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FATE OF *ESCHERICHIA COLI* O157:H7 AND *LISTERIA MONOCYTOGENES* ON FRESH PRODUCE DURING SANITIZER EXPOSURE

By

Stephanie Lynn Rodgers

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ABSTRACT

FATE OF *ESCHERICHIA COLI* O157:H7 AND *LISTERIA MONOCYTOGENES* ON FRESH PRODUCE DURING SANITIZER EXPOSURE

By

Stephanie Lynn Rodgers

Produce (lettuce, strawberries, apples, and cantaloupe) inoculated with *E. coli* O157:H7 and *L. monocytogenes* was exposed to several sanitizer treatments. Ozone (3 ppm) and chlorine dioxide (5 ppm) reduced populations of *L. monocytogenes* and *E. coli* O157:H7 by 5.5 and 5.7 logs, respectively. Chlorine dioxide (3 ppm) and sodium hypochlorite (200 ppm) resulted in maximum reductions of 4.8 logs for *L. monocytogenes* and 5.1 logs for *E. coli* O157:H7. Peracetic acid gave reductions of 4.3 – 4.5 logs for *L. monocytogenes* and *E. coli* O157:H7. Fruit and Vegetable Wash and SCJP 16-162 produced maximum log reductions of 3.3 and 3.4 logs for *L. monocytogenes* and *E. coli* O157:H7, respectively. In comparison, produce treatment with SCJ 16-172 and Viper™ yielded reductions of 3.0 and 2.5 logs for *L. monocytogenes* and *E. coli* O157:H7, respectively. Fit™ was the least effective, giving maximum reductions of only about 1 log for *L. monocytogenes* and *E. coli* O157:H7.

Storage of sanitized produce indicated that the most effective sanitizers for eliminating pathogens, facilitated growth of yeasts and molds and contributed to rapid spoilage of produce (3 ppm ozone and 3 and 5 ppm chlorine dioxide), while peracetic acid (80 ppm) and sodium hypochlorite (100 and 200 ppm) treatment did not adversely affect product shelf life. Sensory analysis using the non-extended triangle test, indicated

that the only statistically significant differences between any of the treated and control samples occurred when whole apples were dipped in sodium hypochlorite (200 ppm) and when shredded lettuce was sprayed with peracetic acid (80 ppm).

The sequential use of copper ion (1 ppm), sodium hypochlorite (100 ppm) and sonication was assessed to decrease numbers of *E. coli* O157:H7 and *L. monocytogenes* during apple cider production. Using the hurdle approach, copper ion water did not significantly reduce populations of either pathogen; however, copper ion water/ sodium hypochlorite (100 ppm) decreased populations of *L. monocytogenes* and *E. coli* O157:H7 by 2.3 and 2.2 log CFU/g, respectively. After juiceration, the pulp contained ~ 1.1 – 1.3 log CFU/g of either pathogen with sonication decreasing the remaining pathogens in the expressed juice by ~ 2 logs CFU/ml. Based on these findings, a 5-log reduction for both pathogens was achievable using 100 ppm sodium hypochlorite followed by juiceration and sonication.

Employing confocal scanning laser microscopy (CSLM) to visualize attachment, colonization, location, and viability of *gfp*-transformed *E. coli* O157:H7 on lettuce and strawberries revealed generalized non-specific attachment to surface structures with some penetration through intact surfaces up to approximately 20μm. *E. coli* O157:H7 attachment to both products was more strongly influenced by water deposition and pooling than by any affinity to stomata or other surface structures. Based on CSLM analysis, viable *E. coli* O157:H7 cells that survived sanitizer treatments did so by organizing into groups or clusters in areas of pooling rather than by penetrating through intact produce surfaces.

To John for all of your love and support

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INTRODUCTION

Consumption of fresh, minimally processed fruits and vegetables has increased as consumers recognize their role in improved health and nutrition. However, new concerns exist about pathogens in raw fruits and vegetables, due to increased numbers of outbreaks of foodborne illnesses (Beuchat, 1998; Altekruse et al., 1998). The recent emergence of various foodborne pathogens, including *E. coli* O157:H7 and *L. monocytogenes*, which can survive and grow on an alarmingly wide range of fresh fruits and vegetables, have proven traditional barriers to be ineffective (Bartz, 1999). Fruits and vegetables were the third leading vehicle in foodborne outbreaks, accounting for 9,413 cases from 1990 to 2001 (CSPI, 2002).

The United States Food Safety Initiative, which was issued in 1997 by President Clinton, addressed public concerns about the safety of the national food supply. The aim was to advance food safety and reduce the incidence of foodborne illness to the greatest extent possible. Replacement of conventional sanitizers to treat or recycle food-processing wastewater with safer, environmentally-friendly, and more effective sanitizers is a goal for the fresh produce industry.

Chlorine, which is the most commonly used sanitizer in the fresh fruit and vegetable industry, is an unpredictable sanitizer, produces by-products that have been known to cause cancer in laboratory animals, and is affected by organic material, temperature, and pH. Therefore, alternative sanitizer treatments are being sought that are more stable over a wide range of temperatures and pH's, not inactivated by the presence

of organic materials, have greater bacteriocidal properties, and do not produce toxic by-products.

Our objective was to determine the efficacy of several proposed alternative sanitizer treatments as compared to traditional chlorine including: ozone, chlorine dioxide, peracetic acid, hydrogen peroxide, commercial organic acid washes, copper ion, and sonication. The produce selected was involved in recent produce outbreaks and represents a wide variety of surface topographies including: strawberries, cantaloupe, apples, apple cider, and lettuce. Known levels of either *Listeria monocytogenes* or *Escherichia coli* O157:H7 were used to artificially contaminate produce samples. The produce was then be exposed to one, or a combination, of the sanitizer treatments in order to determine the most efficacious. Efficacy was verified using selective and non-selective plating media with D-values (time required to reduce the population of viable bacteria by 1 log) for each produce-sanitizer treatment also determined. Finally, confocal microscopy was employed to determine colonization location, details of adhesion, and viability of pathogens on selected produce surfaces.

CHAPTER ONE

LITERATURE REVIEW

INTRODUCTION

In the past two decades, the popularity of diets containing fresh fruits and vegetables in the United States has increased as the distribution of fresh produce expanded to improve availability and consumer's desire for less processed and healthier foods. Fresh whole, cut and minimally-processed fruits and vegetables and juices are well-known as health foods because they are linked to lowered risk for cardiovascular diseases and certain types of cancers (McWilliams, 2001). Advanced worldwide agronomic technologies, which include processing, preservation, distribution, and marketing techniques allow agricultural and food industries to supply consumers with fresh, high quality produce throughout the year. At the same time, however, concerns about pathogens such as *Listeria monocytogenes* and *Escherichia coli* O157:H7 on raw fruits and vegetables has heightened due to the increasing number of foodborne outbreaks caused by fresh produce (Beuchat, 1998).

In its most recent estimate, CDC stated that foodborne disease is responsible for approximately 76 million illnesses, 325,000 hospitalizations and 5,000 deaths annually in the United States. Of those, known pathogens, such as *Salmonella*, *E. coli* O157:H7, *Campylobacter* and *L. monocytogenes* account for approximately 14 million illnesses, 60,000 hospitalizations and 1,800 deaths. Between 1982 and 1994, salad bars containing raw fruits and vegetables ranked as the third leading cause of infection by *E. coli* O157:H7 in the United States. Fruits and vegetables were the third leading cause of

foodborne illness outbreaks and were responsible for 9,413 reported cases between 1990 and 2001 (CSPI, 2002).

Fruits and vegetables can become contaminated with pathogenic microorganisms while growing in fields or orchards, or during harvesting, post-harvest handling, processing, and distribution. Normally, the outer surface of produce acts as a physical barrier, inhibiting bacteria from penetrating to the interior. Once the exterior structure is broken however, bacterial colonization can be rapid (Beuchat, 1992). Thus, mechanical processing, such as cutting, shredding, peeling and juicing may make produce more susceptible to microbial infiltration and growth. Numerous other factors contribute to the increased risks associated with fruits and vegetables including: the use of wastewater for irrigation, increased application of improperly composted manures to soils in which fruits and vegetables are grown, changes in packaging technology such as the use of modified or controlled atmosphere and vacuum packaging, extended time between harvesting and consumption, and changing food consumption patterns (e.g. eating more meals away from home, including greater use of salad bars) (Cliver, 1997; Altekruse et al., 1997).

Increased global trade in raw fruits and vegetables, as well as increased international travel in general, could also increase the risk of produce-associated illnesses (Potter et al., 1997). Finally, the susceptibility of the public to foodborne illnesses is changing due to increased numbers of elderly, immunocompromised, or chronically ill individuals (Hedberg et al., 1994). This changes are likely to lead to increased risk of illness associated with the consumption of raw produce that otherwise may contain certain levels of pathogens innocuous to healthy individuals. In the complex network of produce from the growers to the distributors, it is often difficult to determine the source

of contamination. Complications include wide distribution which disperses product lots, forgotten or unnoticed exposure, short shelf life for raw produce (which rapidly removes produce from store shelves before examination can occur) and the complex organization of growers, packers, shippers, distributors, retailers, and consumers that may involve multiple states (Tauxe et al., 1997). Contamination can occur anywhere in the chain and identification of the source or origin can be nearly impossible.

Although fruits and vegetables are not usually envisioned to be notorious foodborne disease vehicles, human illness caused by ingestion of raw fruits and vegetables has been well documented for over a century (Beuchat, 1998). In 1899, Morse traced celery consumption to a case of typhoid, while in 1903, Warry linked a similar case to watercress grown in sewage-fertilized soil (Block, 2001). In 1912, Creel determined that *Salmonella typhi* persisted on naturally contaminated lettuce and radishes for up to 31 days (Beuchat, 1998). According to Melick in 1917, harvested lettuce and radishes were also found to contain typhoid bacilli that were originally inoculated onto the seeds before planting (Block, 2001). While typhoid and cholera have been controlled by modern sanitation and public health engineering improvements, other foodborne diseases have become concerns.

E. COLI O157:H7

More recently, produce contaminated with pathogens such as *E. coli* O157:H7 and *L. monocytogenes* is a concern since outbreaks involving these two pathogens are occurring more frequently. In the United Sates, *E. coli* O157:H7 infections are usually associated with ground beef due to the presence of *E. coli* O157:H7 in the gastrointestinal

tract of cattle. However, fresh produce also has been implicated in outbreaks of infections with this pathogen.

E. coli O157:H7 was first recognized as a human pathogen in the United States following two hemorrhagic colitis outbreaks in 1982 that were linked to undercooked ground beef hamburgers from the same fast food restaurant chain (Riley et al., 1983). The first of these outbreaks, in Oregon resulted in 26 cases including hospitalizations out of 26 cases. In Michigan, three months later, 21 cases of hemorrhagic colitis including 14 hospitalizations were linked to *E. coli* O157:H7 which was isolated from both patients and a frozen ground beef patty.

E. coli O157:H7 is classified as enterohemorrhagic and produces one or two verotoxins designated VT-1 and VT-2. This disease, characterized by bloody diarrhea and severe abdominal cramps, nausea, and vomiting, has an incubation period of 1-5 days with symptoms persisting for 3 to 7 days. The infective dose for *E. coli* O157:H7 is thought to be extremely low with 1-10 cells capable of causing the illness (Padhye and Doyle, 1992). Some victims (< 15 %), particularly the very young develop hemolytic uremic syndrome (HUS) characterized by renal failure and hemolytic anemia. The disease can lead to permanent loss of kidney function. In the elderly, HUS, plus two other symptoms, fever and neurological symptoms, constitutes thrombotic thrombocytopenic purpura (TTP). This illness can have a mortality rate in the elderly as high as 50%.

E. COLI O157:H7 OUTBREAKS IN PRODUCE

The acid tolerance of *E. coli* O157:H7 is of particular concern, as it facilitates survival and growth in foods that might seem unlikely (Parish, 1997). The acid tolerance response of *E. coli* O157:H7 can be increased by exposure to acid conditions during growth which activates the expression of acid shock proteins and provides protection from normally lethal pH levels (Leyer et al., 1995; Miller and Kaspar, 1994 ; Splittstoesser et al., 1996). Expression of acid shock proteins may further protect the cell from other stress conditions such as heat, osmotic stress, and nisin activity (Leyer et al., 1995).

E. coli O157:H7 produce outbreaks have been reported in lettuce, cabbage, celery, alfalfa sprouts, apple cider, and cantaloupe (Zhao et al., 1993; Beuchat, 1996a). This pathogen has also previously been isolated from cabbage, cauliflower, celery, cilantro, and coriander obtained from markets in Mexico City (Zepeda-Lopez et al., 1995).

In 1991, 23 people became ill in Massachusetts after consuming apple cider that was contaminated with *E. coli* O157:H7 from a traditional cider mill (Besser et al., 1993). Symptoms of infection ranged from self-limited, watery diarrhea to hemorrhagic colitis, HUS, and TTP. Since most of the apples used for cider-making were collected from the ground, contamination was thought to have occurred before harvest from exposure to bovine or deer feces. In 1994 at the University of Texas, 11 people with hemorrhagic colitis were determined to be infected with *E. coli* O157:H7 (Barnett et al., 1995). Assessment of food preparation and storage practices indicated that broccoli and other uncooked salad bar items were cross-contaminated by raw ground beef. In 1995, Ackers et al., (1996) reported that an outbreak of *E. coli* O157:H7 infection involving at least 40

people in Montana was epidemiologically linked to leaf lettuce consumption, which was spray irrigated with contaminated surface water. In a 1996 outbreak involving unpasteurized apple juice in the Pacific northwest, Odwalla brand apple juice and juice mixtures contaminated with *E. coli* O157:H7 were implicated; and all of their apple juice containing products recalled nation wide (FDA, 1996). In 1999, 46 people on Ohio ingested lettuce contaminated with *E. coli* O157:H7 from a salad bar which was contaminated by an ill worker who prepared the lettuce. In March 1999, 72 patrons in a Nebraska restaurant were infected with *E. coli* O157:H7 from iceberg lettuce prepared by a sick worker (Burnett and Beuchat, 2000).

L. MONOCYTOGENES

Contamination of egg, meat, dairy products and raw fruits and vegetables by *L. monocytogenes*, a gram-positive rod that is ubiquitous in the environment, has caused significant public health threats. *L. monocytogenes* can be found on decaying vegetation, soil, animal feces, sewage, silage, and water and can easily contaminate raw fruits and vegetables (Beuchat, 1992). Low level contamination of foods consumed by humans is not uncommon, but contamination of raw fruits and vegetables by *L. monocytogenes* is a particular concern because the pathogen is psychrotrophic and can grow on raw fruits and vegetables during cold storage (~ 4°C) which may not be further processed before consumption. *L. monocytogenes* can also grow over a wide pH and temperature range of 4.1 to 9.6 and 0.5°C to 45°C respectively, which facilitates growth in a large variety of foods (Van Renterghem et al., 1991).

Listeriosis in non-pregnant healthy humans is extremely rare. However, susceptible populations to Listeriosis include those with neoplasm, AIDS, alcoholism, diabetes (Type 1 in particular), cardiovascular disease, renal transplant, and corticosteroid therapy. When a susceptible adult contracts the disease, the most common symptoms include meningitis, meningoencephalitis, or encephalitis. Pregnant women who contract the disease may have symptoms resembling influenza or may be asymptomatic. However, this infection is far more serious to the unborn fetus with abortion, premature birth or delivery of a stillborn infant often the end result. The oral infective dose of *L. monocytogenes* is thought to be fewer than 1,000 total organisms, but it will vary with the strain and susceptibility of the victim. A recent study by Mead et al. (1999) determined that even though listeriosis is a rare human illness, it remains a primary cause of death from a foodborne pathogen.

L. MONOCYTOGENES OUTBREAKS IN PRODUCE

L. monocytogenes contamination has not traditionally been associated with raw produce, yet contamination of fruits and vegetables with the pathogen has become increasingly more common. Incidences of *L. monocytogenes* infection have been associated with fresh produce including: coleslaw, lettuce, cabbage, bean sprouts, cucumbers, potatoes, celery, radishes, salads, and tomatoes (Beuchat, 1996b).

In 1979, at least 23 hospitalized patients in Boston were diagnosed with listeriosis. Hospital food was epidemiologically linked as the vehicle of infection in this outbreak, and patients who consumed lettuce, carrots, and radishes were more likely to contract the illness. In the Maritime Provinces in Canada, an outbreak of *L.*

monocytogenes occurred in 1981 demonstrating, for the first time, that *L. monocytogenes* was a foodborne pathogen (Schlech et al., 1983). Thirty-four cases of perinatal listeriosis and seven cases of adult disease were diagnosed with symptoms of septicemia, meningitis, and encephalitis. It was subsequently determined that coleslaw obtained from the refrigerator of a patient was positive for *L. monocytogenes.* An investigation, revealed that fresh sheep manure was used in fields where the cabbage was grown.

Since *L. monocytogenes* is a natural inhabitant of soil, it is found on a wide range of produce. Sizmur and Walker (1988) identified *L. monocytogenes* in 4 of 60 pre-packaged salads in the U.K., which contained cabbage, celery, lettuce, cucumber, onion, leeks, watercress and fennel. Similarly, Beckers et al. (1989) identified *L. monocytogenes* in 11 of 25 samples of sliced raw vegetables in the Netherlands and Harvey and Gilmour (1993) reported that 7 of 66 samples of salad vegetables and salads produced in Northern Ireland also tested positive for the organism. Moreover, *L. monocytogenes* has been isolated from tomatoes and cucumbers in Pakistan (Vahidy, 1992), and Arumugaswamy et al. (1994) determined that bean sprouts (85%), sliced cucumbers (80%), and leafy vegetables (22.7%) examined in Malaysia tested positive for *L. monocytogenes.* Numerous studies have documented that *L. monocytogenes* can grow on raw produce stored at refrigeration temperatures. Asparagus, broccoli, and cauliflower stored at 4°C (Berrang et al., 1989), and on lettuce stored at 5°C can support growth of *L. monocytogenes* (Beuchat and Brackett, 1990; Steinbrugge et al., 1988).

SANITIZERS

Harvested fruits and vegetables often contain microbial populations of 10^4- to 10^6 CFU/ g (Bracket et al., 1994). While water alone can effectively remove organic matter from produce, incorporation of a sanitizer is necessary to reduce microorganisms by more than 1-2 logs (Abdelnoor et al., 1983). The use of chemicals to enhance the safety of fresh and processed fruits and vegetables is of great interest to the food industry. The most commonly used sanitizer is chlorine, but alternative sanitizers such as peracetic acid, chlorine dioxide, hydrogen peroxide, copper ion, and ozone, are gaining interest for application in the food industry. Table 1 lists the effectiveness of many emerging sanitizers that have been tested on raw fruits and vegetables contaminated with *L. monocytogenes* and *E. coli* O157:H7. Sanitizers that can be used to wash fruits and vegetables are regulated by the U.S. Food and Drug Administration in accordance with the Federal Food, Drug and Cosmetic Act as outlined in the Code of Federal Regulations, Title 21, Ch. 1, Section 173.315 (CFR, 1994).

In response to public concerns about the safety of the national food supply, the President of the United States and Congress issued a new federal initiative in 1997 (entitled the President's Food Safety Initiative) to improve the nation's food safety system and our environment. One of the steps in improving food safety is the identification of safer and more effective sanitizers that are able to replace traditional sanitizing agents to treat or recycle food-processing wastewater. The use of sanitizers by the food industry is continually evolving through research that expands our understanding of their application and efficacy. The challenge is to attain the 5-log reduction standard set by the Food and Drug Administration (FDA) for selected commodities. Research and

Table 1. Examples of Sanitizers Used to Inactivate E. coli O157 H7 and L. monocytogenes on Raw Fruits and Vegetables

Sanitizer	

Table 1. Examples of Sanitizers Used to Inactivate *E. coli* O157:H7 and *L. monocytogenes* on Raw Fruits and Vegetables

Sanitizer	Concentration	Pathogen	Produce	Log Reduction	Reference
Sodium hypochlorite	200 ppm	E. coli O157:H7	Iceberg lettuce	0.3 logs	Takeuchi and Frank (2001)
Sodium hypochlorite	200 ppm	E. coli O157:H7	Iceberg lettuce	0.7 logs	Takeuchi and Frank (2000)
Sodium hypochlorite	200 ppm	L. monocytogenes	Lettuce	1.3 to 1.7 logs	Zhang and Farber (1996)
Sodium hypochlorite	200 ppm	L. monocytogenes	Cabbage	0.9 to 1.2 logs	Zhang and Farber (1996)
Sodium hypochlorite	100 ppm	E. coli O157:H7	Strawberry	1.3 logs	Yu et al. (2001)
Sodium hypochlorite	200 ppm	E. coli O157:H7	Strawberry	1.34 logs	Yu et al. (2001)
Hydrogen peroxide	1%	E. coli O157:H7	Strawberry	1.2 logs	Yu et al. (2001)
Hydrogen peroxide	3%	E. coli O157:H7	Strawberry	2.18 logs	Yu et al. (2001)
Sodium hypochlorite	200 ppm	E. coli O157:H7	Cantaloupe	2.78 logs	Park and Beuchat (1999)
Sodium hypochlorite	2000	E. coli O157:H7	Cantaloupe	3.38 logs	Park and Beuchat (1999)
Hydrogen peroxide	0.20%	E. coli O157:H7	Cantaloupe	1.08 logs	Park and Beuchat (1999)
Hydrogen peroxide	1.00%	E. coli O157:H7	Cantaloupe	2.38 logs	Park and Beuchat (1999)
Peracetic acid	40 ppm	E. coli O157:H7	Cantaloupe	3.08 logs	Park and Beuchat (1999)
Peracetic acid	80 ppm	E. coli O157:H7	Cantaloupe	2.9 logs	Park and Beuchat (1999)
Sodium hypochlorite	200 ppm	E. coli O157:H7	Honeydew melon	2.6 logs	Park and Beuchat (1999)
Sodium hypochlorite	2000 ppm	E. coli O157:H7	Honeydew melon	2.6 logs	Park and Beuchat (1999)
Hydrogen peroxide	0.20%	E. coli O157:H7	Honeydew melon	1.40 logs	Park and Beuchat (1999)
Hydrogen peroxide	1.00%	E. coli O157:H7	Honeydew melon	1.6 logs	Park and Beuchat (1999)
Peracetic acid	40 ppm	E. coli O157:H7	Honeydew melon	2.6 logs	Park and Beuchat (1999)
Peracetic acid	80 ppm	E. coli O157:H7	Honeydew melon	2.6 logs	Park and Beuchat (1999)
Chlorine dioxide	5 ppm	L. monocytogenes	Lettuce	1.1 logs	Zhang and Farber (1996)
Sodium hypochlorite	200 ppm	E. coli O157:H7	Apples	2.1 logs	Wright et al. (2000)

Table 1. Cont.

Sanitizer	Concentration	Pathogen	Produce	Log Reduction	Reference
Hydrogen peroxide	3%	E. coli O157:H7	Broccoli	2 logs	Peters (1995)
Hydrogen peroxide	3%	E. coli O157:H7	Tomatoes	4 logs	Peters (1995)
Peracetic acid	80 ppm	E. coli O157:H7	Apples	2.5 logs	Wright et al. (2000)
Chlorine dioxide	0.3 ppm	L. monocytogenes	Green pepper	1.87 logs	Han et al. (2001)
Chlorine dioxide	3.0 ppm	L. monocytogenes	Green pepper	3.67 logs	Han et al. (2001)
Sodium hypochlorite	200 ppm	L. monocytogenes	Brussels sprouts	2 logs	Brackett (1987)

commercial applications have indicated that alternative sanitizers including ozone, chlorine dioxide, hydrogen peroxide, peracetic acid, copper ion, as well as physical treatments such as sonication may replace chlorine with more benefits. In evaluating potential sanitizers as alternatives to chlorine, the Food Manufacturing Coalition (1996) stated: "Any new technology should be as effective as chlorinated solutions in reducing (especially bacterial) contamination and able to meet USDA standards for microbial count reduction in the particular industry, whether poultry, dairy, beef or other."

The efficacy of a sanitizer is a function of both time and concentration such that, in general, the more concentrated the sanitizer and longer the treatment time, the more rapid its action. For example, Park and Beuchat (1999) found that 2000 ppm of chlorine reduced aerobic microorganisms > 2 logs in 3 minutes on honeydew melons, but 200 ppm of chlorine reduced populations by only 1 log. However, a threshold can be reached and a further increase in concentration or time may give no further improvement in effectiveness, since the relationship between time or concentration and effectiveness is not linear. Rather, it is exponential, e.g. doubling the concentration does not merely double the effectiveness, but increases it ten times. Mazollier (1999) studied the ability of chlorine solutions to decrease total counts and fecal coliforms on green salad leaves during washing. Total microbial counts were reduced by 2-logs when concentrations of free chlorine were 50 ppm on lettuce, but higher concentrations (up to 200 ppm) did not reduce the counts any further. Hence, there is an optimum concentration of sanitizer, below which the effectiveness is reduced, and above which there is no further improvement.

Temperature affects the efficacy of a sanitizer, such that all sanitizers have an optimum temperature. For chlorine compounds, increasing temperature reduces the required contact time, which was demonstrated by El-Kest and Marth (1988) who found that the rate of diffusion of chlorine into microbial cells increased at higher temperatures. Similarly, Zhang and Farber (1996) found that for lettuce, the bacteriocidal effect of chlorine on *L. monocytogenes* was higher at 22°C than at 4°C.

The effectiveness of some sanitizers is influenced by the pH of a solution. For chlorine, as the pH of the solution increases, the effectiveness of chlorine decreases due to the inability of the hypochlorite ion to pass through the bacterial cell wall. For example, Adams et al. (1989) found that lowering the pH from 9 to 5 increased the antimicrobial effect of chlorine 4-fold on lettuce.

The Association of Official Analytical Chemists (AOAC) definition of an efficacious sanitizer is one that can reduce a viable population of organisms by 5 logs within thirty seconds (Aram, 1995). According to the AOAC, an ideal sanitizer should have the following properties: (1) broad spectrum of activity, (2) organisms do not develop resistance, (3) readily soluble in water, as all sanitizers are applied in the aqueous phase, (4) chemically stable during storage, so that antimicrobial properties are not diminished over time, (5) non-toxic to humans so that sanitizers can be applied safely, (6) resistant to inactivation by organic matter, (7) non-corrosive to construction materials, (8) possess surfactant activity, (9) odorless, and (10) available in large quantities at a reasonable cost.

D-values are often used to report the efficacy of a particular sanitizer. When a graph is drawn of the logarithm of the number of surviving cells against time, a line is

obtained. From this graph a d-value (decimal reduction time) can be obtained by determining the time required to decrease the population of bacteria by 1 log (90%). This demonstrates that the death rate is analogous to a first-order reaction, i.e. the number of cells dying is proportional to the number of cells present.

TYPES OF SANITIZERS

Chlorine Compounds. Chlorine was discovered in 1774 by the Swedish chemist Scheele with the discovery of hypochlorites by the French chemist Berthollert following in 1789 (Block, 2001). Chlorine was the first chemical that was found to be effective against noxious odors, which, at the time, were thought to be the cause of contagious diseases. Later in 1825, the Frenchman Labarraque reportedly used calcium hypochlorite to sanitize morgues, sewers, and hospital wards. Currently, chlorine is the most widely used sanitizer in the food industry for disinfection of fruits and vegetables (Nguyen-the and Carlin, 1994). Chlorine is typically used at concentrations of 5 to 200 ppm with a contact time of 1-2 minutes for raw fruits and vegetables (Beuchat, 1996b). Hypochlorites such as calcium hypochlorite ($CaCl_2$) and sodium hypochlorite (NaOCl) are produced when a chlorophor (chlorine-containing compound) is dissolved in water. Chlorine is then released to form hypochlorous acid (HOCl) which is the effective germicide.

The reaction can be expressed by:

$$Cl_2 + H_2O \rightleftharpoons HOCl + HCl$$

Which is accompanied by the secondary reaction

$HOCl \rightleftharpoons ClO^- + H+$

At pH ≤ 2, chlorine is in the elemental form, yet when the pH increases above 10, chlorine is in the hypochlorite ion form (Beuchat, 1996b). At pH 5-10, roughly equal amounts of both the hypochlorous acid and hypochlorite ion forms are present which greatly reduces the bacteriocidal activity of chlorine. The dissociation of HOCl depends on the pH. As the pH of the solution is reduced, the equilibrium is in favor of HOCl. However, since metal containers and processing equipment are often susceptible to corrosion at low pH, a pH of 6.0-7.5 is typically most effective without damaging equipment surfaces. The percentages of chlorine as HOCl at pH 6.0 and 8.0 are about 97% and 23% respectively, at 20°C. Toxic chlorine gas (Cl_2) is formed at a pH below 4 (Beuchat, 1996).

At a given pH, equilibrium is in favor of HOCl as the temperature is decreased. This is because chlorine vaporizes as the water temperature increases. Chlorine rapidly loses activity when in contact with organic matter or exposed to air, light, or metals. A concern among people who use chlorinated water as a disinfectant is that prolonged exposure to chlorine vapors can cause irritation to the skin and respiratory tract. Maximum solubility of chlorine is achieved in water at about 4°C. However, the temperature of the chlorinated water should ideally be at least 10°C higher than that of fruits or vegetables to achieve a positive temperature differential, thereby minimizing the uptake of wash-water through stem tissues and open areas in the skin or leaves, whether due to mechanical assault or naturally present (Burnett et al., 2000). Eliminating the uptake of wash-water that may contain microorganisms, including those that may cause

human illness, would be considered a critical control point in handling, processing, and disinfection of raw fruits and vegetables.

The "available chlorine content" (strength of hypochlorite solutions) is a measure of the amount of chlorine that can be released from hypochlorite. The stability of free available chlorine in solution depends largely on the following factors: a) chlorine concentration, b) presence and concentration of catalysts or reducing agents, c) pH of the solution, d) temperature of the solution, e) presence of organic material, and f) ultraviolet radiation. Any of these factors, alone or in combination, may greatly affect the stability of free available chlorine in solution. The most stable free available chlorine solutions are those having the following characteristics: (a) low chlorine concentration, (b) low or nonexistent levels of copper, cobalt, nickel, or other catalysts, (c) high alkalinity, (d) low temperature, (e) absence of organic material and (f) storage in dark closed containers (i.e. shielded from ultraviolet light). The most common method for measurement of available chlorine content is the iodometric method. This technique is based on the principal that free chlorine liberates iodine in the acidified test solution containing potassium iodide (KI) and the liberated iodine is titrated with a standard sodium thiosulfate solution to a starch endpoint.

Chlorination is an effective means for inactivating pathogens on fruits and vegetables. Nguyen-the and Carlin, (1994) found that dipping Brussels sprouts inoculated with 10^6 CFU/g of *L. monocytogenes* into 200 ppm chlorine solution for 10 seconds decreased *L. monocytogenes* populations by approximately 2 logs CFU/g. Wright et al. (2000) also showed that a 2 minute exposure to 200 ppm sodium hypochlorite reduced *E. coli* O157:H7 populations by about 2 logs on whole apples.

Although shown to be an effective sanitizer, the mechanism by which chlorine kills bacterial cells is not fully understood. It has been previously shown that the sanitizing ability of chlorine decreases with an increase in pH and vice versa, which correlates with the concentration of undissociated hypochlorous acid. Therefore, hypochlorous acid is likely the active ingredient. Many researchers support the theory that chlorine damages the bacterial cell membrane which leads to leakage of cell components, and the formation of substitution products with proteins and amino acids (Banwart, 1981). The method of chlorine inactivation requires that chlorine must first diffuse through the bacterial cell wall with oxidation of the bacterium's enzymes by hypochlorous ion resulting in death. The diffusion of chlorine in the form of hypochlorous acid (HOCl) into the cytoplasm of the bacterium is a process that is highly time dependent.

Chlorination is relatively inexpensive, and the free residual chlorine content of treated water protects against pathogens surviving the actual treatment period and causing re-contamination. Unfortunately, chlorine compounds are corrosive, inherently unstable, and produce trace amounts of organochlorine compounds (chemicals that have been shown to cause cancer in laboratory animals) including chloroform, trihalomethane (THM), bromodichloromethane, and MX [3-chloro-4-(dichloromethyl)-5-hydroxyl-2(5H)-furanone] (Richardson, 1998). THM is formed by the reaction of free chlorine (HOCL, OCl^-) with soluble organic compounds. In 1979, the U.S. Environmental Protection Agency established a maximal THM limit in drinking water of 100 ug/L (Richardson et al., 1998).

Chlorine compounds are also rapidly inactivated by organic material, which is inherent to raw produce surfaces. Chlorine has a strong tendency to acquire extra electrons which makes it a very powerful oxidizing agent. Since chlorine is also a non-selective oxidant, almost any reduced substance in water will react and consume chlorine (NH_3, CN^-, organics, Fe^{2+}, Mn^{2+}, S^{2-}). This effect is commonly referred to as the chlorine demand which may be defined as the difference between the chlorine applied and the chlorine remaining in water after some has been consumed by water impurities including inorganic substances. For instance, Park and Beuchat (1999) found that treatment of cantaloupe with 2000 ppm chlorine treatment reduced *E. coli* O157:H7 populations < 10-fold and reasoned that the very high level of organic matter on the cantaloupe neutralized the chlorine before its action was manifest.

The effectiveness of chlorine compounds is decreased at low temperatures. As temperature increases, the rate of diffusion of disassociated chlorine through the cell wall increases while at the same time, the percent of hypochlorous acid decreases. Thus, the sanitizers effectiveness increases with the temperature as the rate of diffusion increases.

The effectiveness of chlorine compounds in reducing microorganisms on produce is often unpredictable. For example, Park and Beuchat (1999) found that spray treatment of cantaloupe with 200 ppm chlorine was no more effective in eliminating viable *E. coli* O157:H7 cells than spraying with water. Similarly, a chlorine dip of 200 ppm reduced the population of *L. monocytogenes* about 2 logs, whereas dipping in water alone reduced populations 1 log on Brussels sprouts (Bracket, 1987). Therefore, the same concentration of chlorine may result in significantly different log reductions depending on the type of produce examined.

Examination of alternative sanitizers has demonstrated that some alternative sanitizers may be more effective than chlorine at lower concentrations. This was illustrated by Junli et al. (1997), who compared the bacteriocidal effectiveness of chlorine with chlorine dioxide. At pH 8.5, 0.25 ppm chlorine dioxide caused a 99% destruction of *E. coli* O157:H7 in 15 sec, whereas 5 minutes was required for chlorine. Similarly, Korich et al. (1990) examined purified *Cryptosporidium* oocysts that were exposed to ozone, chlorine dioxide, and chlorine. Greater than 90% inactivation was achieved by treatment with 1 ppm ozone for 5 minutes, with 1.3 ppm chlorine dioxide yielding 90% inactivation after 1 hour and 80 ppm sodium hypochlorite required approximately 90 minutes for 90% inactivation. Park and Beuchat (1999) reported that treatment of cantaloupes with 200 ppm chlorine was as effective as 80 ppm peracetic acid, while Zhang and Farber (1996) obtained a maximum $\log_{10}$ reduction of 1.3 – 1.7 log *L. monocytogenes* CFU/g on shredded lettuce using 200 ppm chlorine, which is similar to water rinsing. El-Kest and Marth (1988) determined that bacterial cells are not intrinsically resistant to chlorine, but rather its ineffectiveness may be due to other factors such as insufficient wetting of the hydrophobic surface of the waxy cuticle of fruits and vegetables as suggested by Adams et al. (1989).

Chlorine Dioxide. Chlorine dioxide was first used for disinfection in the water industry in 1940 by the Mathieson Chemical Company who produced a powdered sodium chlorite which, when added to water, produced chlorine dioxide. Niagara Falls, New York recognized its potential and was the first water treatment plant to incorporate chlorine dioxide in their water purification process in 1944 (White, 1972). More recently,

chlorine dioxide has received attention as a sanitizer for fruits and vegetables due to its advantages over chlorine. It is a very powerful oxidizer derived from sodium chlorite, with an oxidation capacity that is almost 3 times greater than chlorine. Chlorine dioxide is effective over a wide pH range, but not affected by high levels of organic matter, and does not dissociate rapidly in water (White, 1972). It has been shown to kill a large number of microorganisms, including spores that are resistant to treatment with chlorine (Richardson et al., 1994). Chlorine dioxide does not react with ammonia to form chloramines as does chlorine. Since chlorine dioxide is less reactive than aqueous chlorine in interacting with organic compounds, fewer toxic, mutagenic by-products are produced (Richardson et al., 1998). Additionally, the number of disinfection by-products observed for chlorine dioxide by Richardson et al. (1998) was 3-5 times lower compared to chlorination.

The Food and Drug Administration (FDA 1998) amended the food additive regulations to allow the safe use of chlorine dioxide as an antimicrobial agent in water to wash fruits and vegetables in an amount not exceeding 3 ppm residual chlorine dioxide. The U.S. Environmental Protection Agency (EPA) approved use of chlorine dioxide as a disinfectant for potable water treatment limiting the residual to 1 ppm (U.S. Federal Rgister, 2000).

