28 ^{bz}	4.43±0.59 ^{ax}	1.70±1.70 ^{bz}	4.11±0.46 ^{bxy}
	2	0.82±0.82 ^{by}	2.77±0.50 ^{cx}	0.00±0.00 ^{by}	3.69±0.70 ^{bx}	0.00±0.00 ^{by}	2.59±1.30 ^{bx}
Sporan®	0.5	4.52±0.09 ^{axy}	5.50±0.22 ^{ax}	0.00±0.00 ^{cz}	4.35±0.31 ^{bxy}	2.90±1.45 ^{by}	4.18±0.06 ^{bxy}
	1	2.95±1.48 ^{bx}	2.56±1.28 ^{bxy}	0.88±0.88 ^{by}	2.62±1.36 ^{bx}	2.24±1.21 ^{bxy}	3.06±1.67 ^{cx}
	1.5	3.21±1.61 ^{bx}	0.00±0.00 ^{cz}	0.00±0.00 ^{bz}	2.41±1.21 ^{bxy}	1.29±1.29 ^{bcyz}	0.00±0.00 ^{dz}
	2	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	1.27±1.27 ^{cx}	0.96±0.96 ^{bx}	0.00±0.00 ^{cx}
Acetic acid	0.5	4.69±0.20 ^{ax}	4.28±0.23 ^{ax}	2.45±1.28 ^{by}	4.27±0.21 ^{bx}	4.99±0.27 ^{ax}	3.84±0.55 ^{bxy}
	1	1.33±1.33 ^{bxy}	0.80±0.80 ^{cx}	1.01±1.01 ^{bxy}	0.00±0.00 ^{cy}	1.08±1.08 ^{bxy}	2.42±1.26 ^{cx}
	1.5	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}	0.00±0.00 ^{dx}
	2	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}

* Counts (log CFU/g) ± standard deviation; ^{abc} Means with different letter in the column within the treatment are significantly different (P < 0.05); ^{xyz} Means with different letter in the row are significantly different (P < 0.05).

5.4.3 Inactivation of *Salmonella* in soil after 28 days

In general, *Salmonella* populations were reduced further with nearly all treatments after 28 days of incubation at room temperature (22°C). *Salmonella* Negev populations detected after 28 days in soil treated with 0.5% Ecotrol®, eugenol or acetic acid were significantly lower than those detected after 7 days. Likewise, populations of Tennessee and Typhimurium serovars detected in soil treated with 1% Ecotrol®, eugenol, or Sporan® were significantly lower than those recovered after 7 days. Occasional increase in populations of some serovars was observed such as of *S. Braenderup* with 1% eugenol and *S. Newport* with 1.5% Ecotrol® treatment. Overall, the increased concentration of the essential oils in the soil was associated with increased bacterial inhibition. *Salmonella* populations were undetectable in soil treated with 1.5 or 2% acetic acid, 2% Sporan®, or 0.5-2% cinnamaldehyde. All but Newport serovars were undetectable in soil treated with 2% Ecotrol®, whereas only *S. Braenderup* populations were undetectable when soil was treated with 2% eugenol.

Table 5.3: Impact of essential oils and acetic acid on *Salmonella* in soil after 28 days

Treatment	Conc. %	Populations of <i>Salmonella</i> serovars in soil treated with oils*					
		Braedenrup	Typhimurium	Negev	Newport	Thompson	Tennessee
control	0	4.91±0.35 ^{ax}	4.8±0.29 ^{ax}	4.01±0.94 ^{ax}	4.63±0.55 ^{ax}	4.66±0.51 ^{ax}	4.67±0.48 ^{ax}
cinnamaldehyde	0.5	0.00±0.00 ^{bx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{bx}	0.00±0.00 ^{bx}
	1	0.00±0.00 ^{dx}	0.00±0.00 ^{cx}	0.00±0.00 ^{dx}	0.00±0.00 ^{cx}	0.00±0.00 ^{cx}	0.00±0.00 ^{dx}
	1.5	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}
	2	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{bx}
Ecotrol®	0.5	4.40±0.05 ^{ax}	4.25±0.20 ^{ax}	1.96±1.00 ^{by}	4.00±0.09 ^{ax}	4.05±0.34 ^{ax}	4.09±0.22 ^{ax}
	1	4.19±0.14 ^{abx}	3.74±0.26 ^{bx}	2.54±0.25 ^{bx}	3.94±0.06 ^{bx}	4.42±0.26 ^{ax}	4.13±0.24 ^{ax}
	1.5	3.69±0.16 ^{ax}	2.97±0.23 ^{bx}	0.56±0.56 ^{by}	2.69±0.03 ^{bx}	3.23±0.88 ^{ax}	2.15±1.08 ^{bxy}
	2	0.00±0.00 ^{bx}	0.72±0.72 ^{bcx}	0.00±0.00 ^{bx}	1.02±1.02 ^{bcx}	0.00±0.00 ^{bx}	0.00±0.00 ^{bx}
eugenol	0.5	4.01±0.36 ^{ax}	4.00±0.73 ^{ax}	0.90±0.90 ^{bcy}	4.12±0.64 ^{ax}	3.94±0.61 ^{ax}	4.34±0.67 ^{ax}
	1	3.92±0.22 ^{bx}	3.92±0.47 ^{bx}	1.20±1.20 ^{bcdy}	3.83±0.33 ^{bx}	4.09±0.35 ^{ax}	4.30±0.42 ^{ax}
	1.5	4.21±0.16 ^{ax}	2.38±1.24 ^{byz}	0.90±0.90 ^{bz}	2.47±1.27 ^{byz}	3.88±0.59 ^{axy}	2.86±1.47 ^{bxy}
	2	0.00±0.00 ^{by}	2.41±0.24 ^{bx}	1.46±0.79 ^{bxy}	2.63±1.40 ^{bx}	1.30±1.30 ^{bxy}	1.21±1.21 ^{bxy}
Sporan®	0.5	4.84±0.48 ^{ax}	4.57±0.48 ^{ax}	0.00±0.00 ^{cy}	4.63±0.33 ^{ax}	4.87±0.38 ^{ax}	4.79±0.46 ^{ax}
	1	2.18±0.48 ^{cxyz}	0.90±0.90 ^{cz}	1.82±0.91 ^{bcyz}	3.32±0.00 ^{bxy}	1.70±0.00 ^{byz}	3.68±0.00 ^{bx}
	1.5	0.56±0.56 ^{bxy}	0.00±0.00 ^{cy}	0.00±0.00 ^{by}	2.22±1.11 ^{bx}	0.00±0.00 ^{by}	0.00±0.00 ^{cy}
	2	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{bx}
acetic acid	0.5	4.11±0.05 ^{ax}	3.23±0.42 ^{ax}	0.96±0.96 ^{bcy}	3.79±0.04 ^{ax}	4.40±0.23 ^{ax}	4.02±0.54 ^{ax}
	1	1.28±1.28 ^{cdxy}	1.43±1.43 ^{cxy}	0.82±0.82 ^{cdxy}	0.00±0.00 ^{cy}	0.00±0.00 ^{cy}	1.69±1.69 ^{cx}
	1.5	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}
	2	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{cx}	0.00±0.00 ^{bx}	0.00±0.00 ^{bx}

* Counts (log CFU/g) ± standard deviation

^{abc} Means with different letter in the column within the treatment are significantly different (P < 0.05)^{xyz} Means with different letter in the row are significantly different (P < 0.05)

5.5 Discussion

Salmonella is an enteric bacterium; animals shed the bacteria in their feces, and soil that contains fresh or incompletely composted manure from wild or domesticated animals can act as a reservoir for the bacteria. Islam et al. (2004) found in their study that the survival profiles of *Salmonella* on vegetables and soil samples contaminated by irrigation water were similar to those observed when contamination occurred through compost. Hence, both contaminated manure compost and irrigation water can play an important role in contaminating soil and root vegetables with *Salmonella* for several months. Multiple studies have shown that *Salmonella* can be isolated from fresh produce, and the prevalence of *Salmonella* in healthy whole fresh vegetables can be as high as 8% (Beuchat, 1996; Doyle, 2000). Therefore, it is of great importance to observe some measures of safety during the preharvest. This is the first study that demonstrates the efficacy of essential oils against salmonella in organic soil.

The inhibitory effect of cinnamaldehyde against *Salmonella* at room temperature was greater than the inhibitory effect of other oils used in this study, exhibiting up to 6 log reduction in *Salmonella* at all concentrations (0.5, 1.0, 1.5, and 2.0%), and at all times (24hr, 7 days and 28 days). Obaidat and Frank (2009) reported that cinnamaldehyde inactivated *Salmonella* and *E. coli* O157:H7 on sliced tomato at 4°C. Raybaudi-Massilia et al. (2009) reported that 0.7% cinnamon oil on fresh cut melons reduced *Salmonella enteritis* by more than 4 log in 21 days. Helander and others (1998) concluded that trans-cinnamaldehyde gained access to the periplasm and to the deeper parts of the bacterial cell, resulting in cell death. Gill and Holley (2004) indicated that cinnamaldehyde

produced a decrease in the intracellular ATP by ATPase activity, resulting in enough disruption of cell membrane to disperse the proton motive force by leakage of small ions (Raybaudi-Massilia et al., 2009).

The effect of Eugenol on *Salmonella* was inconsistent during the trial. For example, up to ca 4 log reduction in *S. Typhimurium* was observed within 24 h in soil treated at 0.5% eugenol, however, its population increased after 7 days. The similar results were observed with Braedenrup serovar in soil treated with 1.5% eugenol. Increase in *Salmonella* populations after 7 days could be due to the repair of injured cells. Smith-Palmer et al. (2001) also reported initial inhibition of *S. enteritidis* with clove oil followed by recovery of this pathogen during the subsequent storage period. Kim and others (1995) also found that eugenol could kill initial bacterial populations by affecting the cellular structures or biochemical reactions of the growing bacterial cells, but once the bacteria overcame the inhibitory effect they multiplied rapidly. The active compound in Ecotrol®, rosemary, is known to possess antimicrobial effect. Some researchers have shown that essential oils of rosemary, sage, and thyme were the most active against *E. coli* (Ouattara et al., 1997; Smith-Palmer et al., 1998).

Sporan® was superior to Ecotrol® and eugenol in reducing *Salmonella* in soil. This could be attributed to the synergetic effects of the active compounds such as rosemary oil, clove oil and thymol present in Sporan®. The bactericidal effect of Sporan® on *Salmonella* serovars was noticeable after 24 h when 1.0% Sporan® was used. Juven et al. (1994) suggested that the inhibition of *S. typhimurium* and *Staphylococcus aureus* by thyme oil was due to the hydrophobic and hydrogen bonding of its phenolic constituents to cell membrane proteins, thereby altering the membrane permeability. Thymol

dissolves in the hydrophobic domain of cytoplasmic membrane and increases the permeability to ATP that results in lethal damage to bacterial cell (Ultee et al., 1999; Burt, 2004).

Salmonella populations reduced over time with increasing concentrations of the various treatments. The study showed a strong correlation between the oil concentration and the antimicrobial efficiency. Acetic acid, Sporan®, Ecotrol® and eugenol showed dose related increases in reducing *Salmonella* in soil samples. Organic soil, due to their complexity and their composition in nutrients might explain the necessity of high concentration of essential oils. Smith-Palmer et al. (2001) found that higher concentrations of oils were needed to completely inhibit *S. enteritis* in high fat cheese. The complex nature of foods compared to laboratory media may allow rapid recovery of injured bacteria (Gill et al, 2002, Rasooli, 2007). Therefore, greater concentrations of essential oils are needed in food, and possibly in soil to achieve the same effect of bacterial inhibition (Smid and Gorris, 1999, Rasooli, 2007).

Although Tween 20 was used in the preparation of the essential oil solution to increase the solubility of the hydrophobic compound and to aid its penetration into bacterial cell wall and membrane, the low efficacy of some of these treatments could be due to the lack of solution homogeneity. Zaika (1988) reported that test medium (i.e. water content, liquid medium, solid medium, food or beverage), oil and its active components (i.e. the process of oil extraction, concentration, geographic origin, climate) and microorganisms tested (inoculation size, origin of culture, strain difference, spore forming) influenced the antimicrobial activity of spices and their extracts, essential oils or active components.

In the absence of antimicrobials, *Salmonellae* were reduced in organic soil by ca 1 log CFU/g ($P < 0.05$) in 28 days which is not uncommon, as it has been reported to survive up to 968 days in soil (Jones, 1986). Other studies have indicated that soil is a possible reservoir for enteric pathogens (Santamaria and Toranzos, 2003) demonstrating that soil can be a possible source of contamination of agricultural products.

The widespread use of pesticides have significant drawbacks including increased cost, handling hazards, concern about pesticide residues on food, and threat to human health and environment (Paster and Bullerman, 1988). Public demand of safer produce has increased interest in investigating on alternative soil preservative to replace synthetic chemical pesticides and to have a synergetic effect with compost. One such alternative is the use of essential oils with pesticidal activity, as well as they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (Paranagama et al., 2003). Soil provides a wealth of nutrients that can be utilized by a variety of microorganisms. Association with soil particles can provide bacteria with high concentration nutrients, due to the release of both organic molecules from attached algal cells, and protection against predation (Fish and Pettibone, 1995). For example, adhesion of *Salmonella* cells to soil particles correlates with cell surface hydrophobicity (Stenstrom, 1989) which is manifested by the modification of the bacterial outer membrane in response to changes in environmental conditions (Winfield and Groisman, 2003). Therefore it is not surprising that enteric bacteria are capable to survive in soil. Many times, cells growing in soils are in a viable but not cultivable state and can easily be resuscitated by internalizing in vegetables, by earthworms, or by coming across a mammalian host (Williams et al., 2006). Therefore, it is important to find a natural

solution for the treatment of organic soil to reduce enteric pathogens. From our results, the use of essential oils, their synergistic effects, and their application in soil should be further evaluated.

5.6 Conclusion

The antimicrobial activity of essential oils in controlling pathogens on fresh produce and other foods has been demonstrated. Soil is one of the major sources of fresh produce contamination at the farm level. In the absence of pathogen kill step in fresh produce processing, it is necessary to minimize its contamination at the pre-harvest level. This study indicated that essential oils can be exploited as an ideal technique for future good agricultural practices. Use of these oils will significantly reduce potential transfer of pathogens from soil to fresh produce and consequently, will help reduce fresh produce related outbreaks and recalls.

Chapter 6: Essential Oils Reduce *Escherichia coli* O157:H7 and *Salmonella* on Spinach Leaves

(Published in Yossa et al. Journal of Food Protection 75 (3) (2012), 488-496).

6.1 Overview

The efficacy of cinnamaldehyde and Sporan® in reducing *E. coli* O157:H7 and *Salmonella* on spinach leaves was investigated. Spinach leaves were inoculated with a five-strain cocktail of *Salmonella* or *E. coli* O157:H7, air-dried for ca. 30 min, and then immersed in a treatment solution containing 5 ppm free chlorine, cinnamaldehyde or Sporan® (800 and 1000 ppm) alone or in combination with 200 ppm acetic acid (20%) for 1 min. Treated leaves were spin-dried and analyzed immediately (day 0) and periodically up to 14-days storage at 4°C. Inoculated leaves washed with water were used as control. The samples were spiral plated on appropriate media for *Salmonella*, *E. coli* O157:H7, gram-negative enteric, mesophilic and psychrotrophic bacterial populations and for yeasts and fungi. Color and texture characteristics of treated leaves were analyzed. Sporan® alone (1000S) or in combination of acetic acid (1000SV) and 800 ppm cinnamaldehyde-Tween (800T) reduced *E. coli* O157:H7 by more than 3 log CFU/g ($P < 0.05$) on spinach leaves following treatment. Likewise, 1000SV treatment reduced *Salmonella* by 2.5 log CFU/g at day 0. *E. coli* O157:H7 and *Salmonella* populations in treated spinach leaves were reduced during storage at 4°C. The 1000SV treatment was superior to chlorine and other treatments in reducing *E. coli* O157:H7 during storage. Saprophytic microbiota on spinach leaves increased during storage at 4°C, but remained lower on Sporan® (800S) and Sporan®-acetic acid (1000SV) treated spinach leaves than on the control spinach leaves. The quality parameters (color and texture) of Sporan®-

treated leaves at 14 days were not significantly different from control-treated spinach leaves. Results show that Sporan® in combination with acetic acid could be used to reduce *E. coli* O157:H7 and *Salmonella* on spinach without affecting the color and texture of spinach leaves.

6.2 Introduction

Contamination of food in the United States results in 9.4 million illnesses, 55,961 hospitalizations, and 1,351 deaths each year (Scallan et al., 2011). Foodborne outbreaks associated with the consumption of some types of fresh and fresh-cut produce have increased in recent years, leading FDA to recognize high risk commodities, such as leafy greens, cantaloupes, tomatoes, and green onions (Burt, 2004). Surveillance data from 1996 to 2008 indicated that about 82 foodborne illness outbreaks were associated with consumption of fresh produce; and 28 of these outbreaks were linked to leafy greens (FDA, 2009). During this period, leafy greens-associated outbreaks accounted for 949 illnesses and 5 deaths. Foodborne illnesses in most of these outbreaks (85.7%) were caused by *E. coli* O157:H7 (FDA, 2009). Outbreaks associated with *Salmonella* and *E. coli* O157:H7 received major attention due to the severity of the illness and occurrence of deaths (Matthews, 2009). Since the 2006 spinach outbreak that sickened 205 people and killed 4 people nationwide, considerable effort and expense by produce growers, handlers and governmental agencies has been expended to enhance programs implementing good agricultural practices and hazard analysis critical control point systems to improve food safety (Doering et al., 2009).

Several factors contributing to the high risk ranking of leafy greens include the fact that they grow in open fields, close to soil that may have been amended with products

containing contaminated animal manure or irrigated with poor quality (Yossa et al., 2011). Transfer of pathogens may also occur directly from animals, birds, and insects (Steele and Odemeru, 2004) or by handling of produce during harvest and immediately post-harvest (Doering, et al., 2009; Matthews, 2009). Because fresh produce often is consumed raw, sanitizing washes are used to clean and disinfect the surfaces of these products. Chlorine is a commonly used sanitizer in washing solutions with initial concentrations ranging from 50 to 200 ppm free chlorine at pH 6.5 with contact times of 20 sec to 2 min (Parish et al., 2003). However, the presence of organic matter in wash water rapidly depletes the concentration of free chlorine available for sanitizing product, and creating the need for frequent replenishment of chlorine (Adams et al., 1989). The cumulative addition of chlorine has raised concerns about the potential for formation of chlorine byproducts on chlorinated wash-water treated commodities (Keskinen et al., 2009).

The efficacy of various produce wash formulations in reducing populations of human pathogens on inoculated spinach has been studied. Lee and Baek (2008) found that spinach treated with 100 ppm chlorine dioxide (ClO_2) or sodium hypochlorite (NaOCl) for 5 min decreased *E. coli* O157:H7 by 2.6 and 1.1 CFU/g, respectively, after 24 h. However, *E. coli* O157:H7 populations in their studies increased during 7 days storage from 2.86 to 6.24 CFU/g with ClO_2 , and from 4.35 to 7.43 CFU/g with NaOCl treatment (Lee and Baek, 2008). Spinach treated with low concentration electrolyzed water (pH 6.2-6.5, 5 ppm available chlorine), strong acid electrolyzed water (pH 2.5-2.7, 50 ppm available chlorine), aqueous ozone (5 ppm), and 1% citric acid for 3 min reduced *E. coli* O157:H7 by 1.60, 1.50, 0.42, and 0.70 CFU/g, respectively (Rahman et al., 2010).

Spinach treated with neutral oxidizing electrolyzing water at 4 ppm and 20 ppm total residual chlorine and a contact time of 10 min reduced *E. coli* populations by 0.44 and 2.62 CFU/g, and *Salmonella* Typhimurium populations by 3.41 and 2.14 CFU/g, respectively (Guentzel et al., 2008). Izumi (1999) found that the microbial load on macerated spinach rinsed with electrolyzed water containing 50 ppm available chlorine for 4 min was reduced by 1.6 CFU/g. Results of these studies showed that the efficacy of the treatment is related to the concentration of chlorine and the contact time. However, recent studies show that pathogens can lodge in stomatal and cut tissue openings that are inaccessible to chlorine sanitizers in wash water (Kordali, 2005).

