AN ABSTRACT OF THE THESIS OF

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Title: Efficacy of Electrolyzed Oxidizing Water and Ozonated Water for Microbial Decontamination of Fresh Strawberries (*Fragaria x ananassa*).

Abstract approved:

Mark A. Daeschel Yanyun Zhao

The objectives of this project were to evaluate the efficacies of electrolyzed oxidizing (EO) and ozonated waters as antimicrobial agents for enhancing the microbiological safety of fresh strawberries (*Fragaria x ananassa*). The influence of sodium chloride (NaCl) concentrations used for preparing EO water was evaluated on their bactericidal activities against naturally occurring aerobic mesophiles on strawberries with a contact time of 5, 10, or 15 min. EO water and ozonated water containing about 1.90 ppm ozone were evaluated and compared with sodium hypochlorite (NaOCl) solution on their capabilities to inactivate and control the growth of *Listeria monocytogenes* and *Escherichia coli* O157:H7 inoculated onto strawberries stored at 4 ± 1 °C for up to 15 d, respectively. Post-treatment neutralization of fruit surfaces by washing was also investigated. More than 2 $\log_{10}$ CFU/g reduction of mesophilic aerobic bacteria was achieved in samples washed for

10 or 15 min in EO water prepared from 0.10% (w/v) NaCl solution. Bactericidal activity of treatment solutions against *L. monocytogenes* and *E. coli* O157:H7 was not affected by post-treatment neutralization, and their effectiveness against both pathogens in whole fruit tissues did not significantly increase with increasing exposure time. The EO water had an equivalent antibacterial effect as compare with NaOCl in eliminating *L. monocytogenes* and *E. coli* O157:H7 on whole strawberry tissues. Fruit surfaces washing with distilled water resulted in 1.90 and 1.27 $\log_{10}$ CFU/ml of rinse fluid reduction of *L. monocytogenes* and *E. coli* O157:H7, respectively, whereas ≥ 2.60 $\log_{10}$ CFU/ml of rinse fluid reduction of *L. monocytogenes* and up to 2.35 and 3.12 log reduction of *E. coli* O157:H7 were observed on fruit surfaces washed with EO water and NaOCl solution, respectively. However, EO water and NaOCl solution treatments did not exhibit a higher microbicidal activity than water treatment during refrigeration storage. The ozone treatment on inoculated strawberries was not remarkably effective in removing and eliminating pathogens on the whole fruit tissues, but the populations of *L. monocytogenes* and *E. coli* O157:H7 were significantly decreased after ozone treatment regardless of the exposure time. The number of *L. monocytogenes* and *E. coli* O157:H7 on fruit surfaces was decreased by 2.17 and 2.02 $\log_{10}$ CFU/ml of rinse fluid, respectively, after washing with ozonated water for 10 min.

Efficacy of Electrolyzed Oxidizing Water and Ozonated Water for Microbial Decontamination of Fresh Strawberries (*Fragaria x ananassa*)

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Pathima Udompijitkul, Author

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CHAPTER 1

Introduction

Strawberries (*Fragaria x ananassa*) are perceived by the consumers as a functional food since they contain various nutritional and bioactive compounds such as vitamin C, folate, potassium, anthocyanins, quercetin, and ellagic acid that may play a significant role in preventing certain diseases as well as promoting health (CSC 2007). This leads to the steady increase in the production and consumption of strawberries in the United States during the last 3 decades (Bertelson 1995; Cook 2002). Strawberries may be consumed fresh or further processed into various forms as ingredients in jam, jelly, syrup, juice drinks, ice cream, yogurt, and bakery and confectionery products (ERS 2005; Flessa and others 2005).

Strawberries may be contaminated during harvesting, postharvest handling and processing, the most common pathogens is hepatitis A virus introduced via an application of contaminated irrigation water or by contact with infected food handlers (Dougherty and others 1965; Niu and others 1992; CDC 1997; Hutin and others 1999; Harris and others 2003; Notermans and others 2004). Natural reservoirs of enteric bacteria such as *Salmonella* and *Escherichia coli* O157:H7 are found with those of

hepatitis A virus and are spread by animal feces, while *Listeria monocytogenes* is widely spread in soil and plant matter. Hence, these pathogenic bacteria may contaminate strawberries that grow in close association with soil and harvested by hand (Brackett 1999; Harris and others 2003).

Strawberries have a delicate and complex surface structure. Once they become contaminated, decontamination is difficult (Flessa and others 2005). Moreover, strawberries are normally consumed fresh or added to products that do not receive thermal treatment after their addition (Flessa and others 2005). Foodborne pathogenic bacteria of public health concern such as *Salmonella* spp., *E. coli* O157:H7, *L. monocytogenes*, and *Shigella flexneri* have been reported to survive in fresh, fresh-cut, and frozen strawberries beyond their expected shelf-life (Knudsen and others 2001; Flessa and others 2002, 2005). These facts have raised the concern that there is a risk of illness from consumption of fresh and non-thermally processed strawberries. This has prompted the search for effective and practical technologies that can reduce contamination and increase safety to consumers.

Use of conventional disinfection treatments such as water or chlorine are not effective for reducing or eliminating both naturally occurring and pathogenic microorganisms associated with fresh strawberries (Gulati and others 2001; Yu and others 2001; Koseki and others 2004). Electrolyzed oxidizing (EO) water generated by an anodic electrolysis of a dilute saline solution has been reported to have a strong bactericidal activity against most pathogenic bacteria in vitro including *E. coli* O157:H7, *L. monocytogenes*, *Salmonella* spp., *Campylobacter jejuni*, *Vibrio* spp.,

Bacillus cereus (Venkitanarayanan 1999; Kim and others 2000a, 2000b; Park and others 2002; Fabrizio and Cutter 2003; Liu and others 2006; Ren and Su 2006). The application of EO water as a surface disinfectant has been extensively studied in various types of vegetables and sprouts with an equivalent or higher antimicrobial effect compared to traditional methods (Koseki and others 2001, 2004; Park and others 2001; Bari and others 2003; Sharma and Demirci 2003). Ozone is a naturally occurring strong oxidizing and disinfecting agent, and has been approved by the United States Food and Drug Administration (FDA) to apply as an antimicrobial agent in direct contact with foods for treatment, storage, and processing purposes (Khadre 2001). Ozone exhibits a potent bactericidal activity on most food-related pathogens (Broadwater and others 1973; Rastaino and others 1995; Kim and Yousef 2000; Selma and others 2006). The uses of ozone to disinfect various types of fresh produce has been investigated with varying results, suggesting that the efficacy of ozone must be individually assessed for each type of commodity (Kim and others 1999; Pérez and others 1999; Achen and Yousef 2001; Zhang and others 2005; Koseki and Isobe 2006; Selma and others 2006).

The objectives of this study were (1) to evaluate the antibacterial activity of EO water prepared from high and low sodium chloride (NaCl) concentrations against indigenous bacteria associated with fresh strawberries, (2) to compare the efficacy of EO water and other conventional disinfectants in controlling the survival and growth of *L. monocytogenes* and *E. coli* O157:H7 inoculated onto strawberries during refrigeration storage, and (3) to evaluate the antimicrobial effect of ozonated water

treatment against *L. monocytogenes* and *E. coli* O157:H7 inoculated onto strawberries and the ability of ozone to control survival and growth of both investigated pathogens and spoilage microorganisms during cold storage.

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CHAPTER 2

Literature Review

2.1 Strawberries

Strawberries have become a growing important part of the United States fresh fruit industry during past 20 years, and are now ranked as the second fruit crop after apples in value (Cook 2002). Bertelson (1995) reported that production of strawberries in the United States rose from 316 million kg in 1970 to 494 million kg in 1993. During that period, the per capita consumption of fresh strawberries increased from 850 g in 1970 to 1,750 g in 1992. Cook (2002) also reported that this value increased to 2,200 g in the year 2000.

Strawberries provide numerous benefits to consumer health. For instance, they are low in calories and a good source of many bioactive phytochemicals, iron, vitamin C, folic acid, fiber, potassium, and cancer-fighting antioxidants. Also, they are excellent in organoleptic quality. Nonetheless, strawberries may have a negative impact to human health as well. Besides biological hazard, strawberries may provide chemical and physical hazards such as allergens, pesticide residual, and filth or foreign matter like soil (Notermans and others 2004).

2.1.1 Microbial safety concerns of fresh strawberries

Many pathogenic microorganisms are ubiquitous in an environment and they can exist as native microflora in intestinal tracts of animal and human. Strawberries can become contaminated via infiltration of sewage water into field, contaminated irrigation water, presence of animals in the field, application of unsuitable composted organic fertilizers, contact with infected harvesters or commingling in processing facilities (Harris and others 2003; Notermans and others 2004).

The risk of foodborne disease outbreaks from consumption of fresh and processed strawberries has not commonly occurred; however, strawberries contaminated with hepatitis A virus and norwalk-like virus have been reported in outbreaks in the United States (Dougherty and others 1965; Niu and others 1992; CDC 1997; Hutin and others 1999; Notermans 2004). *Cyclospora cayetanensis* contamination in a mixture of blackberries, raspberries, and strawberries were considered as a causative agent in an outbreak occurred in Canada in 1999 (CDC 1998). Not only had the strawberry-associated outbreaks taken place in North America but also in Europe and Australia. More than 8,000 persons became ill after the consumption of hepatitis A contaminated frozen strawberries in the Czech Republic and Slovakia. In 2001, a *Salmonella* Typhimurium outbreak had been associated with a pastry-filled custard tart topped with strawberry jelly; however, the report did not confirm that strawberries were the vehicle of the infection (Notermans and others 2004). Examples of documented strawberry-associated outbreaks are summarized in Table 2.1.

Table 2.1 Reported foodborne outbreaks associated with consumption of strawberries (Data adopted from Harris and others 2003; Notermans and others 2004)

Pathogen	Year	Location	Suspected vehicle	No. of cases
Hepatitis A	1990	United States	Frozen strawberries	57
Hepatitis A	1997	United States	Frozen strawberries	256
Hepatitis A	1998	United states	Frozen strawberries	29
Hepatitis A	1998	United States	Strawberries, honey dew melon	41
Norwalk-like virus	1999	United States	Pasta salad, strawberries	63
Hepatitis A	2000	United States	Strawberries	8
C. cayetanensis	1999	Canada	Blackberries, raspberries, and strawberries	94
Hepatitis A	1997	Europe continent	Frozen creams with strawberries	> 8,000
S. Typhimurium	2001	Australia	Pastry-filled custard tart topped with strawberries in jelly	5

2.2 Foodborne pathogens of *Escherichia coli* O157:H7 and *Listeria monocytogenes*

Enteric bacteria such as *Salmonella* spp. and *E. coli* O157:H7 have similar natural reservoirs as hepatitis A virus, the most frequently reported causative agent for strawberry-associated outbreaks in the United States. This suggested that *E. coli* O157:H7 may be an occasional contaminant of strawberries (Knudsen and others 2001; Harris and others 2003). *L. monocytogenes* is widely spread in an environment especially in soil and plant matter suggesting that the presence of this pathogenic bacterium in plants grown in close association with soil is not uncommon (Brackett 1999a). This hypothesis was supported by the currently reported observation that *L. monocytogenes* was found on strawberry samples obtained from retail markets in Norway (Johannessen and others 2002). Strawberries are commonly consumed fresh

or added to other products that do not receive further heat treatment after their addition and previous studies which indicated that pathogenic bacteria such as *E. coli* O157:H7 and *L. monocytogenes* were able to survive in whole, fresh-cut , and frozen strawberries beyond the expected market shelf-life (Knudsen and others 2001; Flessa and others 2005) . All of these reasons suggest that there is a risk of illness from consumption of fresh or processed strawberries contaminated with both pathogens and effective and practical methods to reduce populations of *E. coli* O157:H7 and *L. monocytogenes* is desirable.

2.2.1 Escherichia coli O157:H7

2.2.1.1 General properties of *Escherichia coli* O157:H7

Escherichia coli, a short-rod shaped, Gram-negative facultative anaerobic bacterium, is one of the most commonly found bacterium in human and warm-blooded intestinal tracts. It is normally harmless to the host; however, there are some types of *E. coli* referred to as a diarrheagenic *E. coli* or commonly as pathogenic *E. coli* can cause disease in humans (Meng and others 2001; Feng and Weagant 2002). According to their unique virulence factors, pathogenic *E. coli* can be classified into enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAHE), and other groups that are not currently well characterized. Among of these, only ETEC *E. coli*, EPEC *E. coli*, EHEC *E. coli*, and

EIEC *E. coli* are mainly responsible for the occurrence of food or waterborne outbreaks (Feng and Weagant 2002).

The specific characteristic of EHEC is that it is capable of producing verotoxin or Shigatoxin (Stx). Hemorrhagic colitis (HC) or bloody diarrhea and hemolytic uremic syndrome (HUS) is caused by the production of Stx1 and Stx2. Although various serotypes are also able to produce Stx, only those that have been clinically associated with HC are specified as EHEC (Feng and Weagant 2002).

E. coli O157:H7 belongs to prototypic EHEC had involved in the outbreaks of disease worldwide (Feng and Weagant 2002). *E. coli* O157:H7 is a Gram-negative bacillus in which "O" refers to the somatic and "H" refers to the flagella, antigen (Griffin and Tauxe 1991; Buchanan and Doyle 1997). It has been the major cause of HC and HUS in the United Kingdom and the United States since more than 90% of EHEC strains isolated belongs to serotype O157:H7 or O157:H^- (Smith and others 1987; Karmali 1988). This pathogenic bacterium is one of the most important public health concerns since its infectious dose is as low as 10-100 cells (Feng and Weagant 2002).

The inability of *E. coli* O157:H7 to ferment sorbitol within 24 h as well as the lack of β -glucuronidase activity are the major characteristics used to differentiate *E. coli* O157:H7 from other *E. coli* groups and isolate this pathogen from foods (Wells and others 1983; Doyle and Schoeni 1984; Griffin and Tauxe 1991; Feng and Weagant 2002). This bacterium grows quickly between 30 – 42 °C with a poor growth at 44 – 45 °C and there is no growth within 48 h at 10 or 45.5 °C (Doyle and Schoeni 1984;

Raghubeer and Mathes 1990). Cytotoxicity assays on vero or HeLa tissue culture cells or commercially available ELISA or RPLA kits can be used for detecting the production of Stx1 and Stx2 (Feng and Weagant 2002).

2.2.1.2 Foodborne diseases associated with *Escherichia coli* O157:H7

E. coli O157:H7 was first identified as a human pathogen in 1982 after it had associated with 2 HC outbreaks occurred in Oregon and Michigan resulting from the consumption of fast food hamburgers (Doyle 1991; Abdul-Raouf and others 1993; Boyce and others 1995; Altekruse and others 1997; Buchanan and Doyle 1997). It is recognized as a common cause of bloody and non-bloody diarrhea in North America (Boyce and others 1995) and also known as the important cause of painful bloody diarrhea (hemorrhagic colitis) and renal failure (hemolytic uremic syndrome) in humans (Doyle 1991; Knabel 1995; Altekruse and others 1997; Kim and others 2000b). While most outbreaks had been involved with the consumption of undercooked ground beef, drinking raw and unpasteurized milk was also implicated as a cause of the illness though less frequently (Doyle 1991; Abdul-Raouf 1993; Conner and Kotrola 1995; Beuchat 1998). Dairy cattle, especially young animals, have been recognized as a major natural reservoir for *E. coli* O157:H7 (Doyle 1991; Abdul-Raouf 1993; Beuchat 1998). However, an apple cider product was also implicated as the vehicle of transmission in the 1991 outbreak of *E. coli* O157:H7 in Massachusesetts (Besser and others 1993; Conner and Kotrola 1995). Furthermore, *E. coli* O157:H7 was also identified as a waterborne bacterium since water was

considered as an infection source of a large community outbreak with approximately 240 cases in the United States in 1990 (Doyle 1991).

It is estimated that *E. coli* O157:H7 causes 20,000 cases of infection with 250 deaths each year in the United States (Council for Agricultural Science and Technology 1994; Boyce and others 1995). Furthermore, cases of human infection with *E. coli* O157:H7 have been reported from more than 30 countries on 6 continents. In Scotland, Canada, and the United States, the annual incidence rates of 8 per 100,000 people or greater have been reported (Griffin and Tauxe 1991).

2.2.2 *Listeria monocytogenes*

2.2.2.1 General properties of *Listeria monocytogenes*

The genus *Listeria* belongs to the *Clostridium* family with a specific phylogenetic position as low G+C DNA content (36 - 42%) (Swaminathan 2001). *L. monocytogenes* is one of 6 species in the genus *Listeria,* which includes *L. monocytogenes, L. ivanovii, L. innocua, L. seeligeri, L. welshimeri,* and *L. grayi* (Bille and others 1992; Rocourt 1999). *L. monocytogenes* and *L. ivanovii* are pathogenic for mice and other animals, but only *L. monocytogenes* is important as a causative agent for listeriosis in human (Swaminathan 2001; Hitchins 2002; Jay and others 2005).

L. monocytogenes is a short, rod-shaped, Gram-positive, non-spore forming bacterium. It can also appear coccoidal or filamentous in older culture (Rocourt 1999). This bacterium can grow in both aerobic and anaerobic conditions, but the microaerophilic environment is preferred (Ryser and Donnella 2001).

Multiplication of *Listeria* can occur at temperatures ranging from 1 - 45 °C (Junttila and others 1988) with the most optimum temperature range of 30 - 37 °C. Thus, *L. monocytogenes* is classified as psychrotrophic bacterium. The growth of *L. monocytogenes* occurs over the pH range of 4.1 to around 9.6 with maximum growth occurring at pH 6 - 8 (Jay and others 2005). Furthermore, it is one of a few of foodborne pathogens that can grow at a water activity (a_w) of 0.90 (Miller 1992; Jay and others 2005). It has the characteristic growth as an umbrella shape in tubed motility media when incubated at 25 °C, but not at 35 °C and tumbling motility can be seen in wet mounts (Ryser and Donnella 2001). Hemolysis is useful for differentiating *L. monocytognes* (hemolytic and pathogenic) from *L. innocua* (nonhemolytic and nonpathogenic), the two species most frequently isolated from foods (Bille and others 1992; Swaminathan 2001). The CAMP (Christie-Atkins-Munch-Peterson) test is useful for presumptive isolation of *L. monocytogenes* which gives CAMP positive with *Staphylococcus aureus* and CAMP negative with *Rhodococcus equi* except for some rare strains of *L. monocytogenes* that have a CAMP positive result with *R. equi* (Hitchins 2002).

2.2.2.2 Foodborne diseases associated with *Listeria monocytogenes*

L. monocytogenes was well recognized as a cause of meningtitis and parinatal septicemia in the United States since 1935 (Ryser and Donnelly 2001), but its role as causative agent for foodborne disease was just identified in the 1980s after the first documented of listeriosis outbreaks in Canada associated with consumption of

contaminated coleslaw (Knabel 1995; Schelech 1996; Ryser and Donnelly 2001; Swaminathan 2001). Its ability to survive and grow at refrigeration temperature has caused public concern about the risk of outbreak from consumption of refrigerated foods (George and others 1988; Beuchat 1992). Moreover, *L. monocytogenes* is able to develop impenetrable biofilms on processing equipment which are resistant to decontamination by various cleaning chemicals, antibiotics, heat, light, and drying (Hsu and others 2004).

Unlike most other foodborne pathogens, *L. monocytogenes* is ubiquitous in nature especially in soil, water, animal faces, sewage, silage, and decayed plant materials. It survives well in adverse environmental conditions including low pH, and high NaCl concentration as well as its ability to grow and survive in a wide range of temperature. These allow the bacterium to survive for a long period in both food products and food processing plants (Fenlon 1999; Swaminathan 2001). Listeriosis is now one of the most important foodborne illnesses of public health concern due to the severity of the symptom as well as the high mortality rate (approximately 20-30% of cases), a long incubation period range from a few days to 3 weeks, and its pathogenicity to susceptible individuals (Gellin and Broome 1989; FDA 1992; Swaminathan 2001). The United States Department of Agriculture (USDA) estimated that *L. monocytogenes* infections cause 2,500 cases of listeriosis with nearly 2,300 hospitalization and 500 deaths in the United States each year (FSIS 2000; CDC 2001).