Chlorine dioxide, a yellow-green gas, was first produced by Chenevix in 1802. It is generated by an oxidative process involving addition of concentrated hydrochloric acid to sodium chlorite which can then be incorporated into de-ionized water. The reaction is as follows:

$$2NaClO_2 + Cl_2 \rightarrow 2ClO_2 + 2NaCl$$

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Chlorine dioxide then undergoes the following reaction in water:

$$ClO_2 + 2H+ + 3e \rightleftharpoons ClO- + H_2O$$

The lethality of chlorine dioxide against bacteria involves the loss of membrane permeability control with non-specific oxidative damage to the outer cell membrane. This is followed by destruction of the trans-membrane ionic gradient and disruption of protein synthesis (Berg et al., 1986).

The efficacy of chlorine dioxide is high when studied in model aqueous systems. For example, Junli et al. (1997) reported that *E. coli* populations in an aqueous suspension decreased >3 logs after 1 minute of exposure to 3 ppm chlorine dioxide, while Roller et al. (1980) found that 2 ppm of chlorine dioxide resulted in a 3.5 log reduction of *Escherichia coli* O157:H7 populations in distilled water within 30 seconds.

Chlorine dioxide has been used to reduce microorganisms on lettuce and cabbage (Zhang and Farber, 1996), fish (Lin et al, 1996), and beef (Cutter and Dorsa, 1995), and reduce microbial populations in poultry chiller water (Tsai et al., 1992) and cucumber wash water (Reina et al., 1995). When Zhang and Farber (1996) inoculated *L. monocytogenes* onto the surface of shredded lettuce and cabbage, a 30-second exposure to 5 ppm chlorine dioxide reduced the levels of *L. monocytogenes* by more than 1 log. Similarly, Wisniewsky et al. (2000) reported that 80 ppm chlorine dioxide reduced *E. coli* O157:H7 populations by approximately 4 logs on whole apples after 10 minutes.

Lillard (1979) compared the effectiveness of chlorine and chlorine dioxide in reducing the number of bacteria present in poultry processing water. She found that 5 ppm chlorine dioxide was as effective as 34 ppm chlorine. Results of this study are

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similar to those of Reina et al. (1995) who found that 1.3 ppm chlorine dioxide reduced the numbers of total aerobes in cucumber wash water by 2-6 log CFU/mL.

Unfortunately, the use of chlorine dioxide for sanitizing fruits and vegetables has several disadvantages over traditional chlorination. Chlorine dioxide is unstable and must be generated on site. It can be explosive when concentrated and decomposes at temperatures greater than 30°C.

Hydrogen Peroxide. Hydrogen peroxide was first discovered in 1818 by the French chemist Thenard. In 1858, English physician B.W. Richardson used it to remove foul odors from sewer gases and suggested using it as a sanitizer (Block, 2001). Hydrogen peroxide is classified as GRAS and is approved for direct application onto food for certain specified applications (CFR, 1994). It is easily destroyed by heat, catalase and peroxidase to give the innocuous end products oxygen and water. Pure hydrogen peroxide typically contains stabilizers that inactivate contaminants and eliminate decomposition.

Hydrogen peroxide is effective against a large number of organisms including bacteria, yeasts, fungi and viruses. Generally, hydrogen peroxide is more effective against gram-negative than gram-positive bacteria. It is not affected by changes in pH or presence of organic debris. For example, Sagripanti and Bonifacino, (1997) determined that addition of 25% fetal bovine serum to hydrogen peroxide did not affect its sporicidal action. Dipping sprouts in 200 and 500 ppm chlorine or 2% and 5% hydrogen peroxide, led to similar reductions in *Salmonella* populations after 2 minutes (Beuchat, 1998).

Slightly more than a 2 log reduction was observed after treatment with 200 ppm chlorine or 2% hydrogen peroxide.

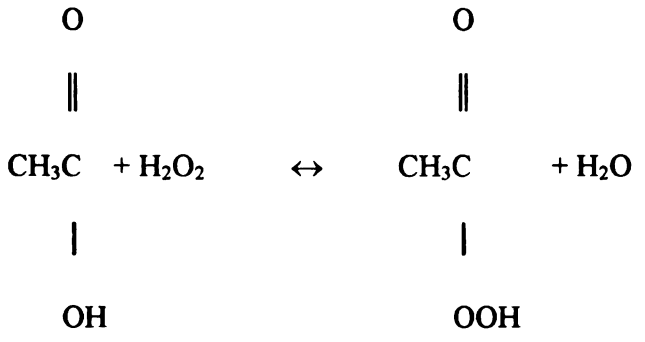
The same concentrations of chlorine and hydrogen peroxide were less effective against *Salmonella* on cantaloupe cubes causing reductions of less than 1 log. A study by Peters (1995) reported that 3% hydrogen peroxide decreased *E. coli* O157:H7 populations on broccoli and tomatoes by 2 and 4 logs in 5 minutes, respectively. A study by Yu et al. (2001) examined the ability of hydrogen peroxide to inactivate *E. coli* O157:H7 on strawberries. They reported 1.2 and 2.1 log CFU/g reductions after 1 minute of treatment using 1% and 3% hydrogen peroxide solutions, respectively. Results from studies on a limited number of fruits and vegetables indicate that hydrogen peroxide has high potential for use as a sanitizer. Park and Beuchat (1999) observed that 1% hydrogen peroxide reduced *E. coli* O157:H7 on cantaloupes by 2.3 logs compared to a water rinse of inoculated fruit. Some fruits and vegetables (mushrooms, some types of berries, and lettuce) however, may not be good candidates for hydrogen peroxide due to undesirable changes in produce color. Dipping freshly-cut green bell pepper, cucumber, zucchini, cantaloupe, and honeydew melon in hydrogen peroxide solution had no adverse effect on appearance, flavor, or texture, but it induced severe browning of shredded lettuce (Beuchat, 1996b).

Peracetic acid. The sanitizing power of peracetic acid was first reported in 1902 by Freer and Novy, who were impressed by the "the excellent disinfecting and cold sterilizations actions of peracetic acid" (Block, 2001). Peracetic acid did not become commercially available until much later in the United States after development of

commercial processes for production. It is increasingly used in clean-in-place sanitizing in beverage and dairy plants because of its effectiveness against yeasts and molds (Marriott, 1994). More recently, peracetic acid has been used effectively as an alternative to chlorine for disinfection of raw fruits and vegetables. Peracetic acid was approved by the FDA in 1986 allowing for its use as an indirect food additive in sanitizing solutions.

The antimicrobial effect of organic acids including peracetic acid has been attributed to depression of pH below the growth range and metabolic inhibition by the undissociated acid molecule which is the lethal species (Taormina and Beuchat, 1999). Unlike chlorine, peracetic acid is not corrosive to processing equipment, produces no toxic disinfection by-products, remains effective in the presence of organic matter, and is not affected by changes in temperature. Dilute peracetic acid solutions are highly unstable; a 1% peracetic acid solution loses half of its strength through hydrolysis in six days (Greenspan et al., 1955).

Peracetic acid is produced by the reaction of acetic acid with hydrogen peroxide in the presence of sulfuric acid, which acts as a catalyst as shown:



The only residuals produced are acetic acid, oxygen, water, hydrogen peroxide, and dilute sulfuric acid.

Peracetic acid is effective against a large number of microorganisms commonly found on produce. Zhang and Farber (1996) reported that numbers of *L. monocytogenes* decreased by up to 1.4 logs in 5 minutes using 1% acetic acid, with 90 ppm peracetic acid reducing in total counts and fecal coliforms by 2 logs on lettuce, which was as effective as 100 ppm chlorine. Similarly, Wisniewsky et al. (2000) reported a 3-log reduction of *E. coli* O157:H7 on apples treated with 80 ppm peracetic acid for 5 minutes. Wright et al. (2000) examined the effectiveness of peracetic acid in reducing of *E. coli* O157:H7 populations on apples. Use of 80 ppm peracetic acid resulted in a 2.5 logs reduction after 2 minutes. Park and Beuchat (1999) reported that peracetic acid (80 ppm) and sodium hypochlorite (200 ppm) were not significantly different from each other on cantaloupe with *E. coli* O157:H7 populations decreasing 2.8 logs. Beuchat (1998) also reported that treatment of pre-packaged salads with 90 ppm peracetic acid reduced total mesophilic bacterial counts by 1 log which was not significantly different than treatment with 100 ppm sodium hypochlorite.

Ozone. Ozone has been used for many years as a disinfectant in water and wastewater treatments. In 1988, a U.S. patent was issued to Fewson for an apparatus to produce ozone for deodorizing sewer gases. Ozone was first introduced as a chemical disinfectant in water treatment in 1893 in Oudshourn, Netherlands (Rice et al., 1982). Ozone treatment of water has been shown to reduce the microbial load, remove color, remove odor and taste, control algae, control macrofuling, and oxidize organic and inorganic compounds. In 1902, Siemens and Halske built the first full scale ozone-generating plant for water treatment in Germany (Kozhinov, 1968). In 1906,

commercial-scale disinfection of potable water with ozone was put into practice in Nice, France. The population there increased from 150,000 to 250,000 by 1956, and water disinfected daily by ozone increased to 20 million gallons (Lebout, 1959). Ozonation has been adopted as standard practice for water treatment and disinfection in many cities in France, the Netherlands, Germany, Austria, and Switzerland. The first potable water treatment plant to use ozone continuously in the U.S. was installed in Whiting, Indiana in 1940. Currently, more than 200 water and wastewater treatment plants employ the use of ozone for treatment of water supplies in the U.S.

Ozone is a more efficient sanitizer than chlorine, for inactivating large numbers of organisms, including chlorine-resistant *Cryptosporidium* and *Giarda* oocysts, both of which have invaded food and water supplies and caused deaths in recent years (Kim et al., 1999). Ozone can destroy pesticides and chemical residues, such as chlorinated by-products (Graham, 1997) and convert non-biodegradable organic materials into biodegradable forms (Kim et al., 1999). Unlike chlorine, the efficacy of ozone is not affected by the solution pH and ozone is non-corrosive to processing equipment (Graham, 1997). Ozone can also chemically remove ethylene gas to slow down the ripening process of fruits and vegetables, thus extending shelf life (Rice et al., 1982). The economic advantages of using ozone over chlorine were examined by Forsythe and Waldroup (1994) and included reduced water purchases, reduced sewage treatment costs, and savings in electrical energy from recycling ozonated water.

Ozone is formed by the excitation of molecular oxygen (O_2) into atomic oxygen (O) in an energizing environment that allows the recombination of atoms into O_3. Ozone production is enhanced at low temperatures since high temperatures encourage thermal

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decomposition of ozone. The ozone molecule decomposes spontaneously to diatomic oxygen reducing the accumulation of inorganic waste in the environment (Kim et al., 1999). Richardson et al. (1998) determined that the by-products of ozone are less likely to cause deleterious health effects than the by-products of chlorine treatment. Ozone is produced in the following reaction:

$$3O_2 \rightleftarrows 2O_3 + \text{heat and light}$$

Ozone is usually produced using the corona discharge method which uses a high-voltage alternating current across a discharge gap through which dry air or O_2 is passed. Oxygen electrons are excited with oxygen molecules splitting and recombining with other oxygen molecules to form ozone. The half-life of molecular ozone in air is relatively long (about 12 hrs). However, in aqueous solutions the half life of ozone is decreased depending on temperature, pH, UV light, initial O_3 concentration and concentration of radical scavengers. Ozone concentrations are typically measured using the indigo colorimetric method which is the recommended method standard (American Public Health Association et al., 1992). This method relates the stepwise decolorization of sulfonated indigo dye across the carbon-carbon double bond by ozone to determine ozone concentration with the change in absorbance measured spectrophotometrically.

Ozone oxidizes in water through two different pathways: direct oxidation by molecular ozone, and indirect oxidation by free radical species formed from the autodecomposition of ozone, and from reactions between ozone and some inorganic and organic compounds (Hoigne and Bader, 1976; Hoigne, 1982; Forni et al., 1982). Autodecomposition of ozone in water is initiated by hydroxide ions and accelerated by

various free radicals and anions which are formed as intermediates, and act as chain carriers of the reactions (Tomiyasu et al., 1985). At low pH, direct oxidation is the predominant pathway, but as the pH of solutions containing dissolved ozone increases, the decomposition rate of molecular ozone to hydroxyl free radicals also increases, such that at pH 10, ozone decomposes instantaneously. Although the resultant hydroxyl free radical is a more powerful oxidant, its half-life is so short (microseconds) that significant concentrations do not occur. Thus, the presence of molecular ozone is necessary to ensure microbial sanitation (Hunt and Marinas, 1997).

Oxidation products found in water after application of ozone include decomposition products of ozone and oxidation products from the organic materials present. Decomposition products from ozone include oxygen (O_2), the ozonide radical anion ($-O_3$), superoxide anion (O^{2-}), perhydroxyl anion (HO_2^-), and the hydroxyl free radical (OH). In addition, hydrogen peroxide is produced in small quantities by decomposition of ozone in water, or as a by-product of ozone oxidation of dissolved organic materials. Most of these intermediates are highly reactive, particularly in water at ambient temperatures, and their rapid decomposition leads to oxygen or hydroxide ions as final, stable decomposition products in aqueous solutions. The hydroxyl free radical is unusually reactive, having an oxidation potential greater than that of ozone itself. Consequently, deliberate formation of hydroxyl free radicals can assist in oxidizing organic materials. Decomposition of ozone in water is initiated by hydroxide ion, ultraviolet radiation, or hydrogen peroxide to produce these intermediate species, particularly the hydroxyl free radical.

The efficacy of ozone occurs by oxidation of the microorganisms' cell membranes due to its high oxidizing potential. (Graham, 1997). This results in a change in cell permeability, eventually leading to cell lysis and death (Murray et al., 1965). The cytoplasm from the lysed organism is dispersed in water with this material contributing to the total organic carbon (TOC) content of the water. Therefore, while ozone is destroying microorganisms, it is simultaneously oxidizing other dissolved organic matter. The removal of trace amounts of TOC is of interest because it further removes undefined dissolved organic materials- e.g. a potential food source for microorganisms. Ozone may also inactivate microorganisms by causing damage to their DNA. In studies by Prat et al. (1968) and Scott (1975), the pyrimidine bases from *E. coli* were modified by ozonation, with thymine being more sensitive to ozone than cytosine and uracil.

In 1997, ozone was awarded Generally Recognized as Safe (GRAS) status by an independent panel of experts sponsored by the Electric Power Research Institute (EPRI) for broad food applications (Graham, 1997). Recent investigations support the efficacy of ozone for reducing microorganisms on fruits and vegetables. Kim et al. (1999) reported that exposing lettuce to 1.3 ppm ozone resulted in a 4-log reduction of mesophilic bacteria. Similarly, Montecalvo (1998) demonstrated a 4-log decrease for populations of *E. coli* O157:H7 on lettuce containing initial concentrations of 8.6 log CFU/g after a 3 minute treatment with 3 ppm ozone. Spotts and Cervantes (1992) proposed that differences in effectiveness of ozone may be due to the presence of surface wounds on tissues that protect bacterial cells residing in areas of damage. According to Kim et al. (1999) bacterial cells were unable to form resistance to treatment with ozone, therefore, bacteria that survive ozone treatment do so only by lack of contact (i.e. protection by

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penetration, or intimate association with damaged tissues) with the ozone. Furthermore, the degree of agitation and turbulence affected the efficacy of ozone in solution, since water with bubbled ozone in solution was more effective than residual ozone in a water suspension. This is supported by the film theory whereby a liquid film forms at the gas-liquid interface so that ozone is more concentrated in the liquid film than in the bulk liquid. Hence, microbial inactivation would be greater when organisms are in contact with ozone bubbles or when present within liquid film than with bulk liquid since smaller bubbles provide a larger surface area and greater inactivation. Longley et al. (1978) proposed that the normal configuration of bacterial cells in clusters rather than individual cells is protective during sanitizer exposure. Ozone bubbles and agitation may aid in enhancing the activity of ozone by helping to break up clusters and increase the accessibility of ozone to individual bacterial cells.

Ozone is also effective against highly resistant parasites such as *Cryptosporidium parvum* oocysts with greater than 90% inactivation achieved after 5 minutes of exposure to 1 ppm ozone (Korich et al., 1990). In contrast, approximately 90 minutes of exposure to 80 ppm chlorine were required to achieve similar results.

Ozone treatment can effectively extend the shelf life of many fresh produce items including blackberries (Barth et al., 1995), grapes (Sarig et al., 1996), black pepper (Zhao and Cranston, 1995), broccoli, carrots, and tomatoes (Hampson and Fiori, 1998). For example, fungal development on blackberries was reportedly suppressed during storage at 2°C in air with 0.3 ppm ozone, while 20% of the control fruit showed decay (Barth et al., 1995). Baranovskaya et al. (1979) also demonstrated that the shelf life of potatoes could be extended up to 6 months at 6-14°C and 93-97% relative humidity with 2 ppm

ozone, without affecting potato quality. Surface oxidation of food may result from excessive use of ozone and enhance oxidative spoilage. Ozone discolored the surfaces of peaches, carrots, and broccoli florets and decreased the ascorbic acid content in broccoli florets and thiamin content in wheat flour (Farooq et al, 1977). Ozone also had a negative effect on the sensory qualities of some grains, ground spices, milk powder, and fish cake due to lipid oxidation (Graham, 1997). However, some researchers reported that ozone treatment improved the sensory quality of beef and eggs, while other researchers found that ozone did not significantly affect the sensory quality of some fruits and vegetables. Therefore, alterations in the sensory attributes depend on the chemical composition of food, ozone dose, and treatment conditions. Using the Ames test with Salmonella strains TA 100 and TA 98, Zhurkov et al. (1997) observed mutagenicity in chlorinated water but not in water subjected to ozone treatment. Levels of mutagens in chlorinated water could be effectively reduced by subsequent treatment with ozone at 0.5 ppm.

Copper Ion. Copper is an essential trace element for humans and animals and plays a role in plant metabolism. It occurs in biologic complexes such as pheophytin (an analogue of chlorophyll), hemocyanin and tyrosinase. Copper is most commonly used in the United States for electrical conductors such as wire and switches. It is also used in producing alloys such as bronze and brass, and for cooking utensils, plumbing pipes, and building construction. In agriculture, copper compounds are used in insecticides, fungicides, herbicides, and algicides. The bacteriocidal properties of such metal ions as copper and silver have been known for many centuries, and their effectiveness has been documented at low concentrations (Takayama et al., 1994). In ancient times, copper-

bottomed ships were often employed since it was widely known that they resisted growth of barnacles and algae (Yeager, 1991).

Copper and other metals are essential participants and cofactors in enzyme function and is also responsible for assisting in the formation of charge and concentration gradients across membranes that may be used in transport processes, intracellular compartmentation, and osmotic responses. Copper also aids in the stabilization of cellular structures, including cell walls, organelles, and membranes, and biomolecules such as enzymes, proteins, and nucleic acids.

Copper ions have been used in combination with various sanitizers to synergistically enhance the destruction of bacterial cells. The presence of copper influences microorganisms by affecting their growth, morphology, and biochemical activities. Toxicity results from blocking functional groups of important molecules (e.g. enzymes, polynucleotides, transport systems for essential nutrients, and ions), displacement or substitution of essential ions from cellular sites, denaturation and inactivation of enzymes, and disruption of cellular and organellar membrane integrity (Gadd, 1992). The disinfecting action of copper is attributed to the positively charged copper ions, which complex with the chemical sanitizing agent and form electrostatic bonds to the negatively charged sites on the bacterial cell surface. This allows the copper-sanitizer complex to more easily penetrate the cell membrane and destroy bacteria (Takayama et al., 1994).

The use of copper ions on fruits and vegetables has several advantages over chlorine. Copper ion is non-corrosive to process equipment even at high temperatures and does not volatilize. In addition, copper ion is odorless and, therefore, does not contribute

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off-tastes and odors to produce. Finally, copper ion does not produce toxic by-products like THM which have been determined to cause cancer.

Copper ion is can be electrolytically generated in water. A direct current is applied across the copper electrode at a specific dose rate so that the concentration of ions builds quickly producing a stable residual that is unaffected by heat, sunlight, or evaporation (Superior Water Solutions Inc., 2000).

Little information exists on the use of copper ion for removing pathogens from produce surfaces. Fortunately, several in vitro studies indicate that copper ion has potential as a produce sanitizer. For example, Kutz et al. (1988) reported 4.2 log reduction of *E. coli* O157:H7 after 1 minute of exposure to copper ion (0.4 ppm).

Sonication. An alternative to chemical sanitizers is the use of sonication, which is a process that involves emission of high frequency sound waves (sound waves pitched above the levels of human hearing) through a liquid medium to dislodge organic debris and mechanically disrupt bacteria. Unlike chlorine and other chemical sanitizers, sonication is not adversely affected by organic material, pH, and temperature, and does not produce by-products. The combination of sonication and heat was used to inhibit lipolytic activity and completely eliminate bacterial contaminants in human milk (Martinez et al., 1992). Harvey and Loomis (1929) and Chambers and Gaines (1932) used ultrasonic vibrations to pasteurize milk. Although sonication is currently being used to clean medical and dental equipment as well as jewelry, it has not yet seen widespread use in the food industry (Rutala et al., 1998; Villasenor et al., 1993).

Sonication destroys bacteria by cavitation, a phenomenon in which mechanical vibrations of high frequency cause alternate compressions and expansions of millions of

microscopic bubbles. The bubbles expand, and then implode violently, releasing large amounts of energy and generating high temperatures and pressures with the resulting high shear forces leading to cell breakage (Shukla, 1992).

Sonication is currently being investigated for controlling microbiological contamination in food. Most of these sonication studies have addressed the inactivation of gram-negative organisms in highly perishable animal-derived foods such as poultry skin and milk (Lillard, 1994). Lee et al. (1989) reported a 4-log reduction in *Salmonella* with a 10-minute ultrasonic treatment in peptone water, and a 0.78-log reduction in chocolate milk treated for 30 minutes.

Some studies indicate that the effectiveness of sonication may be enhanced by the addition of sanitizers such as chlorine. For example, sonication of an *S. typhimurium* cell suspension (10^8 cells/ ml of peptone) at 20 kHz for 55 minutes decreased organism to nondetectable levels (Lillard, 1993). This same study showed that salmonellae, which were attached to broiler skin decreased 1-1.5 log following 30 minutes of sonication at 20 kHz in peptone, <1 log using 0.5 ppm chlorine and 2.4-4 log by combining sonication with 0.5 ppm chlorine. These results are similar to those reported by Garcia et al. (1989), where sonication alone in liquid media had little or no effect on decreasing heat resistance of *B. subtilis* spores, whereas sonication combined with heat sensitized the spores to further heat treatment. Burleson et al (1975) determined that ozone in combination with sonication had a synergistic effect on the inactivation of viruses and bacteria in secondary effluent since, they believed, sonication enhanced interphase transport, broke up particulate organic material and clusters of bacteria, and produced cavitation that decreased the high surface tension that was caused by the presence of

organic matter. Yet, Kim and Yousef (1998) reported that sonication in combination with ozone did not enhance the removal of *Pseudomonas fluorescens* from the surface of fresh lettuce. These results are promising since the effects of sonication alone have been largely ineffective, presumably since ultrasonic waves are transmitted more efficiently over flat surfaces which are not inherent to raw produce (Miller, 1982).

CONFOCAL SCANNING LASER MICROSCOPY

The interaction of bacteria on food surfaces has been studied with microscopy as a key tool (Firstenberg-Eden, 1981; Gaonkar, 1995). While light and electron microscopy have been used to visualize cells on food surfaces and study colonization and details of adhesion, these methods are limited by poor resolution and extensive sample preparation that can cause distortion and artifacts. Confocal scanning laser microscopy (CSLM) provides greater resolution over conventional light microscopy by elimination of out-of-focus plane scatter light and allows for observation in a fully hydrated state once targets are fluorescently marked or dyed. The major advantage of CSLM over other microscopic methods is that laser light is focused within a very specific depth, allowing image collection within internal portions of biological samples or solid foods in order to perform three-dimensional analyses (Whallon, 1998). CSLM has been applied to food microbiology related research (Vodovotz et al., 1996) to study location and viability of microorganisms in foods (Seo and Frank, 1999).

The recent isolation of green fluorescent protein (GFP) from the jellyfish *Aequorea Victoria* has positively impacted cell and molecular biology and allowed for advanced study of microbial cells and attachment. In jellyfish, GFP produces green light

when energy is transferred from a Ca2+-activated phosphoprotein to GFP. The GFP gene has been cloned, characterized, and expressed in heterologous systems. The 27-kDa GFP has a major excitation peak at 396 nm, a minor excitation peak at 475 nm, and an emission peak at 509 nm.

GFP-transformed *E. coli* O157:H7 E318, isolated from ground beef, was developed by Mansel Griffith, University of Guelph, Ontario, Canada. This organism contains a pGFPuv plasmid which can be excited at 488 nm. The GFP-transformed *E. coli* O157:H7 is inherently fluorescent and illumination with blue light can quantitatively determine its presence or absence. The use of GFP-transformed *E. coli* O157:H7 for examination by CSLM is superior to commercially purchased antibody stains since GFP is highly stable to heat, alkaline pH, detergents, and many proteases with low toxicity. In addition, its fluorescence is not dependent on the presence of any other endogenous and exogenous factors provided that the supply of oxygen is adequate. GFP has advantages over fluorescent dyes because cells can be studied nondestructively, without any exogenous substrates or processing (Prachaiyo and McLandsborough, 2000).

Seo and Frank (1999) visualized *E. coli* O157:H7 on lettuce after chlorine treatment using CSLM. They observed that preferential bacterial attachment occurred on cut rather than intact surfaces. Inactivation by chlorine was more effective on intact leaf surfaces than inside the stomata indicating incomplete penetration. Similarly, Takeuchi and Frank (2000) found that 200 ppm chlorine treatment for 5 minutes inactivated *E. coli* O157:H7 primarily on intact, rather than cut leaf lettuce surfaces more effectively than 20 ppm. Han et al. (2000) examined the inactivation of *E. coli* O157:H7 green peppers using chlorine dioxide gas. Using CSLM, they determined that bacterial cells preferentially

attached to injured sites on the surfaces of green peppers. These sites of injury offered protection for the bacterial cells from inactivation by chlorine dioxide gas. Takeuchi and Frank (2000) determined that penetration of *E. coli* O157:H7 into lettuce tissues is affected by the temperature of the inoculum such that lower temperatures of 4°C demonstrated greater penetration as compared with 7, 25, and 37°C using CSLM.

CHAPTER TWO

A COMPARISON OF DIFFERENT CHEMICAL SANITIZERS FOR INACTIVATION OF *E. COLI* O157:H7 AND *L. MONOCYTOGENES* ON APPLES, LETTUCE, STRAWBERRIES, AND CANTALOUPE

ABSTRACT

The ability of ozone (3 ppm), chlorine dioxide (3 and 5 ppm), sodium hypochlorite (100 and 200 ppm), and peracetic acid (80 ppm) to reduce populations of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in an aqueous model system and on inoculated fresh produce including apples (whole and sliced), strawberries, cantaloupe, and lettuce (whole and shredded) was determined by selective plating. Initially, samples of each sanitizer solution were inoculated to contain approximately 10^6 CFU/ml of either pathogen after which aliquots were removed at 15 second intervals over a period of 5 minutes and appropriately plated to determine D-values. Therefore, produce was inoculated by dipping to contain approximately 10^6 *E. coli* O157:H7 or *L. monocytogenes* CFU/g, held overnight, submerged in each sanitizer solution for up to 5 minutes and then examined for survivors. In the model system, populations of both pathogens decreased > 5 logs following 2 to 5 minutes of sanitizer exposure. Based on D-values, ozone (3 ppm) was most effective (15 s) in the model system followed by chlorine dioxide (3 and 5 ppm) (19-21 s), sodium hypochlorite (100 and 200 ppm) (25-27 s), and peracetic acid (80 ppm) (70-75 s). On produce ozone (3 ppm) and chlorine dioxide (5 ppm) were the two most effective treatments, reducing populations of *L. monocytogenes* and *E. coli* O157:H7 5.5 and 5.7 logs, respectively. In comparison, treating produce with chlorine dioxide (3 ppm) and sodium hypochlorite (200 ppm) resulted in maximum reductions of 4.8 logs for *L. monocytogenes* and 5.1 logs for *E. coli*

O157:H7. Peracetic acid was the least effective sanitizer, giving reductions of 4.3 – 4.5 logs for *L. monocytogenes* and *E. coli* O157:H7. After sanitizing, produce samples were stored at 4°C for 9 days and quantitatively examined for *E. coli* O157:H7, *L. monocytogenes*, mesophilic aerobic bacteria, yeasts and molds. Populations of both pathogens remained relatively unchanged during storage; whereas numbers of mesophilic bacteria decreased approximately 2 logs. Although mold and yeast populations were significantly lower on sanitized produce, final counts at day 9 were significantly higher than initial counts for chlorine dioxide- and ozone-treated produce. When a sensory analysis was performed on uninoculated produce exposed to the various sanitizer treatments using the non-extended triangle test, only whole apples dipped in sodium hypochlorite (200 ppm) and shredded lettuce sprayed with peracetic acid (80 ppm) yielded results that were statistically different from the remaining treated and control samples.

INTRODUCTION

Over the past two decades fresh cut and ready-to-use fruits and vegetables have gained popularity as healthy convenience foods. However, safety concerns regarding raw fruits and vegetables have heightened due to increasing numbers of foodborne disease outbreaks. Between 1982 and 1994, salad bars containing raw fruits and vegetables ranked as the third leading cause of infection by *E. coli* O157:H7 in the United States (Beuchat, 1996). Fruits and vegetables were the third leading vehicle in foodborne outbreaks, accounting for 9,413 cases from 1990 to 2001 (CSPI, 2002). One such outbreak of *E. coli* O157:H7 infection involving at least 40 persons in Montana was epidemiologically linked to consumption of leaf lettuce, which was spray-irrigated with contaminated surface water (Ackers et al., 1996). *Salmonella* and *E. coli* O157:H7 have been associated with a wide range of products including lettuce, apple cider, alfalfa sprouts, bean sprouts, watermelon, radish sprouts, cabbage, celery, cucumbers, potatoes, radishes, and tomatoes and cantaloupe (Zhao et al., 1993; Beuchat, 1996a; Beuchat, 1996b).

Listeriosis, a serious foodborne illness, was first recognized in 1981 when an outbreak was traced to coleslaw in the Maritime provinces of Canada (Schlech et al., 1983). Thirty-four cases of perinatal listeriosis and seven cases of adult disease were diagnosed. Investigation revealed that the coleslaw was made from cabbage that was fertilized with raw manure that came from a flock of *L. monocytogenes*- infected sheep. In the U.K. *L. monocytogenes* was detected in 4 of 60 pre-packed, ready-to-eat salads containing cabbage, celery, lettuce, cucumber, onion, leeks, watercress, and fennel (Sizmur and Walker, 1988) with Beckers et al (1989) also identifying the pathogen in 11

of 25 samples of fresh-cut vegetables in the Netherlands. Numerous studies have documented the ability of *L. monocytogenes* to grow on fresh produce such as lettuce, asparagus, broccoli, and cauliflower at refrigeration temperatures (Berrang et al., 1989; Beuchat and Brackett, 1990; Steinbrugge et al., 1988).

The increase in raw produce-associated outbreaks involving *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* has prompted the development and testing of numerous sanitizing treatments for fresh fruits and vegetables. While the most commonly used sanitizer is still chlorine at levels of 100 – 200 ppm, other alternative sanitizers are gaining popularity for both industrial and home use.

Chlorination is relatively inexpensive with the free residual chlorine content in treated water remaining active against bacterial pathogens. However, chlorine compounds can react with trace amounts of organic material on fresh produce to form various carcinogenic (chemicals that have been shown to cause cancer in laboratory animals) organochlorine compounds including chloroform, trihalomethane, bromodichloromethane, and 3-chloro-4-(dichloromethyl)-5-hydroxyl-2(5H)-furanone (Richardson, 1998). In addition, chlorine compounds are also rapidly inactivated by organic material, which is inherent to raw produce surfaces.

The effectiveness of chlorine compounds in reducing microorganisms on produce is often unpredictable. According to Park and Beuchat (1999), spray treatment of cantaloupe with 200 ppm chlorine was no more effective in eliminating viable *E. coli* O157:H7 cells than spraying with water. Similarly, a chlorine dip of 200 ppm reduced populations of *L. monocytogenes* only ~2 logs on Brussels sprouts, whereas dipping in water alone reduced the population 1 log (Bracket, 1987).

In some cases, chlorine is less effective than alternative sanitizers at the same concentration. This was illustrated by Junli et al. (1997), who compared the bacteriocidal effectiveness of chlorine with chlorine dioxide. At pH 8.5, 0.25 ppm chlorine dioxide decreased *E. coli* O157:H7 populations 99% in 15 sec, whereas 5 minutes was required for chlorine to achieve the same results. Similarly, Zang and Farber (1996) found that treatment of shredded lettuce with 100 ppm chlorine was not significantly different than treatment with tap water alone in reducing populations of *L. monocytogenes.*

The Food and Drug Administration (FDA 1998) amended the food additive regulations to allow treatment of fruits and vegetables with chlorine dioxide at levels not exceeding 3 ppm residual in the wash water. Chlorine dioxide is a very powerful oxidizer with an oxidation capacity 3 times greater than chlorine. Chlorine dioxide is effective over a wide pH range, but not affected by high levels of organic matter, and does not dissociate rapidly in water (White, 1972). It has been shown to kill a wide variety of microorganisms, including bacterial spores that are resistant to chlorine (Richardson et al., 1994). Since chlorine dioxide does not react with ammonia to form chloramines as does chlorine and is less reactive towards organic compounds than aqueous chlorine, fewer toxic, mutagenic by-products are produced (Richardson et al., 1998).

Peracetic acid was approved as an indirect food additive by the FDA in 1986 for use in sanitizing solutions. Unlike chlorine, peracetic acid is not corrosive to processing equipment, produces no toxic by-products, remains effective in the presence of organic matter, and is not affected by changes in temperature.

Ozone is superior to chlorine for inactivating a wide range of microorganisms, including chlorine-resistant oocysts of *Cryptosporidium* and *Giardia*, both of which have

invaded food and water supplies and caused deaths in recent years (Kim et al., 1999). In addition, ozone can destroy pesticides and chemical residues such as chlorinated by-products (Graham, 1997) and convert non-biodegradable organic materials into biodegradable forms (Kim et al., 1999). Unlike chlorine, the efficacy of ozone is not affected by the solution pH with ozone also being non-corrosive to processing equipment.

This study was designed to compare the effectiveness of alternative sanitizers as compared to traditional chlorine for decontaminating apples, strawberries, lettuce and cantaloupe. Chlorine dioxide, ozone, and peracetic acid were compared to sodium hypochlorite for inactivation of *L. monocytogenes* and *E. coli* O157:H7 on inoculated raw produce with a storage study also conducted to assess product shelf-life following sanitizer exposure. Finally, a sensory analysis using the triangle test was performed on non-inoculated produce exposed to the various sanitizers to determine consumer acceptance.

MATERIALS AND METHODS

Bacterial Strains. Three strains each of *E. coli* O157:H7 (AR, AD 305, AD 317) and *L. monocytogenes* strains, (CWD 95, CWD 249, and CWD 201) were obtained from C.W. Donnelly (Dept. of Nutrition and Food Sciences, University of Vermont, Burlington, VT). Stock cultures were maintained at -70°C in tripticase soy broth (TSB) (Difco Laboratories, Detroit, MI) containing 10% (v/v) glycerol and subcultured twice in TSB broth containing 0.6% (w/v) yeast extract (TSBYE) at 35°C/ 18-24 h before use.

Preparation of Inoculum

Equal volumes (10 ml) of culture were combined to produce a three-strain cocktail of *E. coli* O157:H7 and *L. monocytogenes*. These cocktails were then centrifuged at 10,000 x g/ 15 min/ 4°C and re-suspended in sterile tap water (30 mls) to simulate commercial conditions.

The laboratory was equipped with negative air pressure to prevent airborne contamination. Sterile latex gloves were worn while handling bacterial cultures. Laboratory equipment was sanitized prior to and after use of *E. coli* O157:H7 and *L. monocytogenes* with Cidex (2.4 % gluteraldehyde) (Ethicon Corp., Ervine, CA).

Sanitizers. Four sanitizer solutions were used in this study (concentrations were similar to those used in industry settings) in addition to distilled water as the control:

1. 80 ppm Tsunami 100 (Ecolab, Mendota Heights, MN), a commercial peroxyacetic acid-based solution
2. Fruit and Vegetable Wash containing 100 and 200 ppm chlorine (S.C. Johnson Professional, Racine, WI)

3. 3 and 5 ppm chlorine dioxide (S.C. Johnson Professional, Racine, WI)

4. 3 ppm ozone

Fruit and Vegetable Wash was prepared by adding 1.13 and 2.26 grams of the powdered product to 1 liter of sterile distilled water (SDW) water to obtain 100 and 200 ppm active chlorine. Total residual chlorine was measured using a chlorine colorometric test kit (Hach Co., Ames, Iowa).

Chlorine dioxide was generated in the laboratory using the manufacturer's (S.C. Johnson Professional; Racine, WI) instructions as follows: 100 ml of the stock 2% Oxine FP solution was added to a 200 ml French square screw-capped bottle; 25 ml of 75% w/w food grade phosphoric acid was added, the bottle was sealed, and the mixture was allowed to generate chlorine dioxide for 5 min with a magnetic stirrer to ensure thorough mixing. The final concentration of chlorine dioxide was determined using the Hach Colorimeter (model CN-66, Hach) before and after each sampling run. A 1:2000 dilution of unactivated Oxine FP solution was used as a control blank.