The demand for safe food, associated with consumer preferences for foods free of synthetic additives, has increased the interest in use of natural preservatives derived from plants. Essential oils possess antibacterial, antifungal, antiviral insecticidal and antioxidant properties (Burt, 2004; Kordali et al., 2005; Prabuseenivasan et al., 2006). Treatment with 1% cinnamaldehyde reduced *Salmonella enterica* serovar. Newport by 1 log CFU/g in celery (Ravishankar et al., 2010). Up to 2 log reduction in *Salmonella* was reported when iceberg lettuce was treated with 1000 ppm myrtle oil for 1 min (Gundez et al., 2008). The antimicrobial effect of 75 ppm oregano oil against *Salmonella enterica* serovar. Typhimurium on lettuce was comparable to the antimicrobial effect of 50 ppm chlorine (Gundez et al., 2010). Sporan®, a broad spectrum, proprietary fungicide commercially available for use on agricultural crops, contains a mixture of essential oils (18 % rosemary, 10 % thyme, and 10 % cloves oil) along with non-fungicidal ingredients. The antibacterial activity of rosemary, thyme and clove oils in broth systems has been reported (Smith-Palmer et al., 1998). The bactericidal concentrations of clove,

thyme, and rosemary oils were 0.075, 0.04, and > 1%, respectively, for *Salmonella enteritidis*; and 0.1, 0.1, and > 1%, respectively, for *E. coli* O157:H7 (Smith-Palmer et al., 1998). Other reports describe evaluations of the antimicrobial activities of essential oils including cinnamaldehyde (MahMoud, 1994; Masuda, 1998; Du et al., 2009; Escalona et al., 2010), cloves (Rhayour et al., 2003; Moreira et al., 2007), rosemary (Del Campo et al., 2000; Moreno et al., 2006; Klancnik et al., 2009) and thymol (Manou et al., 2002) *in vitro* and on some fresh produce. However, no studies have demonstrated their antimicrobial effects on spinach leaves. Furthermore, the effects of Sporan® on fresh produce have not been reported. The purpose of this study was to evaluate the antimicrobial effects of cinnamaldehyde and Sporan® alone, and in combination with acetic acid against *E. coli* O157:H7, *Salmonella*, and the native microbiota of spinach leaves and the resultant food quality parameters of the treated spinach leaves.

6.3 Materials and methods

6.3.1 Preparation of essential oil suspensions

Cinnamaldehyde (> 93%, Sigma-Aldrich, St. Louis, MO), and Sporan® (EcoSMART Technologies, Alpharetta, GA) were used to prepare 800 and 1000 ppm cinnamaldehyde (800C, 1000C) and Sporan® (800S, 1000S) in sterile distilled water (wt/vol), and 800 ppm cinnamaldehyde in 0.5% Tween 20 (800T). Additionally, these oils were used in combination with acetic acid (20%, Fleischmann's Inc., Baltimore, MD) as 800 ppm cinnamaldehyde +200 ppm acetic acid (1000CV) and 800 ppm Sporan® +200 ppm acetic acid (1000SV). A 5 ppm free chlorine solution was made immediately before use by diluting an aliquot of sodium hypochlorite into deionized water and the chlorine concentration was adjusted with a CP-15 chlorine photometer (Chlorine Scientific, Inc.,

Fort Myers, FL). The pH of the chlorine solution was adjusted with 5 ppm acetic acid to 6.5 (Oakton Instruments, Vernon Hills, IL).

6.3.2 Preparation of inocula

Five nalidixic acid resistant strains of *E. coli* O157:H7 and five *Salmonella* strains were used in the study. The *E. coli* strains RM 4406, RM 4688, and RM 1918 (clinical isolates from lettuce outbreaks), RM 4407 (clinical isolate from spinach outbreak), and RM 5279 (bagged vegetable isolate) were obtained from the U.S. Department of Agriculture (Albany, CA). *Salmonella* enterica serovars included Braenderup (CDC clinical isolate # 95-682-997), Newport and Negev 26 H (Thyme isolates), Thompson 2051H and Tennessee 2053N (our laboratory culture collection). Two successive transfers of -80°C cryopreserved cultures were made in tryptic soy broth (TSB, Acumedia, Lansing, MI) and TSB supplemented with 50 ppm nalidixic acid (TSBN), for *Salmonella* and *E. coli* strains, respectively, and then incubated at 37°C for 24 h. Actively growing overnight cultures were centrifuged (7500 g for 10 min, 10°C), and cell pellets were suspended in 0.1 M sterile phosphate buffer (PBS, pH 7.0) to obtain OD_{600} of 1. Equal volumes of individual strains were mixed to prepare cocktails of *E. coli* O157:H7 and *Salmonella* for inoculation studies. Cell concentrations of individual strains were verified by spiral plating (Microbiology International, Frederick, MD) on TSA (Acumedia).

6.3.3 Spinach inoculation and treatment

Bagged spinach was purchased at a retail grocery store and kept at 4°C before the onset of the experiments. Undamaged leaves were aseptically placed on a sterile tray, five spots of 10 μl of the multistrain cocktail (7 log CFU/ml) were distributed on the adaxial surface of spinach leaf and allowed to air dry for ca. 30 min under the hood. Approx. 20 g of air-dried spinach leaves were placed in a beaker containing 60 ml of treatment solution and

washed for 1 min with manual agitation. Treated spinach leaves were air dried in a salad spinner for 1 min and then stored in sterile filter bags (Nasco Whirl-Pak, Fort Atkinson, WI) at 4°C for 14 days. Spinach leaves treated with sterile water served as control.

6.3.4 Enumeration of *E. coli* O157:H7 and *Salmonella*

Surviving populations of *E. coli* O157:H7 and *Salmonella* were determined immediately after wash treatment (day 0) and after storage at 2, 7, and 14 days at 4°C. Five grams of spinach leaves were transferred into stomacher bags containing 45 ml sterile peptone water, sonicated for 30s and then stomached for 2 min (Interscience, St. Norm, France). A 100 µl aliquot of appropriately diluted suspensions were spiral plated (Whitley Scientific, West Yorkshire, England) on XLT4 agar (Acumedia) for *Salmonella*, and on Sorbitol MacConkey media (Acumedia) supplemented with 0.05 mg/l of cefixime, 2.5 mg/l of potassium tellurite and 50 ug/ml nalidixic acid (Sigma-Aldrich, CTSMAC-N) for *E. coli* O157:H7 in duplicate. After 37°C incubation overnight, presumptive *Salmonella*, *i.e.*, black colonies on XLT4, and presumptive *E. coli* O157:H7 straw-color colonies with dark center were then counted using an automated colony counter (Microbiology International Inc., Frederick, MD).

6.3.5 Preparation and enumeration of total microbiota

The effect of cinnamaldehyde and Sporan® on the naturally-occurring culturable microbiota on spinach also was examined. Uninoculated spinach aliquots (20 g) were washed in the different treatment solutions (800C, 800S, 800T, 1000C, 1000CV, 1000S, and 1000SV) as described above and stored at 4°C for up to 14 days. At days 0, 2, 7, and 14, serially diluted suspensions of 5 g spinach leaves were prepared as described above and spiral plated onto TSA (Acumedia) to enumerate mesophilic (incubation at 37°C for 24 h) and psychrotrophic bacteria (incubation at 4°C for up to 8 days); MacConkey Agar

(MAC; Acumedia, incubation at 35°C for gram-negative enteric bacteria), and Dichloran Rose Bengal chloramphenicol (DRBC; Acumedia, incubation at 23°C for 2 - 5 days) for yeast and fungi. Colonies were counted using the automated system described above.

6.3.6 Texture measurement

Texture analysis was conducted on samples treated with essential oils 800C, 800S, 1000C, 1000S and 1000SV at 0, 2, 7, and 10 days. Texture (maximum force measurement values) was determined using the TA-XT2i texture analyzer (Texture Technology Corp. Scarsdale, NY). A 5 g sample was placed into the press holder and a Kramer shear with 5 blade plunger was moved down at 2 mm/s to 1 cm below the bottom of the holder. Maximum peak force (N) was recorded using Texture expert software (version 1.22. Texture Technology Corp.). At least 10 measurements per treatment were recorded.

6.3.7 Color measurement

Color values (L^* , a^* , b^*) of spinach leaves treated with essential oils were measured on days 0, 2, 7, and 14 using a CR-400 chroma meter (Minolta, Inc. Tokyo, Japan). Illuminant D65 and 10° observer angle were used. The instrument was calibrated using a Minolta standard white reflector plate. Five measurements were made on each essential oil-treated spinach leaf (800C, 800S, 1000C, 1000S, and 1000SV).

6.3.8 Statistical analysis

The experiment was repeated three times for each treatment and storage period. Colony counts of *E. coli* O157:H7, *Salmonella*, and native microbiota for each sampling period were converted to log CFU/g. Data were analyzed by a three-way ANOVA using the “Proc Mixed” procedure (SAS 8.2, Cary, NC) for effects of oils, oil concentrations, sampling time, and their interactions. Color and texture data obtained at each sampling

period were analyzed by the 'Proc Mixed' procedure. In all cases, the level of statistical significance was $P < 0.05$.

6.4 Results

6.4.1 Antimicrobial effects of cinnamaldehyde and Sporan® against *E. coli* O157:H7

Initial *E. coli* O157:H7 populations on inoculated spinach leaves were 4.77 log CFU/g. Washing spinach with sterile distilled water (control) removed ~1.5 log CFU/g *E. coli* O157:H7 from inoculated spinach samples. Cinnamaldehyde at 800 ppm alone or in combination with acetic acid did not significantly reduce *E. coli* O157:H7 on spinach leaves compared to control (Table 6.1). Likewise, *E. coli* O157:H7 populations recovered from 800 ppm Sporan®-treated spinach (2.30 log CFU/g) were not different ($P > 0.05$) from control (sterile water) treated spinach (3.27 log CFU/g). *E. coli* O157:H7 populations were further reduced with increased concentrations of cinnamaldehyde and Sporan® from 800 to 1000 ppm; however, the difference was not significant. Combination of 200 ppm acetic acid with cinnamaldehyde did not influence reduction of *E. coli* O157:H7 on spinach; however, the effect of acetic acid was evident when combined with Sporan®. *E. coli* O157:H7 populations of Sporan® + acetic acid (1000SV) treated leaves (1.38 log CFU/g) were significantly lower than the populations of only Sporan® (800S) treated leaves. The most effective treatments were Sporan® + acetic acid (1000SV) and cinnamaldehyde with Tween (800T) that reduced *E. coli* O157:H7 populations on spinach leaves by 3.39 and 3.23 log CFU/g, respectively, from initial *E. coli* O157:H7 populations (4.77 log CFU/g, data not shown). More than 2.5 log reduction was observed with Sporan® (1000S) and 5 ppm chlorine treated spinach

leaves. Lower concentrations of cinnamaldehyde and Sporan® (≤ 600 ppm) were not effective at all in reducing *E. coli* O157:H7 on spinach leaves (data not shown).

At day 2, *E. coli* O157:H7 populations recovered from control samples (2.59 log CFU/g) were similar ($P > 0.05$) to those recovered at day 0. Reduced recovery of *E. coli* O157:H7 was observed by 2 days storage in all treated samples. Populations of 5 ppm chlorine, 800C, 800T, and 1000S-treated spinach recovered at 2 days were significantly lower than their corresponding populations detected at day 0. *E. coli* O157:H7 populations of 800T, 1000S and 1000SV treated spinach were at least 2 log CFU/g lower than the populations of control samples.

E. coli O157:H7 on spinach leaves were reduced additionally during storage for 7 days at 4°C. *E. coli* O157:H7 populations of control spinach (1.39 log CFU/g) were significantly lower than the populations recovered at day 0 (3.27 log CFU/g). Likewise, *E. coli* O157:H7 populations of treated spinach leaves stored for 7 days were significantly lower than the populations of correspondingly treated spinach leaves recovered at day 0. *E. coli* O157:H7 populations recovered from spinach leaves following treatment with 5 ppm chlorine (0.33 log CFU/g), 800T (0.23 log CFU/g) and 1000SV (non-detectable) were significantly lower than those from the control samples (1.39 log CFU/g). By day 14, *E. coli* O157 populations again were reduced on all but the 5 ppm chlorine-treated samples. *E. coli* O157:H7 populations on control spinach leaves (1 log CFU/g) were similar ($P > 0.05$) to the populations on 7 day-stored spinach. *E. coli* O157:H7 was non-detectable by 14 days on stored spinach when treated with Sporan® alone (800 or 1000 ppm) or in combination with 200 ppm acetic acid, and cinnamaldehyde at 1000ppm. Populations on all treated spinach were not significantly different when compared to populations of

corresponding treated samples at day 7. Recovery of *E. coli* O157:H7 was significantly lower for all samples after 14 days compared to the *E. coli* O157:H7 recovered on day 0.

Table 6.1: *E. coli* O157:H7 populations on spinach leave immediately after treatment with essential oils and upon subsequent storage at 4°C ^A

Treatment	<i>E. coli</i> O157:H7 in log CFU/g				
	Conc. (ppr	Day 0	Day 2	Day 7	Day 14
Control	0	3.27±0.51 ^{ax}	2.59±0.31 ^{ax}	1.39±0.09 ^{aby}	1.00±0.65 ^{ay}
Chlorine	5	2.12±0.46 ^{bcdx}	0.80±0.72 ^{cdefy}	0.33±0.57 ^{cy}	0.96±0.24 ^{ay}
Cinnamaldehyde	800 C	2.60±0.36 ^{abcx}	1.39±1.20 ^{bcdy}	0.39±0.68 ^{bcyz}	0.23±0.40 ^{az}
Cinnamaldehyde +Tween	800 T	1.54±0.53 ^{dx}	0.39±0.68 ^{edefy}	0.23±0.40 ^{cy}	0.23±0.00 ^{ay}
Sporan®	800 S	2.30±0.67 ^{abcdx}	1.35±0.56 ^{bcdxy}	0.66±0.65 ^{bcyz}	ND ^{az}
Cinnamaldehyde	1000 C	2.15±0.67 ^{bcdx}	1.67±0.90 ^{abcxy}	0.70±0.70 ^{abcyz}	ND ^{az}
Sporan®	1000 S	1.78±0.75 ^{cdx}	0.23±0.40 ^{ey}	0.23±0.40 ^{cy}	ND ^{ay}
Cinnamaldehyde + acetic ac	1000CV	3.04±0.75 ^{abx}	2.07±0.41 ^{abxy}	1.69±0.18 ^{ayz}	0.39±0.68 ^{az}
Sporan® + acetic acid	1000SV	1.38±1.28 ^{dx}	0.55±0.95 ^{dexy}	ND ^{cy}	ND ^{ay}

^a Initial *Salmonella* populations on unwashed spinach were 4.77 log CFU/g

^b Values are means ± SD. Each experiment was replicated three times. Within a row, means not followed by the same letters (xyz) are significantly different; within a column, means not followed by same letters (abcd) are significantly different (P < 0.05). ND, not detected (below detection limit of 0.23 g CFU/g).

6.4.2 Antimicrobial effects of cinnamaldehyde and Sporan® against *Salmonella*

Initial *Salmonella* populations on inoculated spinach leaves were 4.80 log CFU/g, and washing with sterile deionized water (control) removed ~1 log CFU/g (Table 6.2). Treatment with 5 ppm chlorine reduced *Salmonella* on spinach leaves by 0.7 log CFU/g compared to control; however, the difference was not statistically significant. While all treatments reduced *Salmonella* populations compared to those in the control, the effect only was significant with 1000C and 1000SV. Increased concentration of acetic acid (up to 500 ppm) in combination with 800 ppm cinnamaldehyde or Sporan® did not influence *Salmonella* population reduction on spinach leaves (data not shown).

By day 2, *Salmonella* populations on control leaves (3.40 log CFU/g) were not different from the populations recovered on day 0 (3.67 log CFU/g). All treatments further reduced *Salmonella* populations on spinach leaves on day 2 compared to control. The difference was significant when spinach was treated with 5 ug/ml chlorine (1.5 log CFU/g) or Sporan® + acetic acid (1000SV, 2.18 log CFU/g)). *Salmonella* populations recovered from chlorine (5 ppm) or 1000S – treated spinach leaves after 2 days of storage were significantly lower than their corresponding populations recovered on day 0.

At day 7, *Salmonella* populations on control spinach leaves (2.45 log CFU/g) were similar to all treatment samples with the exception of the 5 ppm chlorine treatment. In most cases, recovery of *Salmonella* at day 7 of storage was not significantly different from the *Salmonella* recovered at day 2 from the same treatments. All treatments reduced *Salmonella* compared to control (1.92 log CFU/g) by 14 days storage; the effect of 1000 SV (0.66 log CFU/g) was significantly different from control samples.

Salmonella populations detected by 14 days were significantly lower compared to the populations recovered at day 0 for all treatments, including the control. Up to 2 log additional reduction was observed by 14 days storage in spinach leaves treated with Sporan® (800 or 1000 ppm) or cinnamaldehyde with Tween-20 (800 T).

Table 6.2: *Salmonella* populations on spinach leaves immediately after treatment with essential oils and upon subsequent storage at 4°C^A

Treatment	<i>Salmonella</i> in log CFU/g				
	Conc. (ppm)	Day 0	Day 2	Day 7	Day 14
Control	0	3.67±0.24 ^{ax}	3.40±0.26 ^{axy}	2.45±0.72 ^{ayz}	1.92±0.87 ^{az}
Chlorine	5	2.96±0.07 ^{abcx}	1.50±1.48 ^{cy}	0.97±1.14 ^{by}	0.96±0.24 ^{aby}
Cinnamaldehyde	800 C	3.08±1.14 ^{abcxy}	3.37±0.47 ^{ax}	2.28±1.50 ^{ayz}	1.34±1.79 ^{abz}
Cinnamaldehyde + Tween	800 T	3.29±0.78 ^{abcx}	2.69±0.91 ^{abx}	2.69±0.96 ^{ax}	1.18±1.29 ^{aby}
Sporan®	800 S	3.07±0.81 ^{abcx}	2.82±0.06 ^{abx}	3.02±1.05 ^{ax}	0.96±0.87 ^{aby}
Cinnamaldehyde	1000 C	2.61±1.21 ^{bcx}	2.64±0.77 ^{abx}	2.19±1.18 ^{ax}	1.66±1.53 ^{abx}
Sporan®	1000 S	3.59±0.55 ^{abx}	2.54±0.27 ^{aby}	2.61±0.62 ^{ay}	1.53±0.61 ^{abz}
Cinnamaldehyde + acetic acid	1000CV	3.06±1.07 ^{abcx}	2.86±0.46 ^{abxy}	2.97±0.96 ^{ax}	1.85±1.08 ^{ay}
Sporan® + acetic acid	1000SV	2.38±1.22 ^{cx}	2.18±0.90 ^{bcx}	2.24±0.10 ^{ax}	0.66±0.57 ^{by}

^a Initial *Salmonella* populations on unwashed spinach were 4.80 log CFU/g

^b Values are means ± SD. Each experiment was replicated three times. Within a row, means not followed by the same letters (xyz) are significantly different; within a column, means not followed by same letters (abcd) are significantly different (P < 0.05). ND not detected (below detection limit of 0.23 g CFU/g).

6.4.3 Effect of cinnamaldehyde and Sporan® on natural microbiota of spinach

The effect of antimicrobials on different bacterial groups (mesophilic, gram-negative enteric, psychrotrophic), as well as yeasts and fungi on spinach are shown in Tables 6.3-6. The initial effect of cinnamaldehyde and Sporan® on natural microbiota was not significantly different compared to control (water) or chlorine.

Mesophilic bacterial populations in spinach leaves were 6.40 log CFU/g. Mesophilic bacterial populations recovered in cinnamaldehyde- or Sporan®-treated spinach leaves were lower ($P > 0.05$) compared to those in the control or chlorine treatments. Combination of Sporan® with acetic acid (1000SV) was the most effective treatment resulting in 0.6 log/g reduction of mesophilic bacteria. Similar to day 0 results, populations of treated spinach leaves were marginally lower to that of control samples (6.72 log CFU/g) by 2 days storage at 37°C. Recovery of mesophiles reached its low limit in 1000C-treated spinach leaves (5.92 log CFU/g) by 2 days storage. The mesophilic bacterial population increased during storage irrespective of control or treatment. Mesophilic bacteria counts in 14 day-stored spinach leaves were significantly greater in all treated samples compared to those from 0 day samples. Up to 2.2 log CFU/g increase in bacterial populations was observed at 14 days storage in some treated samples.

Cinnamaldehyde and Sporan® marginally reduced gram negative enteric bacterial populations compared to those from control or 5 ppm chlorine treatment. Combination of acetic acid did not influence Gram negative enteric bacterial populations of spinach. Likewise, increase in concentrations of cinnamaldehyde and Sporan® from 800 to 1000 ppm did not lead to additional reductions in the populations of these bacteria. Similar to

mesophilic bacterial populations, Gram negative enteric bacterial counts also increased with storage. By 2 days, recovery of Gram negative bacteria was low (5.79 log CFU/g) in 1000 ppm cinnamaldehyde-treated spinach leaves. Gram negative bacterial populations increased significantly by 14 days compared to day 0 in all control and treated spinach leaves. More than 2 log/g increase in these populations was observed by 14 days storage in cinnamaldehyde or Sporan® treated spinach leaves.

None of the treatments significantly reduced psychrotrophs from spinach leaves (Table 6.5). Chlorine (5 ppm) and 1000 ppm cinnamaldehyde were the most effective treatments in reducing psychrotrophic bacteria at day 0, however, populations of psychrotrophs increased during storage. Psychrotroph populations recovered in 1000 ppm cinnamaldehyde-treated spinach leaves at 2 (6.36 log CFU/g) and 7 days storage (7.19 log CFU/g) were lower ($P > 0.05$) than the populations of other stored, treated samples. By 14 days of storage, psychrotrophic bacterial counts were marginally lower on spinach treated with 800 ppm Sporan® alone (7.87 log CFU/g) or in combination with acetic acid (7.81 log CFU/g) compared to the counts on spinach washed with sterile water (8.29 log CFU/g). Psychrotrophic bacteria counts at 14 days were significantly higher in all treatment samples compared to day 0 with the exception of 800 ppm Sporan®.

