

INACTIVATION OF *SALMONELLA* SPP. AND *ESCHERICHIA COLI* O157:H7 ON
DIFFERENT FOOD COMMODITIES BY VOLATILE ANTIMICROBIALS

by

MOHAMMAD M. OBAIDAT

(Under the Direction of Joseph F. Frank)

ABSTRACT

We determined the ability of carvacrol, cinnamaldehyde and ally isothiocyanate (AIT) in their vapor phase to inactivate *E. coli* O157:H7 and *Salmonella* spp. in whole and sliced tomatoes and their effect against *E. coli* O157:H7 on the surface and within lettuce and spinach tissues. Cultural and microscopic methods were employed to confirm inactivation of internalized cells on lettuce. Tomatoes samples were treated with various concentrations of antimicrobial in the vapor phase at 4, 10, and 25 °C in a closed container. Lettuce and spinach were treated at 0, 4, and 10 °C. On tomatoes, AIT was the most effective antimicrobial followed by cinnamaldehyde. An 8.3 µl/liter AIT reduced *Salmonella* and *E. coli* O157:H7 between tomato slices by 1.0 to 3.5 log depending on storage temperature. The same concentration reduced the pathogens to detection limit on tomatoes surface. On lettuce surface, 4 µl/liter AIT, 40 µl/liter carvacrol and cinnamaldehyde reduced O157:H7 by > 4.0 log at 0 and 4 °C in 4 days and at 10 °C in 2 days. However, within lettuce tissue, 16 µl/liter AIT, 80 µl/liter carvacrol and cinnamaldehyde reduced the pathogen by 4.0 log at 0 °C and 2.0 to 4.0 log at 4 °C in 4 days. These concentrations also reduced the pathogen population by 1.0 to 3.0 log at 10 °C in 2 days.

Inactivating the pathogen on spinach was 1.0 log and 3.0 log less on the surface and within the tissue, respectively, compared to lettuce. Surface disinfection and CSLM micrographs supported the results obtained on lettuce tissue. Following surface disinfection more reduction occurred on infiltrated pathogens compared to water washed samples. The data supports using antimicrobials in the vapor phase may improve the safety of refrigerated sliced tomatoes and leafy greens marketed in packages containing enclosed headspace.

INDEX WORDS: *Salmonella* spp., *Escherichia coli* O157:H7, Surface, Tissue, Tomato, Lettuce, Spinach, Allyl isothiocyanate, Carvacrol, Cinnamaldehyde, Vapor-phase

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DEDICATION

Dr. Joe Frank

. The man who spread the earth his mattress and rolled the sky his blanket. His advices, encouragements, undying patience, and smile were the light that guided me throughout this journey.

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CHAPTER 1

INTRODUCTION

Tomatoes and leafy greens have been associated with several foodborne outbreaks within the last two decades. *Salmonella* spp. and *E. coli* O157:H7 being the most frequent causative agents of these outbreaks. Several researchers attributed contaminated soil and irrigation water as being the most important sources of produce contamination; however other researchers believe post harvest contamination is the major source. Nevertheless, pathogen infiltration into leafy green and tomato fruits is well documented. This situation created a need to prevent fresh produce contamination and to control pathogen growth on fresh produce. Several decontamination procedures have been tested and used in fresh produce industry to reduce the population of pathogenic and spoilage microorganisms on produce surface to improve the fresh produce safety and delay their spoilage. The efficacy of these decontamination procedures is influenced by microorganism's location, the type of the produce and water acidity. Furthermore, pathogenic microorganisms may reside in protected sites on fresh produce, making them protected from the sanitizers' action. Chlorinated water, chlorine dioxide gas, electrolyzed water, hydrogen peroxide, peroxyacetic acid and ozone are currently approved for use in fresh produce industry. Nevertheless, the available data indicate that these chemical disinfectants added to wash or cooling water cannot be relied upon to eliminate human pathogens from leafy vegetables when used at levels that don't cause adverse effect on produce quality.

Volatile antimicrobials may reach areas in tomatoes and leafy greens other liquid antimicrobials cannot reach. Therefore, the use of these volatile antimicrobials is promising giving that previous research showed their strong bactericidal effect against *Salmonella* spp. and *E. coli* O157:H7. Thus, this dissertation tested selected antimicrobials in their vapor phase; namely carvacrol, cinnamaldehyde and allyl isothiocyanate, to inactivate these pathogens on fresh produce with emphasis to study their ability to inactivate the pathogens within the produce tissues. The first study tested the ability of the vapor of these antimicrobials to inactivate *Salmonella* spp. and *E. coli* O157:H7 on tomato surface and between tomato slices. The second study tested the ability of these antimicrobials to inactivate lettuce surface-attached and tissue-infiltrated *E. coli* O157:H7. Selected treatments were tested against the pathogen on spinach. The third study confirmed that the antimicrobials' vapor kills infiltrated pathogen by using surface disinfection procedure followed by the antimicrobials treatment and by examining treated lettuce samples under confocal scanning laser microscopy.

CHAPTER 2

LITERATURE REVIEW

I. Fresh Produce Industry and Consumption

The fresh produce industry witnessed a rapid growth due to globalization and increased demand by consumers for healthy, convenient, ready-to-eat products. The improvement in packaging technologies has been largely responsible for the availability of different varieties of bagged fresh produce and ready to eat salads (29). The Produce Marketing Association (PMA) reported that the United States fresh produce industry retail sales increased from \$32.5 billion in 1998 to \$56.3 billion in 2006, with tomatoes accounts for 8.9%, lettuce for 4.0% and spinach for 0.3% of the retail sale of 31 vegetables (2). The United States is also ranked as number one importer and exporter of fresh produce worldwide and accounts for 18% of the \$40 billion in fresh produce world trade (21). Among the world's leading lettuce exporter, the United States ranked first and account approximately 60% of the top lettuce exporters share by quantity in 2006 (14). This exported lettuce accounted for only 12% of the United States production.

American consumers are spending more on fresh produce, where the yearly expenditure on fresh produce jumped from \$138 in 1995 to \$357 in 2005. Meanwhile, the price of lettuce has been highly variable through the years, however by 2004 the prices were in the least expensive years category, with head lettuce price was \$15.53 per cwt and leaf and romaine lettuce price was \$17.65 per cwt (29). The per capita consumption of fresh fruits and vegetables increased 6% between 1987 and 1995, and 8% between 1995 and 2000 (47). Consumption of romaine lettuce

peaked in 1989 at 27.8 pound per capita and decreased on 2004 to 22.5 pound. Meanwhile, romaine lettuce consumption was 0.7 pound per capita on 1985 and increased to 8.1 pounds on 2004, nearly doubled from 2001 to 2004 (*115*). Per capita consumption of lettuce reached 34.5 pounds in 2004. Iceberg lettuce is the most consumed lettuce variety, however per capita consumption of iceberg lettuce is decreasing while that of romaine and loose leaf lettuce is increasing (*29*). With this increase in consumption and development of new products, more foodborne illnesses have been linked to consumption of fresh produce (*21, 23, 109*). However, the probability of contracting a foodborne illness from lettuce is still slim.

II. Fresh produce processing/ production

Nearly 100 percent of the lettuce consumed in the United States is produced domestically (*56*), with California accounting for 73% and Arizona for 26% of the total production (*29*). Lettuce and spinach are cooled after harvest to improve their shelf-life. They are cooled either by hydro-cooling or vacuum cooling, where vacuum cooling is the preferred way. Hydro-cooling uses chlorinated water as coolant, where the produce is submerged with ice water (32 °F). Lettuce also should be stored at high relative humidity, by topping it with ice, due to its high respiration rate.

The United States is considered the second producer of tomatoes after China in the whole world, with a 15% growth in the production between 2003 and 2007 (*15*). However, the United States imports a very small amount of tomatoes (*15*). California is responsible for 94% of the United States production and Florida for 2%, and other 4% being distributed between several other states (*15*). Tomatoes are grown in two distinct ways; poles (stakes) or bushes without support. Some of the tomatoes is grown on poles and harvested at the breaker fruit stage and

marketed as vine ripe. Most tomatoes are grown as bushes and harvested with one and sometimes two picks at the mature green stage. Sandy soil is preferred for early planting as planting in sandy soil in wet winter is easier and also sand warms up more rapidly in spring time which promotes early growth. Clay soil may be used if it is well drained and irrigated with care. All fresh market tomatoes are irrigated; with surface drip irrigation used for pole production, whereas in bushes production one-third of the acreage is irrigated by subsurface drip irrigation (drip tape buried 5-30 cm deep, one line per bed), and two third utilizes furrow irrigation. Drip irrigation is efficient and allows hand harvesting at regular intervals. Subsurface drip irrigation has the added advantage of dry bed surface and reduced weed germination.

Polegrown tomatoes are harvested by hand lug boxes at the breaker stage one to three times a week. Hand-picked tomato then transported to sheds for washing and grading (size and color) and then packing. Bush-grown tomatoes are hand harvested when 10 to 15 of the fruit is red. These tomatoes are picked into buckets then dumped into pins or gondolas to be transported from the field to the packing shed. At the shed, tomatoes are rinsed and grouped by size and grade. After harvest, tomatoes are cooled rapidly to optimize their postharvest quality. Vine-ripened tomato are cooled by forced air and stored at less than 10 °C to extend their shelf life. Room cooling involves placing the containers in a cool room where the cooled air being move around the containers. This system uses an air flow of 200-400 cubic feet/min at high relative humidity (90 to 95%). Forced-air cooling is similar to room cooling where produce is placed in a cold room, however it forces air through the containers rather than around them. Mature- green tomatoes can be ripened immediately, slowly or stored before ripening. Normal ripening temperatures are 18 to 21 °C, while slow ripening temperatures are 14 to 16 °C. Mature green tomatoes are stored for 2 weeks before ripening without adverse effect on the ripening rate,

sensory quality and color development. Mature green tomatoes are usually treated with 100 to 150 ppm ethylene for 24 to 48 h at 20 to 25 °C before shipment to guarantee uniform ripening (106).

III. Sources of produce contamination

Pathogenic and spoilage microorganisms contaminate fresh produce by several routes and at different points in the production chain both pre- and post-harvest. Pre-harvest contamination can occur from contaminated irrigation water, soil, insects and employees; while postharvest contamination may occur from washing water, human handling, transport containers and processing equipment. This along with the increasing number of fresh produce–associated outbreaks provided incentive for the United States Food and Drug Administration to increase actions to ensure the safety of fresh produce. On February 25, 2008, the FDA released its final guidance document entitled “Guidance for Industry: Guide to Minimize Microbial Food Safety Hazards of Fresh-cut Fruits and Vegetables”. The guidance is intended to assist all fresh-cut produce processing firms, both domestic firms and firms importing or offering fresh-cut product for import into the United States, to minimize the microbial food safety hazards common to the processing of most fresh-cut fruits and vegetables sold to consumers and retail establishments in a ready-to-eat form. The draft complements FDA's Current Good Manufacturing Practice (cGMP) requirements for foods by providing specific guidance on the processing of fresh-cut produce (10).

Animal manure is considered the most important source of pre-harvest contamination, giving that the livestock industry in the United States is very huge and includes cattle, hogs, chicken and turkey. This livestock produces about 1.36 billion tons/ year of manure, with more than 90% attributed to cattle (9). Livestock can harbor and shed in their feces some foodborne pathogens

such as *Campylobacter jejuni*, *Salmonella* and *Escherichia coli* O157:H7. The prevalence of *E. coli* O157:H7 in cattle feces has been extensively studied for the pathogens' association with meat products outbreaks. These studies showed that the prevalence of *E. coli* O157:H7 in cattle feces depends on the geographical location and the season. In the United States, the prevalence of *E. coli* O157:H7 in cattle ranges from 0.4% to 7% as showed by studies performed between 1994 to 1999 (49). Other studies show that the prevalence of *E. coli* O157:H7 in cattle was as high as 36.8% (37). The seasonal influence is clear, with higher shedding of the pathogen in feces occurs in spring and summer (27). Several studies tested the survival of *E. coli* O157:H7 in cattle feces. A study done in Ireland examined the survival *E. coli* O157:H7 in feces in grass under ambient weather conditions in Ireland. The pathogen was able to survive for 99 days in feces and in underlying soil (28). Another study in the United Kingdom reported the *E. coli* O157:H7 survived in cattle feces for >50 days, but shorter survival was observed in cattle slurry (83). Similar findings were reported by Himathongkham et al. (67), where the survival of *E. coli* O157:H7 and *Salmonella* Typhimurium was found better in cow manure than in cow slurry, with 6 days to 3 weeks decimal reduction in manure and 2 days to 5 weeks decimal reduction in the slurry. Wang et al. (122) reported that *E. coli* O157:H7 survives in bovine feces for 42 to 49 days at 37 °C, for 49 to 56 days at 22 °C, and for 63 to 70 days at 5 °C (122).

Researchers have also examined the survival of foodborne pathogens in soil and irrigation water. Recent studies showed that *Escherichia coli* O157:H7 persist in the field for 4 to 8 weeks (70, 85). Previous research also showed that *E. coli* O157:H7 persists for up to 5 to 7 months in soil contaminated with the pathogen-contaminated poultry or bovine compost or irrigation water (71). In addition to that, the proximity of domestic or wild animals to irrigation water may serve as a vehicle for *E. coli* O157:H7 to gain access to produce during preharvest operations (118).

Watkins and Sleath (124) found that *Salmonella* persist for up to 5 weeks in sewage applied to agricultural soil. Consequently, the United States Department of Agriculture (USDA), in an attempt to decrease the risk of “manure-borne” pathogens, requires that at least 120 days elapse between noncomposted manure application and harvest of organic crops with edible portions exposed to soil particles (13). Furthermore, the FDA considers prevention of contamination as the most effective control strategy since pathogen growth is not required to cause illness and antimicrobial rinses are not sufficiently effective at killing or removing attached pathogens.

Leafy greens’ post-harvest contamination may occur as a result of using contaminated water or ice (75), improper handling by workers or consumers, and contaminated slicers and shredders (54). In addition, few contaminated leaves can cross contaminate a large mass of uncontaminated leaves (65, 117). This contamination might lead to pathogens’ attachment to lettuce surface, which can remain viable for long period and can grow depending on the storage temperature, moisture content and leaves’ age (30, 43). Weissinger et al. (126) showed that an initial population of 3.3 and 3.4 log CFU/g of *Salmonella* Baildon on lettuce and tomatoes reduced by 2.0 log after storage at 4 °C for 12 days. However, an initial inoculum of 0.28 log CFU/g, was not reduced to undetectable limit. Furthermore, they found that *S. Baildon* possesses unusual tolerance to acidic pH. Low temperature storage of food contaminated with *Salmonella* Typhimurium increase the pathogen acid tolerance, if they were in the stationary phase. Furthermore, surface association also increased the acid tolerance of the pathogen in the exponential and stationary phases (55).

Postharvest contamination of tomatoes has been studied extensively. *Salmonella* Montevideo produces extracellular polymers on tomato cuticles after 10 h at 22 and 30 °C under high relative humidity (97%) leading to a well-defined biofilm after 4 days (72). Lukasik et al. (82) showed

that *Salmonella* spp. and *Escherichia coli* O157:H7 attached preferentially to rough surface rather than smooth surfaces of tomatoes. Deposition of *Salmonella* Montevideo on the surface of tomatoes and tomatillos could result in attachment and subsequent colonization under suitable conditions. *Salmonella* can attach to the tomato surface in 90 min (72). In a study by Allen et al. (17), *Salmonella* spp. remained detectable on tomato surface for ≤ 28 days under condition that simulate packinghouses conditions in Florida (late spring 30 °C/80% RH; Fall/winter 20 °C/90% RH). At spring conditions, *Salmonella* spp. declined to undetectable level in 11 days on all tested surfaces (stainless steel, polyvinyl chloride, sponge rollers, and conveyor belts), except on oak on which *Salmonella* spp. was detected till day 21. However, under fall/winter conditions, stainless steel, PVC and wood surfaces supported the survival of the pathogen over 28 days sampling period.

IV. Infiltration and attachment of pathogens on fresh produce

In 1999, the FDA reviewed the potential for human pathogens and other bacteria to infiltrate into fresh fruits and vegetables, and examined the ability of conventional surface decontamination measures to reduce contamination once this event occurs. The FDA concluded that different opportunities exist for internalization, and that surface treatments are generally ineffective in eliminating internalized pathogens (1).

Escherichia coli O157:H7 may infiltrate lettuce and spinach either pre- or post-harvest. Pre-harvest internalization may occur by cellular uptake through the root and transport through the vascular system (102). Solomon et al. (101) reported that *E. coli* O157:H7 was transmitted to lettuce plants through spray and surface irrigation. Islam et al. (71) also detected *E. coli* O157:H7 in lettuce and parsley that were grown on the pathogen-contaminated soil. However, Barak et

al.(20) contaminated soil with water-*Salmonella* spp. mixture and reported poor natural attachment capacity and subsequent contamination of *Salmonella* for lettuce and tomato via soil directly compared to *Brassicaceae* and carrot. This study concluded that soil may be not the route for pre-harvest contamination and tomato cultivar might have characteristics that deter *Salmonella* attachment and survival in the phyllosphere (20). However, Hora et al. (68) and Jablasone et al. (73) showed that *E. coli* O157:H7 was unable to infiltrate the tissues of mature lettuce and spinach from contaminated growth media, even when roots were damaged mechanically or biologically by coinoculation with plant pathogens.

Postharvest internalization may occur during vacuum cooling or through the cut surface during washing (76). Internalization and pathogen growth are also enhanced by the presence of phytopathogens due to cuticle destruction and release of nutrients (65) and influenced by the storage temperature and the atmosphere of the package. For example, Takeuchi and Frank (110) showed that the infiltration into lettuce was greater at 4 °C than at 37 °C. Seo and Frank (99) observed that *E. coli* O157:H7 attach preferentially to cut edges compared to the intact leave surface. Thorough surface sterilization using chlorine, ethanol, and 1% silver nitrate solution are unable to remove internalized *E. coli* O157:H7 and *Salmonella* Typhimurium(51).

The exact way by which tomatoes got contaminated with pathogens is not well-understood, but experimental evidence suggests pathogen internalization is possible. Various research findings indicate that pathogens can infiltrate into whole tomatoes (16, 17, 66, 130, 131) when subjected to a temperature differential between the tomato and washing water temperature, and by hydrostatic pressure when tomatoes are submerged in the dump tank (22, 23). Zhuang et al. (130) demonstrated that washing tomatoes in water contaminated with *Salmonella* at temperature cooler than that of tomatoes result in the pathogen to infiltrate in the stem scar tissue.

Furthermore, bacterial infiltration increases in the presence of wounds and punctures on the tomato. Tomatoes also can be internally contaminated when tomato stem and flowers are inoculated with *Salmonella* (58). Shi et al. (100) demonstrated that *Salmonella* serovars inoculated into flower of grown tomato were detected in and on developing tomato fruit. *S. Montevideo* is the most adapted to survive within tomatoes among Javiana, Newport, Dublin, Enteritidis, Hadar, Infantis, Typhimurium and Senftenberg. However, some of these serovars were able to grow and persist on unripened tomato. Meanwhile, growth and survival on and in ripened tomato were serovar dependent, with Hadar, Montevideo and Newport being more adapted. Previous research also showed that pathogens on the surface of tomatoes may contaminate internal tissues during slicing, and survive or grow in the slices (78). Guo et al. (59) found *Salmonella* Montevideo to persist for 49 days in tomatoes after tomato flower inoculation.

The behavior of pathogens on tomatoes is affected by pathogen location on the fruit, tomato quality, storage temperature, packaging type, and relative humidity. Tomatoes usually have sufficient acid to limit pathogen growth when stored under refrigeration temperatures. However, fungal and yeast infection of raw tomatoes or natural mycoflora increase the pH of the pericarp tissues to a level that favor pathogen's growth (119, 120). As storage temperatures increase above the refrigeration temperature, *Salmonella* spp. will survive and/or grow more rapidly on tomato skin, stem scar and in chopped tomatoes (18, 41, 119, 126, 131).

V. Tomato and lettuce-associated outbreaks by *E. coli* O157:H7 and *Salmonella* spp.

A foodborne disease outbreak is defined as the occurrence of two or more cases of a similar illness resulting from the consumption of a common food. The number and magnitude of fresh produce-associated outbreaks has been increasing in recent years. Lettuce/leafy greens are

responsible for about 30% of these outbreaks, followed by tomatoes with 17% (31). *E. coli* O157:H7 associated mostly with lettuce, spinach and alfalfa sprouts, while *Salmonella* spp. has been associated mostly with tomatoes, cantaloupe and water melon (32).

Escherichia coli O157:H7 became nationally notifiable disease in 1994. Generally, outbreaks caused by *E. coli* O157:H7 peaks during summer months of the year, between May and November. This attributed to increased prevalence of the pathogen in cattle during summer. Between 1982-2002, 52% of *E. coli* O157:H7 outbreaks were foodborne and 48% occurred via other routes such as drinking water, person-to-person transmission. Ground beef accounted for 21%, produce for 11%, unknown food vehicle for 12% of the total *E. coli* O157:H7 outbreaks (84). The data showed *E. coli* O157:H7 outbreaks were firstly associated with ground beef, but on July 1995, an outbreak associated with leafy lettuce was reported in Montana with 74 cases. Implicated lettuce in this outbreak was traced back to two sources: a local Montana farm and six farms in Washington State which shipped the lettuce under the same label (16). This outbreak was followed by one in Maine on September with 37 cases. On May and June of 1996, an outbreak associated with Mesclun lettuce happened in Connecticut and Illinois with 47 cases. The implicated lettuce was traced back to a single grower-processor, where cattle were found near the lettuce fields. Generic *E. coli* was cultured from wash water and finished lettuce. Furthermore, forward tracing identified three other states that received the implicated lettuce, in which an isolates from one patient matched the outbreak-associated PFGE isolate (66). Then, lettuce-associated outbreak occurred on October of 1999 in Ohio and Indiana. Romaine-lettuce was associated with an outbreak in August 2002 in Washington with 32 cases and pre-packaged lettuce-associated outbreak occurred in Minnesota in 2005. Prepackaged fresh spinach was associated with a multi-state outbreak involved 26 states and Canada. This outbreak occurred in

2006 and caused 206 reported cases with 3 deaths (84). In the same year, two multistate lettuce-associated outbreaks occurred in November causing in total 132 cases (3). Another outbreak was associated with spinach occurred in New Mexico and another associated with lettuce occurred in New York (3). *Salmonella* Typhimurium was also associated with lettuce outbreak in England and Wales in 2000 (69). Lettuce-associated outbreaks of *Salmonella* were reported in Europe in 2000 in five European countries, in 2004 in the United Kingdom, in 2005 in Austria, and in 2005 in Finland. Several fresh produce associated *Salmonella* outbreaks occurred in the United States in 2006, four of them associated with tomatoes and two associated with lettuce (3).

On the last two decades, tomatoes arose as a vehicle for salmonellosis. The first large multistate outbreak of *Salmonella* infection was linked to tomatoes in 1990, where *Salmonella* Javiana caused 176 illnesses (12). Tomatoes-associated outbreak of *Salmonella* Montevideo occurred in the United States in 1993 leading to 100 cases. Another *Salmonella* species, Baildon, was associated with tomatoes in the United States in 1999 leading to 86 cases with 3 deaths. In this outbreak, raw restaurant-prepared tomatoes was implicated as the cause and contamination likely occurred on the farm or during packing (39). Roma tomatoes-associated outbreak caused by *Salmonella* Javiana occurred in the United States in 2002 causing 159 cases. In 2002, tomatoes grown and packed in eastern shores of Virginia contaminated with *S. Newport* caused 510 cases in 26 states. The same strain, as determined by PFGE, caused 72 cases in 16 states in July-November 2005. The source of tomatoes in the 2005 outbreak was traced back to eastern shores of Virginia, where the strain was isolated from pond water used to irrigate the tomatoes. Identifying the same strain in irrigation bond 2 years apart suggest persistence contamination of field tomatoes (57). The same strain with identical PFGE pattern was implicated with tomato outbreak in July-November 2006, however the source was not identified (12). Three Roma-

tomatoes associated outbreak of salmonellosis occurred in United States and Canada in 2004. This outbreak caused 555 cases, 390 of them by Javiana and 125 by Braenderup (40). In one of the outbreaks, multiple *Salmonella* serotypes (Javiana, Typhimurium, Anatum, Thompson, and Muenchen) were isolated from multiple locations of a chain delicatessen, but only Anatum isolates from patients were indistinguishable by PFGE from the tomato isolates (11, 95). The other two outbreaks were associated with a single serotypes; Braenderup in United States and Javiana in Canada. A single tomato packing house in Florida was common to all three outbreaks (11, 60). The investigation of the multistate outbreak of *S. Braenderup* revealed that multiple potential animal reservoir of *Salmonella* were present in and adjacent to the drainage ditches in the farm, although the strain was not isolated from the drainage ditches (12). One multistate tomato-associated outbreak in September-October 2006 was confirmed to be caused by *S. Typhimurium* and traced back into a single packing house in Ohio supplied by three tomato growers from 25 fields, however tomato production had ended by the time the packing house was identified which did not help to investigate the tomato fields (12). These outbreaks demonstrate the potential of large outbreaks to be associated with tomatoes and leafy greens. Tomato and leafy greens contamination occurs early in the production chain rather than in restaurants. However, restaurants handle these commodities in a way that allows pathogen growth (12).

V1. Antimicrobials in fresh produce industry

A. Chlorinated water

Currently, 50 to 200 ppm sodium hypochlorite is the most commonly used chemical sanitizer in produce wash water for their ease of use, low cost, and wide spectrum activity against

bacteria, mold, yeast, viruses, algae, spores and protozoa. Generally, Gram-negative bacteria are more susceptible than Gram-positive ones, bacterial vegetative cells more susceptible than their spores, and bacteria more susceptible than viruses (113).

The activity of chlorine depends on the hypochlorous acid (HOCl) concentration. HOCl is the form of available free chlorine that has the highest bactericidal activity against microorganisms commonly found in fresh fruits and vegetables (97). In water, the equilibrium between HOCl and OCl^- depends on the water pH, with the HOCl concentration increases as the pH decreases. Typically, a pH between 6.0 and 7.5 is used to minimize corrosion of equipments and yield acceptable chlorine efficacy. Besides hypochlorous acid level and water pH, the bactericidal activity of chlorine depends on water temperature, and presence of organic matter, contact time, light, air, or metals.

Chlorine as a sanitizer has some drawbacks. Several studies showed that chlorine is as effective as deionized water or a slightly more effective in removing *E. coli* O157:H7 from lettuce surface and does not kill infiltrated pathogens (86, 87, 98, 126). Populations of *E. coli* O157:H7 inoculated on lettuce leaves declined by < 1.0 after 5 min dip in 100 ppm free chlorine compared to tap water (24). Li et al. (77) showed that submersing lettuce pieces-contaminated with *E. coli* O157:H7 in 20 ppm chlorine for 90 min at 20 or 50 °C was not significantly different from non-chlorine treated lettuce pieces. Zhang and Farber (129) showed that 200 ppm of chlorine was able to reduce the population of *Listeria monocytogenes* on lettuce and cabbage by 1.3-1.7 and 0.9 to 1.2 log, respectively. Increasing treatment time from 1 min to 10 min increased the amount of reduction marginally. Chlorine can also be inactivated by organic material leaching from tissues of fresh cut produce. Furthermore, its activity is affected by the topography of the fruit surface; where microorganisms can hide in crevices, pocket and natural opening in

fresh produce surface preventing chlorine from reaching hiding pathogens (25). In addition to that, the important waterborne pathogens (*Giardia lamblia* and *Cryptosporidium parvum*) are resistant to chlorine at concentrations typically applied for water treatment (35, 91).

Many processors in fresh produce now use the oxidation-reduction potential (ORP) to standardize water as a reflection of the antimicrobial potential of water. ORP is measured in millivolts (Mv), where a value of 650 to 700 mV kill free-floating bacteria, spoilage bacteria and pathogens such as *E. coli* O157:H7 and *Salmonella* in 30 s (108).

B. Chlorine Dioxide

Chlorine dioxide (ClO_2) has strong oxidizing ability, which is 2.5 times the oxidizing ability of chlorine, and its disinfecting power is relatively constant within a pH range of 6 to 10. ClO_2 is also effective against most microbes at concentrations of 3 to 5 ppm in clean water (107). ClO_2 is approved for use on uncut produce as antimicrobial and should be followed by potable water rinse (7). A maximum of 200 ppm ClO_2 is allowed for sanitizing processing equipment and 3 ppm allowed for contact with whole produce.

There is insufficient information about the effectiveness of ClO_2 compared to chlorine as sanitizer of fresh produce. Han et al. (63) showed that ClO_2 gas treatment at 0.6 and 1.24 ppm achieved 3.0 and 6.4 log reductions of *E. coli* O157:H7 on green peppers, however only 3.0 log reduction on injured green pepper surface. Another study showed that ClO_2 is highly effective against *L. monocytogenes* and *E. coli* O157:H7 on the surface of whole produce, but not on shredded or sliced produce (94). Pao et al. (88) showed that 20 ppm chlorine dioxide in 2 s reduced more than 5.0 log of *Salmonella enterica*. The same concentration required 1 min to achieve the same reduction on freshly spot-inoculated tomatoes, however this concentration after

1 min did not reduced the population significantly after drying the inoculum for 24 h at 24 ± 1 °C. Reina et al. (93) showed that 2.8 ppm ClO_2 is effective in killing planktonic cells in cooling water used to treat pickles, but had little effect on or in the fruit. Similar results were obtained by Costilow et al. (37) where 2.5 ppm was effective against microorganisms in water but not on and in cucumbers.

Application of 4 mg/liter ClO_2 gas (4000 ppm) to apples reduced the population of *Listeria monocytogenes* by 5.5 log and 3 mg/liter reduced the pathogen population by 7.4 log/5 g on green peppers. In addition to that, 1.2 mg/liter reduced *E. coli* O157:H7 population by 6.4 log on surface-injured green peppers compared to 1.5-1.7 log reduction by water wash. The use of 5 mg/liter (5000 ppm) chlorine dioxide gas for 1 h was significantly more effective against *Salmonella* spp. on the stem scar than aqueous solutions of 200 ppm sodium hypochlorite (2 min exposure) and 1200 ppm acidified sodium chlorite (2 min exposure) (128). Treating *Salmonella* spp.-inoculated tomatoes with aqueous solutions of 200 ppm chlorine, 1200 acidified sodium chlorite and 87 ppm peroxyacetic acid reduced the population > 1.0 log at tomatoes stem scar and > 2.0 log at puncture wounds. However, chlorine dioxide gas reduced the population to undetectable limit at the stem scar but had no apparent effect against the pathogen at puncture wounds (128). The need for on-site generation, specialized worker safety programs, and closed injections systems for containment of concentrate leakage and fumes makes ClO_2 relatively expensive for produce applications.

C. Ozone (O_3)

Ozone is formed by high-energy output that splits the oxygen (O_2) molecule, and then the single oxygen (O) molecules rapidly combine with the available O_2 to form O_3 . The United

States Food and Drug Administration considers ozone generally recognized as safe (GRAS) for treating drinking water with specific limitation and the United States Department of Agriculture (USDA) considers it as GRAS for reconditioning recycled poultry chilling water. Furthermore, the FDA in 2001 approved ozone to be used as an antimicrobial for treating, storing, and processing food in gas and liquid phase in direct contact with food, including raw and minimally processed fruits and vegetables.

The biocidal ability of ozone attributed to its high oxidation potential and its ability to diffuse through the bacterial cell membrane. Ozone showed to be effective against a wide range of microorganisms including *Listeria monocytogenes*, *Salmonella* Typhimurium, *Staphylococcus aureus*, *Yersinia enterocolitica*, enteric viruses, *Aspergillus*, *Cryptosporidium* and *Giardia* oocytes. The use of 10 mg/liter ozone completely inactivated (7.0 log CFU/tomato) *Salmonella* Enteritidis on the surface of cherry tomatoes after 15 min following 1 h attachment and after 20 min following 4 h attachment, however this concentration changed tomatoes color (41). Exposing lettuce to 1.3 mM ozone for 5 min reduced the microbial load by up to 4.6 log CFU/g (74). Unfortunately, this long exposure time is likely to be impractical in food applications. Moreover, in the same study, ozone treatment (~3 to 10 ppm) was ineffective in reducing *Pseudomonas fluorescens* inoculated (24 h prior to treatment) on lettuce, resulting in < 1.0 log reduction. Moreover, 200 ppm chlorinated water was superior to 2 mg/liter ozonated water in reducing *Salmonella* Typhimurium on tomato surface after 2 min treatment. The former treatment reduced 3.6 log, while the latter one reduced 2.5 log (36). However, chlorine-ozone combination has a beneficial effect on the shelf life of salad. Garcia et al. (53) showed that chlorine, ozone, and chlorine-ozone combination reduced aerobic plate count on Iceberg lettuce by 1.4, 1.1, and 2.5 log, respectively.