Ozone was produced using a laboratory research ozone generator (Allegheny Teledyne Inc., Newport Beach, CA) connected to a 2.5 gallon paint tank equipped with a pressure gauge, pressure regulator, safety release valve, liquid withdrawal tube, and gas inlet tube fitted with a removable sparger. Ozone was bubbled through the sparger (i.e. bubbles of ~10 mm i.d.) into 990 ml of distilled water under 25 psi at 15 SCFH (standard cubic feet per hour) of oxygen until 3 ppm ozone was attained. Ozone concentrations were determined using the indigo colorimetric method as described in Standard Methods for the Examination of Water and Wastewater (1987). Ozonated water was collected in a

100 ml volumetric flask containing 10 ml of the indigo reagent to minimize loss of ozone. A separate volumetric flask was filled with distilled water containing 10 ml of indigo reagent to serve as a blank. The solutions were mixed thoroughly and the absorbance of each solution was immediately measured at 600 nm in a 1 cm cell. The concentration of ozone (milligrams per liter) was calculated using the following formula

$$\text{mg of } O_3/L = (1000A)/(fbV)$$

A= absorbance difference between the sample and blank solution

b = path length (1 cm)

V= sample volume (90 ml)

f = a constant value of 0.42.

Ozone was prepared and used on treated produce under a chemical fume hood to prevent inhalation. Sterile rubber gloves were also worn during handling of ozone treated produce to prevent contact with skin.

Peracetic acid (80 ppm) (Ecolab) was prepared by adding SDW water to a predetermined volume of concentrated sanitizer according to label instructions. Peracetic acid was stored, mixed, and used on treated produce under a chemical fume hood to prevent inhalation. Sterile latex gloves were also worn during handling of peracetic acid treated produce to prevent contact with skin.

Aqueous Model System Study. Sterile centrifuge tubes containing 30 ml aliquots of each sanitizer solution were inoculated from the three strain cocktail to obtain approximately 10^6 *E. coli* O157:H7 and *L. monocytogenes* CFU/ml. Initially and at 15 second intervals, 1 ml aliquots were removed over a period of 5 minutes, serially diluted

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and surface-plated on TSAYE (Difco) to determine numbers of *E. coli* O157:H7 and *L. monocytogenes*. Each treatment was performed in triplicate. The D-values for each antimicrobial treatment were then determined by linear regression.

Produce. Golden Delicious apples (whole and sliced), leaf lettuce (whole and shredded), cantaloupe (whole), and strawberries (whole) were obtained from local suppliers. Unwashed produce was stored for 24 h at 4°C before use. Before inoculation, the outer three or four lettuce leaves were discarded. Lettuce leaves were either used whole or shredded into ½" strips using a sterile razor blade. Golden Delicious apples were unwaxed, blemish-free, and of uniform size and shape (6 to 6 ½ cm diam.). Apples were either used whole or cut into 1" wedges before use. Fresh strawberries and cantaloupe were obtained and treated whole.

Inoculation. Sterile polyethylene bags (25 cm X 20 cm) containing 300 ml of SDW were inoculated with the three strain cocktail as described previously to obtain 10^8 CFU/ml for produce inoculation. Inner lettuce leaves (100 g) were placed in a sterile polyethylene bag and shaken manually for 20 minutes to ensure even distribution of the organism in the product. Batches of 6-8 apples and 20-25 strawberries were immersed in the inoculum and agitated by stirring with a sterile glass rod for 20 minutes to ensure a uniform inoculation. Whole cantaloupes were submerged individually in the inoculum and agitated by stirring with a glass rod for 20 minutes. All produce was then air dried in a laminar flow hood for 18-24 h at 24°C before being subjected to the various sanitizer treatments.

Sanitizer Exposure. Produce samples were subjected to the following wash treatments before (for sensory examination) or after inoculation: 100 and 200 ppm chlorine, 3 and 5 ppm chlorine dioxide, 80 ppm peracetic acid, 3 ppm ozone and tap water (control). Whole products were completely immersed for up to 5 minutes in sterile whirl pack bags containing 100 ml of sanitizer. Produce samples were removed every 15 seconds for microbial analysis in order to determine D-values. Apple slices and shredded lettuce were sprayed (Solo 456, 1.5 gal. garden sprayer, Sindelfingen, Germany) with 100 ml of each treatment solution at ambient temperature for up to 5 minutes. Samples were not rinsed in potable water after sanitizer exposure.

Application of sprayed sanitizers was performed under a Class II biohazard safety cabinet to minimize aerosols. After treatment the cabinet was sanitized with Cidex.

Microbial Analysis. Strawberry and lettuce samples (40 g each) were drained and placed in sterile whirl pack bags containing 100 ml of sodium thiosulfate (0.1 N) stock solution prepared by dissolving 25 g of sodium thiosulfate (Sigma Chemical Co., St. Louis, MO) in 1 L of sterile distilled water (Rand et al., 1975) to neutralize residual chlorine, chlorine dioxide, and ozone. These samples were then homogenized in a stomacher (Model SD-45, Tekmar Co., Cincinnati, OH) for 2 minutes. Triplicate samples of the produce wash water were serially diluted in 0.1% peptone and surface-plated on TSAYE, Sorbitol MacConkey Agar (SMAC) (Difco), Modified Oxford Agar (MOX) (Difco), and Rose Bengal agar (Difco) to quantitate *E. coli* O157:H7, *L. monocytogenes*, and yeasts and molds respectively.

Single apples were drained and placed in individual bags containing 100 ml of 0.1% peptone and vigorously shaken for 5 minutes followed by rubbing/massaging for

another 10 minutes. Triplicate samples of apple wash water were serially diluted and surface plated on TSAYE, SMAC or MOX and Rose Bengal agar.

Cantaloupes were analyzed by removing four 5 X 5 cm square sample areas from the dry surface of the fruit using a flame-sterilized razor blade. Samples were placed in individual bags containing 100 ml of 0.1% peptone and then stomached for 2 minutes. Triplicate samples of cantaloupe wash water were serially diluted and plated in duplicate on TSAYE, SMAC or MOX and Rose Bengal agar. All plates were counted after 48 h of incubation at 37°C. Replicate samples of all treated produce were placed in Cryovac bags and stored aerobically at 2-4° C to simulate home storage conditions. Samples of each produce item before treatment, immediately following treatment, and after 3, 5, 7 and 9 days of refrigerated storage were assessed using the aforementioned microbial procedures for each produce variety.

Sensory Analysis. Sensory analysis was performed on the various samples using the classical, non-extended triangle test. Produce samples that were not contaminated with pathogens were subjected to the various sanitizer treatments at previously mentioned concentrations. After treatment, the samples were air dried for 15-20 minutes and dispensed into individual sample cups which were wrapped in plastic film and refrigerated at 3°C for 48 hours before being given to panelists. Panelists (50 untrained students) were presented with one individual treatment at each panel session (i.e. all produce treated with chlorine dioxide was evaluated in one panel session, all produce treated with ozone was evaluated at another panel session, etc.). The same students participated in all but one of the panel sessions. The students were presented with

individual trays containing: 1) the coded samples; 2) a ballot with instructions regarding evaluation of samples and ; 3) a legal consent form (See Appendix A). All data was analyzed for statistical differences at the 0.05 and 0.01 probability levels, using statistical tables developed by Roessler et al. (1978).

Statistics. All microbial data were analyzed using a factorial ANOVA on duplicate samples at a significance level of $p < 0.05$. Statistical results were subjected to a Bonferroni adjustment for conservative analysis. Statistics were performed using Stat View computer software program.

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RESULTS

Aqueous Model System Studies. Peracetic acid (80 ppm) had the highest D-values (65 and 70 s), while chlorine dioxide (5 ppm) and ozone (3 ppm), which were not significantly different from each other, had the lowest D-values (15-19 s) respectively, for *E. coli* O157:H7 and *L. monocytogenes* (Table 1). D-values for sodium hypochlorite (200 ppm) and chlorine dioxide (3 ppm) (22 - 27 s) were significantly higher than the D-values for chlorine dioxide (5 ppm) and ozone (3 ppm) for both *E. coli* O157:H7 and *L. monocytogenes*. The D-values determined in the model system were significantly lower than those determined for the produce study.

Produce Inoculation Studies. Chlorine dioxide (5 ppm) and ozone (3 ppm) were not significantly different from each other and had the lowest D-values (22 – 96 s), while peracetic acid had the highest D-values for *L. monocytogenes* on all produce types (79 – 131 s) (Figure 1). Sodium hypochlorite (200 ppm) and chlorine dioxide (3 ppm) were not significantly different from each other and had similar D-values (30 – 100 s), regardless of the type of produce. Sodium hypochlorite (100 ppm) was significantly different from all other treatments on whole apples, sliced apples, and whole lettuce, but D-values (41 – 118 s) were significantly lower than those for peracetic acid. D-values (39 – 60 s) for strawberries and cantaloupe treated with sodium hypochlorite (100 ppm) were not significantly different from those treated with sodium hypochlorite (200 ppm) or chlorine dioxide (3 ppm). Treatment of shredded lettuce with sodium hypochlorite (100 and 200 ppm), chlorine dioxide (3 and 5 ppm) and ozone (3 ppm) yielded D-values that were not significantly different from each other (20 – 39 s).

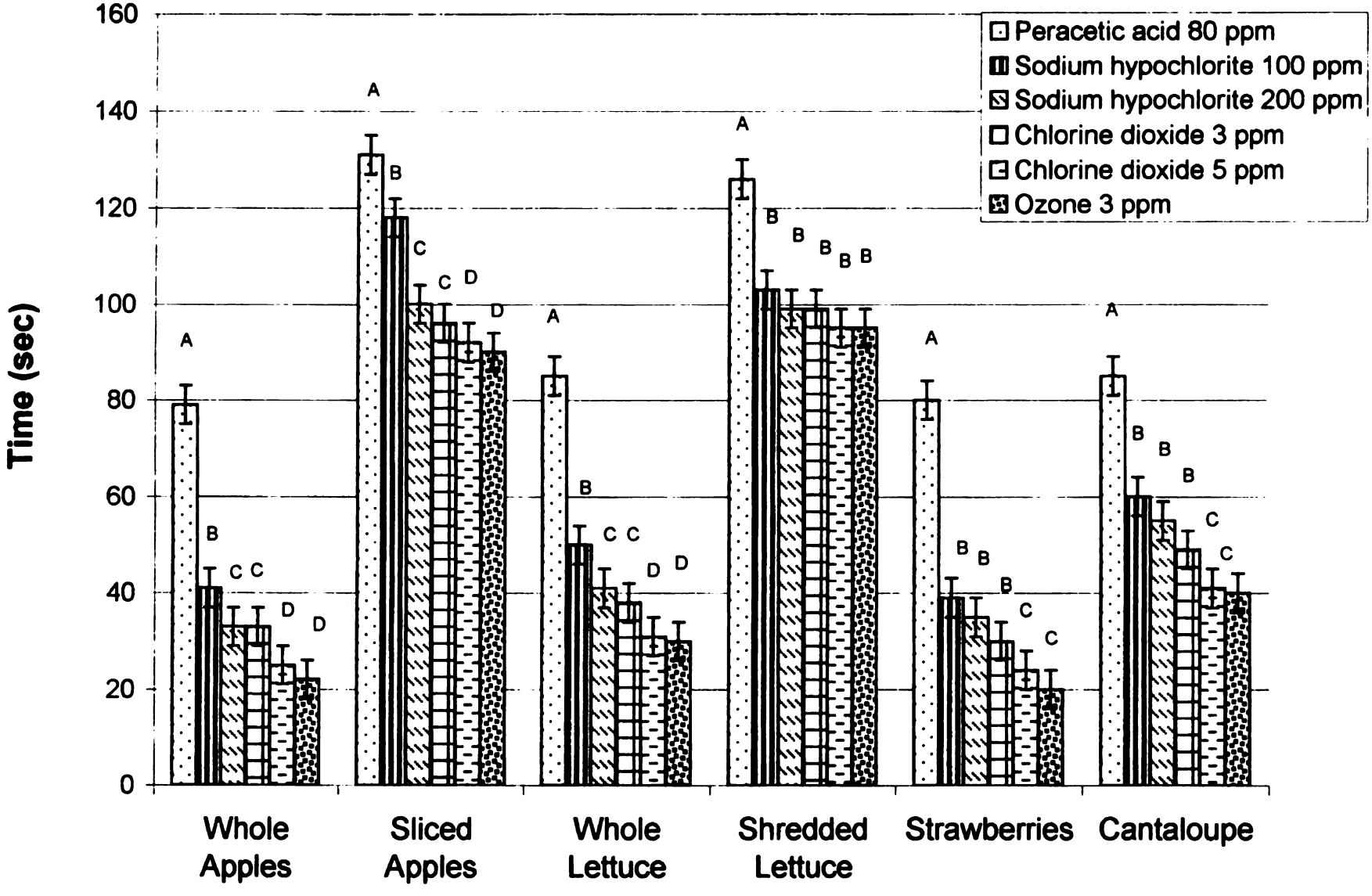
Table 1. D-values (s) for *E. coli* O157:H7 and *L. monocytogenes* populations in a model system exposed to various sanitizers

Treatment	*E. coli* O157:H7	*L. monocytogenes*
Peracetic acid (80 ppm)	65 ± 0.21[a] a	70 ± 0.17[b] a
Sodium hypochlorite (100 ppm)	31 ± 0.13 b	35 ± 0.32 b
Sodium hypochlorite (200 ppm)	22 ± 0.19 c	27 ± 0.18 c
Chlorine dioxide (3 ppm)	24 ± 0.20 c	25 ± 0.21 c
Chlorine dioxide (5 ppm)	18 ± 0.31d	19 ± 0.24 d
Ozone (3 ppm)	16 ± 0.31d	15 ± 0.26 d

[a] Data represent means ± standard deviation for triplicate samples per experiment from three experiments

[b] Data followed by different letters are significantly different by least significant difference at $P < 0.05$

Figure 1. D-values for *L. monocytogenes* on Fresh Produce Exposed to Various Sanitizers



Using *E. coli* O157:H7 as the test organism, peracetic acid yielded the highest D-values (73 – 132 s) for all produce types, while chlorine dioxide (5 ppm) and ozone (3 ppm) were not significantly different from each other and had the lowest D-values (20 – 92 s) (Figure 2). Chlorine dioxide (3 ppm) and sodium hypochlorite (200 ppm) had similar D-values (31 – 101 s) and were not significantly different from each other for whole apples and shredded lettuce. However, chlorine dioxide (3 ppm) had significantly lower D-values (28 – 90 s) than sodium hypochlorite (200 ppm) for sliced apples, whole lettuce, strawberries, and cantaloupe. D- values (49 – 112 s) for 100 ppm sodium hypochlorite on sliced apples, whole lettuce, and shredded lettuce were significantly higher than those for 200 ppm sodium hypochlorite, but on whole apples, strawberries, and cantaloupe D-values for sodium hypochlorite (100 and 200 ppm) were not significantly different from each other (33 – 58 s). D-values for chlorine dioxide (3 and 5 ppm) and ozone (3 ppm) on whole lettuce and cantaloupe were not significantly different from each other (30 – 44 s). D-values for sodium hypochlorite (100 and 200 ppm) and chlorine dioxide (3 ppm) on whole apples were not significantly different from each other (31 – 40 s).

Sliced apples and shredded lettuce, which were sprayed rather than dipped, yielded significantly higher D-values than all other produce types.

Storage Studies. Results of the storage studies indicate that microbial counts generally followed similar trends on all produce types (Table 2).

Treatment of produce with water alone was significantly different from all other treatments. Water removed approximately 1 log CFU/g of *E. coli* O157:H7,

Figure 2. D-Values for *E. coli* O157:H7 on Fresh Produce Exposed to Various Sanitizers

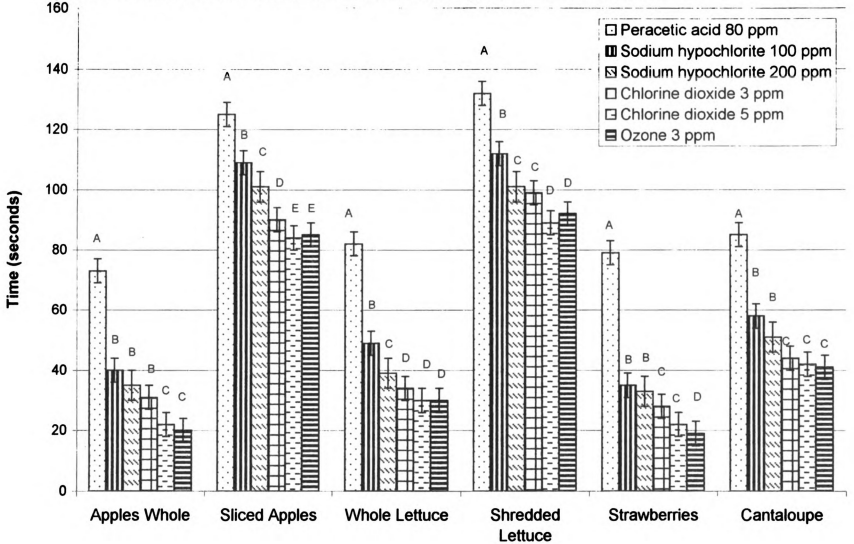


Table 2. Microbial Counts on Inoculated and Treated Fresh Produce During Storage at 4°C

Control - Water / 5 Min.

Produce	*L. monocytogenes* (Log CFU/g)						*E. coli* O157:H7 (Log CFU/g)						Mesophilic Bacteria (Log CFU/g)						Yeasts (Log CFU/g)						Molds (Log CFU/g)					
	Mi	Days					Mi	Days					Mi	Days					Mi	Days					Mi	Days				
	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9
Whole Apples	6.0	5.2	5.1	5.0	5.1	5.1	6.0	5.3	5.2	5.3	5.3	5.2	6.7	6.0	6.6	6.8	6.9	7.0	3.0	3.0	2.6	2.9	3.0	3.1	1.2	1.1	1.5	1.8	1.9	2.1
Sliced Apples	5.9	5.1	5.2	5.2	5.2	5.2	6.1	5.6	5.5	5.4	5.5	5.5	6.6	5.6	6.4	6.6	6.8	6.9	3.0	3.0	3.7	4.1	6.0	6.4	2.3	1.0	2.0	2.5	3.6	5.0
Whole Lettuce	6.1	5.3	5.2	5.3	5.1	5.3	6.1	5.2	5.2	5.2	5.1	5.1	6.5	5.8	6.5	6.7	6.8	6.9	3.2	3.3	3.8	4.0	5.1	5.9	2.4	1.4	1.9	2.6	3.4	5.1
Shredded Lettuce	6.2	5.3	5.2	5.2	5.2	5.2	6.0	5.5	5.4	5.5	5.4	5.5	6.6	5.7	6.5	6.7	6.9	7.0	3.3	3.3	3.9	4.1	5.4	6.0	1.5	1.3	2.0	2.8	3.5	5.4
Strawberries	6.0	5.1	5.0	5.1	5.0	5.0	5.9	5.1	5.1	5.2	5.0	5.0	6.5	5.9	6.6	6.8	6.9	7.2	3.8	3.7	4.0	4.2	6.0	6.1	1.7	1.5	2.1	3.0	4.7	6.0
Cantaloupe	5.9	5.0	5.1	5.1	5.1	5.0	6.0	5.2	5.2	5.3	5.2	5.2	6.4	5.7	6.4	6.7	7.0	7.2	3.9	3.8	3.9	4.3	5.2	5.4	2.0	1.7	2.0	2.9	3.4	5.2

Sanitizer - Peracetic Acid 80 ppm / 5 Min.

Produce	*L. monocytogenes* (Log CFU/g)						*E. coli* O157:H7 (Log CFU/g)						Mesophilic Bacteria (Log CFU/g)						Yeasts (Log CFU/g)						Molds (Log CFU/g)					
	Mi	Days					Mi	Days					Mi	Days					Mi	Days					Mi	Days				
	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9
Whole Apples	6.0	<1	<1	<1	<1	<1	6.0	<1	<1	<1	<1	<1	6.4	2.1	2.9	3.5	4.2	5.0	3.5	2.0	2.2	2.2	2.3	2.4	2.3	<1	1.1	1.1	1.2	1.3
Sliced Apples	5.9	1.5	1.4	1.5	1.4	1.4	6.0	1.6	1.5	1.5	1.6	1.6	6.7	2.8	3.1	3.9	4.7	5.3	3.4	2.0	1.9	2.0	2.1	2.2	2.4	<1	1.9	2.4	2.8	3.6
Whole Lettuce	5.9	<1	<1	<1	<1	<1	5.8	<1	<1	<1	<1	<1	6.6	2.0	2.6	3.8	4.2	4.8	3.3	1.9	2.0	2.1	2.2	2.4	2.2	1.1	1.4	2.2	3.4	3.7
Shredded Lettuce	6.0	1.4	1.4	1.4	1.4	1.4	5.9	1.6	1.5	1.5	1.6	1.6	6.8	2.8	3.5	3.8	4.3	4.9	3.4	1.8	2.1	2.2	2.3	2.4	2.2	1.0	2.0	2.5	3.0	3.9
Strawberries	5.9	<1	<1	<1	<1	<1	5.9	<1	<1	<1	<1	<1	6.9	3.9	4.2	4.4	5.0	5.7	3.1	2.0	2.4	2.6	2.7	2.8	2.3	1.3	2.1	3.0	3.4	4.1
Cantaloupe	5.9	<1	<1	<1	<1	<1	5.9	<1	<1	<1	<1	<1	6.4	2.0	2.3	2.6	4.0	4.9	3.6	1.9	2.6	2.7	2.9	3.1	2.4	1.6	2.2	3.1	3.7	4.4

Sanitizer - Sodium Hypochlorite 100 ppm / 5 Min.

Produce	*L. monocytogenes* (Log CFU/g)						*E. coli* O157:H7 (Log CFU/g)						Mesophilic Bacteria (Log CFU/g)						Yeasts (Log CFU/g)						Molds (Log CFU/g)					
	Mi	Days					Mi	Days					Mi	Days					Mi	Days					Mi	Days				
	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9
Whole Apples	6.0	<1	<1	<1	<1	<1	5.9	<1	1.0	1.0	<1	<1	6.6	2.1	2.1	2.8	3.4	4.8	3.3	2.3	2.5	2.5	2.7	3.0	2.6	<1	1.1	1.2	1.3	1.4
Sliced Apples	5.9	1.3	1.4	1.3	1.3	1.3	6.0	1.2	1.2	1.3	1.2	1.2	6.4	2.5	2.5	2.8	3.4	3.9	3.4	2.1	3.0	3.0	3.4	4.1	2.2	<1	1.9	2.4	3.4	4.0
Whole Lettuce	5.9	<1	<1	<1	<1	<1	6.1	<1	<1	<1	<1	<1	6.7	3.2	3.7	4.0	4.4	4.6	3.3	2.2	2.9	3.1	3.5	4.9	2.5	<1	1.9	2.4	3.2	3.8
Shredded Lettuce	6.0	1.4	1.4	1.3	1.3	1.4	6.0	1.3	1.3	1.2	1.3	1.2	6.8	2.0	2.5	3.1	4.2	4.7	3.4	2.0	2.5	2.7	3.3	4.9	2.3	1.0	2.0	2.5	3.0	4.1
Strawberries	5.8	<1	<1	<1	<1	<1	5.9	<1	<1	<1	<1	<1	6.7	2.0	2.0	2.9	3.5	5.0	3.1	2.4	3.1	3.3	4.0	4.7	2.2	<1	1.3	2.1	3.0	4.4
Cantaloupe	6.0	<1	<1	<1	<1	<1	6.0	<1	<1	<1	<1	<1	6.8	2.0	2.9	3.4	3.9	4.4	3.2	2.6	3.2	3.4	4.1	5.0	2.3	1.1	2.3	3.0	4.1	5.0

Table 2 (cont'd).

Sanitizer - Sodium Hypochlorite 200 ppm / 5 Min.					
Produce	***L. monocytogenes***	***E. coli* O157:H7**	**Mesophilic Bacteria**	**Yeasts**	**Molds**

Table 2 (cont'd).

Sanitizer - Sodium Hypochlorite 200 ppm / 5 Min.

Produce	*L. monocytogenes* (Log CFU/g)						*E. coli* O157:H7 (Log CFU/g)						Mesophilic Bacteria (Log CFU/g)						Yeasts (Log CFU/g)						Molds (Log CFU/g)					
	Mi		Days				Mi		Days				Mi		Days				Mi		Days				Mi		Days			
	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9
Whole Apples	6.0	<1	<1	<1	<1	<1	6.0	<1	<1	<1	<1	<1	6.6	2.6	3.0	3.1	4.4	5.3	3.4	2.0	2.1	2.2	2.4	3.0	2.4	<1	1.2	1.3	1.5	1.5
Sliced Apples	5.9	1.3	1.2	1.2	1.3	1.3	6.0	1.2	1.2	1.1	1.2	1.2	6.5	2.6	3.2	3.4	4.0	4.4	3.3	2.1	3.0	3.1	3.3	3.9	2.5	<1	1.6	2.5	3.1	3.6
Whole Lettuce	6.0	<1	<1	<1	<1	<1	5.9	<1	<1	<1	<1	<1	6.4	2.7	3.4	4.0	4.9	5.2	3.2	1.9	2.4	2.6	3.1	4.0	2.6	<1	1.3	2.2	3.0	3.9
Shredded Lettuce	5.9	1.3	1.3	1.2	1.4	1.3	5.9	1.3	1.2	1.3	1.3	1.3	6.5	2.6	3.0	3.5	4.2	4.9	3.3	1.9	2.6	2.8	3.0	3.6	2.5	<1	1.4	2.7	3.6	4.4
Strawberries	6.0	<1	<1	<1	<1	<1	6.0	<1	<1	<1	<1	<1	6.5	2.8	3.4	4.3	5.0	5.3	3.3	1.8	2.9	3.1	3.4	3.9	2.5	<1	2.0	2.6	4.1	4.8
Cantaloupe	6.0	<1	<1	<1	<1	<1	6.0	<1	<1	<1	<1	<1	6.5	2.8	3.4	3.9	4.4	5.3	3.4	2.0	3.0	3.3	3.7	4.2	2.6	<1	1.8	2.6	4.0	4.6

Sanitizer - Chlorine Dioxide 3 ppm / 5 Min.

Produce	*L. monocytogenes* (Log CFU/g)						*E. coli* O157:H7 (Log CFU/g)						Mesophilic Bacteria (Log CFU/g)						Yeasts (Log CFU/g)						Molds (Log CFU/g)					
	Mi		Days				Mi		Days				Mi		Days				Mi		Days				Mi		Days			
	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9
Whole Apples	6.0	<1	<1	<1	<1	<1	5.8	<1	<1	<1	<1	<1	6.7	2.4	3.1	4.4	4.9	5.4	3.3	2.0	2.1	2.3	3.1	3.9	2.5	<1	1.0	1.1	1.3	1.3
Sliced Apples	5.9	1.2	1.0	1.1	1.0	1.2	5.8	<1	<1	<1	1.0	<1	6.6	2.7	3.0	3.3	3.9	4.5	3.4	2.1	2.9	3.1	3.9	4.5	2.6	< 1	1.6	2.9	4.1	5.0
Whole Lettuce	5.9	<1	<1	<1	<1	<1	5.8	<1	<1	<1	<1	<1	6.8	2.5	3.0	3.4	4.0	4.9	3.3	2.0	2.8	3.1	4.0	4.8	2.5	<1	1.5	3.0	4.2	5.4
Shredded Lettuce	6.0	1.3	1.2	1.3	1.3	1.3	5.9	1.1	1.0	<1	1.1	1.0	6.7	2.5	3.1	3.7	4.1	4.8	3.4	1.9	3.0	3.2	4.1	5.1	2.6	<1	1.7	3.1	4.0	5.1
Strawberries	5.8	<1	<1	<1	<1	<1	6.1	<1	<1	<1	<1	<1	6.5	2.7	3.0	3.4	4.5	5.2	3.3	1.8	2.7	3.1	4.2	5.0	2.4	<1	1.9	3.2	4.5	5.9
Cantaloupe	6.0	<1	<1	<1	<1	<1	6.0	<1	<1	<1	<1	<1	6.4	2.7	3.1	3.4	4.3	5.0	3.5	2.1	2.9	3.0	4.1	5.2	2.2	<1	2.0	2.9	4.0	5.3

Sanitizer - Chlorine Dioxide 5 ppm / 5 Min.

Produce	*L. monocytogenes* (Log CFU/g)						*E. coli* O157:H7 (Log CFU/g)						Mesophilic Bacteria (Log CFU/g)						Yeasts (Log CFU/g)						Molds (Log CFU/g)					
	Mi		Days				Mi		Days				Mi		Days				Mi		Days				Mi		Days			
	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9
Whole Apples	6.0	<1	<1	<1	<1	<1	6.0	<1	<1	<1	<1	<1	6.6	2.5	2.9	3.5	4.4	5.6	3.4	1.9	2.1	2.3	2.8	3.0	2.4	<1	1.0	1.1	1.3	1.5
Sliced Apples	5.9	<1	<1	<1	<1	<1	6.0	1.1	1.1	1.0	1.2	1.0	6.5	2.4	3.0	3.1	3.9	4.2	3.5	1.9	2.0	2.3	2.7	3.4	2.3	<1	1.1	2.2	2.9	3.6
Whole Lettuce	5.8	<1	<1	<1	<1	<1	6.0	<1	<1	<1	<1	<1	6.7	2.3	2.9	4.0	4.9	5.2	3.4	2.0	2.2	2.5	3.0	3.5	2.2	<1	1.0	2.6	3.5	4.4
Shredded Lettuce	5.9	1.2	1.2	1.1	1.1	1.2	5.9	<1	1.0	1.0	<1	<1	6.6	2.4	2.9	3.8	4.1	4.8	3.4	1.8	1.9	2.3	2.9	3.2	2.2	<1	1.2	2.8	3.3	4.5
Strawberries	5.9	<1	<1	<1	<1	<1	5.9	<1	<1	<1	<1	<1	6.6	2.4	3.1	4.2	5.4	5.8	3.3	2.0	2.1	2.5	3.1	3.5	2.2	<1	1.0	2.9	3.8	4.7
Cantaloupe	6.0	<1	<1	<1	<1	<1	6.0	<1	<1	<1	<1	<1	6.6	2.4	2.4	3.2	3.9	4.3	3.5	2.0	2.2	2.6	3.2	3.6	2.2	<1	1.3	2.9	3.7	4.3

Table 2 (cont'd).

Sanitizer - Ozone 3 ppm / 5 Min.

Produce	*L. monocytogenes* (Log CFU/g)						*E. coli* O157:H7 (Log CFU/g)						Mesophilic Bacteria (Log CFU/g)						Yeasts (Log CFU/g)						Molds (Log CFU/g)					
	Mi		Days				Mi		Days				Mi		Days				Mi		Days				Mi		Days			
	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9	0	5	3	5	7	9
Whole Apples	6.0	<1	<1	<1	<1	<1	6.0	<1	<1	<1	<1	<1	6.5	1.3	1.7	2.1	3.3	4.9	3.5	1.9	2.1	2.7	3.0	3.8	2.5	<1	<1	1.3	1.9	2.1
Sliced Apples	6.0	<1	<1	<1	<1	<1	5.9	<1	<1	<1	<1	<1	6.5	2.3	2.4	3.0	3.7	4.2	3.4	1.9	2.3	2.6	3.1	4.0	2.5	<1	1.0	2.1	3.5	4.2
Whole Lettuce	5.9	<1	<1	<1	<1	<1	5.9	<1	<1	<1	<1	<1	6.4	2.2	2.4	2.9	3.4	3.9	3.4	1.8	2.0	2.5	3.2	3.9	2.4	<1	1.1	2.4	3.7	5.0
Shredded Lettuce	6.0	<1	<1	<1	<1	<1	6.0	<1	<1	<1	<1	<1	6.4	2.4	2.7	3.1	4.0	4.7	3.4	1.8	2.1	2.4	3.0	3.9	2.4	<1	1.2	2.6	3.6	4.7
Strawberries	6.0	<1	<1	<1	<1	<1	6.0	<1	<1	<1	<1	<1	6.5	2.0	2.5	0.3	3.7	5.0	3.5	1.9	2.2	2.9	3.6	4.1	2.5	<1	1.0	1.9	3.9	4.9
Cantaloupe	6.0	<1	<1	<1	<1	<1	6.0	<1	<1	<1	<1	<1	6.5	2.3	2.6	3.0	3.9	4.5	3.4	2.0	2.4	2.8	3.7	4.2	2.6	<1	1.1	2.3	3.2	4.4

L. monocytogenes, and mesophilic bacteria on all produce types with subsequent growth not exceeding 1 log during 9 days of storage. Treatment of produce with tap water for 5 minutes reduced mold populations <1 log on the various produce types. After water treatment, mold populations on produce increased 3.5 - 4 logs during storage with these counts eventually 3-4 logs greater than initial counts after 9 days of storage. Treating produce with tap water for 5 minutes did not significantly reduce yeast populations. Yeast counts doubled after 9 days of storage, except for whole apples where yeasts did not increase significantly during storage.

Populations of *E. coli* O157:H7 and *L. monocytogenes* decreased to non-detectable levels on whole apples, whole lettuce, strawberries and cantaloupe after 5 minutes of exposure to 80 ppm peracetic acid. Populations of *L. monocytogenes* and *E. coli* O157:H7 on sliced apples and shredded lettuce were reduced significantly, but approximately 1.5 logs remained on these commodities after a 5 min treatment with 80 ppm peracetic acid. Peracetic acid reduced the populations of mesophilic bacteria by about 4 logs after 5 min. Numbers of mesophilic bacteria increased 2-3 logs during 9 days of storage and were approximately 1-2 logs lower than initial counts. Peracetic acid reduced mold populations 1.0 - 2.5 logs in 5 minutes. Molds grew during storage and were 1-2 logs higher than initial counts after 9 days of refrigerated storage. Yeast populations were affected less than molds by treatment with 80 ppm peracetic acid. Populations of yeasts decreased 1.0-1.5 logs after a 5 minute exposure to peracetic acid with numbers approximately 1 log lower than initial counts 9 days after refrigerated storage.

Treatment of produce with 100 ppm sodium hypochlorite for 5 minutes reduced populations of *L. monocytogenes* and *E. coli* O157:H7 to non-detectable levels in whole apples, whole lettuce, strawberries, and cantaloupe, while approximately 1 log CFU/g of *L. monocytogenes* and *E. coli* O157:H7 remained on sliced apples and shredded lettuce. Populations of mesophilic bacteria decreased 4.0 – 4.5 logs after treatment with 100 ppm sodium hypochlorite and then increased about 2.5 logs during storage and were approximately 1.5 – 2.5 log CFU/g lower than initial counts after 9 days of refrigerated storage. Treatment of produce with 100 ppm sodium hypochlorite reduced mold populations from approximately 2.5 logs to non-detectable levels, except for shredded lettuce and cantaloupe which harbored mold populations of 1 log CFU/g. During 9 days of storage, mold counts approximately doubled in all commodities except whole apples where mold populations increased 1.3 – 2.2 logs by the end of storage. Sodium hypochlorite (100 ppm) reduced yeast populations 1.0 – 1.5 logs after 5 minutes with numbers increasing about 1.5 logs during 9 days of refrigerated storage.

Following a 5 minute exposure to 200 ppm sodium hypochlorite, *E. coli* O157:H7 and *L. monocytogenes* were non-detectable on whole apples, whole lettuce, strawberries and cantaloupe. Approximately 1 log CFU/g *E. coli* O157:H7 and *L. monocytogenes* remained on sliced apples and shredded lettuce. Populations of both pathogens remained constant throughout 9 days of refrigerated storage. Numbers of mesophilic bacteria decreased > 3 logs immediately after treatment with 200 ppm sodium hypochlorite with samples containing levels that were approximately 1.5 logs lower than initial levels after 9 days of refrigerated storage. Yeast counts decreased approximately 1.5 logs after a 5 minute exposure to 200 ppm sodium hypochlorite and then steadily increased during

storage, attaining populations slightly above initial levels after 9 days. Mold counts decreased approximately 2.5 logs after 5 minutes of exposure to 200 ppm sodium hypochlorite and were approximately 1.5 logs higher than the initial inoculum by the 9^{th} day of storage.

Chlorine dioxide (3 ppm) reduced populations of *L. monocytogenes* to non-detectable levels in whole apples, whole lettuce, strawberries, and cantaloupe, while approximately 1 log CFU/g of *L. monocytogenes* remained on sliced apples and shredded lettuce. Populations of *E. coli* O157:H7 were reduced to non-detectable levels on whole apples, sliced apples, whole lettuce, strawberries and cantaloupe after a 5 minute treatment with 3 ppm chlorine dioxide. Approximately 1 log CFU/g *E. coli* O157:H7 remained on lettuce after a 5 minute exposure to 3 ppm chlorine dioxide. Populations of mesophilic bacteria decreased approximately 4 logs after treatment with 3 ppm chlorine dioxide and then increased > 3 logs during the remaining 9 days of storage. Chlorine dioxide (3 ppm) reduced mold populations from approximately 2.5 log CFU/g to non-detectable levels. Mold populations doubled during 9 days of storage in all commodities except whole apples where mold counts increased to only half the initial levels. Treatment of produce with 3 ppm chlorine dioxide reduced populations of yeasts by approximately 1 log CFU/g after 5 minutes. Yeast populations increased approximately 2 logs in whole apples, 2.5 logs in sliced apples and whole lettuce, and approximately 3 logs in shredded lettuce, strawberries, and cantaloupe until the numbers were higher than initial counts for all commodities.