Any food product of animal or plant origin may harbor *L. monocytogenes* in varying numbers as observed by the varieties of foods that have been documented as

vehicles of transmission for listeriosis outbreaks including coleslaw, pate, pork tongue in jelly, raw milk, milk contaminated after pasteurization, soft cheese made with inadequately pasteurized milk, unpasteurized queso fresco, ice cream, raw meat, raw vegetables, raw and smoked fish, shellfish, undercooked chicken and uncooked hotdogs (FDA 1992; Schlech 1996; Mead and others 1999; Jay and others 2005). Jay and others (2005) reported that foods implicated as a source of human listeriosis typically contain *L. monocytogenes* more than 1,000 CFU/g or ml.

Owing to the high severity and case-fatality rate of the disease, the 'zero tolerance' policy for *L. monocytogenes* in foods has been in placed in the United States since 1989 by the USDA and the FDA (Schlech 1996; Altekruse and others 1997). *L. monocytogenes* was designated as an adulterant which means its presence in any ready-to-eat food is prohibited and those products are subjected to recall and/or seizure (Jay and others 2005).

2.3. Chemical disinfectants

The incidences of numerous disease outbreaks from consumption of fresh fruit and vegetables contaminated with foodborne microorganisms have prompted requirements to improve methods for surface decontamination of fresh produce (Beuchat 1996, 1998; Parish and others 2003, Sivapalasingam and others 2004). A variety of methods have been studied to reduce populations of microorganisms including biological control, irradiation, natural antimicrobial compounds, and the application of chemical treatments such as chlorine and chlorine containing

compound, ozone, hydrogen peroxide, organic acid, and trisodium phosphate. Table 2.2 summarizes various documented methods used to decontaminate fresh strawberries. Chlorine is still the most widely used sanitizer in produce industry (Beuchat 1998; Parish and others 2003), and is discussed in the following sections.

Table 2.2 Microbial decontamination on fresh strawberries by various chemical disinfectants

Treatment	Microorganisms	Concentration	Exposure time	Other conditions	Log reduction	Reference
ClO_2 (gaseous)	*Salmonella* spp.	100 mg	60 min	Ambient temp	> 4.76 $\log_{10}$ CFU/fruit	Yuk and others (2006)
ClO_2 (gaseous)	*Salmonella* spp.	8 ppm	120 min	85 - 88% RH, 23 ± 1 °C	3.76 $\log_{10}$ CFU/g	Sy and others (2005)
ClO_2 (gaseous)	Yeasts and molds	8 ppm	120 min	85 - 88% RH, 23 ± 1 °C	4.16 $\log_{10}$ CFU/g	Sy and others (2005)
NaOCl solution	Aerobic mesophiles	150 ppm free chlorine	10 min	Ambient temp	0.9 $\log_{10}$ CFU/fruit	Koseki and others (2004)
NaOCl solution	Fungi	150 ppm free chlorine	10 min	Ambient temp	1.7 $\log_{10}$ CFU/fruit	Koseki and others (2004)
NaOCl solution	*E. coli* O157:H7	65 ppm free chlorine	1 min	23 °C	1.3 $\log_{10}$ CFU/g	Yu and others (2001)
NaOCl solution	*E. coli* O157:H7	130 ppm free chlorine	1 min	23 °C	1.3 $\log_{10}$ CFU/g	Yu and others (2001)
NaOCl solution	*E. coli* O157:H7	1,300 ppm free chorine	1 min	23 °C	1.7 $\log_{10}$ CFU/g	Yu and others (2001)
Acetic acid	*E. coli* O157:H7	2% (v/v)	1 min	23 °C	1.6 $\log_{10}$ CFU/g	Yu and others (2001)
Acetic acid	*E. coli* O157:H7	5% (v/v)	1 min	23 °C	1.6 $\log_{10}$ CFU/g	Yu and others (2001)
Sodium phosphate	*E. coli* O157:H7	2% (w/v)	1 min	23 °C	1.6 $\log_{10}$ CFU/g	Yu and others (2001)
Sodium phosphate	*E. coli* O157:H7	5% (w/v)	1 min	23 °C	1.6 - 1.9 $\log_{10}$ CFU/g	Yu and others (2001)
Hydrogen peroxide	*E. coli* O157:H7	1% (v/v)	1 min	23 °C	1.2 - 1.4 $\log_{10}$ CFU/g	Yu and others (2001)

Table 2.2 (Continued)

Treatment	Microorganisms	Concentration	Exposure time	Other conditions	Log reduction	Reference
Hydrogen peroxide	*E. coli* O157:H7	3% (v/v)	1 min	23 °C	2.2 $\log_{10}$ CFU/g	Yu and others (2001)
Peroxyacetic acid	*E. coli* O157:H7	80 ppm	5 min	21 - 23 °C	> 4.9 $\log_{10}$ CFU/g	Rodgers and others (2004)
Chlorinated trisodium phosphate	*E. coli* O157:H7	100 ppm free chlorine	5 min	21 - 23 °C	> 4.9 $\log_{10}$ CFU/g	Rodgers and others (2004)
Chlorinated trisodium phosphate	*E. coli* O157:H7	200 ppm free chlorine	5 min	21 - 23 °C	> 5.0 $\log_{10}$ CFU/g	Rodgers and others (2004)
ClO_2 (aqueous)	*E. coli* O157:H7	3 ppm free chlorine	5 min	21 - 23 °C	> 5.1 $\log_{10}$ CFU/g	Rodgers and others (2004)
ClO_2 (aqueous)	*E. coli* O157:H7	5 ppm free chlorine	5 min	21 - 23 °C	> 4.9 $\log_{10}$ CFU/g	Rodgers and others (2004)
Peroxyacetic acid	*L. monocytogenes*	80 ppm	5 min	21 - 23 °C	> 4.9 $\log_{10}$ CFU/g	Rodgers and others (2004)
Chlorinated trisodium phosphate	*L. monocytogenes*	100 ppm free chlorine	5 min	21 - 23 °C	> 4.8 $\log_{10}$ CFU/g	Rodgers and others (2004)
Chlorinated trisodium phosphate	*L. monocytogenes*	200 ppm free chlorine	5 min	21 - 23 °C	> 5.0 $\log_{10}$ CFU/g	Rodgers and others (2004)
ClO_2 (aqueous)	*L. monocytogenes*	3 ppm free chlorine	5 min	21 - 23 °C	> 4.8 $\log_{10}$ CFU/g	Rodgers and others (2004)
ClO_2 (aqueous)	*L. monocytogenes*	5 ppm free chlorine	5 min	21 - 23 °C	> 4.9 $\log_{10}$ CFU/g	Rodgers and others (2004)

2.3.1 Chlorine

Chlorine has been used for treating drinking water and wastewater as well as a sanitizer in food processing for many years (Beuchat 1998; Parish and others 2003). Because it is highly effective and inexpensive, it is still the most widely used sanitizer in food industry (NTP 1992; Parish and others 2003; Ayebah and others 2006). Chlorine and chlorine-based compounds are frequently applied as wash, spray, and flume waters to sanitize fruit and vegetables and to reduce microbial loads in water used in washing and packing operations (Beuchat 1998; Suslow 2001; Parish and others 2003).

Chlorine is commercially available in various forms with different antimicrobial activities (Kim and others 2000a) in which liquid chlorine and hypochlorite are most commonly used in food industry (Izumi 1999; Suslow 2001; Parish and others 2003; Sapers 2006). Use level of chlorine is dependent on allowable levels, commodity type, and the anticipated microbial load (Sapers 2006). Water containing 50-200 ppm available chlorine is commonly used as a sanitizer to sanitize produce surfaces and processing equipment with the exposure time of 1 – 2 min (Beuchat 1998; Parish and others 2003; Rodgers and others 2004; Sapers 2006). The United States Food and Drug Administration specified an allowable level for washing fruit and vegetables not to exceed 0.2% when followed by a potable water rinse (CFR 2003). Nonetheless, a concentration of up to 20,000 ppm in the form of calcium hypochlorite was approved for washing alfalfa seeds destined for sprout production (Cherry 1999; NACMCF 1999; Rodgers and others 2004).

Chlorine concentration in wash water can be expressed in terms of total available chlorine which is defined as a combination of combined residual chlorine and free residual chlorine present in the solution. It can also be expressed as free available chlorine which is referred to as the sum of elemental chlorine (Cl_2), hypochlorous acid (HOCl), and hypochlorite ions (OCl^-). Total and free available chlorine in wash water can be determined by commercially available test kits based on colorimetry or by measurement of oxidation-reduction potential (ORP) (Beuchat 1992; Sapers 2006).

Hypochlorous acid is produced when chlorine gas or hypochlorite solution are added into water as described by the following reactions (Beuchat 1992; Eifert and Sanglay 2002).

$$Cl_2 + H_2O \longrightarrow HOCl + H^+ + Cl^-$$

$$NaOCl + H_2O \longrightarrow HOCl + NaOH$$

Hypochlorous acid is identified as the most active antimicrobial component and responsible for the bactericidal activity of chlorinated water (Izumi 1999, Eifert and Sanglay 2002; Parish and others 2003). Its efficacy as a sanitizing agent is 80 times more effective than an equivalent concentration of OCl^- (Kim and others 2000a; Eifert and Sanglay 2002). Thus, the amount of HOCl present in water is one of the important factors affecting the antimicrobial activity of chlorine. In water, HOCl can further dissociate to produce a hydrogen ion (H^+) and OCl^- and the equilibrium between HOCl and OCl^- is pH dependent (Eifert and Sanglay 2002; Parish and others

2003). Sapers (2006) suggested that at pH 6.0, approximately 97% of free available chlorine is in the form of HOCl, whereas, at pH 9.0, 97% of free chlorine will be present as OCl^-.

Although chlorine has a broad spectrum in inactivating microorganisms including bacteria, some spore forming bacteria, yeast, mold and virus, it has a limited efficacy when used as a sanitizer for raw produce. Chlorine is highly reactive with leaves, soil, and plant matter; thus, reducing the amount of active chlorine compound in wash water rapidly (Suslow 1997; Sapers 2006). The limited disinfection effect of chlorine on fresh produce may be also attributed to the readily neutralization of chlorine in contact with organic components leaching from tissues of cut produce surfaces before it reaches the microbial cells embedded in tissues, cracks, and crevices of fruit and vegetables (Parish and others 2003).

Adam and others (1989) suggested that water containing 100 ppm free available chlorine should be applied as a working concentration since the elevated level of chlorine could cause product tainting and equipment corrosion. Moreover, application of chlorine compounds even at low concentration may lead to a deleterious effect on sensory quality of the treated products (Kim and others 1999b). However, the effectiveness of chlorine as a disinfectant is rapidly lost on contact with organic matter or exposure to air, light, or metals and failure to maintain the sufficient concentration of active chlorine in wash water may result in transfer of pathogenic microorganisms from process water to produce or from infected produce to healthy ones. Hence, wash water should be monitored periodically and fresh chlorinated

water should be added if necessary in order to maintain the sufficient concentration of active HOCl (Beuchat 1992, 1998; Ayebah and others 2006). Another concern about applying chlorine or chlorine-containing compound as a disinfecting agent is that the prolonged exposure to chlorine vapors can result in the irritation to the skin and respiratory tract of workers (Beuchat 1998). Yet another disadvantage of using chlorine is its reaction with naturally occurring organic material forming chlorinated organic compounds. These disinfection byproducts found in United States drinking water include trihalomethanes, haloacetic acid, haloketones, and haloacetonitriles (NTP 1992). Trihalomethanes were reported to be a cause of tumors in rodents and linked to a higher rate of cancer, and classified as possible human carcinogens by the United States Environmental Protection Agency (EPA) (NTP 1992; Kim and others 2000b).

2.3.2 Antimicrobial mechanism of chlorine

Although the exact mode of action of chlorine and chlorine-containing compounds against microorganisms is not fully understood, theories have been advanced by researchers (Beuchat 1992). In one theory, Green and Stumpf (1946) hypothesized that HOCl killed bacterial cells by inhibiting glucose oxidation through chlorine-oxidizing sulhydryl groups of certain enzymes which are important in carbohydrate metabolism. Due to the essential nature of aldolase in metabolism, it is considered to be the major site of action. Beuchat (1992) suggested that N-chloro

compounds, which are the product from the combination of chlorine and cell membrane proteins, will interfere with the cell metabolism.

2.4 Newly developed disinfection technologies

Although the best method to eliminate pathogens from fresh produce is to prevent contamination in the first place, this is sometimes difficult or impractical in the real situation. Hence, decontamination practices to remove and inactivate microorganisms attached on fruit and vegetable surfaces are required (Parish and others 2003). Since conventional methods such as washing with chlorinated water has limited efficacy as well as presents a negative impact to processing equipment, products being treated, and human health, several intervention alternatives to chlorine have been investigated (Beuchat 1998; Parish and others 2003; Guan and others 2006). The EO water is considered as a novel chlorination technology, and ozone is the naturally occurring disinfecting agent. These two disinfection technologies are actively being investigated for their potential uses in fruit and vegetable industries.

2.4.1 Electrolyzed oxidizing water (EO water)

Electrolyzed water is a novel disinfection product developed in Japan (Venkitanarayanan and others 1999a, 1999b; Bari and others 2003, Fabrizio and Cutter 2003; Al-Haq and Sugiyama 2004). However, the first demonstration occurred in Russia in the 1970s (Jay and others 2005). It has recently gained much interest as a promising non-thermal food sanitation technique applied in medicine, agriculture, and

food processing (Al-Haq and Sugiyama 2004; Yoshida and others 2004; Guan and others 2006).

Previous studies have shown that water collected from the anode side during the electrolysis of a dilute sodium chloride solution possessed strong bactericidal, fungicidal, and virucidal properties (Venkitanarayanan and others 1999a, 1999b; Morita and others 2000; Al-Haq and others 2002; Buck and others 2002; Al-Haq and Sugiyama 2004; Huang and others 2006; Liu and others 2006). Therefore, it has been used for treating wounds or disinfecting medical equipment (Venkitanarayanan and others 1999a). In 2002, EO water was approved by the Health, Labor and Welfare Ministry as an indirect food additive (bactericidal agent) in Japan (Yoshida and others 2004).

2.4.1.1 Generation of EO water

The EO water is generated by passing a dilute salt solution through a cell containing both inert positively charged (anode) and negatively charged (cathode) electrodes, separated by a membrane. When these electrodes are subjected to a direct current voltage, hydroxide ions and chloride ions, the negatively charged ions in the saline solution, move to the anode side, in with each ion releasing an electron to become a radical. Chloric and hydroxyl radicals then combine together to form HOCl, whereas the combination of two chloric radicals produces Cl_2 . Moreover, oxygen, OCl^-, and hydrochloric acid are also separated from the anode side. During electrolysis, hydrogen and sodium ions, the positively charged ions, move to the

cathode side to take up electrons, and form hydrogen gas and sodium hydroxide (Venkitanarayanan and others 1999a; Hsu 2003, 2005). From the reactions that occur, there are two types of water with the different characteristics generated. An electrolyzed reducing solution possessing pH >11 and oxidation-reduction potential (ORP) < -800 mV is produced from the cathode compartment, while the electrolyzed oxidizing solution (EO water) possessing pH < 2.8 and ORP >1,100 mV along with the presence of HOCl is produced from the anode compartment (Hsu 2003). Electrolyzed reducing water has a high reducing potential in which may provide benefits in reducing free radicals in biological system as well as treating organ malfunction (Kim and others 2000b). Koseki and others (2004) suggested that electrolyzed reducing water acted like a surfactant to reduce the hydrophobicity on the surface of fresh produce being treated; therefore, the bactericidal capability of EO water may be enhanced if electrolyzed reducing water was applied as a prewashing solution. In addition, one study indicated a strong inhibiting effect on lipid oxidations by electrolyzed reducing water (Miyashita and others 1999).

2.4.1.2 Properties of EO water

Since EO water is produced by simple electrolysis of sodium chorine containing water, the need for handling, transportation, and storage of high concentrations of hazardous chemicals is eliminated (Koseki and others 2004; Ayebah and others 2006; Liao and others 2007). This may limit adverse impacts on the environment (Kim and others 2000a; Koseki and others 2002). Al-Haq and Sugiyama

(2004) suggested that the generation of EO water can be modified to reduce the available chlorine concentration while still maintaining the antimicrobial effectiveness of EO water; therefore, health concerns about the negative impact of chlorination is declined. Moreover, EO water reverts back to normal water after application without release of great amounts of harmful gases such as chlorine gas. Finally, the application of EO water provides economical benefits. After the initial investment of an electrolysis apparatus, the operational expenses are minimal. Grech and Rijkenberg (1992) estimated costs of various sources of chlorination and reported that the unit cost per kilogram of chlorine (100% free available) production was $1.60 for liquefied chlorine gas, $2.70 for NaOCl solution (15% w/w), $2.87 for dry calcium hypochlorite (70% w/v), and 34 cents for electrically generated chlorine.

However, the application of EO water as a sanitizing agent may have some negative impacts. The bactericidal activity of EO water is reduced over storage time due to loss of chlorine (Koseki and Itoh 2001). Since EO water contains free chlorine, it may be phytotoxic to plant and may damage plant tissues (Grech and Rijkenberg 1992; Schubert and others 1995). The presence of chlorine gas from water electrolysis may cause discomfort to the operator (Al-Haq and Sugiyama 2004).

2.4.1.3 Antimicrobial mechanism of EO water

So far, the antimicrobial mechanisms of EO water have not been completely clarified (Park and others 2002a). However, some researchers believe that the aggregation of three distinct physicochemical characteristics of EO water including

high ORP, low pH, and presence of free chlorine, contribute to its effectiveness against microorganisms (Venkitanarayanan and others 1999a, 1999b; Liu and others 2006). Len and others (2000) suggested that residual chlorine present in the form of HOCl was the primary bactericidal agent in EO water and there was a strong correlation between HOCl concentration and the bactericidal activity of EO water. Furthermore, morphological changes of *Pseudomonas aeruginosa* exposed to EO water were observed by transmission electron microscopy. Kiura and others (2002) observed breaks and blebs formed on the outer membrane of bacteria and contact with EO water containing higher free chlorine concentration increased the number of breaks and blebs created in bacterial cells.

The oxidation reduction potential (ORP) of a solution is defined as the ability to oxidize or reduce. The higher and positive ORP values indicate a greater oxidizing potential (Venkitanarayanan and others 1999a, 1999b; Park and others 2004). Jay and others (2005) also defined that ORP is the ability to gain or lose electron and each group of bacteria requires certain range of ORP to growth. The optimum ORP for the growth of aerobic bacteria is in a range of 200 - 800 mV, whereas anaerobic bacteria are grown in a range of -200 to -400 mV. Previous studies suggested that the lethal effect of EO water was primarily due to the extreme ORP rather than other factor such as the presence of HOCl (Fabrizio and Cutter 2003; Jay and others 2005; Liao and others 2007). Fabrizio and Cutter (2003) explained that the antimicrobial mechanism of ORP is when bacterial cells exposed to extremely high or low ORPs, their cellular membrane become unstable, and then facilitate the penetration of antimicrobial agents

to disturb metabolic process. Kim and others (2000a, 2000b) also supported that ORP may play the most important role in microbial inactivation.

However, there also have other theories developed for explaining the microbicidal activity of EO water as a result from its low pH generated. Venkitanarayanan and others (1999a, 1999b) hypothesized that the outer a membrane of the bacterial cell is sensitized by the low pH in EO water, thereby facilitating the penetration of active antimicrobial compound into bacterial cells. Len and others (2000) also supported that pH played an important role in enhancing the decontamination capability of EO water since pH is involved in the change of the relative distribution of chlorine species in the EO water. According to their study, EO water adjusted to pH 4 provided the maximum concentration of HOCl and gave the highest log reduction of *Bacillus cereus* F4431/73.