Spotts and Cervantes (104) showed that ozonated water is unable to control decay in wound inoculated pears and unable to reduce fungal infection in inoculated wounds of apples. They attributed this inability to ozone reacting with plant tissue and extracellular biochemicals at wound sites and thus failing to inactivate microorganisms attached to or embedded in plant tissue. Furthermore, the strong oxidizing activity of ozone may cause physiological injuries of produce (89). Ozone is also highly corrosive to equipments and lethal to human with prolonged exposure at concentrations above 4 ppm. Effective, but safe concentrations of ozone are hard to maintain in typical postharvest applications because a highly reliable automated detection system is not available yet (109).

D. Electrolyzed water (EO)

Electrolyzed water (EO) is generated by electrochemical disassociation of dilute NaCl water solution between anode and cathode electrodes separated by a membrane. This dissociation splits salt water into two separate streams, acidic (anode) and alkaline (cathode) water. The acidic stream has antimicrobial properties that include low pH (approx. 2.5), high oxidation-reduction potential (approx 1,100 mV), and chlorine-based reactants (10 to 90 ppm) (90). The concentration of chlorine reactants depends on the amperage of electric generator. Electrolytic oxidizing water (acidic electrolyzed water) is approved for direct and indirect food contact applications (4, 5, 7). The EPA has given EO approval for washing raw foods that are intended to be consumed without processing (6).

Several researchers tested the bactericidal effect of the EO on pathogens associated with fresh produce. Venkitanarayanan et al. (116) reported a reduction of $> 5.0 \log \text{CFU}/100 \text{ cm}^2$ of *E. coli* O157:H7 and reduction to undetectable levels of *L. monocytogenes* on cutting boards. Park et al.

(90) reported 2.6 log per lettuce leaf reduction of *L. monocytogenes* inoculated on whole lettuce leaves after 3 min treatments. Deza et al. (46) concluded that neutral EO containing 89 mg/l chlorine for 30 s is effective treatment to kill *E. coli* O157:H7, *Salmonella* Enteritidis and *Listeria monocytogenes* on tomato surface without affecting the sensory quality. These treatments reduced the population by more than 4.0 log regardless of the pathogen genus. Yang et al. (127) reported a 2.0 and 2.1 log CFU/g reduction, when fresh-cut romaine lettuce was treated with 300 ppm EO water at pH 7 and 30 °C, of *Salmonella* Typhimurium and *L. monocytogenes*, respectively. In this study, they treated the inoculated lettuce after 24 h of storage at 7 °C. Using scanning microscopy, bacterial film, a sticky and threadlike substance surrounding bacterial cells, was observed on the inoculated lettuce sample after 24 h storage. Therefore, biofilm formation affects the efficacy of EO water. Stopforth et al. (105) showed that EO is as effective as chlorine in reducing *E. coli* O157:H7, *Salmonella* spp, and *Listeria monocytogenes* on leafy greens.

E. Other Chemical and non-chemical disinfectants

Several other disinfectants have been evaluated for use in fresh produce industry such as peroxyacetic acid (PPA), hydrogen peroxide, irradiation and essential oils. Peroxyacetic acid is approved by the FDA as food-grade sanitizer at concentrations not more than 100 µg/ml. PPA is formed by reacting hydrogen peroxide with acetic acid. However, its efficacy against pathogens on fresh produce is not studied extensively. Beuchat et al. (26) showed that 80 ppm Tsunami reduced approximately 1.0 log CFU/g of *L. monocytogenes* on shredded and Romaine lettuce pieces at 3 to 4 °C in 15 s. Furthermore, Rodgers et al. (94) showed that 80 ppm PAA was the least effective sanitizer in reducing *E. coli* O157:H7 and *L. monocytogenes* on whole and sliced

lettuce and whole and sliced apple. PAA reduced 4.4 log CFU/g on produce, whereas 5 µg/ml ClO₂ reduced 5.6 log CFU/g, and chlorinated trisodium phosphate (200 µg/ml chlorine and 3 µg/ml chlorine dioxide) reduced 4.5 log CFU/g.

Hydrogen peroxide (H₂O₂) is approved by the FDA as generally recognized as safe, but not allowed for washing fresh produce by itself, unless used at low concentrations and combined with acetic acid to form peroxyacetic acid (PAA). The United States Environmental Protection Agency (EPA) exempted H₂O₂ at concentration ≤ 1 % applied to postharvest agricultural food from the tolerance requirements. Several researchers tested hydrogen peroxide as sanitizer on vegetables and fruits. Ukuku and Sapers (114) showed that 5% H₂O₂ reduced *Salmonella* Stanley by 2.0 log CFU/cm². Treating apples with 1% H₂O₂ reduced *E. coli* O157:H7 by approximately 3.0 CFU at 30 and 40 °C and was not significantly different from reducing the pathogen using 5% H₂O₂ which achieved 3.5 log CFU reductions. However, 200 ppm chlorine reduced 2.6 log of the pathogen. Sapers and Jones (98) reported that 1% H₂O₂ treatment of tomato, at 20 °C for 15 min or 60 °C for 2 min, contaminated with *E. coli* O157:H7 and *Salmonella* spp. reduced the population by ≤ 1.3 log. Increasing the treatment to 5% H₂O₂ at 60 °C for 5 min resulted in larger reduction, but this treatment affected the sensory quality of the tomatoes.

The United States FDA has allowed the use of irradiation for pork, poultry, red meats, fruits, vegetables, herbs, spices, grains, seeds for sprouting, and shell eggs. Current United States regulations limit the use of irradiation for fresh fruits and vegetables up to 1 kGy and specifically for disinfestations and inhibition of produce growth and maturation (8). However, dry or dehydrated vegetables derived spices, seasonings, flavorings and coloring agents may be irradiated to 30.0 kGy (8). Foods treated with ionizing radiation must be labeled with the Radura symbol or with the statement “*Treated by irradiation*” or “*Treated with radiation*” (8). Most of

the research on irradiation of produce focused on determining dose-response relationship while preserving the product quality. In a study by Farkas et al. (50), *L. monocytogenes* and spoilage bacteria were reduced by approximately 4.0 and 5.0 log on pre-cut bell peppers and carrot cubes, respectively, when treated with 1.0 kGy. Hagenmaier and Baker (62) indicated that 0.19 kGy significantly reduced the normal microflora and moderately increased respiration on commercially prepared fresh-cut lettuce. The study demonstrated that eight days after irradiation, the unirradiated lettuce had 5.3 log CFU/g, while the irradiated lettuce had 2.5 log CFU/g. Prakash et al. (92) treated cut romaine lettuce with irradiation at 0.35 kGy which decreased the aerobic plate counts by 1.5 log and yeast and molds by 1.0 log; these differences were maintained through 22 day storage at 4 °C. Niemera (87) tested the effect of several doses of irradiation ranged from 0.25 to 1.5 kGy to inhibit internalized *E. coli* O157:H7 within lettuce leaves. He found that the D₁₀ value of irradiation differ among lettuce varieties and ranged from 0.30 kGy for iceberg lettuce to 0.45 kGy for Boston lettuce. Moreover, he found that internalized cells were more resistant to irradiation than surface-associated ones, with D₁₀ values ranged from 0.12 to 0.14 kGy. However, no surface sterilization technique was performed in his studies after vacuum perfusion to separate surface associated cells from the infiltrated ones (86, 87).

The antimicrobial activities of essential oils and their active compounds are well documented. Wan et al. (121) reported that washing lettuce with 0.1% (v/v) and 1.0% (v/v) suspensions of basil essential oil resulted in 2.0 and 2.3 log reduction of the total viable bacteria on fresh cut lettuce, respectively. Furthermore, washing lettuce spiked with *Shigella sonnei* and *Shigella flexneri* with 0.5% thymol or carvacrol for 2 min showed inhibitory effect against these pathogens, with thymol causing 4.0 log reduction, while carvacrol causing 2.0 log reduction at 7 °C. While, the same treatments resulted in 3.0 log reduction at room temperature (19). Lopez et

al. (81) tested the vapor of cinnamon, oregano and thyme against Gram-positive pathogens (*Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and *Enterococcus faecalis*), Gram-negative pathogens (*Escherichia coli*, *Salmonella Choleraesuis*, *Yersinia enterocolitica* and *Pseudomonas aeruginosa*), mold (*Aspergillus penicillium* and *Penicillium Islandicum*) and yeast (*Candida albicans*). In this study, oregano had the lowest minimum inhibitory concentration (MIC). The antimicrobial component of these essential oils namely, carvacrol and cinnamaldehyde and thymol showed the lowest MIC among 12 tested essential oil components in vapor phase. Santurio et al. (96) compared these components in microdilution technique and found that oregano had the strongest antimicrobial effect against *Salmonella* spp., followed by thyme then cinnamaldehyde. Gutierrez et al. (61) found oregano to be the most effective essential oil against *B. cereus*, *E. coli*, *L. monocytogenes* and *P. aeruginosa* followed by thyme among 7 tested essential oils. Combination of oregano with other essential oils had additive effect against *B. cereus*, but its combination only with basil, thyme and marjoram has additive effect against *E. coli* and *P. aeruginosa*. The study showed that EO are more effective at pH 5 compared to pH 6 or 7, and in media rich in protein compared to media rich in carbohydrate and oil. This lead to the conclusion that a combination of some antimicrobials has additive effect which can be employed as a strategy to minimize the use of high concentrations of a single EO and the subsequent adverse sensory effect. Moreover, the EO can be employed in food with acidic pH and/or high in protein and low in carbohydrate and fat.

Friedman et al. (52) found that the three most effective EO against *E. coli* O157:H7 and *Salmonella enterica* were oregano, thyme and cinnamon among 96 studies ones. The major antimicrobial component of oregano, thyme and cinnamon are carvacrol, thymol and cinnamaldehyde, respectively. Friedman et al. (52) also reported that the three most effective oil

compounds, among 16 compounds tested against *E. coli* O157:H7 were carvacrol, cinnamaldehyde, and thymol; and the most effective compounds against *Salmonella enterica* were thymol, cinnamaldehyde and carvacrol (52). These compounds are phenolic ones and exhibit their antimicrobial activity by disrupting the bacterial cell membrane integrity and proton motive force (34).

Isothiocyanates is another class of plant compounds that have potent antimicrobial activity. Isothiocyanates are glucosinolates-derivative of mustard plants formed by the action of myrosinase on the glucosinolates upon plant injury (42). Isothiocyanate inhibits bacterial enzymes through direct reaction with disulfide bonds or by thiocyanate ion (SCN^-) reaction to sulfhydryl enzymes (44). Lin et al. (79) suggested that gaseous AIT inhibits *Salmonella* Montevideo and *E. coli* O157:H7 by disruption of cell membrane followed by leakage of cellular metabolites. However, their low water solubility limited their use as antimicrobial in food system (80), meanwhile their vapor was studied. Lin et al. (79) concluded that AIT vapor is effective against the stationary and exponential growth stages. This makes AIT vapor applicable to inhibit pathogens in fresh produce since generally pathogens have low metabolic activity in produce due to low storage temperature. Ward et al. (123) prepared horseradish essential oil distillate (90% allyl isothiocyanate) and applied it to the head space of cooked roast beef. Two $\mu\text{l/liter}$ of air of the distillate inhibited *E. coli* O157:H7 and *L. monocytogenes* on agar plates, while 20 $\mu\text{l/liter}$ was required to inhibit the two pathogens on the beef.

VII. Microscopical examination of bacterial cells in plant tissues

Confocal scanning laser microscopy (CSLM) has been widely utilized in food microbiology research to improve understanding of the pathogen-food interaction. In CSLM, a laser

illumination of mixed- argon gas- krypton ion laser is used to excite flourophores with long wavelength excitation. This laser provide lines at 488, 568 and 647 nm which enable analyzing samples labeled with dyes having different excitation and emission spectra (112). The CLSM microscope lens focuses laser light on one point (the focal point) of the sample. Then, the laser moves rapidly from one point to another, producing a scanned image. The microscope and scanner head focus the light emitted from the focal point into a second point, the confocal point. On the confocal point, a pinhole aperture allows light from the confocal point to pass through the detector. This reduces out-of-focus light leading to improvement in resolution along the z-axis and allows optical sectioning of the sample. The optical cross sectioning is an effective way to study the infiltration of pathogens in plant openings or tissue. Furthermore, fully hydrated samples can be visualized facilitating accurate determination of microbial viability within plant tissues. The CSLM equipped with detectors such as photomultiplier tube and charge-couple-device (CCD) that allows detection of low level signals, therefore improving the detection limit. For detailed information about the CSLM usage in food research, the author recommends Takeuchi and Frank (112) published review entitled “Confocal microscopy and microbial viability detection for food research”.

For visualizing pathogens in food matrices, a gene encoding for green fluorescent protein is inserted in the pathogens’ genome. Green fluorescent protein was originally derived from *Aequorea victoria*, a naturally fluorescent jellyfish. This wild-type GFP has major excitation peak at 395 nm and emission peak at 503 nm. Several mutants of this protein has been developed such as red-shifted enhanced GFP (EGFP), in which the maximum excitation peak was shifted to 490 nm, resulting in more efficient detection by argon laser. GFP and its mutant has been a useful reporter gene and viability marker for their bright clear fluorescent that can be detected by

CSLM even in one cell. This protein also facilitates nondestructive visualization of pathogens within food. Takeuchi and Frank (111) showed that *E. coli* O157:H7 on lettuce leaves and cauliflower retained GFP at high frequency. Burnett et al. (33) utilized CSLM to study the attachment of GFP-tagged *E. coli* O157:H7 at various depths within healthy and puncture apple. Using this approach they verified quantitatively that the pathogen infiltration of intact tissue and natural openings is more under negative than positive temperature differential. Solomon et al. (103) also utilized CSLM and showed that GFP-tagged *E. coli* O157: H7 was transmitted from contaminated manure to internal tissue of lettuce leaves. Duffy et al. (48) also used CSLM to confirm internalization of GFP-tagged *Salmonella* on parsley. They found that *Salmonella* internalize into parsley regardless of the dip solution temperature (5, 25 or 35 °C) and dipping period (3 or 15 min), however more internalization and attachment was observed at longer time.

Along with GFP, viability stains are used to visualize dead cells after antimicrobial treatment of pathogens within food, with propidium iodide (PI) being the most commonly used viability stain. PI is nucleic acid stain and impermeable to cell membrane, therefore it presumptively stains dead cells and emits red fluorescence (617 nm) when excited with green light (535 nm). Seo and Frank (100) stained lettuce with propidium iodide to determine the viability of *E. coli* O157:H7 after 20 mg/liter chlorine treatment for 5 min to conclude that many viable cells were found in stomata and on cut edges. Propidium iodide has also been used in conjunction with purified antibodies to *E. coli* O157:H7 labeled with Alexa Fluor 488 (110) and fluorescein isothiocyanate (64, 99) to detect viable and dead cells. Using GFP-tagged pathogens and PI, Warriner et al. (124) reported the survival of *E. coli* O157:H7 and *Salmonella enterica* within and on surface of bean sprouts after sodium hypochlorite treatment.

REFERENCES

1. (U.S. FDA] Food and Drug Administration. 1999. Potential for infiltration, survival and growth of human pathogens within fruits and vegetables. Available online at:
<http://www.cfsan.fda.gov/~comm/juicback.html>. Accessed June 12/2008.
2. [PMA] Produce Marketing Association. 2008. Retail fresh produce industry sales. Accessed July 22/ 2007. Available at
http://www.pma.com/cig/intl/documents/Retail_Sales_2007.pdf.
3. [CDC] Centers for Disease Control and Prevention. 2006. Summary statistics for foodborne outbreaks, 2006. Available at:
http://www.cdc.gov/foodborneoutbreaks/documents/2006_line_list/summary.pdf Accessed July 24/2008
4. [CFR] Code of Federal Regulations. 2004. Title 21, Part 172. Food additives permitted for direct addition to food for human consumption. Available at:
<http://www.gpoaccess.gov/cfr/index.html>. Accessed July 17/2008.
5. [CFR] Code of Federal Regulations. 2004. Title 21, Part 178. Indirect food additives: Adjuvants, production aids, and sanitizers. Available at:
<http://www.gpoaccess.gov/cfr/index.html>. Accessed July 17/2008.
6. [CFR] Code of Federal Regulations. 2004. Title 40, Part 180. Tolerances and exemptions from tolerances for pesticides chemicals in food. Available at:
<http://www.gpoaccess.gov/cfr/index.html>. Accessed July 17/2008.

7. [CFR] Code of Federal Regulations. 2005. Title 21, Part 173. Secondary direct food permitted in foods for human consumption. Available at:
<http://www.gpoaccess.gov/cfr/index.html>. Accessed July 12/2008.
8. [CFR] Code of Federal Regulations. 2005. Title 21, Part 179. Irradiation in the production, processing, and handling of food. Available at:
<http://www.gpoaccess.gov/cfr/index.html>. Accessed July 12/2008.
9. [EPA] Environmental Protection Agency. 2000. Proposed regulations to address water pollution from concentrated animal feeding operations. Report No. 833-F-00-016. Office of Water, Environmental Protection Agency, Washington, D.C.
10. [FR] Federal Register. 2008. 73 FR 10037. Guidance for Industry: Guide to minimize food safety hazards for fresh-cut fruits and vegetables. Available at: www.wais.access.gpo.gov. Accessed July 24/2008.
11. [MMWR] Morbidity and Mortality Weekly Report. 2005. Outbreaks of *Salmonella* infections associated with eating Roma tomatoes--United States and Canada, 2004. Available online at: <http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5413a1.htm>. Accessed July 28/2008. 54:325-328.
12. [MMWR] Morbidity and Mortality Weekly Report. 2007. Multistate outbreaks of *Salmonella* infections associated with raw tomatoes eaten in restaurants--United States, 2005-2006. Available at: <http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5635a3.htm>. Accessed July 28/2008. 56.
13. [USDA] United States Department of Agriculture. Agricultural Marketing Services. 2000. National organic program. 7 CFR Part 205:203. USDA, Washington, D.C.

14. [USDA] United States Department of Agriculture. Foreign Agricultural Service. 2007. The U.S. and world situation: lettuce. Available at <http://www.fas.usda.gov/http/2007%20Lettuce.pdf>. Accessed July 22/ 2007.
15. [USDA]. United States Department of Agriculture. 2007. The U.S. and world tomato situation. Available at: http://www.fas.usda.gov/http/2008_Tomatoes.pdf. Accessed July 25/2008.
16. Ackers, M. L., B. E. Mahon, E. Leahy, B. Goode, T. Damrow, P. S. Hayes, W. F. Bibb, D. H. Rice, T. J. Barrett, L. Hutwagner, P. M. Griffin, and L. Slutsker. 1998. An outbreak of *Escherichia coli* O157:H7 infections associated with leaf lettuce consumption. *J. Infect. Dis.* 177:1588-1593.
17. Allen, R. L., B. R. Warren, D. L. Archer, S. A. Sargent, and K. R. Schneider. 2005. Survival of *Salmonella* spp. on the surfaces of fresh tomatoes and selected packing line materials. *HortTechnology*. 15:831-836.
18. Asplund, K., and E. Nurmi. 1991. Growth of salmonellae in tomatoes. *Int. J. Food Microbiol.* 13:177-181.
19. Bagamboula, C. F., M. Uyttendaele, and J. Debevere. 2004. Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards *Shigella sonnei* and *S. flexneri*. *Food Microbiol.* 21
20. Barak, J. D., A. Liang, and N. E. Narm. 2008. Differential attachment and subsequent contamination of agricultural crops by *Salmonella enterica*. *Appl. Environ. Microbiol.*
21. Barbosa-Cánovas, G. V., J. J. Fernández-Molina, S. M. Alzamora, M. S. Tapia, A. López-Malo, and J. W. Chanes. 2003. Handling and preservation of fruits and vegetables by combined methods for rural areas. *Technical Manual FAO Agricultural Services Bulletin 149*. Food and Agriculture Organization of the United Nations. Rome.

22. Bartz, J. A. 1982. Infiltration of tomatoes immersed at different temperatures to different depths in suspensions of *Erwinia carotovora* subsp. *carotovora*. *Plant Disease*. 66:302-306.
23. Bartz, J. A., and R. K. Showalter. 1981. Infiltration of tomatoes by aqueous bacterial suspensions. *phytopathology*. 71:515-518.
24. Behrsing, J., S. Winkler, P. Franz, and R. Premier. 2000. Efficacy of chlorine for inactivation of *Escherichia coli* on vegetables. *Postharv. Biol. Technol.* 19:187-192.
25. Beuchat, L. R. 2000. Use of sanitizers in raw fruit and vegetable processing, p. 63-78. In: Alzamora, S.M., M.S. Tapia, and A. Lopez-Malo (eds.) *Minimally Processed Fruits and Vegetables: Fundamental Aspects and Applications*. Gaithersburg, MD: Aspen.
26. Beuchat, L. R., B. A. Adler, and M. M. Lang. 2004. Efficacy of chlorine and a peroxyacetic acid sanitizer in killing *Listeria monocytogenes* on iceberg and romaine lettuce using simulated commercial processing conditions. *J. Food Prot.* 67:1238-1242.
27. Blanco, J., M. Blanco,, J. E. Blanco, A. M. Alonso, E. A. Gonzalez, and M. I. Bernardez. 2001. Epidemiology of VTEC in ruminants. In Verocytotoxigenic *E. coli* ed. Duffy, G., P. Garvey, and D. A. McDowell, pp. 113–148. Trumbull, CT: Food Science and Nutrition Press, Inc.
28. Bolton, D. J., C. M. Byrne, J. J. Sheridan, D. A. McDowell, and I. S. Blair. 1999. The survival characteristics of a non-toxigenic strain of *Escherichia coli* 0157:H7. *J. Appl. Microbiol.* 86:407-411.
29. Borris, H., and Brunke, H. 2005. Commodity profile: Lettuce. *Agricultural Marketing Resource Center. University of California*. Available at <http://aic.ucdavis.edu/profiles/lettuce-2005.pdf>. Accessed July 22/2008.

30. Brandle, M. T., and R. Amundson. 2008. Leaf age as a risk factor in contamination of lettuce with *E. coli* O157:H7 and *Salmonella enterica*. *Appl. Environ. Microbiol.* 74:2298-2306.
31. Buchanan, R. 2006. Spinach outbreak as part of broader concerns about produce safety—a FDA perspective. U.S. Food and Drug Administration, Washington, D.C. Available at: <http://www.foodprotection.org/meetingsEducation/Rapid%20Response%20Presentations/Buchanan%20Robert.pdf>. Accessed July 25/2008.
32. Buck, J. W., R. R. Walcott, and L. R. Beuchat. 2003. Recent trends in microbiological safety of fruits and vegetables. Plant Management Network. Available at: <http://www.apsnet.org/online/feature/safety/Buck.pdf>. Accessed July 28/2008.
33. Burnett, S. L., J. Chen, and L. R. Beuchat. 2000. Attachment of *Escherichia coli* O157:H7 to the surfaces and internal structures of apples as detected by confocal scanning laser microscopy. *Appl. Environ. Microbiol.* 66:4679-4687.
34. Burt, S. 2005. Essential oils: their antibacterial properties and potential applications in foods—a review. *Int. J. Food Microbiol.* 94:223-253.
35. Carpenter, C., R. Fayer, J. Trout, and M. J. Beach. 1999. Chlorine disinfection of recreational water for *Cryptosporidium parvum*. *Emerging Infectious Disease.* 5:579-584.
36. Chaidez C., J. L., J. Vidales, and N.C. Campo. 2007. Efficacy of chlorinated and ozonated water in reducing *Salmonella typhimurium* attached to tomato surfaces. *Int J Environ Health Res.* 17:311-318.
37. Chapman, P. A., C. A. Siddons, A. T. Cerdan Malo, and M. A. Harkin. 1997. A 1- year study of *Escherichia coli* O157:H7 in cattle, sheep, pigs, and poultry. *Epidemiol. Infect.* 119:245-250.

38. Costilow, R. N., M. A. Uebersax, and P. J. Ward. 1984. Use of chlorine dioxide for controlling microorganisms during handling and storage of fresh cucumbers. *J. Food Sci.* 49:396-401.
39. Cummings, K., E. Barrett, J. C. Mohle-Boetani, J. T. Brooks, J. Farrar, T. Hunt, A. Fiore, K. Komatsu, S. B. Werner, and L. Slutsker. 2001. A multistate outbreak of *Salmonella enterica* serotype Baildon associated with domestic raw tomatoes. *Emerging Infectious Disease.* 7:1046-1048.
40. D'Aoust, J.-Y., and Maurer, J. 2007. *Salmonella* spp, p. 249-270. In M. P. Doyle and L. R. Beuchat (eds.). *Food Microbiology: Fundamentals and Frontiers*. 3rd ed. ASM Press, Washington, D.C.
41. Das, E., G. C. Gurakan, and A. Bayindirli. 2003. Effect of controlled atmosphere storage, modified atmosphere packaging and gaseous ozone treatment on the survival of *Salmonella enteritidis* on cherry tomatoes. *Food Microbiol.* 23:430-438.
42. Davidson, P. M., and Taylor, T. M. 2007. Chemical preservatives and natural antimicrobials compounds, p. 713-746. In Doyle, M. P., and L. R. Beuchat (eds). *Food microbiology: fundamentals and frontiers*. 3rd ed. ASM Press. Washington, D.C.
43. Delaquis, P., S. Bach, and L. Dinu. 2007. Behavior of *Escherichia coli* O157:H7 in leafy vegetables. *J. Food Prot.* 70:1966-1974.
44. Delaquis, P. J., and G. Mazza. 1995. Antimicrobial properties of isothiocyanates and their role in food preservation. *Food Technol.* 49:73-84.
45. Delaquis, P. J., and P. L. Sholberg. 1997. Antibacterial activity of gaseous allyl isothiocyanate. *J. Food Prot.* 60:943-947.

46. Deza, M. A., M. Araujo, and M. J. Garrido. 2003. Inactivation of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *S. Enteritidis*, and *Listeria monocytogenes* on the surface of tomatoes by neutral electrolyzed water. *Lett. Appl. Microbiol.* 37:482-487.
47. Dimitri, C., A. Tegene, and P. R. Kaufman. 2003. U.S. fresh produce markets: marketing channels, trade practices, and retail pricing behavior Available at <http://www.ers.usda.gov/Publications/AER825/> Accessed July 9/2008.
48. Duffy, E. A., L. Sneros-Zevallos, A. Castillo, S. D. Pillai, S. C. Ricke, and G. R. Acuff. 2005. Survival of *Salmonella* transformed to express green fluorescent protein on italian parsley as affected by processing and storage. *J. Food Prot.* 68:687-695.
49. Duffy, G. 2003. Verocytotoxic *Escherichia coli* in animal faeces, manures and slurries. *J. Appl. Microbiol.* 94:94S-103S.
50. Farkas, J., T. Saray, C. Mohacsi-Farkas, K. Hortik, and E. Andrassy. 1997. Effects of low-dose gamma radiation on shelf-life and microbiological safety of pre-cut/prepared vegetables. *Adv. Food sci.* 19:111-119.
51. Franz, E., A. A. Visser, A. D. Diepeningen, M. M. Klerks, A. J. Termorshuizen, and A. H. C. Bruggen. 2007. Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. *Food Microbiol.* 24:106-112.
52. Friedman, M., P. R. Henika, and R. E. Mandrell. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J. Food Prot.* 65:1545-1560.
53. Garcia, A., J. R. Mount, and P. M. Davidson. 2003. Ozone and chlorine treatment of minimally processed lettuce. *J. Food Sci.* 68:2747-2751.

54. Garg, N., J. J. Churey, and D. F. Splittstoesser. 1990. Effects of processing conditions on the microflora of fresh-cut vegetables. *J. Food Prot.* 53:701-703.
55. Gawande, P. V., and A. A. Bhagwat. 2002. Free content protective effects of cold temperature and surface-contact on acid tolerance of *Salmonella* spp. *J. Appl. Microbiol.* 93:689-696.
56. Glaser, L. K., G. D. Thompson, and C. R. Handy. 2001. Recent changes in marketing and trade practices in the U.S. lettuce and fresh-cut vegetable industries Available at <http://www.ers.usda.gov/Publications/AIB767/>. Accessed July 9/2008.
57. Greene, S. K., E. R. Daly, E. A. Talbot, L. J. Demma, S. Holzbauer, N. J. Patel, T. A. Hill, M. O. Walderhaug, R. M. Hoekstra, M. F. Lynch, and J. A. Painter. 2008. Recurrent multistate outbreak of *Salmonella* Newport associated with tomatoes from contaminated fields, 2005. *Epidemiol. Infect.* 136:157-165.
58. Guo, X., J. Chen, R. E. Brackett, and L. R. Beuchat. 2001. Survival of *Salmonellae* on and in tomato plants from the time of inoculation at flowering and early stages of fruit development through fruit ripening. *Appl. Environ. Microbiol.* 67:4760-4764.
59. Guo, X., J. Chen, R. E. Brackett, and L. R. Beuchat. 2002. Survival of *Salmonella* on tomatoes stored at high relative humidity in soil, and on tomatoes in contact with soil. *J. Food Prot.* 65:274-279.
60. Gupta, S. K., K. Nalluswami, C. Snider, M. Perch, M. Balasegarama, D. Burmeister, J. Lockett, C. Sandt, R. M. Hoekstra, and S. Montgomery. 2007. Outbreak of *Salmonella* Braenderup infections associated with Roma tomatoes, northeastern United States, 2004: a useful method for subtyping exposures in field investigations. *Epidemiol. Infect.* 35:1165-1173.

61. Gutierrez, J., C. Barry-Ryana., and P. Bourke. 2008. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *Int. J. Food Microbiol.* 124:91-97.
62. Hagenmaier, R. D., and R. A. Bake. 1997. Low-dose irradiation of cut iceberg lettuce in modified atmosphere packaging. *J. Agric. Food Chem.* 45:2864-2868.
63. Han, Y., D. M. Sherman, R. H. Linton, S. S. Nielson, and P. E. Nelson. 2000. The effects of washing and chlorine dioxide gas on survival and attachment of *Escherichia coli* O157:H7 to green pepper surfaces. *Food Microbiol.* 17:521-533.
64. Han, Y., R. H. Linton, S. S. Nielsen, and P. E. Nelson. 2000. Inactivation of *Escherichia coli* O157:H7 on surface-injured and –injured green pepper (*Capsicum annum* L.) by chlorine dioxide has as demonstrated by confocal scanning laser microscopy. *Food Microbiol.* 17:643-655.
65. Heaton, J. C., and K. Jones. 2007. Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review. *J. Appl. Microbiol.* 104:613-626.
66. Hilborn E. D., J. H. M., P. A. Mshar, J. L. Hadler, A. Voetsch, C. Wojtkunski, M. Swartz, R. Mshar, M. A. Lambert-Fair, J. A. Farrar, M. K. Glynn, and L. Slutsker. 1999. A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. *Arch Intern Med.* 159:1758-1764.
67. Himathongkhama, S., S. Baharia, H. Riemanna, and D. Clivera. 1999. Survival of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in cow manure and cow manure slurry. *FEMS Microbiol. Lett.* 178:251-257.

68. Hora, R., K. Warriner, B. J. Shelp, and M. W. Griffiths. 2005. Internalization of *Escherichia coli* O157:H7 following biological and mechanical disruption of growing spinach plants. *J. Food Prot.* 68:2506-2509.
69. Hornby, P. W., S. J. O'Brien, G. K. Adak, C. Graham, J. I. Hawker, P. Hunter, P. Lane, A. J. Lawson, R. T. Mitchell, M. H. Reacher, E. J. Threlfall, L. R. Ward, and behalf of the the PHLS Outbreak Investigation Team. 2003. A national outbreak of multi-resistant *Salmonella enterica* serovar Typhimurium definitive phage type (DT) 104 associated with consumption of lettuce. *Epidemiol. Infect.* 130:169-178.
70. Ibekwe, A. M., Watt, P. M., Shouse, P. J., and Grieve, C. M. 2004. Fate of *Escherichia coli* O157:H7 in irrigation water on soils and plants as validated by culture method and real-time PCR. *Can. J. Microbiol.* 50:1007-1014.
71. Islam, M., M. P. Doyle, S. C. Phatak, P. Millner, and X. P. Jiang. 2004. Persistence of enterohemorrhagic *Escherichia coli* O157:H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. *J. Food Prot.* 67:1365-1370.
72. Iturriaga, M. H., M. L. Tamplin, and E. F. Escatín. 2007. Colonization of tomatoes by *Salmonella* Montevideo is affected by relative humidity and storage temperature. *J. Food Prot.* 70:30-34.
73. Jablasone, J., K. Warriner, and M. W. Griffiths. 2005. Interactions of *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes* with plants cultivated in a gnotobiotic system. *Int. J. Food Microbiol.* 99:7-18.
74. Kim, J., A. Yousef, and G. W. Chism. 1999. Use of ozone to inactivate microorganisms on lettuce. *J. Food Saf.* 19:17-34.

