SURVIVAL AND GROWTH OF *LISTERIA MONOCYTOGENES* ON ROMAINE LETTUCE AS INFLUENCED BY CRISPING AND MISTING

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ABSTRACT OF THE THESIS

Survival and Growth of *Listeria monocytogenes* on

Romaine Lettuce as Influenced by Crisping and Misting

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Listeria monocytogenes is a foodborne pathogen of particular concern for manufacturers of refrigerated fresh produce because of its wide distribution in the environment and its ability to grow at refrigeration temperature. Approximately, two-thirds of supermarkets implement crisping and misting to enhance product appearance and quality. Based on the FDA food code, water used for soaking, submersion, hydrating, or crisping does not need to contain chemical sanitizers. When product is submerged in water, cross-contamination may occur. Therefore, the impact of crisping in water with and without sanitizer was investigated.

The study was coupled with determining the influence of misting on growth and survival of *L. monocytogenes.* Romaine lettuce was inoculated with *L. monocytogenes* cocktail to achieve initial population of ca. 5.5 log CFU/g, heads were

submerged in tap water or tap water containing electrolyzed water for 5 min and then held at 5 ^{o}C for 2 h to crisp. Half of the crisped lettuce heads were placed in a refrigerated commercial display cabinet and misted for 24 h. Lettuce was then transferred to a refrigerator (5 ^{o}C and 15 ^{o}C) and held for 7 days. The population of *L. monocytogenes* and psychrotrophic bacteria was determined at day 0, 1, 4, and 7 for each treatment and temperature.

The results showed that crisping in electrolyzed water significantly reduced (P<0.05) the population of *L. monocytogenes* by 2.93 log compared to tap water alone (1.32 log reduction). An additional 1 log reduction in population of *L. monocytogenes* occurred after 24 h misting. The population of *L. monocytogenes* remained relatively constant during 7 days of refrigerated storage. There was no significant difference in population of *L. monocytogenes* on lettuce held at 5^{o}C or 15^{o}C. Crisping and misting treatments reduced the population of psychrotrophic bacteria on lettuce. The psychrotrophic bacteria population was greater on lettuce held at 15^{o}C, reaching 6.99-7.54 log CFU/g after 7 days.

Results of the present study suggest that crisping treatment, especially with electrolyzed water sanitizer significantly reduced the population of *L. monocytogenes* on romaine lettuce. Misting also had a negative effect on the survival and growth of *L. monocytogenes*. Based on the methods used in the present study, the practice of crisping with a commercial sanitizer and misting may enhance the microbial safety of commodities sold at retail supermarkets.

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DEDICATION

To my parents,

Ming Guo and Hongqi Wu

And my husband,

Yi Cao

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1. INTRODUCTION

Fresh produce consumption in the United States increased substantially during the past decades due to an increased awareness of consuming healthy diets. However, along with the growing demands for fresh produce consumption, there are increasing numbers of foodborne illness outbreaks associated with fresh produce throughout the world. According to the Center for Science in the Public Interest, leafy greens were regarded as one of the top riskiest FDA-regulated foods in 2009 (CSPI, 2009). Specific types of fruits and vegetables have been identified as vehicles for foodborne pathogens including *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes*. Several notable causes for contamination have been proposed. Leafy greens can be contaminated through the soil, animal fecal matter and agricultural water for example irrigation water. Cross-contamination during handling and processing of fresh produce has also been identified as a source (Brackett, 1999).

L. monocytogenes is one of the deadliest foodborne pathogen which is associated with a high hospitalization rate and the mortality rate is 20 to 25% (Montville and Matthews, 2005). *L. monocytogenes* is commonly found in soil and linked to decaying plants. The pathogens' cells can under appropriate conditions enter into the plant through cuts, abrasions and natural openings. Several foodborne outbreaks have been linked to the contamination of *L. monocytogenes* on fresh vegetables and fruits. A particularly devastating outbreak was associated with cantaloupe in United States which caused 33 deaths among 147 infected cases (CDC,

2012). Different from other pathogenic organisms, *L. monocytogenes* can survive and grow at refrigeration temperatures. Several studies demonstrate the ability of *L. monocytogenes* strains to thrive on the fresh vegetables at refrigerator conditions (Jacxsens et l., 2002; Carrasco *et al.*, 2008; Francis and Beirne, 1997). Therefore, *L. monocytogenes* represents a key microbiological hazard for RTE vegetables or raw produce stored under refrigeration conditions.

To minimize cross contamination incidences during handling and crisping of fresh produce at retail stores, addition of chemical sanitizers to process water is recommended by the FDA guide for fresh fruits and vegetables (FDA, 2008). Nevertheless, the trend is toward moving away from the use of chlorine due to its by-products and other chemical sanitizers, and because of associated environmental risks (Gil *et al*., 2009). Electrolyzed water has been investigated as a chlorine alternative for fresh produce washing. Ascribe to the neutral pH value, slightly acidic electrolyzed water is more eco-friendly and causes less impact on the users' health (Huang *et al.*, 2008). The bactericidal efficacy of neutral electrolyzed water has been investigated on fresh produce (Issa-Zacharia *et al.*, 2011; Park *et al.*, 2001; Yang, Swem and Li, 2003). But most experiments were carried out under laboratory conditions and applied on cut leaves of vegetables. Misting cabinets are now widely implemented in the retail supermarkets to keep the moisture and improve the appearance of fresh produce. However, there are limited studies describing the impact of misting on the survival and growth of a specific pathogen types on the leafy green vegetables.

This study evaluated the antimicrobial efficacy of electrolyzed water on whole heads of romaine lettuce under simulated commercial crisping conditions. The impact of commercial misting and storage temperature on the survival and growth of *L. monocytogenes* on romaine lettuce was investigated as well.