2.4.1.4 Application of EO water in fruit and vegetables

The EO water had been used extensively as a disinfecting agent for fresh and minimally-processed fruit and vegetables based on its strong bactericidal effect (Venkitanarayanan and others 1999a; Al-Haq and Sugiyama 2004). Izumi (1999) observed that rinsing various fresh-cut vegetables with EO water (pH 6.8) containing 20 ppm free available chlorine for 3 min can decrease the total microbial count by 0.8 to 2.1 $\log_{10}$ CFU/g. Moreover, the disinfection activity of EO water increased with available chlorine in the ranges of 15- 50 ppm. Tissue pH, surface color, and overall appearance of fresh-cut vegetables were not affected by treatment with EO water.

Park and others (2001) examined the efficacy of EO water and acidified chlorinated water containing 45 ppm free available chlorine against *E. coli* O157:H7 and *L. monocytogenes* on lettuce. Rinsing lettuce leaf with EO water up to 3 min at 22 °C significantly reduced populations of *E. coli* O157:H7 and *L. monocytogenes* by 2.41 and 2.65 $\log_{10}$ CFU/g, respectively as compared to water wash only. However, the antibacterial capacity of EO water and acidified chlorinated water was comparable. Changes in a quality of treated lettuce exposed to different sanitizers during 2 weeks storage were not obvious.

The EO water (pH 2.6, ORP 1,140 mV, and 30.3 ppm available chlorine) was reported to have a very strong inactivation effect against *Salmonella* Enteritidis, *E. coli* O157:H7, and *L. monocytogenes* inoculated onto the surfaces of fresh whole tomatoes. After a 40 s treatment with EO water, populations of these pathogenic bacteria were reduced by 7.85, 7.46, and 7.54 $\log_{10}$ CFU/tomato, respectively. No significant change on taste, color, and appearance of tomatoes treated with EO water and stored for 6 hr at room condition was observed (Bari and others 2003).

In addition to the inactivation of spoilage or pathogenic bacteria on fresh produce, EO water may also be used as an effective fungicide on fruit (Guan and Hoover 2006). Brown rot caused by *Monilinia fructicola* is one of the important destructive diseases occurred on stone fruit (DeVries-Peterson and others 1991). Al-Haq and others (2001) reported that immersion of intact peachs inoculated with *Monilinia fructicola* in EO water for 5 min yield the greatest reduction in the incidence and severity of the disease. Peachs treated with EO water and stored at 2 °C

and 50% relative humidity up to 8 d did not develop brown rot until treated fruits were transferred and held at 20 °C with 95% relative humidity. No chlorine-induced phytotoxicity was observed on the treated fruit. The disinfection effect of EO water was also evaluated on wounded pears inoculated with *Botryosphaeria berengeriana* spores. The EO water was able to suppress the incidence and severity of Bot rot with the maximum efficiency observed on fruit immersed in EO water for 10 min (Al-Haq and others 2002). Results from this study suggested that EO water can be used as surface sanitizer to possibly reduce postharvest fungal rot development.

In addition to fresh produce, EO water was successfully applied as a disinfectant on other food products including seafood, meat, poultry, eggs, and rice. The EO water also exhibited a strong efficacy in inactivating disease causing bacteria contaminated on various food contact surfaces including plastic kitchen cutting boards. Moreover, it had been proved to prevent cross contamination from food preparation surface (Venkitanarayanan and others 1999b; Park and others 2002b; Fabrizio and Cutter 2005; Isobe and others 2005; Park and others 2005; Liu and others 2006; Ozer and Demirci 2006). .

2.4.2 Ozone

Ozone (O_3), which is an unstable allotropic form of gaseous oxygen, is comprised of 3 atoms of oxygen combined together to form the molecule O_3. It is considered as a naturally occurring strong oxidizing and disinfecting agent (Xu 1999; WQA 2000). The first production and characterization of ozone was performed by

C.F. Schonbein, a German scientist, in 1840 (EPRI 1997). Ozone is formed naturally in the upper atmosphere by ultraviolet light and by atmospheric electrical discharges. It has a boiling point of -111.9 ± 0.3 °C and a melting point of -192.5 ± 0.4 °C at 1 atm. Ozone is the second most powerful common oxidizing agent with ORP of 2.07 V (WQA 2000;Guzel-Seydim and others 2004). It is nearly colorless at ambient temperature with a pungent odor referring as "fresh air after thunderstorm" (Coke 1993). Its detectable limit by most people is at about 0.01 or 0.02 ppm. Ozone is in the gaseous state at ambient and refrigerated temperature and slightly dissolves in water at approximately 0.88 volumes per 100 volumes (EPRI 1997).

2.4.2.1 Generation of ozone

The general principle for ozone generation was described by Rice and others (1981). The first step is to spilt oxygen molecules providing free radical oxygen atoms which are free to react with diatomic oxygen. Then, the triatomic ozone molecules are generated. Meanwhile, a great amount of energy in the forms of radiation, electricity, or heat is required to break the O-O bond (Rice and others 1981; Greene and others 1993). Ultraviolet radiation and corona discharge are the two major commercial methods normally used for ozone generation (WQA 2000). Ozone is usually generated at the point of use and in closed systems (Kim 1998). The ultraviolet (UV) generator produces ozone at low concentration (0.03 ppm) from oxygen in the air by radiation at the wavelength of 185 nm (Ewell 1946). This method is quite simple, economical, but limited in output capacity with a maximum

concentration of ozone being produced is about 0.10% (w/w) (WQA 2000). For larger amounts of ozone production, corona discharge (CD) generators are required (Graham 1997; Kim 1998; WQA 2000). When a high voltage current is applied across the discharge gap by which air or oxygen is passed through, oxygen electrons are excited leading to the split of oxygen molecules. Oxygen atoms combine with other oxygen molecules to form ozone molecules. The production of ozone is dependent on voltage, current frequency, dielectric material property and thickness, discharge gap, and an absolute pressure inside the discharge gap. Heat removal system is necessary in order to optimize ozone generation and up to 4% (w/w) of ozone concentration can be produced (Rosen 1972). Kim (1998) suggested that ozone can also be produced by other technologies including chemical, thermal, chemonuclear, electrolytic methods, and electrochemical methods.

2.4.2.2 Stability and other functional properties of ozone

The application of ozone as a disinfecting agent provides numerous benefits. Unlike chlorine, the reaction of ozone with organic matter does not provide any toxic disinfection by-products such as thihalomethanes and haloacetic acids (Bott 1991; EPRI 1997; WQA 2000). Moreover, the half-life of ozone in water at room temperature is only about 20 - 30 min depending on temperature, pH, and amount of ozone demanding substances present in water (WQA 2000) before it decomposes back into oxygen. Therefore, no ozone residual remains in the environment for a long period of time and the concern about consumption of residual ozone in food products

after its application is eliminated (Bott 1991; Graham 1997; Khadre and others 2001). Temperature strongly affects ozone autodecomposition rate back to oxygen. At 35 °C, the forward and reverse reactions of the following are in equilibrium (Graham 1997).

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

Kim (1998) suggested that pH had a great effect on the stability of ozone in aqueous solution. At pH 8.0, the half-life of dissolved ozone was approximately 5 min comparing with about 20 min at pH 6.0 (WQA 2000). The amount of ozone demanding substances present in water being ozonized is the other critical factor impacting on the stability of ozone. Half-life of molecular ozone can be as short as seconds in dirty water or wastewater, whereas in clean water the half-life may be as long as an hour (Graham 1997).

Since the reaction of ozone with organic substances is 3,000 times faster than that of chlorine, there is a significant reduction in treatment time as well as elimination of the holding tank requirement (EPRI 1997). Additionally, ozonated wash water could also be recaptured and treated by a combination of ozonation and filtration after treatment. This treated water can be recycled in the washing system to decrease the amount of water usage (Xu 1999).

However, there are also disadvantages of using ozone as a disinfecting agent. Due to its short half-life, ozone needs to be generated on-site, and cannot be produced and transported from a central production plant (Bott 1991; EPRI 1997). Another drawback is the corrosive effect of ozone against many contact materials such as copper, rubber, and some kinds of plastic under certain condition (Bott 1991).

Furthermore, human exposure to ozone at relatively high concentration for a sufficient duration causes acute symptoms such as watery eyes, tightness in the chest, shortness of breath, irritated throat, and headache (Xu 1999). The occupational Safety and Health Administration (OSHA) limits the concentration of ozone use in the atmosphere workplace at 0.1 ppm for 8 h period. For a shorter period of exposure, the safety limit is 0.3 ppm for 15 min (EPRI 1997).

2.4.2.3 Antimicrobial mechanism of ozone

The mechanisms of microbial inactivation by ozone is complicated since ozone attacks numerous cellular constitutes including proteins, unsaturated lipids, and respiratory enzymes in cell membranes, peptidoglycans in cell envelopes, enzymes and nucleic acids in the cytoplasm, as well as proteins and peptidoglycans in spore coats and virus capsids (Khadre and others 2001). Some studies reported that molecular ozone is the primary inhibitor responsible for inactivation of microorganisms. Other studies indicated that numerous active free radicals such as $\cdot OH$, $\cdot O_2$, and $HO_3^{\cdot}$ produced from the decomposition of ozone in water are the active sanitizing agents for killing bacteria (Chang 1971; Harakeh and Butler 1985; Glaze and Kang 1989; Bablon and others 1991; Hunt and Marinas 1997). Even though these free radicals are more powerful oxidants than molecular ozone, their half-life is extremely short in the range of microseconds. Graham (1997) concluded that the presence of free radicals was not significant in controlling microbes.

Numerous components of cell envelopes are oxidized by exposing to ozone including polyunsaturated fatty acids, membrane-bound enzymes, glycoproteins and glycolipids. These result in the leakage of cell constituents and subsequently causing lysis (Murray and others 1965; Khadre and others 2001). Ozone oxidizes double bonds of unsaturated lipids as well as the sulfhydryl groups of enzymes and leads to the disruption of normal cellular activity such as cell permeability. Microbial cells are then inactivated (Khadre 2001). Nevertheless, much research has suggested the microbicidal action of ozone is via the inhibition of enzymes. Chang (1971) suggested that enzymes inactivation by ozone was probably due to the oxidation of sulfhydryl groups in cysteine residues. Ozone may damage nucleic materials inside microbial cells by chemical modification of nucleic acids. In vitro experiments demonstrated that thymine is more sensitive than cytosine and uracil (Scott 1975; Ishizaki and others 1981).

2.4.2.4 Regulation of ozone used as an antimicrobial agent

The first use of ozone as disinfecting agent for water treatment was date back to 1893 in the Netherlands (EPRI 1997). It was not until 1906 that the first commercial-scale ozone treatment for potable water came into practice in France (EPRI 1997; Graham 1997). Since then, it has been the primary sanitizer for disinfecting public water supply systems in Europe (Gomella 1972; Graham 1997). In the United States, ozone was first introduced as water disinfectant in 1940 (Graham 1997).

Ozone was affirmed as generally recognized as safe (GRAS) by the FDA in 1982, with specific limitations, for use as a disinfecting agent in bottled water in accordance with the good manufacturing practices (FDA 1982, Graham 1997). Meanwhile, it was not until 1997 that USDA approved ozone to be applied for reconditioning recycled poultry chilling water (Guzel-Seydim and others 2004). In the same year, it was self affirmed as a GRAS disinfectant for foods by petition of an expert panel (EPRI 1997; Graham 1997; Guzel-Seydim and others 2004; CFR 2006). Ozone in gas and aqueous phases were approved by FDA to use as an antimicrobial agent in direct contact with foods including fresh and minimally fruits and vegetables for treatment, storage, and processing in 2001 (Khadre and others 2001)

2.4.2.5 Application of ozone in fruit and vegetables

Ozone has less of an antimicrobial effect when applied on food surfaces than in low ozone-demand liquid media. Kim and others (1999a) postulated that the natural microflora inactivation by ozone on food products is dependent on the nature and composition of food surfaces, types of microbial contaminant, and the degree of attachment or association of microorganisms with food.

The effect of ozonated water treatment on microbiological and sensory qualities of fresh-cut celery was evaluated during storage at 4 °C. Washing with 0.18 ppm of ozonated water was successful in reducing populations of spoilage microorganisms, as well as maintaining sensory quality of fresh-cut celery up to 9 d in cold storage (Zhang and others 2005). Koseki and others (2004) reported that 5 ppm

of ozonated water treatment for 10 min was capable of reducing the number of naturally present aerobic mesophiles, coliform bacteria, and fungi on cucumber by 0.7, 1.5, and 0.8 $\log_{10}$ CFU/ cucumber, respectively. The same treatment was applied to strawberries, the native aerobic mesophilic bacteria and fungi were decreased by 0.4 and 0.9 $\log_{10}$ CFU/strawberry, respectively.

Ozonated water at a concentration of 3, 5, and 10 ppm residual ozone was used to treat iceberge lettuce for 5 min at ambient temperature. The maximum reduction of aerobic mesophiles observed was 1.4 $\log_{10}$ CFU/g. Furthermore, the combination treatment of hot water (50 °C, 2.5 min) followed by ozonated water (5 ppm, 2.5 min) was also studied on their antimicrobial effect on iceberg lettuce and resulted in 1.4 $\log_{10}$ CFU/g reduction on naturally present bacteria. However, the strong oxidizing property of ozone promoted the onset and progression of browning on treated lettuce (Koseki and Isobe 2006). Results obtained from the study of Kim and others (1999b) demonstrated that bubbling gaseous ozone (4.9% v/v; 0.5 l/min) to a lettuce-water mixture led to a 1.5-1.9 $\log_{10}$ CFU/g reduction of microbial contaminants within 5 min. In this study, various mechanical actions including sonication, stirring, and stomaching were also tested to enhance the microbicidal effect of ozone during treatment. The most efficient ozone delivery method was reported to be bubbled ozone on lettuce-water mixture along with high-speed stirring.

Potatoes inoculated with *Yersinia enterocolitica* were washed with ozonated water (5 ppm) for various contact times. The results revealed that the highest reductions of 1.6 $\log_{10}$ CFU/g were obtained at the first 30 s treatment and

prolongation of the exposure time up to 5 min did not further reduce the bacterial counts. While uninoculated potatoes were also tested to evaluate the effectiveness of ozonated water to decrease populations of naturally present microorganisms, the number of mesophilic aerobic bacteria, psychrotrophic bacteria, coliforms and *L. monocytogenes* were reduced by 1.1, 0.7, 1.5, and 0.8 $\log_{10}$ CFU/g after receiving 1 min washing in 5 ppm ozonated water (Selma and others 2006).

Ozone treatment also has benefits in extending shelf-life of some fruits. Ozone at 0.10-0.30 ppm in atmosphere during storage of blackberries resulted in suppression of fungal development for 12 d at 2 °C and no injury or defects on treated fruit was observed (Barth and others 1995). Pérez and others (1999) reported that 0.35 ppm gaseous ozone at 2 °C was partially effective in controlling fungal growth in strawberries. Ozone- treated strawberries had 15% less fungal decay than the untreated fruit after 2 days storage at 20 °C. Ozone treatment (8 ppm) for 20 min exposure succeeded in lowering populations of bacteria, fungi and yeast. Fungal decay following refrigeration storage of treated grapes declined resulting in increased shelf-life (Sarig and others 1996).

The application of ozone in both gaseous and aqueous forms has been studied in various types of food products other than fresh produce including seafood, meat and poultry products, eggs, dry foods, and cheese. Furthermore, it was also used for sanitizing processing plants and food contact surface in dairy industry (Sheldon and Brown 1986; Chen and others 1987; Rusch and Kraemaer 1989; Whistler and Sheldon

1989; Greene and others 1993; Kim and others 1999a; Guzel-Seydim and others 2000).

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CHAPTER 3

Antimicrobial Effect of Electrolyzed Oxidizing Water against *Escherichia coli* O157:H7 and *Listeria monocytogenes* on Fresh Strawberries (*Fragaria x ananassa*)

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ABSTRACT

Antibacterial activity of electrolyzed oxidizing (EO) water prepared from 0.05% or 0.10% (w/v) sodium chloride (NaCl) solutions against indigenous bacteria associated with fresh strawberries (*Fragaria x ananassa*) were evaluated. Efficacy of EO water and sodium hypochlorite (NaOCl) solution in eliminating and controlling the growth of *Listeria monocytogenes* and *Escherichia coli* O157:H7 inoculated onto strawberries stored at 4 ± 1 °C up to 15 d were investigated at exposure time of 1, 5, or 10 min. Post-treatment neutralization of fruit surface was also determined. More than 2 $\log_{10}$ CFU/g reduction of aerobic mesophiles were obtained in fruit washed for 10 or 15 min in EO water prepared from 0.10% (w/v) NaCl solution. Bactericidal activity of the disinfectants against *L. monocytogenes* and *E. coli* O157:H7 was not affected by post-treatment neutralization, and increasing exposure time did not significantly increase the antibacterial efficacy against both pathogens. Washing fruit surfaces with distilled water resulted in 1.90 and 1.27 $\log_{10}$ CFU/ml of rinse fluid reduction of *L. monocytogenes* and *E. coli* O157:H7, respectively. While ≥ 2.60 $\log_{10}$ CFU/ml of rinse fluid reduction of *L. monocytogenes*, and up to 2.35 and 3.12 $\log_{10}$ CFU/ml of rinse fluid reduction of *E. coli* O157:H7 were observed on fruit surfaces washed with EO water and NaOCl solution, respectively. *L. monocytogenes* and *E. coli* O157:H7 populations decreased over storage regardless of prior treatment. However, EO water and aqueous NaOCl did not show higher antimicrobial potential than water treatment during refrigeration storage.

INTRODUCTION

Strawberries have a fresh market life of 1-2 wk depending on postharvest handling, variety, and maturity of the fruit (Mitcham and Mitchell 2002). Although the incidence of foodborne outbreaks associated with consumption of berries is rare, raw raspberries and possibly blackberries imported from Guatemala have been involved in numerous large outbreaks of *Cyclospora cayetanensis* (Herwaldt and others 1997; Harris and others 2003). Ingestion of commercially frozen strawberries has also been implicated as a cause of outbreaks associated with hepatitis A virus, an organism spread in human feces and contaminated berries via infected harvesters or contaminated irrigation water (Dougherty and others 1965; Niu and others 1992; CDC 1997; Hutin and others 1999; FDA 2001a; Knudsen and others 2001; Harris and others 2003; Flessa and others 2005). Enteric bacteria such as *Salmonella* and *E. coli* O157:H7 have a similar environmental reservoir as *C. cayetanennsis* and the hepatitis A virus (Knudsen and others 2001). Moreover, the ubiquity of *L. monocytogenes* in the environment, especially in soil and plant matter makes possible the presence of this Gram-positive bacterium in fruit and vegetables grown in close association with soil (Beuchat and Ryu 1997; Brackett 1999a). Studies also supported that *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* were able to survive in fresh and frozen strawberries beyond the expected shelf-life of the fruit (Knudsen and others 2001; Flessa and others 2005). Therefore, contamination of strawberries with foodborne bacteria during harvesting or processing may pose a particular hazard to consumers.

The traditional methods for reducing microorganisms on produce is washing with water, cleaning chemicals, or mechanical treatment of the surface by brush or spray washers following by rinsing with potable water (FDA 2001b). Washing fresh produce with cold running tap water is the recommended method for reducing indigenous microflora before consumption (FSIS 2006). However, the efficacy of water treatment in eliminating or reducing naturally occurring and pathogenic microorganisms on fresh produce is limited (Brackett 1999b). According to Koseki and others (2004), washing with tap water failed to reduce the microbial load on cucumber and strawberries. Mohd-Som and others (1995) reported that the aerobic plate count, coliforms, yeast, and mold populations were decreased by approximately 1 $\log_{10}$ CFU/g after treating fresh broccoli with cold running tap water. Treatments with chlorine and chlorine containing compounds are still the most widely used method for reducing or eliminating pathogens in produce industries due to their ease of use and relatively low cost. However, their efficacy at the permitted concentration in most cases was limited to about 1-2 log reductions (Nguyen-the and Carlin 1994; Cherry 1999; Xu 1999; Suslow 2001). Additionally, the use of higher levels of chlorine causes other detrimental effects on product sensory quality, the environment, and human health (Suslow 2001). For instance, chlorine application resulted in formation of reaction products of concern, such as trihalomethanes (THMs) and other chemical residuals formed in wastewater returned to the environment (Richardson and others 1998; Xu 1999, Rodgers and others 2004). In accordance with the necessity to reduce the incidence of foodborne outbreaks as well as to maintain a safer

environment and food supply, more practical and effective antimicrobial treatments are desirable (Graham 1997).