The effect of cinnamaldehyde or Sporan® was not significant on yeast and fungal populations, nevertheless, these oils were superior to chlorine in reducing yeasts and fungi (Table 6.6). Spinach treated with 1000 ppm cinnamaldehyde (5.75 and 6.12 log CFU/g), 800 ppm Sporan® alone (6.04 and 6.19 log CFU/g) or in combination with acetic acid (5.69 and 6.15 log CFU/g at 1000SV) reduced the yeast and fungal loads compared to unwashed (6.21 and 6.67 log CFU/g) and sterile water-washed leaves (6.24

and 6.32 log CFU/g) at day 0 and 2, respectively. Yeast and fungal counts were significantly greater 1 on day 14 compared to the initial counts (day 0) on control or treated spinach leaves.

Table 6.3: Mesophilic aerobic bacteria

Treatment	Concn	Time (days)			
		0	2	7	14
Unwashed		6.48±0.12ax	6.66±0.13ax	6.78±0.50ax	7.59±0.04ax
Control	0ppm	6.49±0.20ay	6.72±0.12ay	7.39±0.68axy	8.13±0.33ax
Chlorine	5ppm	6.24±0.17ay	6.24±0.17ay	7.01±0.57axy	7.98±0.53ax
Cinnamaldehyde	800C	6.13±0.14az	6.29±0.07ayz	7.36±0.75axy	8.40±0.66ax
Cinnamaldehyde + Tween	800T	6.08±0.48az	6.46±0.05ayz	7.44±0.74axy	8.29±0.74ax
Sporan®	800S	6.05±0.14az	6.36±0.57ayz	7.40±0.53axy	7.61±0.36ax
Cinnamaldehyde	1000C	5.87±0.14ay	5.92±0.06ay	7.41±0.78ax	8.21±0.90ax
Cinnamaldehyde + Acetic acid	1000CV	6.11±0.19az	6.32±0.49ayz	7.40±0.75axy	8.29±0.50ax
Sporan®	1000S	6.12±0.28az	6.57±0.36ayz	7.31±0.52axy	8.37±0.70ax
Sporan® + Acetic acid	1000SV	5.80±0.41az	6.34±0.34ayz	7.26±0.45axy	7.77±0.07ax

Values abc represent difference among the treatments, and xyz difference of the treatment during the time.

Table 6.4: Gram negative bacteria

Treatment	Concn	Time (days)			
		0	2	7	14
Unwashed		6.25±0.29ax	6.32±0.40ax	6.74±0.14ax	7.25±0.01bx
Control	0ppm	6.04±0.24ay	6.18±0.44ay	7.12±0.98axy	7.83±0.58abx
Chlorine	5ppm	5.96±0.38ay	5.96±0.38ay	6.69±0.41axy	7.61±1.25abx
Cinnamaldehyde	800C	5.63±0.02ay	5.98±0.51ay	7.35±0.82ax	8.36±0.74abx
Cinnamaldehyde + Tween	800T	5.74±0.48az	6.43±0.05ayz	7.19±1.06ay	8.37±0.67abx
Sporan®	800S	5.58±0.27az	6.16±0.54ayz	7.08±0.55axy	7.64±0.13abx
Cinnamaldehyde	1000C	5.33±0.57ay	5.79±0.07ay	7.43±0.82ax	8.41±0.64abx
Cinnamaldehyde +	1000CV	5.66±0.02az	6.10±0.60az	7.31±0.87ay	8.49±0.81ax

Acetic acid

Sporan®	1000S	5.85±0.12az	6.19±0.59ayz	7.17±0.49axy	8.22±0.62abx
Sporan® + Acetic acid	1000SV	5.68±0.22az	6.11±0.38ayz	7.06±0.35axy	7.65±0.04abx

Values abc represent difference among the treatments, and xyz difference of the treatment during the time.

Table 6.5: Psychrotrophic bacteria

Treatment	Concn	Time (days)			
		0	2	7	14
Unwashed		7.21±0.86ax	7.15±0.62ax	7.10±0.32ax	7.77±0.22ax
Control	0ppm	7.12±0.98axy	6.53±0.25ay	7.45±0.54axy	8.29±0.33ax
Chlorine	5ppm	6.21±1.52ay	6.71±0.78ay	7.27±0.71axy	8.13±0.71ax
Cinnamaldehyde	800C	6.69±1.32ay	6.75±0.45ay	7.42±0.88axy	8.42±0.54ax
Cinnamaldehyde + Tween	800T	6.59±1.53ay	7.08±0.43ay	7.49±0.79axy	8.32±0.91ax
Sporan®	800S	6.79±1.08axy	6.68±0.15ay	7.56±0.65axy	7.87±0.27ax
Cinnamaldehyde	1000C	6.21±1.33ay	6.36±0.79ay	7.19±1.16ay	8.84±0.02ax
Cinnamaldehyde + Acetic acid	1000CV	6.61±1.09ay	7.02±0.40ay	7.26±0.80ay	8.64±0.73ax
Sporan®	1000S	6.69±1.06ay	6.87±0.76ay	7.40±0.45axy	8.50±0.49ax
Sporan® + acetic acid	1000SV	6.49±1.36ay	6.84±0.04axy	7.50±0.61axy	7.81±0.48ax

Values abc represent difference among the treatments, and xyz difference of the treatment during the time.

Table 6.6: Yeast and molds

Treatment	concn	Time (days)			
		0	2	7	14
Unwashed		6.21±0.29 ^{ay}	6.67±0.87 ^{axy}	6.71±0.05 ^{axy}	7.58±0.20 ^{ax}
Control	0ppm	6.24±0.29 ^{ay}	6.32±0.04 ^{ay}	7.01±0.21 ^{axy}	7.93±0.41 ^{ax}
Chlorine	5ppm	6.22±0.43 ^{ay}	6.42±0.77 ^{ay}	7.12±0.15 ^{axy}	7.89±0.76 ^{ax}
Cinnamaldehyde	800C	6.05±0.46 ^{az}	6.47±0.45 ^{ayz}	7.32±0.57 ^{axy}	8.31±0.53 ^{ax}
Cinnamaldehyde + Tween	800T	5.95±0.67 ^{az}	6.67±0.77 ^{ayz}	7.40±0.73 ^{axy}	8.12±0.57 ^{ax}
Sporan®	800S	6.04±0.44 ^{ay}	6.19±0.10 ^{ay}	7.20±0.26 ^{axy}	7.73±0.14 ^{ax}
Cinnamaldehyde	1000C	5.75±0.52 ^{ay}	6.12±0.77 ^{ay}	7.45±0.64 ^{ax}	8.29±0.52 ^{ax}
Cinnamaldehyde + Acetic acid	1000CV	6.02±0.48 ^{az}	6.72±0.53 ^{ayz}	7.38±0.46 ^{axy}	8.30±0.70 ^{ax}
Sporan®	1000S	6.03±0.40 ^{az}	6.74±0.65 ^{ayz}	7.25±0.18 ^{axy}	8.11±0.34 ^{ax}
Sporan® + acetic acid	1000SV	5.69±0.70 ^{az}	6.15±0.09 ^{ayz}	7.23±0.10 ^{axy}	7.71±0.09 ^{ax}

Values abc represent difference among the treatments, and xyz difference of the treatment during the time.

6.4.4 Quality parameters of cinnamaldehyde and Sporan® treated spinach leaves

The effect of antimicrobials on the texture (maximum force measurements, N) of treated spinach leaves are shown in Table 6.7. The initial N value for control spinach (113) was not significantly different from the N values for cinnamaldehyde- or Sporan®-treated spinach leaves (101-118). The N values of day 2 samples were similar ($P > 0.05$) to N values of corresponding treatment samples at day 0. At 7 days, the N values of spinach treated with 800 ppm cinnamaldehyde (105) were significantly lower than the N values of 1000 ppm cinnamaldehyde-treated spinach (120) and 1000SV-treated spinach (123). The maximum force required for breakage of leaves treated with Sporan® at different concentrations was not different for 0, 2, and 7 and 10 days at their corresponding concentrations. In general, more force was required to break the leaves when samples were analyzed after 10 days storage at 4°C in all samples. Cinnamaldehyde affected the texture of spinach leaves when stored for 10 days. After 10 days of storage, maximum force requirements (N) for spinach leaves treated with cinnamaldehyde at 800 or 1000 ppm (126) were significantly higher than the initial N values of spinach leaves treated with 800 or 1000 ppm cinnamaldehyde (105).

The changes in color of spinach leaves following treatment were measured using Hunter L, a, b values (Table 6.8). The lightness of the standard (L) was not significantly affected by cinnamaldehyde or Sporan® at day 0 and throughout the storage study of 14 days. The spinach treated with 800 ppm Sporan® had the lowest L value ($L = 38$) compared to spinach treated with 800 ppm cinnamaldehyde ($L = 43$). The greenness values of treated spinach leaves were not different ($P < 0.05$) from

control at day 0 with the exception of 1000SV treated spinach leaves (-22). The values of the greenness (a value) ranged from -17 to -18 for control during the entire storage period. Likewise, greenness values of treated samples were not different from those of control samples at day 2, 7, and 14. The yellowness (b value) of control leaves at day 0 was 28, which was significantly different from the 'b' values of 1000 ppm cinnamaldehyde-treated leaves (25). Spinach treated with 1000SV resulted in yellowing of the leaves (b = 30) at day 0, and this value was significantly greater than the 'b' values of leaves treated with Sporan® alone (b=25). Acetic acid seemed to be responsible for yellowing the leaves when combined with Sporan®. In addition, there was a significant difference in yellowness values of treated leaves compared to control leaves. On day 7, samples treated with 800 ppm Sporan® had a yellowness value of 31, which was significantly greater than the yellowness values of 800 ppm Sporan®-treated samples obtained at day 0 (25) and day 14 (26).

Table 6.7: Maximal Force (N) measurements of spinach leaves treated with cinnamaldehyde and Sporan® ^A

Treatment	Conc (ppm) ^C	Maximum Force			
		Values (N) ^B			
		Day 0	Day 2	Day 7	Day 10
control	0 ppm	113±13 ^{ax}	108±12 ^{ax}	110±16 ^{abx}	124±13 ^{ax}
cinnamaldehyde	800C	105±14 ^{ay}	108±18 ^{ay}	105±05 ^{by}	126±10 ^{ax}
Sporan®	800S	102±08 ^{ax}	108±19 ^{ax}	108±19 ^{abx}	118±13 ^{ax}
cinnamaldehyde	1000C	105±11 ^{ay}	103±15 ^{ay}	120±17 ^{abxy}	126±14 ^{ax}
Sporan®	1000S	110±19 ^{ax}	110±16 ^{ax}	109±09 ^{abx}	124±11 ^{ax}
Sporan® -acetic a.	1000SV	118±24 ^{ax}	110±22 ^{ax}	123±12 ^{ax}	124±12 ^{ax}

^A Each experiment was replicated ten times.

^B Values in the same row not followed by the same letters (xyz) are significantly different; values in the same column not followed by same letters (ab) are significantly different (P < 0.05).

^C 800C – 800 ppm cinnamaldehyde, 800S –800 ppm Sporan®, 1000C – 1000 ppm cinnamaldehyde, 1000S – 1000 ppm Sporan®, 1000SV - 800 ppm Sporan®+ 200 ppm acetic acid

Table 6. 8: Color measurements of spinach leaves treated with cinnamaldehyde and Sporan® ^A
Color Parameters ^B

Conc. ^C	Day 0			Day 2			Day 7			Day 14		
	L*	a*	b*	L*	a*	b*	L*	a*	b*	L*	a*	b*
Control	42±2 ^{abx}	-18±1 ^{abx}	28±2 ^{abcx}	44±3 ^{ax}	-18±2 ^{ax}	28±3 ^{ax}	43±5 ^{ax}	-18±1 ^{ax}	29±6 ^{ax}	43±2 ^{ax}	-18±1 ^{ax}	30±2 ^{ax}
800C	43±2 ^{ax}	-19±2 ^{abx}	29±3 ^{abx}	42 ^a ±3 ^x	-17 ^a ±1 ^x	27±2 ^{ax}	43±4 ^{ax}	-18±1 ^{ax}	29±3 ^{ax}	43±3 ^{ax}	-18±1 ^{ax}	30±4 ^{ax}
800S	38 ±3 ^{by}	-17±2 ^{abx}	25±5 ^{bcy}	42±3 ^{axy}	-17±1 ^{ax}	26±3 ^{axy}	45±4 ^{ax}	-19±2 ^{ax}	31±5 ^{ax}	42±1 ^{axy}	-17±1 ^{ax}	26±2 ^{ay}
1000C	40±4 ^{abx}	-16±2 ^{ax}	25±4 ^{cy}	42±2 ^{ax}	-17±1 ^{ax}	26±3 ^{axy}	43±3 ^{ax}	-18±1 ^{ax}	29±3 ^{ax}	44±3 ^{ax}	-17±2 ^{ax}	29±4 ^{ax}
1000S	42±2 ^{abx}	-18±1 ^{abx}	29±1 ^{abcx}	43±3 ^{ax}	-18±1 ^{ax}	28±2 ^{ax}	44±2 ^{ax}	-17±1 ^{ax}	29±3 ^{ax}	44±4 ^{ax}	-18±1 ^{ax}	29±3 ^{ax}
1000SV	43±1 ^{abx}	-22±4 ^{cy}	30±5 ^{ax}	43±4 ^{ax}	-17±2 ^{ax}	28±4 ^{ax}	43±4 ^{ax}	-18±1 ^{ax}	29±3 ^{ax}	45±7 ^{ax}	-18±4 ^{ax}	30±6 ^{ax}

^A Each experiment was replicated ten times.

^B Values in the same row for the same parameter not followed by the same letters (xyz) are significantly different; values in the same column not followed by same letters (ab) are significantly different (P < 0.05).

^C 800C – 800 ppm cinnamaldehyde, 800S –800 ppm Sporan®, 1000C – 1000 ppm cinnamaldehyde, 1000S – 1000 ppm Sporan®, 1000SV - 800 ppm Sporan®+ 200 ppm acetic acid

6.4 Discussion

Sporan® and cinnamaldehyde alone and in combination with acetic acid were evaluated to determine the survival of *E. coli* O157:H7, *Salmonella* and native bacteria, yeasts, and fungi on spinach leaves following 1 min wash and during 14 days storage at 4°C. The antimicrobial efficacy of these compounds was influenced by their concentrations, pathogens, and storage period. Washing spinach leaves in 5 ppm free chlorine for 1 min reduced *E. coli* and *Salmonella* populations by 1.15 and 0.71 log CFU/g, respectively, compared to the controls. Chlorine was used at pH 6.5 because at that pH hypochlorous acid (HOCl) is highly biocidal and this pH is commonly used in commercial leafy green washing solutions. Some researchers have increased the contact time and sanitizer concentration to achieve a significant reduction in pathogens. For example, Guentzel et al. (2008) found that 10 min dip treatment of spinach leaves in 120 ppm chlorine resulted in a 79-100% reductions in populations of all organisms tested. Another study also reported increased effectiveness of the near neutral electrolyzed water with an increase in contact time (Jirovetz et al., 2006).

Among 11 essential oils analyzed, Gutierrez et al. (Izumi, 1999) found that oregano and thyme oils had the highest antimicrobial activity against *Bacillus cereus*, *Escherichia coli*, *Lactobacillus* spp., *Listeria* spp., *Pseudomonas* spp.. Moreira et al. (2005) evaluated the antimicrobial activity of the essential oils of eucalyptus, tea tree, rosemary, mint, rosa moschata, clove, lemon, oregano, pine, and sweet basil on *E. coli* O157:H7 and found that minimum inhibitory and bactericidal concentration for clove was 0.25 % and 0.3 %, respectively. However, increased concentrations are needed to achieve the same effects in food systems as in *in vitro* assays (Shelef, 1983; Smid and Gorris, 1999; Keskinen et al., 2009). The greater availability of

nutrients in foods compared to laboratory media may enable bacteria to repair damaged cells faster (Guentzel et al., 2008). Moreira et al. (2007) found that 3 minimum inhibitory concentrations (MIC) of clove oils were needed to achieve a 2 log reduction in *E. coli* O157:H7 populations in blanched spinach during storage at 20-22 °C. The mode of action of these essential oils has not been studied in details. It has been suggested that the antimicrobial activity of essential oils is attributed to more than one mechanism (Burt, 2004; Moreira et al., 2005). Thus combining essential oils could lead to synergistic effect against both spoilage and pathogenic target organism. A concentration of 1000S reduced *E. coli* populations by 1.49 and 2.36 at day 0 and 2 compared to the control. On the other hand, the same concentration did not reduce ($P < 0.05$) *Salmonella* populations. In our study, *E. coli* O157:H7 strains were more sensitive to Sporan® than was *Salmonella*. It is possible that one or more of the *Salmonella* strains used in cocktail may be resistant to Sporan®. Yossa et al. (2011) reported differences in sensitivity of *Salmonella* serovars to Sporan® when inoculated in organic soil. Differential attachment of these *Salmonella* strains to fresh produce has been reported (Patel and Sharma, 2010). Since Sporan® is a mixture of oils composed of phenolic compounds such as thymol, carvacrol, p-cymene (thyme), eucalyptol, camphor (rosemary), and eugenol (cloves), its mechanism of action could be due to more than one compound. Previous studies have reported that essential oils like thymol and carvacrol may disrupt cell membranes of *E. coli* O157:H7 and *Salmonella* Typhimurium (Karatzas et al., 2001). P-cymene is the biological precursor of carvacrol that causes swelling of the cytoplasmic membrane to a greater extent than does carvacrol (Ultee et al., 2002). It has been stated that eugenol disintegrates the cell membrane of *S. Typhimurium* and *S. Typhi* and increases its permeability, which subsequently causes death of the bacterium (Guentzel et al., 2008; Devi et al., 2010). It has also been reported that eugenol

collapses fungal cell membranes (Atsumi, et al., 2001). Sporan® works as a cell membrane disruptor of fungal hyphae and spores (Anonymous, 2008). The mechanism of action of Sporan® could be similar to that of phenolic compounds, which involves disruption of the cell membrane, increase in membrane permeability, and leakage of vital intracellular constituents or impairment of bacterial cellular enzyme.

Synergistic factors have suggested for use with essential oils include, reduced pH, addition of organic acids, reduced oxygen tension with modified atmospheres (Burt, 2004). In this study, acetic acid was combined with 800 ppm Sporan® to control pathogens on washed spinach leaves. The antimicrobial activity of 800 ppm Sporan® against *E. coli* O157:H7 and *Salmonella* was not significantly different from that of control; however addition of 200 ppm acetic acid with 800 ppm Sporan® (1000SV) resulted in significant reductions of these pathogens compared to control treatment. Chang and Fang (2007) stated that acetic acid at 0.05% to 0.5% concentration did not reduce *E. coli* O157: H7 on lettuce. In our study, further reduction of these pathogens in 1000SV treatment after washing and during storage could be due to the combined effect of acetic acid. Smith-Palmer et al. (2009) postulated that the lower water content on foods compared to laboratory media may hamper the progress of antibacterial agents to the target site in the bacteria cell. During *in vitro* assays, microorganisms and essential oils come into close contact, but *in vivo*, the food matrix has cell membranes that act as physical barriers between oil and microorganism contact, resulting in reduced effect of antimicrobials (Moreira et al., 2005).

The effect of cinnamaldehyde in reducing *E. coli* O157:H7 and *Salmonella* on spinach leaves was variable. For example, the effect of 1000 ppm cinnamaldehyde against *Salmonella* was significantly different from control after washing but not during the storage. However, the same concentration reduced significantly *E. coli* O157:H7 populations during storage. Likewise,

recovery of *E. coli* O157:H7 with 800 ppm cinnamaldehyde was significantly different during storage (2 and 7 days). Kim et al. (1995) reported complete reduction (7 log CFU/g) of *E. coli* O157:H7 populations in the presence of 1000 ppm cinnamaldehyde for 2 h. Previous studies have demonstrated that not only general intrinsic factors of food such as pH, salt content, water activity, fat, protein, antioxidants, preservatives, but also extrinsic factors like temperature, packaging, characteristics of microorganisms, time can influence bacterial sensitivity (Smith-Palmer et al., 2001; Parish et al., 2003; Burt, 2004). Some researchers found that the acidity of tomatoes may enhance the antimicrobial effects of essential oils while sprouts may provide additional protection for pathogens (Obaidat and Frank, 2009). Antimicrobial activity of cinnamaldehyde is attributed to the carbonyl group that binds proteins (Wendakoon and Sakaguchi, 1995). Incorporation of Tween-20 to cinnamaldehyde (800T) significantly reduced *E. coli* O157:H7 populations by 1.73 and 1.06 log CFU/g compared to the control and 800C, respectively at day 0. Tween-20 has been used previously to increase the solubility of essential oils and this helps in their penetration into bacterial cell walls and membranes (Burt, 2004, Klancnik et al., 2009). However, Tween-20 did not enhance *Salmonella* reduction in our study. Yossa et al. (2011) found that 2% cinnamaldehyde was required to reduce *Salmonella* Thompson and *S. Tennessee* serovars in soil, whereas other *Salmonella* strains were inactivated at 0.5 % cinnamaldehyde concentration. In this study, some of the *Salmonella* serovars used in the cocktail may be resistant to cinnamaldehyde and other oils. Acetic acid did not improve the effects of cinnamaldehyde in reducing *E. coli* O157:H7 and *Salmonella* populations on spinach leaves.