75. Kim, J. K., and M. A. Harrison. 2008. Transfer of *Escherichia coli* O157:H7 to romaine lettuce due to contact water from melting ice. *J. Food Prot.* 71:252-256.
76. Li, H., T. Mehrdad, and I. O. Bennie. 2008. Impact of vacuum cooling on *Escherichia coli* O157:H7 infiltration into lettuce tissue. *Appl. Environmen. Microbiol.* 74:3138-3142.
77. Li, Y., R. E. Brackett, J. Chen J, L. R. Beuchat. 2001. Survival and growth of *Escherichia coli* O157:H7 inoculated onto cut lettuce before or after heating in chlorinated water, followed by storage at 5 °C or 15 °C. *J. Food Prot.* 64:305-309.
78. Lin, C.-M., and C.-I. Wei. 1997. Transfer of *Salmonella* Montevideo onto the interior surfaces of tomatoes by cutting. *J. Food Prot.* 60:858-862.
79. Lin, C. M., J. F. Preston, and Wei, C. I. 2000. Antibacterial mechanism of allyl isothiocyanate. *J. Food Prot.* 63:727-734.
80. Lin, C. M., Kim, J. M., Du, W. X. and Wei, C. I. 2000. Bactericidal activity of isothiocyanate against pathogens on fresh produce. *J. Food Prot.* 63:26-30.
81. López, P., C. Snchez, R. Batlle, and C. Nerín. 2007. Vapor-phase activities of cinnamon, thyme, and oregano essential oils and key constituents against foodborne microorganisms *J. Agric. Food Chem.* 55:4348-4356.
82. Lukasik, J., M. L. Bradley, T. M. Scott, W.-Y. Hsu, S. R. Farrah, and M. L. Tamplin. 2001. Elution, detection and quantification of polio I, bacteriophages, *Salmonella* Montevideo, and *Escherichia coli* O157:H7 from seeded strawberries and tomatoes. *J. Food Prot.* 64:292-297.
83. Maule, A. 2000. Survival of verocytotoxigenic *Escherichia coli* O157 in soil, water and on surfaces. *Symposium Series for Society of Applied Microbiology* 29:71S-78S.

84. Meng, J., M. P. Doyle, T. Zhao, and S. Zhao. 2007. Enterohemorrhagic *Escherichia coli*. In M. P. Doyle and L. R. Beuchat (eds.) *Food Microbiology: Fundamentals and Frontiers*. 3rd ed. ASM Press, Washington, D.C.:249-270.
85. Nicholson, F. A., S. J. Groves, and B. J. Chambers. 2005. Pathogen survival during livestock manure storage and following land application. *Bioresour.Technol.* 96.
86. Niemera, B. A. 2007. Relative efficacy of sodium hypochlorite wash versus irradiation to inactivate *Escherichia coli* O157:H7 internalized in leaves of Romaine lettuce and Baby spinach. *J. Food Prot.* 70:2526-2532.
87. Niemera, B. A. 2008. Irradiation compared with chlorination for elimination of *Escherichia coli* O157:H7 internalized in lettuce leaves: Influence of lettuce variety. *J. Food Sci.* 73:208-213.
88. Pao, S., D. F. Kelsey, M. F. Khalid, and M. R. Ettinger. 2007. Using aqueous chlorine dioxide to prevent contamination of tomatoes with *Salmonella enterica* and *Erwinia carotovora* during fruit washing. *J. Food Prot.* 70:629-634.
89. Parish, M. E., L. R. Beuchat, T. V. Suslow, L. J. Harris, E. H. Garrett, J. N. Farber, and F. F. Busta. 2003. Methods to reduce/eliminate pathogens from fresh and fresh-cut produce. *Comprehensive Rev. Food Sci. and Food Saf.* 2:161-173.
90. Park, C.-M., Y.-C. Hung, M. P. Doyle, and C. Kim. 2001. Pathogen reduction and quality of lettuce treated with electrolyzed oxidizing and acidified chlorinated water. *J. Food Sci.* 66:1368-1372.
91. Peteers, J. E., E. Ares Mazás, W. J. Masschelein, I. Villacorta Martinez de Maturana, and E. Debacker. 1989. Effect of disinfection of drinking water with ozone or chlorine dioxide on survival of *Cryptosporidium parvum* oocysts. *appl. Environ. Microbiol.* 55:1519-1522.

92. Prakash, A., A. R. Guner, F. Caporaso, and D. M. Foley. 2000. Effects of low-dose gamma irradiation on shelf life and quality characteristics of cut romaine lettuce packaged under modified atmosphere. *J. Food Sci.* 65.
93. Reina, L. D., H. P. Fleming, and E. G. Humphries. 1995. Microbiological control of cucumber hydrocooling water with chlorine dioxide. *J. Food Prot.* 58:541-546.
94. Rodgers, S. T., J. N. Cash, M. Siddiq, and E. T. Ryser. 2004. A comparison of different chemical sanitizers for inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes* in solution and on apples, lettuce, strawberries, and cantaloupe. *J. Food Prot.* 67:721-731.
95. Sandt, C. H., D. A. Krouse, C. R. Cook, A. L. Hackman, W. A. Chmielecki, and N. G. Warren. 2006. The key role of pulsed-field gel electrophoresis in investigation of a large multiserotype and multistate food-borne outbreak of *Salmonella* infections centered in Pennsylvania. *J. Clin. Microbiol.* 44:3208-3212.
96. Santurio, J. M., D. F. Santurio, P. Pozzatti, C. Moraes, P. R. Franchin, and S. H. Alves. 2007. Antimicrobial activity of essential oils from oregano, thyme and cinnamon against *Salmonella enterica* serovars from avian source. *Cient. Rural.* 37.
97. Sapers, G. M. 2003. Washing and sanitizing raw materials for minimally processed fruits and vegetables, p. 221-253. In Novak, J.S., G.M. Sapers, and V.K. Juneja. (eds). *Microbial Safety of Minimally Processed Foods*. Boca Raton, FL: CRC Press.
98. Sapers, G. M., and D. M. Jones. 2006. Improved sanitizing treatments for fresh tomatoes. *J. Food Sci.* 71:252-256.
99. Seo, K. H., and J. F. Frank. 1999. Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. *J. Food Prot.* 62:3-9.

100. Shi, X., A. Namvar, M. Kostrzynska, R. Hora, and K. Warriner. 2007. Persistence and growth of different *Salmonella* serovars on pre- and postharvest tomatoes. *J. Food Prot.* 70:2725-2731.
101. Solomon, E. B., C. J. Potenski, and K. R. Matthews. 2002. Effect of irrigation method on transmission to and persistence of *Escherichia coli* O157:H7 on lettuce. *J. Food Prot.* 65:673-676.
102. Solomon, E. B., S. Yaron, and K. R. Matthews. 2002. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Appl. Environ. Microbiol.* 68:397-400.
103. Solomon, E. B., S. Yaron, and K. R. Matthews. 2002. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Appl. Environ. Microbiol.* 68:397-400.
104. Spotts, R. A., and L. A. Cervantes. 1992. Effect of ozonated water on postharvest pathogens of pear in laboratory and packinghouse tests. *Plant Disease.* 76:256-259.
105. Stopforth, J. D., T. Mai, B. Kottapalli, and M. Samadpour. 2008. Effect of acidified sodium chlorite, chlorine, and acidic electrolyzed water on *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* inoculated onto leafy greens. *J. Food Prot.* 71:625-628.
106. Strange, M. L., Schrader, W. L., and T. K. Hartz. 2000. Fresh-market tomato production in California. *Vegetable Research and Information Center. University of California. Publication 8017*. Available at <http://anrcatalog.ucdavis.edu/pdf/8017.pdf> Accessed July 22/ 2008.
107. Suslow, T. V. 1997. Postharvest chlorination: Basic properties and key points for effective disinfection. Available at: <http://anrcatalog.ucdavis.edu>. Accessed July 13/2008.

108. Suslow, T. V. 1998. Water disinfection: A practical approach to calculating dose values for preharvest and postharvest applications. Available at:
<http://anrcatalog.ucdavis.edu/pdf/7256.pdf>. Accessed July 5/2008.
109. Suslow, T. V. 2004. Ozone applications for postharvest disinfection of edible horticultural crops. Available at: <http://anrcatalog.ucdavis.edu/pdf/8133.pdf>. Accessed June 12/2008.
110. Takeuchi, K., and J. F. Frank. 2000. Penetration of *Escherichia coli* O157:H7 into lettuce leaves as affected by inoculum size and temperature, and the effect of chlorine treatment on cell viability. *J. Food Prot.* 63:434-440.
111. Takeuchi, K., and J. F. Frank. 2001. Expression of red-shifted green fluorescent protein by *Escherichia coli* O157:H7 as a marker for the detection of cells on fresh produce. *J. Food Prot.* 64:298-304.
112. Takeuchi, K., and J.F. Frank. 2001. Confocal microscopy and microbial viability detection for food research. *J. Food Prot.* 64:2088-2102.
113. Troller, J. A. 1993. Sanitation in food processing. Academic Press, Inc., San Diego, C.A.
114. Ukuku, D. O., and G. M. Sapers. 2001. Effect of sanitizer treatments on *Salmonella* Stanley attached to the surface of cantaloupe and cell transfer to fresh-cut tissues during cutting practices. *J. Food Prot.* 64:1286-1291.
115. USDA/ERS. 2005. U.S. Lettuce statistics. Available at
<http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1576>
Accessed July 22/ 2007.

116. Venkitanarayanan, K. S., G. O. Ezeike, Y. C. Hung, and M. P. Doyle. 1999. Inactivation of *Escherichia coli* O157:H7 and *Listeria monocytogenes* on plastic kitchen cutting boards by electrolyzed oxidizing water. *J. Food Prot.* 62:856-860.
117. Wachtel, M. R., and A. O. Charkowski. 2002. Cross-contamination of lettuce with *Escherichia coli* O157:H7. *J. Food Prot.* 65:465-470.
118. Wachtel, M. R., L. C. Whitehand, and R. E. Mandrell. 2002. Association of *Escherichia coli* O157:H7 with preharvest leaf lettuce upon exposure to contaminated irrigation water. *J. Food Prot.* 65:18-25.
119. Wade, W. N., and L. R. Beuchat. 2003. Proteolytic fungi isolated from decayed and damaged tomatoes and implications associated with changes in pericarp pH favorable for survival and growth of foodborne pathogens. *J. Food Prot.* 66:911-917.
120. Wade, W. N., R. Vasdinnyei, T. Deak, and L. R. Beuchat. 2003. Proteolytic yeasts isolated from raw, ripe tomatoes and metabiotic association of *Geotrichum candidum* with *Salmonella*. *Int. J. Food Microbiol.* 86:101-11.
121. Wan, J., A. Wilcock, and M. J. Coventry. 1998. The effect of essential oils of basil on the growth of *Aeromonas hydrophila* and *Pseudomonas fluorescens*. *J. Appl. Microbiol.* 84:152-158.
122. Wang, G., T. Zhao., and M. P. Doyle. 1996. Fate of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces. *Appl. Environ. Microbiol.* 62 2567-2570.
123. Ward, S. M., P. J. Delaquisb, R. A. Holleya, and G. Mazza. 1998. Inhibition of spoilage and pathogenic bacteria on agar and pre-cooked roast beef by volatile horseradish distillates. *Food Res. Int.* 31:19-26.

124. Warriner, K., S. Spaniolas, M. Dickinson, C. Wright, and W.M. Waites. 2003. Internalization of bioluminescent *Escherichia coli* and *Salmonella* Montevideo in growing bean sprouts. *J. Appl. Microbiol.* 95:719-727.
125. Watkins, J., and K. P. Sleath. 1981. Isolation and enumeration of *Listeria monocytogenes* from sewage, sewage sludge and river water. *J. Appl. Bact.* 50:1-9.
126. Weissinger, W. R., W. Chantarapanont, and L. R. Beuchat 2000. Survival and growth of *Salmonella* Baildon in shredded lettuce and diced tomatoes, and effectiveness of chlorinated water as a sanitizer. *Int. J. Food Microbiol.* 62:123-131.
127. Yang, H., B. L. Swem, and Y. Li. 2003. The effect of pH on inactivation of bacteria on fresh-cut lettuce by dipping treatment with electrochemically activated water. *J. Food Sci.* 68:1013-1017.
128. Yuk, H.-G., J. A. Bartz, and K. R. Schneider. 2005. Effectiveness of individual or combined sanitizer treatments for inactivating *Salmonella* spp. on smooth surface, stem scar, and wounds of tomatoes. *J. Food Sci.* 70:409-414.
129. Zhang, S., and J. M. Faber. 1996. The effects of various disinfectants against *Listeria monocytogenes* on fresh-cut vegetables. *Food Microbiol.* 13:311-321.
130. Zhuang, R. Y., and L. R. Beuchat. 1995. Fate of *Salmonella* Montevideo on and in raw tomatoes as affected by temperature and treatment with chlorine. *Appl. Environ. Microbiol.* 61:2127-2131.
131. Zhuang, R. Y., and L. R. Beuchat 1995. Fate of *Salmonella* Montevideo on and in raw tomatoes as affected by temperature and treatment with chlorine. *Appl. Environ. Microbiol.* 61:2127-2131.

CHAPTER 3

INACTIVATION OF *SALMONELLA* SPP. AND *ECHERICHIA COLI* O157:H7 ON SLICED AND WHOLE TOMATOES BY ALLYL ISOTHIOCYANATE, CARVACROL AND CINNAMALDEHYDE IN VAPOR-PHASE¹

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ABSTRACT

Little is known about the effectiveness of antimicrobials in the vapor phase for control of pathogens on the surface of fresh produce. We determined the activity of allyl isothiocyanate (AIT), cinnamaldehyde and carvacrol against *Salmonella* and *E. coli* O157:H7 on sliced and whole tomatoes. Samples were treated with various concentrations of antimicrobial in the vapor phase at 4, 10 and 25 °C in an enclosed container. AIT exhibited the greatest antimicrobial activity followed by cinnamaldehyde. The lowest level of AIT used (8.3 µl/liter of air) inactivated *Salmonella* on sliced tomatoes by 1.0 and 3.5 log at 4 and 10 °C, respectively, in 10 days and by 2.8 log at 25 °C in 10 h. This level of AIT inactivated *Salmonella* on whole tomatoes to the detection limit of < 2 log CFU/tomato at 4 and 10 °C in 10 days and by 1.3 log at 25 °C in 10 h. AIT also inactivated *E. coli* O157:H7 on sliced tomatoes by 3.0 log at 4 and 10 °C in 10 days with no inactivation at 25 °C in 10 h. AIT reduced *E. coli* O157:H7 on whole tomatoes surface by 3.0 and 1.0 log at 4 and 10 °C, respectively, in 10 days and by 2.0 log at 25 °C in 10 h. Overall, greater inactivation occurred at 10 than at 4 °C and on the tomato surface than between slices. Antimicrobials in vapor phase may be useful for controlling pathogens on fresh tomatoes marketed in packages containing enclosed headspace.

INTRODUCTION

Recent foodborne outbreaks associated with tomatoes created a need for understanding the sources of contamination, the survival and/or growth of pathogens, and to develop innovative control technologies. Generally, pathogens on tomatoes are controlled by preventing the contamination during growth and harvesting, by using antimicrobial rinses, and by low temperature storage. Prevention of contamination is the most effective control strategy since pathogen growth is not required to cause illness and antimicrobial rinses are not sufficiently effective at killing or removing attached pathogens. Therefore, introducing additional control measures could be of value.

The behavior of pathogens on tomatoes is affected by pathogen location on the fruit, tomato quality, storage temperature, packaging type, and relative humidity. Tomatoes usually have sufficient acid to limit pathogen growth when stored under refrigeration temperatures. However, fungal and yeast infection of raw tomatoes or natural mycoflora increase the pH of the pericarp tissues to a level that favor pathogen growth (21, 22). As storage temperatures increase above the refrigeration temperature, *Salmonella* spp. will survive and/or grow more rapidly on tomato skin, stem scar and in chopped tomatoes (1, 5, 21, 24, 26). *Salmonella* Montevideo produces extracellular polymers on tomato cuticles after 10 h at 22 and 30 °C under high relative humidity (97%) leading to a well-defined biofilm after 4 days (12). Pathogens on the surface of tomatoes may contaminate internal tissues during slicing, and survive or grow in the slices. *Salmonella* spp. that had infiltrated tomatoes grew at 25 °C (10). Various research findings indicate that bacterial pathogens can infiltrate into whole tomatoes (2, 3, 8, 10, 26) when subject to a temperature differential between the tomato and washing water temperature, and by hydrostatic

pressure when tomatoes are submerged in the dump tank (2, 4). Bacterial infiltration increases in the presence of wounds and punctures on the tomato. Infiltrated pathogens are not removed by normal washing practices.

The main benefit of adding antimicrobial chemicals to wash water for tomato is to control the spread of pathogens by killing those released from the produce, as the pathogen-reducing benefits of antimicrobial washes on the tomato are limited. Chlorinated water, hydrogen peroxide, peroxyacetic acid, and electrolyzed water have been studied for their ability to reduce pathogens on tomatoes during the washing process. These treatments have limited effectiveness, presumably because active agents do not sufficiently contact the target pathogens.

Recent research indicates that antimicrobial chemicals in vapor phase can significantly reduce pathogen populations on the tomato surface. The use of 5 mg/liter chlorine dioxide gas for 1 h was significantly more effective against *Salmonella* spp. on the stem scar than aqueous solutions of 200 ppm sodium hypochlorite (2 min exposure) and 1200 ppm acidified sodium chlorite (2 min exposure) (25). The use of 10 mg/liter ozone completely inactivated (7 log CFU/tomato) *Salmonella* Enteritidis on the surface of cherry tomatoes after 15 min for 1 h attachment and 20 min for a 4 h attachment, however this concentration changes tomatoes color (5). Since vapor-phase antimicrobials may be effective against attached bacteria at locations on the fruit not reached by active agents in aqueous solution, their use in packaged produce or during produce processing could provide an added pathogen control benefit.

Essential oils are able to inactivate pathogens of concern in fresh produce. Of 96 tested essential oils; the three most effective against *E. coli* O157:H7 and *Salmonella enterica* were oregano, thyme and cinnamon; the three most effective oil compounds, among 16 compounds

tested against *E. coli* O157:H7 were carvacrol, cinnamaldehyde, and thymol; and the most effective compounds against *Salmonella enterica* thymol, cinnamaldehyde and carvacrol (7).

The efficacy of antimicrobials in vapor phase is expected to depend on the temperature, time of the exposure and concentration. The aim of this project is to elucidate the effect of these parameters on pathogen inactivation on tomato skin (using whole tomatoes) and on tomato pulp (using sliced tomatoes). Allyl isothiocyanate (AIT, from mustard and horseradish), carvacrol (from oregano), and cinnamaldehyde (from cinnamon) were the volatile antimicrobials selected for this study.

MATERIALS AND METHODS

Inoculum preparation. *Salmonella* spp. and *E. coli* O157:H7 strains isolated from produce-associated outbreaks were used to inoculate the tomatoes. A five-strain cocktail of *Salmonella* spp. (Table 1.1) and a four-strain cocktail of *E. coli* O157:H7 (Table 1.2) were prepared. The strains were grown to stationary-phase by transferring each twice in tryptic soy broth (TSB) at 24 h intervals with incubation at 35 °C. The cocktails were prepared by mixing equal volumes of each pathogen strain. Then, the cocktails were centrifuged at 4500 rpm for 25 min at 4° C, the supernatant fluid discarded, and the pellet suspended in 0.1% peptone water. The cell suspensions contained approximately 9 log CFU/ml.

Inoculation of sliced tomatoes. Fresh light red tomatoes (*Lycopersicon esculentum*, Mill) were purchased at retail from a local grocery store in Athens, GA on the day of the experiment. The pH of the tomatoes was 4.2. Tomatoes were immersed in chlorinated water (200 ppm) for 5 min,

and then rinsed under running tap water to remove chlorine residues. The tomatoes were sliced by using a manual tomato slicer (Lincoln Pro Tomato - 1/4" Cut, Lincoln Foodservice product, Model 0644N, Lincoln Foodservice, Fort Wayne, IN), which was sanitized by submersion in 200 ppm chlorine for 10 min, and then rinsed under running hot tap water. Each tomato was sliced into 10 slices, each of 0.64 cm thickness. The outer two slices were discarded. Two adjacent slices were selected and cut into four pieces ($4.5 \times 4.5 \times 4.5$ cm) with a sterile knife. One pair of the quartered slices was placed with sterile forceps in one autoclave-sterilized septa jar (120 ml volume) (Short Amber WM Septa-Jar TM, Item # S240-0120, I-ChemTM Certified 200 series, Chase Scientific Glass, Inc, Rockwood, TN). Working in a biosafety cabinet, a 100 μ l of 6.7 log CFU/ml inoculum was deposited between the two adjacent quartered tomato slices inside the septa jars.

Inoculation of whole tomatoes. Whole grape tomatoes (*Lycopersicon esculentum*) were used for surface inoculation experiments as this size provided for the desired head space. Fresh grape tomatoes were purchased from a grocery store in Athens, GA the same day of the experiment. Tomatoes were immersed in 200 ppm chlorine for 5 min, and then rinsed under running tap water to remove chlorine residues. Working in a biosafety cabinet, a 3-cm diameter circle was drawn on each tomato with a liquid blocker super pap pen (Daido Sangyo Co., Ltd. Tokyo, Japan). Each tomato was spot-inoculated inside the circle with 100 μ l of 9.0 log CFU/ml inoculum (the 100 μ l were placed in 10 separate spots) (11). This high initial inoculum was used as the preliminary work showed that *Salmonella* and *E. coli* O157:H7 population decreases during the drying period. Lang et al. (14) also observed that *E. coli* O157:H7 and *Salmonella* spp. population on tomato surface decrease by 3.2 and 2.2 log CFU, respectively during drying at

22 ± 2 °C after 24 h (14). The inoculated tomatoes were left under the biosafety cabinet to dry for 24 h at 22 °C (14). Then, one grape tomato was aseptically placed in each speta jar.

Antimicrobial application. The inoculated sliced and whole tomatoes were exposed to the antimicrobial compounds in similar manner. Autoclave-sterilized filter paper pieces (2×2 cm, Whatman Inc., Clifton, NJ) were saturated with sterile deionized water by immersion. One wet filter paper was placed on the top inner side of each speta jar (with caution taken to not contact the tomato sample) to create high relative humidity inside the jars. The antimicrobial compounds were allowed to equilibrate to room temperature. Then 5, 10, and 15 µl (equivalent to 41.5, 83.3, and 125 µl antimicrobial/liter of air of 97% ≥ purity carvacrol or ≥ 98% purity cinnamaldehyde, or 1, 2, 4 µl (equivalent to 8.3, 16.6, and 33.3 µl of antimicrobial/liter of air) of ≥ 98% purity AIT (Sigma- Aldrich, St. Louis, MO) was deposited on a second filter paper of the same size previously attached with double-stick tape (Henkel Consumer Adhesives, Inc, Avon, OH) on the inner upper side of the septa jar. The location of the filter was designed to avoid direct contact of the antimicrobial compound with the tomato sample. Samples in the sealed jars were stored at 4, 10, and 25 °C.

Microbiological analysis. Samples held at 4 and 10 °C were analyzed after 0, 4, 7, and 10 days of incubation. Samples held at 25 °C were analyzed after 0, 4, 7, and 10 h. The entire tomato sample was removed aseptically from the jar and placed in a filter stomacher bag (Nasco. Inc., Ft. Atkinson, WI). For the sliced tomatoes samples, the jars were also rinsed with peptone water to include any juice dripping in the analysis. The weight of each bag was brought to 100 g and then stomached for 1 min at high speed by a stomacher (Stomacher 400 Laboratory Blender,

Seward, UK). Numbers of *Salmonella* spp. were determined by spiral plating (Spiral Biotech, Inc., Norwood, MA) appropriate dilutions on MacConkey agar (Becton, Dickinson and Company, Sparks, MD), while numbers of *E. coli* O157:H7 were determined by spiral plating on sorbitol MacConkey agar (SMAC) (Becton, Dickinson and Company, Sparks, MD). MacConkey plates were incubated at 35 °C for 18 h, while SMAC plates were incubated at 37 °C for 18 h. Only typical colonies were counted. Data was calculated as CFU/inoculated site, since the whole inoculated sample was analyzed.

Statistical analysis. The study employed a factorial random block design. The experimental design consisted of three antimicrobials (AIT, carvacrol, and cinnamaldehyde), four concentrations (0, 8.3, 16.6, and 33.3 µl/liter for first antimicrobial and 0, 41.5, 83.3, and 125 µl/liter for latter two antimicrobials), three temperatures (4, 10, and 25 °C), four sampling times (0, 4, 7, and 10 days for 4 and 10 °C samples, and 0, 4, 7, and 10 h for 25 °C samples), two inoculation methods (on whole tomato and between slices), and two pathogens (*Salmonella* spp. and *E. coli* O157:H7). Each experiment was replicated four times. Data was analyzed using SAS 9.1.3 (SAS Institute, Inc., Cary, NC). Multiple comparisons were evaluated by analysis of variance (ANOVA) using general linear model. A 5% significance level was employed for all analyses.

RESULTS

In the preliminary work, carvacrol and cinnamaldehyde inactivated *Salmonella* spp. on sliced tomatoes at a concentration of 150 µl/liter of air with no adverse effect on aroma as determined

by informal evaluation using four to five untrained panelists. Equivalent or lower concentrations (41.5, 83.3, and 125 $\mu\text{l/liter}$ of air) of carvacrol and cinnamaldehyde were used in this study. Similar concentrations of AIT evaluated in preliminary research produced detectable odor when the containers were opened. Therefore, lower concentrations of 8.3, 16.6, and 33.3 $\mu\text{l/liter}$ of air of AIT were used in this study.

Inactivation of *Salmonella* spp. in the presence of vapor-phase antimicrobials at 4 °C. In the absence of the antimicrobials (control samples), the population of *Salmonella* spp. decreased by ca. 1.0 log CFU on sliced tomatoes in 10 days at 4 °C (Fig 1.1A through 1.1C). The presence of the antimicrobials caused significant greater reduction. AIT exhibited the greatest inactivation ability followed by carvacrol and then cinnamaldehyde. Reduction of *Salmonella* spp. at 4 °C by all antimicrobials increased with time of incubation, but only AIT exhibited concentration-dependent inactivation. The reduction caused by AIT and carvacrol was observed after 4 days of incubation and continued until the tenth day, but the inactivation by cinnamaldehyde was not observed until the tenth day.

In the absence of the antimicrobials (control samples), the population of *Salmonella* spp. on whole tomato decreased significantly (>3 log) in 10 days at 4 °C (Table 1.3). The lowest concentration used of each antimicrobial inactivated *Salmonella* spp. to below the detection limit (4 log units less than the control) within 4 days.

Inactivation of *Salmonella* spp. in the presence of vapor-phase antimicrobials at 10 °C. In the absence of the antimicrobials, the population of *Salmonella* spp. increased on the tomato slices by approximately 2.4 log units between the 4th and 7th days of incubation at 10 °C (Fig

1.2A through 1.2C). All antimicrobials reduced populations of *Salmonella* spp. on sliced tomatoes significantly when compared to the control depending on the concentration, with the AIT reduced the pathogen at all concentrations used and the carvacrol and cinnamaldehyde able to reduced cells only at the highest concentration used. However, carvacrol and cinnamaldehyde prevented growth of *Salmonella* spp. during the first 7 days of incubation when used at lower concentrations.

In the absence of the antimicrobials, the population of *Salmonella* spp. on whole tomato did not change during the 10 days at 10 °C (Fig 1.3A through 1.3C). However, the presence of any the antimicrobials decreased the *Salmonella* population. AIT exhibited the most inactivation ability followed by cinnamaldehyde and then carvacrol. Inactivation increased with increasing concentration for all the antimicrobials. For example, 16.6 and 33.3 µl/liter of AIT inactivated *Salmonella* spp. to the detection limit within 10 and 7 days, respectively.

Inactivation of *Salmonella* spp. in the presence of vapor-phase antimicrobials at 25 °C. In the absence of the antimicrobials, the population of *Salmonella* spp. on sliced tomatoes increased significantly by about 2.9 log CFU over 10 h at 25 °C (Fig 1.4A through 1.4C) after a 4 hour lag phase. The presence of the antimicrobials did not cause any significant reduction in the population at any concentration when samples were incubated for over 10 h. Carvacrol and cinnamaldehyde had no effect on the growth of *Salmonella* spp. on sliced tomatoes during the first 7 hours of incubation and no significant inhibitory effect after 10 h providing a total increase of ca. 2.4 log units. However, AIT prevented growth of *Salmonella* spp. at the lowest concentration used.

The population of *Salmonella* spp. on whole tomato increased by 0.8 log CFU/g after 10 h at 25 °C (Fig 1.5A through 1.5C) when no antimicrobial was used. However, the presence of any of the antimicrobials caused significant inactivation of *Salmonella* population, and the amount of inactivation increased with an increase antimicrobial concentration. Cinnamaldehyde and AIT exhibited the strongest inactivation ability against *Salmonella* spp. producing a significant 2.8 log reduction at highest concentration used (125 µl/liter and 33.3µl/liter of air, respectively) over the 10 h incubation, whereas carvacrol produced a significant 2.0 log reduction at its highest concentration (125 µl/liter) compared to the control over the same time.

Inactivation of *E. coli* O157:H7 in the presence of vapor-phase antimicrobials at 4 °C. No significant change in the population of *E. coli* O157:H7 occurred on sliced tomatoes after 7 days, but a significant increase was observed after 10 day of incubation at 4 °C. The presence of carvacrol and cinnamaldehyde caused a significant reduction in population from 7 to 10 days of storage ($P < 0.05$) (Fig. 1.6A and 1.6B). AIT (Fig. 6C) produced the greatest inactivation ($P < 0.05$) of all the antimicrobials at all concentrations used. After 7 days, concentrations of 16.6 and 33.3 µl/liter of air inactivated the pathogen by over 2 log units, and at a concentration of 8.3 µl /liter AIT was able to reduce the pathogen population by almost 1 log unit compared to the 0 time control.

In the absence of the antimicrobials, the population of *E. coli* O157:H7 on whole tomato decreased by 1.6 log at 4 °C, with most of the decrease occurring during the first 7 days (Table 1.4). All of the antimicrobials inactivated *E. coli* O157:H7 on the tomato surface to below the detection limit (3 log units less than the control) by the 4th day.

Inhibition of *E. coli* O157:H7 in the presence of vapor-phase antimicrobials at 10 °C. In the absence of the antimicrobials, *E. coli* O157:H7 grew on the sliced tomatoes to achieve a 4 log unit increase in 10 days at 10 °C (Fig 1.7A through 1.7C). The presence of the antimicrobials did not reduce the population, but slowed or prevented growth in a concentration dependent manner, with AIT exhibiting the greatest ability to inhibit growth, and carvacrol able to inhibit growth when used at a concentration of 125 µl /liter of air. Carvacrol was effective at preventing growth of *E. coli* O157:H7 during the first 4 days of incubation, with the lower two concentrations losing effectiveness after that time. In contrast the lowest two concentrations of AIT allowed about 1 log unit of growth during the first 4 days, after which growth was completely inhibited. AIT was the most effective growth inhibitor for *E. coli* O157:H7 on the tomato slices at 10 °C incubation as it provided a slower increase in the population from the antimicrobial-free sample resulting in 3.0 to 4.7 log unit lower population than the control after 10 days of treatment.

E. coli O157:H7 maintained a stable population on the tomato surface at 10 °C over the 10 days incubation (Figure 1.8A through 1.8C). Addition of antimicrobials to the headspace inactivated the pathogen. AIT produced the most inactivation ability followed by cinnamaldehyde and then carvacrol. Inactivation increased with increasing concentration of the antimicrobial. For example, the presence of 8.3, 16.6, and 33.3 µl/liter of air AIT inactivated *E. coli* O157:H7 by 1.1, 2.8, and 4.4 log, respectively after 10 days when compared to the control. The presence of 41.5, 83.3, and 125 µl/liter cinnamaldehyde inactivated *E. coli* O157:H7 by 1.5, 2.0, and 2.7 log, respectively, compared to the control by the 10th day. However, the presence of 41.5, 83.3, and 125 µl/liter of carvacrol inactivated *E. coli* O157:H7 by 0.7, 1.4, and 2.5 log, respectively, compared to the control by the 10th day.

Inactivation of *E. coli* O157:H7 in the presence of vapor-phase antimicrobials at 25 °C. In the absence of the antimicrobials, the population of *E. coli* O157:H7 on sliced tomatoes increased about 1.0 log at 25 °C in 10 h after a 4 to 7 hour lag phase (Fig. 1.9A through 1.9C). The presence of 33.3 µl/liter of AIT decreased *E. coli* O157:H7 by 0.7 log compared to the control after 10 h. No other antimicrobial treatments significantly reduced levels of *E. coli* O157:H7 on sliced tomato during the 10 h treatment period.

In the absence of the antimicrobials, the population of *E. coli* O157:H7 on whole tomato remained stable at 25 °C over the 10 h incubation (Fig. 1.10A through 1.10C). All of the antimicrobial treatments achieved inactivation of the pathogen and inactivation increased with increasing antimicrobial concentration. AIT and cinnamaldehyde exhibited significant inactivation ability at all concentrations applied. Inactivation was observed after 4 hours for these antimicrobials. Inactivation of *E. coli* O157:H7 by AIT on the whole tomatoes diminished after 4th h when it was applied at 8.3 and 16.6 µl/liter concentration, but the compound maintained activity when applied at a 33.3 µl/liter. Inactivation by cinnamaldehyde and carvacrol also diminished after four hours when they were applied at a concentration of 41.5 and 83.3 µl/liter but they maintained activity when applied at 125 µl/liter of air.