2. HYPOTHESIS AND OBJECTIVE

The hypothesis of this research is that utilizing sanitizer during the soaking step of crisping will significantly limit cross-contamination and improve microbial safety of romaine lettuce, and misting may increase survival and growth of *L. monocytogenes* on romaine lettuce during retail display and in-home storage.

The specific objectives are:

1. To evaluate the antimicrobial efficacy of electrolyzed water against *L. monocytogenes* on romaine lettuce
2. To determine the impact of electrolyzed water crisping on microbial quality of romaine lettuce during 7 days storage
3. To identify the impact of misting treatment and storage temperature on the survival and growth of *L. monocytogenes* on romaine lettuce during 7 days

3. LITERATURE REVIEW

3.1 Fresh Produce Industry

In recent decades, there has been an increased demand for leafy green vegetables and Ready-to-Eat (RTE) salad mixes since consumers eating habits are changing as part of a healthier lifestyle. Changes in income distribution, age demographics and household size have also contributed to the growing demand for fresh produce since 1980s (Cook, 1990). U.S. fresh produce markets have grown markedly since the 1980s. Producers have responded with increased domestic production, increased importation and improvement in methods to maintain the quality of produce. According to USDA report, per capita consumption of fresh fruits and vegetables increased 6 percent between 1987 and 1995, and 8 percent between 1995 and 2000 (Dimitri, Tegene and Kaufman, 2003). The most recent data indicated that fresh produce was an estimated $27 billion market in 2015, and retail dollar and volume sales are still increasing (PMA, 2014).

Leafy greens are full of antioxidants, vitamin C, polyphenols, vitamin E and carotenoids. It has already been demonstrated that consumption of leafy greens is closely associated with a lowered risk of cancer and cardiovascular diseases common in the Western countries. Leafy greens' carotene or provitamin A content contribute to preventing the cellular damage leading to the development of cancer (Colditz e*t al.*, 1985; Nicolle e*t al.*, 2004). Research also shows that fresh vegetables and fruits play a positive role in decreasing the risk of heart disease, stroke, hypertension, and

cataracts. Besides, leafy greens are rich sources of macro and micro elements such as calcium and phosphorus (Duyn and Pivonka, 2000; Gupta *et al.*, 1989). Lettuce consumption may result in increased cholesterol metabolism and antioxidant status due to its rich fiber moiety and antioxidants (Nicolle e*t al.*, 2004).

However, along with the growing demand for fresh produce, there is increasing concern about safety (chemical and biological) issues because fruits and vegetables, especially raw or minimally processed leafy greens, are considered to be vehicles for human pathogens capable of causing illness. Foodborne pathogen outbreaks associated with contaminated fresh produce haven been increasingly recognized all over the world. For example, several *Salmonella* outbreaks have been linked to the consumption of tomatoes, and tomatoes have been well documented as a vehicle for *Salmonella*. *E.coli* O157: H7, *Listeria*, *Cyclospora*, *Shigella*, etc. are all commonly reported to be associated with leafy green vegetables (Table 1). Leafy greens were identified as the most risky food regulated by FDA (CSPI, 2009). The sources of contamination can be wide and complex. Fresh produce can be easily contaminated with pathogenic organisms through contact with soil, untreated irrigation water or sewage, animal, or cross-contaminated during any processing step. Therefore, fresh produce can become contaminated at any point from the farm to the table (Berger *et al*., 2010; Cook, 1990; Doyle and Erickson, 2008; Taban and Halkman, 2011; Lynch et al., 2009).

Table 1. Examples of some outbreaks of infections epidemiologically associated with leafy green vegetables (Taban and Halkman, 2011).

Type of leafy green vegetable	Microorganism	Year	Location
Lettuce	*Escherichia coli* O157:H7	1995	USA
	Listeria monocytogenes	1979	USA
	Shigella sonnei	1983	USA
	Salmonella Typhimurium DT104	1994	Norway
	Salmonella Newport	2005	Spain
	Campylobacter jejuni	1996	USA
Baby lettuce leaves	*Cyclospora cayatenansis*	1997	USA
Shredded lettuce	*Shigella sonnei*	1986	USA
Iceberg lettuce	*Shigella sonnei*	1994	Norway, Sweden, UK
	Escherichia coli O157:H7	1995	Canada
Lettuce salad	Hepatitis A	1986	USA
	Giardia	1989	USA
Parsley	*Shigella sonnei*	1998	USA
Basil	*Salmonella* Senftenberg	2007	Israel
	Cyclospora cayatenansis	1997	USA

Due to the growing concern about the microbial safety of fresh produce, FDA has issued a series of guidance documents on how to handle fresh produce. Based on the 2013 food code issued by FDA, raw fruits and vegetables shall be thoroughly washed in water to remove soil and other contaminants before being cut (FDA, 2013). The FDA food code points out the importance of using antimicrobial chemicals to minimize the potential for microbial contamination (FDA, 2008). Guidance on crisping and cold storage for industry and retailers was developed by the Produce Marketing Association (PMA) (PMA, 2006). Two farmers markets and two retail supermarkets in the New Brunswick area were surveyed on handling practices for whole heads of fresh lettuce. Typically, lettuce heads were submerged in tap water

for about 5-10 minutes and then placed in a cooler for several hours depending upon type of leafy greens to give a fresh and crisp look. Leafy greens including romaine lettuce are often crisped since they easily dehydrate during transport. However, there is no specific control measure that will completely eliminate the risk of cross-contamination. Therefore, the fresh produce industry has the challenge to improve the microbial safety of leafy green vegetables (Taban and Halkman, 2011).