Electrolyzed oxidizing water (EO water) has gained great interest as a novel non-thermal processing technology. EO water is the product from the anodic electrolysis of a dilute saline solution and has been used for sanitation purposes in medicine, agriculture, and food (Buck and others 2002; Park and others 2004; Guan and Hoover 2006). The strong bactericidal, fungicidal, and virucidal activities of EO water have been documented in previous studies (Venkitanarayanan and others 1999a, 1999b; Morita and others 2000; Al-Haq and others 2002; Buck and others 2002; Huang and others 2006; Liu and others 2006). Furthermore, the efficacy of EO water in reducing or eliminating both the natural microflora and pathogenic microorganisms on fresh vegetables and sprouts was extensively studied (Izumi 1999; Koseki and others 2001, 2002, 2004; Kim and others 2003; Sharma and others 2004; Lin and others 2005). Nevertheless, bactericidal activity of EO water against foodborne pathogens *E. coli* O157:H7 and *L. monocytogenes* on acidic fresh fruits, such as strawberries has not been reported.

The objectives of this study were to evaluate the antibacterial activity of EO water prepared from high and low sodium chloride (NaCl) concentrations against indigenous bacteria associated with fresh strawberries and to compare the efficacy of EO water and other conventional disinfectants in controlling the survival and growth of *L. monocytogenes* and *E. coli* O157:H7 inoculated onto strawberries during refrigeration storage.

MATERIALS AND METHODS

Bacterial cultures

Three strains each of *E. coli* O157:H7 (ATCC 43894, ATCC 43895, and F 4546) and *L. monocytogenes* (Scott A, ATCC 19115, and DA-1) were used in this study. All cultures had been kept frozen at -80 °C before revival in 10 ml tryptic soy broth (TSB) (EMD Chemicals Inc., Gibbstown, N.J., U.S.A.). A 0.1 ml broth culture of individual bacterial strains were daily subcultured in 10 ml of TSB for 2 successive 24-h intervals at 37 °C, and then grown individually in 100 ml of TSB for 24 h before preparing the test inoculum. Final broth cultures were grown to ~10^9 CFU/ml of each strain. The 3-strain cocktail of each tested bacteria was prepared by combining equal portions of each strain to obtain the final cell concentration of ~10^9 CFU/ml and used as the inoculum. The bacterial count of the test inoculum was determined by surface plating 0.1 ml of the appropriate dilution in duplicate to TSB supplemented with Bacto- Agar (15 g/l) (Difco, Detroit, Mich., U.S.A.). In order to verify the initial inoculum levels, plates were incubated at 37 °C for 24 h before enumeration.

Strawberries

Fresh strawberries of uniform size, color, and maturity with a weight range of 20-30 g per fruit were purchased from a local market in Corvallis, Ore., U.S.A., immediately after arrival at the store (either the day before or the day of an experiment). Fruit was held at ambient temperature (22 ± 1 °C) at least 2 h prior to inoculation with pathogens.

To meet the specific goals of each experiment, strawberries were left whole, unhulled, or unwashed. For microbial challenge studies, the calyx of the strawberries was removed by hand wearing sterile disposable gloves (Diamond Grip Plus, Reno, Nev., U.S.A.) before placed in 17.78 x 30.48 cm sterile sampling bag (VWR International Inc., Brisbane, Calif., U.S.A.).

Preparation of treatment solutions

For investigating the antibacterial efficacy of EO water prepared from different concentrations of saline solution, EO water was generated from a batch type JED - 007 Super Water Mini generator (Altex Janix, Kanagawa, Japan) containing 0.05% (w/v) or 0.10% (w/v) NaCl solution with an electrolysis time of 15 min. The EO water used in the microbial challenge study was prepared from a Hoshizaki ROX 20TA-U continuous generator (Hoshizaki Electric Co. Ltd., Toyoake, Aichi, Japan) at a setting of 10 V and 14.0 ± 0.2 A in which deionized water and a 13.6% (w/v) NaCl solution were pumped into the apparatus simultaneously. The EO water was collected from the anode side, and then stored in screw-capped sterile bottles. The EO water was prepared on the day of an experiment and used within 1 h after production. NaOCl solution was prepared by diluting the appropriate amount of commercial bleach solution containing approximately 6% (w/w) of NaOCl (Clorox® Regular Bleach, Clorox Company, Oakland, Calif., U.S.A.) with distilled water (DW). DW as a control was collected from the laboratory supply line instantly before its application and stored in screw-capped sterile bottles. The pH, oxidation reduction potential

(ORP), dissolved oxygen (DO), and free and total chlorine concentration of treatment solutions were determined. The pH was measured by a pH meter (Accumet Research AR 10, Fisher Scientific, Pittsburgh, Pa., U.S.A.) coupled with pH electrode (Symphony pH electrode, Thermo Electron Corp., Waltham, Mass., U.S.A.) while ORP was determined with a dual scale pH/ORP meter (Corning 125, Medfield, Mass., U.S.A.) equipped with the platinum redox electrode model 96-78-00 (Thermo Electron Corp., Beverly, Mass., U.S.A.). The DO was measured by using the YSI Model 95 dissolved oxygen meter (YSI Inc., Tellow Springs, Ohio, U.S.A.). Free and total chlorine concentrations were determined by DPD (N, N-diethyl-p-phenylenediamine) colorimetric method with a Pocket Colorimeter^{TM} chlorine test kit (Hach Company, Loveland, Colo., U.S.A.).

Treatment of uninoculated strawberries with EO water

Four randomly selected strawberries (95 ± 10 g) were put into a sterile 600 ml beaker with the aid of sterile forceps. EO water prepared from 0.05% (w/v) or 0.10% (w/v) NaCl solution or DW (control) was added into the beaker. A fruit to treatment solution ratio of 1:3 by weight and contact time of 5, 10, and 15 min were applied. During the test, the strawberries-sanitizer mixture was agitated on a rotary shaker (Environ shaker, Lab-Line, Melrose Park, Ill., U.S.A.) at 150 rpm to facilitate the exposure between fruit and treatment solutions. Immediately after reaching the contact time, EO water solution was decanted from the strawberries, and the same amount of DW was added to inactivate the bactericidal activity of EO water.

Fruit-water mixture was then shaken with a rotary shaker for 2 min. Treated berries were put into sterile sampling bag with 99 ml Butterfield's phosphate buffer (BP) (pH 7.2) (Hardy Diagnostics, Santa Maria, Calif., U.S.A.) and pummeled in a stomacher (Stomacher 400 Circulator, Seward, London, England) at 230 rpm for 2 min. Fruit homogenate was serially diluted in BP, and then 1 ml or 0.1 ml aliquots of appropriate dilution were pour-plated with plate count agar (PCA) (Becton Dickinson and Co., Sparks, Md., U.S.A.) in duplicate. Populations of mesophilic aerobic bacteria were counted after incubation at 35 °C for 48 h. To obtain the baseline data, untreated samples were combined with 99 ml BP and macerated in the stomacher blender for 2 min at 230 rpm. The recovery and enumeration of bacterial populations were done with the same procedure as described early.

Inoculation of strawberries

For inactivating the surface microflora of strawberries, samples at 22 ± 1 °C were washed in NaOCl solution containing 250 ppm total chlorine prepared by diluting commercial bleach solution (approximately 6% (w/w) NaOCl) with DW, with a fruit to aqueous chlorine ratio of approximately 1:3 by weight for 30 s. Fruit samples were rinsed with DW for 1 min twice to remove the residual chlorine. No chlorine residual was ensured by testing the second rinsing water with the chlorine test kit. Mixed strain cocktail prepared as described above was further diluted with BP to yield a final cell concentration of approximately 10^7 CFU/ml in a gallon-size Ziploc bag (Ziploc®; Johnson & Son, Racine, Wis., U.S.A.). The fruit to bacterial suspension ratio (weight)

was 1:3. Prewashed strawberries were then dip-inoculated with agitation on the rotary shaker at 100 rpm for 15 min to ensure uniform inoculation. The suspension was decanted and strawberries were placed on a sterile aluminum screen under a biosafety class Π hood (Fisher Hamilton Inc., Two Rivers, Wis., U.S.A.) for 30 min at room temperature (22 ± 1 °C) before washing with different solutions.

The drying time applied in this work was comparatively short to those used in other challenge studies with strawberries in which at least 1 h holding period was applied (Knudsen and others 2001; Lukasik and others 2003; Sy and others 2005). However, results from our preliminary experiments indicated that drying inoculated strawberries under the biosafety hood with air movement for 1 h did not give a significant difference in the number of pathogens recovered compared with a 30 min drying period. Flessa and others (2005) also reported that *L. monocytogens* was absorbed into the strawberries within 20 min after inoculation. Therefore, 30 min was selected as a drying time for all challenge studies in this study.

Treatment of strawberries inoculated with *L. monocytogenes* or *E. coli* O157:H7

Four inoculated strawberries (100 ± 20 g) were randomly selected and placed into quart-size Ziploc bags (Ziploc®; Johnson & Son, Racine, Wis., U.S.A.). To obtain the initial population of *L. monocytogenes* or *E. coli* O157:H7 on strawberries, 4 inoculated fruits were put into a sterile sampling bag with 99 ml of BP. After strawberries were pummeled for 2 min at 230 rpm in a stomacher, fruit homogenate was subjected to the microbiological analysis. The disinfection effect of DW

(control), NaOCl solution, and EO water against *L. monocytogenes* and *E. coli* O157:H7 was determined in whole fruit tissues and on fruit surfaces only. Studies were also conducted to evaluate the rinsing effect after sanitizing. Disinfecting agents were added to strawberries in the Ziploc bag with fruit to treatment solution ratio of 1: 3 by weight and contact time of 1, 5, or 10 min at room temperature (22 ± 1 °C). After the exposure, strawberries were immediately removed from the sanitizer, and then immersed in the neutralizing buffer solution (Becton Dickinson Co., Sparks, Md., U.S.A.) with an equal amount of sanitizer in another Ziploc bag for 2 min with 100 rpm agitation to inactivate the bactericidal action of the sanitizers. Treated fruits were transferred to a sterile sampling bag and mixed with 99 ml of BP. In the experiment without a neutralization step, treated samples were drained and placed into a sterile sampling bag with 99 ml of phosphate buffer. Fruits and BP were homogenized at 230 rpm for 2 min. Tissue homogenates were serially diluted in 9 ml BP and duplicate samples of 0.1 ml aliquots were spread on the appropriate agar medium. A 1 ml of neutralizing buffer was also serially diluted and 0.1 ml aliquots were surface plated in duplicate to enumerate the viable cells recovered from the surface of the samples.

Neutralization is one of important steps for determining the efficacy of chemical disinfectant treatment (Beuchat and others 2001). The purpose of neutralization is to inactivate the bactericidal and bacteriostatic effect of chlorine as well as to reduce the ORP reading of NaOCl and EO water. Moreover, the industrial sanitation practice usually includes rinsing produce after exposure to sanitizers (FDA 2001b). Therefore, the results presented in this study would be beneficial to evaluate the effect of

neutralization step to the bactericidal activity of chlorinated water and EO water on strawberries.

Storage study of inoculated strawberries with *L. monocytogenes* or *E. coli* O157:H7

After inoculation and drying at room condition for 30 min, 16 randomly selected strawberries with known weight were placed into a gallon-size Ziploc bag. To determine the initial population of pathogens inoculated onto strawberries, 4 contaminated fruits were put into a sterile sampling bag with 99 ml of BP. After pummeled for 2 min at 230 rpm, fruit homogenate was subjected to microbiological analysis. For treatments, when samples were immersed in NaOCl solution or EO water with a fruits to treatment solution ratio of approximately 1:3 by weight for 5 min at ambient temperature (22 ± 1 °C); they were agitated by shaking on a rotary shaker at 100 rpm to ensure even exposure. Fruit treated with DW under the same conditions were used as a control. In this experiment, samples were not rinsed by neutralizing buffer after exposure to the disinfectants. After washing, 4 fruits were randomly selected to determine the number of microorganisms immediately following the treatment. The remaining fruits were placed on a sterile aluminum screen under the biosafety class Π hood for 10 min. Then, sets of 4 strawberries were randomly selected and put into sterile sampling bags and stored at 4 ± 1 °C for 5, 10, and 15 d before conducting microbiological analysis. Populations of *L. monocytogenes* or *E. coli* O157:H7, mesophilic aerobic bacteria, yeasts, and molds were enumerated.

Microbiological analyses

Spread plate enumeration technique was used for all experiments except as indicated. Populations of *L. monocytogenes* were recovered on modified Oxford agar (MOX) (Becton Dickinson and Co., Sparks, Md., U.S.A.) with 48 h incubation at 37 °C. MacConkey sorbitol agar (Becton Dickinson and Co., Sparks, Md., U.S.A.) supplemented with cefixime-tellurite (Dynal Biotect A.S.A, Oslo, Norway) (CT-SMAC) was used as a selective media for *E. coli* O157:H7 and plates were incubated at 37 °C for 24 h before colonies were counted. The number of mesophilic aerobic bacteria was determined by plating on PCA, and incubated at 37 °C for 48 h. Dichloran Rose Bengal Chloramphenicol agar (DRBC) (EMD Chemicals Inc., Gibbstown, N.J., U.S.A.) was used to enumerate yeasts and molds. The DRBC agar plates were incubated in the dark at 25 °C for 5 d before yeast and mold colonies were counted. Preliminary experiments for confirming the absence of any microorganisms on laboratory distilled water were done by plating water sample on all the types of media as described above.

Statistical analysis

The effect of NaCl concentration on the antibacterial activity of EO water generated was determined with 4 replications. All trials for challenge studies were performed 6 times. Data were analyzed by general linear model procedure (PROC GLM) of the Statistical Analysis System version 9.1 (SAS Institute, Cary, N.C., U.S.A.). Analysis of variance among treatment was performed and comparisons of

mean values were established by Tukey's Studentized (HSD) multiple comparison test at the significant level of 0.05. When microbial colonies were not detected on plates, a value of 1.00 was assigned to the $\log_{10}$ CFU/g value for the purpose of statistical analysis (Hao and others 1998).

RESULTS AND DISCUSSION

Effect of NaCl concentration in EO water against indigenous bacteria on fresh strawberries

Physicochemical properties of tested EO water prepared from different NaCl concentrations and the antimicrobial effects of EO water against the naturally occurring bacteria on fresh strawberries are presented in Tables 3.1 and 3.2, respectively. The EO water prepared from the higher NaCl concentration (0.10% w/v) was lower in pH, comparable in ORP and DO, but significantly higher in free chlorine concentration than that prepared from 0.05% (w/v) NaCl solution. Kiura and others (2002) also reported that increasing salt concentrations in water used for electrolysis leads to a greater amount of free chlorine in EO water.

Table 3.1 Physicochemical properties of EO water prepared from different NaCl concentrations [a]

Properties	0.05% NaCl [b]	0.10% NaCl [b]
pH	2.33 ± 0.08	2.27 ± 0.04
Oxidation reduction potential (mV)	1,095 ± 37	1,137 ± 26
Dissolved oxygen (ppm)	17.61 ± 2.38	18.34 ± 0.97
Free chlorine (ppm)	39.24 ± 2.72	68.13 ± 6.58

[a] Values were means ± standard deviation of quadruplicate determinations (n = 4).
[b] EO water was prepared from 0.05% or 0.10% (w/v) NaCl solution with the JED-007 Super Water Mini generator.

Among the different washing treatments, distilled water or EO water prepared from 0.05% (w/v) NaCl did not significantly ($p>0.05$) reduce the native microflora as compared with untreated fruit, in which only 0.25-0.41 $\log_{10}$ CFU/g reduction was observed on fruits washed in EO water from 0.05% (w/v) electrolyzed solution (Table 3.2). Washing with EO water generated from 0.10% (w/v) aqueous NaCl resulted in a 1.44 - 2.23 log reduction with the maximum reduction at an exposure time of 10 min. Extending washing time from 5-15 min did not further decrease bacterial number with all tested solutions. These results were consistent with data by Koseki and others (2004) that rinsing strawberries with EO water (pH 2.6, ORP 1,130 mV, and 32.1 ppm free chlorine) for 10 min resulted in a 0.90 $\log_{10}$ CFU/fruit reduction of mesophilic aerobic bacteria comparing with 0.10 $\log_{10}$ CFU/fruit for water treatment.

Table 3.2 Populations of naturally present mesophilic aerobic bacteria ($\log_{10}$ CFU/g) [a] on fresh strawberries after washing with distilled water or EO water prepared from different NaCl concentrations

Treatment solution	Exposure time (min)		
	5	10	15
Distilled water	a3.58 ± 0.43 A	a3.10 ± 0.24 A,B	a3.28 ± 0.43 A,B
EO Water 0.05% [b]	a3.10 ± 0.85 B	a3.06 ± 0.60 A,B	a2.94 ± 0.46 A,B
EO Water 0.10% [b]	a1.91 ± 0.62 C	a1.12 ± 0.20 C	a1.24 ± 0.46 C

[a] Values were the means ± standard deviation of 4 replications (n = 4) in which each replication contained 4 strawberries. All treatment comparisons were based on control (untreated fresh strawberries) with a total mesophlic aerobic count of 3.35± 0.22 $\log_{10}$ CFU/g.

[b] EO water was prepared from 0.05% or 0.10% (w/v) NaCl solution with the JED-007 Super Water Mini generator.

Means followed by the same capital letters in the same column were not significantly different ($p>0.05$).

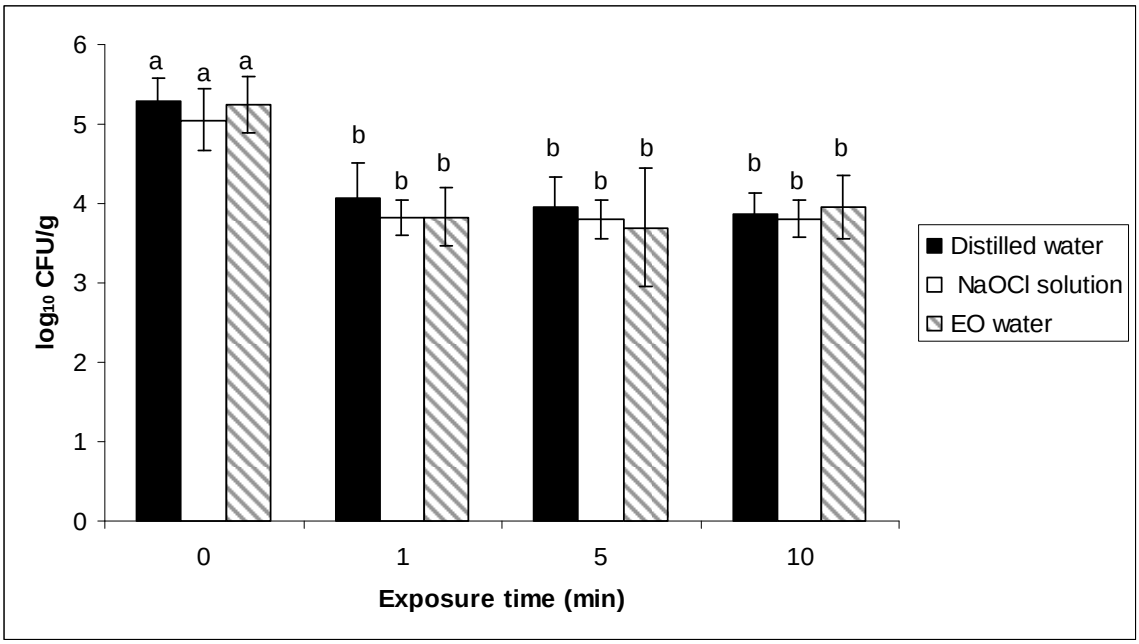
Means preceded by the same lowercase letters in the same row within each treatment solution were not significantly different ($p>0.05$).