DISCUSSION

In the absence of antimicrobials, *Salmonella* spp. and *E. coli* O157:H7 grew more on tomato slices than on the tomato surface as temperature increased. The pathogens also survived better on the whole tomatoes than on tomato slices. For example, the pathogens decreased *ca.* 1.0 log on sliced tomatoes but died by more than 3 log CFU on the tomato surface at 4 °C after 10 days.

Lin et al. (15) found that the survival of *Salmonella* Montevideo on tomato skin was less than that on tomato stem scar at 4 °C after 2 days. Nearly 1-log reduction in *Salmonella* Montevideo on the tomato surface occurred after 2 days at 4 °C. Zhuang et al. (26) observed that *Salmonella* Montevideo population did not change in chopped tomato at 5 °C in 9 days although a decrease of about 0.3 log units was observed, whereas we observed an decrease of 0.8 log units during storage at 4 °C for 10 days using a multistrain cocktail. Survival on tomatoes is serovar dependent; serovars Hadar, Montevideo and Newport are the most adapted to growth and survival in or on the fruit (17). Our study showed that *Salmonella* spp. and *E. coli* O157:H7 grew on sliced tomatoes at 10 °C, but their populations remained stable on the tomato surface, a result similar to that of Zhaung et al. (26) who used *Salmonella* Montevideo. We also found that the two pathogens increased in population on sliced tomato at 25 °C after 10 h but no significant change in numbers was observed on the tomato surface at this temperature. This result is also similar to that of Zhaung et al. (26), who observed that *Salmonella* Montevideo on the tomato surface increased in numbers within 7 days and 1 day at 20 and 30 °C, respectively (26). Difference in pathogen behavior at different locations on tomatoes may be attributed to differences in moisture and nutrient availability. Previous research indicates that growth of *Salmonella* Montevideo on the tomato surface increases at higher relative humidity (12). Stine et al. (19) also observed that survival of several pathogens and viruses on produce (lettuce, cantaloupe and bell pepper) surfaces is influenced by relative humidity.

AIT, carvacrol and cinnamaldehyde in vapor form inactivated and/or inhibited growth of *Salmonella* spp. and *E. coli* O157:H7 on whole and sliced tomato at 4 and 10 °C. Carvacrol and cinnamaldehyde exhibited little inhibition at 25 °C in 10 h but AIT prevented growth of the pathogens in sliced tomatoes and all the antimicrobials inactivated the pathogens on tomato

surface at 25 °C. Although AIT was used at a concentration five times less than carvacrol and cinnamaldehyde, it exhibited the greatest inactivation ability at all temperatures and exposure times.

The antimicrobials in vapor-phase were more effective in inactivating pathogens on the tomato surface than on the flesh (slices). These findings are consistent with those of Lin et al. (15) who found that the vapor of AIT at a concentration of 132 µl/liter of air reduced *Salmonella* Montevideo by 8 log units on the tomato surface, but by only 5 log units on tomato stem scars at 4 °C after 2 days. However, in the present study, lower levels of AIT were evaluated and 8.3 µl/liter produced 1.3 and 2.0 log CFU reduction of *Salmonella* spp. on tomato slices and surface, respectively, after 10 days. The present study employed a cocktail of *Salmonella* spp., whereas Lin et al. (14) used a single strain of *Salmonella* Montevideo. The cocktail was used, in our study, to account for strain differences in persistence and growth on tomatoes and possible antimicrobials susceptibility (17). Another gaseous antimicrobial, chlorine dioxide, also exhibited greater activity at a fruit surface as compared to the tissue (blueberry surface compared to stem scar). The use of 4.1 mg/liter of chlorine dioxide for 25 min reduced *Salmonella* spp. on tomato surface by 4.33 log CFU/tomato, however this level of ClO₂ resulted in adverse sensory effect (20). Chlorine dioxide also produced greater inactivation of *Salmonella* on apple skin than on calyx and stem cavities (6).

Generally, extrinsic factors, such as antimicrobial concentration, time and temperature of exposure, affect the efficacy of an antimicrobial treatment. The inactivation/inhibition activity of AIT, carvacrol and cinnamaldehyde against *Salmonella* spp. and *E. coli* O157:H7 on sliced tomato increased with concentration and time. However, data would need to be collected at shorter time intervals then used in this study to draw an overall conclusion about the effect of

time. Other researchers reported that the ability of chlorine dioxide gas to inactivate *E. coli* O157:H7 on apple surface increased with gas concentration and exposure time (6). Treatment of 12.0 mg/liter for 10 min, 4.8 mg/liter for 20 min, and 3.3 mg/liter were the optimum concentration-exposure time to inactivate *E. coli* O157: H7 on apple surface which reduce the pathogen by 5 log CFU on the skin and 3.0 to 3.7 log CFU on the calyx and stem cavities (6). Chlorine dioxide gas was also used to inactivate *E. coli* O157:H7 on green peppers; where the gas concentration, treatment time, relative humidity (RH), and temperature significantly increased the inactivation ability. The gas concentration was the most important factor and temperature was the least. Furthermore, the gas concentration and RH had a synergistic effect on inactivation (9).

Intrinsic characteristics of the food, of which pH may be the most important in fresh produce, also affect the antimicrobial activity against pathogens. Applying AIT at 1000 and 2000 µg /liter of air did not reduce *Salmonella* spp. on sprouts, but adversely affected the sensory quality, after 11 days of exposure at 10 °C (23). However, in our study, a concentration of 8.3 µl of /liter of air reduced the population on sliced tomato by 1.2 log CFU after 10 days at 10 °C. The difference between the findings of Weissinger et al. (23) and those in this study result from the different food matrix; the acidity of the tomatoes may enhance inactivation and sprouts may provide additional physical protection for pathogens. Previous research indicates that the inherent acidity of fruits enhances antimicrobial activity of lemongrass, cinnamon and geraniol against *Salmonella* Enteritidis, *E. coli* and *L. innocua* (higher in apple and pear juice as compared to melon juice and tryptic soy broth)(16). A concentration of 2 µl/ml of lemongrass, cinnamon or geraniol essential oils was needed to inactivate *Salmonella* Enteritidis, *E. coli* and *L. innocua* in apple and pear juices. However, 8 and 10 µl/ml of cinnamon was required to

inactivate these pathogens in melon and tryptic soy broth, respectively. Meanwhile, a 6 µl/ml of geraniol or 5 µl/ml of lemongrass was needed to inactivate the pathogens. The activity of oregano essential oil against *E. coli* O157:H7 in eggplant salad and against *Salmonella* Enteritidis in taramasalad (cod roe, wet bread, and olive oil salad) was enhanced at lower pH (by adding citric acid) (13, 18). The enhanced activity of essential oils at acidic pH might be attributed to the essential oil becoming more hydrophobic at low pH and therefore exhibiting increased solubility at the lipid layer of the bacterial membrane (18).

The results of this study indicate the potential of using AIT, carvacrol and cinnamaldehyde, individually or in combination, as a kill or growth prevention step in packaged sliced and grape tomatoes. These antimicrobials could be of use to control *Salmonella* spp. and *E. coli* O157:H7 on packaged tomatoes stored at refrigeration temperatures. If there is a breakage in the cold chain, the antimicrobial activity would increase and reduce the potential for pathogen growth. Additional research on the influence of these treatments on sensory quality will need to be completed before practical use. In addition, data validating the application of these treatments in industrial systems is required.

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REFERENCES

1. Asplund, K., and E. Nurmi. 1991. Growth of salmonellae in tomatoes. *Int. J. Food Microbiol.* 13:177-181.
2. Bartz, J. A. 1982. Infiltration of tomatoes immersed at different temperatures to different depths in suspensions of *Erwinia carotovora* subsp. *carotovora*. *Plant Disease*. 66:302-306.
3. Bartz, J. A. 1999. Washing fresh fruits and vegetables: Lessons from treatment of tomatoes and potatoes with water. *Dairy, Food and Environmental Sanitation*. 19:853-864.
4. Bartz, J. A., and R. K. Showalter. 1981. Infiltration of tomatoes by aqueous bacterial suspensions. *phytopathology*. 71:515-518.
5. Das, E., G. C. Gurakan, and A. Bayindirli. 2003. Effect of controlled atmosphere storage, modified atmosphere packaging and gaseous ozone treatment on the survival of *Salmonella enteritidis* on cherry tomatoes. *Food Microbiol.* 23:430-438.
6. Du, J., Y. Han, and R. H Linton. 2003. Efficacy of chlorine dioxide gas in reducing *Escherichia coli* O157:H7 on apple surfaces. *Food Microbiol.* 20:583-591.
7. Friedman, M., P. R. Henika, and R. E. Mandrell. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J. Food Prot.* 65:1545-1560.
8. Guo, X., J. Chen, R. E Brackett, and L. R. Beuchat. 2002. Survival of *Salmonella* on tomatoes stored at high relative humidity, in soil, and on tomatoes in contact with soil. *J. Food Prot.* 65:274-279.

9. Han, Y., J. D. Floros, R. H. Linton, S. S. Nielsen, and E. Nelson. 2001. Response surface modeling for the inactivation of *Escherichia coli* O157:H7 on green peppers (*Capsicum annuum* L.) by chlorine dioxide gas treatments. *J. Food Prot.* 64:1128-1133.
10. Ibarra-Sanchez, L. S., S. Alvarado-Casillas, M. O. Rodriguez-Garcia, N. E. Martinez-Gonzales, and A. Castillo. 2004. Internalization of bacterial pathogens in tomatoes and their control by selected chemicals. *J. Food Prot.* 76:1353-1358.
11. Iturriaga, M. H., E. F. Escartin, L. R. Beuchat, and R. Martinez-Peniche. 2003. Effect of inoculum size, relative humidity, storage temperature, and ripening stage on the attachment of *Salmonella* Montevideo to tomatoes and tomatillos. *J. Food Prot.* 66:1756-1761.
12. Iturriaga, M. H., M. L. Tamplin, and E. F. Escatín. 2007. Colonization of tomatoes by *Salmonella* Montevideo is affected by relative humidity and storage temperature. *J. Food Prot.* 70:30-34.
13. Koutsoumanis, K., K. Lambropoulou, and G-J. E. Nychas. 1999. A predictive model for the non-thermal inactivation of *Salmonella Enteritidis* in a food model system supplemented with a natural antimicrobial. *Int. J. Food Microbiol.* 49:63-74.
14. Lang, M. M., L. J. Harris, and L. R. Beuchat. 2004. Evaluation of inoculation method and inoculum drying time for their effects on survival and efficiency of recovery of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* inoculated on the surface of tomatoes. *J. Food Prot.* 67:732-741.
15. Lin, C. M., J. J. Kim, W. X. Du, and C. I. Wei. 2000. Bactericidal activity of isothiocyanate against pathogens on fresh produce. *J. Food Prot.* 63:25-30.

16. Raybaudi-Massilia, R. M., J. Mosqueda-Melgar, and O. Martín-Belloso. 2006. Antimicrobial activity of essential oils on *Salmonella Enteritidis*, *Escherichia coli*, and *Listeria innocua* in fruit juices. *J. Food Prot.* 69:1579-1586.
17. Shi, X., A. Namvar, M. Kostrzynska, R. Hora, and K. Warriner. 2007. Persistence and growth of different *Salmonella* serovars on pre- and postharvest tomatoes. *J. Food Prot.* 70:2725-2731.
18. Skandamis, P. N., and G. E. Nychas. 2000. Development and evaluation of a model predicting the survival of *Escherichia coli* O157:H7 NCTC 12900 in homemade eggplant salad at various temperatures, pHs, and oregano essential oil concentrations. *Appl. Environ. Microbiol.* 66:1646-1653.
19. Stine, S. W., I. H. Song, C. Y. Choi, and C. P. Gerba. 2005. Effect of relative humidity on preharvest survival of bacterial and viral pathogens on the surface of cantaloupe, lettuce, and bell peppers. *J. Food Prot.* 68:1352-1358.
20. Sy, K. V., M. B. Murray, M. D. Harrison, and L. R. Beuchat. 2005. Evaluation of gaseous chlorine dioxide for killing *Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and yeasts and molds on fresh and fresh-cut produce. *J. Food Prot.* 68:1176-1187.
21. Wade, W. N., and L. R. Beuchat. 2003. Proteolytic fungi isolated from decayed and damaged tomatoes and implications associated with changes in pericarp pH favorable for survival and growth of foodborne pathogens. *J. Food Prot.* 66:911-917.
22. Wade, W. N., R. Vasdinnyi, T. Deak, and L. R. Beuchat. 2003. Proteolytic yeasts isolated from raw, ripe tomatoes and metabiotic association of *Geotrichum candidum* with *Salmonella*. *Int. J. Food Microbiol.* 86:101-11.

23. Weissinger, W. R., K. H. McWatters, and L. R. Beuchat. 2001. Evaluation of volatile chemical treatments for lethality to *Salmonella* on alfalfa seeds and sprouts. *J. Food Prot.* 64:442-450.
24. Weissinger, W. R., W. Chantarapanont, and L. R. Beuchat 2000. Survival and growth of *Salmonella* Baildon in shredded lettuce and diced tomatoes, and effectiveness of chlorinated water as a sanitizer. *Int. J. Food Microbiol.* 62:123-131.
25. Yuk, H.-G., J. A. Bartz, and K. R. Schneider. 2005. Effectiveness of individual or combined sanitizer treatments for inactivating *Salmonella* spp. on smooth surface, stem scar, and wounds of tomatoes. *J. Food Sci.* 70:M409-M414.
26. Zhuang, R. Y., and L. R. Beuchat 1995. Fate of *Salmonella* Montevideo on and in raw tomatoes as affected by temperature and treatment with chlorine. *Appl. Environ. Microbiol* 61:2127-2131.

TABLE 1.1. *Salmonella* spp. strains* used in this study

Isolate	Outbreak-associated
<i>S. Poona</i>	Cantaloupe associated outbreak
<i>S. Stanley</i> H 1256	Alfalfa sprout associated outbreak
<i>S. Baildon</i>	Tomato associated outbreak
<i>S. Typhimurium</i> DT 104	Multiple antibiotic resistant
<i>S. Montevideo</i>	Tomato associated outbreak

* Obtained from Dr. Mark Harrison at Department of Food science and Technology at the University of Georgia, Athens, GA.

TABLE 1.2. *Escherichia coli* O157:H7 strains* used in this study

Isolate	Outbreak-associated
C7927	Apple cider associated outbreak
F4556	Alfalfa sprout associated outbreak
H1730	Lettuce associated outbreak
SEA 13B88	Un-pasteurized apple juice associated outbreak

* Obtained from Dr. Larry Beuchat at Center for Food Safety at University of Georgia, Griffin, GA.

TABLE 1.3. Behavior of *Salmonella* spp. on whole tomatoes stored at 4 °C for 10 days in presence of vapor-phase antimicrobials. (Data calculated as log CFU/inoculation site $\pm$ standard deviation.)

	Day 0	Day 4	Day 7	Day 10
Control	7.09 $\pm$ 0.17	5.39 $\pm$ 0.81	4.04 $\pm$ 0.11	3.59 $\pm$ 0.57
Carvacrol (41.5 μ l/liter)	----*	< 3.0	< 2.0	< 2.0
Cinnamaldehyde (41.5 μ l/liter)	----	< 3.0	< 2.0	< 2.0
AIT (8.3 μ l/liter)	----	< 3.0	< 2.0	< 2.0

* ----; same as day 0 control

TABLE 1.4. Behavior of *E. coli* O157:H7 on whole tomatoes stored at 4 °C for 10 days in presence of vapor-phase antimicrobials. (Data calculated as log CFU/inoculation site $\pm$ standard deviation.)

	Day 0	Day 4	Day 7	Day 10
Control	7.84 $\pm$ 0.15	5.73 $\pm$ 0.1	5.24 $\pm$ 0.07	5.17 $\pm$ 0.1
Carvacrol (41.5 μ l/liter)	----*	< 3.0	< 2.0	< 2.0
Cinnamaldehyde (41.5 μ l/liter)	----	3.16	< 2.0	< 2.0
AIT (8.3 μ l/liter)	----	< 3.0	< 2.0	< 2.0

* ----; same as day 0 control

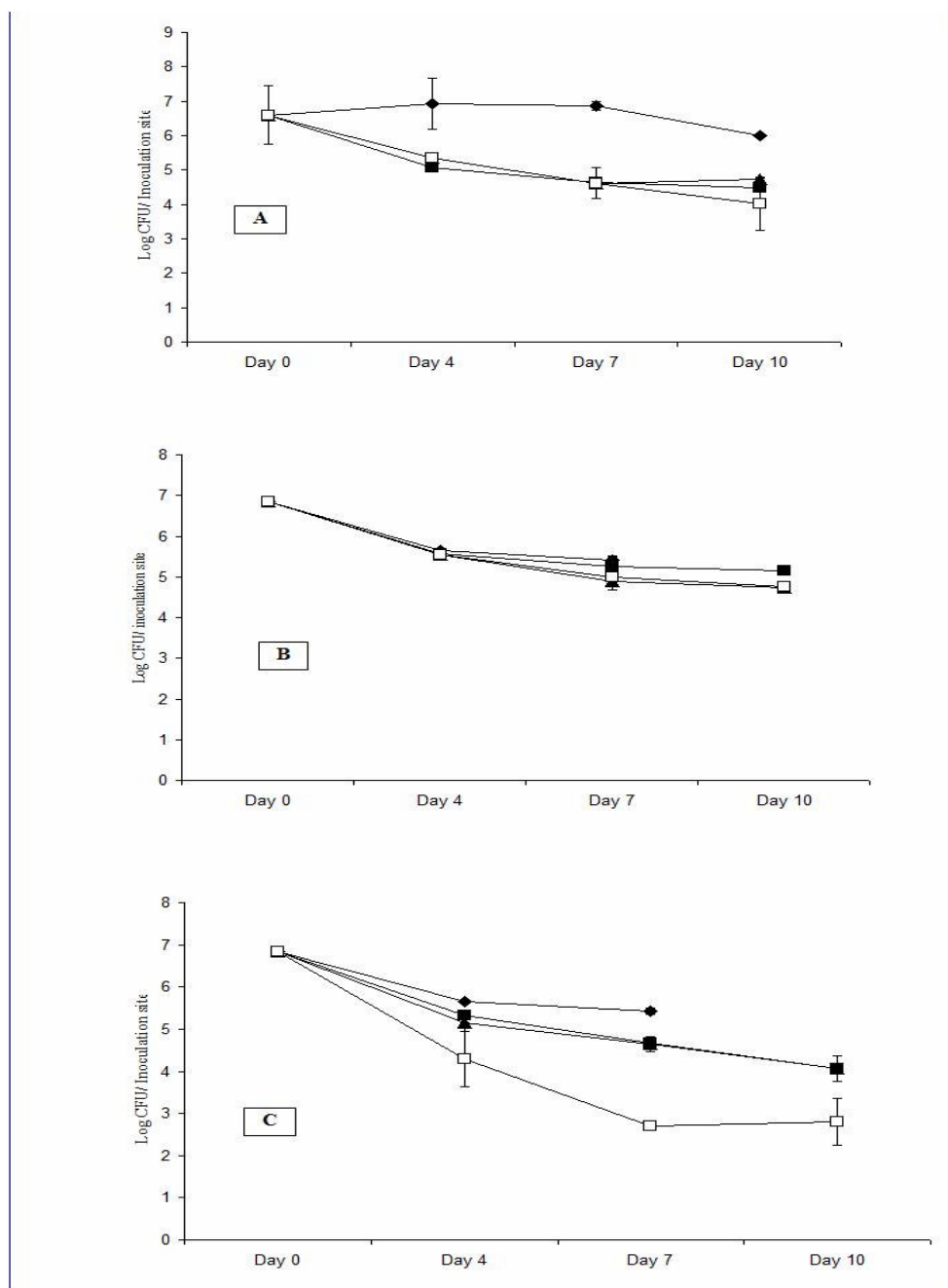


FIGURE 1.1. Behavior of *Salmonella* spp. on sliced tomatoes stored at 4 °C for 10 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (◆ control, ■ 41.5 µl/liter for A,B, 8.3 µl/liter for C, ▲ 83.3 µl/liter for A,B, 16.6 µl/liter for C, □ 125 µl/liter for A,B, 33.3 µl/liter for C). Detection limit was 100 cfu/inoculation site.

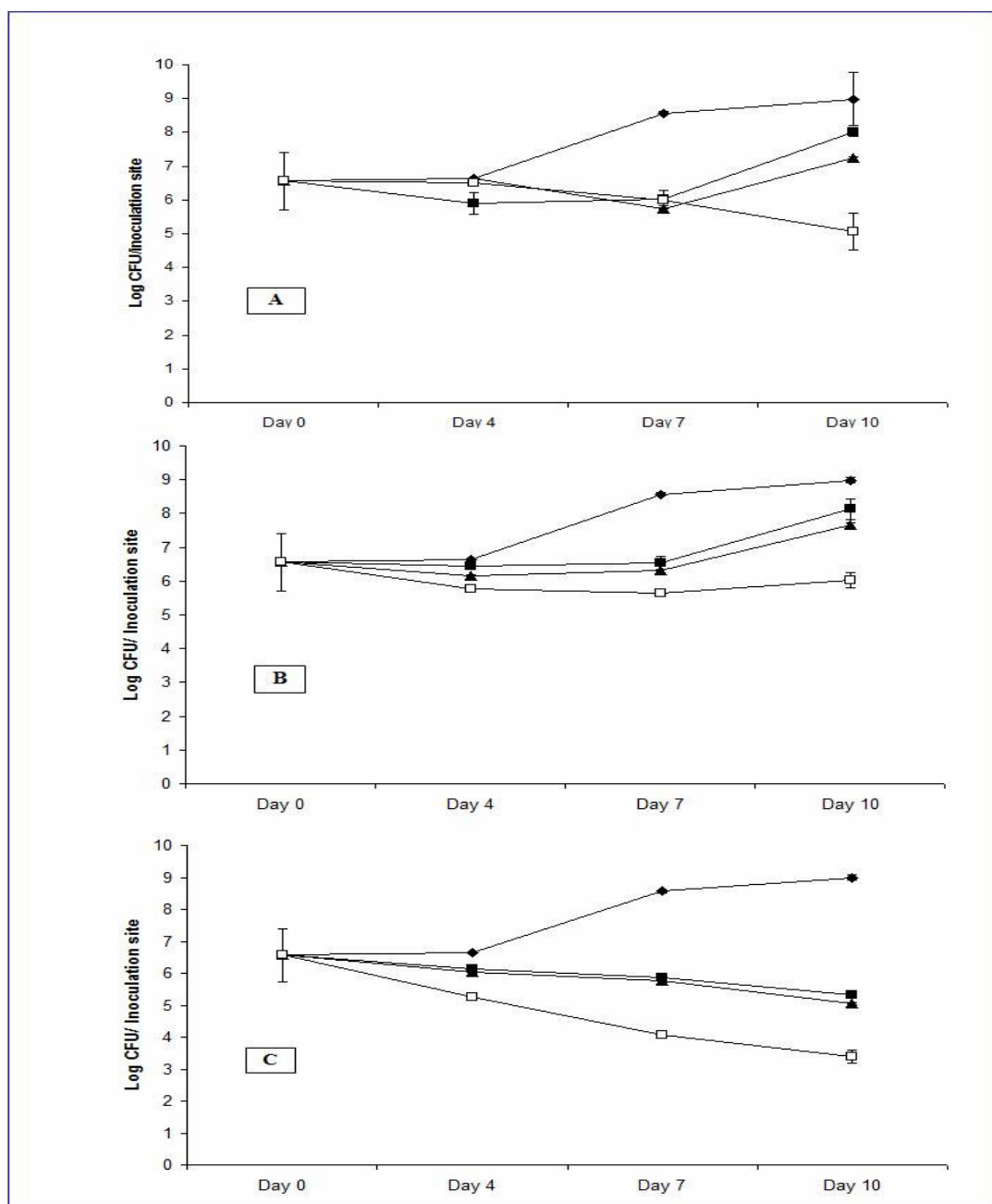


FIGURE 1.2. Behavior of *Salmonella* spp. on sliced tomatoes stored at 10 °C for 10 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (◆ control, ■ 41.5 µl/liter for A,B, 8.3 µl/liter for C, ▲ 83.3 µl/liter for A,B, 16.6 µl/liter for C, □ 125 µl/liter for A,B, 33.3 µl/liter for C). Detection limit was 100 cfu/inoculation site.

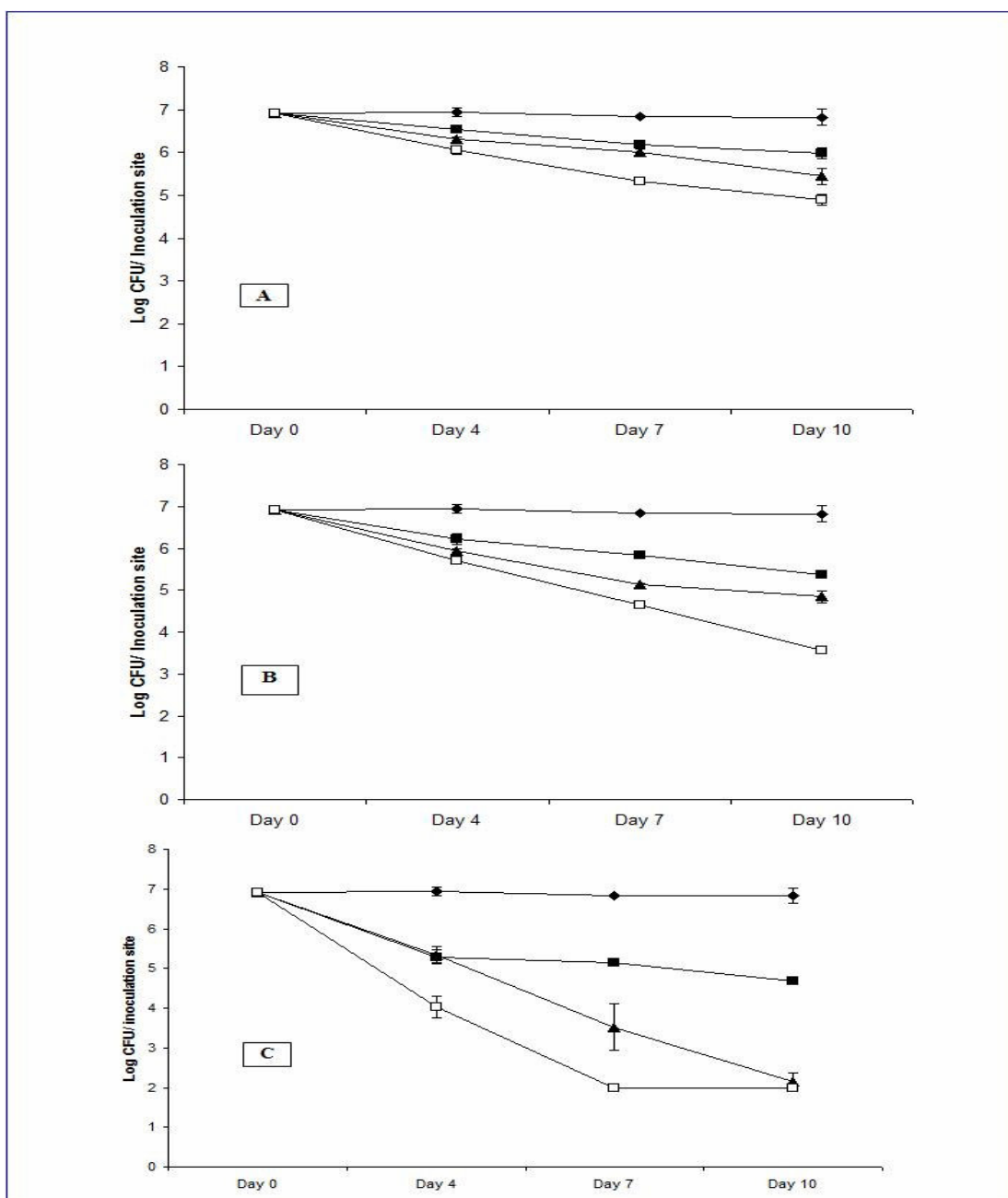


FIGURE 1.3. Behavior of *Salmonella* spp. on whole tomatoes stored at 10 °C for 10 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (◆ control, ■ 41.5 µl/liter for A,B, 8.3 µl/liter for C, ▲ 83.3 µl/liter for A,B, 16.6 µl/liter for C, □ 125 µl/liter for A,B, 33.3 µl/liter for C). Detection limit was 100 cfu/inoculation site.

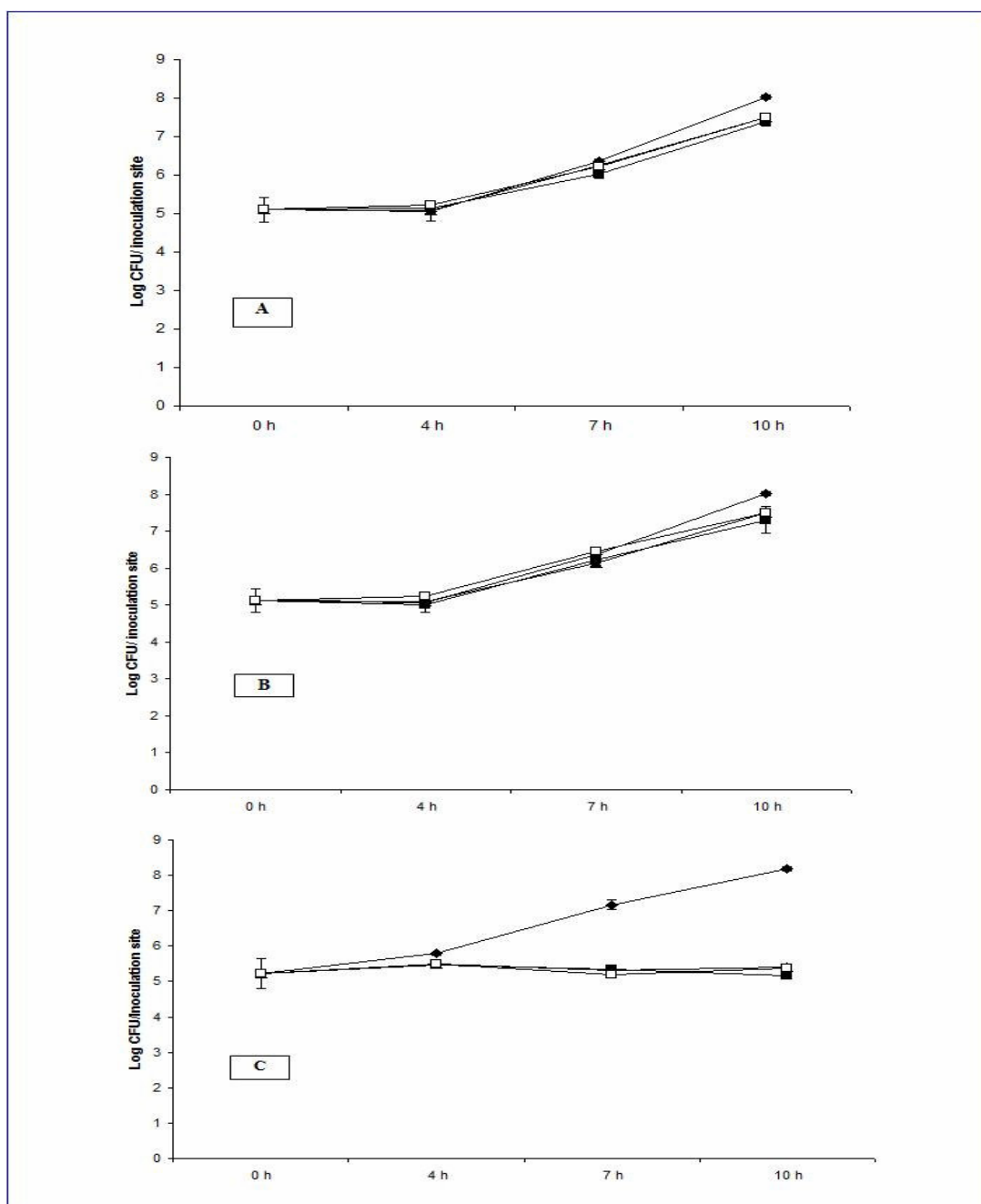


FIGURE 1.4. Behavior of *Salmonella* spp. on sliced tomatoes stored at 25 °C for 10 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (♦ control, ■ 41.5 µl/liter for A,B, 8.3 µl/liter for C, ▲ 83.3 µl/liter for A,B, 16.6 µl/liter for C, □ 125 µl/liter for A,B, 33.3 µl/liter for C). Detection limit was 100 cfu/inoculation site.