3.2 L. monocytogenes

3.2.1 Listeriosis

L. monocytogenes is a gram-positive, nonsporeforming, facultatively anaerobic rod-shaped bacterium which can grow between -0.4 and 50 ^{o}C (Farber and Peterkin, 1991). Besides, *L. monocytogenes* is one of the top three deadliest foodborne pathogens, the other two are *Salmonella* and *Toxoplasma*. *Listeria* infections were associated with a high hospitalization rate and caused around 27% of reported foodborne illness related deaths (CDC, 2011; Mead *et al*., 1999).

Listeriosis has become a major foodborne disease which can be caused by the consumption of the food contaminated with *L. monocytogenes*. Listeriosis incidence rates are higher in older adults, pregnant women, neonates and those who with certain immunocompromising and chronic conditions (Cartwright *et al*., 2013). *L. monocytogenes* can cause mild gastroenteritis, septicemia, and meningitis in non-pregnant adults. Infected pregnant women may suffer non-specific flu-like illness including fever, headache or even may remain asymptomatic. But it can result in

stillbirth and cause abortion of the fetus. Though listeriosis is rare, it's severe and little is known about sporadic listeriosis which in fact, causes the majority of human infections (Allerberger and Wagner, 2010; Schuchat *et al.*, 1992). Different from other foodborne pathogens which excrete toxins in blood, *L. monocytogenes* can manipulate host cell actin polymerization and spread directly to nearby cells. The intracellular growth and cell-to-cell spread of *L. monocytogenes* are mediated by the membrane-associated bacterial protein ActA (Robbins *et al.*, 1999; Gaillard *et al*., 1991).

3.2.2 Distribution in environments and specific foods

L. monocytogenes is widely spread in the environment. It is present in many animals including humans. Therefore, it can be isolated from feces of these animals. *L. monocytogenes* probably is the most prevalent disease-causing microorganism found in soil and it has been demonstrated to be found in sewage sludge or irrigation water (Beuchat and Ryu, 1997). So it is not surprising that the organism occurs on fresh produce and minimally processed vegetables due to the contamination from decaying vegetation, soil, sewage water or food processing plants. The possible cycle of *L. monocytogenes* between vegetables and humans is illustrated in Figure 1 (Montville and Matthews, 2005; Al-Ghazali and Al-Azawi, 1990; Beuchat, 1996).

There is limited research describing the internalization of *Listeria* in plants, most studies have been carried out on *E. coli and Salmonella*. But there are two potential pathways for *L. monocytogenes* entering plant tissues. One is through the

natural opening and through wounds of physical damage on the plant surface. Alternatively, the organisms may enter through the root system (Deering, Mauer and Pruitt, 2012; Berger *et al*., 2010; Rees, Dodd and Nwaiwu, 2013).

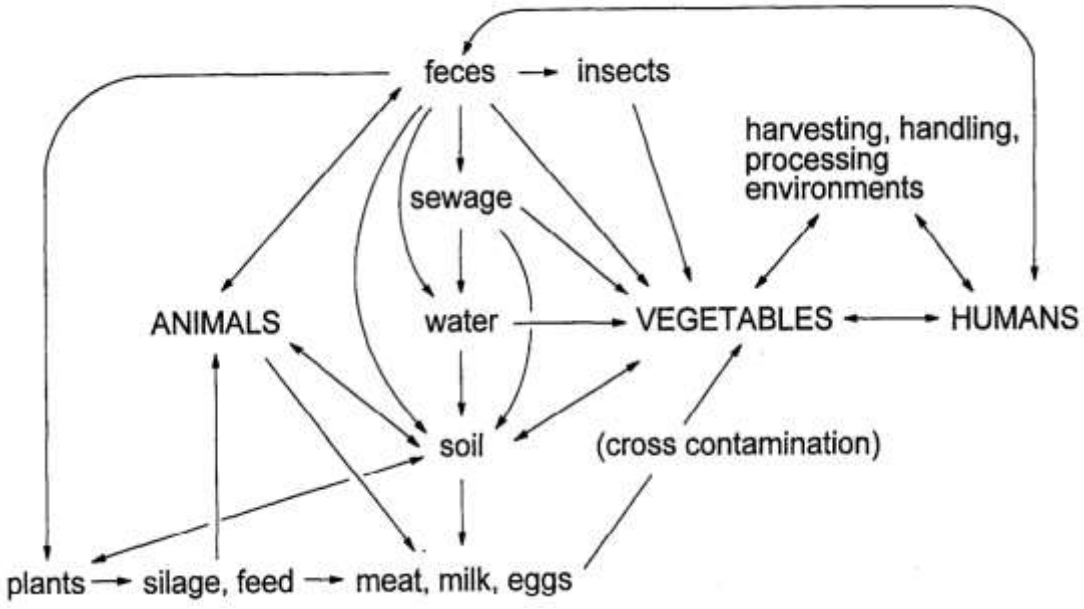


Figure 1. Potential Pathway of *L. monocytogenes* between vegetables and human (Beuchat, 1996)

L. monocytogenes has been shown to be associated with a diverse array of food sources and it's a major cause of recalls in United States (Donnelly, 2001). It's a foodborne pathogen of particular concern for manufacturers of refrigerated Ready-to-Eat (RTE) foods such as salad, soft cheeses, deli meat or frankfurters. *L. monocytogenes* can grow at refrigeration temperature, which is unique from most other foodborne pathogens and makes it a concern for food products stored under refrigeration (milk products, meat products or fresh vegetables) (Szabo *et al.*, 2003). It has been demonstrated that the minimum growth temperature could be as low as -0.1 to -0.4 ^{o}C for *L. monocytogenes* strains in chicken broth and/or UHT milk (Walker, Archer and Banks, 1990). Fate of *L. monocytogenes* in processed meat products at refrigeration temperature also has been studied. A 10^3 to 10^4 CFU *L. monocytogenes* /g increase within 6 weeks at 4.4 ^{o}C on ham or other meat products has been demonstrated, depending upon the type of meat and pH of the products (Glass and Doyle, 1989). Many different types of vegetables have been reported for the presence of *L. monocytogenes* as well (Farber and Peterkin, 1991).