Chlorine dioxide (5 ppm) was significantly more effective in reducing numbers of molds and yeasts than 3 ppm chlorine dioxide. Populations of *L. monocytogenes* and *E.*

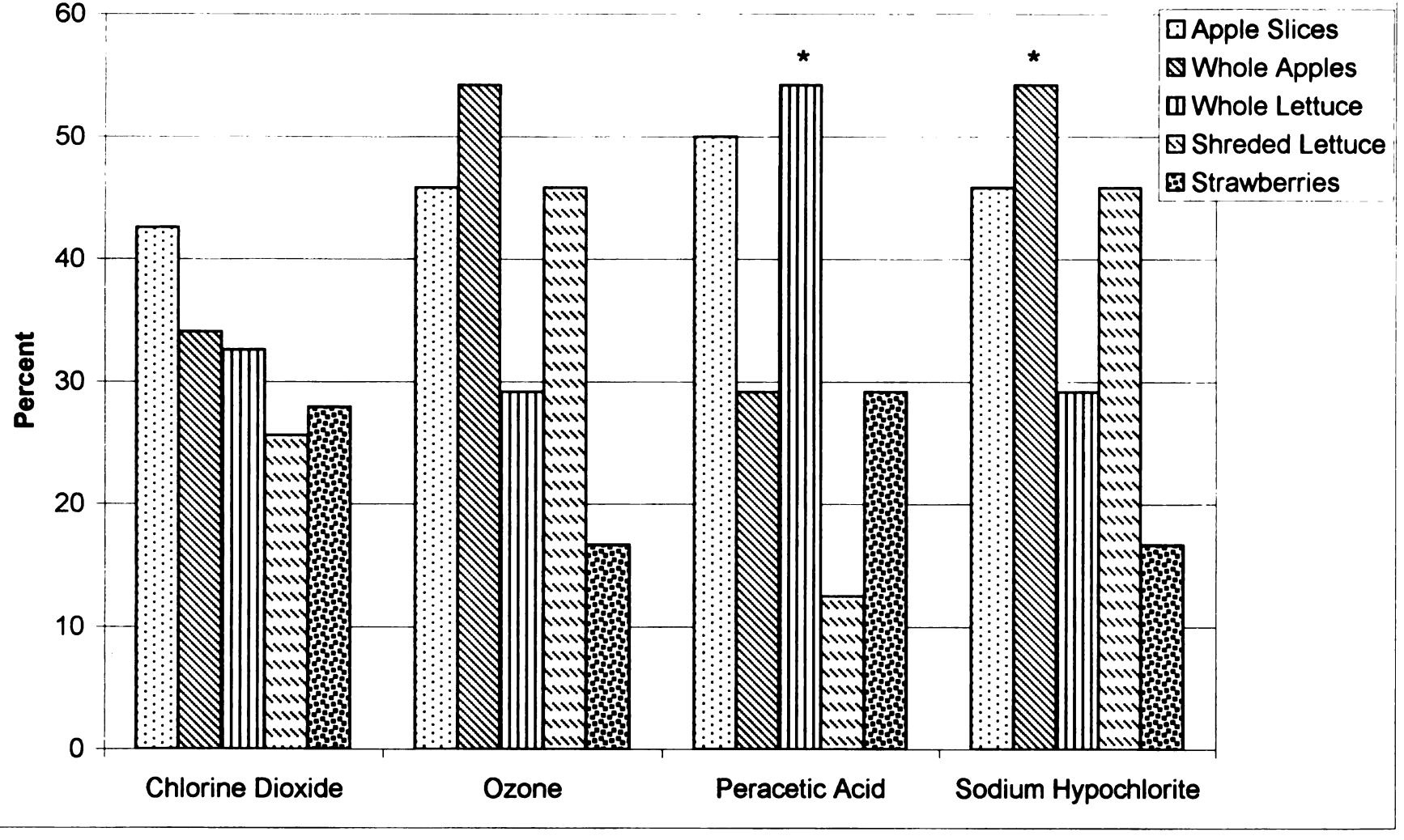
coli O157:H7 decreased to non-detectable levels following a 5 minute exposure to 5 ppm chlorine dioxide, except in shredded lettuce with *L. monocytogenes* and sliced apples with *E. coli* O157:H7. Chlorine dioxide (5 ppm) was less effective for these latter two commodities with 1 log CFU/g of *E. coli* O157:H7 and *L. monocytogenes* remaining immediately after treatment and persisting during 9 days of refrigerated storage. Chlorine dioxide (5 ppm) reduced populations of mesophilic bacteria by approximately 4 logs. Therefore, populations of mesophilic bacteria rose steadily during storage, but were approximately 1 log lower after 9 days as compared to initial levels. Mold counts decreased about 2 logs to non-detectable levels after a 5 minute exposure to 5 ppm chlorine dioxide. Mold counts increased during storage and were 1.5 – 2 logs higher after 9 days of storage compared to initial levels, except for whole apples in which the mold count was approximately 1 log lower than initial levels. Treatment of produce with 5 ppm chlorine dioxide for 5 minutes reduced yeast populations about 1.5 logs. Populations of yeasts rose during storage and were approximately the same as initial levels after 9 days of refrigerated storage.

Using ozone (3 ppm), *E. coli* O157:H7 and *L. monocytogenes* were no longer detected on any produce samples after 5 minutes. Populations of either pathogen did not rise above non-detectable levels during 9 days of refrigerated storage. Numbers of mesophilic bacteria decreased approximately 4 - 5 logs after a 5 minute exposure to 3 ppm ozone and then increased approximately 3 logs during storage. Yeast counts decreased about 1 log after treatment with 3 ppm ozone and then increased approximately 2 logs during storage until populations were slightly higher than initial levels at day 9. Mold populations decreased approximately 2.5 logs following a 5 minute exposure to 3

ppm ozone. Mold populations increased significantly during storage and were approximately 5 logs higher by day 9 than initial levels, except for whole apples in which mold populations increased only 2 logs during 9 days of refrigerated storage.

Sensory Analysis. The only statistically significant differences between any of the treated samples and the control samples occurred when whole apples were dipped in the sodium hypochlorite treatment or when shredded lettuce was sprayed with peracetic acid (Figure 3). In both instances the minimum number of correct responses was recorded for the samples to be statistically different at the 0.05 probability level but not at the 0.01 level.

Figure 3. Percentage of sensory panelists who identified correct odd sample in Triangle Test



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DISCUSSION

In the model system, D-values were significantly lower than on actual produce. Produce inherently contains large amounts of organic matter on its surfaces which negates the lethal effect of many sanitizers, and since the model system tested contained no organic matter, the sanitizers were optimally efficient. Similarly, Kim (1998) reported that ozone is more effective when used on a pure cell suspension than on raw fruits and vegetables. Consequently, the effectiveness of these sanitizers in the model system does not absolutely represent their effectiveness under actual processing conditions on raw fruits and vegetables and should be used only as an indicator of possible usefulness.

Treatment of produce with water alone was significantly different from all other treatments, decreasing bacterial populations only about 1 log on produce. These results are similar to those reported by Wright et al. (2000) who found that treatment of *E. coli* O157:H7-inoculated apples with water decreased populations 1.1 log. Similarly, Brackett (1987) reported a 1 log reduction for *L. monocytogenes* on Brussels sprouts dipped in water. Water alone contains no antimicrobial activity. Hence, the reductions observed in this study can be attributed to the effect of washing bacteria from the produce surface. Clearly washing in water is of limited use since this method would not be effective for heavily contaminated produce.

Populations of *L. monocytogenes* and *E. coli* O157:H7 remained relatively constant throughout 9 days of storage. Reductions of approximately 1 log CFU/g have been reported by numerous other researchers and are inevitably the result of removal of some bacteria due to the surface washing effect as discussed earlier. Abdoul-Raouf et al. (1993) also reported that populations of *E. coli* O157:H7 on shredded lettuce did not

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change by more than 1 log after 10 days of storage at 5°C. In addition, Richert et al. (2000) reported that populations of *E. coli* O157:H7 inoculated onto sliced cucumbers were unchanged after 7 days of refrigerated storage. The inability of *L. monocytogenes* and *E. coli* O157:H7 to grow on our produce during refrigerated storage may be attributed to the presence of competitive microflora. Mold and yeast growth on produce during 9 days of storage was not inhibited by treatment with water with spoilage of strawberries enhanced.

Peracetic acid (80 ppm) had the highest D-values and was the least effective sanitizer examined in the model system and on produce for both *E. coli* O157:H7 and *L. monocytogenes*. Maximum log reductions for peracetic acid on produce were 4.3 logs for *L. monocytogenes* and 4.5 logs for *E. coli* O157:H7 after 5 minutes. According to Wright et al. (2000), *E. coli* O157:H7 populations on apples decreased about 2.5 logs after a 2 minute exposure to 80 ppm peracetic acid. Similarly, Wisniewsky et al. (2000) reported a 3-log reduction for *E. coli* O157:H7 on apples treated with 80 ppm peracetic acid for 5 minutes. Results of the storage study indicate that peracetic acid significantly reduced levels of *E. coli* O157:H7, *L. monocytogenes* and mesophilic bacteria on all produce types and prevented re-growth during 9 days of refrigerated storage. Reduced microbial growth has been reported using 90 ppm peracetic acid on pre-packaged salads and was attributed to the residual effects of acetic acid released by the degradation of peracetic acid. Peracetic acid was significantly more effective than chlorine dioxide (3 and 5 ppm), ozone (3 ppm) and sodium hypochlorite (100 and 200 ppm) for reducing mold and yeast populations. Peracetic acid is increasingly being used in clean-in-place systems within

beverage and dairy plants because of its effectiveness against yeasts and molds with our work demonstrating a few benefits for raw produce.

Sodium hypochlorite (100 and 200 ppm) decreased populations of *E. coli* O157:H7 and *L. monocytogenes* on raw produce with maximum log reductions of 4.8 for *L. monocytogenes* and 5.1 for *E. coli* O157:H7 after a 5 minute exposure. Overall, 200 ppm sodium hypochlorite was significantly more effective than 100 ppm sodium hypochlorite, with 200 ppm sodium hypochlorite also not significantly different from 3 ppm chlorine dioxide. These results are similar to those of Wright et al. (2000) who reported that *E. coli* O157:H7 populations decreased 2.1 logs on apples after a 2 minute exposure to 200 ppm sodium hypochlorite.

Inactivation of bacterial cells using chlorine compounds has been examined by many researchers with its effectiveness now generally regarded as being somewhat unpredictable. For example, Park and Beuchat (1999) found that 200 ppm chlorine was as effective as peracetic acid (80 ppm) for treating cantaloupes, while Zhang and Farber (1996) reported a maximum log reduction of 1.3 – 1.7 for *L. monocytogenes* on shredded lettuce treated with 200 ppm chlorine.

The ineffectiveness of chlorine may be explained by differences in the inoculation method (spot, dipping, spraying), concentration of the initial inoculum, procedures for preparing the inoculum methods for removal (stomaching, homogenizing, washing) and enumeration of surviving cells (selective vs. non-selective plating media). Also, when the temperature of the chlorinated wash water is at least 10°C higher than that of the inoculated fruits and vegetables, minimum uptake of wash water through tissues is achieved resulting in a greater log reduction by chlorine (Beuchat, 1998). The

ineffectiveness of chlorine may also be due to insufficient wetting of the hydrophobic surface (waxy cuticle) of fruits and vegetables.

The efficacy of chlorinated compounds is strongly influenced by the presence of organic debris with its effectiveness in commercial processing facilities being inconsistent. Commercial fruit and vegetable processors typically recycle wash and flume water. Since the concentration of organic debris in recycled flume water often increases significantly over time, the chlorine concentration would have to be constantly monitored and adjusted. Previous studies have documented similar results with greater bacterial inactivation occurring at higher concentrations of chlorine. For example, Park and Beuchat (1999) reported that 2000 ppm chlorine was more effective than 200 ppm chlorine in eliminating *E. coli* O157:H7 from cantaloupe, indicating that higher sanitizer concentrations may further inactivate microorganisms.

Results of our storage study indicate that reductions in the population of mesophilic bacteria using sodium hypochlorite (100 and 200 ppm) were not significantly different from chlorine dioxide (3 and 5 ppm) and ozone (3 ppm), but were significantly greater than peracetic acid. Sodium hypochlorite (100 and 200 ppm) was significantly more effective than ozone and chlorine dioxide in suppressing yeast and mold growth during storage, but was significantly less effective than peracetic acid. This indicates that the amount of residual chlorine remaining on produce during storage was not as effective in suppressing yeast and molds as the acetic acid residual left by peracetic acid.

Chlorine dioxide was highly effective against *L. monocytogenes* and *E. coli* O157:H7 on surface-inoculated produce. Overall, 5 ppm chlorine dioxide was significantly more effective than 3 ppm chlorine dioxide, with 5 ppm chlorine dioxide not

significantly different from ozone. Results of this study are similar to those of Reina et al. (1995) who found that 1.3 ppm chlorine dioxide reduced the total aerobic flora in cucumber wash water by 2-6 logs. These results indicate that chlorine dioxide is as effective as sodium hypochlorite in decreasing the numbers of *E. coli* O157:H7 and *L. monocytogenes* on raw produce. Chlorine dioxide provides processors with a safer alternative to chlorine for disinfection, since the number of chlorine dioxide by-products is 3-5 times lower than that for chlorine (Richardson et al., 1998). While highly effective against *L. monocytogenes* and *E. coli* O157:H7, storage study results indicate that 3 ppm chlorine dioxide decreased the shelf life of selected commodities due to enhanced growth of yeasts and molds. This may be the result of rapid decomposition of residuals left by chlorine dioxide which accelerates growth of yeasts and molds.

This study demonstrated that ozone (3 ppm) was extremely effective against *L. monocytogenes* and *E. coli* O157:H7 on raw produce. Kim et al. (1999) also reported that 1.3 ppm ozone was highly effective on fresh lettuce with > 4 log reductions for mesophilic bacteria after 5 minutes of exposure. In addition, Montecalvo (1998) demonstrated a 4-log decrease in populations of *E. coli* O157:H7 on lettuce containing initial concentrations of 8.6 log CFU/g after a 3 minute treatment to 3 ppm ozone.

According to Kim et al. (1999), bacterial cells are unable to develop resistance to ozone; therefore, surviving bacteria do so only by lack of ozone contact (i.e. protection by penetration, or intimate association with damaged tissues). The degree of agitation and turbulence also affects the efficacy of ozone in solution, since ozonated water that was bubbled in solution was more effective than residual ozone suspended in water. Our findings are based on an ozone delivery system with a sparger that continuously

generated small bubbles in solution. Small bubbles possess a larger surface area for sanitizer action and therefore, greater inactivation.

Longley et al. (1978) proposed that bacterial cells are rarely present as discrete or individual particles, but rather exist in clusters possessing high surface tension which protect the cells from the lethal effects of sanitizers. Hence, bubbles and agitation enhance the activity of ozone by breaking up these clusters. Decomposition of ozone is very rapid with its antimicrobial action mainly occuring at the surface of produce since the water phase of foods accelerates its decomposition. Therefore, treatment with ozone does not result in any residual antimicrobial activity. This effect could clearly be seen in the storage study results in which molds and yeasts grew to high levels after 9 days of refrigerated storage. Thus, while ozone was highly effective against *L. monocytogenes* and *E. coli* O157:H7 it was not effective against yeasts and molds. Growth of these spoilage microorganisms might relate to the removal of microflora from the produce that naturally inhibit molds and yeasts.

Although ozonated water does not appear to extend the shelf life of produce, ozone gas has been shown to increase the shelf life of apples and oranges (Horvath et al.,1985) and grapes (Sarig et al., 1996). The effect of ozone gas has been attributed to the oxidation of ethylene, the inactivation of spoilage microorganisms, and the removal of other metabolic products (Horvath et al., 1985) According to some researchers, ozone may contribute to the deterioration of food quality by excessive surface oxidation (Rice et al., 1982).

Using a colorimeter, Liew and Prange (1994) determined that ozonation of carrots increased the total color difference with ozonated carrots appearing lighter in color

compared to the control. However, these color changes were not detected in a sensory panel. Scott and Lesher (1963) reported that 0.02 to 0.04 ppm ozone could be detected by humans as a sweet, pleasant odor whereas 3 ppm ozone was used for treatment in our study. Thus, the time between ozonation of produce and sensory examination was likely sufficient for the break down of ozone, thus negating any odor. Overall, consumers would not reject most sanitized produce based on taste alone

Shredded and cut produce yielded significantly higher D-values compared to whole produce. The large areas of damage on shredded lettuce and cut apples greatly increased the surface area for bacterial attachment which in turn enhanced survival during the sanitizer treatment. In addition to incomplete sanitizer contact from spraying as opposed to immersion, the large cut surface area likely introduced large amounts of organic matter into the wash water which would have decreased sanitizer effectiveness. Many sanitizers also react with plant tissues and extracellular biochemical components at wound sites and are unable to inactivate bacterial cells attached to or embedded in plant tissue (Kim et al., 1999). In addition, the hydrophobic structure of the waxy cuticle of fruit and vegetable skins provides a natural barrier to attachment and penetration of bacteria into flesh tissue. Cut produce is not able to offer this protection with bacteria readily penetrating into the product.

Populations of molds and yeasts on shredded lettuce and sliced apples were consistently higher than on whole produce and were enhanced by storage at refrigeration temperatures. These results may be explained by the effect of pH on cut produce. Abdoul-Raouf et al. (1993) reported that the pH of sliced cucumbers and carrots stored at refrigeration temperatures decreased significantly after 14 days. This pH decrease was

attributed to the fermentation of sugars that were released as a result of cutting. Since low pH values favor the growth of yeasts and molds, this effect may have favored mold and yeast growth on sliced apples and shredded lettuce stored at refrigeration temperatures, as well as higher moisture content and increased nutrient availability on cut produce also playing a role (Richert et al., 2000).

The varied surface topographies of fresh produce such as cantaloupe and strawberries provide sites for greater attachment and production of biofilms that are able to resist removal by various wash treatments (Cherry, 1999; Lund, 2000). According to Yu et al. (2001), the relative ineffectiveness of many sanitizers on strawberries is probably due to the rough surface and the presence of numerous surface-borne achenes (seeds), which serve as bacterial attachment sites and decease accessibility to sanitizing solutions. Areas that are highly textured also provide sites where bacteria can preferentially attach and form pools which dry into stacks of bacterial cells. Other work in our laboratory using confocal scanning laser microscopy indicates that *E. coli* O157:H7 cells surviving on inoculated lettuce and strawberries after ozone, sodium hypochlorite, and chlorine dioxide were arranged primarily in stacks. Hence, these bacterial cell clusters likely afford some protection against chlorine and other sanitizers.

In conclusion, the results of this study indicate that peracetic acid (80 ppm), sodium hypochlorite (100 and 200 ppm), chlorine dioxide (3 and 5 ppm), and ozone (3 ppm) effectively decreased the numbers of *E. coli* O157:H7 and *L. monocytogenes* on fresh produce. Chlorine dioxide (3 and 5 ppm) and ozone (3 ppm) were more effective against *E. coli* O157:H7 and *L. monocytogenes* compared to the other sanitizers, but were less effective at preventing growth of yeasts and molds during the storage study. Sodium

hypochlorite (100 and 200 ppm) and peracetic acid (80 ppm) were less effective against *E. coli* O157:H7 and *L. monocytogenes*, but were better at retarding the growth of yeasts and molds during produce storage. Sensory panelists detected the use of peracetic acid (80 ppm) on chopped lettuce and sodium hypochlorite (200 ppm) on whole apples. Selection of one particular sanitizer for pathogen reduction on all produce is not practical with selection based on antimicrobial activity, surface properties of the produce, effect on shelf-life and consumer acceptance.

CHAPTER THREE

USE OF POST-HARVEST WASHES AND SANITIZERS TO REDUCE MICROBIAL POPULATIONS ON SELECTED HORTICULTURAL PRODUCTS

ABSTRACT

The ability of Fruit and Vegetable Wash (containing 25 ppm chlorine), Viper™ (containing 1000 ppm hydrogen peroxide), Fit™ (containing 4,000 ppm citric acid and 450 ppm sodium lauryl sulfate), SCJP 16-172 (containing 1,000 ppm lactic acid and 750 ppm sodium lauryl sulfate), and SCJP 16-162 (containing 3000 ppm lactic acid and 3000 ppm ethanol) to reduce populations of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in an aqueous model system and on inoculated fresh produce including apples (whole and sliced), strawberries, and lettuce (whole and shredded) was assessed. Samples of each sanitizer solution were inoculated to contain approximately 10^6 CFU/ml of either pathogen after which aliquots were removed at 15 sec intervals over a period of 5 min and appropriately plated on selective media to determine D-values. Alternatively, produce was inoculated by dipping to contain approximately 10^6 *E. coli* O157:H7 or *L. monocytogenes* CFU/g, held overnight, submerged in each sanitizer solution for up to 5 min and then examined for survivors. In the model system, D-values ranged from 25 - 197 seconds following 5 min of sanitizer exposure. Based on D-values, Fruit and Vegetable Wash and SCJP 16-162 were the most effective (25 – 31 sec) followed by SCJ 16-172 and Viper™ (31 – 37 sec), and Fit™ (184- 197 sec). Fruit and Vegetable Wash and SCJP 16-162 treatment of all produce resulted in maximum log reductions of 3.3 and 3.4 logs for *L. monocytogenes* and *E. coli* O157:H7, respectively. In comparison, produce treatment with SCJ 16-172 and Viper™ yielded reductions of 3 and 2.5 logs for

L. monocytogenes and *E. coli* O157:H7, respectively. Fit^{TM} was the least effective sanitizer, giving maximum reductions of only about 1 log for *L. monocytogenes* and *E. coli* O157:H7.

INTRODUCTION

The potential for fruits and vegetables to become contaminated with pathogenic microbes, including *L. monocytogenes* and *E. coli* O157:H7, is high due to many produce contamination sources during growing, harvesting, processing, and distribution, including irrigation water and animal waste fertilizers (FDA, 1998; Madden, 1992). After harvest, fruits and vegetables often contain microbial populations of 10^4- to 10^6-CFU/ g (Bracket et al., 1994).

Since 1990, fruits and vegetables were the third leading cause of foodborne illness accounting for 9,413 reported cases. Between 1982 and 1994, salad bars containing raw fruits and vegetables ranked as the third leading cause of infection by *E. coli* O157:H7 in the United States (CPSI, 2002). *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 have been associated with a wide range of products including lettuce, apple cider, alfalfa sprouts, bean sprouts, watermelon, radish sprouts, cabbage, celery, cucumbers, potatoes, radishes, and tomatoes and cantaloupe (Zhao et al., 1993; Beuchat, 1996a; Beuchat, 1996b).

The United States Food Safety Initiative, which was issued in 1997 by President Clinton, addressed public concerns about the safety of the national food supply. The aim was to improve food safety and reduce the incidence of foodborne illness to the greatest extent feasible. Replacement of traditional sanitizers to treat or recycle food-processing wastewater with safer, environmentally-friendly, and more effective sanitizers is of great concern for the fresh produce industry. Identification of alternative sanitizers by the food industry is continually evolving through research that expands the understanding of their

application and efficacy. The challenge is to attain the 5-log kill recommendation set by the Food and Drug Administration (FDA) for selected commodities.

While water alone can effectively remove organic matter from produce, incorporation of a sanitizer is necessary to reduce microorganisms by more than 1-2 logs (Abdelnoor et al., 1983). The most commonly used sanitizer is chlorine. Unfortunately, chlorine compounds are corrosive, inherently unstable, produce trace amounts of organochlorine compounds (chemicals that have been shown to cause cancer in laboratory animals) including chloroform, trihalomethane (THM), bromodichloromethane, and MX [3-chloro-4-(dichloromethyl)-5-hydroxyl-2(5H)-furanone], and are affected by the presence of organic debris, temperature, and pH (Richardson, 1998). The effectiveness of chlorine for sanitizing raw fruits and vegetables is unpredictable and the same concentrations of chlorine may result in significantly different log reductions depending on the type of produce treated. For example, a chlorine dip of 200 ppm reduced the population of *L. monocytogenes* by about 2 logs on Brussels sprouts, whereas dipping in water alone reduced the population by only 1 log (Bracket, 1987). However, using 200 ppm chlorine, Zhang and Farber (1996) reported maximum reductions for *L. monocytogenes* on shredded lettuce of 1.3-1.7 log CFU/g, which is similar to treatment with water. Therefore, identification of sanitizers that are effective regardless of the pH, temperature, presence of organic debris, and type of produce examined is essential. Research and commercial applications have indicated that alternative sanitizers including organic acids and hydrogen peroxide may offer more benefits than chlorine.

Hydrogen peroxide is effective against a large number of organisms including bacteria, yeasts, fungi, and viruses. Generally, hydrogen peroxide is more effective against gram-negative than gram-positive bacteria. It is not affected by changes in pH or presence of organic debris. A study by Peters (1995) reported that 3% hydrogen peroxide decreased numbers of *E. coli* O157:H7 on broccoli and tomatoes by 2 and 4 log CFU/g, respectively. In another study, Yu et al. (2001) reported *E. coli* O157:H7 reductions of 1.2 and 2.1 log CFU/g on strawberries using 1% and 3% hydrogen peroxide, respectively. Results from studies on a limited number of fruits and vegetables indicate that hydrogen peroxide has high potential for use as a sanitizer.

Organic acids have received significant attention as sanitizers since they are naturally present in fruits and vegetables as normal accumulation products during fermentation to retard bacterial growth. The antimicrobial effect of organic acids has been attributed to depression of pH below the growth range and metabolic inhibition by the undissociated acid which can most readily penetrate bacterial cells (Taormina and Beuchat, 1999). Unlike chlorine, organic acids are not corrosive to processing equipment, produce no toxic by-products, remain effective in the presence of organic matter, and are not affected by changes in temperature. A wide range of organic acids, including acetic, lactic, citric, and propionic acid have been tested for their efficiency in disinfection of raw fruits and vegetables. Application of lemon juice (citric acid) to cut fruits and vegetables inhibits both browning and growth of pathogens (Beuchat, 1998). Similarly, a 2% acetic acid was shown to reduce *Yersinia enterocolitica* on parsley by >6 logs. However, other studies have demonstrated far more variable results. For example, Shapiro and Holder (1960) reported that treatment of salad vegetables with 1500 ppm

citric acid did not inhibit bacterial growth during 4 days of storage at 10°C and results were not significantly different from those obtained by treatment with water.

In response to consumer concerns over the safety of raw fruits and vegetables, the industry begun to develop and market several new fruit and vegetable sanitizer washes including Fit™ (citric acid/sodium lauryl sulfate) (Proctor and Gamble, Inc.), which is no longer produced, and Viper™ (1,000 ppm hydrogen peroxide) (SCJP, Sturtevant, WI). Other experimental sanitizers are being developed that contain organic acids in combination with other additives to create a synergistic effect. Despite claims of effectiveness by the various manufacturers, antimicrobial activity of most of these alternative sanitizers has not yet been independently confirmed. The objective of this study was to compare five proposed alternative sanitizers- SCJ Experimental Wash (1000 ppm lactic acid / 750 ppm sodium lauryl sulfate), SCJ Fruit and Vegetable wash (25 ppm hypochlorite), Fit™ (4,000 ppm citric acid, 450 ppm sodium lauryl sulfate), SCJ 16-162 (3000 ppm lactic acid, 3000 ppm ethanol), and Viper™ (1,000 ppm hydrogen peroxide) to traditional chlorine for activity against *E. coli* O157:H7 and *L. monocytogenes*. Produce (strawberries, apples, and lettuce) was selected based on involvement in recent outbreaks along with variable surface characteristics.

MATERIALS AND METHODS

Bacterial Strains. Three *E. coli* O157:H7 strains (AR, AD 305, AD 317) as well as three *L. monocytogenes* strains (CWD 95, CWD 249, and CWD 201) were obtained from C.W. Donnelly (Dept. of Nutrition and Food Sciences, University of Vermont, Burlington, VT). Stock cultures were maintained at -70°C in tripticase soy broth containing 10% (v/v) glycerol and subcultured twice in tripticase soy broth containing 0.6% (w/v) yeast extract (TSB-YE) (Difco Laboratories, Detroit, MI) at 35°C/ 18-24 h before use.

Preparation of Inoculum. Equal volumes of culture were combined to produce one three-strain cocktail of *E. coli* O157:H7 and *L. monocytogenes*. The cocktails were then centrifuged at 10,000 x g/ 15 min/ 4°C and re-suspended in sterile tap water to simulate commercial conditions.

Sanitizers. The following five sanitizer solutions were compared to a water control:

1. Fruit and Vegetable Wash containing 25 ppm chlorine (S.C. Johnson Professional (SCJP), Racine, WI)
2. Viper™ containing 1000 ppm hydrogen peroxide (SCJP)
3. Fit™ containing 4,000 ppm citric acid and 450 ppm sodium lauryl sulfate (Proctor and Gamble, Cincinnati, OH)
4. SCJP 16-172 containing1,000 ppm lactic acid and 750 ppm sodium lauryl sulfate (SCJP)

5. SCJP16-162 containing 3000 ppm lactic acid and 3000 ppm ethanol (SCJP)

The Fruit and Vegetable Wash was prepared by adding 0.68 grams of the powdered product to 1 liter of sterile distilled (SDW) water to obtain 25 ppm active chlorine. Total residual chlorine was measured using a chlorine colorometric test kit (Hach Co., Ames, Iowa). Fit™ was prepared by adding 5.25 gm of powder to 1 liter of SDW according to the manufacturers directions. Viper™ containing1000 ppm hydrogen peroxide was prepared by adding of 15.6 mls of the concentrate to 1 liter of SDW. SCJP 16-172 and 16-162 were prepared by adding 5.9 and 10 ml respectively, of the liquid concentrate to 1 liter of SDW.

Model System Study. Test tubes containing aqueous solutions (9 ml) of each sanitizer were inoculated from the three-strain cocktail to obtain approximately 10^6 *E. coli* O157:H7 or *L. monocytogenes* CFU/ml. Initially and at 15 second intervals, 1 ml aliquots were removed over a period of 5 minutes. A sodium thiosulfate (0.1 N) stock solution was prepared by dissolving 25 g of sodium thiosulfate (Sigma Chemical Co., St. Louis, MO) in 1 L of autoclaved distilled water (Rand et al., 1975). The solution was used to neutralize residual chlorine with 1 ml aliquots added to the first tube for serial dilution. Samples were serially diluted and surface-plated on tripticase soy agar containing 0.6% (w/v) yeast extract (TSAYE) (Difco) to determine numbers of *E. coli* O157:H7 and *L. monocytogenes*. Each treatment was performed in triplicate. D-values for each antimicrobial treatment were then determined by linear regression.

Produce Study. Golden Delicious apples (whole and sliced- to simulate commercial packaging applications), leaf lettuce (whole and shredded- to simulate commercial packaging applications), and strawberries (whole) were obtained from local suppliers. Unwashed produce was stored for 24 h. at 4°C before use. Before inoculation, the outer three or four lettuce leaves were discarded. Lettuce leaves were either used whole or shredded into ½" wide strips using a sterile razor blade. Golden Delicious apples were unwaxed, blemish-free, and of uniform size and shape. Apples were either used_whole or cut into 1" thick wedges before use. Fresh blemish-free strawberries of uniform size and shape were obtained and treated whole.

Inoculation. Sterile polyethylene bags (25 cm X 20 cm) containing 300 ml of SDW were inoculated with the three-strain cocktail as described previously to obtain 10^8 CFU/ml for produce inoculation. Inner lettuce leaves (100 g) were placed in the bag and manually shaken for 20 minutes to ensure even distribution of the organism on the product. Batches of 6-8 apples and 20-25 strawberries were immersed in the inoculum and agitated by stirring with a sterile glass rod for 20 minutes to ensure uniform inoculation. All produce was then air dried in a laminar flow hood for 18-24 h. at 24°C before being subjected to the various sanitizer treatments.

Sanitizer Exposure. Produce samples were subjected to the following wash treatments after inoculation: water, Fruit and Vegetable Wash, Viper™, Fit™, SCJP 16-172, and SCJP 16-162. All products were completely immersed in sterile polyethylene

bag containing 100 ml of sanitizer for 5 minutes and were not subjected to a potable water rinse after sanitizer exposure.

Microbial Analysis. Strawberry and lettuce samples (40 g each) were drained and placed in sterile polyethylene bags containing 100 ml of sodium thiosulfate (0.1 N) stock solution to neutralize residual chlorine. These samples were then homogenized in a stomacher (Model SD-45, Tekmar Co., Cincinnati, OH) for 2 minutes. Triplicate samples of the produce wash water were serially diluted in 0.1% peptone and spiral-plated (400 Autoplate Automated Spiral Plater, Spiral Biotech, Inc., Dethexda, MD) on Sorbitol MacConkey Agar (SMAC) (Oxoid, Basingstoke, Hampshire, UK), Modified Oxford Agar (MOX) (Difco Laboratories, Detroit, MI) to determine numbers of *E. coli* O157:H7 and *L. monocytogenes* respectively. Apples were drained, individually placed into separate bags containing 100 ml of 0.1% peptone and vigorously shaken for 5 minutes followed by rubbing/massaging for another 10 minutes. Triplicate samples of apple wash water were serially diluted and spiral plated on SMAC or MOX.

RESULTS

Statistical Analysis of Microbial Data. All microbial data were analyzed using a factorial ANOVA on triplicate samples at a significance level of $p<0.05$. Statistical results were subjected to Bonferroni adjustment for conservative analysis.

Aqueous Model System Study. D-values for *E. coli* O157:H7 and *L. monocytogenes* in the aqueous model system after exposure to various sanitizers are shown in Figure 1. Fit™ had the highest D-value of 197 and 184 seconds for *E. coli* O157:H7 and *L. monocytogenes*, respectively. SCJ 16-172 and Viper™ were not significantly different from each other and had similar D-values (31- 37 seconds). SCJ Fruit and Vegetable Wash and SCJ 16-162 were not significantly different from each other and had the lowest D-values (25- 27 and 27-31 for *E. coli* O157:H7 and *L. monocytogenes*, respectively.

Produce Inoculation Studies. The D-values for *E. coli* O157:H7 on fresh produce exposed to the same sanitizers are shown in Figure 2. SCJ Fruit and Vegetable Wash and SCJ 16-162 were not significantly different from each other and had the lowest D-values (33 – 64 seconds), while Fit™ had the highest D-values for *E. coli* O157:H7 on all produce types (80 - 132 seconds). SCJ 16-172 and Viper™ were not significantly different from each other and had similar D-values (39 – 79 seconds), regardless of the type of produce. Sliced apples and shredded lettuce yielded slightly higher D-values for all sanitizers (60 – 132 seconds).