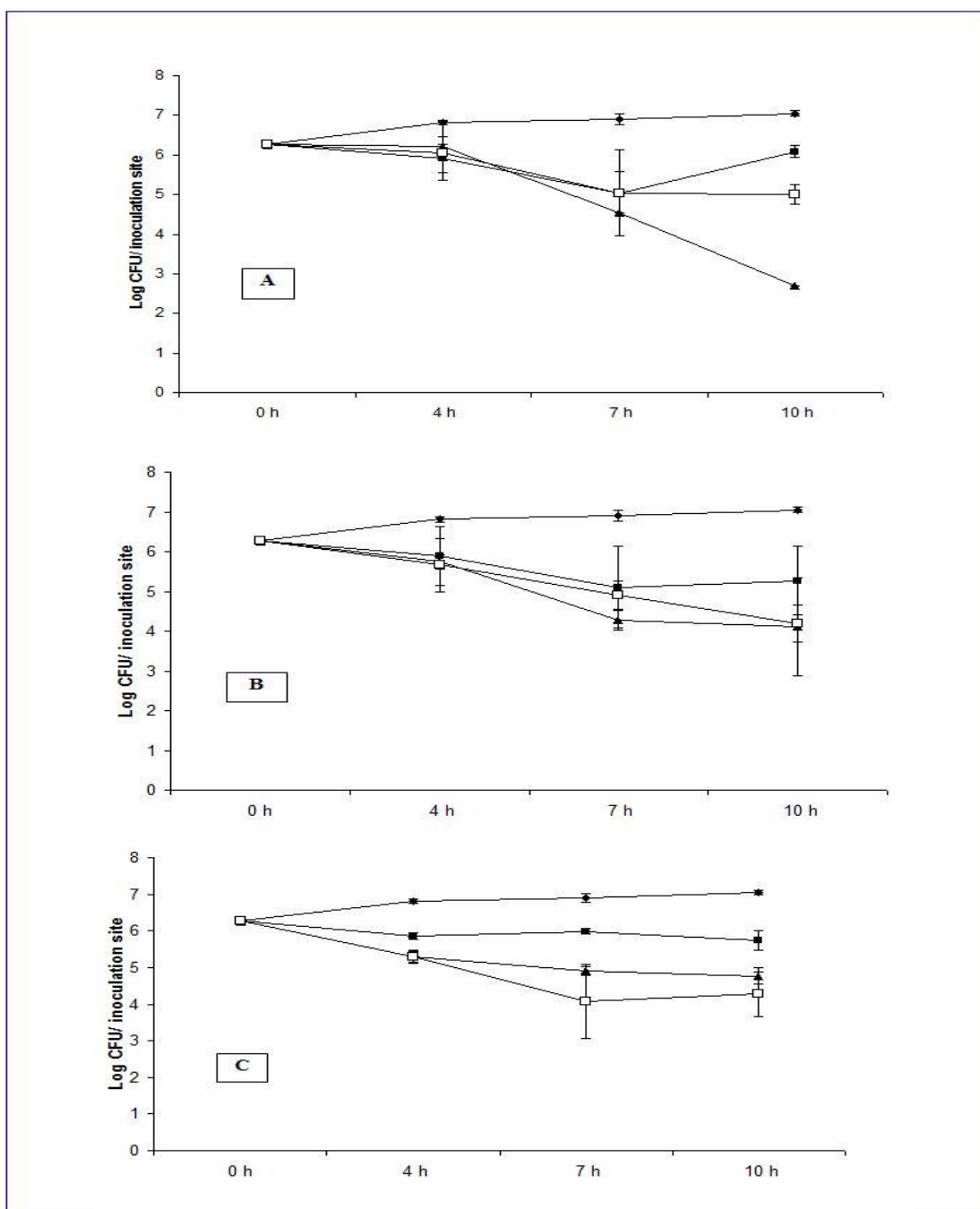


FIGURE 1.5. Behavior of *Salmonella* spp. on whole tomatoes stored at 25 °C for 10 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (◆ control, ■ 41.5 µl/liter for A,B, 8.3 µl/liter for C, ▲ 83.3 µl/liter for A,B, 16.6 µl/liter for C, □ 125 µl/liter for A,B, 33.3 µl /liter for C). Detection limit was 100 cfu/inoculation site.

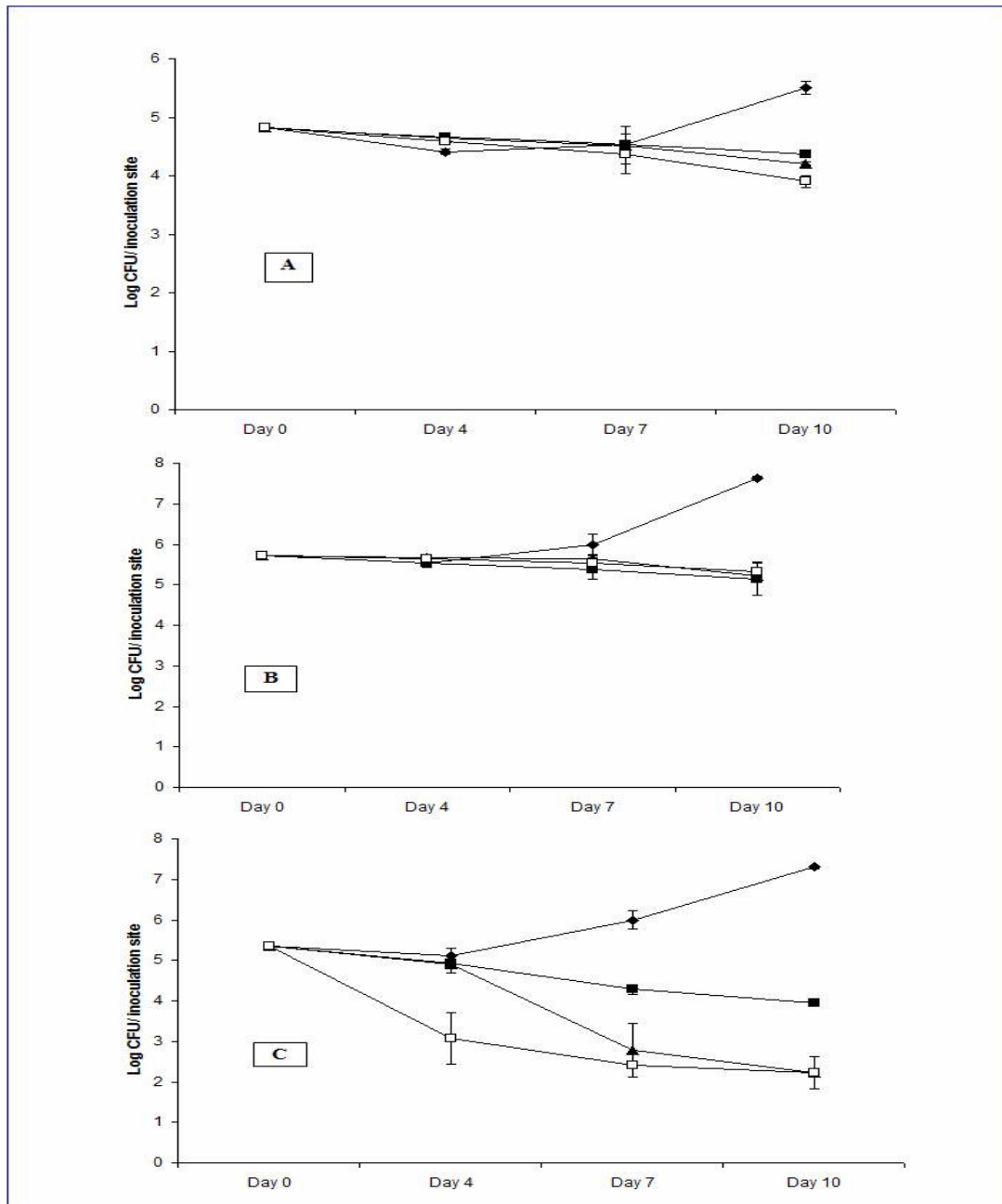


FIGURE 1.6. Behavior of *E. coli* O157:H7 on sliced tomatoes stored at 4 °C for 10 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (◆ control, ■ 41.5 μl/liter or A,B, 8.3 μl/liter for C, ▲ 83.3 μl/liter for A,B, 16.6 μl/liter or C, □ 125 μl for A,B, 33.3 μl/liter for C). Detection limit was 100 cfu/inoculation site.

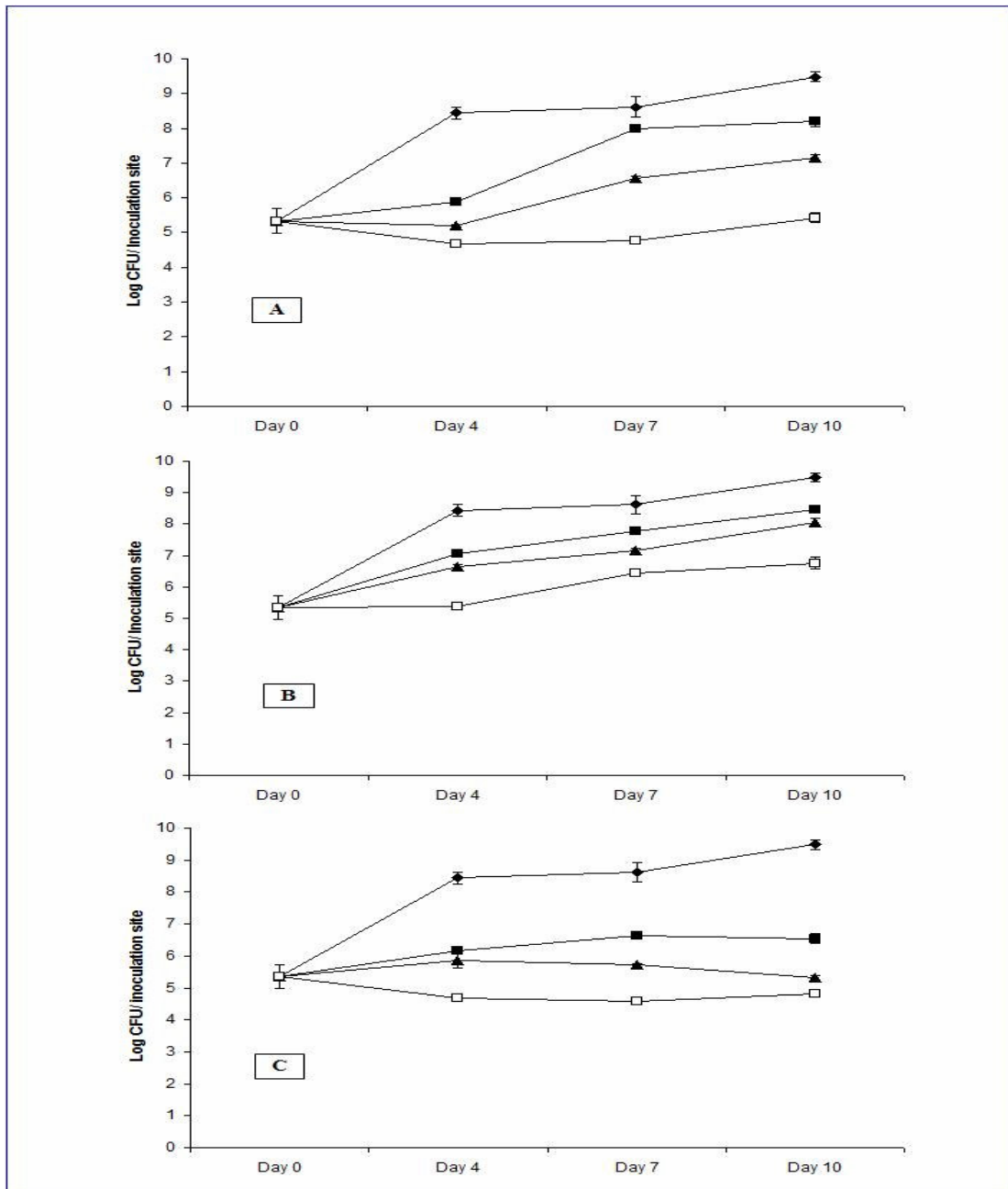


FIGURE 1.7. Behavior of *E. coli* O157:H7 on sliced tomatoes stored at 10 °C for 10 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (◆ control, ■ 41.5 µl/liter for A,B, 8.3 µl/liter for C, ▲ 83.3 µl/liter for A,B, 16.6 µl/liter for C, □ 125 µl for A,B, 33.3 µl /liter C). Detection limit was 100 cfu/inoculation site.

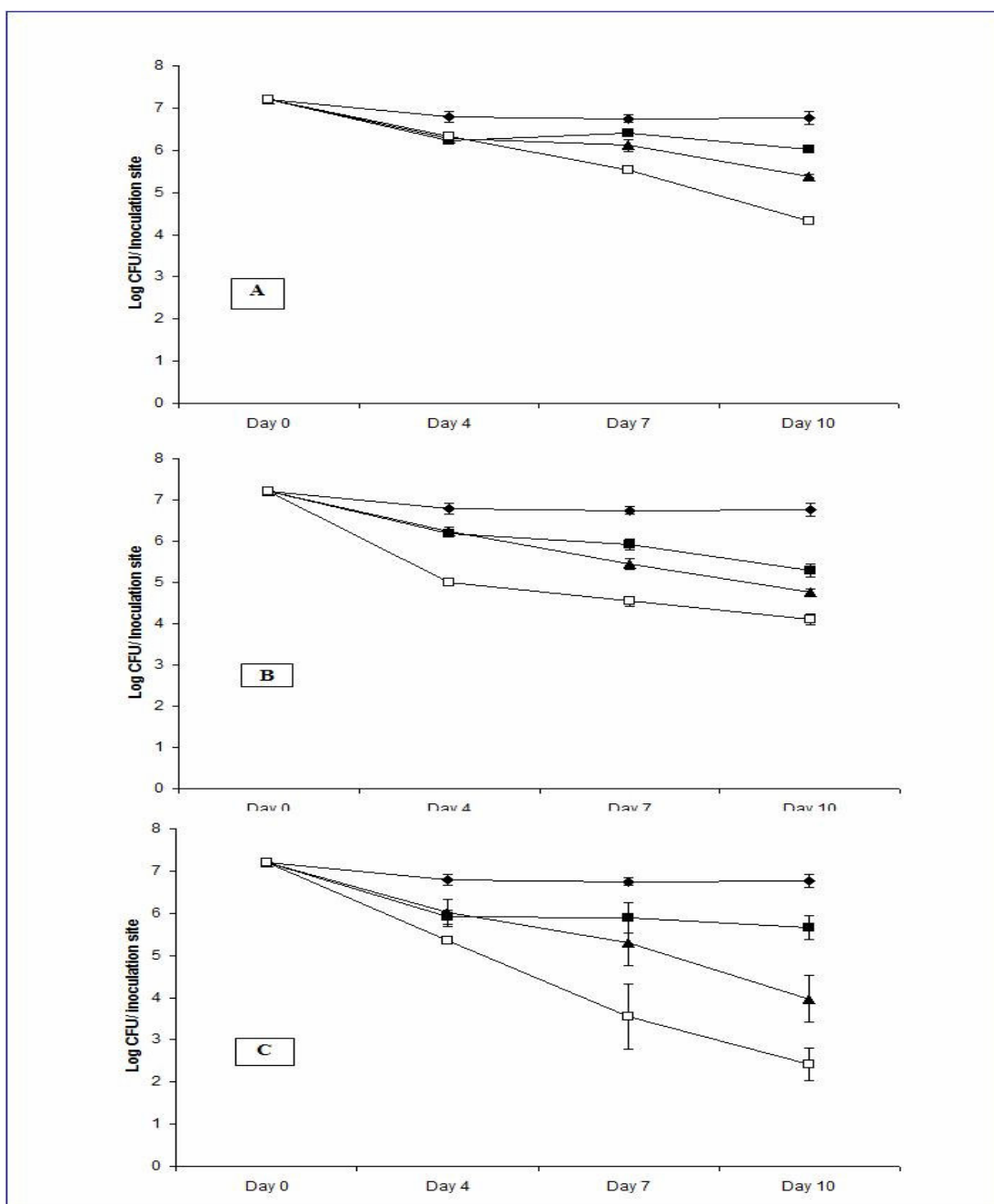


FIGURE 1.8. Behavior of *E. coli* O157:H7 on whole tomatoes stored at 10 °C for 10 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (◆ control, ■ 41.5 µl/liter for A,B, 8.3 µl/liter for C, ▲ 83.3 µl/liter for A,B, 16.6 µl/liter for C, □ 125 µl/liter for A,B, 33.3 µl/liter for C). Detection limit was 100 cfu/inoculation site.

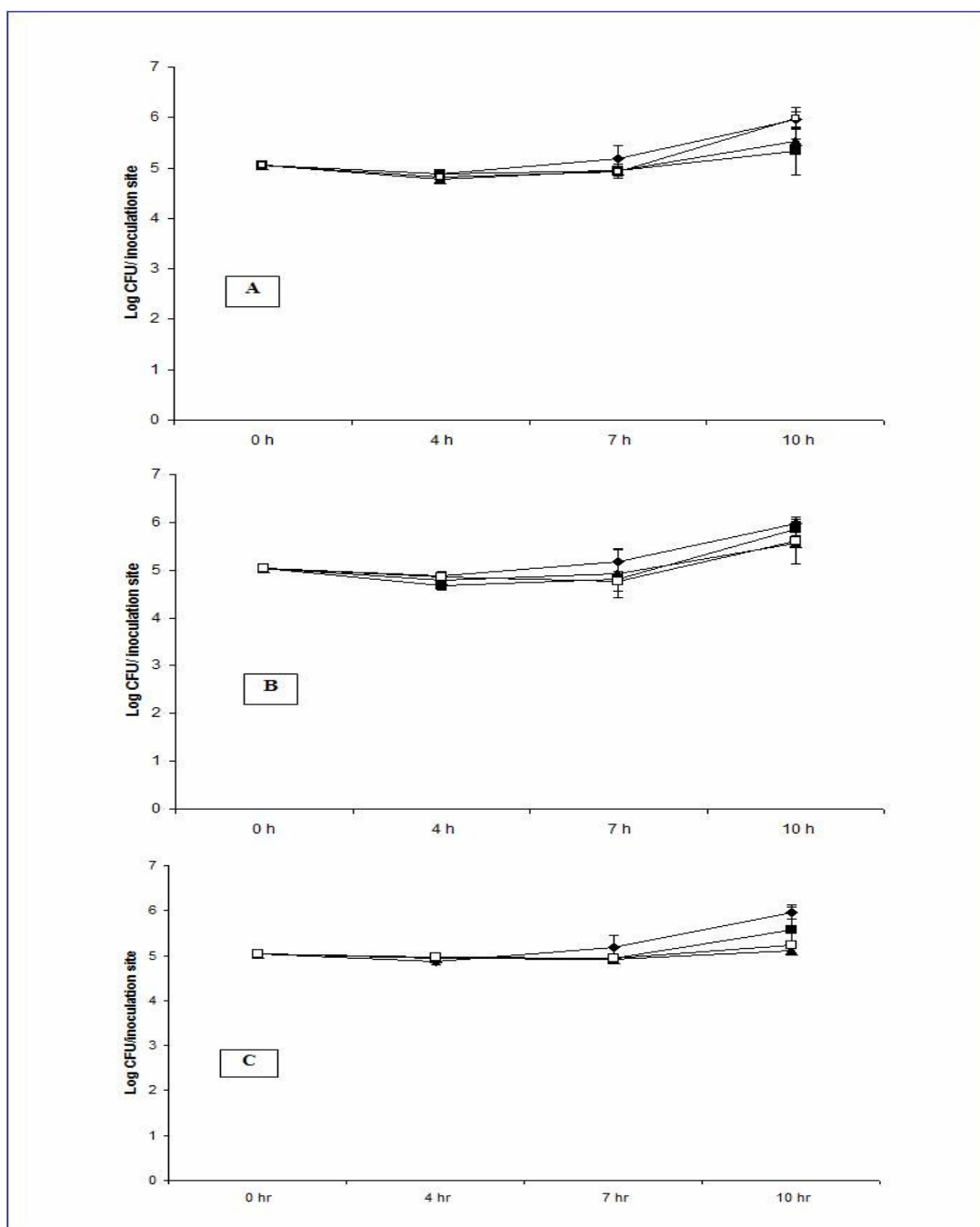


FIGURE 1.9. Behavior of *E. coli* O157:H7 on sliced tomatoes stored at 25 °C for 10 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (♦ control, ■ 41.5 µl/liter for A,B, 8.3 µl/liter for C, ▲ 83.3 µl/liter for A,B, 16.6 µl/liter for C, □ 125 µl/liter for A,B, 33.3 µl/liter for C). Detection limit was 100 cfu/inoculation site.

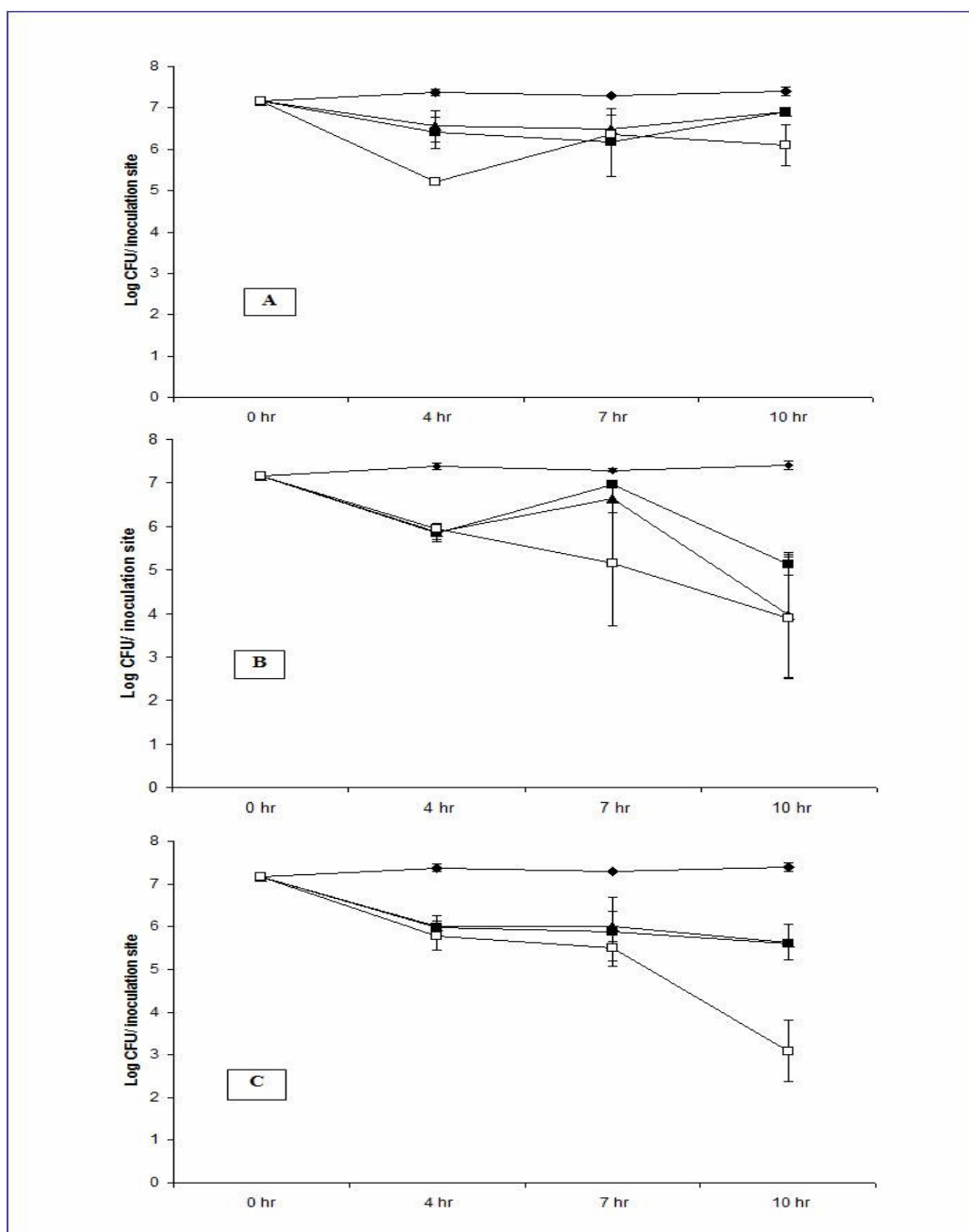


FIGURE 1.10. Behavior of *E. coli* O157:H7 on whole tomatoes stored 25 °C for 10 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (♦ control, ■ 41.5 µl/liter for A,B, 8.3 µl/liter for C, ▲ 83.3 µl/liter for A,B, 16.6 µl/liter for C, □ 125 µl/liter for A,B, 33.3 µl/liter for C). Detection limit was 100 cfu/inoculation site.

CHAPTER 4

INACTIVATION OF SURFACE-ATTACHED AND TISSUE-INFILTRATED *E. COLI* O157:H7 ON LETTUCE AND SPINACH USING ALLYL ISOTHIOCYANATE, CARVACROL AND CINNAMALDEHYDE IN VAPOR PHASE¹

¹Obaidat, M. M., and J. F. Frank. Submitted to Journal of Food Protection.

ABSTRACT

Antimicrobials in the vapor phase might be more effective in controlling *E. coli* O157:H7 attached to leafy greens surface or infiltrated within the tissue than aqueous antimicrobials. We determined the activity of allyl isothiocyanate (AIT), cinnamaldehyde and carvacrol against *E. coli* O157:H7 on lettuce and spinach surface and tissue. Samples were treated with various concentrations of antimicrobial in the vapor phase at 0, 4, and 10 °C in an enclosed container. On lettuce surface, the vapor of the lowest concentration of these antimicrobials inactivated > 4 log of *E. coli* O157:H7 at 0 and 4 °C in 4 days and at 10 °C in 2 days. However, within lettuce tissue, the highest concentration reduced the population by 4 log at 0 °C and 2 to 4 log at 4 °C in 4 days. These concentrations also reduced the population of the pathogen by 1 to 3 log at 10 °C in 2 days. The pathogen level on spinach surface was reduced 1 log less than it was on lettuce surface. However, reducing the pathogen level on spinach tissue was 2 and 3 log less than within lettuce tissue at 0 and 4 °C, respectively. Overall, greater inactivation occurred on lettuce than spinach leaves and on the vegetable surfaces than within tissue. Using antimicrobials in the vapor phase may improve the safety of refrigerated leafy greens marketed in packages containing enclosed headspace.

INTRODUCTION

Consumption of leafy greens has been associated with foodborne disease outbreaks, many of which have been caused by *E. coli* O157:H7. This pathogen contaminates leafy greens in pre-harvest and possibly post-harvest stages of the production chain. Because of its ability to survive for up to 6 months in soil and irrigation water and on growing vegetables, contaminated soil and irrigation water could be sources of contamination (15). Post-harvest contamination of leafy greens may occur as a result of exposure to contaminated water or ice (17), improper handling by workers or consumers, and contaminated slicers and shredders (11). In addition, a few contaminated leaves can cross contaminate a large mass of uncontaminated product (13, 34). *E. coli* O157:H7 may attach to the lettuce surface, and remain viable or grow depending on the storage temperature, moisture content and leaves' age (4, 5). The behavior of *E. coli* O157:H7 in leafy vegetables has been reviewed by Delaquis et al. (5).

E. coli O157:H7 may infiltrate into lettuce and spinach either pre- or post-harvest. Pre-harvest internalization may occur by cellular uptake through the root and transport through the vascular system (29). However, Hora et al. (14) and Jablasone et al. (16) found that *E. coli* O157:H7 was unable to infiltrate the tissues of mature lettuce and spinach from contaminated growth media, even when roots were damaged mechanically or biologically (by coinoculation with plant pathogens). Post-harvest internalization may occur during vacuum cooling or through the cut surface or from wounds and stomata during washing (20). Internalization and possible pathogen growth can also be enhanced by the presence of phytopathogens due to cuticle destruction and release of nutrients (13), and is influenced by the storage temperature and the atmosphere of the package (31).

Sodium hypochlorite is widely used to disinfect water used in processing leafy greens; however, chlorine is not effective in disinfecting the leaves because of its limited ability to penetrate tissue crevices and its rapid inactivation when in contact with organic matter. Previous research indicates that chlorine is the same or marginally more effective than deionized water in removing *E. coli* O157:H7 from the lettuce leaf, and does not kill infiltrated pathogens (2, 3, 20, 32, 33). Evidence indicates that the surface structure of lettuce protects *E. coli* O157:H7 from inactivation by chlorine (32). Consequently, various alternatives to chlorine have been tested including acidified sodium chlorite (30), fumaric acid (18), ozone, chlorine dioxide (28), electrolyzed water, and UV radiation (5, 35). These studies indicate that the tested chemical disinfectants added to wash or cooling water cannot be relied upon to eliminate *E. coli* O157:H7 from leafy vegetables when used at levels that do not cause adverse effects on produce quality (5). Even a combination of hypochlorite and UV radiation was not effective in killing internalized *E. coli* O157:H7 (20).

Since vapor-phase volatile antimicrobials may contact bacteria at locations on the leaf not reached by chemicals in aqueous solution, their use in packaged leafy greens could provide an added pathogen control benefit. Of 96 tested essential oils; the three most effective against *E. coli* O157:H7 were oregano, thyme and cinnamon; the three most effective essential oil components, among 16 tested against *E. coli* O157:H7 were carvacrol, cinnamaldehyde, and thymol (10). However, this information was obtained on liquid-phase compounds. Only limited information is available on their efficacy in vapor form. Furthermore, allyl isothiocyanate (AIT) has also been suggested as a promising antimicrobial for *E. coli* O157:H7 as an alternative to chlorine (27). AIT in vapor phase inactivated up to 8 log CFU of *E. coli* O157:H7 on lettuce in 2 days at 4 °C (22).

Since the efficacy of volatile antimicrobials is expected to depend on the storage temperature, time of the exposure and antimicrobial concentration, this research aimed to elucidate the effect of these parameters on the ability of carvacrol, cinnamaldehyde and AIT in vapor phase to inactivate attached and infiltrated *E. coli* O157:H7 on lettuce leaves. Based on these results, selected antimicrobial treatments were applied to inoculated spinach leaves to provide a comparison with lettuce.

MATERIALS AND METHODS

Inoculum preparation. *E. coli* O157:H7 strains isolated from produce-associated outbreaks were used to inoculate lettuce. Three GFP_{uv} transformed *E. coli* O157:H7 (K3995; a clinical isolate from 2006 spinach outbreak, K4992; a clinical isolate from a 2006 lettuce outbreak, and F4546; an alfalfa sprout outbreak) were obtained from the Center for Food Safety, University of Georgia, Griffin. The GFP plasmid encoded ampicillin resistance gene which allowed confirmation of isolate identity. The strains were stored at -80 °C in Microbank® beads (Pro-Lab Diagnostics, Richmond Hill, ON, Canada). When needed, one bead of each strain was cultured in TSB-medium supplemented with ampicillin (100 µg/ml) at 37 °C. Then, the strains were grown to stationary-phase by transferring each twice in tryptic soy broth (TSB + ampicillin) at 24 h intervals with incubation at 37 °C. The cocktail was prepared by mixing equal volumes of each strain. Then, the cocktail was centrifuged at 4,500 rpm for 25 min at 4° C, the supernatant fluid discarded, the pellet washed with 0.1% peptone water, and re-suspended in 10 ml of peptone water. This suspension was diluted in 0.1 % peptone to achieve *ca.* 7.0 log CFU/ml cell

suspension for surface inoculation and *ca.* 8.0 log CFU/ ml cell suspension for infiltration inoculation.

Inoculation of lettuce surface. Pre-packaged leafy green lettuce was purchased at retail in Athens, GA on the day of the experiment. Undamaged leaves were selected, rinsed under running tap water, and dried by spinning in salad spinner. The surface of the rinsed leaves was inoculated by placing 50 µl of the cell suspension on each leaf. These inoculated leaves were placed in the biosafety cabinet for 2 h, followed by incubation at 4 °C for 22 h to allow bacterial attachment to the leaf surfaces as recommended by Lang et al. (19).

Vacuum infiltration within lettuce. *E. coli* O157:H7 infiltration within lettuce leaves was accomplished by application of vacuum as developed by Folsom (8). A cut of 2.54 cm was made on the stem end of each leaf to provide a uniform fresh cut that would facilitate bacterial infiltration. Ten leaves were placed with the cut edge submerged in 10 ml of the inoculum cocktail in a 100 ml beaker. The beakers were placed in a vacuum chamber which was evacuated to achieve an absolute pressure of 50 kPa for 20 min (approximately 50 kPa below atmospheric pressure). The vacuum was then released gradually over a 5 min period. The leaves were taken from the beaker, placed in Petri dishes and then rinsed three times with sterile deionized water. The rinsed leaves were placed on stainless steel tray and dried by pressing them gently between KimWipes™ (Kimberly-Clark Corporation, Irving, TX). Uninoculated and inoculated controls were prepared for both surface and infiltration-inoculated samples.

Antimicrobial application and sample storage. Antimicrobials in vapor phase were applied to surface inoculated and pathogen infiltrated samples in similar manner. A Petri dish was filled

with sterile deionized water, in which autoclave-sterilized Whatman® filter paper pieces (previously cut 2×2 cm pieces) (Whatman Inc., Clifton, NJ) were immersed. One wet filter paper was placed on the inner upper side of each septa jar (with caution taken not to touch the sample) to create high relative humidity inside the jars. The antimicrobial compounds were allowed to equilibrate to room temperature. Then 5, 7.5 and 10 µl (equivalent to 40, 60, and 80 µl antimicrobial / liter of air) of carvacrol ($\geq 97\%$ purity) or cinnamaldehyde ($\geq 98\%$ purity) or 0.5, 1, and 2 µl (equivalent to 4, 8 and 16 µl of antimicrobial/ liter of air) of AIT ($\geq 98\%$ purity) (Sigma-Aldrich, St. Louis, MO) were deposited on a second filter paper previously attached on the inner upper side of the septa jar by double-stick tape (Henkel Consumer Adhesives, Inc, Avon, OH) to avoid volatile direct contact with the samples. Samples in the sealed jars were stored at 0, 4, and 10 °C.