The population of natural microbiota of spinach leaves increased during storage irrespective of treatment. Our results are in agreement with the results obtained by other researchers who

observed an increase in background bacteria on spinach and lettuce (Nguyen-The and Carlin, 1994; Doering et al., 2009). In our study, populations of all native microbiota increased over time during storage at 4°C, whereas those of *E. coli* O157:H7 and *Salmonella* decreased. Luo et al. (2009) reported that *E. coli* O157:H7 declined significantly ($P < 0.01$) on products stored at 1 and 5°C for 3 days, whereas mesophilic and psychrotrophic bacteria, yeast and fungi increased significantly over time at all storage temperatures.

The effect of cinnamaldehyde or Sporan® as a produce wash is comparable to that of chlorine. Moreover, Sporan® in combination with acetic acid is superior to chlorine in reducing *E. coli* O157:H7 on spinach leaves. Since Sporan® or cinnamaldehyde-treated spinach leaves are acceptable in color and texture qualities, Sporan® could offer a natural alternative as a produce wash for spinach leaves provided that the sensory qualities are acceptable. In addition, the antioxidant properties of plant essential oils (Longaray et al., 2005) may also confer benefits to consumer health. Further studies will be required on the effect of these plant essential oils on sensory qualities. These oils could be evaluated with additional hurdles to enhance reduction of these pathogens on spinach leaves.

Chapter 7: The Effect of Cinnamaldehyde and Sporan® on the Growth of *E. coli* O157:H7 and *Salmonella* and Native Microflora Associated with Lettuce

Yossa et al. (2012). Submitted International journal of Food Microbiology

7.1 Overview

Foodborne outbreaks associated with the consumption of fresh produce have increased. In an effort to identify natural antimicrobial agents as fresh produce wash; the effect of essential oils in reducing enteric pathogens on iceberg and romaine lettuce was investigated. Lettuce were cut into pieces (3 x 2 cm), inoculated with a five-strain cocktail of *Escherichia coli* O157:H7 or *Salmonella enterica* (5 log CFU/g), air-dried for ca. 30 min, and then immersed in a treatment solution containing 5 ppm free chlorine, cinnamaldehyde or Sporan® (800 and 1000 ppm) alone or in combination with 200 ppm acetic acid (20%) for 1 min. Treated leaves were spin-dried and stored at 4°C. Samples were taken for determining the surviving populations of *E. coli* O157:H7, *Salmonella*, total coliforms, mesophilic and psychrotrophic bacteria, and yeasts and molds during 14 days storage period. The effect of treatments on lettuce color and texture was also determined. Cinnamaldehyde-Tween (800 ppm, 800T) reduced *E. coli* O157:H7 by 2.89 log CFU/g ($P < 0.05$) on iceberg lettuce at day 0; Sporan®-acetic acid (1000SV) reduced *E. coli* O157:H7 and *Salmonella* on iceberg and romaine lettuce by 2.68 and 1.56 log CFU/g ($P < 0.05$), respectively, at day 0. The effect of essential oils was comparable to that of 5 ppm free chlorine in reducing *E. coli* O157:H7 and *Salmonella* populations on iceberg and romaine lettuce throughout the storage time. The natural microbiota on treated lettuce leaves increased during the storage time, but remained similar ($P > 0.05$) to those treated with chlorine and control (water). The texture and the

color of iceberg and romaine lettuce treated with essential oils were not different from the control lettuce after 14 days. This study demonstrates the potential of Sporan® and cinnamaldehyde as effective lettuce washes that do not affect lettuce color and texture.

7.2 Introduction

In recent year, an increase in bacterial foodborne outbreaks linked to the consumption of fresh or minimally-processed leafy greens have been reported (Horby et al., 2003; Sivapalasingam et al., 2004). The Center for Science in the Public Interest identified 365 outbreaks in the United States linked to leafy greens contaminated with *E. coli*, Norovirus, or *Salmonella*, that resulted in 13,568 cases of illness (CSPI, 2009). Beyond their health effects, foodborne illnesses can cause emotional and economic hardship; *Salmonella* alone causes approximately 1 million foodborne infections that cost \$365 million in direct medical expenditures annually (CDC, 2011), and the societal cost of a single fatal case of *E. coli* O157:H7 infection has been estimated at \$7 million (Frenzen et al., 2005). Those foodborne outbreaks cause economical hardship to the farmers and the fresh produce industry, and can contribute to the skepticism of the consumers in regards to the safety of food. For example, after the *E. coli* O157:H7 outbreak that occurred in 2006 in spinach, most consumers stopped eating spinach, and buying other bagged produce as well (Cuite et al., 2007).

Lettuce can be contaminated with enteric pathogens when grown in a farm fertilized with inadequately treated compost (Beuchat, 1999; Solomon et al., 2002; Mukherjee et al., 2004), through flood irrigation with contaminated water or surface runoff (Ackers et al., 1998; Solomon et al., 2002) and through direct contact with mammals, other animals, and bird feces. Fresh produce can also become contaminated through human handlers during harvest and post-harvest, ineffective disinfection practices, and improper packaging (Thunberg et al., 2002).

The control of pathogenic microorganisms on fresh produce plays an important role in maintaining product quality and microbiological safety (Len et al., 2000). For both organic and conventional operations, chlorine based sanitizers are commonly used on produce surfaces and processing equipment (Suslow, 2000), and their effectiveness depends on their chemical and physical state, treatment conditions (such as water temperature, acidity, and contact time), resistance of pathogens, and the nature of the produce surface. In addition to these parameters, plant exudates released during slicing and shredding of fresh produce may react with the chlorine and neutralize its antimicrobial activity (FDA, 2009), requiring frequent monitoring and replenishing of chlorine (Sapers, 2009). Previous research has shown less than 2 log CFU reductions in enteric pathogens when chlorine is used as a produce wash (Singh et al., 2002; Lang et al., 2004). Enteric pathogens may hide at sites inaccessible to sanitizers and therefore limit its effectiveness (Kroupitski et al., 2009, Lopez-Galvez et al., 2010a). Chlorine may also form harmful chlorinated compounds such as chloramines and trihalomethanes in water (Dychdala, 2001; Lopez-Galvez et al., 2010b). Due to these limitations, there is a need for alternative sanitizers in reducing or eliminating microbial loads from produce.

Moreover, consumer awareness and concern regarding synthetic chemical additives have led researchers and food processors to look for natural food additives with a broad spectrum of antimicrobial activity (Marino et al., 2001). Essential oils are the odorous, volatile products of an aromatic plant's secondary metabolism, normally found in plant materials such as bark, buds, herbs, flowers, fruits, leaves, roots, seeds, stem, and twigs (Prabuseenivasan et al., 2006; Oussalah et al., 2007). Essential oils from basil, cinnamon, clove, dill, geranium, ginger, green tea, and other plants have shown *in vitro* antimicrobial effect against microorganisms such as *Aspergillus*, *Bacillus*, *Campylobacter*, *Candida*, *Enterobacter*, *Enterococcus*, *E. coli*,

Lactobacillus, *Listeria*, *Penicillium*, *Pseudomonas*, *Salmonella*, *Staphylococcus*, *Yersinia* and others (Smith-Palmer et al., 1998; Lopez et al., 2005, 2007; Gutierrez et al., 2008). Essential oils have been evaluated in various foods for their antimicrobial and preservative properties (Burt, 2004; Du et al., 2009; Obaidat and Frank, 2009). The antimicrobial effect of basil oil on spoilage bacteria on lettuce was comparable to washing with 125 ppm of chlorine (Wan et al., 1998). Singh et al. (2002) reported the antimicrobial effect of thyme oil in reducing *Salmonella* on romaine lettuce. Gunduz et al. (2009, 2010) found that iceberg lettuce washes with 75 ppm oregano oil and 1000 ppm myrtle oil were comparable to 50 ppm chlorine in reducing *Salmonella* Typhimurium populations on iceberg lettuce. Lemongrass oil (0.5%) reduced *Salmonella* Newport by 1.5 and 2 log CFU/g on organic romaine and iceberg lettuce, respectively (Moore-Neibel et al., 2012). The present study is the first report on antimicrobial activity of cinnamaldehyde and Sporan® on romaine and iceberg lettuce. The objectives of this study were to compare the antimicrobial effects of cinnamaldehyde or Sporan® alone and in combination with acetic acid against *E. coli* O157:H7, *Salmonella* and the native microbiota. The quality parameters of essential oils treated lettuce leaves were also analyzed.

7.3 Materials and methods

7.3.1 Antimicrobials used and preparation of antimicrobial suspensions

Cinnamaldehyde (Sigma-Aldrich, St. Louis, MO) and Sporan® (a proprietary formula containing clove, rosemary and thyme oil) from EcoSMART Technologies (Alpharetta, GA) were used as antimicrobials in this study. Antimicrobial suspensions were prepared as follows; 800 and 1000 ppm cinnamaldehyde (800C, 1000C) and Sporan® (800S, 1000S) in sterile distilled water (w/v), 800 ppm cinnamaldehyde in 0.5% Tween 20 (800T), and combined with acetic acid (20%) as 800 ppm cinnamaldehyde +200 ppm acetic acid (1000CV) and 800 ppm

Sporan® +200 ppm acetic acid (1000SV). A 5 ppm free chlorine solution was freshly made by dissolving sodium hypochlorite in deionized water and the chlorine concentration was adjusted with chlorine photometer (Chlorine Scientific, Inc., Fort Myers, FL). The pH of the antimicrobials, chlorine and control water were 4.0 – 4.7, 6.5, and 6.4, respectively.

7.3.2 Bacterial culture preparation

Five nalidixic acid resistant strains of *E. coli* O157:H7 and five *Salmonella* strains were used for this study. The *E. coli* strains RM 4406, RM 4688, and RM 1918 (clinical isolates from lettuce outbreaks), RM 4407 (clinical isolate from spinach outbreak), and RM 5279 (bagged vegetable isolate) were obtained from our laboratory culture collection. *Salmonella enterica* serovars included Braenderup (clinical isolate), Newport and Negev 26 H (Thyme isolates), Thompson 2051H and Tennessee 2053N (from our culture collection) The cultures were prepared by two successive transfers of cryopreserved cells in tryptic soy broth (TSB, Acumedia, Lansing, MI) and TSB supplemented with 50 ppm nalidixic acid (TSBN), for *Salmonella* and *E. coli* strains, respectively. After overnight incubation at 37°C, cultures were centrifuged (7500 x g for 10 min, 10°C), and cell pellets were suspended in 0.1 M sterile phosphate buffer (PBS, pH 7.0) to obtain OD₆₀₀ of 1. Equal volumes of individual strains were mixed to prepare *E. coli* O157:H7 or *Salmonella* cocktails for inoculation studies. Three independent trials were carried out either with *E. coli* O157:H7 or *Salmonella* cocktail.

7.3.3 Lettuce inoculation and treatment

Iceberg and romaine lettuce were purchased at a retail grocery store and kept at 4°C before the onset of the experiments. The outer leaves of iceberg and romaine lettuce were removed and discarded. Cut lettuce pieces (3 x 2 cm) were each inoculated with *E. coli* O157:H7 or *Salmonella* cocktail (7 log CFU/ml, 5 spots of 10 µl) on the adaxial surface of the pieces and then air dried for 30 min. Approximately 20 g of air-dried lettuce pieces were placed in a beaker

containing 100 ml of treatment solution and washed for 1 min with manual agitation. Treated lettuce pieces were processed in a salad spinner for 1 min and then stored in sterile whirl-pak filter bags (Nasco Whirl-Pak, Fort Atkinson, WI) under aerobic condition at 4°C for 14 days. Samples washed with sterile water served as control.

7.3.4 Enumeration of *E. coli* O157:H7 and *Salmonella*

Samples were analyzed after inoculation and during incubation period at 0 (after wash treatment), 2, 7, and 14 days for surviving populations of *E. coli* O157:H7 and *Salmonella*. Lettuce samples (5 g) were pummeled in 45 ml sterile peptone water for 2 min in a stomacher (Interscience, St. Norm, France), then serially diluted in peptone water and spiral plated (Whitley Scientific, West Yorkshire, England) on Xylose-lysine-tergitol agar (XLT4, Acumedia) for *Salmonella*, and on Sorbitol MacConkey agar (Acumedia) supplemented with 0.05mg/l of cefixime, 2.5mg/l of potassium tellurite and 50 ppm nalidixic acid (Sigma-Aldrich, CTSMAC-N) for *E. coli* O157:H7. Colonies were counted after 24 h of incubation at 37°C using Protocol colony counter (Microbiology International Inc., Frederick, MD).

7.3.5 Enumeration of native microbiota

Uninoculated lettuce pieces (40 g) were washed in 400 ml of the different treatment solutions (800C, 800S, 800T, 1000C, 1000CV, 1000S, and 1000SV) as described above and stored at 4°C for up to 14 days. At days 0, 2, 7, and 14, serially diluted suspensions of 10 g of lettuce pieces were prepared as described above and spiral plated onto TSA (Acumedia) to enumerate mesophilic (incubation at 37°C for 24 h) and psychrotrophic (incubation at 4°C for up to 8 days) bacteria; MacConkey Agar (MAC; Acumedia; incubation at 35°C for 24 h) for total coliforms, and Dichloran Rose Bengal chloramphenicol agar (DRBC; Acumedia; incubation at 23°C for 2 - 5 days) for yeasts and molds. Colonies were counted using Protocol colony counter (Microbiology International Inc.).

7.3.6 Color and texture measurement of treated lettuce

Color values (L, a, b) of lettuce leaves treated with selected essential oils were measured on days 0, 2, 7, and 14 using a CR-400 chroma meter (Minolta, Inc. Tokyo, Japan). Illuminant D65 and 10° observer angle were used. The instrument was calibrated using a Minolta standard white reflector plate. At least five measurements were made on each essential oil-treated lettuce pieces. Texture (maximum force measurement for breakage of leaves, N values) was analyzed on lettuce leaves treated with selected essential oils at 0, 2, 7, and 14 days using the TA-XT2i texture analyzer (Texture Technology Corp. Scarsdale, NY). A 5 g sample was placed into the press holder and a Kramer shear with 5 blade plunger was moved down at 2 mm/s to 1 cm below the bottom of the holder. Maximum peak force (N) was recorded using Texture Expert software (version 1.22. Texture Technology Corp.). At least 10 measurements per treatment were recorded.

7.3.7 Statistical analysis

The experiment was repeated three times for each treatment and storage period. Reduction in *E. coli* O157:H7, *Salmonella*, and background microflora (log CFU/g) from initial populations were compared among treatment-time combinations by a three-way ANOVA using ‘proc-mixed’ procedure (SAS 9.2, Cary, NC). Color and texture data were analyzed similarly by the proc mixed procedure. The level of statistical significance was set at $P < 0.05$ in all cases.

7.4 Results

7.4.1 Antimicrobial effects of cinnamaldehyde and Sporan® against *E. coli* O157:H7 or *Salmonella* on iceberg lettuce

The initial *E. coli* O157:H7 count of inoculated iceberg leaves was 4.39 log CFU/g (data not shown). At day 0, a treatment with 800 ppm cinnamaldehyde+Tween-20 (800T) significantly reduced *E. coli* O157:H7 populations by 2.89 log CFU/g compared to that of 5 ppm chlorine

(1.49 log CFU/g reduction) and water (0.76 log CFU/g reduction) (Fig. 7.1.). *E. coli* O157:H7 populations on iceberg leaves treated with 800S, 1000S and 800T were significantly reduced at 2 days by more than 2.5 log CFU/g compared to that of 800C and water (1.48 and 0.75 log CFU/g reductions, respectively). A treatment with 1000SV and 1000S significantly reduced *E. coli* O157:H7 (2.88 and 2.65 log CFU/g, respectively) in lettuce compared to chlorine (1.20 log CFU/g) and control (0.71 log CFU/g) at 7 days. *E. coli* O157:H7 populations were undetectable in most treatments after 14 days of storage; more than 2.5 log reductions were reported with all treatments except 1000CV and control.

Initial *Salmonella* populations on inoculated iceberg lettuce were 4.62 log CFU/g (data not shown). More than 2.5 log reductions in populations of *Salmonella* were observed with most treatments on day 0; reductions were significant in lettuce treated with 1000S (2.67 log CFU/g) compared to that of 800T (1.73 log CFU/g) and control (1.06 log CFU/g). Likewise, significant reductions in *Salmonella* population were reported with 1000SV treatment compared to 800S, 1000C and control on day 2. *Salmonella* populations were further reduced during storage in all treated samples. Significant reductions were observed on day 14 in lettuce treated with chlorine and 800T (2.87- and 2.65 log reductions, respectively) compared to other treatments and control.

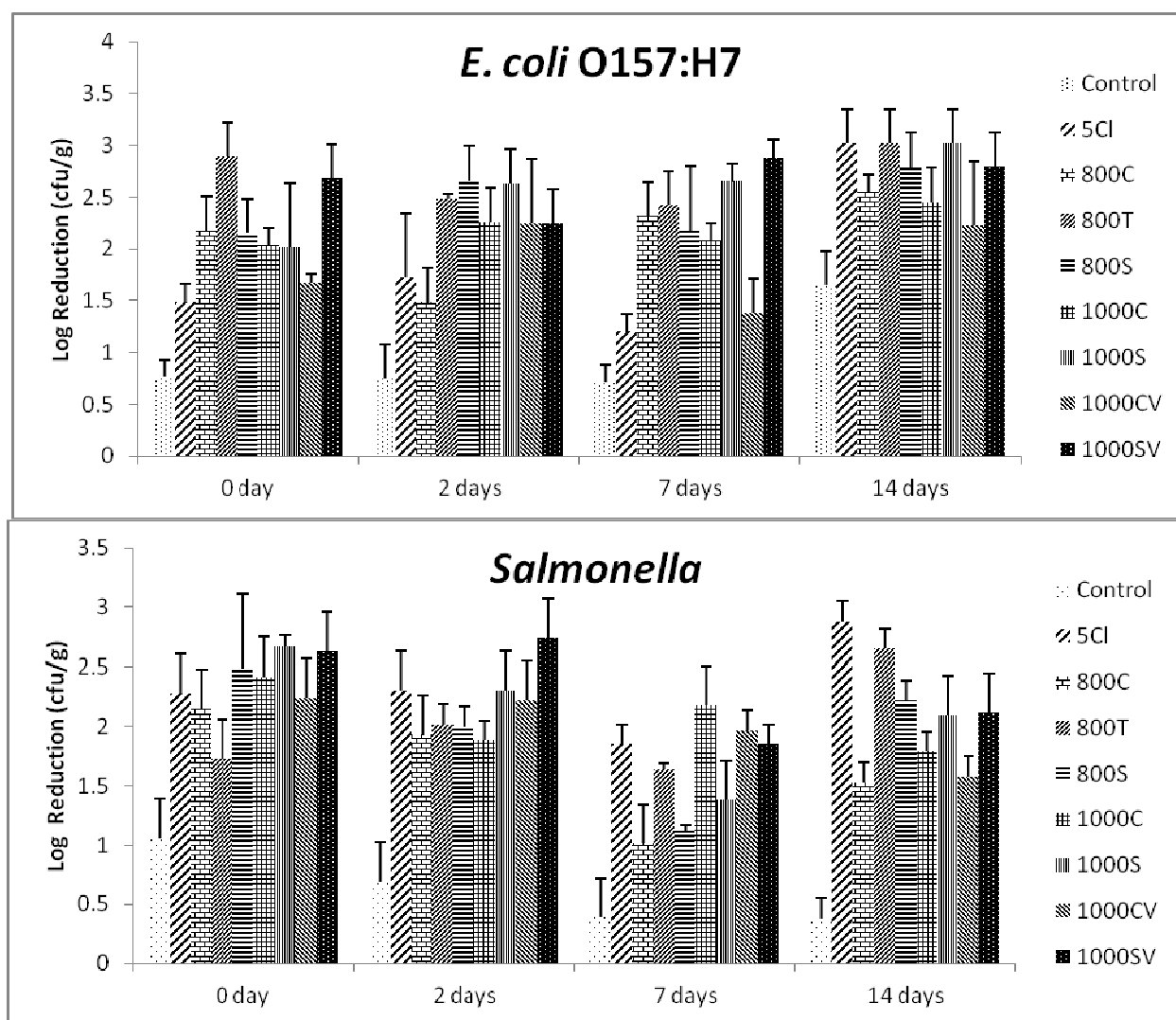


Fig. 7.1: Effect of different antimicrobials on populations of *E. coli* O157:H7 and *Salmonella* in cut iceberg lettuce stored at 4°C.

Each column represents the difference in populations between inoculated control and treatment measured at the sampling periods (*E. coli* O157:H7 populations on inoculated control leaves were 4.39, 3.90, 2.88, and 3.02 log CFU/g at 0, 2, 7, and 14 days, respectively. *Salmonella* populations on inoculated control leaves were 4.62, 4.15, 3.07, and 2.98, log CFU/g at 0, 2, 7, and 14 days, respectively)

Values (log CFU/g) are the mean of three replicates and vertical bars represent the standard errors. Values (log CFU/g) are the mean of three replicates and vertical bars represent the standard errors. 800C – 800 ppm cinnamaldehyde, 800T – 800 ppm cinnamaldehyde in 0.5% tween 20, 800S -800 ppm Sporan®, 1000C – 1000 ppm cinnamaldehyde, 1000CV – 800 ppm cinnamaldehyde + 200 ppm acetic acid, 1000S – 1000 ppm Sporan®, 1000SV - 800 ppm Sporan®+ 200 ppm acetic acid.