Figure 1. D-Values (sec) for *E. coli* O157:H7 and *L. monocytogenes* Exposed to Various Sanitizers in an Aqueous Model System

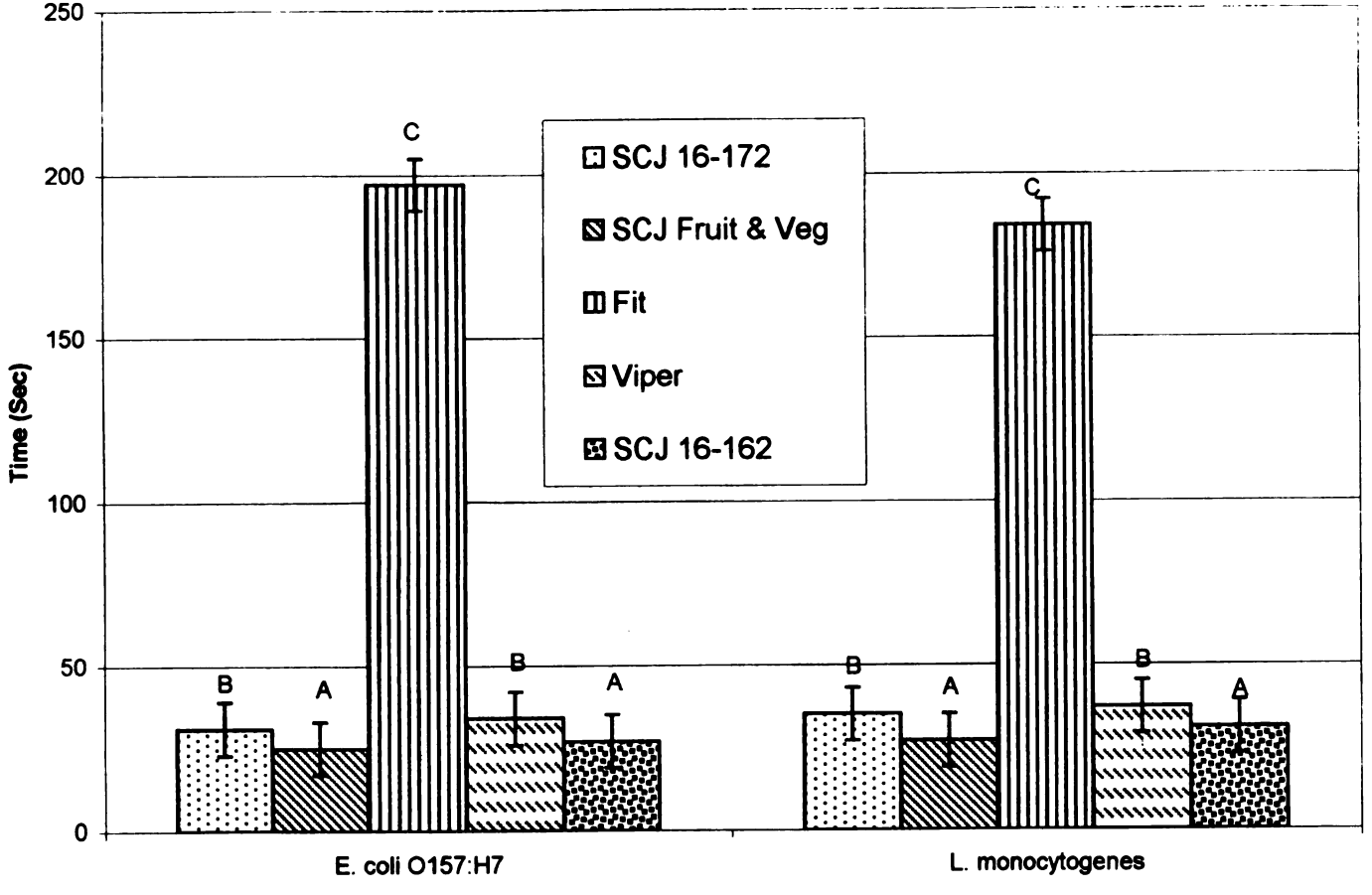
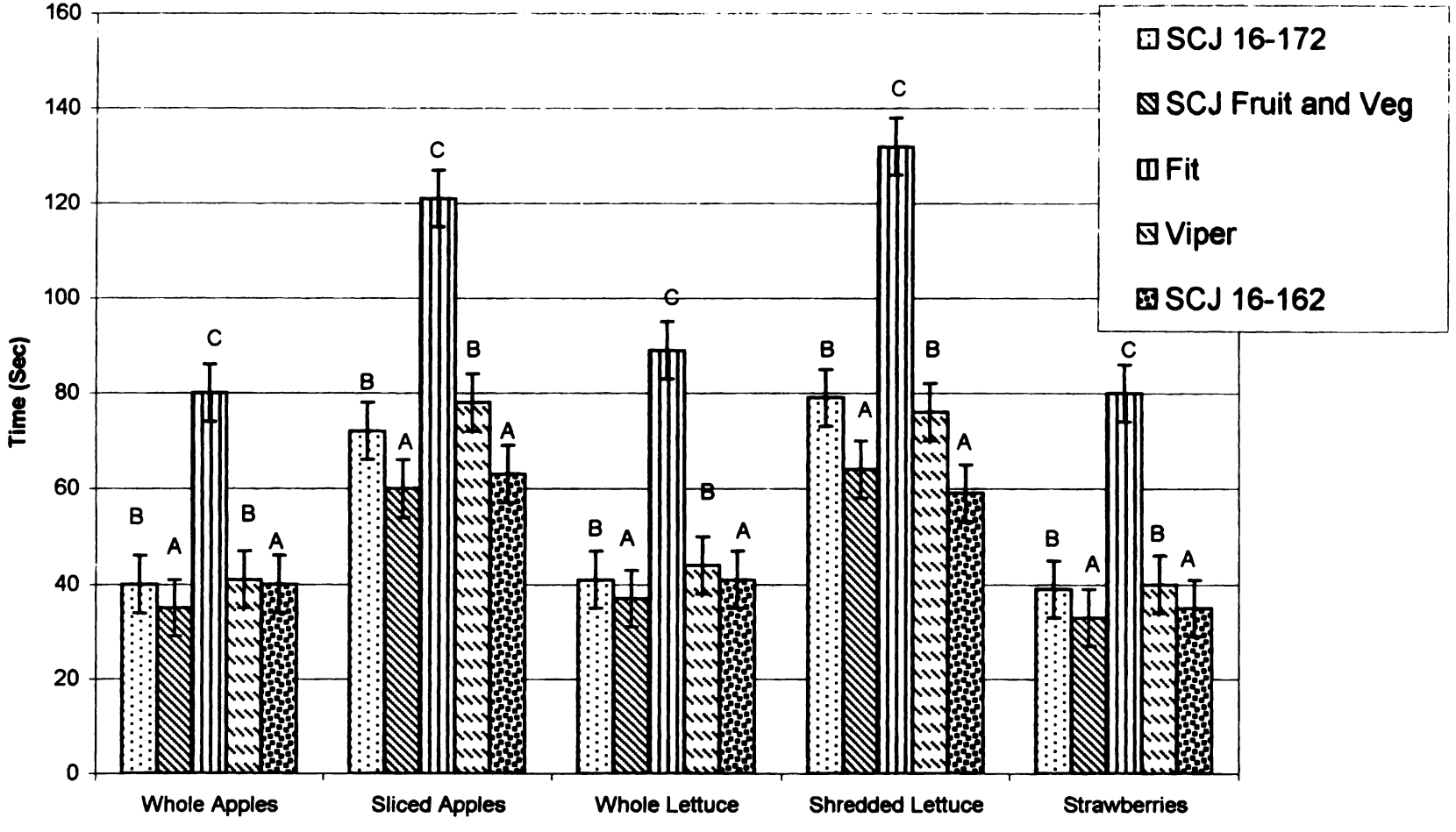


Figure 2. D-Values (sec) for *E. coli* O157:H7 on Fresh Produce Exposed to Various Sanitizers



Using *L. monocytogenes* as the test organism, Fit™ had the highest D-values for all produce types (89 – 140 seconds) (Figure 3). SCJ Fruit and Vegetable Wash and SCJ 16-162 were not significantly different from each other and had the lowest D-values for whole apples, sliced apples, and shredded lettuce (34 – 64 seconds). SCJ 16-172 and Viper™ had similar D-values and were not significantly different from each other on whole apples, sliced apples, whole lettuce, and strawberries. Treatment of whole lettuce and strawberries with SCJ Fruit and Vegetable Wash, SCJ 16-162, SCJ 16-172 and Viper™ produced D-values that were not significantly different from each other for all four treatments. The D-values for SCJ 16-172 and Viper™ on shredded lettuce were 82 and 71 seconds, respectively, and were significantly different from each other.

The log reductions for *E. coli* O157:H7 and *L. monocytogenes* after a 5 minute exposure to the various sanitizers are shown in Table 1. Water and Fit™ were not significantly different from each other and reduced populations of both pathogens by only ~1 log for all produce examined. SCJ 16-172 and Viper™ were not significantly different from each other and reduced populations of *L. monocytogenes* by approximately 2.5 logs and *E. coli* O157:H7 by approximately 3 logs on all produce examined. SCJ Fruit and Vegetable Wash and SCJ 16-162 did not significantly differ from each other and reduced populations of *L. monocytogenes* and *E. coli* O157:H7 by approximately 3.2 logs on all produce examined.

Figure 3. D-Values (sec) for *L. monocytogenes* on Fresh Produce Exposed to Various Sanitizers

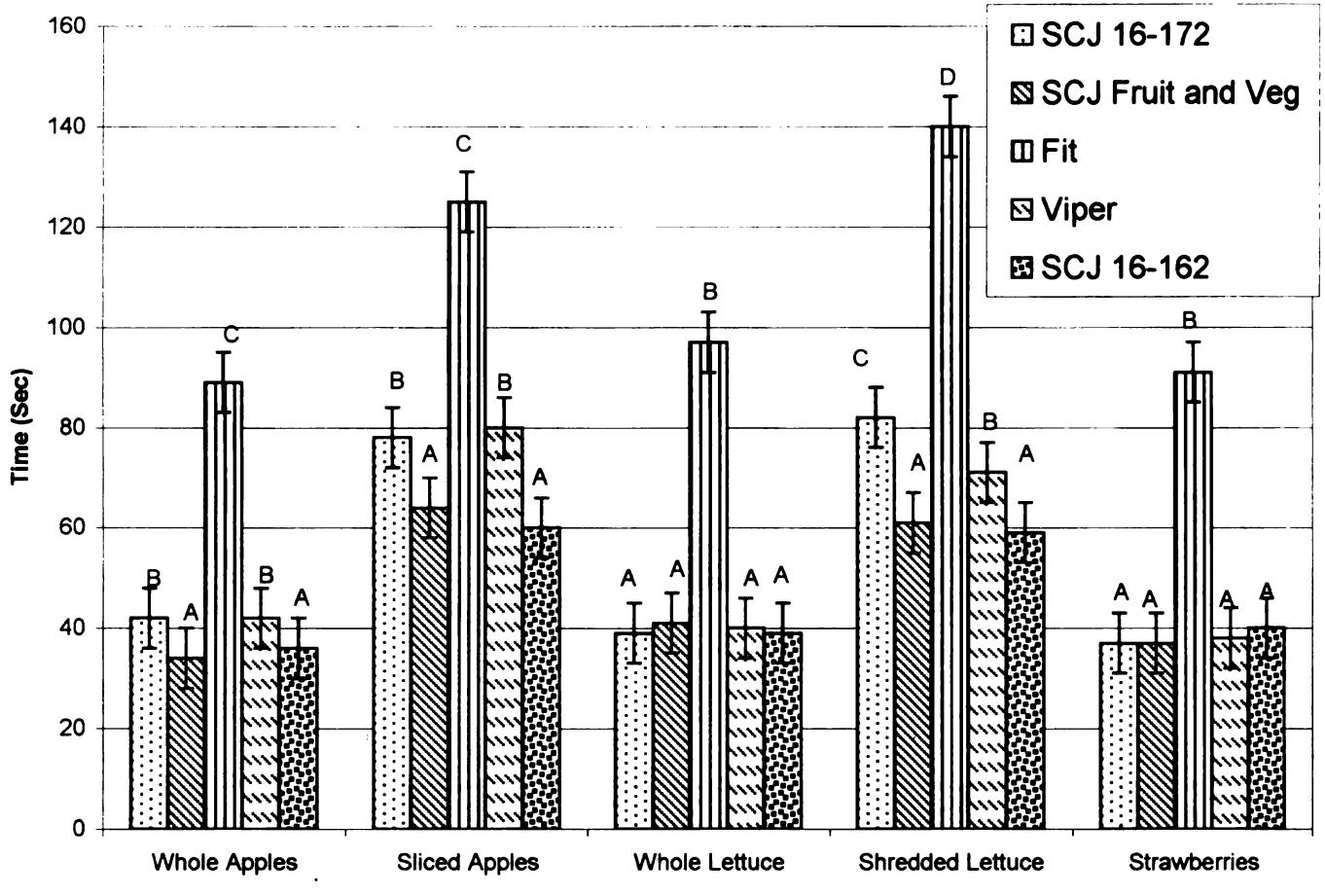


Table 1. *E. coli* O157:H7 and *L. monocytogenes* Reductions on Fresh Produce After a 5 Minute Exposure to Various Sanitizers

Produce	*L. monocytogenes* Initial (CFU/g)	*L. monocytogenes* after sanitizer (CFU/g)	Log Reduction	*E. coli* O157:H7 Initial (CFU/g)	*E. coli* O157:H7 after sanitizer (CFU/g)	Log Reduction
Control - Water / 300 sec.						
Whole Apples	8.2 ± 0.34 a[a]	7.3 ± 0.19 a[b]	0.9 ± 0.16	7.9 ± 0.19 a	7.0 ± 0.15 a	0.9 ± 0.12 a
Sliced Apples	8.0 ± 0.24 a	7.0 ± 0.25 a	1.0 ± 0.22	8.1 ± 0.32 a	6.9 ± 0.16 a	1.2 ± 0.11 a
Whole Lettuce	7.9 ± 0.31 a	6.9 ± 0.18 a	1.0 ± 0.26	8.2 ± 0.14 a	7.2 ± 0.24 a	1.0 ± 0.15 a
Shredded Lettuce	8.1 ± 0.16 a	7.0 ± 0.30 a	1.1 ± 0.19	8.0 ± 0.22 a	7.0 ± 0.34 a	1.0 ± 0.19 a
Strawberries	8.1 ± 0.21 a	7.2 ± 0.32 a	0.9 ± 0.17	8.0 ± 0.33 a	7.1 ± 0.19 a	0.9 ± 0.21 a
Sanitizer - SCJ 16-172 / 300 sec.						
Produce	***L. monocytogenes* Initial (CFU/g)**	***L. monocytogenes* after sanitizer (CFU/g)**	**Log Reduction**	***E. coli* O157:H7 Initial (CFU/g)**	***E. coli* O157:H7 after sanitizer (CFU/g)**	**Log Reduction**
Whole Apples	8.0 ± 0.20 a	5.2 ± 0.18 b	2.8 ± 0.16	7.9 ± 0.18 a	5.1 ± 0.14 b	2.8 ± 0.14 b
Sliced Apples	8.1 ± 0.17 a	5.9 ± 0.16 b	2.2 ± 0.13	8.2 ± 0.23 a	5.2 ± 0.12 b	3.0 ± 0.11 b
Whole Lettuce	8.2 ± 0.32 a	5.8 ± 0.23 b	2.4 ± 0.27	8.1 ± 0.31 a	5.2 ± 0.11 b	2.9 ± 0.13 b
Shredded Lettuce	8.0 ± 0.26 a	5.8 ± 0.25 b	2.2 ± 0.26	8.2 ± 0.27 a	5.3 ± 0.20 b	2.9 ± 0.12 b
Strawberries	7.9 ± 0.15 a	5.7 ± 0.32 b	2.2 ± 0.34	8.0 ± 0.16 a	5.4 ± 0.16 b	2.6 ± 0.18 b
Sanitizer - SCJ Fruit & Veg / 300 sec.						
Produce	***L. monocytogenes* Initial (CFU/g)**	**L. monocytogenes after sanitizer (CFU/g)**	**Log Reduction**	***E. coli* O157:H7 Initial (CFU/g)**	***E. coli* O157:H7 after sanitizer (CFU/g)**	**Log Reduction**
Whole Apples	8.1 ± 0.15 a	5.0 ± 0.25 c	3.1 ± 0.18	7.9 ± 0.24 a	4.8 ± 0.10 c	3.1 ± 0.11 c
Sliced Apples	8.0 ± 0.16 a	4.9 ± 0.20 c	3.1 ± 0.26	7.9 ± 0.29 a	4.7 ± 0.14 c	3.2 ± 0.16 c
Whole Lettuce	8.0 ± 0.26 a	5.0 ± 0.30 c	3.0 ± .015	8.3 ± 0.21 a	4.9 ± 0.17 c	3.4 ± 0.18 c
Shredded Lettuce	7.9 ± 0.31 a	5.1 ± 0.28 c	2.8 ± 0.34	8.2 ± 0.17 a	5.0 ± 0.12 c	3.2 ± 0.17 c
Strawberries	8.2 ± 0.11 a	5.1 ± 0.14 c	3.1 ± 0.16	8.1 ± 0.28 a	5.0 ± 0.12 c	3.1 ± 0.11 c

Table 1. (Cont.)

Sanitizer Fit™ / 300 sec.

Produce	*L. monocytogenes* Initial (CFU/g)	*L. monocytogenes* after sanitizer (CFU/g)	Log Reduction	*E. coli* O157:H7 Initial (CFU/g)	*E. coli* O157:H7 after sanitizer (CFU/g)	Log Reduction
Whole Apples	8.1 ± .023 a	7.3 ± 0.24 a	0.8 ± 0.13	8.2 ± 0.20 a	7.0 ± 0.16 a	1.2 ± 0.13 a
Sliced Apples	8.0 ± 0.31 a	7.0 ± 0.30 a	1.0 ± 0.17	7.9 ± 0.16 a	6.9 ± 0.20 a	1.0 ± 0.14 a
Whole Lettuce	8.0 ± 0.16 a	6.9 ± 0.19 a	1.1 ± 0.22	7.9 ± 0.26 a	7.2 ± 0.11 a	0.7 ± 0.12 a
Shredded Lettuce	7.9 ± 0.24 a	7.0 ± 0.32 a	0.9 ± 0.14	8.0 ± 0.14 a	7.0 ± 0.10 a	1.0 ± 0.11 a
Strawberries	8.2 ± 0.20 a	7.2 ± 0.28 a	1.0 ± 0.23	8.2 ± 0.25 a	7.1 ± 0.22 a	1.1 ± 0.12 a

Sanitizer - Viper / 300 sec.

Produce	*L. monocytogenes* Initial (CFU/g)	*L. monocytogenes* after sanitizer (CFU/g)	Log Reduction	*E. coli* O157:H7 Initial (CFU/g)	*E. coli* O157:H7 after sanitizer (CFU/g)	Log Reduction
Whole Apples	8.1 ± 0.20 a	5.2 ± 0.16 b	2.9 ± 0.14	8.0 ± 0.24 a	4.9 ± 0.12 b	3.1 ± 0.16 b
Sliced Apples	8.3 ± 0.19 a	5.9 ± 0.11 b	2.4 ± 0.13	8.0 ± 0.30 a	5.0 ± 0.13 b	3.0 ± 0.17 b
Whole Lettuce	7.9 ± 0.33 a	5.8 ± 0.14 b	2.1 ± 0.11	8.3 ± 0.19 a	5.3 ± 0.10 b	3.0 ± 0.12 b
Shredded Lettuce	8.0 ± 0.21 a	5.8 ± 0.11 b	2.2 ± 0.10	7.9 ± 0.28 a	5.2 ± 0.11 b	2.7 ± 0.11 b
Strawberries	8.0 ± 0.20 a	5.7 ± 0.20 b	2.3 ± 0.12	8.1 ± 0.31 a	5.4 ± 0.15 b	2.7 ± 0.14 b

Sanitizer - SCJ 16-162 / 300 sec.

Produce	*L. monocytogenes* Initial (CFU/g)	*L. monocytogenes* after sanitizer (CFU/g)	Log Reduction	*E. coli* O157:H7 Initial (CFU/g)	*E. coli* O157:H7 after sanitizer (CFU/g)	Log Reduction
Whole Apples	8.1 ± 0.21 a	5.0 ± 0.16 c	3.1 ± 0.11	8.0 ± 0.31 a	4.7 ± 0.17 c	3.3 ± 0.15 c
Sliced Apples	8.2 ± 0.23 a	4.9 ± 0.17 c	3.3 ± 0.14	8.1 ± 0.18 a	4.7 ± 0.16 c	3.4 ± 0.11 c
Whole Lettuce	8.2 ± 0.30 a	5.0 ± 0.20 c	3.2 ± 0.17	8.2 ± 0.26 a	4.9 ± 0.17 c	3.3 ± 0.10 c
Shredded Lettuce	7.9 ± 0.19 a	5.1 ± 0.13 c	2.8 ± 0.15	8.2 ± 0.30 a	4.9 ± 0.10 c	3.3 ± 0.14 c
Strawberries	8.0 ± 0.22 a	5.1 ± 0.10 c	2.9 ± 0.20	7.9 ± 0.15 a	5.0 ± 0.13 c	2.9 ± 0.16 c

DISCUSSION

In the model system and on all produce, Fit™ had the highest D-values and was the least effective sanitizer for both *L. monocytogenes* and *E. coli* O157:H7 and was not significantly different from the water control. The limited amount of antimicrobial activity associated with Fit™ is reportedly due to citric acid with sodium lauryl sulfate included in the formulation as a surfactant. Previously, researchers have demonstrated that citric acid is of limited use in decontaminating fresh produce. Shapiro and Holder (1960) treated salad vegetables with citric acid at concentrations up to 1,500 ppm and observed no difference in bacterial growth compared to controls. Similarly, treatment of cut lettuce, endive, carrots, celery, radishes, and green onions with 10,000 ppm ascorbic acid resulted in less than a 1 log decrease in numbers of aerobic mesophilic bacteria after 10 days of storage at 4.4°C (Priepke et al., 1976). Addition of surfactants to commercial fruit and vegetable wash water reportedly can enhance the removal of pathogenic microorganisms adhering to the surface of fresh produce. Anionic acid surfactants such as sodium lauryl sulfate aid in removal of surface bacteria by facilitating wetting of the produce surface. In one study, a commercial 1% acid anionic surfactant solution removed approximately 1-2 logs CFU/g of *E. coli* O157:H7 from apple halves (Sapers et al., 1999). Bacterial reductions of approximately 1 log can be attributed to the washing effect, since water alone contains no antimicrobial activity. For example, Wright et al. (2000) found that treatment of *E. coli* O157:H7-inoculated apples with water removed 1.1 log CFU/g. Therefore, when used alone, most surfactants are presumed to be minimally effective. Use of a surfactant in combination with one or more organic acids can create a synergistic effect with more promising results. For example, a 1% commercial surfactant

- phosphoric acid solution removed approximately 2 logs CFU/g of *E. coli* O157:H7 from cut apple surfaces after 5 minutes (Sapers et al., 1999). Similarly, Beuchat et al. (2001) determined that treatment of *Salmonella*-inoculated alfalfa seeds with Fit™ reduced populations by 2.3 log CFU/g after 30 minutes with this treatment as effective as 200 ppm chlorine. In our study, however, a synergistic effect from Fit™, which combines sodium lauryl sulfate and citric acid, was not observed.

In the model system and on all produce types examined, SCJ Fruit and Vegetable Wash and SCJ 16-162 proved to be most effective for reducing populations of *L. monocytogenes* and *E. coli* O157:H7 by approximately 3.2 logs on all produce examined. These results support the effectiveness of chlorine as an antimicrobial agent with chlorine-based sanitizers most widely used for disinfecting fruits and vegetables. For example, Nguyen-the and Carlin (1994) found that immersing Brussels sprouts containing 10^6 CFU/g of *L. monocytogenes* in a 200 ppm chlorine solution for 10 seconds decreased viable populations by about 2 logs. In addition, Wright et al. (2000) reported that 200 ppm sodium hypochlorite decreased *E. coli* O157:H7 populations on apples by 2.1 logs after 2 minutes.

SCJ 16-162, which was comparable to chlorine for pathogen inactivation, may provide a safer alternative to traditional chlorine-based sanitizers. The efficacy of SCJ 16-162 likely results from a synergistic effect caused by the combination of lactic acid and ethanol. Ethanol, which destroys bacteria by targeting the bacterial cell wall, leads to lysis of the cytoplasmic membrane and release of cellular contents (Pethicia, 1958). Ethanol is bacteriocidal at concentrations of 30% or higher, depending on the bacterial species and exposure time (Morton, 1950). The efficacy of ethanol for destruction of *E.*

coli O157:H7 was reported by Rutala (1998), with an aqueous solution of 60% ethanol destroying *E. coli* within 60 seconds. Lactic acid generally has proven less effective for inactivating *E. coli* O157:H7 on fresh produce. (Comes and Beelman, 2002). In studies involving multiple pathogens, *E. coli* O157:H7 which is known for acid tolerance, was shown to be more resistant toward organic acid treatments compared to many other organisms. Lactic acid destroys bacterial cells by decreasing the intracellular pH to lethal levels. The synergistic effect that occurs between ethanol and lactic acid is likely responsible for the effectiveness of SCJ 16-162 that was observed in this study.

SCJ 16-172 and Viper were not significantly different from each other and had D-values in the model system study that ranged from 31 – 37 seconds, indicating that both treatments were significantly more effective than treatment with Fit™. As expected, lactic acid was more effective than citric acid in reducing *E. coli* O157:H7 and *L. monocytogenes* with similar findings reported by Ryu et al. (1999). This effect was attributed to the higher pK_a for lactic acid compared to citric acid (Ryu et al., 1999). SCJ 16-172 and Viper™ were significantly less effective than SCJ 16-162 and SCJ Fruit and Vegetable Wash in the model system. The significantly lower D-values for SCJ 16-172 as compared to SCJ 16-162 are likely the result of formulation differences with SCJ 16-162 containing three times the concentration of lactic acid as SCJ 16-172. Additionally, SCJ 16-162 contained ethanol, which was absent in the formulation for SCJ 16-172. When used on produce inoculated with *E. coli* O157:H7, SCJ 16-172 and Viper™ were not significantly different from each other. Using *L. monocytogenes* as the test organism, SCJ 16-172 and Viper™ were not significantly different from each other for whole apples, sliced apples, whole lettuce, or strawberries; however Viper™ was significantly more

effective than SCJ 16-172 on shredded lettuce. This may be due to the higher amount of organic debris present or the increased surface area from cutting. SCJ 16-172 and Viper™ were not significantly different from each other and reduced populations of *L. monocytogenes* and *E. coli* O157:H7 approximately 2.5 and 3 logs, respectively, on all produce, indicating that it was significantly more effective than Fit™, but significantly less effective than SCJ 16-162 and SCJ Fruit and Vegetable Wash. When Yu et al. (2001) examined the ability of hydrogen peroxide to decrease populations of *E. coli* O157:H7 on strawberries, reductions of 1.2 and 2.1 log were observed using 1 and 3% hydrogen peroxide, respectively. Additionally, Sapers et al. (1999) reported that populations of *E. coli* O157:H7 on inoculated apple halves decreased 3 to 4 logs using 5% hydrogen peroxide.

Treatment of whole lettuce and strawberries with SCJ 16-172, SCJ Fruit and Vegetable Wash, Viper™, and SCJ 16-162 inoculated with *L. monocytogenes* yielded D-values that were not significantly different from each other. These produce types inevitably harbor organic debris and contain many crevices ideal for bacterial attachment. This was also documented by Yu et al. (2001) who indicated that the relative ineffectiveness of many sanitizers on strawberries was probably due to the inherent rough surface texture and the presence of numerous surface-borne achenes (seeds), which provide sites for bacterial attachment making these cells less accessible to sanitizing solutions. Rapid inactivation of chlorine compounds, and possibly SCJ 16-162, in the presence of organic debris may account for their inability to further reduce bacterial populations on these produce types.

Overall, sliced apples and shredded lettuce yielded significantly higher D-values compared to uncut products. Decreased antimicrobial activity was most likely due to leaching of organic material from these surfaces into the wash water since high concentrations of organic matter reportedly decrease the effectiveness of many sanitizers. In addition, many sanitizers react with plant tissues and extracellular biochemical components at wound sites and are unable to inactivate bacterial cells that are attached to or embedded in the plant tissue (Kim et al., 1999). The hydrophobic structure of the waxy cuticle of fruit and vegetable skins also provides a natural barrier which inhibits attachment and penetration of bacteria into flesh tissue. However, cut produce is not able to offer such protection with bacteria being able to penetrate and attach directly to the flesh of fruits and vegetables.

In conclusion, Fit™ was not effective in eliminating *E. coli* O157:H7 or *L. monocytogenes* from raw fruits and vegetables and was not significantly different from that of water alone. SCJ 16-172, SCJ Fruit and Vegetable Wash, Viper™, and SCJ 16-162 were effective in decreasing the numbers of *E. coli* O157:H7 and *L. monocytogenes* on raw produce surfaces. Although these sanitizers did not achieve reductions greater than 3 logs on raw fruits and vegetables, they should be considered useful in sanitation programs as part of a hurdle approach for minimally processed fruits and vegetables.

CHAPTER FOUR

USE OF HIGH FREQUENCY SONICATION, COPPER ION, AND SODIUM HYPOCHLORITE TO REDUCE MICROBIAL POPULATIONS IN RAW APPLES AND FRESH APPLE CIDER

ABSTRACT

The ability of sonication (44 to 48 kHz) to reduce populations of *Escherichia coli* O157:H7 and *Listeria monocytogenes* in inoculated cider was determined by selective plating. In addition, a hurdle approach to reduce populations of *E. coli* O157:H7 and *L. monocytogenes* in apple cider was examined using sodium hypochlorite (100 ppm) and copper ion water (1 ppm) on whole apples followed by juicerating and sonication of expressed cider at 44 to 48 kHz. Commercial cider was inoculated to contain approximately 10^6 CFU/ml of either pathogen after which aliquots were removed at 15 second intervals over a period of 5 minutes and appropriately plated to determine D-values. Alternatively, whole apples were inoculated by dipping to contain approximately 10^6 *E. coli* O157:H7 or *L. monocytogenes* CFU/g, held overnight, and submerged in copper ion water or copper ion water containing 100 ppm sodium hypochlorite for 3 minutes and then examined for survivors. Treated apples were also juicerated and the resulting cider was sonicated for 3 minutes. Populations of both pathogens decreased by 1-2 logs in inoculated cider following 3 minutes of sonication. Using the hurdle approach, copper ion water did not significantly reduce populations of either pathogen; however, copper ion water/ sodium hypochlorite (100 ppm) reduced populations of *L. monocytogenes* and *E. coli* O157:H7 by 2.3 and 2.2 log CFU/g, respectively. Juiceration of these apples reduced populations of either pathogen in the juice by 1.1 – 1.3 log CFU/ml with sonication of the expressed juice further decreasing *L. monocytogenes* and

E. coli O157:H7 by approximately 2 logs CFU/ml. Hence, a 5-log total process reduction for both pathogens was achievable using 1 ppm copper ion and 100 ppm sodium hypochlorite followed by juiceration and sonication.

INTRODUCTION

Recent outbreaks of *E. coli* O157:H7 infection traced to unpasteurized juices have provided significant challenges to both cider producers and regulatory agencies. In September 1980, 14 people who drank unpasteurized apple cider subsequently became ill. This is thought to be the first recorded outbreak of *E. coli* O157:H7 infection linked to unpasteurized cider (Steele et al, 1982). Between 1980 and 1990, at least six documented outbreaks of *E. coli* O157:H7 infection in North America were associated with unpasteurized apple cider with five of the outbreaks occurring over a 3-year period (Besser et al., 1993; CDC 1997). In the largest of these outbreaks, which included 49 cases and 1 fatality, Odwalla-brand apple juice and juice mixtures produced in the Pacific northwest were recalled nationwide (FDA, 1996). A second outbreak of *E. coli* O157:H7 infection occurring in Washington State in 1996 was linked to apple cider that was pressed at a church event. The implicated apples had been washed in chlorinated water, although it is not clear how much chlorine was used (FDA, 1998).

Another potential contaminant of apples is *Listeria monocytogenes*, since this pathogen is commonly found on decaying vegetation and in soil, animal feces, sewage, silage, and water (Beuchat, 1992). *L. monocytogenes* can grow over a wide range of pH (4.1 to 9.6) and temperatures (0.5°C to 45°C), which facilitates growth in a large variety of foods (Van Renterghem et al., 1991).

The increasing frequency of outbreaks associated with cider, has heightened the need for regulations that will enhance the safety of apple cider. In response, the FDA published the juice Hazard Analysis and Critical Control Points (HACCP) final rule on Jan 19, 2001 which requires that fresh juice manufacturers reduce bacterial levels by 5

logs in the final product. All firms except small and very small businesses had to comply by Jan. 22, 2002. Small (employing < 500 people) and very small (total annual sales < $500,000) businesses must comply by Jan. 21, 2003 and Jan. 20, 2004, respectively (FDA, 2001). These regulations, however, do not address the risks associated with consumption of whole apples, which may harbor human pathogens. To date, an outbreak of *E. coli* O157:H7 infection associated with consumption of raw apples has not been reported. However, the potential for infection caused by the consumption of apples harboring *E. coli* O157:H7 exists. Because the number of cells needed to cause illness is low, a single contaminated apple may result in illness, or infect other apples during processing. Chlorine compounds are often used as sanitizers during processing of whole apples. Unfortunately, these chemicals can react to produce trace amounts of various carcinogenic organochlorine compounds and are rapidly inactivated by organic material inherent to raw produce surfaces.

Therefore, new decontamination methods that are effective against a wide range of bacteria, do not produce harmful by-products, and are not affected by the presence of organic matter, are of great interest to the fresh produce industry. One such strategy is sonication, a process that involves emission of high frequency sound waves through a liquid medium to dislodge organic debris and mechanically disrupt bacteria. Sonication destroys bacteria by cavitation, a phenomenon in which mechanical vibrations of high frequencies cause bubbles to expand, and then implode violently, releasing large amounts of energy and generating very high temperatures and pressures resulting in cell breakage (Shukla, 1992). Although routinely used to clean equipment in both medical and dental

offices (Rutala et al., 1998; Villasenor et al., 1993), sonication has not yet seen widespread use in the food industry.

Research indicates that the effectiveness of sonication may be enhanced by adding a sanitizer such as chlorine which creates a synergistic effect. For example, sonicating a *S. typhimurium* cell suspension (10^8 cells/ ml of peptone) at 20 kHz for 55 min decreased the pathogen to nondetectable levels (Lillard, 1993). This same study showed that salmonellae populations attached to broiler skin decreased 1-1.5 logs by sonicating in peptone water at 20 kHz for 30 min; <1 log by chlorine alone; and 2.4 - 4 logs by sonicating in a solution containing 0.5 ppm free residual chlorine.

Copper ion may also have potential for reducing microbial loads on raw produce including apples. Copper ion is non-volatile, non-corrosive to processing equipment even at high temperatures and does not produce any known off- odors off-tastes or toxic by-products.

The bacteriocidal properties of copper have been known for centuries with its effectiveness documented at low concentrations (Takayama et al., 1994). When used in combination with various sanitizers, copper ions act synergistically to inactivate bacteria. The disinfecting action of copper is attributed to the positively charged copper ions, which complex with the sanitizer. The formation of electrostatic bonds to the negatively charged sites on the bacterial cell surface allows the copper-sanitizer complex to more effectively penetrate the bacterial cell membrane (Takayama et al., 1994).

Little information exists on the use of copper ion for decreasing microbial loads on fresh produce. However, several in vitro studies indicate that copper ion has potential

as a produce sanitizer with Kutz et al. (1988) reporting a 4.2 log reduction in *E. coli* O157:H7 after 1 minute of exposure to copper ion (0.4 ppm).

The objective of this study was to assess the sequential use of copper ion, sodium hypochlorite, apple juiceration and sonication to reduce populations of *E. coli* O157:H7 and *L. monocytogenes* during apple cider production.

MATERIALS AND METHODS

Bacterial Strains. Three strains of *E. coli* O157:H7 (AR, AD 305, AD 317) and *L. monocytogenes* (CWD 95, CWD 249, and CWD 201) were obtained from C.W. Donnelly (Dept. of Nutrition and Food Sciences, University of Vermont, Burlington, VT). Stock cultures were maintained at -70°C in tripticase soy broth (TSB) (Difco, Difco Laboratories, Detroit, MI) containing 10% (v/v) glycerol and subcultured twice in tripticase soy broth (9 ml) containing 0.6% (w/v) yeast extract (TSB-YE) (Difco) at 35°C/ 18-24 h before use.

Preparation of Inoculum. Individual cultures were then centrifuged at 10,000 x g for 15 min at 4°C to obtain a pellet which was re-suspended in sterile tap water (30 ml) to simulate commercial practices. These suspensions served as the inoculum for the model system cider. Equal volumes (10 ml) of culture were also combined to produce a three-strain cocktail of *E. coli* O157:H7 and *L. monocytogenes* for use on whole apples. These cocktails were then centrifuged at 10,000 x g for 15 min at 4°C and re-suspended in sterile tap water (30 ml) to simulate commercial conditions.

The laboratory was equipped with negative air pressure to prevent airborne contamination. Sterile latex gloves were worn while handling bacterial cultures. Laboratory equipment was sanitized prior to and after use of *E. coli* O157:H7 and *L. monocytogenes* with Cidex (2.4 % gluteraldehyde) (Ethicon Corp., Ervine, CA).

Inoculation of Cider. Commercially prepared unpasteurized apple cider was obtained locally, stored at 4°C and allowed to come to room temperature (~25°C) before

being inoculated with the three strains of *E. coli* O157:H7 and *L. monocytogenes* at a level of 10^6 CFU/ml.

Inoculation of Apples. Un-waxed, blemish-free Golden Delicious apples of uniform size and shape (2 ½ to 2 ¾ inches in diameter) were obtained locally for cider production. Sterile polyethylene bags (25 cm x 20 cm) containing 300 ml of sterile distilled water (SDW) were inoculated with on 18-24 h-old broth cultures containing a dilute suspension of a three-strain cocktail of *E. coli* O157:H7 or *L. monocytogenes.* Batches of 6-8 apples were placed in the bag and agitated by stirring with a glass rod for 20 minutes to ensure even distribution on the surface. Apples were then air dried in a laminar flow hood for 18-24 hours at 24°C before being subjected to the various sanitizer treatments.

Treatment Preparation and Application. In order to determine optimum conditions for destruction of the three individual strains of *E. coli* O157:H7 and *L. monocytogenes*, the effect of sonicating at 44 to 48 kHz (Vibra cell- Sonics and Materials 600 watt ultrasonic processor; Sonics and Materials, Inc. Newtown, CT) for 5 minutes was assessed. Inoculated cider samples (1.5 mls) were placed in sterile microcentrifuge tubes and sonicated at 44 to 48 kHz for 5 minutes. Initially and at 30 second intervals, 1 ml aliquots were removed over a period of 5 minutes to determine the effect of sonication on destruction of the pathogens. Samples (1 ml) were serially diluted and spiral plated (400 Autoplate Automated Spiral Plater, Spiral Biotech, Inc., Bethesda, MD) on Sorbitol MacConkey Agar (SMAC) (Difco) and Modified Oxford Agar (MOX) (Difco) and

incubated for 24 h at 37°C for enumeration of *E. coli* O157:H7 and *L. monocytogenes*, respectively. From these results the D-values were calculated by linear regression. All treatments were performed in triplicate.