Based on the characteristics of *L. monocytogenes* and its wide distribution, fresh produce and minimally processed vegetables can serve as vehicles for *L. monocytogenes* transmittance.

3.2.3 *Listeria* prevalence and outbreaks

Listeria is prevalent in a wide range of food categories and items from many food categories such as meat, dairy products and produce have been linked to outbreaks of listeriosis. *L. monocytogenes* is widely distributed on raw fruits and

vegetables (Table 2). Listeriosis outbreak in 1981 were the first documented incidence of foodborne pathogen outbreak due to *Listeria* infection (*L. monocytogenes* serotype 4b) and it caused 17 deaths among 41 cases. Coleslaw was recognized as the vehicle for the *Listeria* organism (Lianou and Sofos, 2007). And between 1998 and 2011, there were 36 *Listeria* outbreaks documented in the United States, which caused 330 deaths. *L. monocytogenes* serotype 4b**,** a virulent strain, caused the largest number among all the outbreaks (Cartwright *et al*., 2013; CDC, 2013). Along with the growing concern about listeriosis, an enhanced surveillance system was established by CDC in 2004 for investigations of listeriosis (CDC, 2015).

Table 2. Prevalence of *L. monocytogenes* in fresh produce (Lianou and Sofos, 2007)

Product	Country	Source	Prevalence	Reference
Dried fruits	Portugal	Retailer, producers	1/12 (8.3%)	177
Field cress	USA	Supermarkets	2/11 (18.2%)	247
Frozen vegetables	Portugal	Retailer, producers	35/271 (12.9%)	177
Lettuce	Australia	Supermarkets	1/16 (1.7%)	246
	Spain	Restaurants	1/10 (10.0%; raw)	243
			1/10 (10.0%; RTE)	
	Norway	Markets	1/200 (0.5%)	130
	Ireland	Supermarkets	1/80 (1.3%; iceberg)	88
			4/80 (5.0%; romaine)	
			3/80 (3.8%; radicchio)	
Potatoes	USA	Farmers' markets	4/8 (50.0%)	247
Sprouts				
Alfalfa sprouts	USA	Grocery stores	1/206 (0.5%)	245
Bean sprouts	Ireland	Supermarkets	1/80 (1.3%)	88
Vegetables	Canada	Hospital food service	5/135 (3.7%)	198
	Italy	Plants, supermarkets	33/738 (4.5%)	92
	UK	Catering, retail premises	88/2,934 (3.0%; open)	225
	UK	Supermarkets, shops	90/3,849 (2.3%; bagged)	
	Ireland	Supermarkets	2/80 (2.5%)	88

Vegetable salads	Singapore	Restaurants, supermarkets, manufacturers	2/50 (4.0%)	191
	USA	Restaurants, supermarkets	1/63 (1.6%)	146
	UK	Retail premises	77/2,276 (3.0%)	152
	Cyprus	Production sites,	24.0%	73
	USA	Retail markets	22/2,966 (0.7%)	99
	Ireland	Supermarkets	10/80 (12.5%)	88

Though produce is not frequently identified as source of outbreaks, there are still several *Listeria* outbreaks link to fresh produce consumption and recalls emerging in recent years according to CDC reports. There was one in 2009 caused by sprouts and one in 2010 caused by celery. The cantaloupe outbreak of 2011 in the United States which occurred in multiple states and caused 33 deaths among 147 cases was the worst. Four *L. monocytogenes* serotypes were isolated from the contaminated cantaloupes. An environment investigation found that water containing *Listeria* was on the floor processing and facility equipment. Lack of efficient sanitization and cooling process also contributed to the introduction and spread of *L. monocytogenes* on the cantaloupes (CDC, 2012; Laksanalamai *et al*., 2012; McCollum *et al*., 2013).

Multistate outbreak of listeriosis linked to frozen vegetables produced by CRF frozen food and packaged salads produced by Dole occurred in 2016. These two *L. monocytogenes* outbreaks resulted in several hospitalization cases and 3 deaths. Both Dole and CRF frozen food recalled their products because of possible *L. monocytogenes* contamination. The ability of *L. monocytogenes* to tolerate and survive at low temperature was one of the contributors to the outbreaks (CDC, 2016).

The source of contamination can be complex, occurring in the field and through initial processing (Lynch et al., 2009). According to the investigation of the cantaloupe outbreak, the contaminated processing facility or equipment and subsequent handling of these contaminated produce was one source to allow the amplification and spread of *L. monocytogenes* (Laksanalamai *et al*., 2012). The cells of the pathogen could easily attach and adhere to the surface of processing utensils and equipment. Several studies demonstrate the ability of *L. monocytogenes* to adhere to various food-use materials and form biofilms (Beresford et al., 2001; Blackman and Frank, 1996). Therefore, more efficient sanitizers and enhanced surveillance system should be applied.