The effect of NaCl concentration in EO water preparation on the antibacterial activity may attribute to the different amounts of free chlorine present. Len and others (2000) observed that residual chlorine in the form of hypochlorous acid (HOCl) was the primary bactericidal agent in EO water. Hypochlorous acid and other free chlorine species including hypochlorite ion (OCl^-) and molecular chlorine (Cl_2) are produced at the positive side (anode) during electrolysis of dilute saline solution (Venkitanarayanan 1999a; Hsu 2003, 2005). The lethal effect of EO water against microorganisms can be attributed to oxidation of the outer membrane of bacterial cells, and the degree of cell damage was proportionally correlated with increased free chlorine concentration in EO water (Kiura and others 2002).

Inactivation of EO water on *L. monocytogenes* and *E. coli* O157:H7 on fresh strawberries

Figures 3.1 and 3.2 illustrate the number of viable *L. monocytogenes* and *E. coli* O157:H7 cells recovered from whole fruit homogenates after washing with DW, NaOCl solution, or EO water for various exposure times, and then rinsing with neutralizing buffer (Figs. 3.1(a) and 3.2 (a)) or without neutralization step (Figs. 3.1(b) and 3.2 (b)), respectively. The mean populations of *L. monocytogenes* and *E. coli* O157:H7 on inoculated whole fruits were 5.16 and 4.99 $\log_{10}$ CFU/g, respectively. Antimicrobial activity of tested disinfectants against both investigated pathogens were not affected by neutralization after treatment ($p>0.05$).

a



b

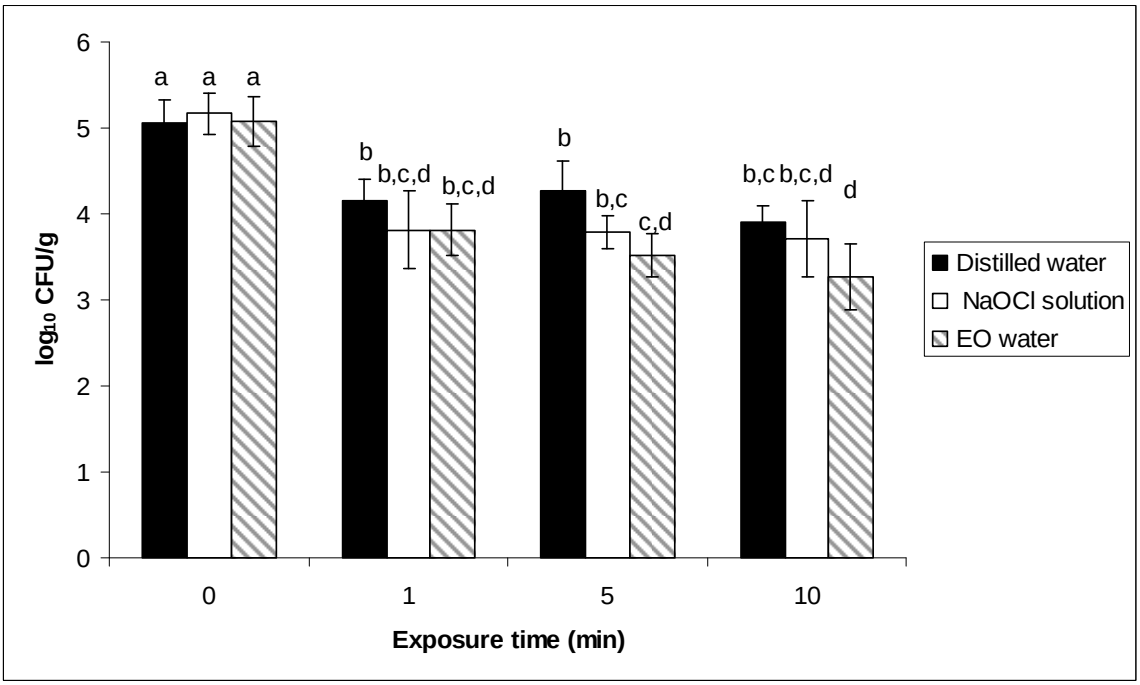
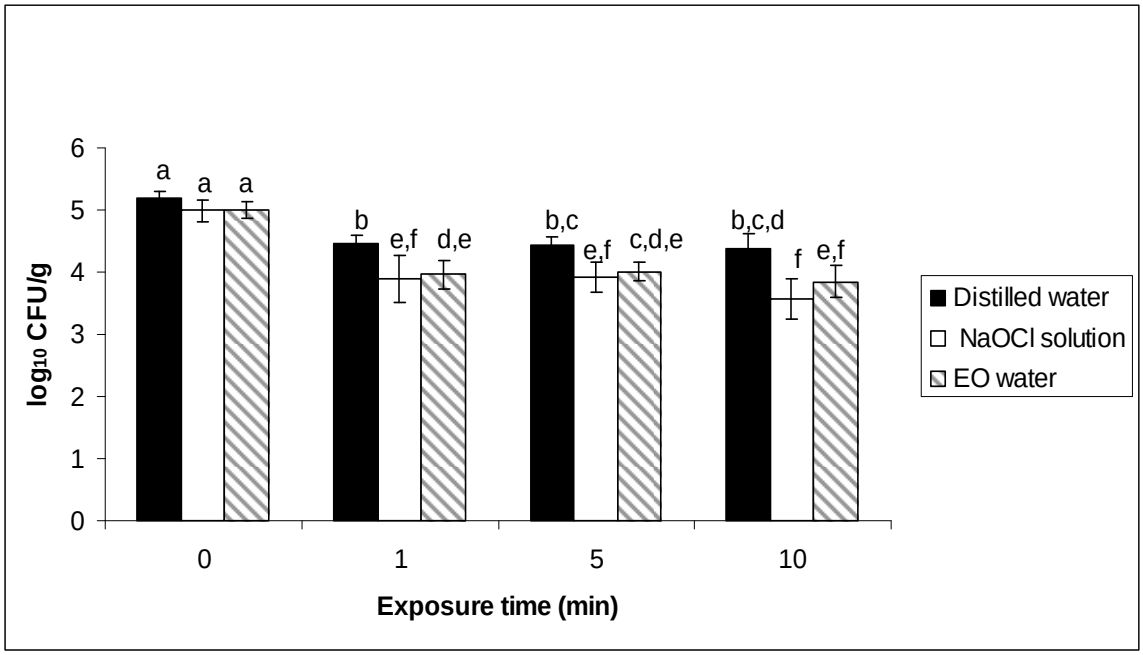


Figure 3.1 Populations of *L. monocytogenes* recovered from whole strawberry tissues after immersed in treatment solutions at various exposure times: (a) rinsing with neutralizing buffer after treatment; (b) without rinsing with neutralizing buffer. Vertical bars indicated standard deviation. Samples with different superscripts were significantly different ($p<0.05$).

a



b

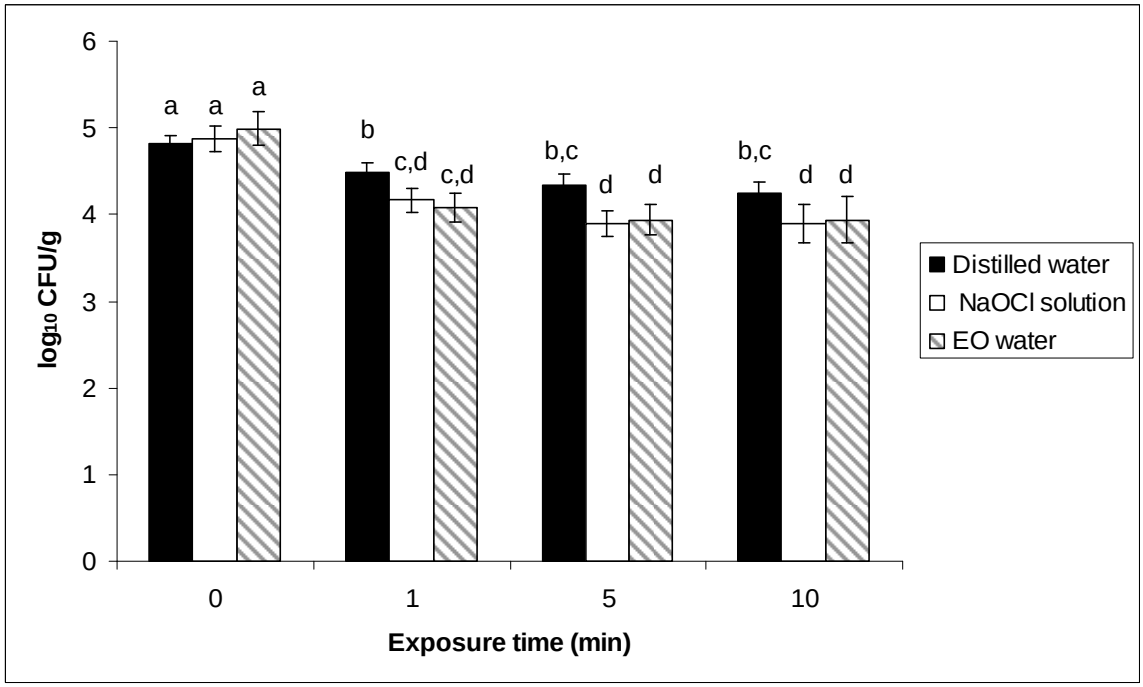


Figure 3.2 Populations of *E. coli* O157:H7 recovered from whole strawberry tissues after immersed in treatment solutions at various exposure times: (a) rinsing with neutralizing buffer after treatment; (b) without rinsing with neutralizing buffer. Vertical bars indicated standard deviation. Samples with different superscripts were significantly different ($p<0.05$).

With neutralization after treatment, EO water succeeded in lowering the viable cells of *L. monocytogenes* on whole fruit comparing with untreated berries; however, its antimicrobial activity was not considerably different ($p>0.05$) from DW and NaOCl solution. For treatments with EO water and NaOCl solution, the highest log reduction was achieved at an exposure time of 5 min, whereas 10 min was required to obtain maximum antibacterial activity of DW. In contrast to the results on *L. monocytogenes*, washing *E. coli* O157:H7 contaminated strawberries with NaOCl solution followed by neutralization (Fig. 3.2(a)) showed a greater decontamination effect than DW at all exposure times, whereas the former had a similar antibacterial activity with EO water at each point of exposure time. Without neutralization (Figs. 3.1(b) and 3.2(b)), NaOCl and EO water solutions demonstrated an equivalent antimicrobial effect against *L. monocytogenes* and *E. coli* O157:H7 at all exposure times.

The number of bacterial cells in the rinsing neutralizing buffer represented the number of target bacteria removed from the surface of untreated and treated strawberries. Figures 3.3 and 3.4 portray the decreases in population of *L. monocytogenes* and *E. coli* O157:H7 on fruit surfaces observed after washing with different disinfectants. Again, the bactericidal activities of aqueous NaOCl and EO water against *L. monocytogenes* were similar at all exposure times and significantly higher than the DW treatment ($p<0.05$). Up to 1.90, 2.60, and 2.76 $\log_{10}$ CFU/ml reduction of *L. monocytogenes* was obtained from rinse water of fruit surfaces washed with DW, NaOCl solution, and EO water, respectively. Even for a 1 min exposure to

NaOCl solution or EO water, the number of *L. monocytogenes* was reduced by 2.03 and 2.11 $\log_{10}$ CFU/ml of rinse fluid, respectively.

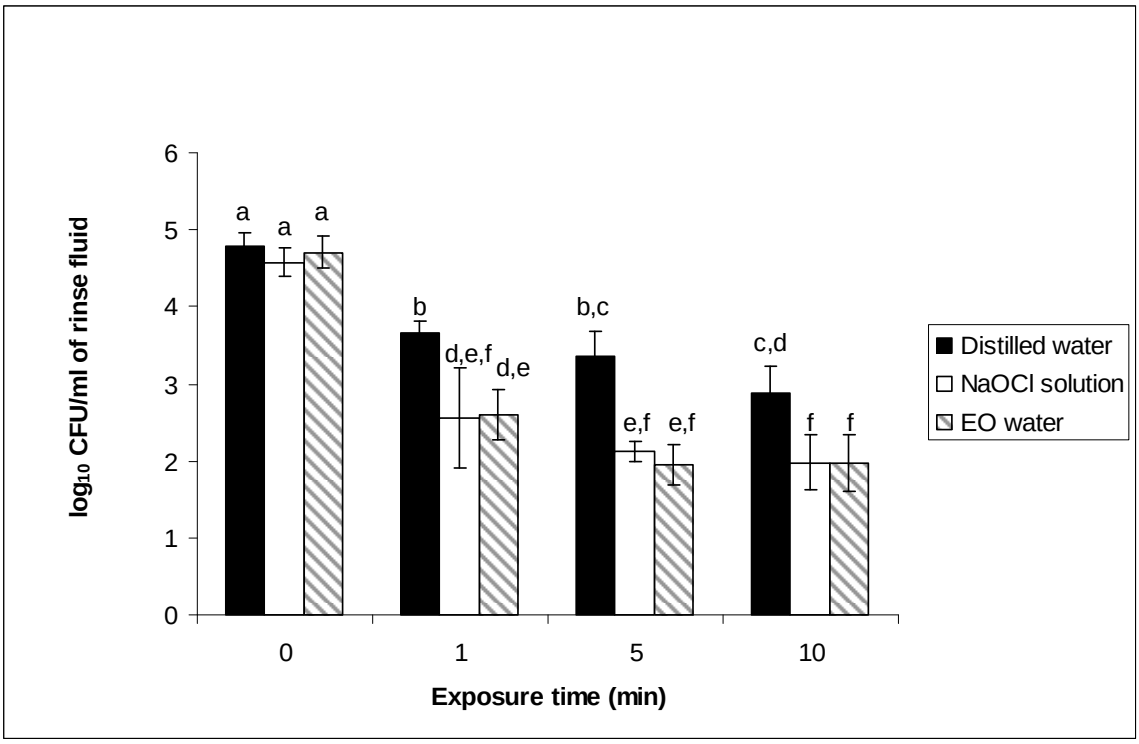


Figure 3.3 Populations of *L. monocytogenes* recovered from strawberry surfaces after immersed in treatment solutions at various exposure times. Vertical bars indicated standard deviation. Samples with different superscripts were significantly different ($p<0.05$).

The EO water and NaOCl solution gave a stronger antimicrobial effect ($p<0.05$) against *E. coli* O157:H7 adhered onto the strawberry surfaces than DW at all points of exposure time (Fig. 3.4) and aqueous NaOCl was able to reduce *E. coli* O157:H7 to a greater degree than that with EO water at 10 min exposure. Up to 1.27, 3.12 and 2.35 $\log_{10}$ CFU/ml of rinse fluid reductions in *E. coli* O157:H7 were received with DW, NaOCl solution, and EO water treatments, respectively. Moreover, same as obtained on *L. monocytogenes*, *E. coli* O157:H7 populations were reduced by 2.10 and 1.77

$\log_{10}$ CFU/ml of rinse fluid after washing with NaOCl and EO water for 1 min, respectively.

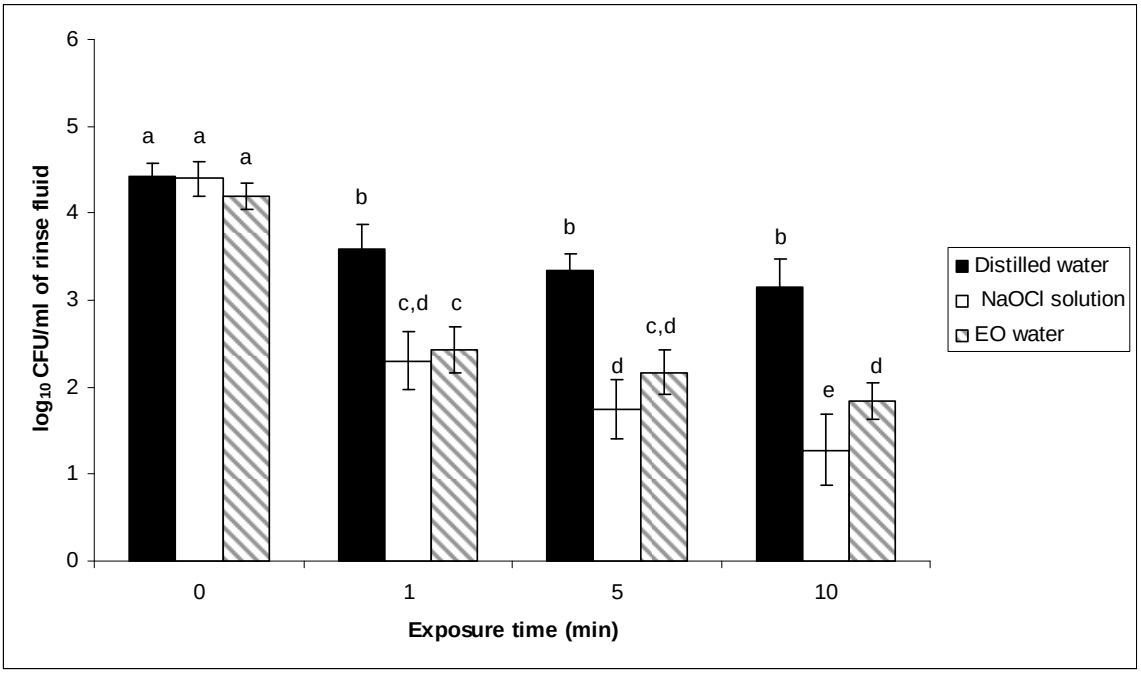


Figure 3.4 Populations of *E. coli* O157:H7 recovered from strawberry surfaces after immersed in treatment solutions at various exposure times. Vertical bars indicated standard deviation. Samples with different superscripts were significantly different ($p<0.05$).

Results obtained from this study indicated that prolongation of exposure time would not lead to a significant reduction in the number of both *L. monocytogenes* and *E. coli* O157:H7 in fruit homogenate for each type of disinfectants. In this experiment, a 5 min treatment gave a comparable bactericidal activity with an extended exposure time; the 5 min exposure time was thus chosen to be a standard exposure time in the storage study.

The insignificant effect of neutralization procedure on inactivation efficacy of tested disinfectants against two pathogens might result from the removal of bacterial

cells loosely attached to fruit during rinsing with neutralizing buffer. At the same time; however, the presence of chemical disinfectant residuals may exhibit a bactericidal effect during sample homogenization or before microbial enumeration.

Data in Table 3.3 represents the physicochemical properties of treatment solutions applied in the challenge study. EO water had the lowest pH, but highest in ORP and DO. The free and total chlorine concentration of DW was negligible whereas these 2 values of NaOCl solution were almost 4 times higher than those of EO water. Even though extensive work has been done to examine the mode of action of EO water against microorganisms, so far it has not been clarified which of the characteristics of EO water (low pH, high ORP, or presence of free available chlorine) were the most important bactericidal factors (Kim and others 2000a, 2000b; Len and others 2000; Park and others 2004; Liao and others 2007). The outer membrane of microorganisms may be sensitized by low pH or it may become unstable by exposure to extremely high ORP of EO water; thereby facilitating sanitizer penetration (Venkitanarayanan and others 1999a, 1999b; Fabrizio and Cutter 2003). At a pH value of 9.55 for NaOCl solution, OCl^- was predominant as free residual chlorine presented. However, OCl^- was 80 times less effective than HOCl at the same concentration (Eifert and Sanglay 2002). In addition, at the acidic pH of EO water, most of the free chlorine present was in the form of HOCl, reported to be the primary component for antimicrobial activity of EO water (Len and others 2000). This observation supported the results in this study that aqueous NaOCl exhibited an equal bactericidal activity with EO water that contains significantly less free available chlorine.