Ear plugs were worn during the operation of the ultrasonic processor to protect hearing.

Preparation of Sanitizer Treatments. A sanitizer solution containing 100 ppm total residual chlorine was prepared by adding 1.13 g of sodium hypochlorite (S.C. Johnson Professional, Racine, WI) to sterile distilled water (SDW). Total residual chlorine was measured using a chlorine colorometric test kit (Hach Co., Ames, Iowa). Copper ion water was generated using a pilot plant-sized copper ion generator which pumped electrolytically generated copper ions into a stream of SDW water (Superior Water Systems Inc., Fort Wayne, Indiana). The copper ion concentration was determined prior to treatment using a colorimetric copper ion test kit (model EC-20; La Motte Chemical Products Co., Inc., Chestertown, MD). Sodium thiosulfate (0.1 N) stock solution was prepared by dissolving 25 g of sodium thiosulfate (Sigma Chemical Co., St. Louis, MO) in 1 L of SDW (Rand et al., 1975). The solution was used to neutralize residual chlorine with 1 ml aliquots added to the first tube for serial dilution.

Hurdle Approach Study. Populations of *E. coli* O157:H7 and *L. monocytogenes* were determined in the following samples: a.) inoculated apples, b.) inoculated apples after sonication/sanitizer treatment, c.) apple pulp after juicerating, d.) apple cider and e.) apple cider after sonication.

To determine the inoculum levels, inoculated apples were drained, placed in individual bags containing 100 ml of 0.1% peptone and vigorously shaken for 5 minutes followed by rubbing/massaging for another 10 minutes. Triplicate samples of apple wash water were serially diluted and spiral plated on SMAC, or MOX and incubated for 24 h at 37°C.

Inoculated whole apples were placed in a 1.4-liter capacity sonicating water bath (22 to 44 kHz Fisher Ultrasonic Cleaner, Model FS140) containing 1 ppm copper ion water or 1 ppm copper ion water with 100 ppm sodium hypochlorite and sonicated for 3 minutes. Apples that were sampled at this step were drained and placed in individual Whirl-pack bags containing 100 ml 0.1% peptone (Difco) or 0.1% sodium thiosulfate (for samples containing chlorine) and vigorously shaken for 5 minutes followed by rubbing for another 10 minutes. Triplicate samples of apple wash water were serially diluted and spiral plated on SMAC and MOX and incubated for 24 h at 37°C to quantitate *E. coli* O157:H7 and *L. monocytogenes*, respectively.

Apples that had been sonicated in copper ion water, sodium hypochlorite, or copper ion water containing sodium hypochlorite were removed from the sonicating water bath and drained. The apples were then placed in a juicerator (Omega OM 9000, Omega Products, Inc., Harrisburg, PA) for 5 minutes to obtain the cider and pulp fractions. Pulp samples (40 g) were placed in individual sterile polyethylene bags containing 100 ml of 0.1% peptone and homogenized in a stomacher (Model SD-45, Tekmar Co., Cincinnati, OH) for 2 minutes. Triplicate samples of apple pulp were serially diluted, spiral plated on SMAC, and MOX and incubated for 24 h at 37°C to quantitate *E. coli* O157:H7 and *L. monocytogenes*, respectively.

One ml samples of the cider were serially diluted in 0.1% peptone, spiral plated on SMAC and MOX and incubated for 24 h at 37°C for enumeration of *E. coli* O157:H7 and *L. monocytogenes*, respectively. All treatments were performed in triplicate.

Lastly, samples (1.5 ml) of the cider fraction were also placed in microcentrifuge tubes and were sonicated at 44 to 48 kHz for 3 minutes. One-ml samples were serially diluted and spiral plated on SMAC and MOX and incubated for 24 h at 37°C for enumeration of *E. coli* O157:H7 and *L. monocytogenes*, respectively. All treatments were performed in triplicate.

Statistical Analysis and Enumeration. D-values for *E. coli* O157:H7 and *L. monocytogenes* in the various treatments were determined by linear regression. All microbial data were analyzed using a factorial ANOVA on duplicate samples at a significance level of $p < 0.05$. Statistical results were subjected to a Bonferroni adjustment for conservative analysis.

RESULTS

Initial concentrations of *E. coli* O157:H7 and *L. monocytogenes* in the inoculated cider ranged from 6.3 – 6.4 log CFU/ml (Figure 1). Sonication reduced *E. coli* O157:H7 and *L. monocytogenes* populations 0.8 – 1.9 and 1 – 1.5 logs, respectively, after three minutes with no additional reductions observed after five minutes. The log reductions for the individual strains of *L. monocytogenes* and *E. coli* O157:H7 were not significantly different from each other throughout the five-minute treatment period.

Inactivation of *E. coli* O157:H7 on apples using copper ion, sodium hypochlorite and sonication is shown in Figure 2. Inoculated apples contained ~ 6.2 log CFU/g *E. coli* O157:H7. When treated with water (control) or 1 ppm copper ion water, no significant reduction in *E. coli* O157:H7 populations was observed on apples; however, treatment with 100 ppm sodium hypochlorite as well as copper ion (1 ppm)/ sodium hypochlorite (100 ppm) significantly reduced *E. coli* O157:H7 populations by 1.5 and 2.3 logs, respectively. Juicerating inoculated apples treated with 1 ppm copper ion water or 1 ppm copper ion water and 100 ppm sodium hypochlorite led to fractionation of *E. coli* O157:H7 between the cider and pulp fractions. Populations of *E. coli* O157:H7 on apples treated with 1 ppm copper ion water were 4.68 and 1.25 log CFU/ g or ml in the cider and pulp fractions, respectively, after juicerating. Sonication of the remaining cider for three minutes resulted in a ~ 2 log reduction of *E. coli* O157:H7. Using sodium hypochlorite (100 ppm) - treated apples, populations of *E. coli* O157:H7 were 3.5 and 1.3 logs CFU/ g or ml in cider and pulp, respectively, after juicerating. Sonication of the juice produced a 1.6 log reduction. Juicerating of apples after treatment with the combination of 1 ppm copper ion water and 100 sodium hypochlorite yielded *E. coli* O157:H7 populations of

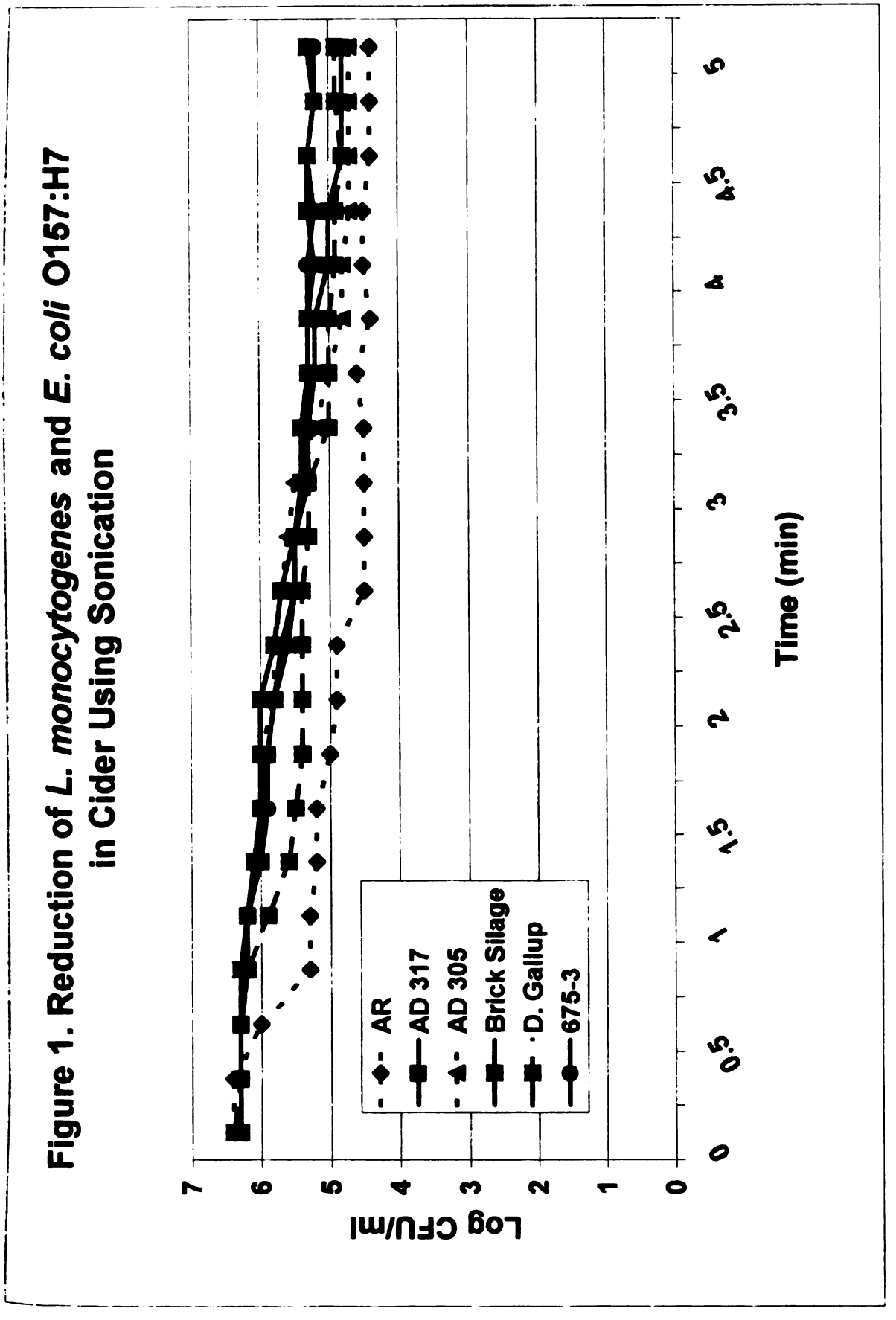
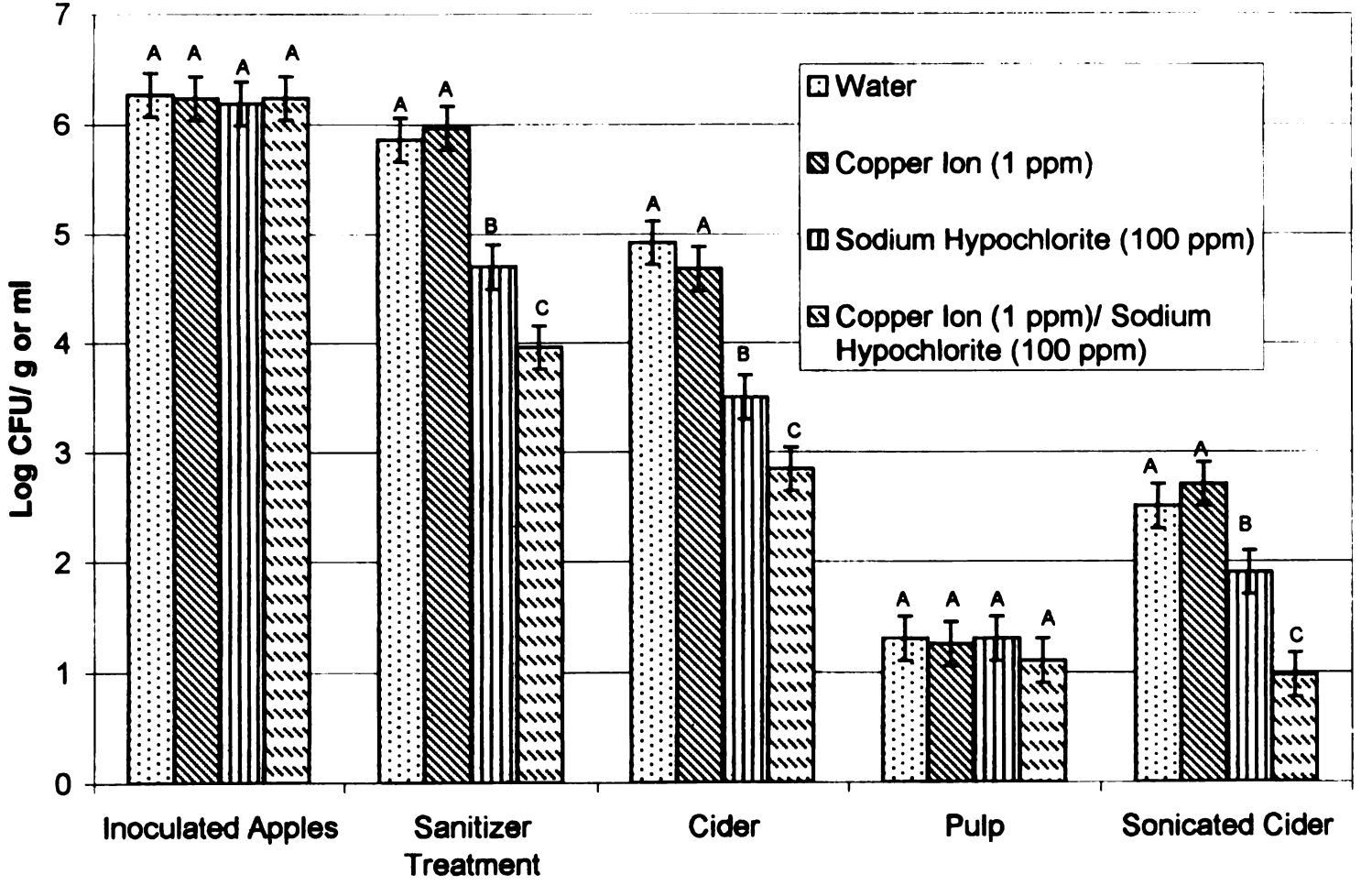


Figure 1. Reduction of L. monocytogenes and E. coli O157:H7 in Cider Using Sonication
Log CFU/ml
Time (min)
AR
AD 317
AD 305
Brick Silage
D. Gallup
675-3

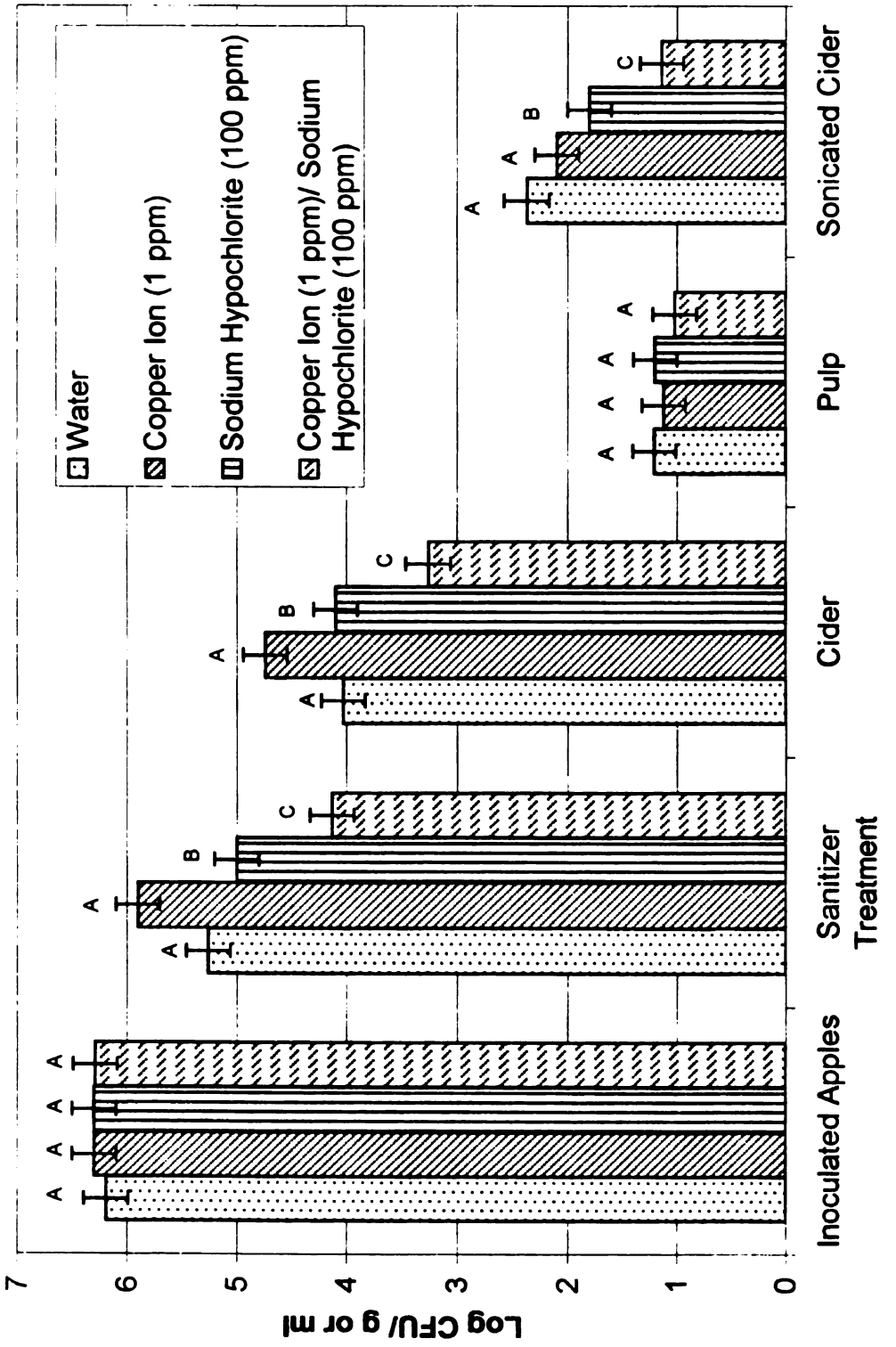
Figure 2. Inactivation of *E. coli* O157:H7 in Apples and Cider Using Copper Ion, Sodium Hypochlorite and Sonication



2.9 and 1.1 log CFU/ g or ml in the cider and pulp, respectively. Further sonication of the resulting cider fraction significantly reduced populations of *E. coli* O157:H7 by 1.8 log CFU/ml.

Figure 3 shows the inactivation of *L. monocytogenes* on apples and in cider using sonication and copper ion or sodium hypochlorite. Inoculated apples initially contained populations of approximately 6.3 logs CFU/g. Although, treatment with water (control) or 1 ppm copper ion water did not significantly reduce numbers of *L. monocytogenes* on inoculated apples, populations decreased 1.3 logs after treatment with 100 ppm sodium hypochlorite and 2.2 logs after treatment with copper ion (1 ppm)/ sodium hypochlorite (100 ppm). Juicerating inoculated apples treated with 1 ppm copper ion water or 1 ppm copper ion water with 100 ppm sodium hypochlorite yielded *L. monocytogenes* populations of 3.26 log CFU/ml and 1.02 logs CFU/g, in the cider and pulp fractions, respectively. *L. monocytogenes* populations were 4.74 logs CFU/ml and 1.12 logs CFU/g after juicerating 1 ppm copper ion water-treated apples into cider and pulp fractions, respectively. Further sonication of expressed cider reduced populations by approximately 2.3 logs CFU/ml. However, numbers of *L. monocytogenes* were 4.1 and 1.2 logs CFU/ g or ml in cider and pulp fractions, respectively after treatment with 100 ppm sodium hypochlorite with cider sonication reducing populations by 2.3 logs. Numbers of *L. monocytogenes* on apples treated with 1 ppm copper ion water and 100 ppm sodium hypochlorite were 3.26 and 1.02 logs CFU/ g or ml in the pulp and cider fractions, respectively, after juiceration. Sonication of the remaining cider for 3 minutes significantly reduced populations of *L. monocytogenes* by 2.12 log CFU/ml.

Figure 3. Inactivation of *L. monocytogenes* in Apples and Cider Using Copper Ion, Sodium Hypochlorite and Sonication



Total log reductions for inoculated apples treated with copper ion (1 ppm) and water into cider were ~3.5 and ~ 4 logs for *E. coli* O157:H7 and *L. monocytogenes*, respectively, and were not significantly different from each other. Sodium hypochlorite (100 ppm) produced total log reductions of 4.3 and 4.5 for *E. coli* O157:H7 and *L. monocytogenes*, respectively. Using the hurdle approach, the combination of copper ion (1 ppm) and sodium hypochlorite (100 ppm) in addition to sonication was the most effective overall treatment and produced total log reductions of 5.3 and 5.2 for *E. coli* O157:H7 and *L. monocytogenes*, respectively.

DISCUSSION

In the model study, sonicating cider for 3 minutes reduced populations of *E. coli* O157:H7 and *L. monocytogenes* by 1.5 – 2.0 and 1.0 – 1.5 logs, respectively. Based on practicality for cider producers, a sonication treatment time of 3 minutes was selected for further study. However, a longer treatment time may have enhanced bacterial log reductions, since Wrigley and Llorca (1992) reported the greatest inactivation of *Salmonella typhimurium* after 30 minutes of sonication. Other researchers also have shown longer sonication treatments to be more effective (Stone and Fryer, 1984). For example, Lee et al. (1989) reported a 4-log reduction for *Salmonella* in peptone water using a 10-minute ultrasonic treatment.

Individual strains of *E. coli* O157:H7 and *L. monocytogenes* did not differ significantly in their susceptibility to sonication. These results indicate that the effectiveness of sonication would produce consistent results in destruction of bacterial cells.

The D-values of *L. monocytogenes* were significantly lower than those for *E. coli* O157:H7 except for *E. coli* O157:H7 strain AR, which had significantly lower D-values than all other strains examined. These results indicate that *L. monocytogenes* may be more susceptible to treatment with sonication than *E. coli* O157:H7 if the treatment time were increased. Most sonication studies have assessed the fate of enteric gram-negative pathogens in highly perishable animal-derived foods such as poultry and milk (Lillard, 1994). Therefore, the effectiveness of sonication on gram positive bacterial pathogens such as *L. monocytogenes* implicates that decontamination by sonication may be

considered for juices with high susceptibility to contamination by gram positive pathogens.

The Food and Drug Administration (FDA) has passed a regulation requiring fruit and vegetable juice processors to implement Hazard Analysis and Critical Control Point Programs. Part of this regulation requires a 5-log reduction for pathogens in the finished product (FDA, 2001). Small and very small cider producers are not required to comply with the new standards immediately and may choose to incorporate alternative methods of decontamination until that time. Furthermore, investigations on the efficacy of methods used to decontaminate raw apples that are consumed whole, may further increase consumer safety and confidence. In this study, the hurdle concept was investigated to achieve a 5-log reduction using copper ion water, sodium hypochlorite, juiceration and sonication. Treatment of *E. coli* O157:H7- and *L. monocytogenes*-inoculated apples with the water control or copper ion water alone only reduced the initial inoculum levels by 0.3 - 0.4 logs. These results are similar to those reported by other researchers using a water treatment to remove pathogens from produce. For example, Wright et al. (2000) found that treatment of *E. coli* O157:H7-inoculated apples with water removed 1.1 log CFU/g. Similarly, Brackett (1987) reported a 1 log reduction for *L. monocytogenes* on Brussels sprouts dipped in water. Reductions observed in this study using copper ion water alone can be attributed to the washing of bacteria from the produce surface rather than the antimicrobial effect of copper ion water since log reductions are no higher than those previously reported for water alone.

Chlorine concentrations ranging from 50 to > 300 ppm are often used by producers to reduce the numbers of microorganisms on apples (Burnett and Beuchat,

2000). However, numerous studies have shown that *E. coli* O157:H7 can still be found on the surfaces of inoculated apples after chlorine treatment. These results indicate that chlorine may be unable to access areas on apples where bacterial cells can reside. In addition, active chlorine concentrations may decrease significantly after contact with organic material. In our study, sodium hypochlorite (100 ppm) reduced populations of *E. coli* O157:H7 and *L. monocytogenes* on apples by 1.5 and 1.3 log CFU/g, respectively. These results are similar to those of Wright et al. (2000) who examined the effects of 200 ppm sodium hypochlorite on removal of *E. coli* O157:H7 from apples and found that populations decreased 2.1 logs on the surface after a 2 minute exposure.

Copper ions have been used in combination with various sanitizers for synergistically inactivating bacterial cells. While information on the use of copper ion for decreasing the microbial load on fresh produce is scarce, several in vitro studies indicate that copper ion has potential as a produce sanitizer. Kutz et al. (1988) reported a 4.2 log reduction for *E. coli* O157:H7 after a 1 minute exposure to 0.4 ppm copper ion. In this study, copper ion water containing 100 ppm sodium hypochlorite significantly reduced the levels of *E. coli* O157:H7 and *L. monocytogenes* on apples by 2.3 and 2.2 log CFU/g, respectively These results indicate that sodium hypochlorite creates a synergistic effect when used with copper ion water. Since cider producers routinely use chlorinated water to wash apples, they may improve sanitation by incorporating copper ion.

The process of juiceration, which fractionates apples into cider and pulp, yielded *E. coli* O157:H7 and *L. monocytogenes* populations of 1.0 – 1.3 and 2.84 – 4.68 and 3.26 – 4.74 in the pulp and cider fractions, respectively. Since populations of *E. coli* O157:H7

and *L. monocytogenes* change as a direct result of juiceration, this effect should be considered during HACCP planning for apple processors.

Higher numbers of both pathogens were consistently observed in the cider as compared to the pulp. Hence, despite significant reductions in the number of total bacteria from apples, populations of *E. coli* O157:H7 and *L. monocytogenes* are still high enough in the cider to cause illness. Therefore, further pathogen reduction strategies must be considered for cider.

Sonication of the resultant cider after pressing reduced levels of *E. coli* O157:H7 and *L. monocytogenes* by 1.8 – 2.0 and 2.2 – 2.6 logs, respectively. These results indicate that populations of *E. coli* O157:H7 and *L. monocytogenes* on apples treated with 0.1 ppm copper ion water followed by sonication of cider resulted in reductions of 3.54 to 4.2 logs for the total process. Reductions of 5.15 and 5.27 logs could be achieved for *E. coli* O157:H7 and *L. monocytogenes*, respectively when apples treated with 0.1 ppm copper ion water containing 100 ppm sodium hypochlorite were juicerated and the cider sonicated.

Reductions of *L. monocytogenes* and *E. coli* O157:H7 after three minutes of sonication were not significantly different from reductions after five minutes of sonication. The model system study was conducted to determine optimal conditions for destruction of *E. coli* O157:H7 and *L. monocytogenes* in cider using the shortest treatment time that produced the greatest destruction. In this case, a treatment time of three minutes was as effective as a five-minute treatment time and was used as the standard treatment time for the remainder of the study.

According to the FDA, processors of unpasteurized juices that are considered small or very small producers, would be free to employ a combination of methods to achieve the required 5-log reduction until the compliance deadline. Furthermore, cider processors that are too small to be regulated by the FDA may choose alternative decontamination methods, such as those presented in our study, that are less costly than pasteurization to enhance safety. Based on the results of this study, the use of chlorine and copper ion water in conjunction with sonication can reduce populations of *E. coli* O157:H7 and *L. monocytogenes* by 5 logs during cider production. Therefore, this process could find use as part of a HACCP plan to reduce the public health risks associated with unpasteurized cider.

CHAPTER FIVE

ASSESSMENT OF GFP TRANSFORMED *E. COLI* O157:H7 INACTIVATION BY SANITIZERS ON LETTUCE AND STRAWBERRIES USING CONFOCAL SCANNING LASER MICROSCOPY

ABSTRACT

Attachment and viability of GFP-transformed *Escherichia coli* O157:H7 (strain E318) on leaf lettuce and strawberries was evaluated in response to a 5-minute exposure to water and four different sanitizers - Fit™ (4,000 ppm citric acid and 450 ppm sodium lauryl sulfate), sodium hypochlorite (200 ppm chlorine), chlorine dioxide (3 ppm), and ozone (2 ppm), using standard plating techniques and confocal scanning laser microscopy (CSLM). Whole lettuce leaves and strawberries were inoculated by submersion in an *E. coli* O157:H7 suspension prepared from 18-24 h broth cultures to obtain 10^8 CFU/g. Visualization of *E. coli* O157:H7 on the surface of lettuce and strawberries by CSLM at an excitation wavelength of 488 nm revealed generalized non-specific attachment to surface structures with some penetration through intact surfaces up to approximately 20μm. *E. coli* O157:H7 attachment to both products was more strongly influenced by water deposition and pooling than by any affinity to stomates or other surface structures. Fit™ was the least effective (~1 log reduction on either product) sanitizer tested and was not significantly different from water, while ozone and chlorine dioxide were the most effective yielding reductions of 3.77 and 3.55 logs on lettuce and 3.28 and 3.17 logs on strawberries, respectively, with these differences not significant. Sodium hypochlorite yielded intermediate results, giving reductions of 2.95 and 2.34 for lettuce and strawberries, respectively. Based on CSLM analysis, viable *E. coli* O157:H7 cells that

survived sanitizer treatments did so by organizing into groups or clusters in areas of pooling rather than by penetrating through intact produce surfaces.

INTRODUCTION

Contamination of raw fruits and vegetables by *E. coli* O157:H7 has become a major public health concern that was prompted by recent outbreaks involving lettuce, radish sprouts, apple cider, and alfalfa sprouts (Ackers et al., 1996; Mermin et al., 1996; Gutierrez, 1997). *E. coli* O157:H7 is able to attach and grow on produce including sliced cucumbers, green peppers, shredded lettuce, cantaloupe, watermelon, and apples (Abdoul-Raouf et al., 1993; delRosario and Beuchat, 1995; Diaz et al., 1996). Richert et al. (2000) determined that *E. coli* O157:H7 could survive and grow on broccoli, cucumbers, and green peppers during storage at 15°C. The presence of *E. coli* O157:H7 on fresh fruits and vegetables has been associated with fecal contamination from cattle or other animals during growing, fertilization, and irrigation with improperly treated flume water identified as another source of contamination during vegetable processing (Beuchat, 1999; FDA, 1998). Use of safer and more effective sanitizers in flume water is important to both producers and consumers. Identification of alternative sanitizers by the food industry is continually evolving with the challenge being to attain a 5-log reduction as promulgated by the Food and Drug Administration (FDA) for selected commodities.

While water alone will effectively remove organic matter from produce, incorporation of a sanitizer is necessary to reduce microbial populations by more than 1-2 logs when used on fresh produce (Abdelnoor et al., 1983). Most commonly used chlorine-based sanitizers possess limited bacteriocidal activity, produce potentially carcinogenic by-products, and are adversely affected by organic material, temperature and pH (Beuchat, 1999; Beuchat, 1998; Adams et al., 1989). Most notably, chlorinated

sanitizers are unable to inactivate microorganisms that have penetrated the surface (Dychdala, 2001).

Sanitizer efficacy is usually evaluated by standard plating techniques; however, this method does not indicate specific details about how or why certain sanitizers are more effective than others. Confocal scanning laser microscopy (CSLM) has been used to study the attachment, location, and viability of *E. coli* O157:H7 on produce surfaces including lettuce, green peppers, and apples (Seo and Frank, 1999; Takeuchi and Frank, 2000; Han et al., 2000; Burnett et al., 2000b). This technique allows visualization of the pathogen without the use of fixatives, which may interfere with viability and attachment. CSLM can also be used to obtain images within solid foods with samples ready for immediate viewing after labeling of the target organism with a fluorescent marker or dye. Light or traditional epifluorescence microscopy has limited resolution and cannot be used to obtain images from solid food products, while electron microscopy requires intensive sample preparation, often involving dehydration which can markedly alter the spatial environment of the food. According to Seo and Frank (1999) and Burnett et al. (2000), *E. coli* O157:H7 can penetrate the surface layer of apples and lettuce as viewed by CSLM with the pathogen then protected from inactivation by chlorine-based sanitizers. Consequently, alternative sanitizer treatments are being sought.

The recent isolation of green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has positively impacted cell and molecular biology and permitted advanced study of microbial cells and attachment. A GFP- transformed *E. coli* O157:H7 was developed by Mansel Griffiths at the University of Guelph, which contains a pGFPuv plasmid that can be excited at 488 nm with illumination by blue light able to

quantitatively determine its presence or absence. Such strains are highly advantageous for CSLM since fluorescent antibody staining is no longer required for visualization. Hence, GFP-labeled cells can be studied nondestructively, without any exogenous substrates or processing (Prachaiyo and McLandsborough, 2000).

The complex surface of many fruits and vegetables provides many sites for bacterial attachment and subsequent penetration. Some fruits, such as strawberries, are not washed before reaching consumers due to their high susceptibility to fungal deterioration, which is promoted by the presence of water (Yu et al., 2001). Lettuce poses other decontamination problems due to its large wrinkled surface area which allows limited access to sanitizers. Understanding the nature of bacterial attachment and areas where bacteria congregate on fruits and vegetables will help better define target areas for decontamination. This information will facilitate the development of specific sanitizers and application strategies for enhanced reduction of microbial pathogens on fresh produce.

In this study, leaf lettuce and strawberries were selected as model samples for fruits and vegetables. Both of these products have highly textured surfaces that are difficult to access by sanitizers or are susceptible to fungal deterioration in the presence of water and are, therefore, less likely to be washed before reaching consumers. The objectives of this study were to examine the attachment and viability of GFP transformed *E. coli* O157:H7 in response to four sanitizer treatments (Fit^{TM}, ozone, chlorine dioxide, and sodium hypochlorite) using plate count analysis and CSLM.

MATERIALS AND METHODS

Produce. Leaf lettuce and strawberries were obtained from local suppliers. Unwashed produce was stored for 24 hrs. at 4°C before use. The outer lettuce leaves were removed and discarded with only whole strawberries and lettuce leaves of uniform size used for inoculation experiments and microscopic observation.

Preparation of inocula. *E. coli* O157:H7 E318, was obtained from Mansel Griffith, University of Guelph, Ontario, Canada. This strain contains a pGFPuv plasmid (Clontech Labs, Inc., Palo Alto CA.) and can be excited at 488 nm. A stock culture was maintained at -79°C in a water-glycerol (90:10, vol:vol) mixture. The organism was transferred monthly to tryptic soy agar (TSA; Difco Laboratories, Detroit, MI) slants containing 100 ug of ampicillin (Sigma Chemicals, St. Louis, MO.) per ml (TSAA), incubated for 24 h at 37°C and stored at 4°C. Cultures were activated by re-streaking on TSAA followed by incubation for 24 h at 37°C. *E. coli* O157:H7 suspensions for inoculation of produce were obtained by flooding each of ten plates with 10 ml of sterile deionized water (SDW) and disrupting colonies with a sterile bent glass rod. The suspensions from ten plates were harvested using a pipette, transferred to sterile 50-ml centrifuge tubes and centrifuged at 10,000 rpm for 15 min. Numbers of *E. coli* O157:H7 in the suspension were determined by surface plating serial dilutions in 0.1% peptone on TSAA.

The laboratory was equipped with negative air pressure to prevent airborne contamination. Sterile latex gloves were worn while handling bacterial cultures.

Laboratory equipment was sanitized prior to and after use of *E. coli* O157:H7 and *L. monocytogenes* with Cidex (2.4 % gluteraldehyde) (Ethicon Corp., Ervine, CA).

Preparation of Sanitizers. Four chemical treatments were evaluated for their ability to inactivate *E. coli* O157:H7 on inoculated lettuce and strawberries: Fit™ (a commercial sanitizer containing the following ingredients water, oleic acid, glycerol, ethanol, potassium hydroxide, citric acid, and distilled grapefruit oil) (Proctor and Gamble, Inc., Cincinnati OH), sodium hypochlorite (S.C. Johnson Professional, Sturtevant, WI), chlorine dioxide (S.C. Johnson Professional, Sturtevant, WI), and ozone. Fit™ was prepared according to the label directions using sterile deionized water (SWD). A solution containing 200 ppm active chlorine was prepared by adding 2.26 grams of powdered sodium hypochlorite to 1 liter of SDW.

Chlorine dioxide was generated in the laboratory using the manufacturer's (S.C. Johnson Professional; Racine, WI) instructions as follows: 100 ml of the 2% stock Oxine FP solution was added to a 200 ml French square screw-capped bottle; 25 ml of 75% w/w food grade phosphoric acid was added, the bottle was sealed, and the mixture was allowed to generate chlorine dioxide for 5 min with a magnetic stirrer to ensure thorough mixing. The final concentration of chlorine dioxide was determined using the Hach Colorimeter (model CN-66, Hach Co., Ames, Iowa) before and after each sampling run. A 1:2000 dilution of unactivated Oxine FP solution was used as a control blank. The active chlorine concentration for both sodium hypochlorite and chlorine dioxide was determined with the chlorine test kits (Hach) before application to produce.

Ozone (2 ppm dissolved in SDW) was produced using a commercial ozone generator (Clear Water Tech., San Louis Obisbo, CA) that utilized oxygen in combination with UV light to produce gaseous ozone that was bubbled into_water through a stainless steel sparger with the ozone bubbles measuring ~10 mm in_diameter. Ozone concentrations in the water were monitored before sampling using the indigo colorimetric method (APHA, 1987).

Ozone was prepared and used on treated produce under a chemical fume hood to prevent inhalation. Sterile rubber gloves were also worn during handling of ozone treated produce to prevent contact with skin.