3.3 Efforts to approach microbial safety of fresh produce

3.3.1 Current usage and studies of sanitizers

With increasing number of Foodborne outbreaks from contaminated fresh produce recognized in many parts of the world, more efforts and research on control of microbial hazard of fresh produce have been carried out. According to the US food code, all fresh produce should be washed thoroughly in tap water and sanitizing agents approved by FDA may be used for submersion washing fruits and vegetables. The use of chemical sanitizers in water intended for the submersion of fresh produce in retail setting is not required (FDA, 2013). At retail supermarkets and grocery stores, the practice of crisping of leafy greens is implemented to remove soil and improve consumer acceptance. Leafy greens for example lettuce are submerged in tap water or water supplemented with chemical sanitizers for several minutes and kept in the

cooler for several hours to give a fresh and crisp look (International Fresh-cut Produce Association, 2006). However, submersion in tap water not containing a chemical sanitizer can result in cross-contamination of fresh produce especially if the water is recycled or reused. For that reason, FDA guide stressed the significance of adequate water quality in fresh-cut processing (FDA, 2008). Transmission of pathogens has been demonstrated during the washing process of fresh-cut leaves and washing water quality was determined as a factor affecting the level of cross-contamination (Allende *et al.*, 2008). Therefore, the addition of efficacious sanitizing agents in the wash water should be managed to reduce the microbial population and circumvent cross contamination (López-Gálvez *et al*., 2009; Brackett, 1999).

Chlorine or chlorine based sanitizers are the most widely used disinfectant in processing of fresh produce. Liquid chlorine disinfectant is usually applied in the 50 to 200 ppm concentration range with a contact time of 1 to 2 min (Parish *et al*., 2003). Chlorine has been investigated for its antimicrobial efficacy with mixed results. For example, Zhuang demonstrated that populations of *Salmonella* on the tomato surface were significantly reduced by dipping in 60 or 110 ppm chlorine while 320 ppm chlorine didn't result in significant reduction (Zhuang et al., 1995). Beuchat stated that treated lettuce with 200 ppm chlorine didn't have significant bactericidal effect compared to deionized water and Nguyen pointed out that inactivation of *L. monocytogenes* on vegetables by chlorine was limited as well (Parish *et al*., 2003; Beuchat, 1999). Studies revealed that the free available chlorine as hypochlorous

acid has the highest bactericidal activity against microorganisms (Beuchat and Ryu, 1997; Luo *et at*., 2011). However, chlorine can react with natural organic matter, which may result in the depletion of free chlorine and formation of by-products including trihalomethanes, haloaceticacids, haloketones and chloropicrin. Due to links to negative human health and environment impacts, there is a trend toward reducing the use of chlorine (Ölmez and Kretzschmar, 2009).

Other commercial sanitizers for washing fresh produce have been studied as well, such as chlorine dioxide, organic acid, hydrogen peroxide, etc. (Table 3). Chlorine dioxide, comparing to chlorine, is more stable over a wide pH range and it is also a powerful oxidant. Chlorine dioxide is only allowed to be used on whole produce at a maximum concentration of 3 ppm (Ölmez and Kretzschmar, 2009). However, low concentration may not guarantee antimicrobial efficacy. A study by Zhang and Farber founded only 1 log reduction of *L. monocytogenes* on lettuce by application of 5 ppm aqueous chlorine dioxide for 10 min (Zhang and Farber, 1996). Organic acid sanitizers are commonly investigated as antimicrobial agents because of the low pH which negatively impacts survival of most microorganisms. The safety of organic acid based sanitizers has been confirmed by both EPA and FDA as a no-rinse sanitizer and many of them are generally recognized as safe (GRAS, 21 CFR Part 184; Gonzalez *et al.*, 2004). The antimicrobial activity of organic acids depends upon a lot on the type of organic acid. Several studies indicated that the combinations of acetic acid, lactic acid with other solutions like chlorine or nisin can increase the antimicrobial efficacy than organic acid sanitizers alone (Bari *et al.*, 2005; Zhang and

Farber, 1996). Physical interventions including irradiation, UV light or ozone gas may be alternatives to the chemical agents for inactivating pathogens on fresh produce. Efficacy of these physical treatments is dependent on the produce type. In spite of this limitation, consumer acceptance and complexity of application at retail level are still questionable (Doyle and Erickson, 2008; Parish *et al*., 2013).

In response to the growing demanding for more efficient and consumer acceptable sanitizers or methods to reduce microbial hazards of fresh produce; a wealth of research is addressing such issues. However, most of studies were conducted under laboratory conditions and results cannot be compared due to different experimental conditions (Gil *et al*., 2009). Therefore, the investigations under realistic industry conditions are required high demand.

Table 3. Selected studies on the evaluation of the efficacy of sanitizing agents on lettuce produce (Gil *et al*., 2009)

Sanitizer treatment/storage conditions	Microorganism	Product	Reference
- Lactic acid (15 g/L) and hydrogen peroxide (H_2O_2) (15 g/L) for 15 min; lactic acid (15 g/L) and H_2O_2 (20 g/L) for 5 min; H_2O_2 (20 g/L) for 60 or 90 s	*E. coli* O157:H7, *Salmonella* spp and *L. monocytogenes*	Lettuce	Lin et al. (2002)
- Chlorine (100 mg/L) and peroxyacetic acid (80 mg/L) for 30 s.	*L. monocytogenes*	Iceberg and romaine lettuce	Beuchat *et al.* (2004)
- Ozonated water (1, 3, 5 mg/L) for 0.5, 1, 3, 5 min and ozonated water (3 mg/l) combined with organic acids (acetic, lactic and citric acids) (10 g/L) for 1 min	*E. coli* O157:H7 and *L. monocytogenes*	Lettuce	Yuk *et al.* (2006)
- Chlorine (100 mg/L) and lactic, citric, acetic and ascorbic acids (5 and 10 g/L) for 2 and 5 min	*E. coli* O157:H7 and *L. monocytogenes*	Iceberg lettuce	Akbas and Ölmez (2007)
- Acidified sodium chlorite (1200 mg/L),	*E. coli* O157:H7,	Leafy greens	Stopforth *et al.*