Microbiological analysis. The treated samples and controls were analyzed after 0.5, 1, 2, and 4 days of holding at 0, 4 °C and after 0.5, 1, and 2 days of holding at 10 °C. The samples were prepared for enumeration by blending (Osterizer® classic blender and Oster® ice crushing blade # 4961, Fisher Scientific, Pittsburgh, PA) the entire leaf for 30 s in 50 ml of 0.1% peptone using 8 oz (237 g) blender jars (Ball quilted crystal jelly jars, Jarden Corp, Muncie Ind.). Appropriate dilutions were plated on Sorbitol MacConkey agar (SMAC)) (Sigma- Aldrich, St. Louis, MO) supplemented with 100 µg/ml ampicillin and 0.1% sodium pyruvate (Sigma- Aldrich, St. Louis, MO). The SMAC plates were incubated at 35 °C for 24 h and then the colonies on the plates were counted.

Statistical analysis. The study used a random factorial block design. The experimental design consisted of three antimicrobials (AIT, carvacrol, and cinnamaldehyde), four concentrations (0,

4, 8, and 16 $\mu\text{l/liter}$ for first antimicrobial and 0, 40, 60, and 80 $\mu\text{l/liter}$ for latter two antimicrobials), three temperatures (0, 4, and 10 $^{\circ}\text{C}$), five sampling times (0, 0.5, 1, 2 ,and 4 days), two inoculation methods (surface inoculation and vacuum infiltration). Data was analyzed using SAS 9.1.3 (SAS Institute, Inc., Cary, NC) by calculating the analysis of variance (ANOVA) using general linear model. Multiple comparisons between the means were calculated using Duncan's test with a 5% significance level. Each experiment was replicated four times.

Inactivation of *E. coli* O157:H7 on spinach. Selected vapor phase antimicrobial treatments were evaluated for inactivation of *E. coli* O157:H7 on spinach to provide a comparison to results obtained on lettuce. Baby spinach was purchased from retail at local grocery store in Athens, GA on the day of the experiment. Inoculum preparation, spinach inoculation (surface inoculation and vacuum infiltration), antimicrobial application, samples storage and analysis were done as previously described for lettuce experiments. The study used a random factorial block design. The experimental design consisted of three antimicrobials (AIT, carvacrol, and cinnamaldehyde), three concentrations (0, 4, and 16 $\mu\text{l/liter}$ for first antimicrobial and 0, 40, and 80 $\mu\text{l/liter}$ for latter two antimicrobials), two temperatures (0 and 4 $^{\circ}\text{C}$), three sampling times (0, 2 ,and 4 days), two inoculation methods (surface inoculation and infiltrated pathogens). To compare inactivating the pathogen on spinach to inactivating it on lettuce, the data were converted to log reduction for each treatment. Transformed data were analyzed using SAS 9.1.3 (SAS Institute, Inc., Cary, NC) by calculating the analysis of variance (ANOVA) using the general linear model. Multiple comparisons between the means were accomplished by using Duncan's test with a 5% significance level. Each experiment was replicated four times

RESULTS

Preliminary work indicated that carvacrol and cinnamaldehyde at a concentration of 125 $\mu\text{l/liter}$ of air and AIT at a concentration of 33.3 $\mu\text{l/liter}$ of air inactivated *E. coli* O157:H7 on lettuce and spinach but imparted browning discoloration of the lettuce leaves as determined by four to five untrained evaluators. Therefore, lower concentrations with no detectable aroma or browning effect (20, 40, and 80 $\mu\text{l/liter}$) of carvacrol and cinnamaldehyde and lower concentrations of AIT (4, 8, and 16 $\mu\text{l/liter}$) were used in this study.

Inactivation of *E. coli* O157:H7 on and in lettuce in the presence of vapor-phase antimicrobials at 0 °C. In the absence of the antimicrobials, the population of *E. coli* O157:H7 on lettuce surface decreased gradually by about 1.0 log over 4 days at 0 °C (Fig 2.1A thru 2.1C). The presence of volatile antimicrobial caused significant inactivation during the 4 days treatment period, where the inactivation amount depended on treatment time. A 1 log reduction of *E. coli* O157:H7 was noticed within the first 12 h of treatment by all antimicrobials at their lowest concentration. The lowest concentration of cinnamaldehyde, carvacrol and AIT reduced the population by 4.81, 4.67 and 3.66 log, respectively by the fourth day of treatment.

In the absence of the antimicrobials, the population of lettuce-infiltrated *E. coli* O157:H7 gradually decreased by 1.2 log over 4 days of incubation at 0 °C (Fig 2.2A thru 2.2C). The presence of any of the antimicrobial at any concentration reduced the population significantly over 4 days. A significant inactivation started after 1 day of treatment; where carvacrol caused the greatest reduction in the population. As the treatment period progressed, cinnamaldehyde exhibited increasing inactivation making it the most effective antimicrobial by the fourth day of treatment followed by carvacrol and then AIT. All concentrations of cinnamaldehyde inactivated

the pathogen to the detection limit (3 log less than the control) by the fourth day of treatment. Only the highest concentration of carvacrol and AIT were able to reduce the pathogen to the detection limit. The inactivation effect of all antimicrobials increased with time and concentration except for the a lack of concentration effect of cinnamaldehyde at the fourth day, where all concentrations studied provided inactivation close to the detection limit.

Inactivation of *E. coli* O157:H7 on and in lettuce in the presence of vapor-phase

antimicrobials at 4 °C. In the absence of the antimicrobials, the population of *E. coli* O157:H7 on lettuce surface decreased gradually by about 1.2 log over 4 days at 4 °C (Fig 2.3A thru 2.3C). The antimicrobials significantly reduced *E. coli* O157:H7 on the lettuce surface; as a significant reduction was observed at the first 12 h of treatment and increased with time. At that point, carvacrol caused the most reduction in the population (1.5 log reduction), while AIT and cinnamaldehyde produced *ca.* 1 log reduction. However, the lowest concentration of all tested antimicrobials reduced *E. coli* O157:H7 by > 4 log within 4 days. Meanwhile, the middle concentration caused similar reduction within 2 days.

Lettuce-infiltrated *E. coli* O157:H7 decreased gradually by 1.0 log in the absence of antimicrobials after 4 days at 4 °C (Fig 2.4A thru 2.4C). Meanwhile, the presence of any of the antimicrobials caused significantly greater reduction during the 4 days treatment period. Generally, cinnamaldehyde caused the greatest inactivation, followed by AIT and carvacrol. The highest concentration of cinnamaldehyde reduced the population to the detection limit (3 log units less than the control). The inactivation effect of all antimicrobials increased with time.

Inactivation of *E. coli* O157:H7 in and on lettuce in the presence of vapor-phase

antimicrobials at 10 °C. In the absence of the antimicrobials, the population of *E. coli* O157:H7 on lettuce surface did not change after 2 days at 10 °C (Fig 2.5A thru 2.5C). However, the presence of the antimicrobials significantly reduced the pathogen population at any concentration applied. Inactivation of the pathogen increased with concentration and time and the antimicrobials differed in their bactericidal activity. A significant inactivation was caused by carvacrol and cinnamaldehyde within the first 12 h of treatment. Furthermore, the lowest concentration of these two antimicrobials inactivated the pathogen to the detection limit (4.5 log less than the control) within 2 days. However, only the highest concentration of AIT could cause the same effect.

The population of lettuce-infiltrated *E. coli* O157:H7 remained stable for 2 days (the storage period) at 10 °C (Fig 2.6A thru 2.6C). The presence of any of the antimicrobials reduced the population significantly which was noticed after 12 h of treatment. In general, AIT and carvacrol were the most effective followed by cinnamaldehyde. The concentration and treatment period influenced the inactivation effect of carvacrol and AIT. Carvacrol reduced the population by 1.0, 1.7, and 3.2 log at 20, 40, and 80 µl/liter of air, respectively, after 2 days of treatment. However, AIT reduced the population by 0.8, 1.7, and 2.7 log units at 4, 8, and 16 µl/liter of air, respectively, after 2 days of treatment. Cinnamaldehyde reduced the population by *ca.* 1.0 log at all concentrations used after 2 days of treatment.

Comparison of vapor-phase activity of carvacrol, cinnamaldehyde and AIT on *E. coli*

O157:H7 on lettuce and spinach. This experiment compared inactivation of *E. coli* O157:H7 on lettuce to inactivation on spinach using vapor phase antimicrobials. Statistical analysis

indicated that there was no significant difference in the activity of the antimicrobials against lettuce surface-attached and tissue-infiltrated *E. coli* O157: H7 in this study compared to the previous study.

Comparison of the efficacy of vapor phase antimicrobial treatments against *E. coli*

O157:H7 on the surfaces of lettuce and spinach stored at 0 °C for 4 days. There was no significant difference in the survival of *E. coli* O157:H7 on the surfaces of lettuce and spinach at 0 °C in 4 days in the absence of antimicrobials (Table 2.1). There was also no significant difference in the efficacy of the antimicrobial treatments applied to surface inoculated lettuce and spinach after 2 and 4 days of treatment, with the exception of the highest concentration of the antimicrobials. The highest concentrations of the antimicrobials reduced the pathogen population to a greater extent on lettuce surface after 2 days of treatment. In addition, more reduction of the pathogen on lettuce and spinach surface was observed after 4 days than after 2 days of treatment by lowest and highest used concentrations.

Comparison of the efficacy of vapor phase antimicrobial treatments against *E. coli*

O157:H7 on the surfaces of lettuce and spinach stored at 4 °C for 4 days. There was no significant difference in the survival of *E. coli* O157:H7 on the surfaces of lettuce and spinach at 4 °C in 4 days in the absence of antimicrobial (Table 2.2). The reduction in pathogen population after the antimicrobials treatment was greater on lettuce than on spinach for both antimicrobial concentrations tested after both after 2 and 4 days of treatment. A similar result was obtained when treating with cinnamaldehyde after 2 days of treatment, but not after 4 days. However, AIT exhibited similar reducing effect of the pathogen at its lowest and highest concentration after 2 and 4 days of treatment.

In general, more reduction of the pathogen was exhibited by the antimicrobials on lettuce and spinach surface after 4 days compared to 2 days of treatment, except for cinnamaldehyde effect against the pathogen on lettuce surface. These findings demonstrate that the inactivation of the pathogen on spinach by vapor phase antimicrobials requires longer exposure time to achieve a reduction similar to that when the pathogen is on lettuce.

Comparison of the efficacy of vapor phase antimicrobial treatments on lettuce and spinach with infiltrated *E. coli* O157:H7 stored at 0 °C for 4 days. *E. coli* O157:H7 survived better when infiltrated in spinach leaves than in lettuce ones at 0 °C after 4 days of incubation when no antimicrobial was applied (Table 2.3). Carvacrol, cinnamaldehyde and AIT produced greater reduction of the *E. coli* O157:H7 population infiltrated into lettuce leaves compared to spinach leaves at the lowest and highest concentrations used after 4 days of treatment. However, only the highest concentration of the antimicrobials produced this reduction after 2 days of treatment.

Generally, more reduction was exhibited after 4 days than after 2 days of the pathogen infiltrated into lettuce by all antimicrobials. However, of the tested antimicrobials, only AIT produced a similar result for spinach.

Comparison of efficacy of vapor phase antimicrobial treatments on lettuce and spinach with infiltrated *E. coli* O157:H7 stored at 4 °C for 4 days. There was no significant difference in the survival of *E. coli* O157:H7 infiltrated into lettuce and spinach leaves stored at 4 °C in 4 days, although a significant increase in the population was observed after 2 days of the pathogen in spinach leaves but not lettuce leaves. In general, the lowest concentration of carvacrol, cinnamaldehyde, and AIT exhibited a similar inactivation effect on the pathogen infiltrated into

lettuce and spinach leaves. However, the highest concentration of all these antimicrobials exhibited greater inactivation against *E. coli* O157:H7 in lettuce leaves compared to the pathogen within spinach leaves after 2 and 4 days of treatment. The inactivation effect was significantly higher after 4 days compared to 2 days at the highest antimicrobial concentration compared to the lowest concentration against the pathogen on both lettuce and spinach tissues.

DISCUSSION

In the absence of antimicrobials, the population of surface-attached and tissue infiltrated *E. coli* O157:H7 decreased by 1 log on lettuce stored at 0 and 4 °C for 4 days, but the population did not change when lettuce was held at 10 °C for 2 days. Furthermore, the behavior of the pathogen on spinach surface and tissue was similar to that on lettuce surface and tissue at 0 and 4 °C. Previously, several studies examined the behavior of *E. coli* O157:H7 on packaged lettuce but these studies differ in the growth conditions, packaging conditions, and pathogen strains. Our findings are similar to those of Abdul-Raouf et al. (1) and Li et al. (21) who found that *E. coli* O157:H7 on lettuce decreased by 1 log at 5 °C in 14 and 18 days, respectively. Lin et al. (22) also found *E. coli* O157:H7 inoculated onto shredded lettuce decreased by 0.5 log at 4 °C in 4 days. Our findings are also similar to those of Delaquis et al. (6) who found that the population of *E. coli* O157:H7 on iceberg lettuce slightly decreased at 1 °C and remained stable at 10 °C in 14 days. However, Francis and O'Beirne (9) concluded that the population of *E. coli* O157:H7 on shredded lettuce did not change significantly at 4 °C in 12 days, although, their data indicate approximately 0.5 log increase in the population after 4 days.

AIT, carvacrol and cinnamaldehyde in vapor phase were able to inactivate *E. coli* O157:H7 infiltrated into lettuce tissue as well as on lettuce surface. The lowest concentration of all antimicrobials reduced the pathogen population on lettuce surface by > 4 log at 0, 4 and 10 °C except for AIT at 10 °C, where the highest concentration was able to do that effect. However, the highest concentration of the antimicrobials reduced the pathogen population within lettuce tissue by 1 to 2 log and ca. 4 log at 0 °C after 2 and 4 days, respectively. These antimicrobials reduced the pathogen within lettuce tissue by 1 to 2 and 2 to 4 logs at 4 °C in 2 and 4 days, respectively. However at 10 °C, they reduced the pathogen population by 1 to 3 logs in 2 days. This indicates the ability of antimicrobials to inactivate greater number of a pathogen on the lettuce surface than within the tissues. These findings are consistent with those of Obaidat and Frank (26) who found volatile antimicrobials to be more effective on inactivating *E. coli* O157:H7 and *Salmonella* spp. on tomato surface than between tomato slices. Previous research showed that chlorine dioxide, another gaseous antimicrobial, exhibits greater activity at a blueberry surface as compared to the tissue. Chlorine dioxide also produced greater inactivation of *Salmonella* on apple skin than on calyx and stem cavities (7). Moreover, Niemira (25) showed that irradiation D₁₀ values for killing lettuce-infiltrated *E. coli* O157:H7 were higher than those to kill the pathogen on lettuce surface.

Generally extrinsic factors, such as antimicrobial concentration, time of exposure and storage temperature, affect the efficacy of an antimicrobial treatment. Of these factors, exposure time was the most important in this study. Perhaps this is due to the narrow range of the antimicrobial concentrations and storage temperatures that were used. Storage temperature had a significant effect in reducing the pathogen on lettuce surface but not within lettuce tissue. A significant difference in inactivation was observed between 0 °C and 4 and 0 and 10 °C, but no

difference between 4 and 10 °C. Other researchers reported that the ability of chlorine dioxide gas to inactivate *E. coli* O157:H7 on apple surface increased with gas concentration and exposure time (7). Chlorine dioxide gas was also used to inactivate *E. coli* O157:H7 on green peppers; where the gas concentration, treatment time, relative humidity (RH), and temperature significantly increased the inactivation ability. The gas concentration was the most important factor and temperature was the least. Furthermore, gas concentration and RH had an interactive effect on inactivation (12). The ability of the antimicrobials in vapor phase to reduce the pathogen population in our study indicates that the antimicrobials were also effective at temperatures where *E. coli* O157:H7 was declining (0 and 4 °C) and stable (10 °C). This is consistent with Lin et al. (23) observation who observed that AIT vapor was effective against *E. coli* O157:H7 on shredded lettuce at both exponential and stationary growth stages (23).

Inactivation of *E. coli* O157:H7 on lettuce surface was approximately 1 log greater than that on spinach surface at 0 and 4 °C. Meanwhile, inactivating the pathogen within lettuce tissue was about 3 log and 2 log greater than that within spinach tissue at 0 and 4 °C, respectively. Spinach leaves are thicker than lettuce leaves and therefore may offer greater protection to internalized cells during treatment with antimicrobial vapor. There could also be other physical or biochemical differences between spinach and lettuce leaves that might influence the inactivation of pathogens by these treatments. Niemira (24) observed that the response of *E. coli* O157:H7 to irradiation was more complex in spinach leaves than in romaine lettuce leaves.

Overall, the bactericidal and bacteriostatic effect of AIT, carvacrol, and cinnamaldehyde against surface-attached and tissue- infiltrate *E. coli* O157:H7 indicates a potential application for improving the safety of refrigerated packaged leafy greens, if they could be incorporated into a packaging system that would provide exposure of the leaves to the antimicrobial vapor.

Additional research on the influence of these treatments on sensory quality will need to be completed before practical application. Moreover, data validating the effectiveness of these treatments in industrial systems is required.

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REFERENCES

1. Abdul-Raouf, U. M., L. R. Beuchat, and M. S. Ammar. 1993. Survival and growth of *Escherichia coli* O157:H7 on salad vegetables. *Appl. Environ. Microbiol.* 59:1999-2006.
2. Beuchat, L. R. 1998. Surface decontamination of fruits and vegetables eaten raw: a review. *Food Safety Issues. Food Safety Unit, World Health Organization.* WHO/FSF/FOS 98.2. 42 pp.
3. Beuchat, L. R. 1999. Survival of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces applied to lettuce and the effectiveness of chlorinated water as a disinfectant. *J. Food Prot.* 62:845-849.
4. Brandle, M. T., and R. Amundson. 2008. Leaf age as a risk factor in contamination of lettuce with *E. coli* O157:H7 and *Salmonella enterica*. *Appl. Environ. Microbiol.* 74:2298-2306.
5. Delaquis, P., S. Bach, and L. Dinu. 2007. Behavior of *Escherichia coli* O157:H7 in leafy vegetables. *J. Food Prot.* 70:1966-1974.
6. Delaquis, P., S. Stewart, S. Cazaux, P. Toivonen. 2002. Survival and growth of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in ready-to-eat iceberg lettuce washed in warm chlorinated water. *J. Food Prot.* 65:459-464.
7. Du, J., Y. Han, and R. H. Linton 2003. Efficacy of chlorine dioxide gas in reducing *Escherichia coli* O157:H7 on apple surfaces. *Food Microbiol.* 20.
8. Folsom, J. P. 18 October 2007. Personal communication. [Email. James.folsom@biofilm.montana.edu].

9. Francis, G. A., and D. O'Beirne. 2001. Effects of vegetable type, package atmosphere and storage temperature on growth and survival of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *J. Ind. Microbiol. Biotechnol.* 27:111-116.
10. Friedman, M., P.R. Henika, and R.E. Mandrell. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J. Food Prot.* 65:1545-1560.
11. Garg, N., J. J. Churey, and D. F. Splittstoesser. 1990. Effects of processing conditions on the microflora of fresh-cut vegetables. *J. Food Prot.* 53:701-703.
12. Han, Y., J. D. Floros, R. H. Linton, S. S. Nielsen, and E. Nelson. 2001. Response surface modeling for the inactivation of *Escherichia coli* O157:H7 on green peppers (*Capsicum annuum* L.) by chlorine dioxide gas treatments. *J. Food Prot.* 64:1128-1133.
13. Heaton, J. C., and K. Jones. 2007. Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review. *J. Appl. Microbiol.* 104:613-626.
14. Hora, R., K. Warriner, B. J. Shelp, and M. W. Griffiths. 2005. Internalization of *Escherichia coli* O157:H7 following biological and mechanical disruption of growing spinach plants. *J. Food Prot.* 68:2506-2509.
15. Islam, M., M. P. Doyle, S. C. Phatak, P. Millner, and X. Jiang. 2004. Persistence of enterohemorrhagic *Escherichia coli* O157:H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. *J. Food Prot.* 67:1365-1370.
16. Jablasone, J., K. Warriner, and M. W. Griffiths. 2005. Interactions of *Escherichia coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes* with plants cultivated in a gnotobiotic system. *Int. J. Food Microbiol.* 99:7-18.

17. Kim, J. K., and M. A. Harrison. 2008. Transfer of *Escherichia coli* O157:H7 to romaine lettuce due to contact water from melting ice. *J. Food Prot.* 71:252-256.
18. Kondo, N., M. Murata, and K. Isshiki. 2006. Efficiency of sodium hypochlorite, fumaric acid, and mild heat in killing native microflora and *Escherichia coli* O157:H7, *Salmonella* Typhimurium DT104, and *Staphylococcus aureus* attached to fresh-cut lettuce. *J. Food Prot.* 69:323-329.
19. Lang, M. M., L. J. Harris, L. R. Beuchat. 2004. Survival and recovery of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* on lettuce and parsley as affected by method of inoculation, time between inoculation and analysis, and treatment with chlorinated water. *J. Food Prot.* 67:1092-1103.
20. Li, H., T. Mehrdad, and I. O. Bennie. 2008. Impact of vacuum cooling on *Escherichia coli* O157:H7 infiltration into lettuce tissue. *Appl. Environ. Microbiol.* 74:3138-3142.
21. Li, Y., R. E. Brackett, J. Chen, and L. R. Beuchat. 2001. Survival and growth of *Escherichia coli* O157:H7 inoculated onto cut lettuce before or after heating in chlorinated water, followed by storage at 5 or 15°C. *J. Food Prot.* 64:305-3-9.
22. Lin, C., J. Kim, W.X. Du, and C.I. Wei. 2000. Bactericidal activity of isothiocyanate against pathogens on fresh produce. *J. Food Prot.* 63:25-30.
23. Lin, C. M., J. F. Preston, and C. I. Cheng. 2000. Antibacterial mechanism of allyl isothiocyanate. *J. Food Prot.* 63:727-734.
24. Niemira, B. A. 2007. Relative efficacy of sodium hypochlorite wash versus irradiation to inactivate *Escherichia coli* O157:H7 internalized in leaves of romaine lettuce and baby spinach. *J. Food Prot.* 70:2526-2532.

25. Niemira, B. A. 2008. Irradiation compared with chlorination for elimination of *Escherichia coli* O157:H7 internalized in lettuce leaves: influence of lettuce variety. *J. Food Sci.* 73:M208-M213.
26. Obaidat, M. M., and J. F. Frank. 2009. Inactivation of *Salmonella* spp. and *Escherichia coli* O157:H7 on sliced and whole tomatoes by allyl isothiocyanate, carvacrol and cinnamaldehyde in vapor-phase. *J Food Prot.* In press.
27. Park, C. M., P.J. Taormina, and L. R. Beuchat. 2000. Efficacy of allyl isothiocyanate in killing enterohemorrhagic *E. coli* O157:H7 on alfalfa seeds. *Int. J. Food Microbiol.* 56:13-20.
28. Singh, N., K. Singh, A. K. Bhunia, and R. L. Stroshine. 2002. Efficacy of chlorine dioxide, ozone, and thyme essential oil or a sequential washing in killing of *E. coli* O157:H7 on lettuce and baby carrots. *Lebensm.-Wiss. Technol.* 35:720-729.
29. Solomon, E. B., S. Yaron, and K. R. Matthews. 2002. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Appl. Environ. Microbiol.* 68:397-400.
30. Stopforth, J. D., T. Mai, B. Kottapalli, and M. Samadpour. 2008. Effect of acidified sodium chlorite, chlorine, and acidic electrolyzed water on *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* inoculated onto leafy greens. *J. Food Prot.* 71:625-628.
31. Takeuchi, K., A. N. Hassan, and J. F. Frank. 2001. Penetration of *Escherichia coli* O157:H7 into lettuce as influenced by modified atmosphere and temperature. *J. Food Prot.* 64:1820-1823.
32. Takeuchi, K., and J. F. Frank. 2000. Penetration of *Escherichia coli* O157:H7 into lettuce leaves as affected by inoculum size and temperature, and the effect of chlorine treatment on cell viability. *J. Food Prot.* 63:434-440.

33. Takeuchi, K., and J. F. Frank. 2001. Quantitative determination of the role of lettuce leaf structures in protecting *Escherichia coli* O157:H7 from chlorine disinfection. *J. Food Prot.* 64:147-151.
34. Wachtel, M. R., and A. O. Charkowski. 2002. Cross-contamination of lettuce with *Escherichia coli* O157:H7. *J. Food Prot.* 65:465-470.
35. Yaun, B. R., S. S. Sumner, J. D. Eifert, and J. E. Marcy. 2004. Inhibition of pathogens on fresh produce by ultraviolet energy. *Int. J. Food Microbiol.* 90:1-8.

TABLE 2.1. Inactivation of *E. coli* O157:H7 on the surface of lettuce and spinach by treatment with vapor phase carvacrol, cinnamaldehyde and AIT at 0 °C in 4 days (n=4, data presented as reduction log CFU/leaf)

Treatment	Day 2		Day 4	
	Lettuce	Spinach	Lettuce	Spinach
Control	0.75 a	0.29 a	0.80 a	0.15 a
Carvacrol (40 µl/air)	3.46 b	2.53 b	4.81 a	3.51 b
Carvacrol (80 µl/air)	3.74 b	1.70 c	4.45 a	3.67 a
Cinnamaldehyde (40 µl/air)	2.98 b	2.82 b	4.81 a	3.51 a
Cinnamaldehyde (80 µl/air)	4.45 a	3.27 b	5.32 a	4.96 a
AIT (4 µl/air)	1.04 b	1.24 b	3.50 a	3.29 a
AIT (16 µl/air)	3.97 a	2.31 b	4.42 a	4.39 a

abc values in the same row that do not share the same lowercase letter are significantly different ($P \leq 0.05$).

TABLE 2.2. Inactivating lettuce and spinach- surface attached *E. coli* O157:H7 by carvacrol, cinnamaldehyde and AIT at 4 °C in 4 days (n=4, data presented as reduction log CFU/leaf)

Treatment	Day 2		Day 4	
	Lettuce	Spinach	Lettuce	Spinach
Control	1.06 a	0.62 a	0.48 a	0.32 a
Carvacrol (40 µl/air)	2.46 d	3.10 c	4.13 a	3.64 b
Carvacrol (80 µl/air)	4.58 a	2.81 c	4.10 ab	3.62 b
Cinnamaldehyde (40 µl/air)	4.26 a	2.60 b	4.60 a	4.54 a
Cinnamaldehyde (80 µl/air)	4.51 a	2.29 b	4.89 a	4.66 a
AIT (4 µl/air)	2.14 b	2.00 b	4.22 a	3.75 a
AIT (16 µl/air)	3.74 ab	2.60 b	4.71 a	4.24 a

abcd values in the same row that do not share the same lowercase letter are significantly different ($P \leq 0.05$).

TABLE 2.3. Inactivation of *E. coli* O157:H7 infiltrated into lettuce and spinach leaves by treatment with vapor phase carvacrol, cinnamaldehyde and AIT at 0 °C in 4 days. (n=4, data presented as reduction log CFU/leaf)

Treatment	Day 2		Day 4	
	Lettuce	Spinach	Lettuce	Spinach
Control	0.54 ab	0.30 b	1.31 a	0.94 ab
Carvacrol (40 µl/air)	1.14 b	1.32 ab	2.74 a	1.58 ab
Carvacrol (80 µl/air)	1.76 b	1.56 b	4.07 a	1.02 b
Cinnamaldehyde (40 µl/air)	1.41 b	1.10 b	4.08 a	1.28 b
Cinnamaldehyde (80 µl/air)	2.81 b	1.26 c	4.83 a	1.70 c
AIT (4 µl/air)	1.30 b	1.13 b	2.47 a	1.03 b
AIT (16 µl/air)	2.20 b	1.10 c	4.28 a	2.55 b

abc values in the same row that do not share the same lowercase letter are significantly different ($P \leq 0.05$).

TABLE 2.4. Inactivation of *E. coli* O157:H7 infiltrated into lettuce and spinach leaves by treatment with vapor phase carvacrol, cinnamaldehyde and AIT at 4 °C in 4 days. (n=4, data presented as reduction log CFU/leaf)

Treatment	Day 2		Day 4	
	Lettuce	Spinach	Lettuce	Spinach
Control	0.82 a	-0.26 b	1.03 a	0.87 a
Carvacrol (40µl/air)	0.99 b	0.96 b	2.20 a	2.64 a
Carvacrol (80µl/air)	1.41 c	0.84 c	4.70 a	2.42 b
Cinnamaldehyde (40 µl/air)	1.54 ab	0.81 b	2.42 a	2.17 a
Cinnamaldehyde (80 µl/air)	1.46 c	0.71 d	4.27 a	2.27 b
AIT (4 µl/air)	1.66 a	0.79 a	1.69 a	0.89 a
AIT (16 µl/air)	1.96 c	1.09 d	4.03 a	3.41 a

abcd values in the same row that do not share the same lowercase letter are significantly different ($P \leq 0.05$).

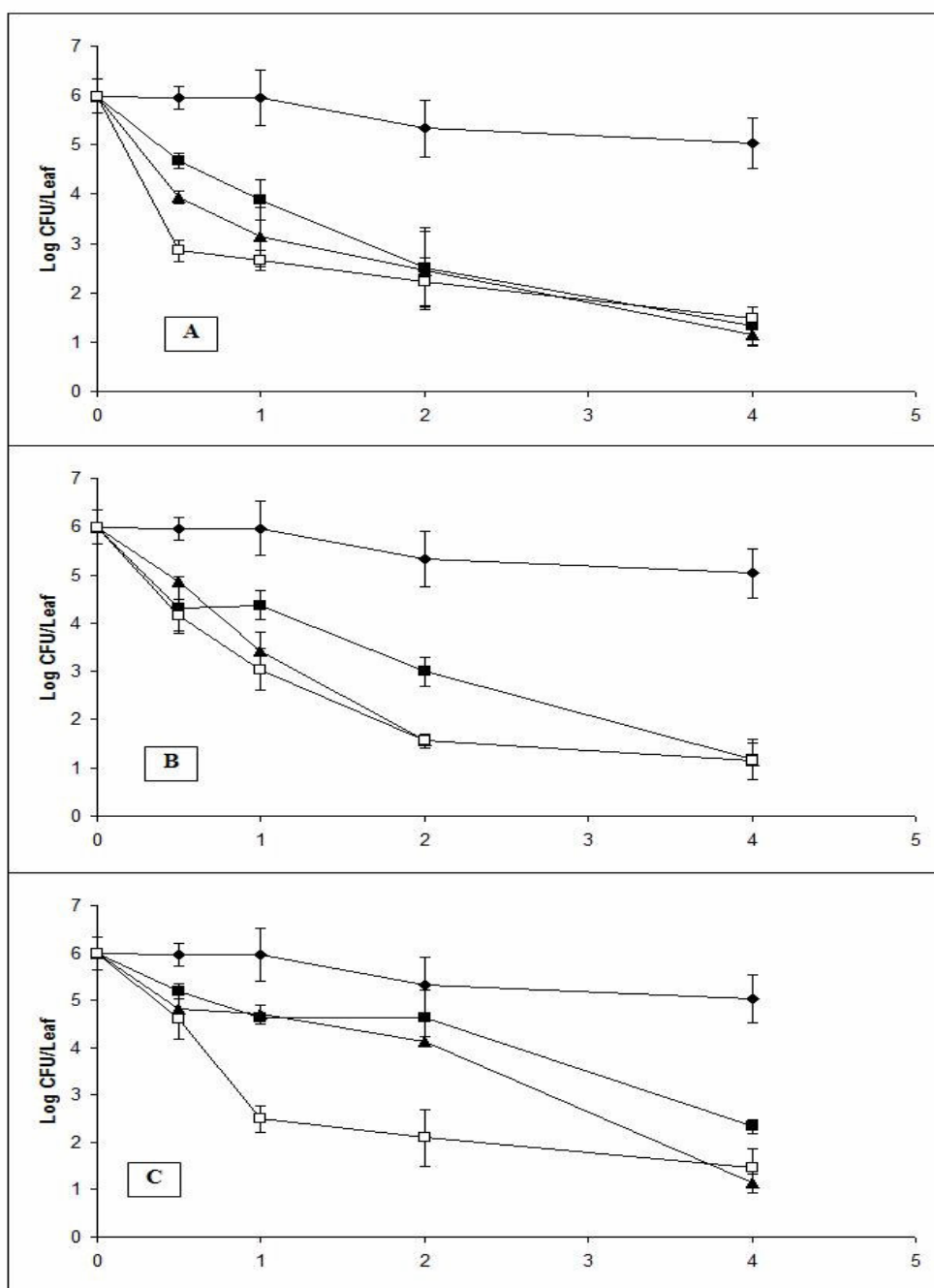


FIGURE 2.1. Inactivation of lettuce-surface attached *E. coli* O157:H7 at 0 °C in 4 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (♦ control, ■ 40 µl for A and B, 4 µl for C, ▲ 60 µl for A and B, 8 µl for C, □ 80 µl for A and B, 16 µl for C).