Table 3.3 Physicochemical properties of treatment solutions used in the microbial challenge study [a]

Treatment solution	pH	ORP [c] (mV)	DO [d] (ppm)	Free chlorine (ppm)	Total chlorine (ppm)
Distilled water	5.30 ± 0.19	452 ± 76	8.63 ± 0.34	< 0.1	< 0.1
NaOCl solution	9.55 ± 0.27	799 ± 156	8.54 ± 0.29	180 ± 16	199 ± 11
EO water [b]	2.60 ± 0.11	1293 ± 147	13.15 ± 0.96	47 ± 7	53 ± 6

[a] Values were means ± standard deviation.
[b] EO water was prepared from a Hoshizaki ROX 20TA-U continuous generator.
[c] Oxidation reduction potential
[d] Dissolved oxygen

Park and others (2004) reported that chlorine is very toxic to both *L. monocytogenes* and *E. coli* O157:H7; thus, the failure to inactivate pathogenic bacteria by EO water and aqueous NaOCl in the current study to a greater degree may suggest that the target bacteria were not directly exposed to the disinfectants. Seo and Frank (1999) suggested that the effectiveness of disinfectants depends on the accessibility between the active sanitizing agent and the target microorganisms. Therefore, microorganisms that may be embedded in cracks, crevices, and stomata or penetrate into interior structures can be protected from the action of disinfectants. The hydrophobicity of microbial cells may aid in their protection against penetration of disinfectants and may also facilitate the attachment to epidermal layer of plant tissue (Burnett and Beuchat 2001).

EO water for controlling the growth of *L. monocytogenes* and *E. coli* O157:H7 on fresh strawberries during refrigeration storage

Populations of *L. monocytogenes*, *E. coli* O157:H7, mesophilic aerobic bacteria, and yeasts and molds recovered from *L. monocytogenes* or *E. coli* O157:H7 inoculated strawberries followed 5 min washing treatment and stored at 4 ± 1°C for up to 15 d are given in Tables 3.4 and 3.5, respectively. A 5 min treatment with DW, NaOCl solution, and EO water resulted in 1.27, 2.06, and 1.65 $\log_{10}$ CFU/g reduction in *L. monocytogenes*, respectively, and 0.45, 0.66, and 0.71 $\log_{10}$ CFU/g reduction in *E. coli* O157:H7, respectively. Contrary to the exposure time study, a 5 min treatment did not provide a significant difference in microbicidal activities among DW, NaOCl solution, and EO water to reduce *L. monocytogenes* and *E. coli* O157:H7 (Tables 3.4 and 3.5). This was perhaps due to the number of fruit per treatment in the storage study which was higher than that used in the exposure time study although the fruit to sanitizer ratio was kept same (about 1 to 3 by weight). Therefore, a longer sanitizing period or a higher ratio of disinfectant to produce might be necessary in order to improve the disinfectant effect of the tested solutions.

In a study by Yu and others (2001), strawberries dipped in DW and NaOCl solution (130 ppm free chlorine) within 1 min reduced *E. coli* O157:H7 populations by approximately 0.8 and 1.3 $\log_{10}$ CFU/g, respectively. These larger reductions, compared with those observed in current study may be attributed to the differences between the sensitivity of single and multiple strains to disinfecting agent. Also, the exposure time during dip-inoculation in the current study was much longer, which may facilitate the internalization of pathogens within sample tissues, thus protecting

them from direct exposure to disinfectants. Lastly, different methods for the recovery of viable cells from treated strawberries may be another factor resulting in the dissimilar results.

Table 3.4 Populations of microorganisms [a] (log_{10} CFU/g) recovered from *L. monocytogenes* inoculated strawberries after treatment and storage at 4 ± 1 °C

Treatment solution	log_{10} CFU/g of *L. monocytogenes* [b]				
	Exposure time (min)		Storage time (d) after 5 min exposure		
	0	5	5	10	15
Distilled water	**a**5.21 ± 0.26 **A**	**b**3.94 ± 0.50 **A(x)**	2.62 ± 0.89 **A(y)**	1.00 ± 0.00 **A(z)**	1.00 ± 0.01**A(z)**
NaOCl solution	**a**5.47 ± 0.48 **A**	**b**3.41 ± 0.45 **A(x)**	1.28 ± 0.27 **A(y)**	1.13 ± 0.20 **A(y)**	1.00 ± 0.00**A(y)**
EO water	**a**5.34 ± 0.28 **A**	**b**3.69 ± 0.47 **A(x)**	1.80 ± 0.37 **A(y)**	1.44 ± 0.43 **A(y)**	1.25 ± 0.31**A(y)**
Treatment solution	log_{10} CFU/g of mesophilic aerobic bacteria [c]				
	Exposure time (min)		Storage time (d) after 5 min exposure		
	0	5	5	10	15
Distilled water	**a**5.66 ± 0.18 **A**	**b**4.69 ± 0.15 **A(x)**	3.10 ± 0.56 **A(y)**	2.02 ± 0.65 **A(z)**	2.25 ± 0.73 **A(z)**
NaOCl solution	**a**5.61 ± 0.18 **A**	**b**4.15 ± 0.08 **B(x)**	2.19 ± 0.68 **B(y)**	1.39 ± 0.37 **A(y)**	1.82 ± 0.55 **A(y)**
EO water	**a**5.61 ± 0.16 **A**	**b**4.41 ± 0.36 **B(x)**	2.65 ± 0.93**A,B(y)**	2.41 ± 0.84 **A(y)**	3.62 ± 1.31 **A(y)**
Treatment solution	log_{10} CFU/g of yeast and mold [d]				
	Exposure time (min)		Storage time (d) after 5 min exposure		
	0	5	5	10	15
Distilled water	**a**3.65 ± 0.22 **A**	**a**3.42 ± 0.35 **A(x)**	3.77 ± 0.34 **A(x)**	4.78 ± 0.63 **A(y)**	5.67 ± 0.50**A(z)**
NaOCl solution	**a**3.85 ± 0.20 **A**	**b**3.35 ± 0.28 **A(x)**	3.77 ± 0.29 **A(x,y)**	4.36 ± 0.37 **A(y)**	5.59 ± 0.63**A(z)**
EO water	**a**3.86 ± 0.47 **A**	**b**3.14 ± 0.13 **A(x)**	3.43 ± 0.17 **A(x)**	4.56 ± 0.49 **A(y)**	5.54 ± 0.59**A(z)**

[a] Values were means ± standard deviation of 6 replications (n = 6) in which each replication contained 4 strawberries.

[b] Populations of *L. monocytogenes* detected by spread plating with MOX.

[c] Populations of mesoplilic aerobic bacteria detected by spread plating with PCA.

[d] Populations of yeast and mold detected by spread plating with DRBC.

Means preceded by the same lowercase letters (a through b) in the same row within each type of microorganism and sanitizing agent were not significantly different ($p>0.05$).

Means followed by the same lowercase letters (x through z) in parentheses in the same row within each type of microorganisms and sanitizing agent were not significantly different ($p>0.05$).

Means followed by the same capital letters in the same column within each type of microorganisms and storage time were not significantly different ($p>0.05$).

Table 3.5 Populations of microorganisms [a] (log_{10} CFU/g) recovered from *E. coli* O157:H7 inoculated strawberries after treatment and storage at 4 ± 1 °C

Treatment solution	log_{10} CFU/g of *E. coli* O157:H7 [b]				
	Exposure time (min)		Storage time (d) after 5 min exposure		
	0	5	5	10	15
Distilled water	**a**4.97± 0.11**A**	**b**4.52 ± 0.19**A(x)**	3.55 ± 0.37**A(y)**	2.42 ± 0.23**A(z)**	2.35 ± 0.77**A(z)**
NaOCl solution	**a**4.92 ± 0.17**A**	**b**4.26 ± 0.47**A(x)**	3.09 ± 0.82**A(y)**	2.39 ± 0.43**A(y,z)**	1.98 ± 0.69**A(z)**
EO water	**a**4.91 ± 0.20**A**	**b**4.20 ± 0.18**A(x)**	3.31 ± 0.69**A(y)**	2.92 ± 0.51**A(y,z)**	2.22 ± 0.71**A(z)**
Treatment solution	log_{10} CFU/g of mesophilic aerobic bacteria [c]				
	Exposure time (min)		Storage time (d) after 5 min exposure		
	0	5	5	10	15
Distilled water	**a**5.03 ± 0.12 **B**	**b**4.55 ± 0.14**A(x)**	3.94 ± 0.31**A(y)**	3.84 ± 0.38**A(y)**	3.57 ± 0.56**A(y)**
NaOCl solution	**a**5.22 ± 0.14 **A**	**b**4.51 ± 0.39 **A(x)**	3.87 ± 0.58**A(y)**	3.41 ± 0.50**A(y)**	3.10 ± 0.31**A(y)**
EO water	**a**5.06 ± 0.10**A,B**	**b**4.36 ± 0.18**A (x)**	3.58 ± 0.41**A(y)**	3.43 ± 0.46**A(y)**	3.79 ± 0.77**A(y)**
Treatment solution	log_{10} CFU/g of yeast and mold [d]				
	Exposure time (min)		Storage time (d) after 5 min exposure		
	0	5	5	10	15
Distilled water	**a**2.67 ± 0.96**A**	**a**2.63 ± 0.84**A(x)**	2.11 ± 0.35**A(x)**	3.25 ± 0.80**A(x)**	3.64 ± 1.01**A(x)**
NaOCl solution	**a**2.79 ± 0.81**A**	**a**1.81 ± 0.59**A(x)**	1.71 ± 0.58**A(x)**	2.26 ± 0.80**A(x)**	2.37 ± 82**A,B(x)**
EO water	**a**2.01 ± 0.62**A**	**a**1.74 ± 0.49**A(x)**	1.90 ± 0.65**A(x)**	2.01± 0.75**A(x)**	1.43 ± 0.37**B(x)**

[a] Values were means ± standard deviation of 6 replications (n = 6) in which each replication contained 4 strawberries.

[b] Populations of *E. coli* O157:H7 detected by spread plating with CT-SMAC

[c] Populations of mesoplilic aerobic bacteria detected by spread plating with PCA

[d] Populations of yeast and mold detected by spread plating with DRBC

Means preceded by the same lowercase letters (a through b) in the same row within each type of microorganism and sanitizing agent were not significantly different ($p>0.05$).

Means followed by the same lowercase letters (x through z) in parentheses in the same row within each type of microorganisms and sanitizing agent were not significantly different ($p>0.05$).

Means followed by the same capital letters in the same column within each type of microorganisms and storage time were not significantly different ($p>0.05$).

Due to the fact that *L. monocytogenes* and *E. coli* O157:H7 are able to grow on PCA, the bacterial populations recovered from the standard plate count procedure were made up of target pathogen and background microflora. This may be attributed to a higher number of aerobic mesophile count recovered. The reduction level of mesophilic aerobic bacteria associated with *E. coli* O157:H7 inoculated strawberries followed a 5 min treatment was similar to those observed with *E. coli* O157:H7 count on CT-SMAC. Moreover, aerobic mesophile populations detected were very close to that of *E. coli* O157:H7 recovered, whereas mesophilic aerobic bacteria associated with *L. monocytogenes* contaminated strawberries that received a 5 min treatment decreased to a lesser degree to that observed on *L. monocytogenes* enumerated with MOX. In addition, the *L. monocytogenes* count was about 0.7 $\log_{10}$ CFU/g lower than the total plate count; therefore, sublethal injury of *L. monocytogenes* may have occurred.

In contrast to the number of yeast and mold enumerated from *E. coli* O157:H7 inoculated strawberries, aqueous NaOCl and EO water reduced contaminating fungi in *L. monocytogenes* inoculated berries following the 5 min washing, but levels of reduction were not significantly different among tested solutions ($p>0.05$). Yeast and mold on *E. coli* O157:H7 inoculated samples were the only microorganisms that were not inactivated by the test solutions after 5 min treatment.

The present study indicated that both *L. monocytogenes* and *E. coli* O157:H7 can survive on strawberries beyond their expected market shelf-life of cold storage at 4 ± 1 °C. However, the recovery levels decreased after the extended storage regardless of

the prior treatment. The NaOCl solution and EO water did not have a significantly superior effect than DW on controlling pathogens during refrigeration storage ($p>0.05$). Beuchat and Brackett (1991) also noted that prior treatment with chlorinated water (210-280 ppm free chlorine) did not affect the survival of *L. monocytogenes* inoculated into tomatoes during storage at 10 °C. We observed a total reduction of approximately 2.6 and 2.1 $\log_{10}$ CFU/g with *L. monocytogenes* and *E. coli* O157:H7, respectively. Results of our study were consistent with that of Roering and others (1999) who observed that the resistance of *E. coli* O157:H7 to the acidic conditions of apple cider (pH 3.3-3.5) was much greater than *L. monocytogenes* at refrigeration temperature. Han and others (2004) observed that acid-sensitive bacteria such as *L. monocytogenes* may be injured and inactivated when exposed to organic acid released from strawberries during stomaching. This contributed to their observations of lower bacterial recovery. Besides the lethal effect of low pH and cold temperature, studies have also suggested that the dryness on fruit surfaces, lack of nutrients, and growth of competitive microorganisms may contribute to decreased number of viable cells during a long-time storage (Knudsen and others 2001; Yu and others 2001; Han and others 2004).

A limited antifungal effect of EO water and NaOCl solution on strawberries inoculated with *L. monocytogenes* and *E. coli* O157:H7 was observed. Residual NaOCl and EO water after treatment did not give a fungicidal effect greater than DW on inoculated fruits with both *L. monocytogenes* and *E. coli* O157:H7. Numbers of yeast and mold obtained from *E. coli* O157:H7 inoculated strawberries at 15 d of

storage after treatment with EO water was the only observation indicating the reduction of yeast and mold populations after low-temperature storage which also exhibited a greater fungicidal activity of EO water as compared with DW. Significant increases in the yeast and mold population were observed with *L. monocytogenes* contaminated berries. Growth of yeast and mold on strawberries after the sanitizing treatment and stored at refrigeration temperature was also reported by Rodgers and others (2004).

Strawberries have an irregular shape and complicated morphological structure. They are comprised of 5 tissue zones, epidermis, hypodermis, cortex, bundle, and pith, also numerous achens (seed) embedded in epidermis rendering the surface uneven and complex (Szczesniak and Smith 1969; Han and others 2004). Koseki and others (2004) reported that the incomplete elimination of naturally occurring microorganisms by EO water and 150 ppm NaOCl solution may result from the complexity of strawberry's surface structure. Yu and others (2001) also pointed out that the rough surface and presence of numerous seeds on the strawberry surfaces may provide the attachment sites for bacteria, and may compromise the effectiveness of sanitizers. Although the microbial load inoculated onto samples in the current study was relatively high, there may be cases where strawberries will be contaminated with similar number of pathogen. Therefore, the investigated sanitizers may be more efficacious in reducing or eliminating disease causing bacteria on strawberries in real world situation.

CONCLUSIONS

Populations of inoculated bacteria on strawberries were significantly reduced after treatment with DW, NaOCl solution, or EO water regardless of the exposure time. The lower chlorine concentration of EO water demonstrated an equivalent antimicrobial activity against *L. monocytogenes* and *E. coli* O157:H7 as that NaOCl solution containing 4 times as much free chlorine may make EO water a promising alternative disinfectant to ensure produce safety as well as reducing concerns about the formation of carcinogenic chlorine by-products. However, the tested sanitizers in the current study cannot be relied on controlling pathogens during refrigeration storage if the numbers of contamination are as higher as the level tested in this study. Therefore, preventing contamination during harvesting, postharvest handling, processing, transportation, and retail handling remain very important strategies to reduce risk of illness from consumption of fresh strawberries.

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CHAPTER 4

Inactivation Effect of Ozonated Water against *Listeria monocytogenes* and *Escherichia coli* O157:H7 on Fresh Strawberries (*Fragaria x ananassa*)

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ABSTRACT

The antibacterial activity of ozonated water was evaluated on fresh strawberries (*Fragaria x ananassa*). Whole strawberries were dip-inoculated (~ 7 $\log_{10}$ CFU/ml) with 3-strain mixtures of *Listeria monocytogenes* or *Escherichia coli* O157:H7 before subjected to washing with ozonated water (approximately 1.90 ppm) for 1, 5, or 10 min. The impact of post-treatment neutralization on the efficacy of aqueous ozone was also determined. Moreover, inoculated and ozone-treated fruit were stored at 4 ± 1 °C for up to 15 d for monitoring the survival and growth of investigated pathogens and spoilage microorganisms. Immediately after washing with aqueous ozone, numbers of *L. monocytogenes* and *E. coli* O157:H7 were significantly decreased regardless of the exposure times employed in both whole fruit tissues and fruit surfaces only. Bactericidal activity of ozonated water against target pathogens was not affected by post-treatment neutralization, and prolongation of exposure time did not indicate further reduction of *L. monocytogenes* and *E. coli* O157:H7 significantly on inoculated strawberries. Surface counts of *L. monocytogenes* and *E. coli* O157:H7 were reduced by 2.17 and 2.02 $\log_{10}$ CFU/ml of rinse fluid after washing with ozonated water for 10 min, respectively.

INTRODUCTION

Fresh strawberries can become contaminated via the application of contaminated irrigation water and the contact with infected harvesters. Meanwhile, the contamination of frozen strawberries may come from an extra human handling during destemming in the field and commingling in the processing facilities (Harris and others 2003; Notermans and others 2004). Besides consuming in fresh and frozen forms, strawberries are also used in others products that were not received additional heat after their addition (Flessa and others 2005). Furthermore, food-related pathogens, such as *Salmonella* spp., *Escherichia coli* O157:H7, and *Listeria monocytogenes* were able to survive in both fresh and frozen strawberries beyond their expected shelf-life (Knudsen and others 2001; Flessa and others 2005). Additionally, the per capita consumption of fresh and processed strawberries increased by 38 percent from 1990 to 2000 (Cook 2002). All of these have raised the concerns about the safety consumption of strawberries and their products. Even though fresh and processed strawberries have been rarely implicated as a vehicle of infection for foodborne illness, outbreaks of hepatitis A virus and Norwalk-like virus were associated with consumption of strawberries in the United States (Dougherty and others 1965; Niu and others 1992; CDC 1997; Hutin and others 1999; Notermans and others 2004). So far, bacterial foodborne illness has never been associated with consumption of strawberries, but the natural reservoir of enteric bacteria such as *Salmonella* and *E. coli* O157:H7 are similar to that of hepatitis A virus (Knudsen and others 2001; Harris and others 2003). Therefore, the contamination of berries with

bacterial pathogens is feasible. Since *L. monocytogenes* is ubiquitous in soil and plant matter, it can be an occasional contaminant in plant grown in close association with soil such as strawberries (Beuchat and Ryu 1997; Brackett 1999).

Application of chlorine and chlorine-based agents has been the practical method for industries to sanitize produce and surfaces, as well as to decrease microbial loads in the water used during the cleaning and packing operations (Delaquis and others 2004). Nevertheless, the reaction of chlorine with naturally present organic matter resulting in the formation of carcinogenic trihalomethanes (THMs) and other chemical residual formed in wastewater returned to the environment (Richardson and others 1998; Xu 1999; Rodgers and others 2004). While the use of chlorine at elevated concentration may deteriorate sensory quality of a product being treated (Suslow 2001), low levels of chlorine may not be effective against certain type of bacteria, protozoan cysts, worm eggs, and viruses (Keswick and others 1984; Beuchat and Brackett 1990; Korich and others 1990). Therefore, there is the current need to search for an alternative disinfecting agent in which has an equivalent or superior in disinfecting effect as well as possess advanced properties to overcome the disadvantages of chlorine usage.

Ozone is the powerful antimicrobial agent that can be applied in both gaseous and aqueous forms. In the United States, it has been recently approved to apply as a direct food additive for treatment, storage, and processing (Khadre and others 2001). Ozone has historically long been used as a sanitizer for water treatment in the European countries since the beginning of this century (Gomella 1972; Pérez and others 1999).

It spontaneously decomposes back to oxygen in a short period of time and leaves no toxic residue; therefore, the concern about chemical residual remained on products after treatment is eliminated (Koseki and Isobe 2006). Many previous studies demonstrated the highly effective of ozone treatment against several food-related pathogenic microorganisms in vitro (Broadwater and others 1973; Rastaino and others 1995; Kim and Yousef 2000; Selma and others 2006); however, the variations in the antimicrobial potential of ozone were reported when it was used to treat fresh and minimally processed fruit and vegetables (Kim and others 1999; Achen and Yousef 2001; Zhang and others 2005; Koseki and Isobe 2006; Selma and others 2006). Pérez and others (1999) suggested that the efficacy of ozone must be individually assessed for each commodity.