chlorine (50 mg/L) and acidicelectrolyzed water (30 – 35 mg/L) for 60 s and 90 s	*Salmonella* spp and *L. monocytogenes*		(2008)
- Sodium hypochlorite (300 and 600 mg/L) for 3 min, and irradiation doses of 0.25 – 1.5 kGy at a rate of 0.098 kGy/min	*E. coli* O157:H7	Lettuce varieties	Niemira (2008)
- Combined treatment of UV/H2O2 (UV at 0.63 mW/cm2 for 60 s and H2O2 at 1.5% v/v sprayed at a rate of 480 ml/min for 60 s) and chlorine (200 mg/L) for 3 min	*E. coli* O157:H7, *Salmonella* spp and *Pseudomonas fluorescens*	Iceberg and romaine lettuces, spinach	Hadjok *et al.* (2008)
- Chlorine (100 mg/L), citric acid (10 g/L) and ascorbic acid (10 g/L) for 5 min. Product stored for 14 days at 3 and 8 °C	*L. innocua* and *E. coli*	Lettuce and coleslaw mix	Francis and O'Beirne (2002)
- Chlorinated water (10, 100, 200 mg/L), hydrogen peroxide (10, 20, 30 ml/L), peroxyacetic acid (40, 60, 80 mg/L) for 2 min and sodium bicarbonate (1, 5, 10%) for 5 min. Product stored for 21 days at 4, 25 and 37 °C	*E. coli* and F-specific coliphage MS2	Lettuce and cabbage	Allwood *et al.* (2004)
- Warm (48 °C) chlorinated water (100 mg/L) for 30 s followed by cold chlorinated water (100 mg/L) for 25 s. Product stored for 18 days at 4 °C	Natural microflora	Lettuce	McKellar *et al.* (2004)
- Sodium hypochlorite (100 mg/l) for 1 min. Product stored for 14 days at 5 °C	Natural microflora	Fresh-cut romaine lettuce	Luo (2007)

3.3.2 Electrolyzed water

Electrolyzed water is a somewhat new sanitizer receiving considerable interest in recent years for application in the food industry. There are two main types of electrolyzed water that have been investigated, acidic electrolyzed water (2.3 < pH < 2.8), neutral or slightly acidic electrolyzed water (pH ~ 6.5) (Hao *et al.*, 2012). Compared to traditional chlorine sanitizers, electrolyzed water, especially neutral electrolyzed water is environmental friendly and regarded as safe because it doesn't contribute to the corrosion of processing equipment and irritation of hands caused by low pH and high chlorine concentration (Hao *et al.*, 2015).

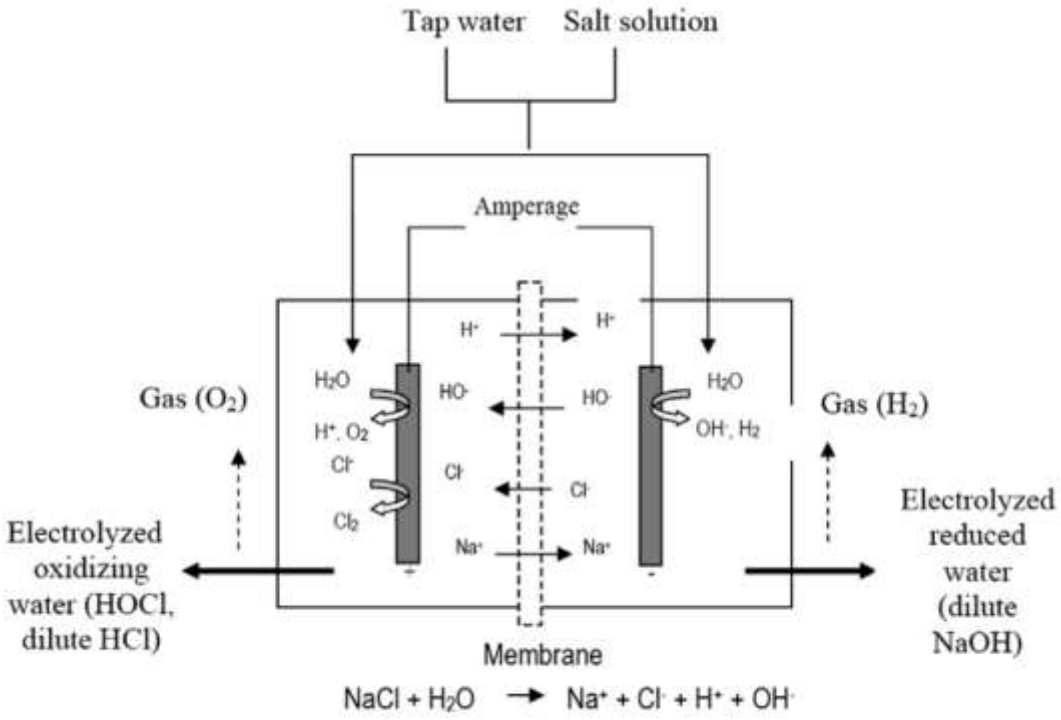
3.3.2.1 Mechanism

Electrolyzed water is produced by combining ordinary tap water with sodium chloride (~ 1% NaCl). These solutions are electrolyzed by passing through an electrolytic cell, within which the anode and cathode are separated by a membrane. During the electrolysis of salt solutions, negatively charged ions including chloride and hydroxide move to the anode and become oxygen gas, chlorine gas, hypochlorite ion, hypochlorous acid and hydrochloric acid. Electrolyzed oxidizing water with relatively low pH (2.3-2.8) and high oxidation-reduction potential (ORP > 1000mV) is generated from the anode side (Figure 1) (Hsu, 2005; Huang *et al.*, 2008). Neutral electrolyzed water is produced similarly as acidic electrolyzed water, but part of the product from the anode side is then redirected into the cathode chamber (Abadias *et al.*, 2008).