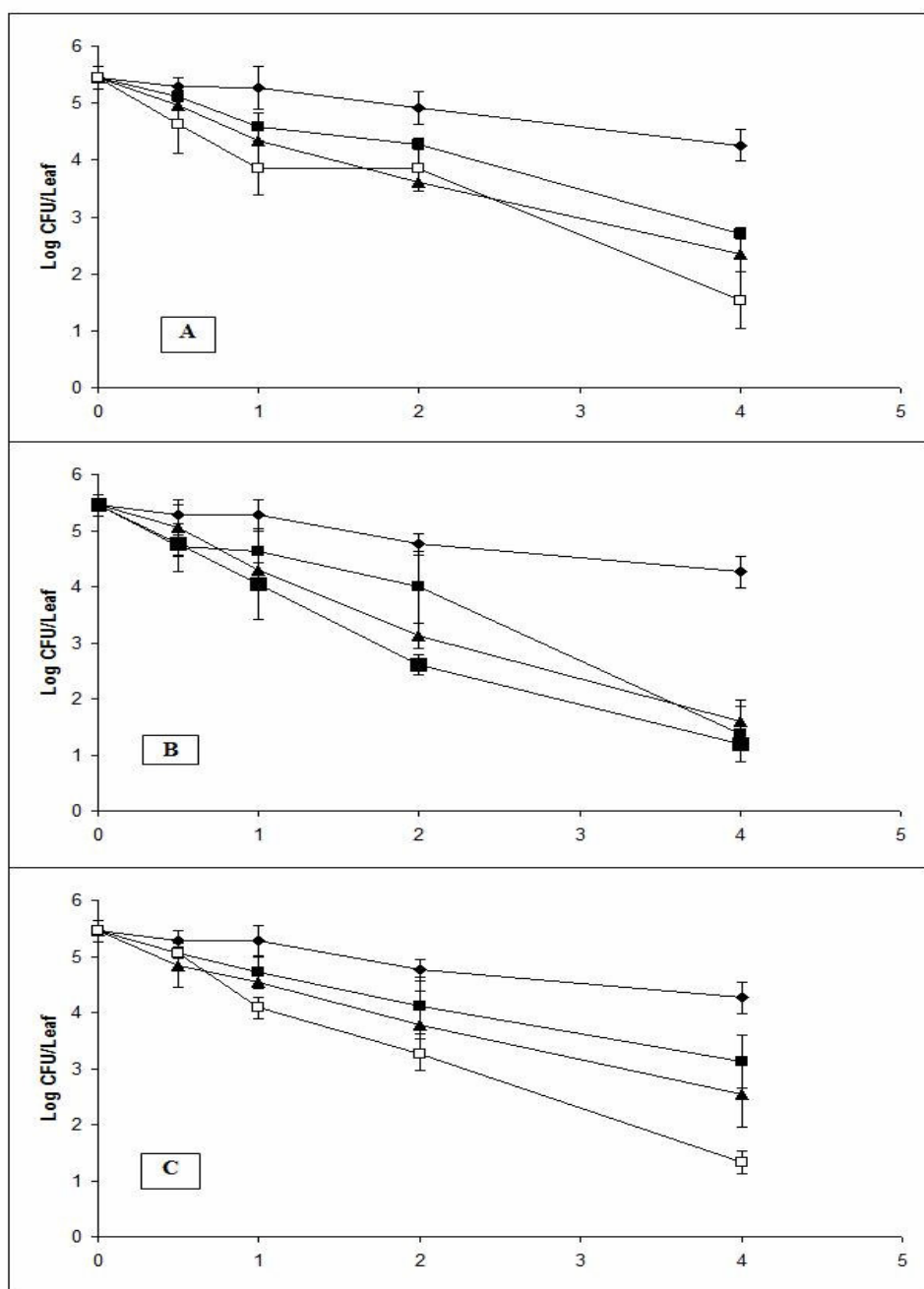


FIGURE 2.2. Inactivation of lettuce infiltrated *E. coli* O157:H7 at 0 °C in 4 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (♦ control, ■ 40 µl for A and B, 4 µl for C, ▲ 60 µl for A and B, 8 µl for C, □ 80 µl for A and B, 16 µl for C).

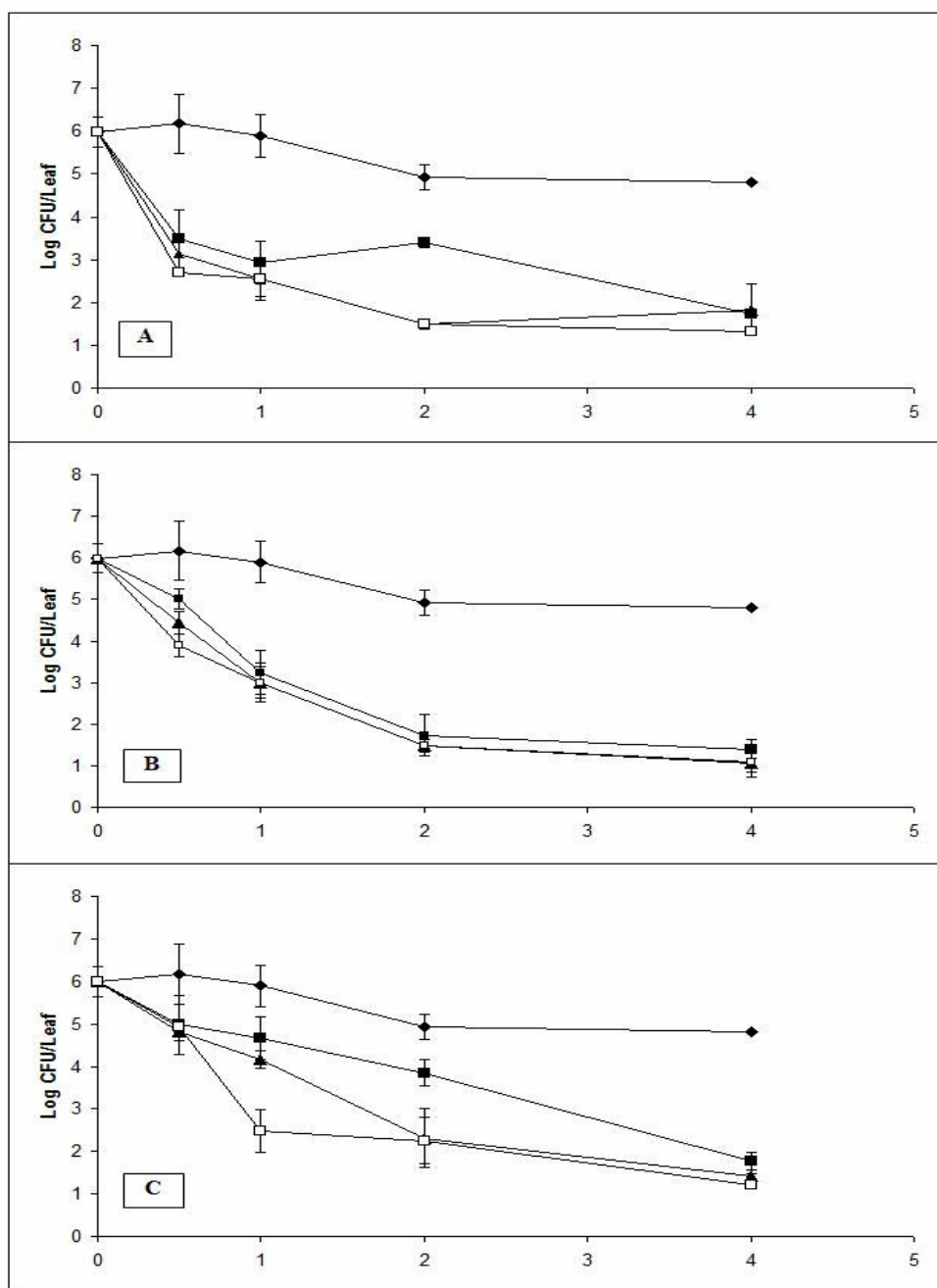


FIGURE 2.3. Inactivation of lettuce-surface attached *E. coli* O157:H7 at 4 °C in 4 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (♦ control, ■ 40 µl for A and B, 4 µl for C, ▲ 60 µl for A and B, 8 µl for C, □ 80 µl for A and B, 16 µl for C).

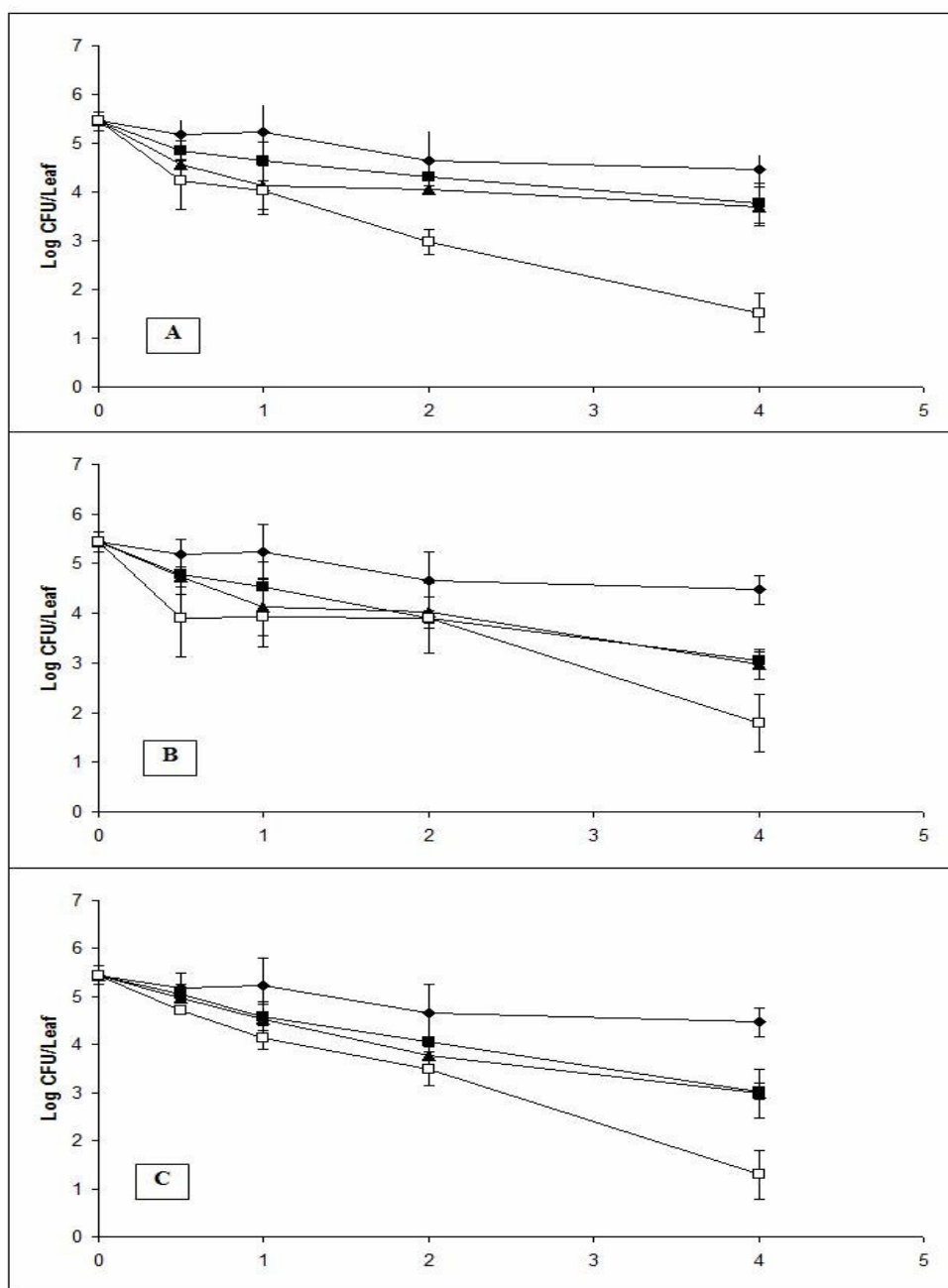


FIGURE 2.4. Inactivation of lettuce infiltrated *E. coli* O157:H7 at 4 °C in 4 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (♦ control, ■ 40 µl for A and B, 4 µl for C, ▲ 60 µl for A and B, 8 µl for C, □ 80 µl for A and B, 16 µl for C).

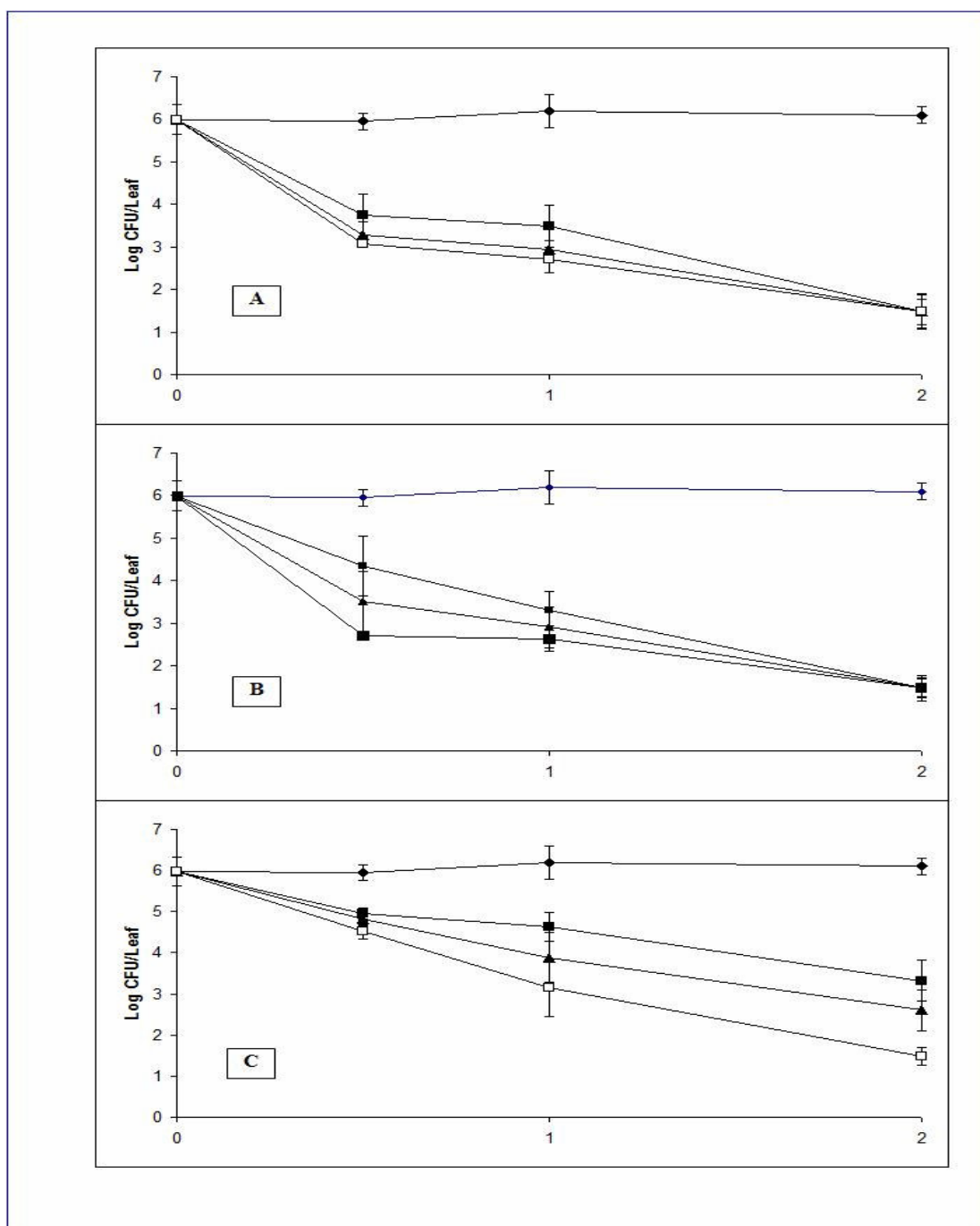


FIGURE 2.5. Inactivation of lettuce-surface attached *E. coli* O157:H7 at 10 °C in 2 days in the presence of different concentrations of vapor-phase A. Carvacrol; B. Cinnamaldehyde; C. Allyl Isothiocyanate. (♦ control, ■ 40 µl for A,B, 4 µl for C, ▲ 40 µl for A,B, 8 µl for C, □ 80 µl for A,B, 16 µl for C).

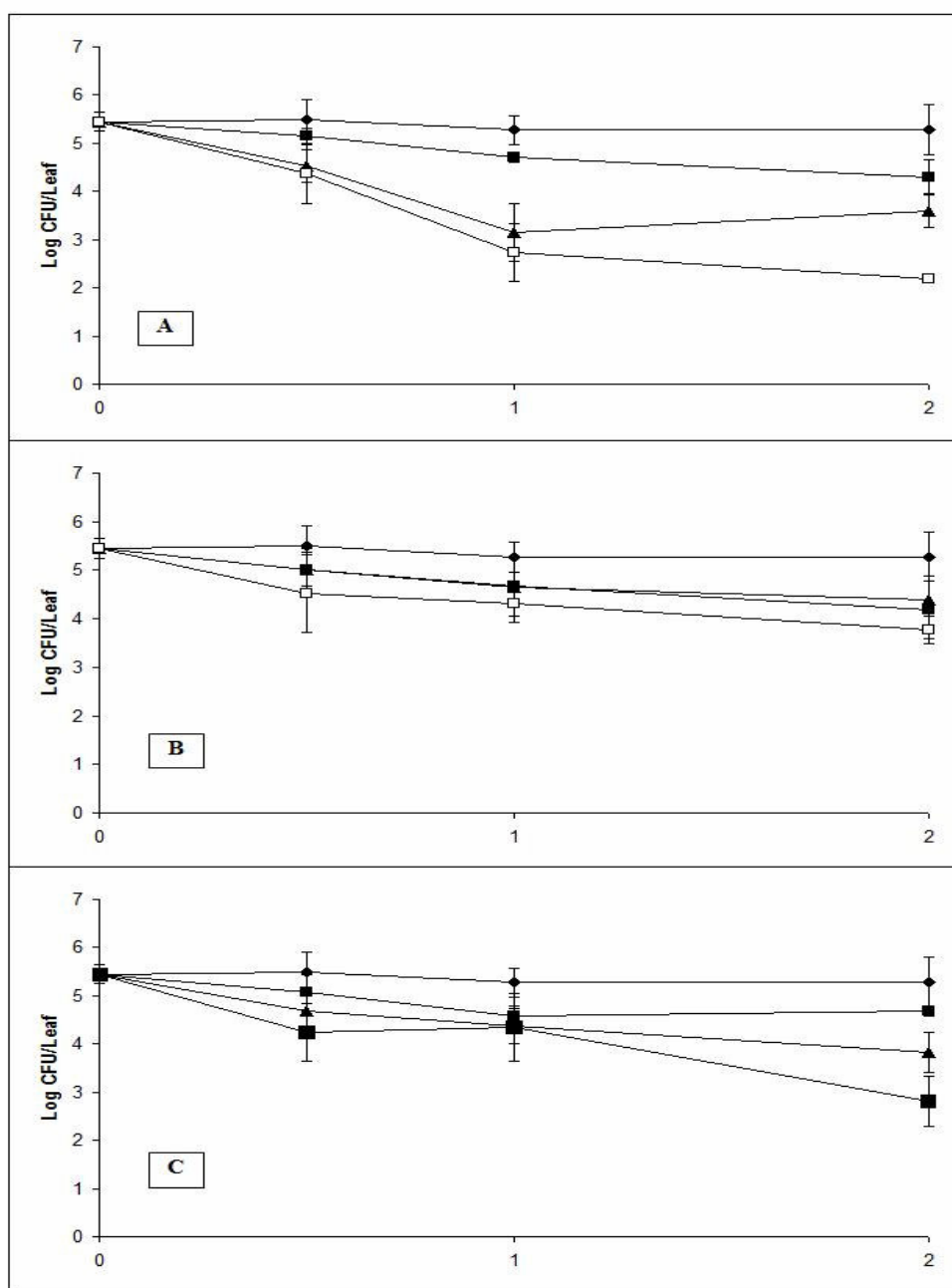


FIGURE 2.6. Inactivation of lettuce infiltrated *E. coli* O157:H7 at 10 °C in 2 days in the presence of different concentrations of vapor-phase carvacrol (A), cinnamaldehyde (B), and allyl Isothiocyanate (C). (♦ control, ■ 40 µl for A and B, 4 µl for C, ▲ 60 µl for A and B, 8 µl for C, □ 80 µl for A and B, 16 µl for C).

CHAPTER 5

VISUALIZING THE EFFECT OF VAPOR-PHASE CARVACROL, CINNAMALDEHYDE, AND ALLYL ISOTHIOCYANATE ON LETTUCE- INFILTRATED *E. COLI* O157:H7 BY CONFOCAL SCANNING LASER MICROSCOPY¹

¹ Obaidat, M. M. and J. F. Frank. To be submitted to Journal of Food Protection

ABSTRACT

Our previous research showed that carvacrol, cinnamaldehyde and allyl isothiocyanate are able to inactivate *E. coli* O157:H7 cells once they infiltrated within lettuce and spinach tissue. In this study, we disinfected the surface of lettuce leaves with silver nitrate solution and employed confocal scanning laser microscopy (CSLM) to validate our previous findings. Surface disinfected and water washed samples (controls) were treated with the antimicrobials' vapor at 0, 4 and 10 °C for 2 days. Some treated and untreated samples were observed under CSLM. The results showed that the vapor of antimicrobials exhibited greater reduction on the surface disinfected samples than on water-washed ones. The antimicrobials vapor reduced the pathogen population on surface disinfected samples by about 1.0 log at 0 °C in 2 days. Only AIT produced 1.0 log reduction on surface disinfected samples at 4 °C in 2 days. However, the antimicrobials reduced the pathogen population by 1.0 to 2.0 logs at 10 °C in 2 days. The CSLM micrographs supported the colony enumeration data for treated and untreated sample. Therefore, this study proved our previous findings that volatile antimicrobials are able to inactivate *E. coli* O157:H7 within lettuce tissue.

INTRODUCTION

Confocal scanning laser microscopy (CSLM) has been widely utilized in food microbiology research to improve the understanding of pathogen-food interaction and to study pathogen penetration below the surface of several food commodities. In the CSLM, a laser illumination of mixed- argon gas- krypton ion laser is used to excite flourophores (20). The CLSM microscope lens focuses laser light on one point (the focal point) of the sample. Then, the laser moves rapidly from one point to another, producing a scanned image. The microscope and scanner head focus the light emitted from the focal point into a second point, the confocal point. On the confocal point, a pinhole aperture allows light from the confocal point to pass through the detector. This reduces out-of-focus light leading to improvement in resolution along the z-axis and allows optical sectioning of the sample. The optical cross sectioning is an effective way to study the infiltration of pathogens in plant openings and tissue. Furthermore, fully hydrated samples can be visualized facilitating accurate determination of microbial viability within plant tissues. For detailed information about the CSLM usage in food research, Tackeuchi and Frank (20) published a review entitled “Confocal microscopy and microbial viability detection for food research”.

For visualizing pathogens in food matrices, a gene encoding for green fluorescent protein or mutants of this gene are inserted in the pathogens' genome. GFP and its mutant has been a useful reporter gene and viability marker for their bright clear fluorescent that can be detected by CSLM even in a single cell. This protein also facilitates nondestructive visualization of pathogens within food. Takeuchi and Frank (21) showed that *E. coli* O157:H7 on lettuce leaves and cauliflower retained GFP at high frequency and used it successfully as a marker to detect *E.*

E. coli O157:H7 on fresh produce when observed by the CSLM. Burnett et al. (1) utilized CSLM to study the attachment of GFP-tagged *E. coli* O157:H7 at various depths within healthy and puncture apple. Using this approach they verified quantitatively that pathogen attachment to intact apple skin and infiltration in lenticels, russet areas and floral tube of apples was greater under negative temperature differential (cold inoculum, warm apple) than under absence of temperature differential. Solomon et al. (18) also used CSLM and showed that GFP-tagged *E. coli* O157:H7 was transmitted from contaminated manure to internal tissue of lettuce leaves. Duffy et al. (3) also used CSLM to confirm internalization of GFP-tagged *Salmonella* on parsley. They found that *Salmonella* internalize into parsley regardless of the dip solution temperature (5, 25 or 35 °C) and dipping period (3 or 15 min).

Along with GFP, viability stains are used to visualize dead cells after antimicrobial treatment of pathogens within food, with propidium iodide (PI) being the most commonly used viability stain. PI is a nucleic acid stain that is impermeable to bacterial cell membrane, therefore it presumptively stains dead cells and emits red fluorescence (617 nm) when excited with green light (535 nm). Seo and Frank (17) stained inoculated lettuce with propidium iodide to determine the viability of *E. coli* O157:H7 after 20 mg/liter chlorine treatment for 5 min to conclude that many viable cells were found in stomata and on cut edges. Using GFP-tagged pathogens and PI, Warriner et al. (23) reported that the survival of *E. coli* O157:H7 and *Salmonella enterica* within and on surface of bean sprouts after sodium hypochlorite treatment. Propidium iodide has also been used in conjunction with purified antibodies to *E. coli* O157:H7 labeled with fluorescein isothiocyanate (8, 17) and Alexa Fluor 488 (19) to distinguish viable from dead cells.

Currently, sodium hypochlorite solution is the most widely used sanitizer in leafy greens processing; however several studies showed that sodium hypochlorite is as effective as deionized

water or a slightly more effective in removing *E. coli* O157:H7 from lettuce surface and does not kill infiltrated pathogens (13, 19). Freidman et al. (7) showed that oregano, thyme and cinnamon are the most effective essential oils against *E. coli* O157:H7; and carvacrol, cinnamaldehyde, and thymol are the most effective essential oil compounds against this pathogen (7). However, this information was obtained on liquid-phase compounds. Only limited information is available on their efficacy in vapor form. Furthermore, allyl isothiocyanate (AIT) has also been suggested as a promising antimicrobial for *E. coli* O157:H7 as an alternative to chlorine(15). In addition, AIT in vapor phase inactivated up to 8 log CFU of *E. coli* O157:H7 on lettuce in 2 days at 4 °C (14). Thus, using essential oils or their active components in their vapor-phase may be effective against bacteria at locations on the fruit not reached by chemicals in aqueous solution and their use in packaged leafy greens could provide an added pathogen control benefit.

This research aimed to confirm that volatile antimicrobials (AIT, carvacrol and cinnamaldehyde) kill lettuce-tissue infiltrated and surface-attached *E. coli* O157:H7. Our approach was to use 1% silver nitrate (AgNO₃) solution as surface disinfectant followed by treatment with AIT, carvacrol and cinnamaldehyde to determine their ability to inactivate cells not killed by the surface treatment. Viable and nonviable internalized *E. coli* O157:H7 were visualized under confocal scanning laser microscopy following the volatile antimicrobials treatment.

MATERIALS AND METHODS

Inoculum preparation. *E. coli* O157:H7 strains isolated from produce-associated outbreaks were used to inoculate lettuce. Three GFP_{uv} transformed *E. coli* O157:H7 strains (K3995; a clinical isolate from 2006 spinach outbreak, K4992; a clinical isolate from a 2006 lettuce

outbreak, and F4546; an alfalfa sprout outbreak) were obtained from the Center for Food Safety, University of Georgia, Griffin. The strains were stored at -80 °C in Microbank® beads (Pro-Lab Diagnostics, Richmond Hill, ON, Canada). When needed one bead of each strain was cultured in TSB-medium supplemented with ampicillin (100 µg/ml) at 37 °C. Then, the strains were grown to stationary-phase by transferring each twice in tryptic soy broth (TSB + ampicillin) at 24 h intervals with incubation at 37 °C. The cocktail was prepared by mixing equal volumes of each strain. Then, the cocktail was centrifuged at 4500 rpm for 25 min at 4° C, the supernatant fluid discarded, the pellet washed with 0.1% peptone, and re-suspended in 10 ml of peptone water. This suspension was 1:10 dilution in peptone water to achieve 8 log CFU/ml pathogen concentration.

Pathogen infiltration within lettuce and surface disinfection. Infiltration was accomplished by vacuum application as developed by Folsom (5). Pre-packaged leafy green lettuce was purchased at retail in Athens, GA on the day of the experiment. Undamaged leaves were selected, rinsed under running tap water, and dried by spinning in salad spinner. A cut of 2.54 cm was made on the stem end of each leaf to provide a uniform fresh cut that would facilitate bacterial infiltration. Ten cut leaves were placed with the cut edge submerged in 10 ml of the inoculum cocktail in a 100 ml beaker. The beakers were placed in a vacuum chamber which was evacuated to achieve an absolute pressure of 50 kPa (approximately 50 kPa below atmospheric pressure). The beakers were placed inside the vacuum chamber for 20 min, and then the pressure was evacuated slowly over a 5 min period. The leaves were taken from the beakers, placed in Petri dishes and then washed three times with sterile deionized water. The washed leaves were placed on stainless steel tray and dried by pressing them gently between KimWipes™

(Kimberly-Clark Corporation, Irving, TX). One leaf was placed in each jar. Silver nitrate solution was used to disinfect the leaf surface after the vacuum infiltration and washing it with deionized water. A 10 g/liter silver nitrate (Fisher Scientific, Pittsburgh, PA) solution was prepared as described by Franz et al. (6). Surface disinfection of the leaves was also employed as described in Franz et al. (6).

Antimicrobials application and sample storage. Pieces (2×2 cm) of Whatman® filter paper (Whatman Inc., Clifton, NJ) were attached on the inner upper side of septa jars (120 ml volume) (Short Amber WM Septa-Jar TM, Item # S240-0120, I-Chem™ Certified 200 series, Chase Scientific Glass, Inc, Rockwood, TN) by double-stick tape (Henkel Consumer Adhesives, Inc, Avon, OH) to avoid volatile direct contact with the lettuce leaves that were in the jars later. Septa jars were autoclaved under vacuum cycle at 121.1 Kpa for 45 min. Then, a Petri dish was filled with sterile deionized water, in which autoclave-sterilized Whatman® filter paper pieces (previously cut 2×2 cm pieces) (Whatman Inc., Clifton, NJ) were immersed. One wet filter paper was placed on the top inner side of each septa jar 12 h before placing the lettuce leaves within the jars and applying the antimicrobials to create high relative humidity inside the jars. A 5 µl (equivalent to 40 µl antimicrobial / liter of air) of ≥ 97% purity carvacrol or ≥ 98% purity cinnamaldehyde or 0.5 µl (equivalent to 4 µl of antimicrobial/ liter of air) of ≥ 98% purity AIT (Sigma- Aldrich, St. Louis, MO) was deposited on a second filter paper previously attached on the inner upper side of the septa jar by double-stick tape. Samples in the sealed jars were stored at 4 °C. Uninoculated and inoculated controls were also prepared. The samples were stored at 0, 4 and 10 °C.

Microbiological analysis. The treated samples and controls were prepared for enumeration of *E. coli* O157:H7 by blending (Osterizer® classic blender and Oster® ice crushing blade # 4961, Fisher Scientific, Pittsburgh, PA) the entire leaf for 30 s in 50 ml of 1 g/liter peptone using 8 oz (236.6 g) blender jars (Ball quilted crystal jelly jars, Jarden Corp, Muncie Ind.). Appropriate dilutions were plated on sorbitol MacConkey agar (SMAC) (Sigma-Aldrich, St. Louis, MO) supplemented with 100 µg/ml ampicillin and 1 g/liter sodium pyruvate (Sigma-Aldrich). The SMAC plates were incubated at 35 °C for 24 h.

Statistical analysis. The study used a random factorial block design. The experimental design consisted of three antimicrobials (AIT, carvacrol, and cinnamaldehyde), two concentrations (0 and 4 µl/liter for first antimicrobial and 0 and 40 µl/liter for latter two antimicrobials), three temperatures (0, 4 and 10 °C), three sampling times (0, 1, and 2 days), one inoculation method (vacuum infiltration) and three washing methods (deionized water, chlorinated water and silver nitrate solution). Data was analyzed using SAS 9.1.3 (SAS Institute, Inc., Cary, NC) by calculating the analysis of variance (ANOVA) using general linear model and multiple comparisons between the means was done by Duncan's test with a 5% significance level. Each experiment was replicated four times.