7.4.2 Antimicrobial effects of cinnamaldehyde and Sporan® against *E. coli* O157:H7 or *Salmonella* on romaine lettuce

The antimicrobial effects of treatments on the populations of *E. coli* O157:H7 and *Salmonella* on romaine lettuce are shown in Fig. 7.2. The initial *E. coli* O157:H7 populations on unwashed romaine lettuce were 5.22 log CFU/g (data not shown). A treatment with Sporan® plus acetic acid (1000SV) significantly reduced *E. coli* O157:H7 (1.56 log CFU/g) on romaine lettuce compared to Sporan® alone (800S, 1.14 log CFU/g), 5 ppm free chlorine (1.07 log CFU/g), or water (0.87 log CFU/g). *E. coli* O157:H7 were further reduced at day 2; 1.66-, 1.59-, 1.48-, 1.45- and 1.38-log reductions were observed for 5 ppm chlorine, 1000CV, 1000SV, 1000C and water, respectively. At day 7, *E. coli* O157:H7 populations in unwashed romaine lettuce were 3.31 log CFU/g (data not shown). The treatments with 1000S, 800T, 1000CV, and 1000SV significantly reduced *E. coli* O157:H7 on romaine lettuce compared to that with water or Sporan® (800S). The antimicrobial effects of 800C, 1000S, and 1000CV (2.93-, 2.46-, and 2.46 log reductions, respectively) were significantly different ($p \leq 0.05$) from the effects of 1000SV, 5 ppm chlorine, water and 800S treatments (1.43-, 1.05-, 0.91-, and 0.33 log reductions, respectively). An increase in Sporan® concentration from 800 ppm to 1000 ppm resulted in an additional ca.2 log reduction of *E. coli* O157:H7 on 14 days. Similarly, addition of acetic acid enhanced the antimicrobial effect of Sporan® against *E. coli* O157:H7 as evident from significant reductions in 1000SV-treated iceberg lettuce on days 2, 7 and 14 compared to that of Sporan® alone.

Salmonella populations were significantly reduced in romaine lettuce following treatment with 5 ppm chlorine and 1000SV (2.58- and 2.28 logs, respectively). Use of acetic acid with Sporan® (1000SV) resulted in significantly higher *Salmonella* reduction (2.28 log CFU/g) than the treatment with Sporan® alone (800S, 0.96 log CFU/g). *Salmonella* populations were reduced during storage in all samples irrespective of treatment. More than 3 log reductions were reported

in 5 ppm chlorine-treated romaine lettuce on 2, 7, and 14 days from their respective initial concentrations. Likewise, 2.38-2.99 log reductions in 1000SV-treated romaine lettuce were observed during 2-14 days storage period from their initial concentrations. The antimicrobial effects of chlorine and 1000SV were significantly different from control and all other treatments during 14-days storage period except on day 14 where *Salmonella* reductions in 800S-treated samples were similar ($P > 0.05$) to those treated with 1000SV or chlorine.

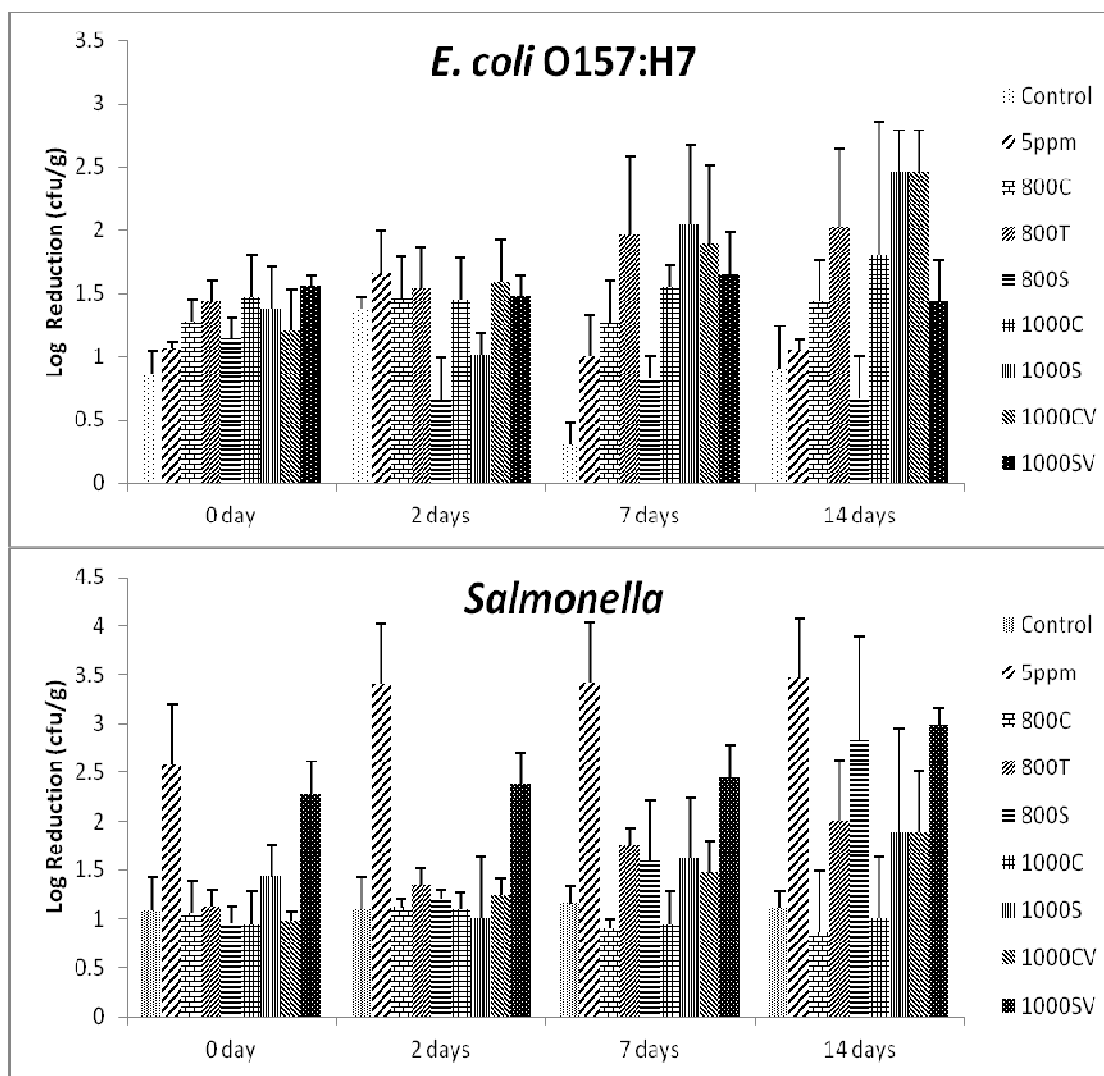


Fig. 7.2: Effect of different antimicrobials on populations of *E. coli* O157:H7 and *Salmonella* in cut romaine lettuce stored at 4°C.

Each column represents the difference in populations between inoculated control and treatment measured at the sampling periods (*E. coli* O157:H7 populations on inoculated control leaves were 5.22, 4.67, 3.31, and 2.99 log CFU/g at 0, 2, 7, and 14 days, respectively. *Salmonella* populations on inoculated control leaves were 5.22, 4.63, 4.18, and 3.46 log CFU/g at 0, 2, 7, and 14 days, respectively)

Values (log CFU/g) are the mean of three replicates and vertical bars represent the standard errors. 800C – 800 ppm cinnamaldehyde, 800T – 800 ppm cinnamaldehyde in 0.5% tween 20, 800S -800 ppm Sporan®, 1000C – 1000 ppm cinnamaldehyde, 1000CV – 800 ppm cinnamaldehyde + 200 ppm acetic acid, 1000S – 1000 ppm Sporan®, 1000SV - 800 ppm Sporan®+ 200 ppm acetic acid.

7.4.3 Antimicrobial effects of cinnamaldehyde and Sporan® against native microbiota on iceberg and romaine lettuce

Populations of mesophilic, psychrotrophic, total coliforms and yeasts and molds on iceberg lettuce were influenced by the treatment and storage period (Table 7.1). In general, populations of native microbiota from treated samples were not different from control or untreated lettuce leaves. Mesophilic populations increased significantly on all iceberg lettuce samples by day 7 except on those samples treated with 1000SV and 5 ppm chlorine. Mesophilic bacterial population on chlorine-treated iceberg lettuce remained constant ($P > 0.05$) throughout the storage time. Total coliforms increased significantly on all treated iceberg lettuce samples at day 7 with the exception of 1000S-treated lettuce. Psychrotrophic bacterial populations also increased significantly at day 7 in most treated samples. Similarly, yeasts and molds recovered from 7 days stored treated lettuce were significantly higher than their corresponding initial populations.

Mesophilic, total coliforms, psychrotrophic, and yeasts and molds populations on treated romaine lettuce were not significantly different from untreated romaine lettuce on day 0 (Table 7. 2). However, populations of this native microbiota increased with storage. Mesophilic bacteria on untreated romaine lettuce, control, and lettuce treated with 5 ppm chlorine, 800S, and 1000SV remained constant ($P > 0.05$) throughout the storage period but increased significantly in 1000S-treated samples at day 14. Similarly, total coliforms recovered from untreated romaine lettuce, control and lettuce treated with chlorine were not different ($P < 0.05$) throughout the storage time. Also, yeasts and molds counts of untreated romaine lettuce, control, and lettuce treated with 5 ppm chlorine and 1000SV were constant ($P > 0.05$) during 14 days of storage.

Table 7.1: Effect of different antimicrobials on populations of native microorganisms in cut iceberg lettuce stored at 4°C

Treatment	Concn (ppm)	Populations (log CFU/g) ^a			
		Day 0	Day 2	Day 7	Day 14
Mesophilic Bacteria					
Unwash		3.47±0.47ax	4.74±0.29axy	6.51±0.26ay	6.58±0.35ay
Control (water)		3.14±0.58ax	4.46±1.00axy	6.24±0.66ay	6.56±0.65ay
Chlorine	5	3.52±0.81ax	4.07±0.17ax	6.14±0.31ax	6.39±0.83ax
Cinnamaldehyde	800C	4.13±1.23ax	4.70±0.14axy	7.23±0.31ay	7.55±0.45ay
Cinnamaldehyde + Tween	800T	3.14±0.94ax	4.30±0.57axy	7.17±0.61ay	7.13±0.58ay
Sporan®	800S	3.59±0.34ax	5.06±0.52axy	6.80±0.23ay	6.88±0.46ay
Cinnamaldehyde	1000C	3.43±0.52ax	4.39±0.49axy	7.21±0.34ayz	7.59±0.21az
Cinnamaldehyde + acetic acid	1000CV	2.94±0.14ax	4.30±0.19axy	7.01±0.41ayz	7.29±0.67az
Sporan®	1000S	3.95±0.79ax	4.54±0.09axy	6.91±0.49ay	7.38±0.31ay
Sporan® + acetic acid	1000SV	3.43±0.35ax	4.23±0.19axy	6.37±0.53axy	6.93±0.16ay
Total Coliform					
Unwashed		3.35±0.50ax	4.51±0.52axy	6.00±0.06axy	6.80±0.12ay
Control (water)		2.96±0.83ax	4.40±0.75axy	6.24±0.94ay	6.60±0.60ay
Chlorine	5	3.11±0.40ax	4.01±0.16axy	6.59±0.59ay	6.59±0.89ay
Cinnamaldehyde	800C	4.12±1.12ax	4.56±0.21axy	7.08±0.37ayz	7.53±0.55az
Cinnamaldehyde + Tween	800T	3.04±0.86ax	4.06±0.63axy	7.22±0.60ayz	6.95±0.39az
Sporan®	800S	3.50±0.49ax	5.01±0.58axy	6.75±0.13ay	7.26±0.18ay
Cinnamaldehyde	1000C	3.40±0.58ax	4.32±0.52axy	6.54±0.52ayz	7.75±0.35az
Cinnamaldehyde + acetic acid	1000CV	2.98±0.19ax	4.10±0.13ax	7.12±0.30ay	7.13±0.29ay
Sporan®	1000S	3.85±0.78ax	4.35±0.24ax	6.68±0.48axy	7.40±0.29ay
Sporan® + acetic acid	1000SV	3.18±0.50ax	4.21±0.21axy	6.49±1.05ay	6.84±0.17ay
Psychrotrophic Bacteria					

Unwashed		3.33±0.51ax	5.08±0.41axy	6.60±0.13ay	6.75±0.18ay
Control (water)		3.33±0.37ax	4.55±0.82axy	6.30±0.63ay	6.77±0.51ay
Chlorine	5	3.55±0.79ax	4.02±0.19axy	6.25±0.31axy	6.63±0.91ay
Cinnamaldehyde	800C	4.13±1.12ax	4.68±0.13axy	7.37±0.15ayz	7.85±0.03az
Cinnamaldehyde + Tween	800T	3.23±0.76ax	4.33±0.44axy	7.13±0.62ayz	7.39±0.75az
Sporan®	800S	3.56±0.59ax	5.00±0.58axy	6.90±0.08ay	7.31±0.22ay
Cinnamaldehyde	1000C	3.50±0.54ax	4.53±0.49axy	7.28±0.26ayz	7.69±0.41az
Cinnamaldehyde + acetic acid	1000CV	2.93±0.30ax	4.06±0.46axy	7.01±0.41ayz	7.43±0.63az
Sporan®	1000S	4.08±0.50ax	4.58±0.28axy	6.82±0.76axy	7.52±0.32ay
Sporan® + acetic acid	1000SV	3.27±0.53ax	3.89±0.31axy	6.30±1.04ayz	7.03±0.18az
Yeasts and Molds					
Unwashed		1.83±1.61ax	1.90±1.65abx	4.56±0.91abxy	5.36±1.03ay
Control (water)		2.29±0.59ax	2.93±0.40abxy	3.38±2.97abxy	5.45±0.59ay
Chlorine	5	2.20±0.46ax	1.90±1.65abx	2.67±2.61bxy	5.34±0.86ay
Cinnamaldehyde	800C	2.78±0.50ax	4.13±1.55axy	4.18±3.62abxy	6.02±0.82ay
Cinnamaldehyde + Tween	800T	2.75±0.23ax	1.80±1.56abxy	5.11±1.38abyz	6.52±0.64az
Sporan®	800S	2.70±0.45ax	2.59±2.24abx	5.01±0.52abxy	6.16±0.13ay
Cinnamaldehyde	1000C	3.14±1.09ax	3.57±0.44abxy	5.63±0.66axy	6.34±0.28ay
Cinnamaldehyde + acetic acid	1000CV	2.09±0.35ax	3.18±0.48abxy	5.36±1.25abyz	6.46±0.31az
Sporan®	1000S	3.08±0.55ax	2.10±1.87abxy	5.39±1.23abyz	6.24±0.22az
Sporan® + acetic acid	1000SV	2.47±0.12ax	1.00±1.73bxy	5.20±0.89abyz	6.11±0.29az

^a Values are mean ± SD. Each experiment was replicated three times.

Values in the same row not followed by the same letters (xyz) are significantly different; values in the same column not followed by same letters (ab) are significantly different (P < 0.05).

800C – 800 ppm cinnamaldehyde, 800T – 800 ppm cinnamaldehyde in 0.5% tween 20, 800S -800 ppm Sporan®, 1000C – 1000 ppm cinnamaldehyde, 1000CV – 800 ppm cinnamaldehyde + 200 ppm acetic acid, 1000S – 1000 ppm Sporan®, 1000SV - 800 ppm Sporan®+ 200 ppm acetic acid.

Table 7.2: Effect of different antimicrobials on populations of native microorganisms in romaine lettuce stored at 4°C

Treatment	Concn (ppm)	Populations (log CFU/g) ^a			
		Day 0	Day 2	Day 7	Day 14
Mesophilic Bacteria					
Unwash		3.83±0.70ax	4.82±0.60ax	5.64±0.31ax	6.12±0.77ax
Control (water)	0	3.74±1.26ax	4.89±0.86ax	6.87±1.75ax	6.39±0.69ax
Chlorine	5	3.88±0.90ax	4.78±1.45ax	5.90±0.69ax	5.62±1.57ax
Cinnamaldehyde	800C	3.75±0.32ax	4.36±0.49axy	7.35±0.26ay	7.72±0.31ay
Cinnamaldehyde + Tween	800T	3.69±0.68ax	4.02±0.61axy	7.24±0.24ay	7.33±0.33ay
Sporan®	800S	3.24±0.76ax	4.92±0.15ax	5.87±0.39ax	6.54±0.96ax
Cinnamaldehyde	1000C	3.30±1.51ax	4.14±0.65axy	7.14±0.27ay	7.33±1.05ay
Cinnamaldehyde + acetic acid	1000CV	3.66±1.10ax	4.25±0.61axy	7.35±0.49ayz	7.76±0.29az
Sporan®	1000S	2.79±0.94ax	4.08±1.20axy	6.14±1.44axy	6.46±1.20ay
Sporan® + acetic acid	1000SV	2.94±0.72ax	4.33±0.61ax	5.69±0.64ax	6.14±0.96ax
Total Coliform					
Unwashed		4.01±0.36ax	5.08±0.43ax	5.90±0.71ax	6.45±0.88ax
Control (water)	0	4.37±0.43ax	4.72±1.11ax	6.76±0.75ax	6.59±1.25ax
Chlorine	5	3.95±0.95ax	5.13±0.82ax	6.29±0.74ax	6.60±1.18ax
Cinnamaldehyde	800C	3.99±0.10ax	4.42±0.62axy	6.00±0.67ayz	7.95±0.35az
Cinnamaldehyde + Tween	800T	3.85±0.72ax	4.24±0.67ax	7.05±1.40axy	7.82±0.72ay
Sporan®	800S	3.21±0.80ax	5.10±0.68axy	6.75±0.13axy	7.13±1.31ay
Cinnamaladehyde	1000C	3.93±0.92ax	4.39±0.55axy	7.56±0.74ay	7.76±1.14ay
Cinnamaldehyde + acetic acid	1000CV	3.79±0.84ax	4.33±0.50axy	7.67±0.53ayz	8.27±0.65az
Sporan®	1000S	2.58±1.05ax	4.35±0.81axy	7.24±0.52ay	6.78±1.42ay
Sporan® + acetic acid	1000SV	2.79±0.96ax	4.70±0.54axy	5.99±0.97axy	6.35±0.92ay

Psychrotrophic Bacteria

Unwashed		4.01±0.56ax	5.25±0.45ax	6.06±0.84ax	6.58±0.61ax
Control (water)	0	4.39±0.30ax	5.22±0.51ax	6.91±0.94ax	7.18±0.79ax
Chlorine	5	3.64±0.81ax	5.12±0.54ax	6.46±1.32ax	7.09±1.58ax
Cinnamaldehyde	800C	3.71±0.39ax	4.77±0.91axy	7.69±0.55ayz	8.30±0.56az
Cinnamaldehyde + Tween	800T	3.35±0.30ax	4.32±0.40axy	7.65±0.70ayz	8.06±0.64az
Sporan®	800S	2.20±1.95ax	5.00±0.83axy	6.75±0.92ay	7.31±0.22ay
Cinnamaldehyde	1000C	3.85±0.99ax	4.64±0.47axy	7.84±0.61ayz	8.22±1.20az
Cinnamaldehyde + acetic acid	1000CV	3.42±0.47ax	4.42±0.52axy	7.92±0.47ayz	8.43±0.70az
Sporan®	1000S	2.27±2.03ax	4.28±1.54axy	7.02±1.09ay	7.42±1.46ay
Sporan® + acetic acid	1000SV	2.50±2.21ax	4.75±0.85axy	6.14±1.02ay	7.12±1.44ay

Yeasts and Molds

Unwashed		3.49±0.26ax	3.83±0.07ax	4.75±0.98ax	5.01±0.28ax
Control	0ppm	3.40±0.70ax	3.57±0.54ax	5.58±0.77ax	5.46±0.47ax
Chlorine	5ppm	2.76±1.14ax	3.64±0.40ax	5.66±1.23ax	5.42±0.78ax
Cinnamaldehyde	800C	3.31±0.40ax	3.06±0.39ax	6.20±0.96ax	6.27±0.36ax
Cinnamaldehyde + Tween	800T	3.34±0.16ax	3.29±0.10ax	6.22±0.53ax	6.34±0.50ax
Sporan®	800S	2.03±1.78ax	3.43±0.14axy	5.58±1.10ay	5.89±0.81ay
Cinnamaldehyde	1000C	3.09±1.02axy	2.28±1.99ax	6.42±0.91ay	6.46±0.68ay
Cinnamaldehyde + Acetic acid	1000CV	2.51±2.18ax	3.19±0.44axy	6.58±0.94ay	6.40±0.32ay
Sporan®	1000S	1.87±1.63ax	2.27±2.00axy	5.54±1.16ay	5.67±1.42ay
Sporan® + acetic acid	1000SV	2.91±0.86ax	2.04±1.80ax	5.47±1.34ax	5.10±0.96ax

^a Values are mean ± SD. Each experiment was replicated three times.