The main effective compounds in neutral electrolyzed water are hypochlorous acid (HOCl) and hypochlorous acidic ion (ClO^-). These active compounds can enter into bacterial cells and high oxidation potential of these compounds can inactivate the microbial cell by damaging the cell membranes, inhibiting glucose oxidation, disrupting protein synthesis and functions (Huang *et al.*, 2008; Liao, Chen and Xiao, 2007). Unlike acidic electrolyzed water, neutral electrolyzed water only contains a trace amount of chlorine because it dissolves poorly at neutral pH. Chlorine is most active in hypochlorous acid form, which predominates when pH of a solution ranges from 5.0 to 6.5. Therefore, the occurrence of Cl_2 off-gassing, the cause of some human health and safety issue, is less in the neutral electrolyzed water (Guentzel e*t*

al., 2008; Rhaman, Ding and Oh, 2010). Besides, it's more stable as chlorine loss decreased dramatically with the increase of pH (2.5-9) (Len *et al.*, 2002).

The main advantage of neutral electrolyzed water is that it's safer and eco-friendly. While electrolyzed water sanitizers have some drawbacks as well. The solution may rapidly lose its antimicrobial efficacy if the active compounds like hypochlorous acid are not continuously supplied by electrolysis, as the oxidation-reduction potential (ORP) and chlorine concentration reduce along with the storage time. Horiba reported decreased bactericidal activity of stored neutral electrolyzed water compared to freshly prepared solution (Horiba *et al.*, 1999). ORP may be regarded as the primary factor affected microbial inactivation because ORP can damage the outer and inner cell membranes. Nevertheless, other investigators noted that free chlorine of EW acted on microorganisms (Hao *et al.*, 2012; Kim, Hung and Brackett, 2000; Huang *et al.*, 2008).



Positive pole: $2H_2O \rightarrow 4H^+ + O^2 \uparrow + 4e^-$

$2NaCl \rightarrow Cl_2 \uparrow + 2e^- + 2Na^+$

$Cl_2 + H_2O \rightarrow HCl + HOCl$

Negative pole: $2H_2O + 2e^- \rightarrow 2OH^- + H_2 \uparrow$

$2NaCl + 2OH^- \rightarrow 2NaOH + Cl^-$

Figure 2. Schematics of electrolyzed water generator and produced compounds (Huang *et al.*, 2008)

3.3.2.2 Application of electrolyzed water in field of fresh produce

In recent years, electrolyzed water has been regarded as an alternative sanitizer to the traditional chlorine products and evaluated as a disinfectant in the food industry. It has been widely used and studied on fresh vegetables for efficacy in inactivating bacteria.

A 5 min dipping treatment in slightly acidic electrolyzed water resulted in the same microbial reduction as sodium hypochlorite solution for *E. coli* and *Salmonella* spp. on Chinese celery, lettuce and sprouts. It also significantly reduced the total aerobic mesophilic bacteria on vegetables (Issa-Zacharia *et al.*, 2011). The results are in agreement with other studies on lettuce. Park observed that electrolyzed water with 45 ppm chlorine could significantly reduce the population of *E. coli* O157:H7 and *L. monocytogenes* by approximately 2.5 log CFU/g compared to water alone. There was no significant quality change of lettuce found among different washing treatments (Park *et al*., 2001). Yang also suggested that dipping into electrolyzed water at a neutral pH retained a best visual quality of lettuce and achieved around 2 log reductions of several pathogens (Yang et al., 2003). No significant difference was observed between neutral and strong acid electrolyzed water on microbial reduction after dipping treatment and neutral electrolyzed water doesn't leave chemical residuals on food (Rahman, Ding and Oh, 2010). Other vegetables have been tested as well. For instance, smooth surface vegetables like fresh tomatoes were washed in neutral electrolyzed water (89 mg/L active chlorine) for 5 min. It was shown as an effective method to control the presence of *E. coli*, *S.* Enteritidis and *L.*

monocytogenes on the surface of tomatoes, without affecting the organoleptic qualities (Deza, Araujo and Garrido, 2003). The electrolyzed water has been proved to be a promising alternative to chlorine in washing broccoli as well.

Several factors would influence the bactericidal effects including contact time, temperature, and structural characteristics of the commodity. For example, *L. monocytogenes* may be more resistant than *E. coli* O157:H7 to the electrolyzed water because of differences in cell wall structure between Gram-negative and Gram-positive bacteria (Kim, Hung and Brackett, 2000). Storage temperature of treated produce influences the bactericidal effect as well. Koseki suggested that 1 °C (among 1, 5 and 10 °C) was the best storage temperature to achieve the best antimicrobial result (Koseki and Itoh, 2001) and efficacy of electrolyzed water ice has been studied as well (Koseki, Isobe and Itoh, 2004).

Electrolyzed water has been proved to be a promising alternative to chlorine. However, most experiments were carried out under laboratory conditions, which examined the need for investigations under commercial processing conditions.

3.4 Misting

Nowadays, more and more supermarkets implement misting cabinets for keeping the moisture, extending shelf-life and promoting produce appearance in retail display. Automatic misting is a type of humidification technology for produce at a constant time interval (Mohd-Som *et al.*, 1995). A retail supermarket in New Brunswick as example, the misting cabinets are set to mist 7-8 seconds every 6 min.