Inoculation of Produce. Whole strawberries and leaf lettuce were submerged in sterile whirl pack bags (25 cm X 20 cm) (Whirl Pak) containing 300 ml of SDW inoculated with 18-24 h broth cultures as described previously or, for controls, in SDW for 24 h at room temperature. Inner lettuce leaves (100 g) were placed in a bag and shaken manually for 20 minutes to ensure even distribution of the organism in the product and batches of 20-25 strawberries were immersed in the inoculum and agitated by stirring with a sterile glass rod for 20 minutes to ensure uniform distribution. Produce was then rinsed in SDW for 1 minute, and drained in sterile petri dishes at room temperature for 1 h under a laminar flow hood at 24°C before being subjected to the various sanitizer treatments. Inoculated produce samples were then treated with Fit™, sodium hypochlorite (200 ppm), chlorine dioxide (3 ppm), ozone (2 ppm), or tap water for 2 minutes, rinsed with SDW for 1 minute and dried at room temperature for 10 minutes.

Plate Count Analysis. Treated strawberry and lettuce samples (40 g each) were drained and placed in sterile whirl pack bags containing 100 ml of sterile phosphate buffer solution (PBS, 10 mM, pH 7.2). Samples were pummeled in a stomacher (Tekmar Co., Cincinnati, OH) for 2 minutes, one ml of strawberry homogenate was removed from the bag, serially diluted and surface plated in duplicate on TSAA. Plates were incubated for 24 h at 37°C, and *E. coli* O157:H7 colonies were counted.

Sample Preparation. Squares (2 x 2 cm) were cut from 4 random areas of each inoculated lettuce leaf while 2 cm-diameter circles were cut from 4 random areas of each inoculated whole strawberry using a sterile double-sided razor blade. Mounts for microscopic analysis were prepared by constructing a square well of Vaseline on a clean microscope slide for confinement. Samples were placed in the center of the Vaseline well using sterile forceps. A coverslip was then placed on the produce specimen with sufficient pressure to facilitate adherence to the Vaseline. Slides were then placed in individual petri dishes for containment and subsequently examined by CSLM.

CSLM. Samples were first examined visually for GFP-labeled *E. coli* O157:H7 using a conventional epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with a mercury bulb, 450-490 nm (blue) excitation filter, and a LP 520 emission filter. Samples were then observed using a Zeiss 210 Laser Scanning Confocal Microscope (Carl Zeiss, Inc.) equipped with a 40x dry objective (numerical aperture = 0.75) and a dual line argon ion laser. The green fluorescence of GFPuv-labeled *E. coli* O157:H7 was detected using an excitation wavelength of 488 nm. Emitted light was

collected through a dichroic mirror, and a band pass 520-560 nm filter or a long pass 520 nm filter. Observations were recorded on a Sony Videoprinter and stored on the hard drive. Samples from random locations on each lettuce and strawberry sample were examined for bacterial attachment. Between 5 and 10 fields per each location for each sample were examined.

Statistical analysis. All trials were replicated at least five times. Two control groups (uninoculated produce and inoculated produce treated with water) were compared with images obtained from treated produce samples to determine treatment effects. Videoprints were selected to represent typical results. Plate count data were subjected to ANOVA using the Student's T-test. Plate count data represents the mean value obtained from three independent trials, each of these being obtained from duplicate samples.

RESULTS

REDUCTION OF E. COLI O157:H7

Lettuce. Populations of *E. coli* O157:H7 decreased by 1.17 log CFU/g after treatment with water as a control on lettuce (Table 1). Reductions of *E. coli* O157:H7 on lettuce by Fit™ were not significantly different ($P<0.05$) from treatment with water alone. Sodium hypochlorite was significantly different from all other treatments and reduced *E. coli* O157:H7 by 2.95 log CFU/g on lettuce. Log reductions of *E. coli* O157:H7 by chlorine dioxide on lettuce were found to be 3.52 log CFU/g, which were not significantly different ($P<0.05$) from ozone (3.77 log CFU/g).

Strawberries. Populations of E. coli O157:H7 decreased by 1.28 log CFU/g after treatment with water as a control on strawberries (Table 2). Reductions of *E. coli* O157:H7 on strawberries by Fit™ were not significantly different ($P<0.05$) than treatment with water alone. Sodium hypochlorite was significantly different from all other treatments and reduced *E. coli* O157:H7 by 2.34 log CFU/g on strawberries. Log reductions of E. coli O157:H7 by chlorine dioxide on strawberries were found to be 3.17 log CFU/g, which were not significantly different ($P<0.05$) from ozone (3.28 log CFU/g).

VISUALIZATION OF PGFPUV *E. COLI* O157:H7 ON INOCULATED LETTUCE AND STRAWBERRIES

The GFP-labeled strain of *E. coli* O157:H7 was easily recognizable by CSLM as fluorescent green rods on inoculated lettuce and strawberries ranging in size from 1-3 um.

Table 1. *E. coli* O157:H7 populations after immersion of inoculated whole lettuce leaves in water, Fit™, sodium hypochlorite, chlorine dioxide, and ozone.

Treatment	Population recovered (log CFU/g)[a]	Population reduction (log CFU/g)
Water	7.06 ± 0.21 a[b]	1.17 ± 0.17 a
Fit	6.98 ± 0.34 a	1.25 ± 0.16 a
Sodium Hypochlorite (200 ppm)	5.28 ± 0.26 b	2.95 ± 0.22 b
Chlorine Dioxide (3 ppm)	4.71 ± 0.18 c	3.52 ± 0.36 c
Ozone (2 ppm)	4.46 ± 0.20 c	3.77 ± 0.13 c

[a] Initial inoculum levels on the lettuce were 8.23 ± 0.19 log CFU/g (mean ± SD) for *E. coli* O157:H7

[b] Data followed by different letters are significantly different by least significant difference at $P < 0.05$

Table 2. *E. coli* O157:H7 populations after dipping inoculated strawberries in water, Fit™, sodium hypochlorite, chlorine dioxide, and ozone aqueous solutions

Treatment	Population recovered (log CFU/g)[a]	Population reduction (log CFU/g)
Water	6.87 ± 0.13a[b]	1.28 ± 0.30 a
Fit	6.91 ± 0.22 a	1.24 ± 0.22 a
Sodium Hypochlorite (200 ppm)	5.81 ± 0.39 b	2.34 ± 0.16 b
Chlorine Dioxide (3 ppm)	4.98 ± 0.28 c	3.17 ± 0.23 c
Ozone (2 ppm)	4.87 ± 0.41 c	3.28 ± 0.27 c

[a] Initial inoculum levels on the strawberries were 8.15 ± 0.19 log CFU/g (mean ± SD) for *E. coli* O157:H7

[b] Data followed by different letters are significantly different by least significant difference at $P < 0.05$

No *E. coli* O157:H7 cells were detected on uninoculated lettuce or strawberries. The intensity of fluorescence varied from dim to bright green. This particular strain exhibited the phenomenon of autoenhancement under the blue laser (488 nm) with fluorescence intensity increasing over time as samples were viewed under CSLM. The surfaces of uninoculated (negative control) and inoculated lettuce (positive control) are shown in Fig. 1(a) and (b), respectively. Uninoculated and inoculated strawberry surfaces are shown in Fig 2 (a) and (b). Although the cell walls and stomates (in red) of lettuce and strawberries autofluoresced, the green color of viable bacterial cells remained visible. These tissues contain porphyrins (e.g. chlorophyll and carotenoids) which cause red autofluorescence, but do not contain much lignin, which is a main source of green autofluorescence in plant cells (Kays, 1991)

ATTACHMENT OF E. COLI O157:H7 TO EXTERNAL SURFACE STRUCTURES

Lettuce. Populations of E. coli O157:H7 used to inoculate produce in this study ranged from 8.4 to 8.58 $\log_{10}$ CFU/ml. Attachment of E. coli O157:H7 cells to intact leaf lettuce was observed on virtually all surface structures including cell surfaces, cell junctions, trichomes, and stomates. E. coli O157:H7 was evenly distributed as seen in Fig. 3 (a). However, stacks of bacterial cells (groups of cells that overlapped each other) also were observed on some surfaces as shown in Fig. 3 (b). E. coli O157:H7 appeared to gather in areas on the produce that were slightly concave due to inherent fluctuations

Figure 1a. Conventional transmitted image of the surface of an uninoculated lettuce leaf. Numerous stomates are seen on the surface of lettuce (arrow). A vein can be viewed on the left side. The round green structures visible through the partially transparent epidermis are chlororplasts.

Figure 1b.Conventional epifluorescence image of inoculated lettuce. Red autofluorescence comes from the chloroplasts. Several E. coli O157:H7 cells (green rods) are visible.

Images in this dissertation are presented in color

Figure 1a.

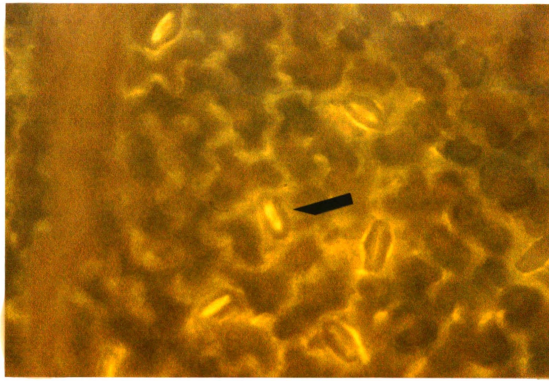


Figure 1b.

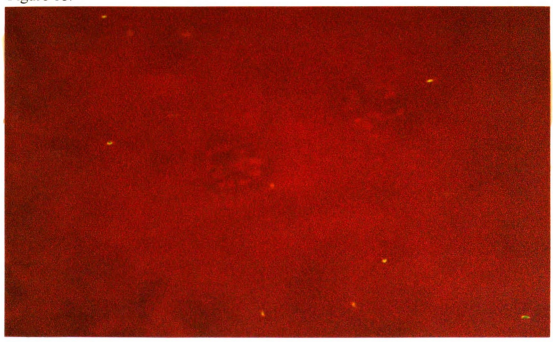


Figure 2. Photograph of conventional epifluorescence of strawberry surface (a) Uninoculated strawberry (negative control). A trichome is growing from the surface of the strawberry. Cell walls and junctions can be seen fluorescing in red. (b) Inoculated strawberry (positive control) containing numerous E. coli O157:H7 cells (green). E. coli O157:H7 colonized trichomes (arrow), cell surfaces, and cell junctions.

Figure 2a.

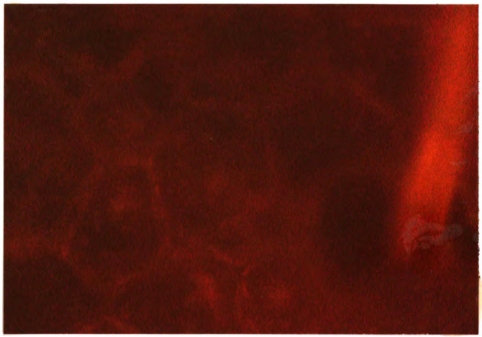


Figure 2b.

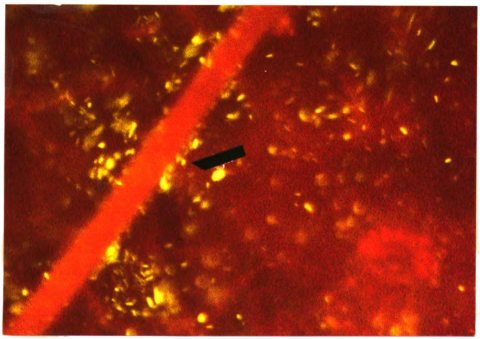


Figure 3. Laser confocal fluorescence image of inoculated untreated lettuce surface showing a) an area of even distribution of E. coli O157:H7 cells on leaf surface. (b) an area where the bacteria are stacked into groups

Figure 3a.

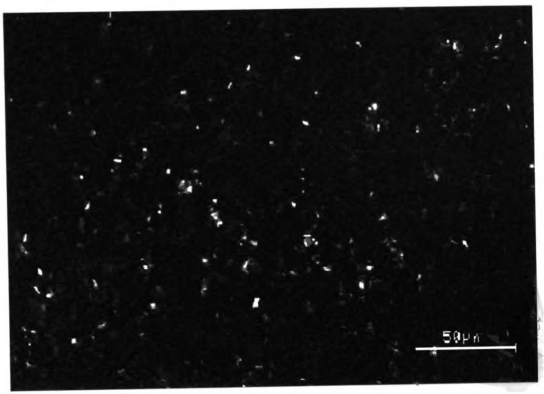
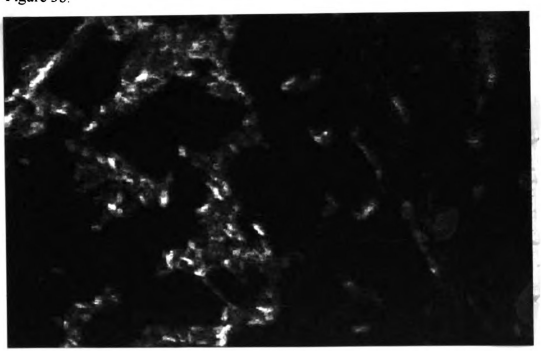


Figure 3b.



in surface topography. Presence of E. coli O157:H7 on particular surface structures was not related to leaf structure, but rather to the presence of pooled liquid. For example, when water was present on the leaf structure in a specific formation as viewed under transmission microscopy, visualization of the liquid under fluorescence revealed the same pattern of bacteria (Fig. 4(a) and (b)). This could also be seen in areas where water had completely dried (Fig. 4(c)). Differences in fluorescence intensity between attached and unattached cells were not observed, since both dim and bright mobile cells were evident. Many viable cells of E. coli O157:H7 were moving on the hydrated surface. Sporadic colonization of the space above the stomates in both lettuce and strawberries indicated that E. coli O157:H7 had not entered the leaf through the stomata (Figs. 5(a) and (b)). Occasional leaf samples contained areas of weak green autofluorescence from pre-existing tissue damage with these areas moderately colonized by E. coli O157:H7. While most E. coli O157:H7 cells were attached to the surface (0 -10 μm down), some penetration through intact surfaces was observed with penetration depths reaching 20 μm (Fig 6(a)). Some E. coli O157:H7 cells were occasionally seen at the same depth as chloroplasts, indicating penetration through the surface (Fig. 6(b)).

Strawberries. Distribution of E. coli O157:H7 on strawberry surfaces included cell walls, cell junctions, trichomes, achenes, and stomates with stacks of bacterial cells observed as previously described. The presence of liquid on the surface of the samples again appeared to influence the positioning of E. coli O157:H7 as described previously. Grains of pollen occasionally found on the surface of strawberries fluoresced yellow and were also colonized by E. coli O157:H7. The thick mat of trichomes protruding from the

Figure 4. Complementary views of an area on the surface of an inoculated lettuce leaf where water is receding. (a) In this laser transmitted image the dark gray line marks the boundary of the water, which is receding to the left. In (b), a laser confocal fluorescence image of the exact same area, large numbers of bacteria are seen, but only in the area still covered by water. (c) Area on lettuce leaf in a characteristic pattern of where a pool of water had completely dried.

Figure 4a.

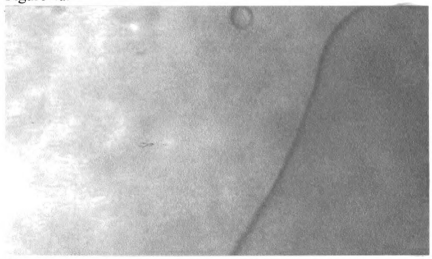


Figure 4b.

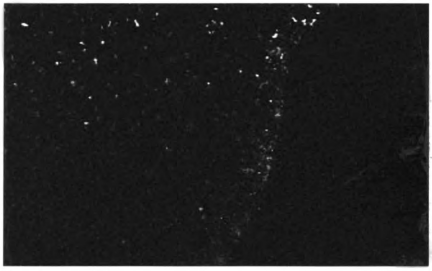


Figure 4c.

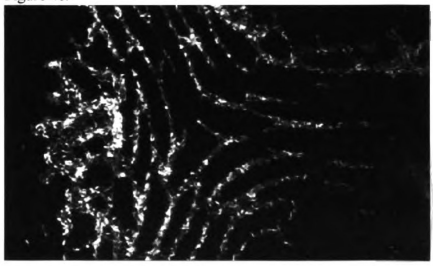


Figure 5. Laser fluorescence image of surface of inoculated lettuce. Arrow indicates a bacterial cell inside the opening of a stomate. (a) LP 520 filter (b) LP 520 and BP 520-560 filters show stomate (red) and E. coli O157:H7 cell (green), respectively.

Figure 5a.

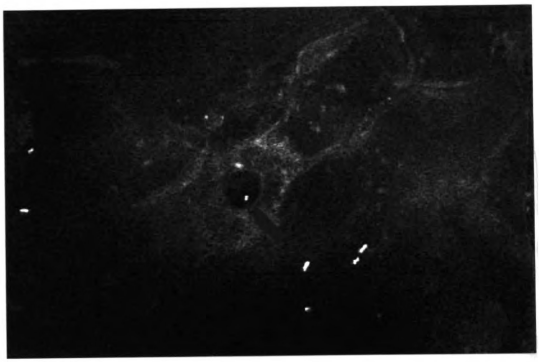


Figure 5b.

Figure 6a. Phi-z section through an inoculated lettuce leaf showing the epidermis and part of the mesophyll. Top arrow indicates the coverslip. Middle arrow indicates the surface of the lettuce sample. Bottom arrow shows 2 bacterial cells (green) that have penetrated through the surface approximately 19 um.

Figure 6b. Laser fluorescence image of inoculated untreated lettuce. Several bacterial cells have penetrated through the leaf surface and are found near the chloroplasts.

Figure 6a.

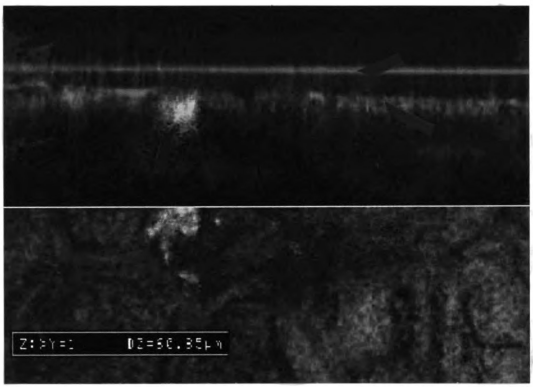
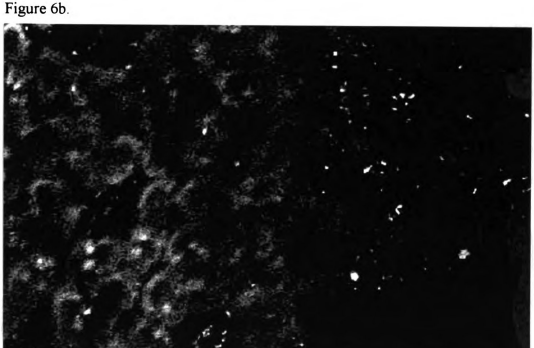


Figure 6b.



surface of strawberries often trapped large numbers of bacteria (Fig. 2(b)). *E. coli* O157:H7 cells were mainly attached to the surface of strawberries (0 -10 μm down), but occasional penetration through intact surfaces (up to 20 μm) was observed.

OBSERVATION OF *E. COLI* O157:H7 ON PRODUCE AFTER SANITIZER TREATMENTS

Lettuce. After inoculation, *E. coli* O157:H7 viability on the surface of produce treated with water, Fit™, sodium hypochlorite, chlorine dioxide, and ozone was assessed using CSLM. None of the sanitizer treatments completely eliminated all *E. coli* O157:H7 from lettuce as demonstrated earlier from the plate counts. Fit™ did not visually reduce the numbers of *E. coli* O157:H7 on inoculated lettuce as compared to water. This finding was consistent with the previous results from colony enumeration. Treatment of inoculated lettuce with sodium hypochlorite visually reduced the numbers of *E. coli* O157:H7 compared to the water control and Fit™. These results were also consistent with the previous log reductions where sodium hypochlorite was significantly more effective than both Fit™ and water on lettuce (1.7 log CFU/g reduction). Following treatment with sodium hypochlorite, viable bacteria were found on all major lettuce surface structures, but populations of *E. coli* O157:H7 were visually reduced compared to the water control. *E. coli* O157:H7 cells that remained on lettuce even after treatment with sodium hypochlorite were observed at various levels of penetration including viable cells that had penetrated as far as 20 μm. Using CSLM, treatment of lettuce with chlorine dioxide and ozone were visually indistinguishable from each other and reduced populations of *E. coli* O157:H7 more effectively than sodium hypochlorite. These

findings were consistent with the aforementioned plate count data in which chlorine dioxide and ozone were not significantly different from each other on lettuce. *E. coli* O157:H7 cells that remained viable on lettuce after treatment with chlorine dioxide and ozone were grouped together in clusters. The distribution of bacteria did not resemble the control samples treated with water, indicating that individual cells were more easily destroyed by sanitizers than large groups of cells or colonies. *E. coli* O157:H7 cells that had penetrated as much as 10 μm into cells on the surface were still viable on lettuce samples even after treatment with chlorine dioxide and ozone.

Strawberries. Visualization using CSLM indicated that *E. coli* O157:H7 cells were present on the surface of strawberries even after treatment with Fit™, sodium hypochlorite, chlorine dioxide, and ozone. Numbers of *E. coli* O157:H7 on strawberries were not visually reduced by treatment with Fit™ compared to water, which supported the previous plate count data. Produce samples subjected to Fit™ were coated with a thin layer of oil, which decreased fluorescence intensity (Fig. 7(a) and (b)). Treatment of inoculated strawberries with sodium hypochlorite visually reduced populations of *E. coli* O157:H7 compared to Fit™ and the water control; however, viable bacterial cells were found on all major strawberry surface structures with occasional penetration observed up to approximately 20 μm. Ozone and chlorine dioxide treatment of inoculated strawberries visually reduced populations of *E. coli* O157:H7 and were indistinguishable from each other which was consistent with results from the previous plate count results. *E. coli* O157:H7 cells on strawberries after treatment with sodium hypochlorite, ozone, and chlorine dioxide were organized into clusters as described previously (Fig. 8 (a), (b), and (c)). Generally, populations of *E. coli* O157:H7 were visually reduced on the surface of

Figure 7. Surface of inoculated strawberry treated with Fit. A thin layer of oil droplets cover the surface of the fruit. (a) Laser transmittance image (b) Laser fluorescence image showing numerous colonies of E. coli O157:H7 on the surface of the fruit within the oil layer.

Figure 7a.

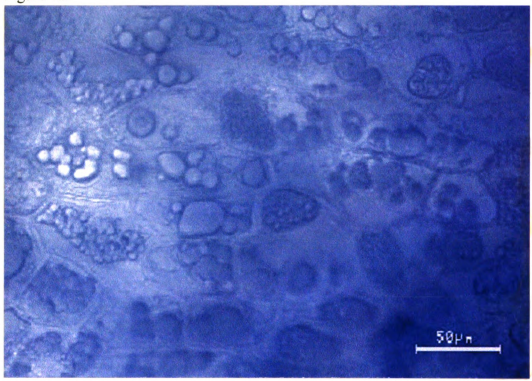


Figure 7b.

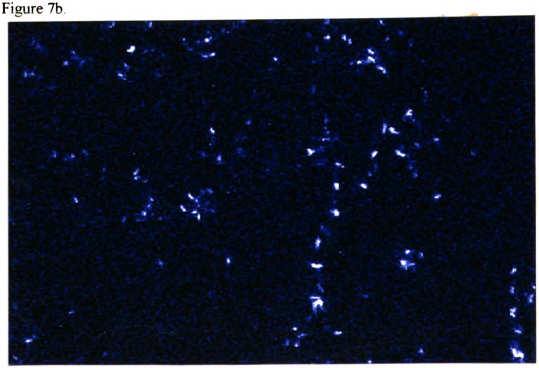


Figure 8. Laser fluorescence image of inoculated strawberries after sanitizer treatment. The level of colonization by E. coli O157:H7 is similar in all. (a) 100 ppm sodium hypochlorite (b) 3 ppm chlorine dioxide (c) 2 ppm ozone

Figure 8a.

Figure 8b.

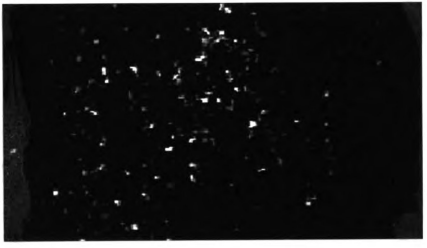
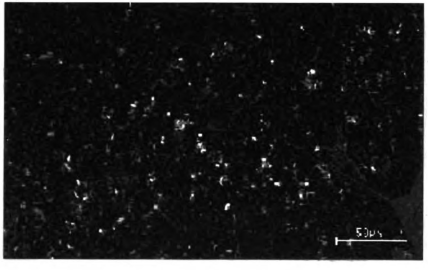


Figure 8c.



lettuce compared to strawberries treated with sodium hypochlorite, chlorine dioxide, and ozone, which supported earlier plate count data.

DISCUSSION

In this study, we devised a system for direct visualization of *gfp-E. coli* O157:H7 interaction with lettuce and strawberry surface structures in response to sanitizers. A major advantage of using GFP in combination with CSLM is the ease of detection without complex sample preparation (e.g. immunofluorescent labeling of cofactors, additional substrates, fixatives and washes) with these cells capable of being studied in situ. Furthermore, the use of selective media for detecting target pathogens also can be eliminated, further decreasing preparation time. By using GFP-*E. coli* O157:H7 the integrity of the plant tissue is not compromised during processing for CSLM.

Previously, Seo and Frank (1999) reported a microscopic method for differentiating viable from nonviable *E. coli* O157:H7 cells on lettuce using CSLM. Their method used fluorescein isothiocyanate-labeled affinity-purified antibody (FITC-Ab) to *E. coli* O157:H7 to stain viable cells green, whereas dead cells were stained red by propidium iodide (PI). Sanitizers like chlorine, that inactivate or destroy bacterial cells by damaging the cell membrane, allow PI to penetrate the cell. Unfortunately, sanitizers that destroy bacteria by other means may not produce this same color differentiation. Han et al. (2000) used the FITC-Ab/ PI stain to assess viability of *E. coli* O157:H7 on green peppers treated with chlorine dioxide gas and observed a two-color differentiation since chlorine dioxide destroys cell membranes. In our study, ozone and Fit™ were examined for their ability to decrease numbers of viable *E. coli* O157:H7 cells on lettuce and strawberries. However, the mechanism for inactivation of bacterial cells by these sanitizers is not fully understood and might not result in cell lysis. Therefore, propidium iodide might not be an accurate indicator of sanitizer efficacy for these two sanitizers.

Consequently, development of a method for assessing sanitizer efficacy without the use of PI would be necessary. Although several researchers have studied the inactivation of *E. coli* O157:H7 on the surface of lettuce and strawberries by various sanitizers using traditional plate count methods, information on location, attachment characteristics and viability after sanitizer treatment is generally lacking. Until now, CSLM studies involving produce have focused on lettuce, apples, and green peppers and have assessed only a single sanitizer.

Unlike Fit^{TM}, sodium hypochlorite, chlorine dioxide, and ozone effectively reduced the numbers of *E. coli* O157:H7 on lettuce and strawberries. The plate count results for *E. coli* O157:H7 indicated that water reduced these populations by about 1 log on lettuce and strawberries. These results are similar to those of Yu et al. (2001) who reported decreases of 0.75 and 0.86 log CFU/g when inoculated strawberries were treated with distilled water. Since water alone contains no antimicrobial activity, this reduction can be attributed to the washing of bacteria from the surface.

The limited ability of Fit^{TM} to reduce microbial populations on fresh produce can be attributed to inclusion of citric acid and sodium lauryl sulfate. Previously, researchers have demonstrated that citric acid is minimally effective as an antimicrobial agent. Shapiro and Holder (1960) treated salad vegetables with 1 to 1500 ppm citric acid and observed no difference in bacterial counts compared to controls. Addition of surfactants to commercial fruit and vegetable wash water can facilitate the removal of pathogenic microorganisms from produce. Anionic acid surfactants such as sodium lauryl sulfate aid in the removal of surface bacteria by facilitating wetting of the product surface which improves contact between the antimicrobial agent and adhering microorganisms.

According to Sapers et al. (1999), a commercial 1% acid anionic surfactant solution decreased *E. coli* O157:H7 populations 1-2 logs CFU/g on apple halves. Reductions of approximately 1 log can be attributed to washing of bacteria from the produce surface, since water alone contains no antimicrobial activity. Therefore, surfactants are likely to be slightly more effective than water. In our study, Fit™ was relatively ineffective as a sanitizer. Takeuchi and Frank (2001b), who examined a prototype sanitizer solution containing ingredients similar to Fit™, reported that the numbers of attached cells were not significantly different between samples treated with water and the prototype wash solution (ethyl alcohol, baking soda, citric acid, sodium lauryl sulfate, oleic acid, and distilled grapefruit oil).

Results from our study indicate that 200 ppm sodium hypochlorite was more effective at reducing *E. coli* O157:H7 populations on strawberries (2.34 logs) than reported by Yu et al. (2001) (1.3 logs). This may be the result of less organic matter inherently present on the surface of strawberries in our study compared to that of Yu et al. (2001). Decreased effectiveness of many sanitizers on strawberries compared to lettuce is likely due to the rough surface of strawberries and the presence of numerous surface-borne achenes (seeds), which provide sites for bacterial attachment making the organism less accessible to sanitizers.

When Takeuchi and Frank (2000) treated lettuce with 200 ppm chlorine for 5 minutes, less attached cells were observed on surfaces (0.7 log CFU/cm^2) than on cut edges (1 log CFU/cm^2); however, high numbers of viable cells remained at both sites. Varying attachment conditions may influence the reduction of *E. coli* O157:H7 on produce by chlorine-based sanitizers. Takeuchi and Frank (2000) also found that 24 h of

incubation at 4°C allowed greater penetration of *E. coli* O157:H7 into cut edges compared to incubation at 7, 22, or 37°C. However, reduced attachment of *E. coli* O157:H7 has been reported at lower temperatures suggesting that the physiological state of the organism at different temperatures likely influences the ease and extent of bacterial attachment (Takeuchi et al., 2001). Additionally, Phillips (1999) demonstrated the synthesis of a unique outer membrane protein by *E. coli* O157:H7 when grown at 10°C. Such alterations in surface structure in response to low temperatures will likely alter cell surface hydrophobicity and capability for attachment. In the study by Takeuchi and Frank (2000), lettuce was incubated for 24 h at 4°C which provided optimal conditions for penetration rather than attachment with sanitizers unable to inactivate cells that had penetrated below the surface. Therefore, their log reductions would be expected to be significantly lower than those reported in our study which used inoculation conditions (25°C/ 24 h) apparently optimal for attachment rather than penetration.

CSLM revealed that *E. coli* O157:H7 cells were randomly distributed on lettuce and strawberries after exposure to sodium hypochlorite with no affinity observed for particular surface structures. Remaining bacteria were organized into large groups or clusters that were scattered over the produce surface. These results are similar to those reported by Han et al. (2000), who observed stacks of bacterial cells on the surface of green peppers. This information indicates that clusters of bacterial cells in close proximity to each other may protect individual cells against sanitizer inactivation

Plate count analysis indicated that *E. coli* O157:H7 populations on lettuce and strawberries decreased 3.77 and 3.28 log CFU/g, respectively, using 2 ppm ozone. These log reductions are similar to those observed by Montecalvo et al. (1998) who observed a

4 log decrease in *E. coli* O157:H7 on lettuce using 0.3 ppm ozone. In contrast, Kim et al. (1999) reported only a 2 log reduction for *E. coli* O157:H7 on lettuce using 1.3 ppm ozone. The discrepancy in the latter study might reflect increased levels of organic debris on the lettuce, since ozone demand by organic nutrients reduces the level of free ozone available for microbial inactivation (Kim et al., 1999). Visual examination indicated that penetration offered little protection from treatment with ozone as *E. coli* O157:H7 was widely distributed on the surface of lettuce and strawberries with no specific attachment sites noted. Bacterial cells that survived on produce after treatment with ozone were arranged in large groupings or clusters as described previously.

Deposition of *E. coli* O157:H7 cells on the surface of lettuce and strawberries was directly related to the presence of liquid on the produce surface. After inoculating in a liquid suspension, the produce samples were rinsed and dried with most *E. coli* O157:H7 cells pooling in concave areas that were the last to dry. This phenomenon resulted in irregular contamination with some areas having few bacteria, and others high numbers due to pooling. Han et al. (2000) examined the viability of *E. coli* O157:H7 on artificially injured and uninjured surfaces of green peppers after treatment with chlorine dioxide with most bacterial cells found in injured locations. Pooling of the inoculum in injured areas was thought to account for this observation. In a study by Burnett et al. (2000b), *E. coli* O157:H7 preferentially attached to clefts on the surface of apples which were 10 to 16 um below the cuticle. However, these attachment sites were also likely the same areas where bacteria-laden water pooled and dried which would naturally lead to areas of higher concentration. These results indicate that specific surfaces on produce surfaces likely play a minor role in determining attachment sites for *E. coli* O157:H7

where the product is artificially contaminated by immersion. In support of this theory, Takeuchi and Frank (2000) noted that fewer specific attachment sites for *E. coli* O157:H7 on lettuce were observed at inoculum levels of 10^9 as compared with 10^8 and 10^7. They concluded that *E. coli* O157:H7 attached to alternate sites after the favored initial attachment sites were unavailable. The implications are that many sanitizer or water rinse treatments may concentrate surviving bacteria in less elevated surface areas. Therefore, the development and application of sanitizers should target areas where water has the potential to pool (blossom and stem ends, etc.) on the surfaces of fruits and vegetables in order to effectively decontaminate produce.

In our work, most bacteria were attached to the surface of lettuce and strawberries (0-10μm down) as seen using CSLM. While penetration of *E. coli* O157:H7 through intact surfaces on both lettuce and strawberries was rare, however penetration up to 20μm was observed. Similarly, Takeuchi and Frank (2001) reported that most *E. coli* O157:H7 cells were imbedded 1-10 μm down from the surface of lettuce. However, Seo and Frank (1999) only observed penetration of *E. coli* O157:H7 at the cut edges of lettuce leaves, which we did not examine in our study. Han et al (2000) found that most viable *E. coli* O157:H7 cells were located 0-8 um above the surface of green pepper. These results indicate that penetration through intact, uninjured surfaces on produce is rare.

In conclusion, GFP-*E. coli* O157:H7 in conjunction with CSLM was a useful tool for assessing specific attachment sites and viability on fresh produce after sanitizer exposure. Statistically significant differences in survival of *E. coli* O157:H7 cells attached to strawberries and lettuce were observed using ozone, Fit™, sodium hypochlorite and chlorine dioxide. Specific attachment sites for *E. coli* O157:H7 on

lettuce and strawberries were difficult to separate from the water pooling effect that was inherently part of the deposition process. Therefore the specific role of water in conjunction with bacterial deposition and attachment should be further investigated with important differences likely to be found between artificially and naturally contaminated produce.

CONCLUSION

The results of this research indicate that some of the alternative sanitizers examined could effectively replace traditional chlorine disinfection for raw produce and offer some additional benefits. Ozone (3 ppm) and chlorine dioxide (5 ppm) were the two most effective sanitizers examined and reduced populations of *L. monocytogenes* and *E. coli* O157:H7 by 5.5 and 5.7 logs, respectively. Treatment of produce with chlorine dioxide (3 ppm) and sodium hypochlorite (200 ppm) resulted in maximum reductions of 4.8 logs for *L. monocytogenes* and 5.1 logs for *E. coli* O157:H7. Peracetic acid was less effective giving reductions of 4.3 – 4.5 logs for *L. monocytogenes* and *E. coli* O157:H7. Fruit and Vegetable Wash and SCJP 16-162 treatment of produce resulted in maximum log reductions of 3.3 and 3.4 logs for *L. monocytogenes* and *E. coli* O157:H7, respectively. In comparison, produce treatment with SCJ 16-172 and Viper™ yielded reductions of 3.0 and 2.5 logs for *L. monocytogenes* and *E. coli* O157:H7, respectively. Fit™ was the least effective sanitizer, giving maximum reductions of only about 1 log for *L. monocytogenes* and *E. coli* O157:H7.

Further storage of sanitized produce indicated that the most effective sanitizers for eliminating *L. monocytogenes* and *E. coli* O157:H7, tended to facilitate growth of yeasts and molds and contribute to rapid spoilage of produce (3 ppm ozone and 3 and 5 ppm chlorine dioxide), while peracetic acid (80 ppm) and sodium hypochlorite (100 and 200 ppm) treatment did not adversely affect product shelf life. Sensory analysis using the non-extended triangle test, indicated that the only statistically significant differences between any of the treated and control samples occurred when whole apples were dipped

in sodium hypochlorite (200 ppm) and when shredded lettuce was sprayed with peracetic acid (80 ppm).