Values in the same row not followed by the same letters (xyz) are significantly different; values in the same column followed by same letters a are not significantly different ($P < 0.05$).

800C – 800 ppm cinnamaldehyde, 800T – 800 ppm cinnamaldehyde in 0.5% tween 20, 800S -800 ppm Sporan®, 1000C – 1000 ppm cinnamaldehyde, 1000CV – 800 ppm cinnamaldehyde + 200 ppm acetic acid, 1000S – 1000 ppm Sporan®, 1000SV - 800 ppm Sporan®+ 200 ppm acetic acid.

7.4.4 The effect of cinnamaldehyde and Sporan® on texture and color of iceberg and romaine lettuce

Effect of the essential oils on texture of fresh cut iceberg and romaine lettuces are shown in Tables 7.3. and 7.4. Treated iceberg lettuce was compared with lettuce washed with water (control). Initial force values (N) of treated samples were not different ($p > 0.05$) from control N values except those washed with 800 ppm cinnamaldehyde where maximum force (164) was significantly lower than the that of control samples (193). During storage at 4°C, no significant differences ($p > 0.05$) were noticed between the texture of the treated samples and the control except iceberg samples treated with 800 ppm and 1000 ppm cinnamaldehyde. At 14 days of storage, the force measurement of 800 ppm cinnamaldehyde-treated iceberg lettuce were higher ($p \leq 0.05$) than those recorded at days 0, 2 and 7. Likewise, initial force values of iceberg lettuce treated with 1000 ppm cinnamaldehyde were significantly higher than those stored at 2 days. The texture measurements of treated romaine lettuce were not significantly different from that of control. In addition, no differences were observed in the force of treated samples and the control throughout the 14 day storage time at 4°C.

The effect of oils on color of lettuces was measured using Hunter L, a, and b values (Tables 5 and 6). Color measurements showed no significant differences ($p > 0.05$) in color coordinate values a (greenness) and b (yellowness) between control and treated iceberg lettuce. However, significant differences ($p \leq 0.05$) were observed in the lightness (L) values. At day 0, the lightness values of the iceberg samples treated with cinnamaldehyde or Sporan® at 800 and 1000 ppm concentrations were different from control iceberg. At day 2 and 7 and after 14 days of storage at 4°C, the lightness values of iceberg samples treated with 800S and 1000SV were similar ($P > 0.05$) to the control, Color coordinate values (L, a, b) of control and treated romaine lettuce were not significantly different throughout the 14 days of storage.

Table 7.3: Maximal Force (N) required for breakage for cut iceberg leaves treated with essential oils and stored for 14 days at 4°C

Treatment	Concentration	Maximum force (N) ^a			
		Day 0	Day 2	Day 7	Day 14
Control	0	193±13 ^{abx}	184±12 ^{abcx}	192±16 ^{abx}	190±13 ^{ax}
cinnamaldehyde	800C	164±14 ^{cy}	169±18 ^{cxy}	176±05 ^{bxy}	182±10 ^{ax}
Sporan®	800S	187±08 ^{abx}	194±19 ^{ax}	188±19 ^{abx}	197±13 ^{ax}
cinnamaldehyde	1000C	195±11 ^{abx}	173±15 ^{bcy}	185±17 ^{abxy}	180±14 ^{axy}
Sporan®	1000S	180±19 ^{bcx}	180±16 ^{abcx}	186±09 ^{abx}	182±11 ^{ax}
Sporan® +acetic	1000SV	200±24 ^{ax}	187±22 ^{abx}	199±12 ^{ax}	197±12 ^{ax}

^a Values are mean ± SD. Each experiment was replicated ten times.

Values in the same row for the same parameter not followed by the same letters (xy) are significantly different; values in the same column not followed by same letters (ab) are significantly different ($P < 0.05$).

800C – 800 ppm cinnamaldehyde, 800T – 800 ppm cinnamaldehyde in 0.5% tween 20, 800S - 800 ppm Sporan®, 1000C – 1000 ppm cinnamaldehyde, 1000CV – 800 ppm cinnamaldehyde + 200 ppm acetic acid, 1000S – 1000 ppm Sporan®, 1000SV - 800 ppm Sporan®+ 200 ppm acetic acid.

Table7.4: Color measurements (L, a, b values) of iceberg leaves treated with essential oils and stored for 14 days at 4°C

Color Values ^a												
Treatment	Day 0			Day 2			Day 7			Day 14		
	L*	a*	b*	L*	a*	b*	L*	a*	b*	L*	a*	b*
0ppm	51±2 ^{cxy}	-19±1 ^{ax}	33±2 ^{ax}	54±3 ^{cy}	-18±2 ^{ax}	34±3 ^{abx}	54±5 ^{cxy}	-20±1 ^{ax}	33±6 ^{abx}	56±2 ^{cdx}	-20±1 ^{abx}	36±2 ^{abx}
800C	62±2 ^{axy}	-20±2 ^{ax}	36±3 ^{axy}	61±3 ^{ay}	-17±1 ^{ax}	35±2 ^{ay}	61±4 ^{axy}	-20±1 ^{ax}	37±3 ^{axy}	65±3 ^{ax}	-20±1 ^{abx}	39±4 ^{ax}
800S	55 ±3 ^{bx}	-20±2 ^{ax}	33±5 ^{ax}	52±3 ^{cx}	-17±1 ^{ax}	30±3 ^{bx}	55±4 ^{bcx}	-18±2 ^{ax}	31±5 ^{bx}	53±1 ^{dx}	-19±1 ^{ax}	32±2 ^{cx}
1000C	59±4 ^{abx}	-19±2 ^{ax}	33±4 ^{ay}	58±2 ^{abx}	-17±1 ^{ax}	33±3 ^{aby}	61±3 ^{ax}	-19±1 ^{ax}	34±3 ^{abxy}	62±3 ^{abx}	-21±2 ^{abx}	37±4 ^{abx}
1000S	58±2 ^{abx}	-21±1 ^{ax}	36±1 ^{ax}	59±3 ^{ax}	-18±1 ^{ax}	35±2 ^{ax}	59±2 ^{abx}	-20±1 ^{ax}	35±3 ^{abx}	59±4 ^{bcx}	-21±1 ^{bx}	37±3 ^{ax}
1000SV	54±1 ^{cx}	-19±4 ^{ax}	32±5 ^{ax}	54±4 ^{bcx}	-17±2 ^{ax}	32±4 ^{abx}	55±4 ^{bcx}	-19±1 ^{ax}	32±3 ^{bx}	55±7 ^{cdx}	-20±4 ^{abx}	34±6 ^{bcx}

^a Values are mean ± SD. Each experiment was replicated ten times.

Values in the same row for the same parameter not followed by the same letters (xy) are significantly different; values in the same column not followed by same letters (ab) are significantly different (P < 0.05).

800C – 800 ppm cinnamaldehyde, 800T – 800 ppm cinnamaldehyde in 0.5% tween 20, 800S -800 ppm Sporan®, 1000C – 1000 ppm cinnamaldehyde, 1000CV – 800 ppm cinnamaldehyde + 200 ppm acetic acid, 1000S – 1000 ppm Sporan®, 1000SV - 800 ppm Sporan®+ 200 ppm acetic acid.

Table 7.5: Maximal Force (N) required for breakage for cut romaine leaves treated with essential oils and stored for 14 days at 4°C

Treatment	Concn	Maximum force (N) ^a			
		Day 0	Day 2	Day 7	Day 14
Control	0ppm	109±12 ^{abx}	114±11 ^{ax}	104±11 ^{ax}	113±09 ^{abx}
Cinnamaldehyde	800C	114±13 ^{ax}	107±10 ^{ax}	111±13 ^{ax}	120±09 ^{ax}
Sporan®	800S	95±08 ^{bx}	105±13 ^{ax}	95±08 ^{ax}	109±12 ^{abx}
Cinnamaldehyde	1000C	101±11 ^{abx}	104±12 ^{ax}	104±15 ^{ax}	100±08 ^{bx}
Sporan®	1000S	99±16 ^{abx}	110±08 ^{ax}	101±06 ^{ax}	113±07 ^{abx}
Sporan® +acetic acid	1000SV	95±14 ^{abx}	107±11 ^{ax}	102±08 ^{ax}	107±10 ^{abx}

^a Values are mean ± SD. Each experiment was replicated ten times.

Values in the same row for the same parameter followed by the same letter x are not significantly different; values in the same column not followed by same letters (ab) are significantly different (P < 0.05).

800C – 800 ppm cinnamaldehyde, 800T – 800 ppm cinnamaldehyde in 0.5% tween 20, 800S - 800 ppm Sporan®, 1000C – 1000 ppm cinnamaldehyde, 1000CV – 800 ppm cinnamaldehyde + 200 ppm acetic acid, 1000S – 1000 ppm Sporan®, 1000SV - 800 ppm Sporan®+ 200 ppm acetic acid

Table: 7.6: Color measurements (L, a, b values) of romaine leaves treated with essential oils and stored for 14 days at 4°C

Color Values ^a												
Treatment	Day 0			Day 2			Day 7			Day 14		
	L*	a*	b*	L*	a*	b*	L*	a*	b*	L*	a*	b*
0ppm	50±5 ^{ax}	-19±2 ^{ax}	30±5 ^{ax}	48±6 ^{ax}	-19±2 ^{ax}	29±6 ^{ax}	48±8 ^{ax}	-19±3 ^{ax}	30±7 ^{ax}	49±8 ^{ax}	-19±2 ^{ax}	32±7 ^{ax}
800C	49±6 ^{ax}	-20±3 ^{ax}	31±6 ^{ax}	51±6 ^{ax}	-20±3 ^{ax}	33±6 ^{ay}	51±7 ^{ax}	-21±4 ^{ax}	36±1 ^{axy}	50±6 ^{ax}	-21±2 ^{ax}	36±2 ^{ax}
800S	46 ±2 ^{ax}	-18±1 ^{ax}	28±3 ^{ax}	46±4 ^{ax}	-19±2 ^{ax}	30±4 ^{ax}	45±1 ^{ax}	-18±1 ^{ax}	28±2 ^{ax}	44±3 ^{ax}	-19±1 ^{ax}	29±2 ^{ax}
1000C	47±4 ^{ax}	-19±2 ^{ax}	31±4 ^{ax}	48±4 ^{ax}	-20±2 ^{ax}	32±5 ^{ay}	48±2 ^{ax}	-21±1 ^{ax}	33±3 ^{axy}	49±4 ^{ax}	-21±2 ^{ax}	35±3 ^{ax}
1000S	46±2 ^{ax}	-19±3 ^{ax}	30±2 ^{ax}	45±4 ^{ax}	-19±1 ^{ax}	30±3 ^{ax}	46±3 ^{ax}	-18±2 ^{ax}	28±3 ^{ax}	47±3 ^{ax}	-20±1 ^{ax}	32±3 ^{ax}
1000SV	44±3 ^{ax}	-19±2 ^{ax}	29±4 ^{ax}	45±2 ^{ax}	-18±2 ^{ax}	28±4 ^{ax}	45±2 ^{ax}	-19±2 ^{ax}	30±3 ^{ax}	46±2 ^{ax}	-20±1 ^{ax}	31±3 ^{ax}

^a Values are mean ± SD. Each experiment was replicated ten times.

Values in the same row for the same parameter not followed by the same letters (xy) are significantly different; values in the same column followed by same letter a are not significantly different (P < 0.05).

800C – 800 ppm cinnamaldehyde, 800T – 800 ppm cinnamaldehyde in 0.5% tween 20, 800S -800 ppm Sporan®, 1000C – 1000 ppm cinnamaldehyde, 1000CV – 800 ppm cinnamaldehyde + 200 ppm acetic acid, 1000S – 1000 ppm Sporan®, 1000SV - 800 ppm Sporan®+ 200 ppm acetic acid.

7.5 Discussion

Washing with water reduced ca. 1 log CFU/g of *E. coli* O157:H7 and *Salmonella* on cut iceberg and romaine lettuce. Similar results were reported by others (Singh et al., 2002; Chang and Fang, 2007, Kim et al., 2011; Moore et al., 2011). Essential oils have been evaluated to reduce pathogen population on fresh produce. Kim et al. (2011) found significant effect of clove extracts in reducing *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* Typhimurium on fresh lettuce. In this study, cinnamaldehyde and Sporan® alone or in combination with acetic-acid were used to reduce pathogenic populations on fresh cut lettuce. The results showed that the antimicrobial efficacy of these oils was dependent on pathogen, type of produce, and storage period. Oussalah et al. (2007) reported that the bacteriostatic concentrations of cinnamaldehyde from bark (87%) were 0.025 and 0.05 % (v/v), respectively, for *E. coli* and *Salmonella* Typhimurium. However, a greater concentration was needed to achieve the same result in food (Shelef, 1983, Smid and Gorris, 1999) than in the laboratory media because the active components could bind with food ingredients such as proteins or fats (Davidson, 1997). The antimicrobial effect of oils against native microflora was not evident in our study. The concentration may not have been sufficient to reduce all mesophilic, psychrotrophic bacteria, total coliforms, yeast and molds. Among coliforms, enteric pathogens were more susceptible to antimicrobials. Several substances have been used to dissolve the essential oils or to stabilize it in water-based culture media, such as ethanol, methanol, Tween 20, acetone, polyethylene glycol, *n*-hexane, dimethyl sulfoxide (Burt, 2004). In this study, use of Tween 20 to dissolve cinnamaldehyde lowered the pH of the solution from 4.17 to 3.83, increased its diffusion on the surface of lettuce, and

contributed to its efficacy. A cinnamaldehyde-Tween treatment (800T) significantly reduced *E. coli* O157:H7 populations on iceberg lettuce at day 2 and *Salmonella* populations on romaine and iceberg lettuce at day 7 and 14, respectively. However, the same effect was not evident when cinnamaldehyde-Tween was used against *E. coli* O157:H7 populations on romaine lettuce. While some researchers have recommended additives to dissolve or stabilize the essential oils in water based culture (Hammer et al., 1999), others have reported reduced activity of oils when emulsifiers and solvents are used (Remmal et al., 1993; Hili et al., 1997; Mann and Markham, 1998). Furthermore, a number of potential synergists have been suggested for use with essential oils, such as low pH, low water activity, chelators, low oxygen tension, mild heat and raised temperature (Burt, 2004). In this study, adding acetic acid (200 ppm) to cinnamaldehyde (800 ppm) lowered the pH from 4.17 to 4.00; however, the inhibitory effect of cinnamaldehyde was not significant when acetic acid was added. The synergistic effect of lower pH may also depend on type of essential oil, oil concentration, pathogenic strain and type of fresh produce used in the study.

It has been demonstrated that cinnamaldehyde disrupts cell membrane causing leakage of small ions (Gill and Holley, 2004). It has been suggested that the antimicrobial activity of essential oils is attributed to more than one mechanism (Burt, 2004; Moreira et al., 2005). Sporan® is a proprietary fungicide for agricultural crops which contains a proprietary blend of 10 % of clove and thyme and 18 % of rosemary essential oils. In our previous studies, antibacterial activity of Sporan® against *E. coli* O157:H7 and *Salmonella* in organic soil was dose dependent (Yossa et al., 2010, 2011). Other researchers achieved a higher inactivation of enteric bacteria on fresh lettuce with high concentration of essential

oils (Gunduz et al., 2010; Kim et al., 2011). However, the effect of oil concentration was not significant on iceberg and romaine lettuce in this study. These results indicated that the antimicrobial effects of these oils were also dependent on bacterial strain, storage period, and type of produce. It also could be that a difference of 200 ppm was not sufficient to expect a significant reduction throughout the study. Addition of 200 ppm acetic acid to Sporan® lowered the pH from 4.7 to 4.21 and contributed to higher antimicrobial activity of Sporan® in some cases. Increased antimicrobial activities of thymol (Juven et al., 1994) and rosemary (Del Campo et al., 2000) were observed when tested at lower pH. Juven et al. (1994) stated that the susceptibility of bacteria to essential oils might increase with lower pH values, since the hydrophobicity of the oils increases at low pH, consequently enabling easier dissolution in the lipids of the cell membrane of *S. Typhimurium*. Sporan® is a fungicide, whose mode of action is to disrupt the cell wall of fungal spores and hyphae (Anonymous, 2008). Its mechanism of action on bacteria is unknown, but since it is made up of clove, rosemary and thyme oil, the mode of action could be disintegration of the cellular membrane followed by leakage of cellular components. Devi et al. (2010) demonstrated that eugenol, the active component in clove, causes the disruption of cytoplasmic membrane, and further increases its permeability, which subsequently causes death of *Salmonella typhi*. Eugenol can also collapse fungal cell membrane (Atsumi et al., 2001). It has been stated that thymol disintegrated the outer membrane of gram negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP (Burt, 2004).

Throughout the storage period, texture of cut iceberg and romaine washed with 800C, 800S, 1000C, 1000SV was not significantly different from control lettuce with the

exception of iceberg samples treated with 800C at day 0. Likewise, color characteristics of treated romaine lettuce were not significantly different from control; however, the lightness of iceberg lettuce was affected by cinnamaldehyde.

7.6 Conclusion

The effectiveness of essential oils used in this study against native microbiota was comparable to that of chlorine. Recovery of *E. coli* O157:H7 populations from lettuce treated with essential oils were significantly lower than in chlorine treated samples at day 0 and 7 for iceberg and day 0 and 14 for romaine lettuce stored at 4°C. The effect of these oils was comparable to chlorine in reducing *Salmonella* populations on iceberg and romaine lettuce throughout the storage period. In addition, the texture and the color of iceberg and romaine leaves treated with oils were not different from control lettuce. The results of this study suggest that Sporan® plus acetic acid has the potential to be used as a produce wash treatment to control enteric pathogens in fresh produce provided that sensory characteristics of treated lettuce are acceptable. Further studies simulating industrial settings will be helpful. The natural antimicrobial combination can also be used as an alternative decontaminant for organic fresh produce.

Chapter 8: Antibacterial Activity of Cinnamaldehyde and Sporan® against *Escherichia coli* O157:H7 and *Salmonella*

Yossa et al., to be submitted International Journal of Applied Microbiology.

8.1 Overview

Fresh produce has been implicated as a vehicle of *E. coli* O157:H7 and *Salmonella* infections in recent years. Natural antimicrobials have been evaluated as produce wash to meet consumers' preference for natural ingredients or less chemicals in food supplies. We evaluated the antimicrobial effect of cinnamaldehyde and Sporan® on *E. coli* O157:H7 and *Salmonella*. A five strain cocktail of *E. coli* O157:H7 and *Salmonella* were inoculated in Luria-Bertoni broth (7 log CFU/ml) containing cinnamaldehyde or Sporan® (800 and 1000 ppm) alone or in combination with 200 ppm acetic acid, and incubated at 37°C for up to 6 h. *E. coli* O157:H7 and *Salmonella* were undetectable after 1 h in presence of 800 ppm cinnamaldehyde. A 1000 ppm Sporan® significantly reduced *Salmonella* and *E. coli* O157:H7 populations by 1.83- and 3.02 log CFU/ ml within 2 and 4 h, respectively. The synergistic effect of acetic acid was not evident as it did not enhance ($P > 0.05$) the bactericidal activity of oils. Cinnamaldehyde was highly effective against both *E. coli* O157:H7 and *Salmonella* whereas the effect of Sporan® was dependent on its concentration, exposure time, and pathogen. *E. coli* O157:H7 was more sensitive to the oils than *Salmonella*.

Scanning and Transmission electron microscopy of oil-treated bacterial cells revealed cell structural damage and leakage of cellular content.

8.2 Introduction

Foodborne illnesses caused by the consumption of food contaminated with pathogenic bacteria have been concern to public health (Burt and Reinders 2003; Oussalah et al. 2007). From 2000 to 2008, *Salmonella* alone caused 380 deaths, 19,000 hospitalizations and 1 million illnesses in the United States. Although, the number of infection with *E. coli* O157:H7 decreased, *E. coli* O157:H7 still remain a concern; ranked fifth in causing 63,000 illnesses, 2100 hospitalizations, and 20 deaths in the United States (CDC, 2011a). The battle against these bacteria remain challenging because they contaminate diverse foods; *Salmonella* can be found in poultry, eggs, ground meat, fruits, vegetables, and processed food such as frozen pot pies, peanut (CDC, 2011b). *E. coli* O157:H7 has been detected from ground meat, poultry, fruits, leafy greens, dough, and processed foods as pizza (CDC, 2011c).

The antimicrobial properties of some essential oils and their components have been described earlier (Shelef 1983; Nychas, 1995). Recent studies have determined the antimicrobial activity of cinnamaldehyde, cloves, thyme, and rosemary against *E. coli* O157:H7 and *Salmonella* (Hammer et al., 1999; Del Campo et al., 2000; Rhayour et al., 2003; Kim et al., 2004; Oussalah et al., 2007). Thyme and clove oils disintegrate outer membrane of gram negative bacteria releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane (Lambert et al., 2001, Devi et al., 2010). Wendakoon and Sakaguchi (1995) reported that the carbonyl group of cinnamaldehyde binds to the proteins, preventing amino acid decarboxylase activity in *Enterobacter aerogenes*. Smid et al. (1996) observed damage to cytoplasmic membrane of *Saccharomyces cerevisiae* when treated with cinnamaldehyde, leading to excessive

leakage of metabolites and enzymes from the cell and finally loss of viability. We evaluated the effects of cinnamaldehyde and Sporan®, a proprietary blend of 10% clove, 18% rosemary and 10% thyme oils against *E. coli* O157:H7 and *Salmonella* strains *in vitro*. The effect of these oils on *E. coli* and *Salmonella* cell structure was observed through the scanning and transmission electron microscopy (SEM and TEM).