Confocal scanning laser microscopy. Vacuum infiltration and antimicrobial treatment for the CSLM were prepared as for the microbiological analysis. The samples were stored at 4 °C for 2 days. Untreated (control) and antimicrobial-treated samples were stained with propidium iodide (Molecular Probe, Eugene, Ore). Leaves were submerged in 10 µM solution for 10 min and then rinsed three times with SDW (16). Each sample was placed in a 60×15 mm Petri dish (Becton,

Dickinson and Company, NJ) and the Petri dish was then filed with sterile deionized water. Samples were observed by Leica SP2 Spectral Confocal Scanning Laser Microscope with Coherent Tisapphire multiphoton laser (Mira Optima 900-F) (Leica Microsystems Inc., Exton, PA) equipped with a 63× water immersion long working distance lens. The excitation of the lettuce samples at 488 nm used for GFP resulted in less green autofluorescence from lettuce cells. Image collection and three-dimensional reconstruction of images from multiple optical sections was accomplished using Leica proprietary software. Optical sections for volume rendering of infected leaves were collected by setting the stepping motor increment and the pixel size at *ca.* 0.2 μm.

RESULTS AND DISCUSSION

Inactivation of lettuce-infiltrated *E. coli* O157:H7 by vapor-phase antimicrobials after water wash or surface disinfection. The lettuce leaves were treated with silver nitrate to inactivate surface attached cells while leaving internalized cells viable. Washing the inoculated lettuce with silver nitrate (surface disinfection) significantly reduced *E. coli* O157:H7 by 0.62 log units compared to washing them with water (Day 0 data; Tables 3.1 to 3.3), an indication of the amount of surface attached cells not removed by water. The population of *E. coli* O157:H7 was significantly reduced in both water-washed (1.0 log) and surface disinfected (0.72 log) samples when stored at 0 °C for 2 days in the absence of vapor phase antimicrobials treatments (Table 3.1). Treating the samples with vapor phase antimicrobials significantly reduced the population of *E. coli* O157:H7 after water wash and surface disinfection (Table 3.1). Comparing water washed to surface disinfected samples, the antimicrobials resulted in greater reduction on the surface-disinfected samples, which provided an indication of the ability of these

antimicrobials to inactivate internalized cells. On water-washed lettuce, AIT exhibited the greatest significant reduction (*ca.* 0.60 log) of O157:H7 population followed by cinnamaldehyde (*ca.* 0.40 log) and carvacrol (*ca.* 0.44 log) after 1 day of treatment, however cinnamaldehyde showed the greatest significant reduction after 2 days by reducing the population by 0.53 log. On the surface disinfected lettuce, both AIT and cinnamaldehyde significantly reduced the population of O157:H7 after 1 day of treatment by 1.24 log and 1.10 log, respectively, meanwhile all antimicrobials produced a significant reduction after 2 days of treatment, where AIT reduced the population by 1.85 log, cinnamaldehyde by 1.16 log, and carvacrol by 0.95 log. The amount of reduction increased with time on the surface-disinfected samples but decreased on the water washed ones.

In the absence of the antimicrobial, lettuce-tissue infiltrated *E. coli* O157:H7 significantly decreased on both water-washed (*ca.* 1.15 log) and surface disinfected samples (*ca.* 1.24 log) at 4 °C in 2 days (Table 3.2). Only AIT, after 1 and 2 days, and carvacrol, after 2 days of treatment, showed greater significant reduction on the surface disinfected samples than on the water-washed ones (Table 3.2). Only AIT significantly reduced *E. coli* O157:H7, which was *ca.* 1.5 log on the surface disinfected samples and *ca.* 0.75 log on the water washed ones compared to their perspective controls. The other antimicrobial-treatment time combinations produced a similar reduction effect on the water-washed and the surface disinfected samples which was not significantly different from their perceptive controls. Only AIT exhibited a significant reduction on water-washed lettuce after 2 days of treatment compared to the control.

In the absence of the antimicrobial, the population of lettuce-infiltrated *E. coli* O157:H7 was significantly reduced on both water-washed (*ca.* 0.42 log) and surface disinfected (*ca.* 0.56 log) samples at 10 °C in 2 days (Table 3.3). Generally, the application of the antimicrobials reduced

the pathogen significantly on the water-washed and on the surface disinfected samples at 10 °C (Table 3.3). Overall, all antimicrobials caused greater significant reduction of the pathogen on the surface disinfected samples than on the water-washed ones. On the water-washed samples, AIT and carvacrol reduced the pathogen significantly by 1.26 and 1.00 log, respectively, after 1 day of treatment and all antimicrobials caused a significant reduction after 2 days of treatment; where AIT reduced the pathogen by 1.22 log, carvacrol by 0.90 log, and cinnamaldehyde by 0.61 log. However, on the surface disinfected samples, all antimicrobials, but cinnamaldehyde after 1 day, showed significant reduction after 1 and 2 days of treatment and the amount of reduction increased with time. AIT produced 1.86 log reduction after 1 day of treatment and 2.51 log reduction after 2 days. Meanwhile, carvacrol produced 0.95 log reduction after 1 day and 1.96 log after 2 days of treatment. Cinnamaldehyde produced 0.68 log reduction after 2 days of treatment. Overall, AIT and then carvacrol produced the greatest reduction on the water-washed and on the surface disinfected samples after both days of treatment compared to their perspective controls. The results also indicate that the amount of pathogen reduction increased with time on the surface-disinfected sample but not on the water washed ones.

The results of this study shows that the antimicrobials cause a greater reduction of pathogens on the surface disinfected samples than on water washed ones. This might be attributed to the surface disinfection by silver nitrate producing injured cells which were killed later by the volatile antimicrobials. Kim et al. (12) showed that a combined and sequential treatments of *Pseudomonas aeruginosa* biofilms with silver ion and tobramycin enhanced antimicrobial efficiency by more than 200%. Jung et al. (10) showed that following treating *E. coli* with silver nitrate solution, the number of cell counted by conventional plating was significantly lower than the number determined by flow cytometry. This indicates that silver nitrate induced a sublethal

injury or active but nonculturable state in bacterial cells (10). The silver ion (Ag^+) of the silver nitrate targets various locations in a bacterial cell such as proteins in the cell wall/membrane, enzymes and DNA which results in protein inactivation, cell wall detachment and DNA condensation (4).

Confocal scanning laser microscopy. CSLM was used to visualize GFP-tagged *E. coli* O157:H7 within lettuce leaves following volatile antimicrobial treatment. To achieve this, green fluorescence from GFP and red fluorescence from propidium iodide stain (PI) were collected separately and then overlaid to produce a two-color image. The PI stains cells lacking a functional membrane and therefore presumed nonviable. Lettuce tissue contains porphyrins such as chlorophyll and carotenoids, which cause red autofluorescence, but only small amounts of lignin, which is the major source of green autofluorescence in plant cells (11). This accounts for the ease of observing GFP-tagged *E. coli* O157:H7 in lettuce tissue (21). No bacterial cells were observed on uninoculated samples (negative control) (data not shown). GFP-tagged *E. coli* O157:H7 infiltrated within lettuce tissue were readily visible under the CSLM by observing the green fluorescence of the GFP (Fig 3.1). Only viable cells were visible in the untreated-inoculated samples as indicated by the lack of cells stained with PI (Fig 3.1). The micrographs also show that *E. coli* O157:H7 cells preferentially attached to the cut edge of lettuce, which confirms previous findings (17, 19, 22). This might attributed to the hydrophilic cell surface of the pathogens cells (2) resulting in preferential attachment to the damaged tissue of lettuce leaves. Three-dimensional volume reconstruction of interior portions of leaves showed that *E. coli* O157:H7 was entrapped 40 to 70 μm below the cut edge (Fig 2). Takeuchi and Frank (19) showed that *E. coli* O157:H7 could penetrate lettuce tissue $73.5 \pm 16.0 \mu\text{m}$ below the surface of cut edge at 4 °C as observed under the CSLM.

The micrographs of the treated samples (Figs 3.3 thru 3.5) show that both dead and live bacterial cells were observed inside lettuce leaves following the antimicrobials treatment and some green-to-yellow cells were observed which indicates questionable viability; possibly either injured and culturable or injured and nonculturable cells. The micrographs show that following antimicrobials treatment, most internalized *E. coli* O157:H7 were inactivated or inhibited as indicated by red color-stained *E. coli* O157:H7 following PI staining, however some remained viable as indicated by their green fluorescence (Figs 3.3 thru 3.5). The micrographs' results are consistent with the colony enumeration data as fewer CFU/leaf were enumerated following the antimicrobials treatment. The CSLM results support previous findings by Obaidat and Frank (submitted for publication) which indicated that the volatile antimicrobials are able to inactivate *E. coli* O157:H7 infiltrated within lettuce leaves. These volatile antimicrobials are superior to other antimicrobial treatments in inactivating infiltrated pathogens. For example, Seo and Frank (17) demonstrated the failure of chlorine treatment to inactivate *E. coli* O57:H7 cells once they penetrate lettuce leaves as confirmed by CSLM. They also observed that some, but not all, cells on the stomata were inactivated by chlorine. Itoh et al. (9) also showed that surface treatment of radish sprouts with mercury chloride failed to inactivate internalized *E. coli* O157:H7.

The findings of this study supports previous finding of Obaidat and Frank (submitted for publication) that the volatile antimicrobials are able to inactivate *E. coli* O157:H7 within lettuce leaves. Overall, these volatile antimicrobials can be used, individually or in combination, to inactivate *E. coli* O157:H7 on lettuce and spinach, if they could be incorporated in a packaging system that would provide an exposure of leaves to the vapor of these antimicrobials. However, a formal sensory study should be completed before practical use in food industry.

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REFERENCES

1. Burnett, S. L., J. Chen, and L. R. Beuchat. 2000. Attachment of *Escherichia coli* O157:H7 to the surfaces and internal structures of apples as detected by confocal scanning laser microscopy. *Appl. Environ. Microbiol.* 66:4679-4687.
2. Dewanti, R., and A. C. L. Wong. 1995. Influence of culture conditions on biofilm formation by *Escherichia coli* O157:H7. *Int. J. Food Microbiol.* 26:147-164.
3. Duffy, E. A., L. Sneros-Zevallos, A. Castillo, A., S. D. Pillai, S. C. Ricke, and G. R. Acuff. 2005. Survival of *Salmonella* transformed to express green fluorescent protein on Italian parsley as affected by processing and storage. *J. Food Prot.* 68:687-695.
4. Feng, Q. L., G. Q. Chen, F. Z. Cui, T. N. Kim, and J. O. Kim. 2000. A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. *J. Biomed. Mater. Res.* 52:662-668.
5. Folsom, J. P. 18 October 2007. Personal communication. [Email. James.folsom@biofilm.montana.edu].
6. Franz, E., A. A. Visser, A. D. Diepeningen, M. M. Klerks, A. J. Termorshuizen, and A. H. C. Bruggen. 2007. Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. *Food Microbiol.* 24:106-112.
7. Friedman, M., P. R. Henika, and R. E. Mandrell. 2002. Bactericidal activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. *J. Food Prot.* 65:1545-1560.
8. Han, Y., R. H. Linton, S. S. Nielsen, and P. E. Nelson. 2000. Inactivation of *Escherichia coli* O157:H7 on surface-injured and –injured green pepper (*Capsicum annum* L.) by chlorine

dioxide has as demonstrated by confocal scanning laser microscopy. *Food Microbiol.* 17:643-655.

9. Itoh, Y., Y. Sugita-Konishi, F. Kasuga, M. Iwaki, Y. Hara-Kudo, N. Saito, Y. Noguchi, H. Konuma, and S. Kumagai. 1998. Enterohemorrhagic *Escherichia coli* O157:H7 present in radish sprouts. *Appl. Environ. Microbiol.* 64:1532-1535.

10. Jung, W. K., H. C. Koo, K. W. Kim, S. Shin, S. H. Kim, and Y. H. Park. 2008. Antibacterial activity and mechanism of action of the silver ion in *Staphylococcus aureus* and *Escherichia coli*. *Appl. Environ. Microbiol.* 74:2171-2178.

11. Kays, S. J. 1991. Development of plants and plant parts, p. 257–334. In Postharvest physiology of perishable plant products. Van Nostrand Reinhold, New York.

12. Kim, J., B. Pitts, P. S. Stewart, A. Camper, and J. Yoon. 2008. Comparison of antimicrobial effect on biofilm of chlorine, silver ion and tobramycin. *Antimicrob. Agents Chemother.* 52:1446-1453.

13. Li, H., T. Mehrdad, and I. O. Bennie. 2008. Impact of vacuum cooling on *Escherichia coli* O157:H7 infiltration into lettuce tissue. *Appl. Environ. Microbiol.* 74:3138-3142.

14. Lin, C., J. Kim, W. X. Du, and C. I. Wei. 2000. Bactericidal activity of isothiocyanate against pathogens on fresh produce. *J. Food Prot.* 63:25-30.

15. Park, C. M., P. J. Taormina, and L. R. Beuchat. 2000. Efficacy of allyl isothiocyanate in killing enterohemorrhagic *Escherichia coli* O157:H7 on alfalfa seeds. *Int. J. Food Microbiol.* 56:13-20.

16. Seo, K. H., and J. F. Frank. 1999. Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. *J. Food Prot.* 62:3-9.

17. Seo, K. H., and J. F. Frank. 1999. Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. *J. Food Prot.* 52:3-9.
18. Solomon, E. B., S. Yaron, and K. R. Matthews. 2002. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Appl. Environ. Microbiol.* 69:397-400.
19. Takeuchi, K., and J. F. Frank. 2000. Penetration of *Escherichia coli* O157:H7 into lettuce leaves as affected by inoculum size and temperature, and the effect of chlorine treatment on cell viability. *J. Food Prot.* 63:434-440.
20. Takeuchi, K., and J. F. Frank. 2001. Confocal microscopy and microbial viability detection for food research. *J. Food Prot.* 64:2088-2102.
21. Takeuchi, K., and J. F. Frank. 2001. Expression of red-shifted green fluorescent protein by *Escherichia coli* O157:H7 as a marker for the detection of cells on fresh produce. *J. Food Prot.* 64:298-304.
22. Takeuchi, K., C. M. Matute, A. N. Hassan and J. F. Frank. 2000. Comparison of the attachment of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella Typhimurium*, and *Pseudomonas fluorescens* to Lettuce leaves. *J. Food Prot.* 63:1433-1437.
23. Warriner, K., S. Spaniolas, M. Dickinson, C. Wright, and W. M. Waites. 2003. Internalization of bioluminescent *Escherichia coli* and *Salmonella* Montevideo in growing bean sprouts. *J. Appl. Microbiol.* 95:719-727.

TABLE 3.1. Inactivation of lettuce-tissue infiltrated *E. coli* O157:H7 after water wash or surface disinfection by silver nitrate in the presence of 40 µl/liter of air of carvacrol, 40 µl/liter of air of cinnamaldehyde and 4 µl/liter of air of AIT vapors at 0 °C for 2 days. (n=4, data presented as log CFU/leaf).

Treatment	Day 0	Day 1	Day 2
DW [*]	5.74 a	5.24 a	4.75 a
SD [‡] followed by silver nitrate	5.12 b	5.10 ab	4.67 ab
DW followed by carvacrol	5.74 a	4.80 c	4.40 ab
SD followed by carvacrol	5.12 b	4.76 c	3.72 c
DW followed by cinnamaldehyde	5.74 a	4.762 bc	4.22 b
SD followed by cinnamaldehyde	5.12 b	3.99 d	3.51 c
DW followed by AIT	5.74 a	4.64 c	4.58 ab
SD followed by AIT	5.12 b	3.86 d	2.82 d

abcd values in the same column that do not share the same lowercase letter are significantly different ($P \leq 0.05$).

^{*} Deionized water

[‡] Surface disinfection

TABLE 3.2. Inactivation of lettuce-tissue infiltrated *E. coli* O157:H7 by 40 µl/liter of air of carvacrol, 40 µl/liter of air of cinnamaldehyde and 4 µl/liter of air of AIT after water wash or surface disinfection by silver nitrate at 4 °C for 2 days. (n=4, data presented as log CFU/leaf).

Treatment	Day 0	Day 1	Day 2
DW [*]	5.74 a	5.27 a	4.59 a
SD [†] followed by silver nitrate	5.12 b	4.87 ab	3.88 bc
DW followed by carvacrol	5.74 a	4.95 ab	4.54 a
SD followed by carvacrol	5.12 b	4.31 bc	3.50 cd
DW followed by cinnamaldehyde	5.74 a	4.80 ab	4.28 ab
SD followed by cinnamaldehyde	5.12 b	4.42 ab	3.87 bc
DW followed by AIT	5.74 a	4.63 ab	3.84 bc
SD followed by AIT	5.12 b	3.55 c	3.27 d

abcd values in the same column that do not share the same lowercase letter are significantly different ($P \leq 0.05$).

^{*} Deionized water

[†] Surface disinfection

TABLE 3.3. Inactivation of lettuce-tissue infiltrated *E. coli* O157:H7 by 40 µl/air carvacrol, 40 µl/air cinnamaldehyde and 4 µl/air AIT after either after water wash or surface disinfection by silver nitrate at 10 °C in 2 days. (n=4, data presented as log CFU/leaf).

Treatment	Day 0	Day 1	Day 2
DW [*]	5.74 a	5.40 a	5.32 a
SD [†] followed by silver nitrate	5.12 b	4.76 bc	4.56 b
DW followed by carvacrol	5.74 a	4.40 cd	4.42 bc
SD followed by carvacrol	5.12 b	3.81 de	2.60 e
DW followed by cinnamaldehyde	5.74 a	5.11 ab	4.71 b
SD followed by cinnamaldehyde	5.12 b	4.69 c	3.88 d
DW followed by AIT	5.74 a	4.24 d	4.10 cd
SD followed by AIT	5.12 b	2.90 e	2.05 e

abcde values in the same column that do not share the same lowercase letter are significantly different ($P \leq 0.05$).

^{*} Deionized water

[†] Surface disinfection

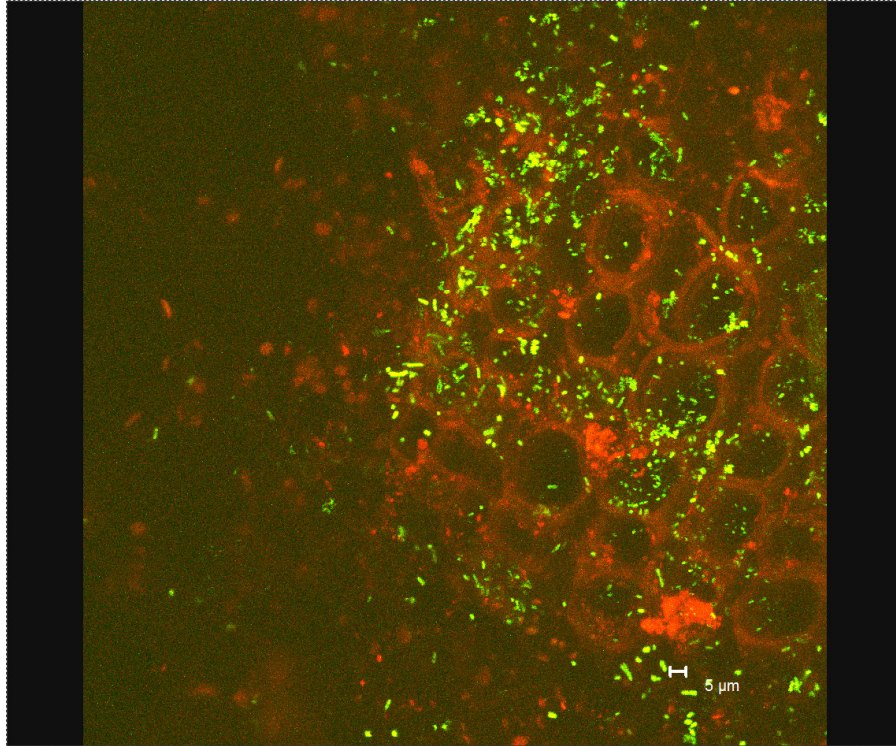


FIGURE 3.1. Micrograph of lettuce leaf infiltrated with GFP-tagged *E. coli* O157:H7 and incubated at 4 °C for 2 days (*micron bar = 5 mm*). The leaf was immersed in 8 log CFU/ml and 50 KPa vacuum was applied for 20 min. Green color indicates viable *E. coli* O157:H7 cells.

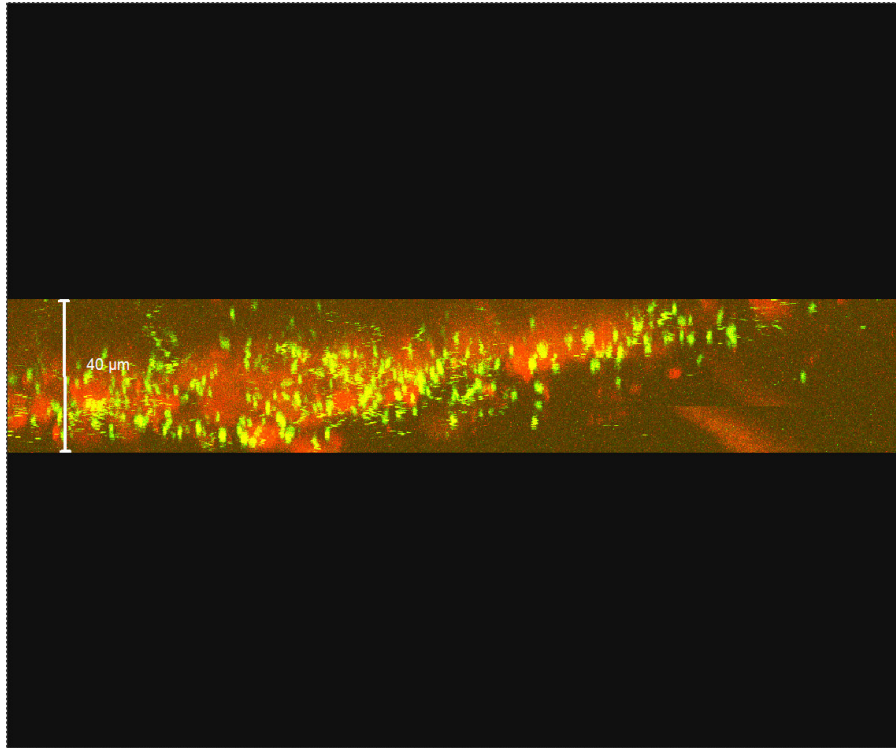


FIGURE 3.2. Three dimensional reconstruction of lettuce leaf infiltrated with GFP-tagged *E. coli* O157:H7 and incubated at 4 °C for 2 days (*micron bar = 40 mm*). The leaf was immersed in 8 log CFU/ml and 50 KPa vacuum was applied for 20 min. The leaf was then stained with 50 μM propidium iodide. Green color indicates viable *E. coli* O157:H7 cells.

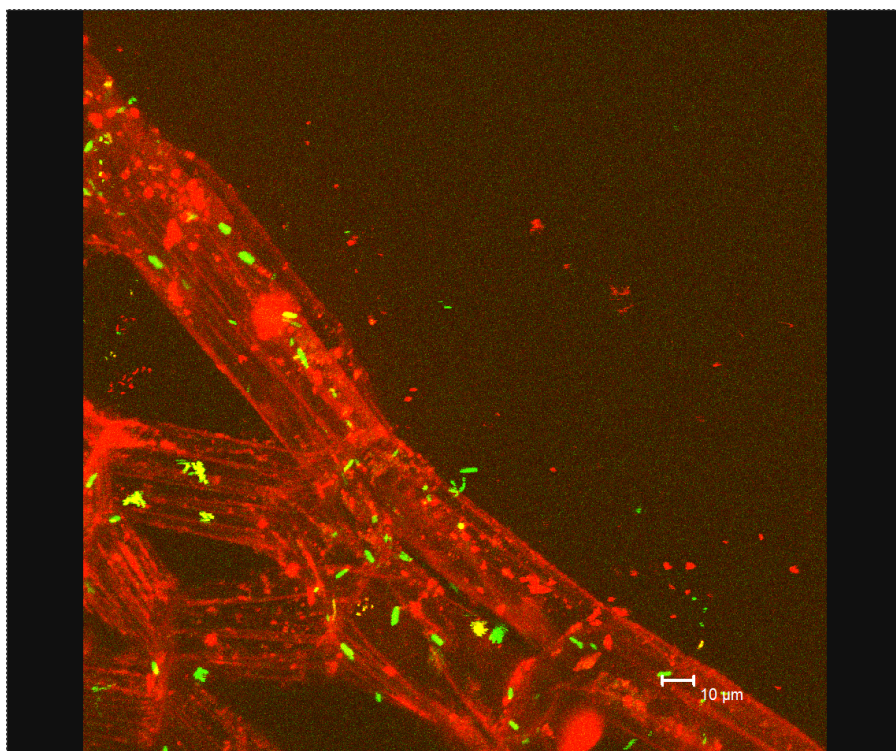


FIGURE 3.3. Micrograph of lettuce leaf infiltrated with GFP-tagged *E. coli* O157:H7 and treated with 40 μ l/liter carvacrol at 4 °C for 2 days (*micron bar = 10 mm*). The leaf was then stained with 50 μ M propidium iodide. Green color indicates viable *E. coli* O157:H7 cells. Red cells indicated killed *E. coli* O157:H7. Yellow cells indicate injured *E. coli* O157:H7.

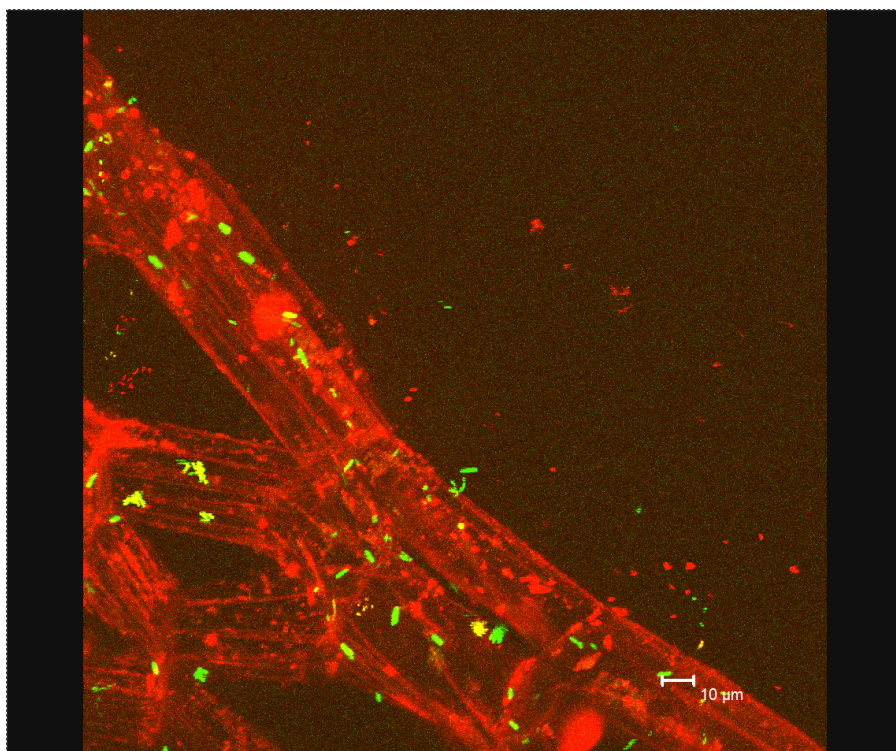


FIGURE 3.4. Micrograph of lettuce leaf infiltrated with GFP-tagged *E. coli* O157:H7 and treated with 40 μ l/liter cinnamaldehyde at 4 °C for 4 days (*micron bar = 10 mm*). The leaf was then stained with 50 μ M propidium iodide. Green color indicates viable *E. coli* O157:H7 cells. Red cells indicated killed *E. coli* O157:H7. Yellow cells indicate injured *E. coli* O157:H7.

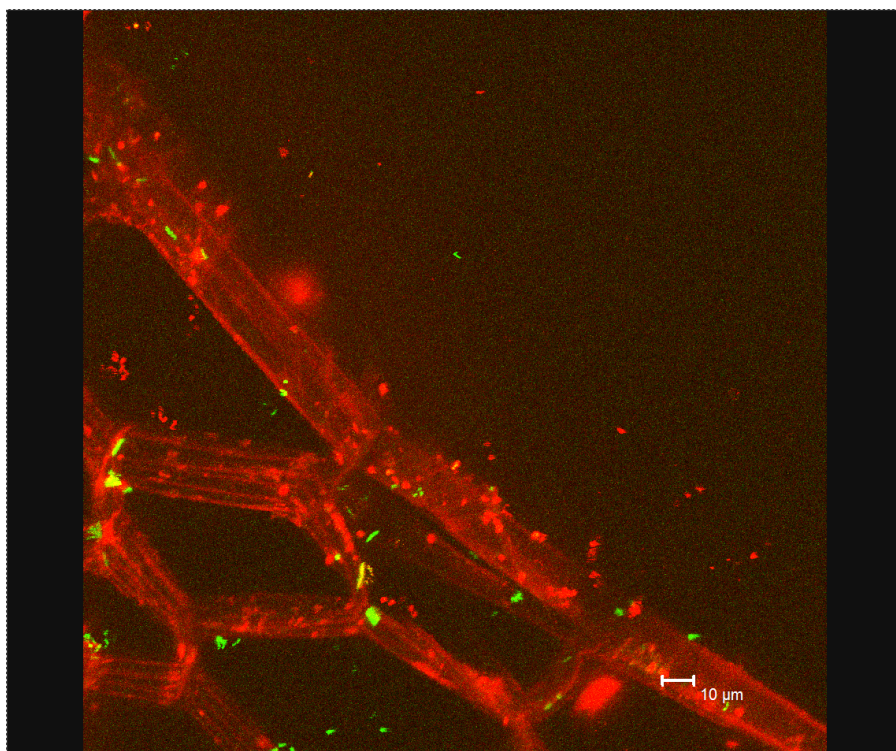


FIGURE 3.5. Micrograph of lettuce leaf infiltrated with GFP-tagged *E. coli* O157:H7 and treated with 40 μl/liter AIT at 4 °C for 4 days. The leaf was then stained with 50 μM propidium iodide. Green color indicates viable *E. coli* O157:H7 cells. Red cells indicated killed *E. coli* O157:H7. Yellow cells indicate injured *E. coli* O157:H7.

CHAPTER 6

CONCLUSION

The findings of these studies indicate the potential of using AIT, carvacrol and cinnamaldehyde, individually or in combination, as a kill or growth prevention step of *Salmonella* spp. and *E. coli* O157:H7 in packaged sliced and grape tomatoes, and packaged lettuce and spinach. AIT showed the greatest reductions of the pathogens followed by cinnamaldehyde with exposure time followed by antimicrobial concentration being the most factors affecting the inactivation ability of the antimicrobials. These antimicrobials could be of use to control *Salmonella* spp. and *E. coli* O157:H7 on packaged tomatoes stored at refrigeration temperatures as they showed the ability to inactivate *Salmonella* spp. and *E. coli* O157:H7 on tomato surface and between slices at 4 °C. An 8.3 µl/liter of AIT reduced *Salmonella* and *E. coli* O157:H7 between tomatoes slices by 1.0 and 3 log, respectively, at 4 °C in 10 days. The same concentration also reduced *Salmonella* and *E. coli* by 2.0 and 3.0 log, respectively, at 4 °C in 10 days. If there is a breakage in the cold chain, the antimicrobial activity would increase and reduce the potential for pathogen growth since the data showed that 8.3 µl/liter of is able to inactivate *Salmonella* and *E. coli* O157:H7 by 2.0 and 1.0 log on tomatoes surface and by ca. 3.0 of the two pathogens between tomatoes slices at 10 °C in 10 days.

The bactericidal and bacteriostatic effect of AIT, carvacrol, and cinnamaldehyde against surface-attached and tissue- infiltrated *E. coli* O157:H7 indicates a potential application for improving the safety of refrigerated packaged leafy greens, if they could be incorporated into a

packaging system that would provide exposure of the leaves to the antimicrobial vapor. A 40 µl/liter of air of carvacrol or cinnamaldehyde or 4 µl/liter of air of AIT reduced *E. coli* O157:H7 by 4.0 log on lettuce surface at 0 and 4 °C and by 4.0 log at 0 °C and 2.0 to 4.0 at 4 °C within lettuce tissue. Higher concentrations of these antimicrobials, 80 µl/liter of carvacrol or cinnamaldehyde or 16 µl/liter of AIT, reduced the pathogen by the same amount at the corresponding temperatures. The same antimicrobials reduce the pathogen by approximately the same amount in 2 days at 10 °C. This indicates that these antimicrobials will inactivate the pathogen in case a breakage in the cold chain occurs during handling packaged leafy greens. Reducing the pathogen on spinach surface was 1.0 log less than on lettuce surface at 0 and 4 °C. However, reducing the pathogen was 2.0 and 3.0 log less within spinach leaves than within lettuce ones at 0 and 4 °C, respectively. This indicates that spinach leaves might provide better protection for the pathogens from the antimicrobials action.

Surface disinfection of the lettuce leaves by silver nitrate solution supported the findings that the volatile antimicrobials are able to inactivate *E. coli* O157:H7 within lettuce leaves as the pathogen population was significantly reduced following the antimicrobials treatment on surface-disinfected samples. CSLM micrographs also supported the findings. The micrographs agreed with the microbiological analysis data as less viable cells were observed under CSLM in the antimicrobials treated samples. Although the concentrations of the antimicrobials employed in this research showed no adverse effect on the sensory quality on tomatoes, lettuce and spinach at the concentrations applied, a formal sensory study should be completed before practical use in food industry.