The sequential use of copper ion (1 ppm), sodium hypochlorite (100 ppm) and sonication was assessed to decrease populations of *E. coli* O157:H7 and *L. monocytogenes* during apple cider production. Using the hurdle approach, copper ion water did not significantly reduce populations of either pathogen; however, copper ion water/ sodium hypochlorite (100 ppm) decreased populations of *L. monocytogenes* and *E. coli* O157:H7 by 2.3 and 2.2 log CFU/g, respectively. After juiceration, the pulp contained ~ 1.1 – 1.3 log CFU/g of either pathogen with sonication decreasing the remaining *L. monocytogenes* and *E. coli* O157:H7 in the expressed juice by ~ 2 logs CFU/ml. Based on these findings, a 5-log reduction for both pathogens was achievable using 1 ppm copper ion and 100 ppm sodium hypochlorite followed by juiceration and sonication.

Employing confocal scanning laser microscopy (CSLM) to visualize attachment, colonization, location, and viability of *gfp*-transformed *E. coli* O157:H7 on lettuce and strawberries revealed generalized non-specific attachment to surface structures with some penetration through intact surfaces up to approximately 20μm. *E. coli* O157:H7 attachment to both products was more strongly influenced by water deposition and pooling than by any affinity to stomata or other surface structures. Fit™ was the least effective (~1 log reduction on either product) sanitizer tested and was not significantly different from water, while ozone and chlorine dioxide were the most effective yielding reductions of 3.77 and 3.55 logs on lettuce and 3.28 and 3.17 logs on strawberries, respectively, with these differences not significant. Sodium hypochlorite yielded

intermediate reductions, giving reductions of 2.95 and 2.34 for lettuce and strawberries, respectively. Based on CSLM analysis, viable *E. coli* O157:H7 cells that survived sanitizer treatments did so by grouping in clusters in areas of pooling rather than by penetrating through intact produce surfaces.

This research indicates that sanitizers, other than chlorine, may be effective for reducing pathogens on raw fruits and vegetables and improving shelf life attributes and taste that is non-detectable to consumers. Incorporation of multiple sanitizers may further reduce bacterial loads on produce and extend shelf life. Additionally, the use of CSLM could be used to examine sanitized produce after prolonged storage to examine physiological horticultural changes that occur over time.

Appendix A

Consent Form:

Department of Food Science and Human Nutrition

Michigan State University

You are being asked to participate in a sensory panel to determine if treatments which may be used for reducing or eliminating pathogenic microorganisms from fruits and vegetables cause flavor differences in the selected products. None of the horticultural products being tested has been exposed to or contaminated with any pathogenic microorganisms but they have been washed in a solution that contained (name of treatment), a commercially available antimicrobial compound, which has been approved for use on fruits and vegetables.

I _____ have read the above project description and agree to serve on this sensory panel on this _____ day of _____. In addition to tasting sample(s) I am aware that I will be asked to complete a brief questionnaire. I understand that I may withdraw my consent and discontinue participation in the panel at any time.

I understand that if I am injured as a result of my participation in this research project, Michigan State University will provide emergency medical care, if necessary, but these and other medical expenses must be paid from my own health insurance program.

Signed_____

Date_____

Example of Possible Sample Combination Codes for Triangle Sensory Panel

Codes for chlorine dioxide triangle sensory panels

T= Chlorine dioxide treated samples

U= Untreated controls

Possible combinations of samples
1=TUU 2=TUT 3=TTU 4=UTT 5=UTU 6=UUT

On the sensory ballots apple slices/1 refers to combination 1 (i.e. TUU), apple slices/2 refers to combination 2, etc.

Apple Slices

T= 211 or 223 U= 251 or 243

Whole Apples

T= 321 or 333 U= 347 or 311

Whole Lettuce

T= 523 or 555 U= 543 or 567

Shredded Lettuce

T= 645 or 631 U= 661 or 673

Strawberries

T= 733 or 701 U= 727 or 745

Sample Sensory Ballot

Product: Apple Slices/1 Name: Date:

You will receive a set of three samples which are numbered. Please taste the samples in order from left to right. You may clear your palate between samples with the water provided if you wish and retasting is permitted. In this set, two samples are identical and one is different. Circle the number of the off sample. Even if you cannot detect any flavor difference between the samples you must choose one and circle it.

211 **251** **243**

Appendix B

SAFETY PRECAUTIONS FOR SANITIZERS

Sodium hypochlorite	Normal safety precautions
Chlorine dioxide	Normal safety precautions
Ozone	Sterile rubber gloves worn to prevent skin contact Prepared under chemical fume hood to prevent inhalation
Peracetic acid	Sterile latex gloves worn to prevent skin contact Prepared under chemical fume hood to prevent inhalation
SCJP 16-162	Normal safety precautions
SCJ 16-172	Normal safety precautions
Viper	Normal safety precautions
Fit	Normal safety precautions
Copper Ion	Normal safety precautions
Sonication	Ear protection to prevent hearing damage

REFERENCES

Abdelnoor, A.M., Batshoun, R., Roumani, B.M. 1983. The bacterial flora of fruits and vegetables in Lebanon and the effect of washing on the bacterial content. Zbl. Bakt. Hyg. L. Abt. Orig. B. 177:342-349.

Abdoul-Roouf, U.M. L. Beuchat, and M.S. Ammar. 1993. Survival and Growth of *E. coli* O157:H7 on Salad Vegetables. Appl. Environ. Microbiol. 59: 1999-2006.

Ackers, M., Mahon, B., Leahy, E., Damrow, T., Barrett, L. 1996. An outbreak of *Escherichia coli* O157:H7 infections associated with leaf lettuce consumption, Western Montana. Abstract K43, 36th Interscience Conference on Antimicrobial Agents and Chemotherapy, Sept. 15-18, 1996. P 258. Washington, DC, American Society for Microbiology.

Adams, M.R., Hartley, A.D., and Cox, L.J. 1989. Factors affecting the efficacy of washing procedures used in the production of prepared salads. Food Microbiol. 6: 69-77.

Aram, B. 1995. Disinfectants. 6.1-6.18. In P. Cuniff (ed.) Official methods of analysis. Vol I. AOAC Intl. MD.

Altekruse, S.F., Ccohen, M.L., and Swerdlow, D.L. 1997. Emerging foodborne diseases. Emerg. Infect. Dis. 3: 285-293.

Altekruse, S.F., Swerdlow, D.L., 1996. The changing epidemiology of foodborne diseases. Am. J. Med. Sci., 311: 23-29.

APHA: American Public Health Association. 1992. Standard methods for the examination of water and wastewater, 18th ed., p. 4-105 – 5-107. American Public Health Association, Ind., New York.

Arumugaswamy, R.K., Rusul, G., Abdul Hamid, S.N. 1994. Prevalence of *Listeria monocytogenes* in foods in Malaysia. Int. J. Food Microbiol. 23: 117-121.

Banwart, G. 1981. Basic Food Microbiology. AVI Publishing Co. Inc. Westport, CT.

Baranovskaya, V., Zapol'skii, O., Ovrutskaya, N., Yushkevich, O. 1979. The use of ozone gas sterilization during storage of potatoes and vegetables Konserun, Ovoshesus. Promst' 4:10-12.

Barnett, B., Schwartze, D., Sweat, S., Lea, J, Taylor, B., Bibb G., Pierce, G., Hendricks, K. 1995. Outbreak of *Escherichia coli* O157:H7 ,Waco, TX. P. 17-18. Epidemic Intelligence Service 44th Annu. Conf. CDC Atlanta, GA.

Barth, M. M., Zhou, C., Mercier, M., and Payne, F. A. 1995. Ozone storage effects on anthocyanin content and fungal growth in blackberries. J. Food Sci. 60:1286-1287.

Bartz, J.A., Showalter, R.K. 1981. Infiltration of tomatoes by bacteria in aqueous suspension. Phytopathology. 72:515-518.

Bartz, J.A. 1999. Washing fresh fruits and vegetables: Lessons from treatment of tomatoes and potatoes with water. Dairy Food Env. Sanit. 12: 853-864.

Beckers, H.J. Veld, P.H., Soentoro, P.S., Delfgou-van Asch, E. 1989. The occurrence of Listeria in food. P. 85-97. In: Foodborne listeriosis: Proc. Symp, Wiesbaden, Germany.

Berg, J.D., Roberts, P., Matin, A., 1986. Effect of chlorine dioxide in selected membrane functions of Escherichia coli. J. Appl. Bacteriol. 60: 213-220.

Berrang, M.E., Brackett, R.E., Beuchat, L.R. 1989. Growth of *Listeria monocytogenes* on fresh vegetables stored under controlled atmosphere. J. Food Prot. 52: 702-712.

Besser, R., Lett, S., Weber, J., Doyle, M., Barrett, T., Wells, J., Griffen, P. 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. JAMA 269: 2217-2220.

Beuchat, L.R., Ward, T., Pettigrew, C.A. 2001. Comparison of chlorine and a prototype produce wash product for effectiveness in killing *Salmonella* and *E. coli* O157:H7 on alfalfa seeds. J. Food Prot. 64: 152-158.

Beuchat, L. 1999. Survival of Enterohemorrhagic *E. coli* O157:H7 in bovine feces applied to lettuce and the effectiveness of chlorinated water as a disinfectant. J. Food Prot. 62: 845-849.

Beuchat, L.R. 1996a *Listeria monocytogenes* : Incidence on vegetables. Food Control. 7:223-228.

Beuchat, L.R. 1996b. Pathogenic microorganisms associated with fresh produce. J. Food Prot. 59: 204-216.

Beuchat, L. 1998. Surface decontamination of fruits and vegetables eaten raw: a review. WHO/FSF/FOS/98.2 World Health Organization, Geneva.

Beuchat, L.R. 1992. Surface disinfection of raw produce. Dairy Food Environ. Sanit. 12: 6-9.

Beuchat, L.R., and Brackett, R.E. 1990. Survival and growth of *Listeria monocytogenes* on lettuce as influenced by shredding, chlorine treatment, modified atmosphere packaging, and temperatures. J. Food Sci. 55: 755-758.

Beuchat, L.R., Brackett, R.E. 1991. Behavior of *Listeria monocytogenes* inoculated into raw tomatoes and processed tomato products. Appl. Environ. Microbiol. 57: 1267-1371.

Beuchat, L.R., Brackett, R.E., Hao, D.Y., Conner, D.E. 1986. Growth and thermal inactivation of *Listeria monocytogenes* in cabbage and cabbage juice. Can. J. Microbiol. 32: 791-795.

Block, S. 2001. Historical review. In: Block, S. ed. Disinfection, sterilization, and preservation. Pg. 3-19. Lippincott Williams and Wilkins, Philadelphia, PA.

Bott, T. 1991. Disinfectant testing in relation to the food industry in Hygiene, U.K.

Brackett, R.E. 1987. Antimicrobial effect of chlorine on *Listeria monocytogenes.* J. Food Prot. 50: 999-1003, 1008.

Brackett, R., Lett, S., Weber, J., Doyle, M. 1994. Ineffectiveness of hot acid sprays to decontaminate *Escherichia coli* O157:H7 on beef. J. Food Prot. 57:198-203
Costerton, J. W., Geesey, G.G., Cheng, K.J. 1978. How bacteria stick. Sci. Amer. 238: 86-95.

Burleson, G.R., Murray, T.M., and Pollard, M. 1975. Inactivation of viruses and bacteria by ozone, with and without sonication. Appl. Microbiol. 29: 340-344.

Burnett, S.L., and Beuchat, R. 2000. Human pathogens associated with raw produce and unpasteurized juices, and difficulties in decontamination. J. Ind. Microbiol. Biotechnol. 25: 281-287.

Burnett, S.L., J. Chen, L. Beuchat. 2000b. Attachment of *E. coli* O157:H7 to the Surfaces and Internal Structures of Apples as Detected by Confocal Scanning Laser Microscopy. Appl. Environ. Micro. 66: 4679-4687.

Centers for Disease Control and Prevention. 1997. Outbreaks of *E. coli* O157:H7 infection and cryptosporidiosis associated with drinking unpasteurized apple cider- Connecticut and New York, October, 1996. Morbid. Mortal. Weekly Rep. 46(1): 4-8.

CFR. 1994. Hydrogen peroxide. Code of Federal Regulations 21, Parts 170-199, Section 184.1366, April 1, U.S. Govt. Print. Office, Washington, D.C.

Chambers, L.S., and Gaines, N.J. 1932. Some effect of intense audible sound on living organisms and cells. J. Cellular Comp. Physiol. 1: 451-473.

Cherry, J.P. 1999. Improving the safety of fresh produce using antimicrobials. Food. Tech. 53: 54-59.

Cliver, D.O. 1997. Foodborne diseases. In: Doyle, M.P., Beuchat, L.R., Montville, T.J. eds. Food microbiology: Fundamentals and frontiers, p. 437-446. Washington, D.C., American Society for Microbiology.

Comes, J.E., and Beelman, R.B. 2002. Addition of fumaric acid and sodium benzoate as an alternative method to achieve a 5-log reduction of *E. coli* O157:H7 populations in apple cider. J. Food Prot. 65: 476-483.

CPSI Center for Science in the Public Interest. 2002. Food Security Guidelines for the Meat and Poultry Industry. U.S. Federal Register at: www.cfsan.fda.gov/~lrd/fr020109.html.

Cutter, C., and Dorsa, W. 1995. Chlorine dioxide spray washes for reducing fecal contamination on beef. J. Food Prot. 58: 1294-1296.

Del Rosario, B.A., and L.R. Beuchat 1995. Survival and Growth of Enterohemorrhagic *E. coli* O157:H7 in Cantaloupe and Watermelon. J. Food. Prot. 58: 105-107.

Diaz, C. and H. Hotchkiss. 1996. Comparative Growth of *E. coli* O157:H7: Spoilage organisms and shelf life of shredded iceberg lettuce stored under modified atmospheres. J. Sci. Food Agric. 70: 433-438.

Dickson, J.S., Cutter, C.G., and Siragusa, G.R. 1994. Antimicrobial effects of trisodium phosphate against bacteria attached to beef tissue. J. Food Prot. 57: 952-955.

Dychdala, G.R. 2001. Chlorine and Chlorine Compounds In. Disinfection Sterilization and Preservation. Ed. Block, S.S. Philadelphia, PA, Lea and Febiger.

Eckert, J.W., Ogawa, J.M. 1988. The chemical control of post-harvest diseases: deciduous fruits, berries, vegetables and root/tuber crops. Annu. Rev. Phytopathol. 26: 433-435.

El-Kest, S. and Marth, E. 1988. Inactivation of *Listeria monocytogenes* by chlorine. J. Food Prot. 51: 520-524.

Farooq, S.E., Chian, S.K., and Engelbrecht, R.S. 1977. Basic concepts in disinfection with ozone. J. Water Pollut. Control Fed. 49: 1818-1831.

Food and Drug Administration. 1998. Guidance to Industry: Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables. CFSAN, Washington, D.C.

Food and Drug Administration. 2001. Hazard analysis and critical control point (HACCP); procedures for the safe and sanitary processing and importing of juices; final rule. Fed. Regist. 66: 6137-6202.

FDA. HHS News. 1996. *Escherichia coli* O157:H7 outbreaks associated with Odwalla brand apple juice production. www.fda.gov

Firstenberg-Eden, R. 1981. Attachment of bacteria to meat surfaces: a review. J. Food Prot. 44: 602-607.

Forni, L., Bahnemann, D., and Hart, E.J. 1982. Mechanism of the hydroxide ion initiated decomposition of ozone in aqueous solution. J. Phys. Chem. 86: 255-259.

Forsythe, R.H., and Waldroup, A.L. 1994. The economics of conservation of poultry processing water using ozone. Poult. Sci. 74 (Suppl. 1): 87

Gadd, G.M. 1992. Metals and microorganisms: a problem of definition. FEMS Microbiol. Lett. 100: 197-204.

Garcia, M.S., Burgos, J., Sanz, B., Ordonez, J.A. 1989. Effect of heat and ultrasonic waves on the survival of two strains of *Bacillus subtilis*. J. Appl. Bacteriol. 67: 619-628.

Gaonkar, A.G. 1995. Characterization of food: emerging methods. Elsevier Science B. V., New York.

Graham, D.M. 1997. Use of ozone for food processing. Food Technol. 51:72-75.

Greenspan, F.P., Johnsen, M.A., Trexler, P.C. 1955. Peracetic acid aerosols. In: Proceedings of the 42nd Annual Meeting of Chemical Special Manufacturing Association. 59-64.

Gutierrez, E. 1997. Japan Prepares as O157:H7 Strikes Again. Lancet. 349:942.

Hampson, B.C., Fiori, S.R. 1998. Application of ozone in food process wash waters: A pilot study. Ozone Sci.

Han, Y., R.H. Linton, S.S. Nielsen, and P.E. Nelson. 2000. Inactivation of *E. coli* O157:H7 on Surface-Uninjured and –Injured Green Pepper (Capsicum Annuum L.) by Chlorine Dioxide Gas as Demonstrated by Confocal Laser Scanning Microscopy. Food Micro. 17:643-655.

Harakeh, S., Illescas, A., Matin, A. 1988. Inactivation of bacteria by Purogene. J. Appl. Bacteriol. 64: 459-463.

Harvey, E.N., and Loomis, A.L. 1929. The destruction of luminous bacteria by high frequency sound waves. J. Bacteriol. 17: 373-376.

Harvey, J., Gilmour, A. 1993. Occurrence and characteristics of *Listeria monocytogenes* in foods produces in Northern Ireland. Int. J. Food Microbiol. 19: 193-205.

Hedberg, C.W., MacDonald, K.L., Osterholm, M.T. 1994. Changing epidemiology of food-borne disease: a Minnesota perspective. Clin. Infect. Dis., 18: 671-682.

Hoigne, J., 1982. Mechanisms, rates and selectivities of oxidations of organic compounds initiated by ozonation of water. P. 341-379. In Handbook of Ozone Technology and Applications, Vol. 1, (Ed. Rice, R.G., and Netzer, A.) Ann Arbor Science Publishers, Ann Arbor, MI

Hoigne, J. and Bader, H. 1976. The role of hydroxyl radical reactions in ozonation processes in aqueous solutions. Water Res. 10: 377-386.

Horvath, M., Blitzky, L., and Huttner, J. 1985. Ozone. P305-312. Elsevier, New York.

Hunt, N.K., and Marinas, B.J. 1997. Kinetics of *Escherichia coli* inactivation with ozone. Water Res. 31: 1355-1362.

Junli, H., Li, W., Nanqi, R., and Fang, M. 1997. Disinfection effects of chlorine dioxide on bacteria in water. Water Res. 31: 607-613.

Karapinar, M., Gonul, S.A. 1992. Removal of *Yersinia enterocolotica* from fresh parsley by washing with acetic acid or vinegar. Int. J. Food Microbiol. 16: 261-264.

Kays, S.J. 1991. Nature and structure of harvested products, p. 23-73. In Postharvest physiology of perishable plant products. Van Nostrand Reinhold, New York.

Kim, J., Ahmed, Yousef, E. 1998. Effective ozone delivery for decontamination of fresh lettuce. Institute of Food Technologists annual meeting: book of abstracts, p 55-56.

Kim, J., Ahmed, Yousef, E., Chism, G. 1999. Use of ozone to inactivate microorganisms on lettuce. J. Food Safety. 19: 17-34.

Korich, D.G., Mead, J.R., Madore, M.S., Sinclair, N.A., and Sterling, C.R. 1990. Effects of ozone, chlorine dioxide, chlorine and monoamine of *Cryptosporidium parvum* oocyst viability. Appl. Env. Microbiol. 56:1423.

Kozhinov, V.F. 1968. Equipment for the ozonation of water. Publishing House on the Literature of Construction, Moscow, Russia.

Kutz, S.M., Landeen, L.K., Yahya, M.T., and Gerba, C.P. Microbiological evaluation of copper: silver disinfection units. Proceedings of the 4th Conference in Progress in Clinical Disinfection. State University of New York, Bighamton, New York April 11-13, 1988.

Lebout, H. 1959. Fifty years of ozonation at Nice. In "Ozone Chemistry and Technology," Adv. In Chemistry Series, Vol 21, ed. R.G. Rice and A. Netzer. Pg. 450-452. Am. Chem. Soc., Washington, D.C.

Lee, B.H., Kermasha, S., Baker, B.E. 1989. Thermal, ultrasonic and ultraviolet inactivation of Salmonella in thin films of aqueous media and chocolate. Food Microbiol. 6: 143-152.

Leyer, G., Wang, L., Johnson, E. 1995. Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. Appl. Env. Microbiol. 61: 3752-3755.

Liew, C.L., and Prange, R.K. 1994. Effect of ozone and storage temperature on postharvest diseases and physiology of carrots (*Caucus carota L.*) J. Am. Soc. Hortic. Sci. 119: 563-567.

Lillard, H.S. 1979. Levels of chlorine and chlorine dioxide of equivalent bactericidal effect in poultry processing water. J. Food Sci. 44: 1594-1597.

Lillard, H.S. 1993. Bacteriocidal effect of chlorine on attached salmonellae with and without sonication. J. Food Prot. 56: 716-717.

Lillard, H.S. 1994. Decontamination of poultry skin by sonication. Food Technol. 48: 72-73.

Lin, W., Huang, T., Cornell, J., Lin, C., Wei, C. 1996. Bacteriocidal activity of chlorine and chlorine dioxide solutions in a fish model system. J. Food Sci. 61: 1030-1034.

Longley, K.E. et al. 1978. The role of mixing in wastewater disinfection. Am. Chem. Soc. J. Am. Water Works Assoc. 74: 376-380.

Lund, B.M. and Snowdon, A.L. 2000. Fresh and processed fruits Pg. 738-758. In B.M. Lund, T.C. Baird-Parker, and G.W. Gould (ed.), The microbiological safety and quality of food, vol. 1. Aspen Publishers, Inc. Gaithersburg, MD.

Madden, J.M. 1992. Microbial pathogens in fresh produce- the regulatory perspective. J. Food. Prot. 55: 821-823.

Marriott, N.G. 1994. Principles of Food Sanitation. Chapman and Hall, Inc., London.

Marshall, K.C., Stout, R., and Mitchell, R. 1971. Mechanisms of the initial events in the sorption of marine bacteria to surfaces. J. Gen Microbiol. 68: 337-348.

Martinez, F.E., Davidson, A.G.F., Anderson, J.D., Nakai, S., Desai, I.D., and Redcliffe, A. 1992. Effects of ultrasonic homogenization of human milk on lipolysis, IgA, IgG, lactoferrin and bacterial content. Nutr. Res. 12: 561-568.

Mazollier, J. Ive gamme. Lavage-desinfection des salades. Infros-Crifl. 1988. 41:19.

McWilliams, M. 2001. Foods: Experimental Perspectives. Prentice Hall. P. 187-193.

Mead PS, Slutsker L, Dietz V, et al. Food-related illness and death in the United States. Emerg Infect Dis 1999;5: 607-25

Mermin, J., P. Mead, K. Gensheimer, and P. Griffin. 1996. Outbreak of *E. coli* O157:H7 Infections Among Boy Scouts in Maine. Abst. K44, p.258. 36th Intersci. Conf. Antimicrob. Agents Chemother. 1996. American Society for Microbiology., Washington, D.C.

Miller, F. 1982. College Physics. 5th ed. Harcourt Brace Jovanovich Inc., New York.

Miller, L., Kaspar, C. 1994. *Escherichia coli* O157:H7 acid tolerance and survival in apple cider. J. Food Prot. 57: 460-464.

Montecalvo, JR, J. 1998. Ozone research results summary. Department of Food Science and Nutrition, California State Polytechnic University, San Luis Obispo.

Morton, M.E. Alcohols. In: Block, S.S. ed. Disinfection, sterilization, and preservation, 3rd ed. Philadelphia: Lea and Febiger, 1983: 225-250.

Murray, R., Pamela, S., Elson, H. 1965. Location of mucopeptide of selection of the cell wall of E. coli and other gram-negative bacteria. C. J. Microbiol. 11: 547-560.

Nakashima, K., Fukumoto, N, Nakano, N., and Ogawa, M. 1980. Removal of bacteria from parsley by sonication. J. Jap. Soc. Food Nutr. 29: 261-268.

Nguyen-the, and C., Carlin, F. 1994. The microbiology of minimally processed fresh fruits and vegetables. Crit. Rev. Food Sci. Nutr. 34: 371-401.

Padhye, N. and Doyle, M. 1992. *E. coli* O157:H7: Epidemiology. Pathogenesis, and methods for detection in food. J. Food Prot. 55: 555-565.

Park, C., and Beuchat, L. 1999. Evaluation of sanitizers for killing *Escherichia coli* O157:H7, *Salmonella*, and naturally occurring microorganisms on cantaloupes, honeydew melons, and asparagus. Dairy Food. Env. Sanit. 19: 842-847.

Parish, M.E. 1997. Public health and nonpasteurized fruit juices. Crit. Rev. Microbiol. 23: 109-119.

Peters, D. 1995. Control of enteric pathogenic bacteria on fresh produce. M.S. thesis. University of Nebraska.

Pethica, B. 1958. Bacterial lysis: lysis by physical and chemical methods. J. Gen. Microbiol. 18: 473-480.

Phillips, C. The epidemiology, detection and control of *E. coli* O157:H7. 1999. J. Sci. Agric.79: 1367-1381.

Potter, M.E., Motarjemi, Y., Kaferstein, F.K. 1997. Emerging foodborne diseases. World Health, Jan – Feb. 16-17.

Prachaiyo, P, P., and McLandsborough, L.A. 2000. A microscopic method to visualize *Escherichia coli* interaction with beef muscle. J. Food Prot. 63: 427-433.

Prat, R., Nofre, C., Cier, A. 1968. Effects de l'hypochlorite de sodium, de l'ozone et des radiations ionisontes dur les constituants pyrimidiques *d'Escherichia coli*. Ann. Inst. Pasteur Paris. 114: 595-607.

Priepke, P.E., Wei, L.S., Nelson, A.L. (1976). Refrigerated storage of prepackaged salad vegetables. J. Food Sci. 41: 379-385.

Reina, L.D., Flemming, H.P., Humphries, E.G. 1995. Microbiological control of cucumber hydrocooling water with chlorine dioxide. J. Food Prot. 58: 541-546.

Restaino, L., Frampton, E.W., Hemphill, J.B., and Palnitkar, P. 1995. Efficiency of ozonated water against various food-related microorganisms. Appl. Env. Microbiol. 61: P. 3471.

Rice, R., Farquhar, J., Bollyky. 1982. Review of the applications of ozone for increasing storage times of perishable foods. Ozone Sci. Eng. 4: 147-163.

Richardson, S.D., Thruston, A., Caughran, T., Collette, T., Patterson, K., Lykins, Majetich, G., Zhang, Y. 1994. Multispectral identification of chlorine dioxide disinfection by-products in drinking water. Environ. Sci. Technol. 28: 592-599.

Richardson, S.D., Thruston, A., Caughran, T., Collette, T., Patterson, K., Lykins, B. 1998. Chemical by-products of chlorine and alternative disinfectants. Food. Tech. 52: 58-61.

Richert, K.J., Albrect, L.B., Bullerman, L.B., and Sumner, S.S. 2000. Survival and growth of *E. coli* O157:H7 on broccoli, cucumber, and green pepper. Dairy Food Environ. Sanit. 20: 24-28.

Riley, L.W., Remis, S.D., Helgerson, H.B., McGee, J.G., Wells, B.R., Davis, R.J., Hbert, R.J., Olcott, E.S., Johnson, N.T., Hargrett, P.A., Blake, P.A., and Cohen, M.L. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med. 308: 681-685.

Roessler, E.B., Pangborn, R.M., Sidel, J.L., and Stone, H. 1978. Expanded statistical tables for estimating significance in paired-preference, paired-difference, duo-trio, and triangle tests. J. Food. Sci. 43, 940-943.

Roller, S., Olivieri, V., Kawata, K. 1980. Mode of bacterial inactivation by chlorine dioxide. Water Res. 14: 635-641.

Rutala, W.A., Gergen, M.F., Jones, J.F., and Weber, D.J. 1998. Levels of microbial contamination on surgical equipment. Amer. J. Infect. Control. 26: 143-145.

Ryu, J.H., Deng, Y., and Beuchat, L.R. 1999. Behavior of acid adapted and unadapted *E. coli* O157:H7 when exposed to reduced pH achieved with various organic acids. J. Food Prot. 62: 451-455.

Sagripanti, J.L., and Bonifacino, A. 1997. Effects of salt and serum on the sporicidal activity of liquid disinfectants. J. AOAC Int. 80: 1198-1207.

Sapers, G.M., Miller, and Mattrazzo. 1999. Effectiveness of sanitizing agents in inactivating *Escherichia coli* on golden delicious apples. J. Food Sci. 64: 734-737.

Sarig, P., Zahavi, Y., Zutkhi, S., Yannai, N., Lisker, Benarie, R. 1996. Ozone for control of post-harvest decay of table grapes caused by *Rhizopus stolonifer*. Physiol. Mol. Plant Pathol. 48: 403-415.

Schlech, W.F., Lavigne, P.M., Bostoulussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S., Broome, C.V. 1983. Epidemic listeriosis-evidence for transmission by food. N. Engl. J. Med. 308: 203-206.

Scott, D. 1975. The effect of ozone on nucleic acids and their derivatives. P 226-240. In W.J. Blogoslawski and R.G. Rice (ed.). Aquatic applications of ozone. International Ozone Institute, Syracuse, N.Y.

Scott, D.B.M., and Lecher, E.C. 1963. Effect of ozone on survival and permeability of *E. coli*. J. Bacteriol. 85:567-576.

Seo, K.H., Frank, J.F. 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.

Shapiro, J.E., and Holder, I.A. 1960. Effect of antibiotic and chemical dips on the microflora of packaged salad mix. Appl. Microbiol., 8:341.

Shukla, T.P. 1992. Microwave ultrasonics in food processing. Cereal Foods World. 37: 332-333.

Sizmur, K., Walker, C.W. 1988. Listeria in prepacked salads. Lancet. I: 1167.

Splittstoesser, D., McLellan, M., Churey, J. 1996. Heat resistance to *Escherichia coli* O157:H7 in apple juice. J. Food Prot. 59: 226-229.

Spotts, R.A., and Cervantes, L.A. 1992. Effect of ozonated water on postharvest pathogens of pear in laboratory and Packinghouse. Plant Disease 76, 256-259.

Standard Methods for Examination of Water and Wastewater, 17th ed., American Public Health Association: New York, 1987, Pg. 162-165, 298-300.

Steele, B.T., Murphy, T.N., and Rance, C.P. 1982. An outbreak of hemolytic uremic syndrome associated with ingestion of fresh apple juice. J. Pediatr. 101: 963-965.

Steinbruegge, E.G., Maxcy, R.B., and Liewen, M.B. 1988. Fate of *L. monocytogenes* on ready to serve lettuce. J. Food Prot. 51:596-599.

Stone, D.L. and Fryer, T.F. 1984. Disruption of bacterial clumps in refrigerated raw milk using an ultrasonic cleaning unit. New Zealand J. Dairy Sci. Technol. 19: 221-228.

Superior Water Solutions Inc. 2000. Superior2o.com/superior.htm.

Takayama, M., Sugimoto, H., Uchida, R., Yamauchi, R., Tanno, K. 1994. Antimicrobial activities of silver and copper ions.

Takeuchi, K., Frank, J. 2001a. Quantitative determination of the role of lettuce leaf structures in protecting *E. coli* O157:H7 from chlorine disinfection. J. Food Prot. 64: 147-151.

Takeuchi, K., Frank, J. 2001b. Direct microscopic observation of lettuce leaf decontamination with a prototype fruit and vegetable washing solution and 1% NaCl-$NaCO_3$. J. Food Prot. 64: 1235-1239.

Takeuchi, K., Frank, J. 2000. Penetration of *Escherichia coli* O157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. J. Food Prot. 63: 434-440.

Takeuchi, K., C. Matute, A. Hassan, and J. Frank. 2000. Comparison of the Attachment of *E. coli* O157:H7, *L. monocytogenes*, *Salmonella Typhimurium*, and *Pseudomonas fluorescens* to Lettuce Leaves. J. Food Prot. 63: 1433-1437.

Taormina, P.J., and Beuchat, L.R. 1999. Behavior of enterohemorrhagic *E. coli* O157:H7 on alfalfa sprouts during the sprouting process as influenced by treatments with various chemicals. J. Food Prot. 62: 850-856.

Tauxe, R. 1997. Emerging foodborne diseases: an evolving public health challenge. Emerg. Infect. Dis. 3: 425-434.

Tiwari, N.P. and Alenrath, S.G. 1990. Occurrence of Listeria species in food and environmental samples in Alberta. J. Can. Inst. Food Sci. Technol. 23: 109-113.

Tomiyasu, H., Fukutomi, H., and Gordon, G. 1985. Kinetics and mechanism of ozone decomposition in basic aqueous solution. Inorg. Chem. 24: 2962-2966.

Tsai, L., Schade, J., Molyneux, B. 1992. Chlorination of poultry chiller water: chlorine demand and disinfection efficiency. Poult. Sci. 71: 188-196.

U.S. Federal Registrar. 2000. 40 CFR Part 141.64. Maximum containment level for disinfection byproducts.

U.S. Department of Agriculture-Economic Research Service. 1998. Foodborne illness costs from seven major pathogens are $5-$9 billion per year. Economic Research Services Food Safety Briefing.

Vahidy, R., 1992. Isolation of *L. monocytogenes* from fresh fruits and vegetables (Abstract). HortScience, 27: 628.

Van Renterghem, B., Huysman, F., Rygole, R., Verstraete, W. 1991. Detection and prevalence of *L. monocytogenes* in the agricultural ecosystem. J. Appl. Bacteriol., 71: 211-217.

Vasse, J. Frey, P., and Trigalet, A. 1995. Microscopic studies of intracellular infection and protoxylem invasion of tomato roots by Pseudomonas solanaceorum. Mol. Plant. Microb. Interact. 8:241-251.

Villasenor, A. Hill, S.A., Seale, N.S. 1991. Comparison of two ultrasonic cleaning units for deterioration of cutting edges and debris removal on dental burs. Pediatric Dent. 326-330.

Vodovotz, Y., Vittadini, E., Coupland, J., McClements, D.J., and Chinachoti, P. 1996. Bridging the gap: use of confocal microscopy in food research. Food Technol. 50, 74-82.

Walmsley, A.D., and Williams, A.R. 1991. Measurement of cavitational activity within ultrasonic baths. J. Dent. 19: 62-66.

Wei, C.I., Huang, T.S., Kim, J.M., Lin, W.F., Tamplin, M.L., Bartz, J.A. 1995. Growth and survival of *Salmonella montevideo* on tomatoes and disinfection with chlorinated water. J. Food Prot. 58:829-836.

Whallon, J. 1998. Introduction to laser scanning confocal microscopy. Michigan State University.

White, G. 1972. Handbook of chlorination for potable water, wastewater, cooling water, industrial processes, and swimming pools. Van Nostrand Reinhold Co., N.Y.

Williams, D.W., Montecalvo, J., Mueller, D., Warls., K., Swanson, Peterson, L. 1995. Ozonation as an alternative disinfectant for carrot washwater. IFT annual meeting book of abstracts. P. 81.

Wisniewski, M., Glatz, B.A., Gleason, M.L., and Reitmeier, C.A. 2000. Reduction of *E. coli* O157:H7 counts on whole fresh apples by treatment with sanitizers. J. Food Prot. 63: 6, 703-708.

Wright, J., Sumner, S., Hackney, C., Pierson, M., Zoecklein, B. 2000. Reduction of *Escherichia coli* O157:H7 on apples using wash and chemical sanitizer treatments. Dairy, Food Env. Sanit. 20: 120-126.

Wrigley, D.M. and Llorca, N. 1992. Decrease of *Salmonella typhimurium* in skim milk and egg by heat and ultrasonic wave treatment. J. Food Prot. 55: 678-680.

Yeager, C.C. Copper and zinc preservatives. 1991. In: Disinfection, Sterilization and Preservation. 4th ed. Philadelphia, Lea and Febiger. P. 358-361.

Yu, K., M. Newman, D. Archbold, T. Hamilton-Kemp. 2001. Survival of *E. coli* O157:H7 on Strawberry Fruit and the Reduction of the Pathogen Population by Chemical Agents. J. Food Prot. 64: 1334-1340.

Zepeda-Lopez, H., Ortega-Rodriguez, Quinonez-Ramirez, E., Vazguez-Salinas, C. 1995. Isolation of *Escherichia coli* O157:H7 from vegetables. Annu. Mtg. Am. Soc. Microbiol. Washington, D.C.

Zhang, S, and Farber, J.M. 1996. The effects of various disinfectants against *Listeria monocytogenes* on fresh cut vegetables. Food Microbiol. 13: 311-321.

Zhao, J., and Cranston, P.M. 1995. Microbial decontamination of black pepper by ozone and the effect of the treatment on volatile oil constituents of the spice. J. Sci. Food Agric. 68: 11-18.

Zhao, T., Doyle, M.P., Besser, R.E. 1993. Fate of entrohemorrhagic *Escherichia coli* O157:H7 in apple cider with and without preservatives. Appl. Environ. Microbiol. 59: 2526-2530.

Zhuang, R.Y., Beuchat, L.R., Angulo, F.J. 1995. Fate of *Salmonella montevideo* on and in raw tomatoes as affected by temperature and treatment with chlorine. Appl. Environ. Microbiol. 61: 2127-2131.

Zhurkov, V.S., Sokolovskii, V.V., Mozhaeva, T.E., Mirkis, V.I., Borisov, V.I., and Akhal'tseva, L.V. 1997. Impact of chlorination and ozonation on the total mutagenic activity of drinking water. Gig. Sanit. 1: 11-13.