8.3 Materials and Methods

8.3.1 Bacteria and essential oils

Five nalidixic acid resistant strains of *E. coli* O157:H7 and five *Salmonella* strains from the laboratory stock were used to investigate the minimal inhibitory concentration and the effect of cinnamaldehyde on the survivability of the bacterial cells. The strains of *E. coli* O157:H7 RM 4406, RM 4688, and RM 1918 (clinical isolates from lettuce outbreaks), RM 4407 (clinical isolate from spinach outbreak), and RM 5279 (clinical isolate, bagged vegetable isolate, outbreak) were kindly provided by Robert Mandrell (U.S. Department of Agriculture, Albany, CA). Five *Salmonella enterica* serovars, *S. Braedenrup* (CDC clinical isolate), *S. Newport* and *S. Negev* 26 H (Thyme isolates). *S. Thompson* 2051H and *S. Tennessee* 2053N were used from our Environmental Microbial and Food Safety Laboratory culture collection. A *dam* mutant *Salmonella* MT 2195 and *E. coli* O157:H7 B 6914 were used for Scanning and Transmission Electron Microscopy studies.

Cinnamaldehyde (> 93%, Sigma-Aldrich, St. Louis, MO, USA), and Sporan® (EcoSMART Technologies, Alpharetta, GA) were used to prepare 800 and 1000 ppm cinnamaldehyde (800C, 1000C) and Sporan® (800S, 1000S) in Luria-Bertani, Broth (LB, Acumedia, Lansing, MI, supplemented with 50 ppm nalidixic acid for *E. coli*, LBN).

Additionally, these oils were used in combination with acetic acid (20%, Fleischmann's Inc., Baltimore, MD) as 800 ppm cinnamaldehyde +200 ppm acetic acid (1000CV) and 800 ppm Sporan® +200 ppm acetic acid (1000SV).

8.3.2 Effect of cinnamaldehyde and Sporan® on the viability of nalidixic acid resistant *E. coli* O157:H7 and *Salmonella*

Bacterial cells were grown overnight in LB or LBN at 37°C. The actively growing overnight cultures were centrifuged (7500 xg, 10 min, 10°C), washed in 0.1 M sterile phosphate buffer solution (PBS, pH 7.0)). Bacterial populations of each strain were adjusted by measuring the OD at 600nm at 0.9 – 1 and cocktails of *Salmonella* and *E. coli* O157:H7 strains were prepared using equal volume of five strains. Three ml of the cocktail (~7 log CFU/mL) were transferred to 27 ml LB broth containing 800, and 1000 ppm of cinnamaldehyde or Sporan® alone or in combination with 200 ppm acetic acid and incubated at 37°C in shaker incubator (75 rpm) for 6 h. Samples (1 ml) were pulled every h and were centrifuged at 12,000 rpm for 5 min, washed with PBS, and spiral plated, or spread on Sorbitol MacConkey media (Acumedia) supplemented with 0.05mg/l of cefixime, 2.5mg/l of potassium tellurite and 50 ug/ml nalidixic acid (Sigma-Aldrich, CTSMAC-N) for *E. coli* O157:H7 and on XLT4 agar (Acumedia) for *Salmonella*, in duplicate. Cells suspended in LB or LBN without oils were used as control.

8.3.3 Cell preparation for microscopy

E. coli O157:H7 B 6914 and *Salmonella* MT 2195, non pathogenic strains were actively cultured for 24 h in tryptic soy broth (TSB, Acumedia). Bacterial cells were harvested by centrifugation as described above and washed with sterile water. Five ml of sterile TSB containing an aliquot of cinnamaldehyde, Sporan® and Sporan® + acetic acid was added

to the cell pellet and incubated for 3 h at 37°C. Following incubation, bacterial cells were washed three times with sterile water and observed under microscopy.

8.3.4 Scanning electron microscopy (SEM)

Low-temperature SEM observations were performed using an S-4700 field emission scanning electron microscope (Hitachi High Technologies America, Inc., Pleasanton, CA) equipped with a Quorum CryoPrep PP2000 (Quorum Technologies, Ltd., East Sussex, UK) cryotransfer system. Bacteria were transferred onto filter paper (Whatman #1) which was attached with a thin layer of Tissue Tek (OCT Compound, Ted Pella, Inc., Redding, CA), which acted as the cryo-adhesive upon freezing to a flat specimen holder consisting of 16 x30 mm copper plate. The samples were frozen conductively, in a Styrofoam box, by placing the plates on the surface of a pre-cooled (-196°C) brass bar whose lower half was submerged in liquid nitrogen (LN₂). After 20-30s, the holders containing the frozen samples were transferred to a LN₂ Dewar for future use or cryotransferred under vacuum to the cold stage in the pre-chamber of the cryotransfer system. Removal of any surface contamination (condensed water vapor) took place in the cryotransfer system by etching the frozen specimens for 10-15 min by raising the temperature of the stage to -90°C. Following etching, the temperature was lowered below -130°C, and a magnetron sputter head equipped with a platinum target, was used to coat the specimens with a very fine layer of platinum. The specimens were transferred to a pre-cooled (-140°C) cryostage in the SEM for observation. An accelerating voltage of 5kV was used to view the specimens. Images were captured using a 4pi Analysis System (Durham, NC).

8.3.5 Transmission electron microscopy (TEM)

Salmonella was fixed for 1 hour at room temperature by immersion in 3% glutaraldehyde / 0.05M NaCacodylate Buffer, pH7.0. This was followed by washing in a .05M NaCacodylate buffer rinse, 3 times over 1 hour, post fixed in 2% buffered osmium tetroxide for 2 hours, dehydrated in ETOH and infiltrated with Spurr's low-viscosity embedding resin. 60-90nm silver-gold sections of the tissue were cut on a Reichert/AO Ultracut microtome with a Diatome diamond knife and mounted onto 400 mesh Ni grids. They were stained with 4% uranyl acetate and 3% lead citrate and viewed in an HT-7700 Hitachi Microscope at 80kV.

8.3.6 Statistical analysis

Colony counts of bacterial cells were converted to log CFU/ml. The experiment was performed in triplicate. Data were analyzed by a 2 way ANOVA using Tukey test for effects of oil concentrations, time, and their interactions. In all cases, the level of statistical significance was $p < 0.05$.

8.4 Results

8.4.1 Effect of cinnamaldehyde and Sporan® on the viability of *E. coli* O157:H7 and *Salmonella*

The growth curves of nalidixic acid resistant *E. coli* O157:H7 and *Salmonella* in LB broth containing cinnamaldehyde and Sporan® are shown in figures 1 and 2.

E. coli O157:H7 populations in control LB broth increased from 5.72 to 8.38 log CFU/ml during the 6 h incubation at 37°C. All treatments reduced *E. coli* O157:H7 at 1 h. The effect of 800C, 1000C and 1000CV on *E. coli* O157:H7 were bactericidal within 1 hour, the *E. coli* O157:H7 populations were undetectable in 1h (< 1 log CFU/ml). *E. coli* O157:H7 populations were reduced by ca. 1.25 log CFU/ml during the first 2 h

incubation followed by increase in their populations when treated with 800 ppm Sporan® alone or in combination with acetic acid. However, populations after 3 and 4 h in 800S-treated LB both (5.06 and 5.71 log CFU/ml) and 1000SV-treated LB broth (5.11 and 5.74 log CFU/ml) were significantly lower than those in control (7.70 and 8.02 log CFU/ml), respectively. Significant reductions in *E. coli* O157:H7 populations were observed throughout the 6 h period when treated with 1000 ppm Sporan®. *E. coli* O157:H7 populations in 1000S-treated LB broth were 4.83-, 5.32-, 4.99-, and 4.46 log CFU/ml, which were significantly lower than their corresponding controls at 3, 4, 5, and 6 h, respectively.

Likewise, the populations of *Salmonella* in control LB broth increased from 6.23 to 8.48 log CFU/ml during the 6 h incubation period (Figure 2). Similar to *E. coli* O157:H7 results, the effects of cinnamaldehyde at all concentrations were bactericidal within 1 h. Nevertheless, a marginal growth of *Salmonella* populations of 0.23 - 0.47 log/CFU ml were observed in cinnamaldehyde treated LB broths at 5-6 hour. *Salmonella* populations were increased in 800S- and 1000SV-treated LB broth after 2 h, the difference in *Salmonella* populations of 800S- and 1000SV- treated LB broth was not significant from those of control LB broth at 4-6 h. *Salmonella* populations were reduced by 1.83 CFU/ml within 2 h in 1000S-treated LB broth followed by increase to reach to initial inoculums level (ca. 6.23 CFU/ml). *Salmonella* populations in 1000S-treated LB broth after 2, 3, 4, and 5 h (4.40, 4.75, 4.99, and 5.69 log CFU/ml), respectively were significantly lower than their corresponding controls.

The effect of 800 ppm Sporan® was insignificant irrespective of presence of acetic acid.

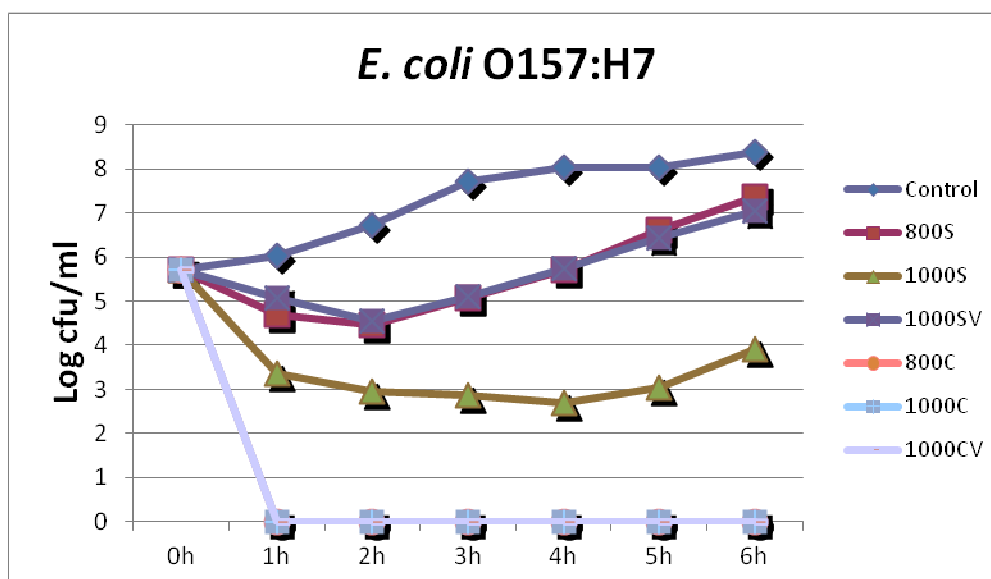


Fig. 8.1: Growth curves of mixed strains of nalidixic acid resistant *E. coli* O157:H7 in presence of cinnamaldehyde, Sporan® and Sporan®-acetic acid.
 800C – 800 ppm cinnamaldehyde, 800S -800 ppm Sporan®, 1000C – 1000 ppm cinnamaldehyde, 1000CV – 800 ppm cinnamaldehyde + 200 ppm acetic acid, 1000S – 1000 ppm Sporan®, 1000SV - 800 ppm Sporan®+ 200 ppm acetic acid.

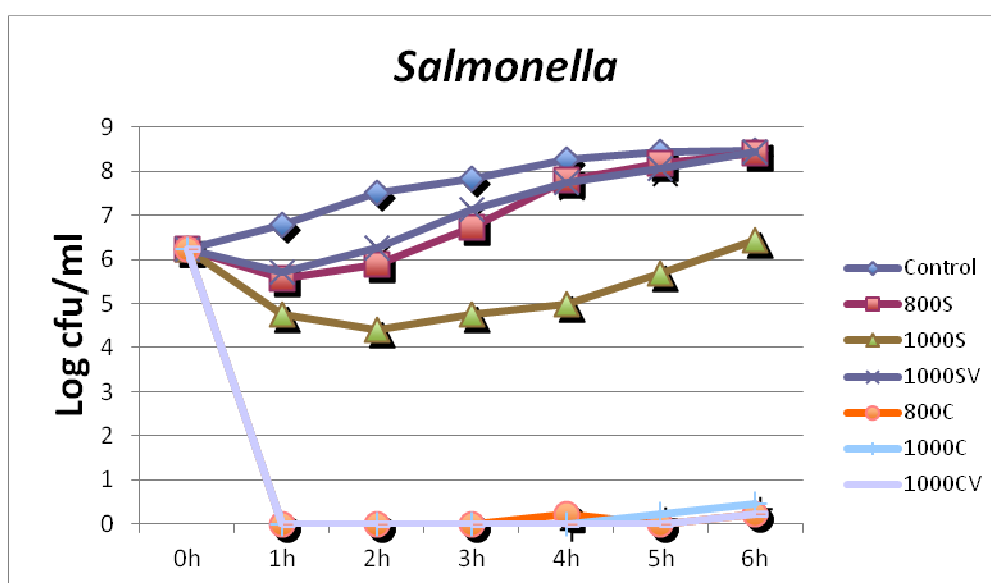


Fig. 8.2: Growth curves of mixed strains of *Salmonella* in presence of cinnamaldehyde, Sporan® and Sporan®-acetic acid.
 800C – 800 ppm cinnamaldehyde, 800S -800 ppm Sporan®, 1000C – 1000 ppm cinnamaldehyde, 1000CV – 800 ppm cinnamaldehyde + 200 ppm acetic acid, 1000S – 1000 ppm Sporan®, 1000SV - 800 ppm Sporan®+ 200 ppm acetic acid.

8.4.2 SEM and TEM images of *E. coli* O157:H7 and *Salmonella* treated with cinnamaldehyde and Sporan®

SEM images of treated *E. coli* O157:H7 showed different surface patterns than the untreated control (Fig. 8.3). The untreated cells presented wave-like structures, whereas the treated cells showed surface deformation. *E. coli* O157:H7 cells treated with cinnamaldehyde, Sporan® and Sporan® + acetic acid appeared wrinkled and shrunken, including morphological rod alterations. In addition, some cells treated with cinnamaldehyde or Sporan® + acetic acid were transparent. Likewise, TEM image of *E. coli* B1914 (Fig. 8.4) of untreated cells showed no alterations of the internal structures while treated cells exhibited a granulated surface for some cells, deformation and even disruption of the outer membrane for other cells.

Similarly, SEM images of treated *Salmonella* cells showed morphological alterations (Fig. 8.5). When compared to untreated cells, all treated cells collapsed and appeared empty of contents. On the other hand, images of TEM of *Salmonella* cells (Fig. 8.6) revealed severe damage caused by the antimicrobial treatment. Cells treated with cinnamaldehyde, Sporan®, and Sporan® + acetic acid showed either empty content without visible changes in the outer membrane, disruption of the plasma membrane, or cell wall lysis.

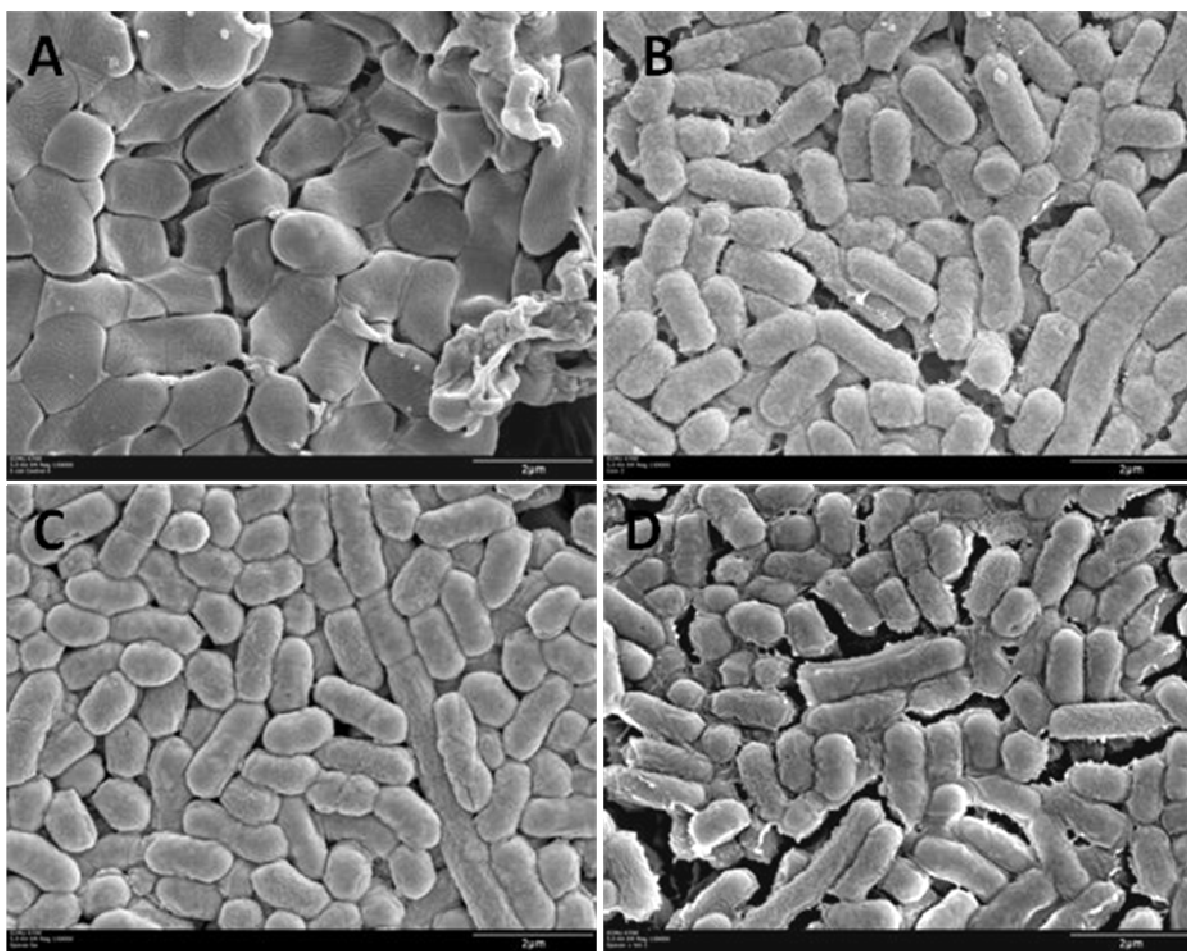


Fig. 8.3: Scanning electron microscopy of *E. coli* B 6914 cells after a 3 h treatment with cinnamaldehyde, Sporan® and Sporan®-acetic acid. Images were viewed in S- 4700 Hitachi.
 (a) Untreated cells;
 (b) Cells treated with 0.4 % cinnamaldehyde;
 (c) Cells treated with 0.4 % Sporan®;
 (d) Cells treated with 0.36 % Sporan® + 0.04 % acetic acid.
 Scale: 2 µm, 5.0 KV EM MAG 7000 X.

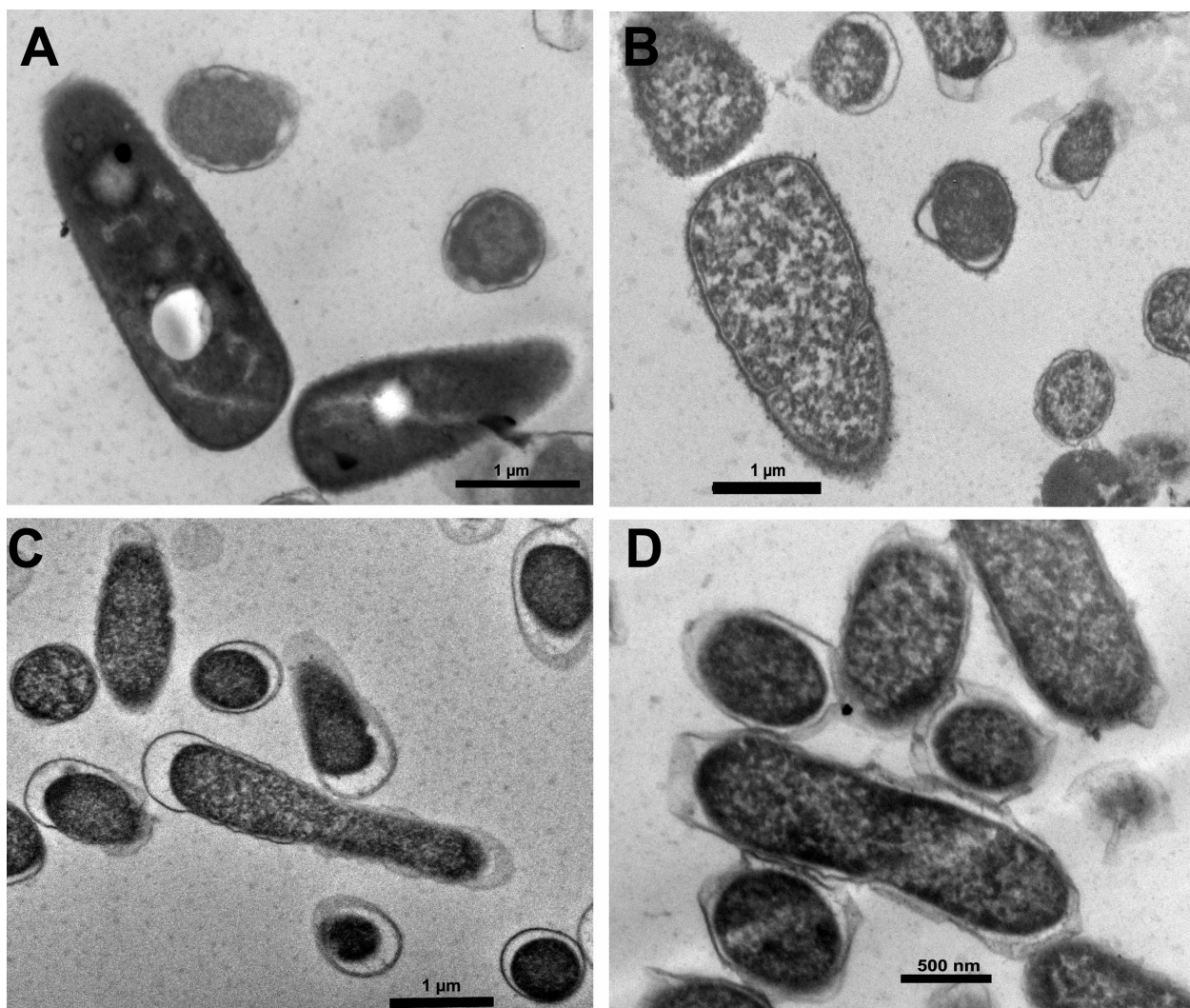


Fig.8.4: Transmission electron microscopy of *E. coli* B 6914 cells after 3 h treatment with cinnamaldehyde, Sporan® and Sporan®- acetic acid. Images are viewed in HT – 7700 Hitachi Microscope at 80 kV.