The purposes of this research were to evaluate the antimicrobial effect of ozonated water treatment at different exposure times and to determine the efficacy of ozone in controlling the survival and growth of *L. monocytogenes* and *E. coli* O157:H7 inoculated onto strawberries during refrigeration storage.

MATERIALS AND METHODS

Bacterial cultures

Three strains each of *L. monocytogenes* (Scott A, ATCC 19115, and DA-1) and *E. coli* O157:H7 (ATCC 43894, ATCC 43895, and F4546) were used in this study. Stock cultures were maintained at -80 °C before revival in 10 ml tryptic soy broth (TSB) (EMD Chemicals Inc., Gibbstown, N.J., U.S.A.). Bacterial cultures for inoculation

were prepared by sub-cultured for 2 successive d in 10 ml TSB at 37 °C. At 24 h before the experiment, a portion (0.1 ml) of individual strain was transferred into 100 ml TSB and incubated at 37 °C. Count at the end of the incubation period was approximately 10^9 CFU/ml of each strain. The 3-strain cocktail of tested bacteria was prepared by combining the equal portion of each strain to obtain the final cell concentration of approximately 10^9 CFU/ml and used as the test suspension. To verify the initial inoculum level, test suspension was surface plated a 0.1 ml of the appropriate dilution in duplicate on to TSB supplemented with Bacto- Agar (15 g/l) (Difco, Detroit, Mich., U.S.A.), and then incubated at 37 °C for 24 h before enumeration.

Strawberries

Fresh strawberries (*Fragaria x ananassa*) with uniform size, color, maturity, and having a weight range of 20-30 g per fruit were purchased from a local supermarket in Corvallis, Ore., U.S.A. immediately after arrival at the store, and stored for maximum 2 d at 1 °C before test. Fruits were transferred to an ambient temperature (22 ± 1 °C) at least 2 h prior to inoculation with pathogenic bacteria. Calyxes of the strawberries were removed by hands with sterile disposable gloves (Diamond Grip Plus, Reno, Nev., U.S.A.) before placed in 17.78 x 30.48 cm sterile sampling bag (VWR International Inc., Brisbane, Calif., U.S.A.).

Preparation of ozone demand-free glassware and water

Ozone demand-free glassware and water were prepared by following the method of Kim and others (1999) with some modifications. Briefly, all glassware were washed with a mild detergent (Liqui-Nox®, Alconox Inc., White Plains, N.Y., U.S.A.) and thoroughly rinsed with tap water and distilled water. They were then autoclaved at 121 °C for 15 min to remove volatile organic compounds and let dry. Ozone demand-free water was prepared by ozonating distilled water and collected in screw-capped ozone demand-free bottles. The ozonated water was autoclaved at 121 °C for 15 min to remove residual ozone and stored until needed.

Ozone generation and measurement

Ozonated water was generated from the corona discharge ozone generator (CD 12, ClearWater Technologies., San Luis Obispo, Calif., U.S.A.) in which dry air was passed through oxygen generator (OXS 100, ClearWater Technologies, San Luis Obispo, Calif., U.S.A.). The feed gas was supplied to the thermally-protected reaction chamber inside the generator with air flow rate of 7.87×10^{-5} m^3/s. Then, some oxygen molecules were split while passing through the high voltage electrical field to form oxygen atoms that recombined with other oxygen molecules providing ozone molecules (Anonymous 2005). Gaseous ozone was drawn and dissolved in about 6,000 ml recirculated distilled water at room temperature (22 ±1 °C) by means of the injection manifold. Ozonation of water was continued for 20 min and the ozonated water was then collected in screw-capped ozone demand-free bottles and used within

5 min after production. All experimental setup for ozone generation was performed inside a chemical hood (Labconco Corp., Kansas city, Miss., U.S.A.) and all necessary safety precautions were followed. To determine the ozone concentration, approximately 10 ml of ozonated water was dispensed into a 50-ml ozone demand-free glass beaker containing 20 ml ozone demand-free water. The dilution mixture was thoroughly mixed with magnetic stirrer bar for 10 s before ozone concentration was determined by the indigo trisulfonate method with the AccuVac® ampuls (Hach Company, Loveland, Colo., U.S.A.). Briefly, residual ozone is able to decolorize potassium indigo trisulfonate ($C_{16}H_7N_2O_{11}S_3K_3$) and its concentration is inversely proportional to color intensity of ozone-indigo trisulfonate mixture. The decrease in absorbance is measured by photometer at 600 ± 10 nm and it is linear with increasing ozone concentration (Bader and Hoigne 1986; APHA 1998). The pH was measured by a pH meter (Accumet Research AR 10, Fisher Scientific, Pittsburgh, Pa., U.S.A.) coupled with pH electrode (Symphony pH electrode, Thermo Electron Corp., Waltham, Mass., U.S.A.) while the oxidation reduction potential (ORP) was determined with a dual scale pH/ORP meter (Corning 125, Medfield, Mass., U.S.A.) equipped with the platinum redox electrode model 96-78-00 (Thermo Electron Corp., Beverly, Mass., U.S.A.). Dissolved oxygen (DO) was measured by using the YSI model 95 dissolved oxygen meter (YSI Inc., Tellow Springs, Ohio, U.S.A.). All of the physicochemical properties of ozonated water were measured within 5 min after production.

Inoculation of strawberries

To remove native microflora, strawberries were pre-washed for 30 s with sodium hypochlorite (NaOCl) solution containing 250 ppm total chlorine, which was prepared by diluting appropriate amount of commercial bleach solution containing approximately 6% (w/w) of NaOCl (Clorox® Regular Bleach; Clorox Company, Oakland, Calif., U.S.A.) with distilled water, with a fruit to aqueous chlorine ratio of about 1:3 by weight. Then, samples were rinsed twice with distilled water for 1 min to remove residual chlorine. No chlorine residual was ensured by testing the second rinsing water with a Pocket Colorimeter™ chlorine test kit (Hach Company, Loveland, Colo., U.S.A.). Mixed strain cocktail prepared as described earlier was further diluted with Butterfield's phosphate buffer (BP) (pH 7.2) (Hardy Diagnostics, Santa Maria, Calif., U.S.A.) to obtain the test suspension at final concentration of approximately 10^7 CFU/ml in a gallon-sized Ziploc bag (Ziploc®; Johnson & Son, Racine, Wis., U.S.A.). The ratio of fruit to bacterial suspension was 1:3 by weight. Pre-washed strawberries were then dip-inoculated with agitation on the rotary shaker (Environ shaker, Lab-Line, Melrose Park, Ill., U.S.A.) at 100 rpm for 15 min to facilitate uniform inoculation. The liquid suspension was decanted and contaminated strawberries were placed on a sterile aluminum screen under the biosafety Class II hood (Fisher Hamilton Inc., Two Rivers, Wis., U.S.A.) for 30 min at room temperature (22 ± 1 °C) prior to sanitation treatment.

Ozone treatment of strawberries inoculated with *L. monocytogenes* or *E. coli* O157:H7

For enumerating the initial populations of target pathogen on strawberries, 4 randomly selected, inoculated strawberries were put inside sterile sampling bag with 99 ml of BP. After fruit were macerated for 2 min at 230 rpm in a stomacher blender (Stomacher 400 Circulator, Seward, England), fruit homogenate was subjected to the microbiological analysis. The bactericidal activity of ozonated water against *L. monocytogenes* and *E. coli* O157:H7 was determined in both whole fruit tissues and on fruit surfaces. The effect of neutralization after decontamination treatment was also evaluated by placing 4 randomly selected, inoculated strawberries (100 ± 20 g) into a quart-sized Ziploc bag (Ziploc®; Johnson & Son, Racine, Wis., U.S.A.), following the addition of ozonated water at a fruit to treatment solution ratio of 1:3 by weight. The experiment was conducted at ambient temperature (22 ± 1 °C) with the exposure time of 1, 5, or 10 min. After reaching the determined exposure time, strawberries were immediately removed from ozonated water and put into a second Ziploc bag containing the neutralizing buffer (pH 7.2) (Becton Dickinson Co. Sparks, Md., U.S.A.) with the same amount as an aqueous ozone. Fruit were agitated for 2 min at 100 rpm to terminate the disinfection action of ozonated water. Treated fruit were transferred to a sterile sampling bag and mixed with 99 ml of BP. In the experiment without the neutralization step, treated fruit were drained from ozonated water and placed into a sterile sampling bag containing 99 ml of BP directly. Fruits and BP mixture was homogenized by using a stomacher blender at 230 rpm for 2 min. Tissue homogenates were serially diluted in 9 ml BP and duplicate samples of 0.1 ml

aliquots were spread on the appropriate agar media. A 1 ml of neutralizing buffer was also subsequently diluted and 0.1 ml aliquots were surface plated in duplicate to enumerate the viable cells recovered from the surface of the treated samples.

Neutralization is one of the important steps required in the protocol for chemical decontamination treatment. The purpose of neutralization is to terminate the bactericidal and bacteriostatic effect of sanitizing agent at the end of treatment time (Beuchat and others 2001). Moreover, the industrial sanitation practice usually includes rinsing produce after exposure to sanitizers (FDA 2001). Therefore, the results present in this study would be valuable to evaluate the effect of neutralization step to the bactericidal activity of ozonated water on strawberries.

Storage study of inoculated strawberries with *L. monocytogenes* or *E. coli* O157:H7

After inoculation and drying at ambient condition for 30 min, 16 randomly selected strawberries with known weight were placed into a gallon-size Ziploc bag. To obtain the baseline data, 4 contaminated fruits were put into a sterile sampling bag with 99 ml of BP. After pummeled for 2 min at 230 rpm in a stomacher, fruit homogenate was subjected to the microbiological analysis. Contaminated strawberries were immersed in ozonated water with a fruit to treatment solution ratio of approximately 1:3 by weight for 5 min at ambient temperature (22 ± 1 °C), where sample and sanitizing agent were agitated by shaking on a rotary shaker at 100 rpm to ensure even exposure. In this experiment, samples were not rinsed by neutralizing buffer after washing with ozonated water. At the end of washing period, 4 fruits were

randomly selected to determine the number of microorganism immediately following the treatment while the remaining fruits were placed on a sterile aluminum screen under the biosafety class Π hood for 10 min. Then, each set of 4 strawberries was randomly selected and put into the sterile sampling bags and stored at 4 ± 1 °C for 5, 10, and 15 d before conducting microbiological analysis. Populations of *L. monocytogenes*, *E. coli* O157:H7, mesophilic aerobic bacteria, yeasts, and molds were assessed by the protocol as indicated later.

Microbiological analyses

Spread plate technique was applied for all experiments in this study. Populations of *L. monocytogenes* were recovered on modified Oxford agar (MOX) (Becton Dickinson and Co., Sparks, Md., U.S.A.) with 48 h incubation at 37 °C. MacConkey sorbitol agar (Becton Dickinson and Co., Sparks, Md., U.S.A.) supplemented with cefixime-tellurite (Dynal Biotect A.S.A, Oslo, Norway) (CT-SMAC) was used as a selective media for recovering *E. coli* O157:H7 and plates were incubated at 37 °C for 24 h before colonies were counted. The number of mesophilic aerobic bacteria was determined by plating on plate count agar (PCA) (Becton Dockinson and Co., Sparks, Md., U.S.A.), and incubated at 37 °C for 48 h. Dichloran Rose Bengal Chloramphenicol agar (DRBC) (EMD Chemicals Inc., Gibbstown, N.J., U.S.A.) was used to enumerate yeast and mold. The DRBC agar plates were incubated in the dark at 25 °C for 5 d before yeast and mold colonies were counted.

Statistical analysis

In the study of the exposure time effect, experiments were performed in triplicate whereas storage study experiment was replicated 6 times. Population means and standard deviations were calculated and analyzed using a GLM procedure of the Statistical Analysis System version 9.1 (SAS Institute, Cary, N.C., U.S.A.). Comparisons of means were determined with Tukey's Studentized Range (HSD) test at a significant level of 0.05.

RESULTS AND DISCUSSION

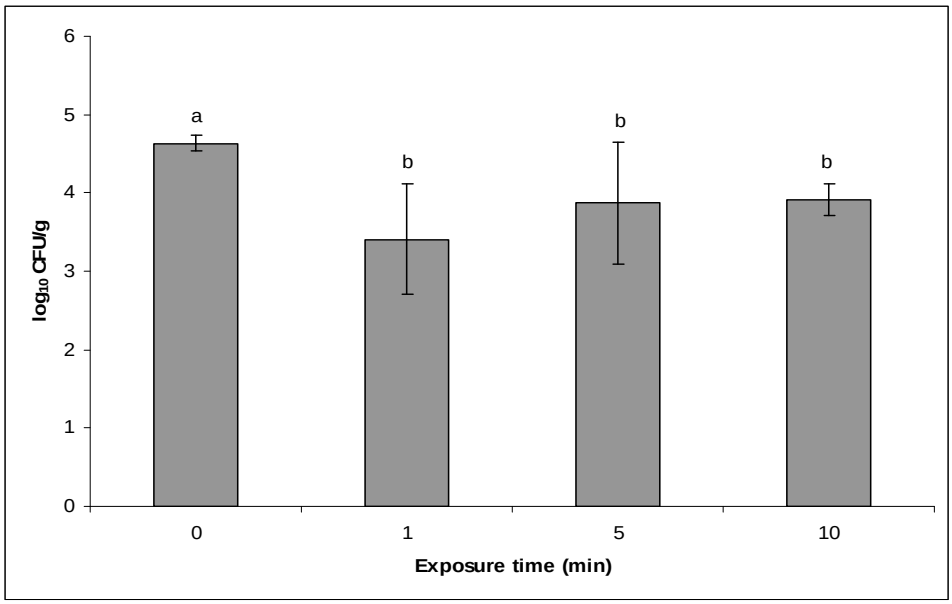
Effect of exposure time with ozonated water treatment

Ozonated water used for decontaminating strawberries in this study has a pH 3.99, ORP 738 mV, DO 8.09 ppm, and ozone concentration of 1.90 ppm. Figures 4.1 and 4.2 show the number of viable *L. monocytogenes* and *E. coli* O157:H7 cells recovered from whole fruit homogenates after washing with ozonated water for various exposure times, and then rinsing with neutralizing buffer (Figs. 4.1(a) and 4.2 (a)) or without neutralization step (Figs. 4.1(b) and 4.2 (b)), respectively. The mean populations of *L. monocytogenes* and *E. coli* O157:H7 on inoculated fruit following inoculation and dry for 30 min were equal at 4.82 $\log_{10}$ CFU/g.

The neutralization following treatment did not significantly affect ($p>0.05$) the bactericidal activity of ozonated water against *L. monocytogenes* and *E. coli* O157:H7. The maximum $\log_{10}$ CFU/g reduction of *L. monocytogenes* and *E. coli* O157:H7 in the whole fruit tissues were 1.22 and 0.74 after washing with ozonated water following

neutralization, respectively (Fig. 4.1(a) and 4.2(a)), while up to 0.68 and 0.84 $\log_{10}$ CFU/g reduction were observed, respectively, on samples without neutralization. The number of bacterial cells in the rinsing neutralizing buffer represented the number of bacteria removed from the surface of untreated and treated strawberries. The populations of *L. monocytogenes* and *E. coli* O157:H7 on fruit surfaces that survived the ozonated water treatment are exhibited in Fig. 4.3. The highest reduction in surface numbers of *L. monocytogenes* and *E. coli* O157:H7 were 2.17 and 2.02 $\log_{10}$ CFU/ml of rinse fluid on samples treated for 10 min, respectively.

a



b

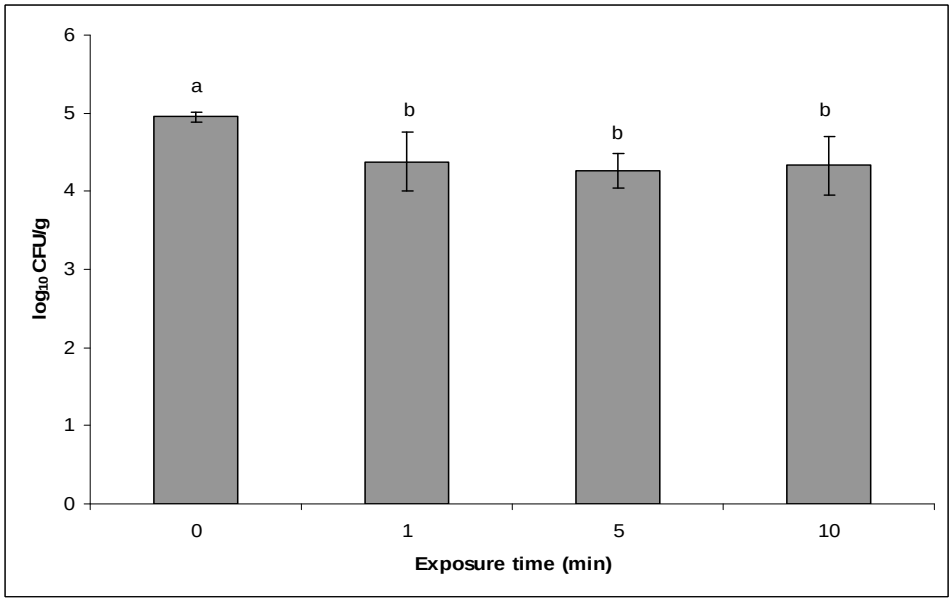
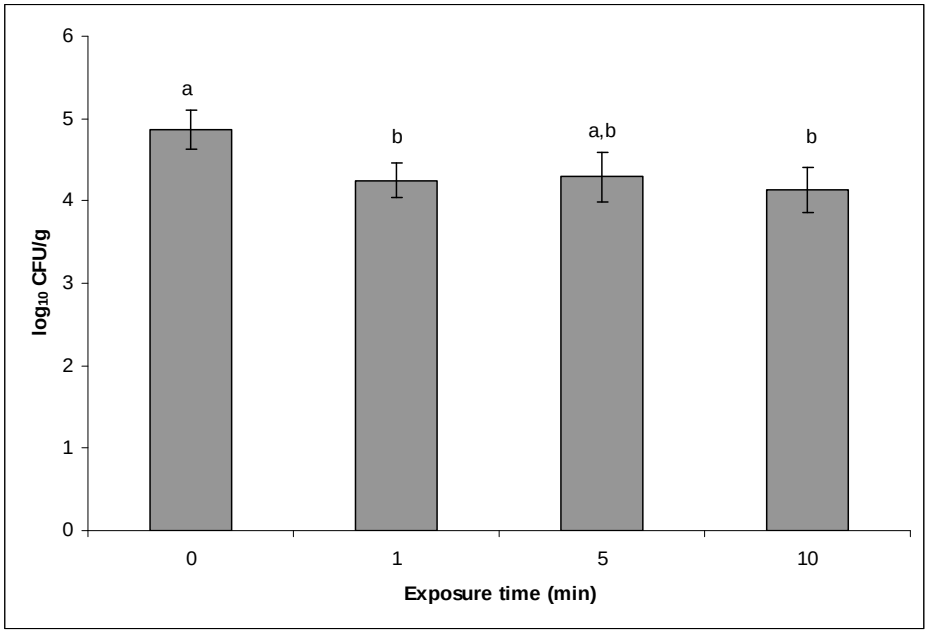


Figure 4.1 Populations of *L. monocytogenes* recovered from whole strawberry tissues after exposure to ozonated water at various times: (a) rinsing with neutralizing buffer after treatment; (b) without rinsing with neutralizing buffer. Vertical bars indicated standard deviation. Samples with different superscripts were significantly different ($p<0.05$).