(a) Untreated cells; Magnification: x 33.0K (x 8.0K)

(b) Cells treated with 0.4 % cinnamaldehyde; Magnification: x 29.0K (x 7.0K)

(c) Cells treated with 0.4 % Sporan®; Magnification: x 25.0K (x 6.0K)

(d) Cells treated with 0.36 % Sporan® + 0.04 % acetic acid; Magnification: x 41.0K (x 10.0K)

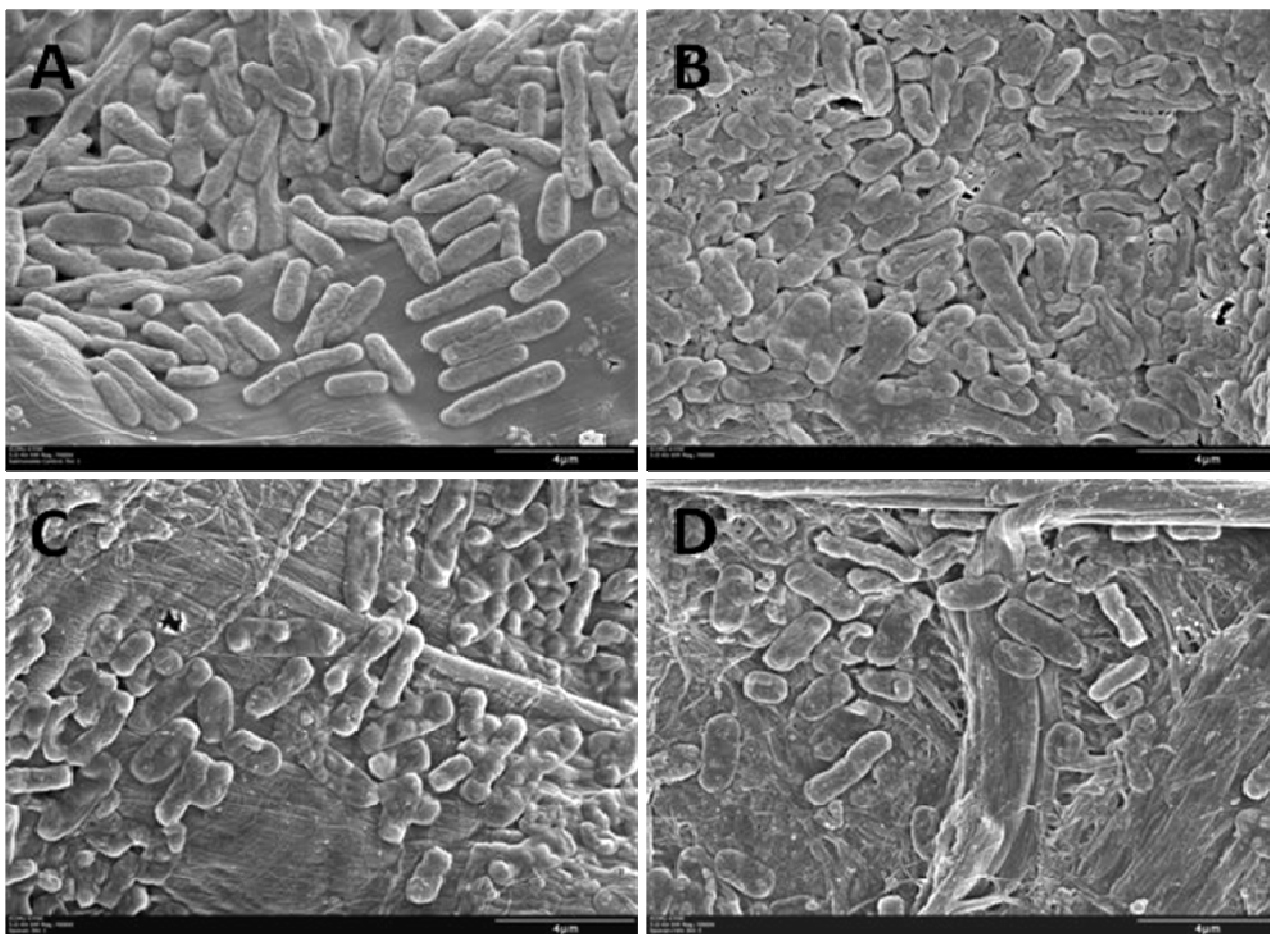


Fig. 8.5: Scanning electron microscopy of *Salmonella* MT 2195 cells after a 3 h treatment with cinnamaldehyde, Sporan® and Sporan®- acetic acid. Images were viewed in S- 4700 Hitachi.

- (a) Untreated cells;
 - (b) Cells treated with 0.4 % cinnamaldehyde;
 - (c) Cells treated with 0.4 % Sporan®;
 - (d) Cells treated with 0.36 % Sporan® + 0.04 % acetic acid.
- Scale: 4 μ m, 5.0 KV EM MAG 7000 X.

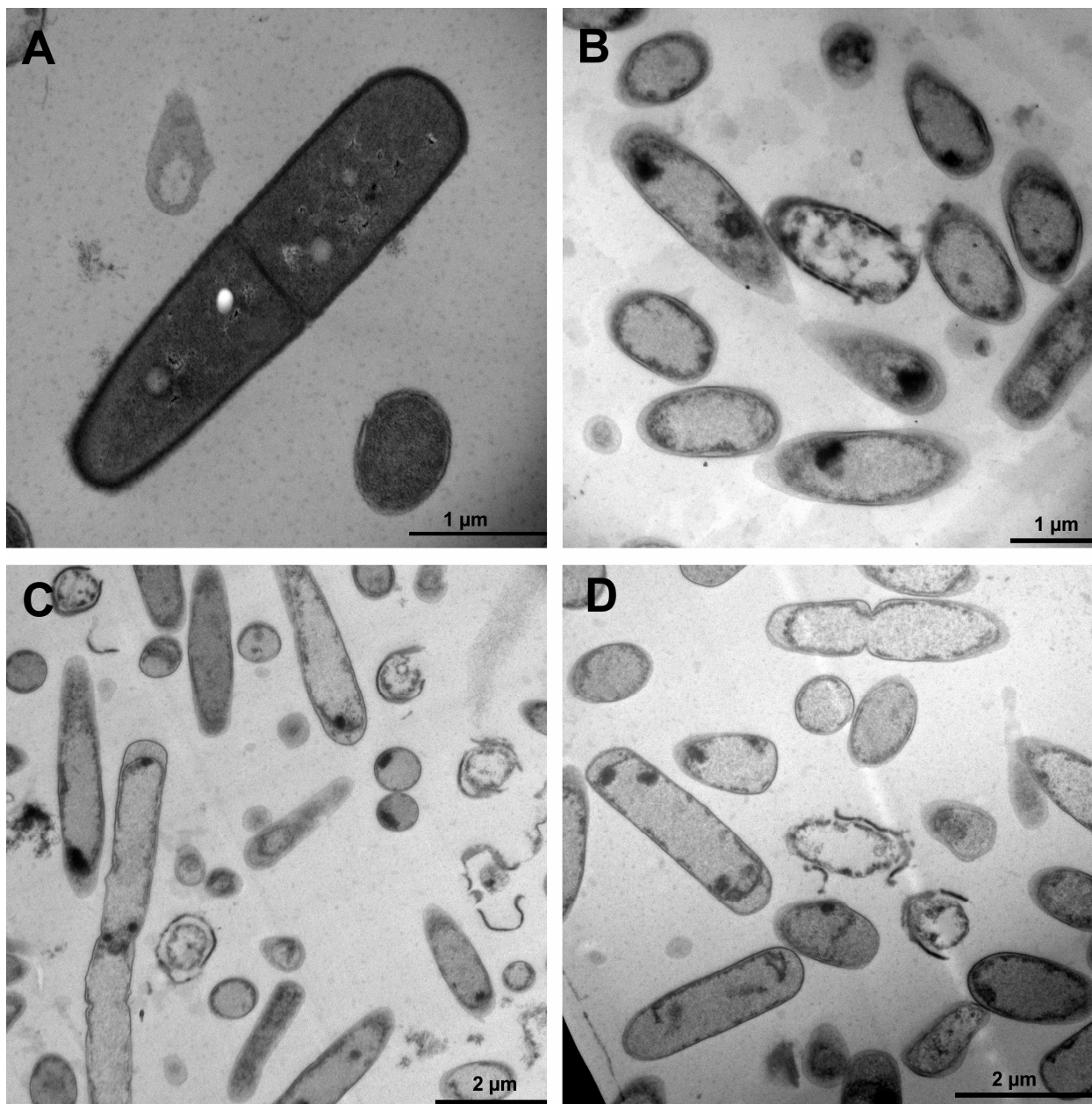


Fig. 8.6: Transmission electron microscopy of *Salmonella* MT 2195 cells after 3 h treatment with cinnamaldehyde, Sporan® and Sporan®- acetic acid. Images are viewed in HT – 7700 Hitachi Microscope at 80 kV.

- (a) Untreated cells (Magnification = x 41.0K);
- (b) cells treated with 0.4 % cinnamaldehyde (Magnification = x 49.0K);
- (c) cells treated with 0.4 % Sporan® (Magnification = 41.0K);
- (d) cells treated with 0.36 % Sporan® + 0.04 % acetic acid (Magnification = x 41.0K).

8.5 Discussion

Essential oils are the odorous volatile products of an aromatic plant's secondary metabolism, found in leaves, bark, fruit, and when they occur in various organs in the same plant, they frequently have different composition profiles (Oussalah et al., 2007). Recently, antimicrobial effects of essential oils against bacteria, yeasts, fungi and viruses have been reported (Reichling et al., 2009). The major active components in essential oils are phenols, terpenes, and aldehydes (Ceylan and Fung, 2004), and these mainly damage the cell cytoplasmic membrane (Di pasqua et al., 2007; Sikkema et al. 1995).

In this study, cinnamaldehyde exhibited strong antimicrobial properties on *E. coli* O157:H7 and *Salmonella*. Our results are in agreement with Kim et al. (2004) who observed complete inhibition of *E. coli* O157:H7 in LB containing 1000 ppm of cinnamaldehyde after 2 h. Ali et al. (2005) observed 3.5 log₁₀ reduction of *E. coli* O157:H7 in broth containing 1 ppm cinnamaldehyde after 75 min. Likewise, Helander et al. (1998) reported inhibition of *E. coli* and *Salmonella* Typhimurium at 132-396 ppm cinnamaldehyde in LB broth. Cinnamaldehyde is a highly effective fungicidal agent (Smid et al., 1996) with minimal mammalian toxicity (Jenner et al., 1964). On the other hand, the inhibitions of bacterial cells by Sporan® were dependent on its concentration, exposure time, and test pathogen. Combination of acetic acid with Sporan® to the treatment did not improve its antimicrobial effects.

SEM images of untreated *E. coli* O157:H7 and *Salmonella* cells revealed a surface with wrinkled protrusions, naturally present structures on the surface of living bacteria. Similar observations of intact cells were reported by Greif et al. (2010). In our study, the cell membranes of *E. coli* O157:H7 were severely affected by antimicrobial treatments rather than its cellular content. This finding is in agreement with those of Di Pasqua et al. (2007) who stipulated that cinnamaldehyde

and thymol caused structural alteration of the outer envelope in *E. coli* O157:H7. The *E. coli* O157:H7 cell envelopes of treated cells were transparent indicating interactions of the antimicrobials compounds with the cells membrane. Helander et al. (1998) reported that cinnamaldehyde penetrated to the periplasm and to the cell interior of Gram-negative bacteria through outer membrane-traversing porin proteins. *E. coli* O157:H7 cells treated with 625 ppm oregano oil collapsed, resulting in leakage of cellular contents (Burt and Reinders, 2003). Cinnamaldehyde and Sporan® damaged outer membrane of *E. coli* O157:H7 cells in our study. We speculate that antimicrobial action of cinnamaldehyde and Sporan® could be different from oregano oil, or the strain variation might have influenced differences in our results.

On the other hand, *Salmonella* seemed to be affected internally and externally. The penetration of antimicrobials in the cell envelope might have caused internal damage to the *Salmonella* cells. Our results are in agreement with Smid et al. (1996) who reported disintegration of the cytoplasmic membrane of *Saccharomyces cerevisiae*, leading to excessive loss of viability. Cellular damage to *Salmonella* by cinnamaldehyde and Sporan® was similar. Sporan® has been reported to disrupt cell membrane of fungal hyphae and spores (Anonymous, 2008).

Most authors have suggested that the modes of action of essential oils depend on the type of microorganisms, mainly on their cell wall structure and to their outer membrane arrangement. They observed cellular damage due to the significant differences in the outer membranes of Gram-negative and Gram-positive bacteria (Rhayour et al., 2003; Shan et al., 2007). In the light of our findings, SEM and TEM images showed us that essential oils could interfere differently with cells belonging to the same bacteria group. Despite these differences, the mechanism of action of cinnamaldehyde and Sporan® involves the cell membrane.

8.6 Conclusion

Some European countries have abandoned the use of hypochlorite for disinfection of foods; environmental friendly natural plant-derived antimicrobials with less mammalian toxicity could be better alternatives. This study shows that cinnamaldehyde and Sporan®® effectively eliminate or reduce enteric pathogens. These antimicrobials should be evaluated for their potential in reducing pathogens in various foods, including poultry, livestock food and water, compost manure as well as irrigation water.

Chapter 9: Conclusion

In organic soil, reduction of *E. coli* O157:H7 and *Salmonella* populations varied with oil concentrations and the strains. The concentration of 2 % of cinnamaldehyde, Sporan® and acetic acid reduced up to 5 log CFU/g and 6 log CFU/g *E. coli* O157:H7 and *Salmonella* in soil. Likewise, essential oils effectively reduced *E. coli* O157:H7 and *Salmonella* populations on produce without affecting the color and texture of leaves. Preliminary investigation was conducted on the effect of antimicrobials on the sensory attributes of spinach and iceberg lettuce. of the untrained panel members were able to identify the treated samples at day 0 (Table 9.1). However, they were unable to distinguish spinach samples treated with 800S and 1000SV and iceberg samples treated with 800C and 1000SV after 2 days of storage at 4 °C (Table 9.2). The study shows the efficacy of essential oils in controlling enteric pathogens in organic soil and the possibility of extending the application of cinnamaldehyde, Sporan® and Sporan®-acetic acid as produce disinfectant. Figure 9.1 shows the potential points of application of essential oils during the production (before and after harvest) chain of produce.

Table 9.1: Sensory evaluation of spinach and iceberg leaves treated with essential oils at day 0

	Spinach			Iceberg		
Samples	accept	reject	%	accept	reject	%
800S	4	14	63.6	5	15	68.2
Control	14	4	18.2	16	4	18.2
Control	14	4	18.2	17	3	13.6
1000SV	6	12	66.7	5	15	65.2
Control	15	3	16.7	17	3	13.0
Control	15	3	16.7	15	5	21.7
800C	5	13	56.5	7	13	61.9
Control	13	5	21.7	16	4	19.0
Control	13	5	21.7	16	4	19.0

N = 18 unlearned panelists

800C – 800 ppm cinnamaldehyde, 800S – 800 ppm Sporan®, 1000SV – 800 ppm Sporan® + 200 ppm acetic acid.

Table 9.2: Sensory evaluation of spinach and iceberg leaves treated with essential oils after 2 days

	Spinach			Iceberg		
Samples	accept	reject	%	accept	reject	%
800S	16	9	34.6	9	16	61.5
Control	15	10	38.5	22	3	11.5
Control	18	7	26.9	18	7	26.9
1000SV	18	7	29.2	13	12	48.0
Control	17	8	33.3	15	10	40.0
Control	16	9	37.5	22	3	12.0
800C	10	15	62.5	16	9	32.1
Control	20	5	20.8	17	8	28.6
Control	21	4	16.5	14	11	39.3

N = 25 unlearned panelists

800C – 800 ppm cinnamaldehyde, 800S – 800 ppm Sporan®, 1000SV – 800 ppm Sporan® + 200 ppm acetic acid.

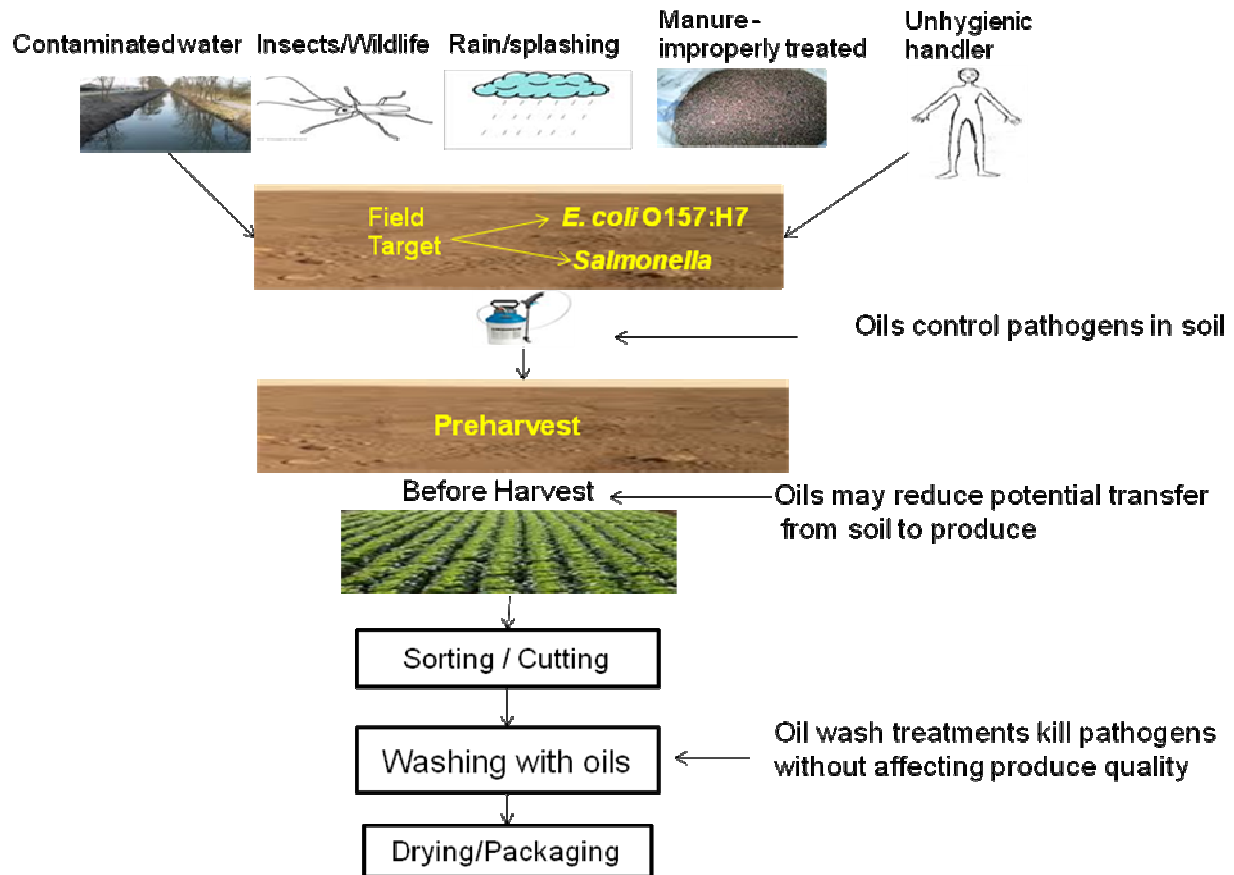


Fig. 9.1: Strategies for preventing/eliminating foodborne pathogens on produce

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