a



b

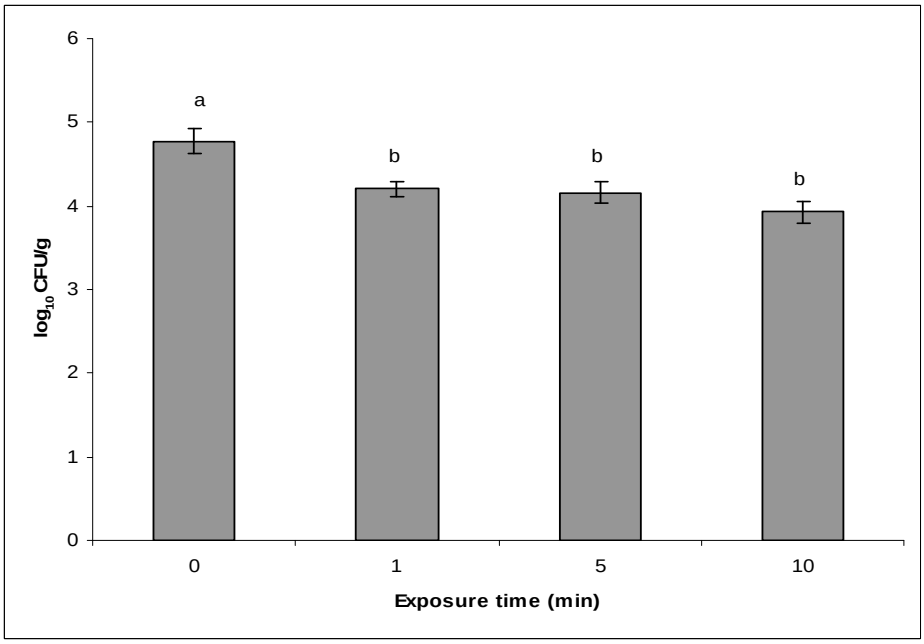
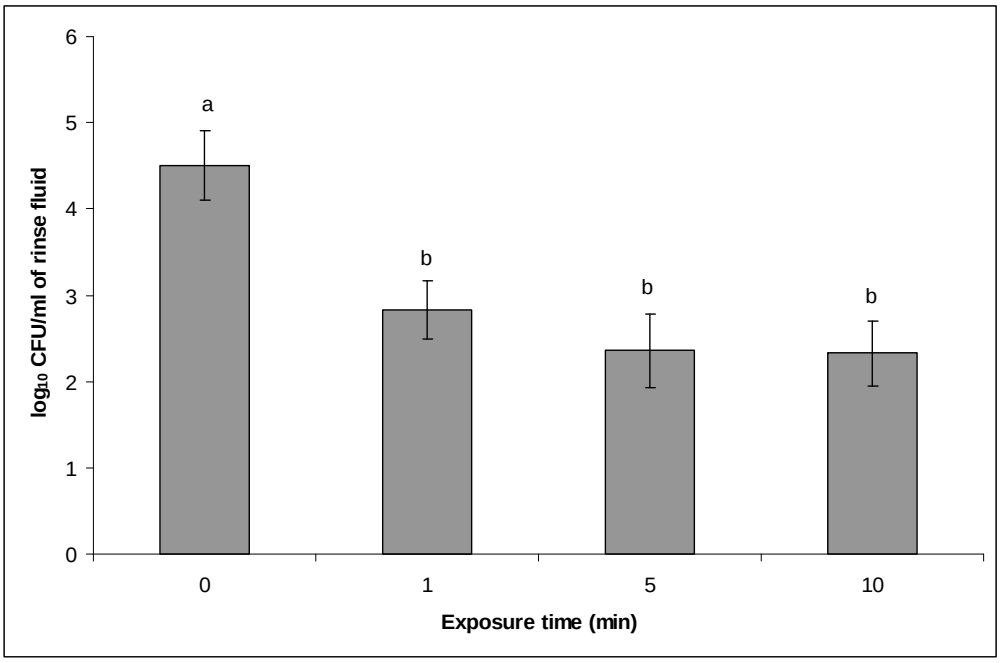


Figure 4.2 Populations of *E. coli* O157:H7 recovered from whole strawberry tissues after exposure to ozonated water at various times: (a) rinsing with neutralizing buffer after treatment; (b) without rinsing with neutralizing buffer. Vertical bars indicated standard deviation. Samples with different superscripts were significantly different ($p<0.05$).

a



b

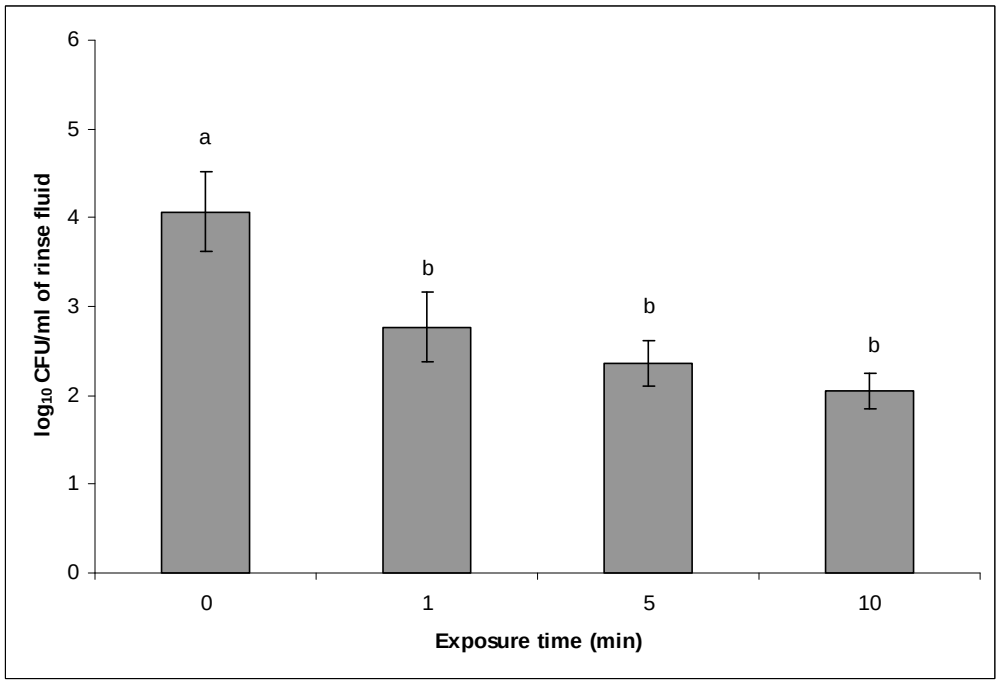


Figure 4.3 Populations of (a) *L. monocytogenes* and (b) *E. coli* O157:H7 recovered from strawberry surfaces after exposure to ozonated water at various times. Vertical bars indicated standard deviation. Samples with different superscripts were significantly different ($p<0.05$).

All experiments in the current study indicated that inactivation of *L. monocytogenes* and *E. coli* O157:H7 on strawberries did not increase significantly ($p > 0.05$) along with increased exposure time in both whole fruit tissues and fruit surfaces regions. These results were consistent with the study of Achen and Yousef (2001) in which dipping apples inoculated with *E. coli* O157:H7 in ozonated water (22-24 ppm ozone) or washing contaminated fruit in bubbling ozone water (21-28 ppm ozone) for 1, 3, or 5 min did not give a significant difference in lowering the number of *E. coli* O157:H7 attached to the surfaces and the stem-calyx areas of apples. Koseki and Isobe (2006) observed that ozone concentration in ozonated water without overflow decreased over time when added produce being treated. In our study, strawberries were dipped in predetermined volume of ozonated water for up to 10 min; thus, gradual reduction in residual ozone concentration during treatment may explain the undistinguished bactericidal activity of aqueous ozone at extended exposure times. Previous studies suggested that the key point to enhance disinfection potential of ozone was to maintain residual ozone concentration during treatment by bubbling ozone to the produce-water mixture rather than dipping foods to the pre-ozonated water (Hurst 1993; Kim and others 1999; Wade and others 2003).

The insignificant differences in the antibacterial efficacy of ozonated water against *L. monocytogenes* and *E. coi* O157:H7 with or without neutralization procedure may be attributed to the removal of bacterial cells loosely attached to fruit during rinsing with neutralizing buffer. At the same time; however, the residual ozone present on

fruit surfaces after washing may exhibit the bactericidal effect during homogenizing fruit sample or before serially dilution.

Ozone exhibited a strong disinfection effect against microorganisms suspended in pure water or buffer solution (Broadwater and others 1973; Korich and others 1990; Rastaino and others 1995; Selma and others 2006); however, the limitation in its efficacy to remove or eliminate naturally occurring and pathogenic microorganisms on fresh produce has been previously reported (Kim and others 1999; Achen and Yousef 2001; Sharma and others 2002; Wade and others 2003; Selma and others 2006). Kim and others (1999) suggested that food may contribute organic matter imposing an ozone demand, and then competes for ozone with microorganisms being inactivated. Cho and others (2003) also proposed that it was difficult to predict ozone reaction in the presence of organic matter since its decomposition was dependent on the types of organic matter present.

The inaccessibility of ozonated water to bacterial cells embedded in the cracks, crevices, stomata, or penetrated to the internal structure of samples may attribute to the limited bactericidal activity of ozonated water against investigated bacteria inoculated onto strawberries. Ogawa and others (1990) reported that spores of *Botrytis cinerea* on the surface of uninjured tomatoes were eliminated when treated with 3.8 ppm ozonated water for 10 min whereas these fungal spores inoculated on injured surfaces were not inactivated. Similar results were demonstrated by Spotts and Cervantes (1992) in which ozone treatment (5.5 ppm) for 5 min cannot control decay in wound-inoculated pear fruits inoculated with *Penicillium expansum*. Seo and Frank (1999)

suggested that efficacy of disinfectant treatment depends on the accessibility between the active sanitizing agent and target microorganisms.

Ozonated water for controlling the growth of *L. monocytogenes* and *E. coli* O157:H7 on fresh strawberries during refrigeration storage

Changes in *L. monocytogenes*, *E. coli* O157:H7, mesophilic aerobic bacteria, and yeast and mold populations recovered from *L. monocytogenes* or *E. coli* O157:H7 inoculated strawberries during storage at 4 ± 1 °C for up to 15 d are summarized in Table 4.1 and 4.2, respectively. Following the 5 min treatment with ozonated water (~ 1.90 ppm), 0.46 and 0.44 $\log_{10}$ CFU/g reduction of *L. monocytogenes* and *E. coli* O157:H7 were observed, respectively. Degree of *L. monocytogenes* and *E. coli* O157:H7 inactivation in the storage study after treatment with ozonated water for 5 min was less than that achieved at the same contact period in the exposure time study. In addition, the number of *L. monocytogenes* did not significantly decrease ($p>0.05$) compared with untreated samples. The higher number of fruit per treatment in the storage study may be attributed to these different results. Although the ratio by weight of strawberry samples to ozonated water was kept the same (approximately 1 to 3), the low level of residual ozone applied in this experiment may not compensate for the increase in organic matter loads. Therefore, a further development for the appropriate treatment protocol for ozone generation, ozone delivering system to produce, ratio of ozonated water to strawberries, and other conditions during washing is desirable.

Table 4.1 Populations of microorganisms [a] (log_{10} CFU/g) recovered from *L. monocytogenes* inoculated strawberries after treatment with ozonated water and storage at 4 ± 1 °C

Type of microorganisms	Exposure time (min)		Storage time (d) after 5 min exposure		
	0	5	5	10	15
L. monocytogenes [b]	5.40 ± 0.45 **A**	**a** 4.94 ± 0.53 **A**	**b** 3.73 ± 0.69	**c** 2.41 ± 0.73	**c** 2.52 ± 0.70
Mesophilic aerobic bacteria [c]	5.51 ± 0.27 **A**	**a** 5.00 ± 0.30 **B**	**A,b** 4.31 ± 0.43	**b** 3.80 ± 0.79	**b** 3.75 ± 0.58
Yeast and mold [d]	3.25 ± 0.52 **A**	**a** 3.08 ± 0.79 **A**	**a** 3.25 ± 0.57	**a** 3.22 ± 0.71	**a** 3.27 ± 0.50

[a] Values were means ± standard deviation of 6 replications (n = 6) in which each replication contained 4 strawberries.
[b] Populations of *L. monocytogenes* detected by spread plating with MOX.
[c] Populations of mesoplilic aerobic bacteria detected by spread plating with PCA.
[d] Populations of yeast and mold detected by spread plating with DRBC.
Means preceded by the same lowercase letters in the same row within each type of microorganism were not significantly different ($p>0.05$).
Means followed by the same capital letters in the same row within each type of microorganism were not significantly different ($p> 0.05$).

Table 4.2 Populations of microorganisms [a] (log_{10} CFU/g) recovered from *E. coli* O157:H7 inoculated strawberries after treatment with ozonated water and storage at 4 ± 1 °C

Type of microorganisms	Exposure time (min)		Storage time (d) after 5 min exposure		
	0	5	5	10	15
E. coli O157:H7 [b]	5.07 ± 0.15 **A**	**a** 4.63 ± 0.28 **B**	**b** 3.39 ± 0.64	**c** 2.12 ± 0.58	**c** 1.90 ± 0.38
Mesophilic aerobic bacteria [c]	5.11 ± 0.13 **A**	**a** 4.59 ± 0.18 **B**	**b** 3.80 ± 0.45	**c** 3.08 ± 0.37	**b,c** 3.21 ± 0.46
yeast and mold [d]	2.69 ± 0.14 **A**	**a** 2.48 ± 0.28 **A**	**a** 2.66 ± 0.56	**a** 3.10 ± 0.60	**a** 3.20 ± 0.51

[a] Values were means ± standard deviation of 6 replications (n = 6) in which each replication contained 4 strawberries.
[b] Populations of *E. coli* O157:H7 detected by spread plating with CT-SMAC.
[c] Populations of mesoplilic aerobic bacteria detected by spread plating with PCA.
[d] Populations of yeast and mold detected by spread plating with DRBC.
Means preceded by the same lowercase letters in the same row within each type of microorganism were not significantly different ($p>0.05$).
Means followed by the same capital letters in the same row within each type of microorganism were not significantly different ($p> 0.05$).

Populations of *L. monocytogenes* and *E. coli* O157:H7 decrease significantly ($p<0.05$) with the long-term storage at 4 ± 1°C. The total $\log_{10}$ CFU/g reductions after 15 d storage were 2.42 and 2.73 for *L. monocytogenes* and *E. coli* O157:H7, respectively. Mesophilic aerobic bacteria declined to the similar extent as the target inoculated bacteria after a 5 min treatment with ozonated water. Owing to the fact that *L. monocytogenes* and *E. coli* O157:H7 are able to grow on PCA, bacterial counts referring as mesophilic aerobic bacteria present in this study were made up of the intentionally inoculated bacteria and background microflora that survived pretreatment with 250 ppm NaOCl solution. As observed in Table 4.1 and 4.2, populations of aerobic mesophile counts were very similar to the numbers of *L. monocytogenes* and *E. coli* O157:H7, respectively, at the beginning of storage time. However, as inoculated fruit stored at refrigeration temperature for a long period of time, both *L. monocytogenes* counts on MOX and *E. coli* O157:H7 counts on CT-SMAC were considerably lower than the number of bacteria detected on PCA. These may result from the growth of native microflora competed with pathogenic bacteria or the latter were injured due to low temperature or acidic condition of fruit samples.

The antifungal property of ozone was very minimal in the present study. Treatment with ozonated water (~ 1.90 ppm) for 5 min failed to significantly decrease ($p>0.05$) yeast and mold populations on *L. monocytogenes* and *E. coli* O157:H7 inoculated strawberries compared to untreated fruit. Increasing in the number of yeast and mold on fruit samples inoculated with both pathogenic bacteria during

refrigeration storage was observed; however, differences in counts over the prolongation of storage were not significant ($p>0.05$). Gaseous ozone treatment (0.35 ppm) was reported to be inefficient in preventing fungal decay in strawberries after 4 d storage at 20°C (Pérez and others 1999). Moreover, re-growth of yeast and mold on strawberries after washing with ozonated water (3 ppm) and subsequently storing at refrigerated condition also informed by Rodgers and others (2004).

Besides the presence of naturally occurring organic matter from the produce being treated as described earlier, the inaccessibility of ozone to sites harboring target cells on food surfaces and the attachment of inoculated bacteria on the rough surface areas were the other reasons explaining for the limited efficacy of ozone treatment on produce (Kim and others 1999; Achen and Yousef 2001; Wade and others 2003). Achen and Yousef (2001) reported the lesser efficiency of ozone when used to treat the stem/calyx region of apples compared with the fruit surfaces. Strawberries have an irregular shape and complicated morphological structure containing of 5 tissue zones, epidermis, hypodermis, cortex, bundle, and pith, also numerous achens (seed) embedded in epidermis rendering surface uneven and complex (Szczesniak and Smith 1969; Han and others 2004; Koseki and others 2004). The complex and delicate nature of strawberry surfaces hampered the removal of microorganisms after contamination (Flessa and others 2005). Typically, 1-2 log reductions were achieved after treatment depending on the sanitizers used and types of inoculated organisms (Yu and others 2001; Lukasik and others 2003). The minimal efficiency of ozone used to decontaminate strawberries was also reported by Koseki and others (2004);

populations of naturally present aerobic mesophiles and fungi were reduced by only 0.4 and 0.9 $\log_{10}$ CFU/ fruit after washing in ozonated water (5 ppm) for 10 min, respectively. The rough surfaces and presence of numerous seeds of strawberries had been reported to provide attachment sites for bacteria, and then diminish the efficacy of chemical sanitizers (Yu and others 2001).

CONCLUSIONS

The ozone treatment on strawberries inoculated with *L. monocytogens* or *E. coli* O157:H7 was not remarkably effective in removing and eliminating pathogens on the whole fruit tissues. Meanwhile, pathogenic bacteria populations were significantly reduced following the treatment compared to the untreated fruit regardless of exposure time. More than 2 $\log_{10}$ CFU/ml of rinse fluid reduction of *L. monocytogenes* and *E. coli* O157:H7 on fruit surface regions were achieved with ozonated water washing. Sanitizing with ozone cannot be relied on controlling spoilage and pathogenic microorganisms on strawberries during refrigeration storage if fruit were contaminated with a high level of bacteria as demonstrated in this study. Further study to improve the reliable method for enhancing the antimicrobial properties of ozone on fresh strawberries is required.

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CHAPTER 5

General Conclusion

Electrolysis of dilute sodium chloride (NaCl) solution generates electrolyzed oxidizing (EO) water within the anode compartment of an electrolyzed water generator. It contains the aggregation of three distinct antimicrobial factors including low pH, presence of active chlorine species, and high oxidation reduction potential. This study concluded that EO water generated from the electrolysis of higher NaCl solution concentration (0.10% w/v) had a greater antibacterial effect against naturally occurring aerobic mesophiles on strawberries than that prepared from lower NaCl level (0.05% w/v). In comparison with sodium hypochlorite (NaOCl) solution that contained almost 4 times higher in chlorine concentration than EO water, EO water had an equivalent bactericidal activity against *Listeria monocytogenes* and *Escherichia coli* O157:H7 on inoculated strawberries. This result may support the application of EO water as an alternative disinfection method of chlorine to improve the microbiological safety of fresh strawberries.

The study on the bactericidal activity of ozonated water containing approximately 1.90 ppm ozone against *L. monocytogenes* and *E. coli* O157:H7 on

inoculated strawberries resulted in significant reduction in both pathogens regardless of the exposure time. However, extension of exposure time from 1-10 min did not indicate an increase in antibacterial activity of ozone treatment and the limited effect on inactivating both pathogens on whole strawberry tissues was observed. Therefore, the antimicrobial efficacy of ozonated water at higher ozone concentration should be further studied. More studies are necessary to develop ozone treatment procedure for improving its ability to eliminate pathogenic bacteria contaminated on fresh strawberries as well as for simple and practical operation.

Data obtained from this study also indicated that prior washing inoculated strawberries with disinfecting agents did not affect the survival of *L. monocytogenes* and *E. coli* O157:H7 during refrigeration storage beyond the expected market shelf-life. Therefore, preventing contamination in the field and during postharvest handling is essential to reduce the risk of foodborne disease outbreaks from strawberry consumption.

For further understanding the effect of disinfecting agents on the quality of surface structures of strawberries as well as the former microbicidal effects on microorganisms embedded in the natural openings or interior tissues, microscopic observations on the surface and sub-surface regions of strawberries after washing should be performed Additional research should also be conducted to determine the effect of EO water and ozonated water treatments on sensory characteristics of strawberries.

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