Misting treatment has been illustrated to improve loss of ascorbic acid, chlorophyll and green color retention of broccoli under market display conditions compared to non-misted broccoli (Barth *et al.*, 1992). However, the impact of misting treatment on the microbial growth on fresh produce under commercial display conditions has not been intensively studied and the results of current research are not consistent.

Reduced aerobic plate counts, coliforms, yeast and mold were found on misted broccoli stored at refrigerated temperature during 5 days compared to non-misted broccoli. Misting intervals were set as 4 s every 4 min. The washing effect of misting and residual chlorine in the tap water may explain the reduced microbial counts (Mohd-Som *et al.*, 1995). In the contrast, Rossman suggested that misting could increase the level of aerobic plate counts, yeast and mold on the retail leaf lettuce. The increased microbial growth was attributed to the increased water due to the application of misting (Rossman et al., 2012). The study of Rossman agrees with the result of Quinlin's investigation on the impact of misting. She demonstrated that there is an increase in the number of microorganisms on leaf lettuce which has been misted rather than non-misted lettuce (Quinlin, 2004). Most investigations were carried out on the growth of natural microflora on the surfaces of fresh produce, but no specific foodborne pathogen has been learnt. In this study, the impact of misting treatment on the growth of *L. monocytogenes* and natural microflora on the lettuce was investigated.

The storage condition is also an important factor to minimize the distribution

and proliferation of both pathogenic and spoilage organisms on the fresh produce. Increased growth rate of foodborne pathogens has been demonstrated in several studies to be associated with temperature abuse and it is recommended to keep the products at 4 ^{o}C to minimize the microbial hazards of produce (Jacxsens, Devlieghere and Debevere, 2002; Carrasco *et al.*, 2008). Therefore, temperature control is critical at every stage of postharvest handling of fresh produce (Beuchat and Ryu, 1997). But *L. monocytogenes* has been demonstrated to survive and thrive at refrigeration temperature. The impact of storage temperature on the survival and growth of *L. monocytogenes* and psychrotrophic bacteria on the lettuce was studied.

4. MATERIALS AND METHODS

4.1 Lettuce samples

Cases of romaine lettuce (Hiji Bros., California) were purchased from local farmers' market one day before use and stored at 5 °C. The lettuce was grown in the United States and it was not subjected to any treatment before purchase. Any heads that were visibly damaged were discarded. The outer leaves were trimmed and a thin slice of the butt-end of the lettuce head was removed and discarded. After trimming, the average weight of a whole head of romaine lettuce was approximately 500 g.

4.2 Strains used and preparation of inoculum

L. monocytogenes strains L008 (Canadian cole slaw/cabbage outbreak, serotype 4b), L2624 (cantaloupe outbreak, serotype 1/2b) and L2625 (cantaloupe outbreak, serotype 1/2a) were used in present study. Prior to each experiment, one isolated colony of each working culture was transferred into 10 mL fresh brain heart infusion (BHI, Difco) and incubated at 37 °C for 20 h to prepare the inoculum. Cells of each strain were collected by centrifugation (10 min at 4,500 rpm) (Allegra^{TM} 21R, Beckman Coulter) and washed with 5 mL of 0.1% sterile peptone water (SPW, Difco). The pellets were collected again by centrifugation and re-suspended in 5 mL of 0.1% peptone water. Equal volumes (2 mL) of three cultures were mixed and diluted in 30 mL of 0.1% peptone water to achieve an inoculum containing approximately 10^8 -10^9 CFU/mL of *L. monocytogenes* cells.

4.3 Inoculation of lettuce samples

Seventy-two heads of trimmed romaine lettuce were hung on 4-tier shelf which was covered with aluminum foil. Whole heads of romaine lettuce were spot inoculated with the *L. monocytogenes* inoculum to give an initial population of approximately 5 log CFU/g per head. Each head was inoculated at 20 different spots (5 µL each) on the outer surface and 10 spots on the inside of leave. The inoculated lettuce was air-dried for 1 h. Two heads of lettuce were sampled to determine *L. monocytogenes* population.

4.4 Crisping and misting

Four stainless steel sinks used in the present study were cleaned with a commercial detergent and rinsed with tap water. Two sinks were filled with 100 L of tap water and other two were filled with 100 L commercial electrolyzed water solution (EW, containing 50-60 ppm free chlorine) which was automatically dispended through a delivery system (Produce Fresh^{TM}, Sterilox). The temperature (Traceable^{TM}, Fisher Scientific), pH value (accumet^{TM} AB15, Fisher Scientific) and free chlorine concentration (Palintest 1000 Chlorometer, Palintest Ltd) were measured before and after crisping. Forty-eight heads of romaine lettuce (twelve heads in each sink) were submerged either in tap water or EW solution for 5 min and kept in a refrigerator for 2 h to drain excessive water and crisp.

Half of the lettuce samples (thirty heads, including ten non-misted, ten water-misted and ten EW-misted heads) were then placed in a three shelf

commercial chilled display case (Hussmann, Ingersoll Rand) for 24 h. Misting intervals were set as 10 s every 8 min, providing a total of 40 mL water each interval. Non-misted samples were stored in commercial plastic bags at 5 °C and 15 °C respectively. After 24 h misting, lettuce was separated and stored at 5 °C and 15 °C for 7 days. The treatment scheme is shown on Figure 3. Photos of outer leaves and butt-ends of each treated lettuce were taken before microbiological analysis for a simple evaluation of the lettuce appearance change.

4.5 Microbiological analysis

Four samples (25 g) from two replicate heads of lettuce of each treatment and storage condition were processed for microbiological analysis. A whole head of lettuce was chopped and mixed thoroughly, and two 25 g of one chopped lettuce were randomly selected and placed into sterile stomacher bags. A volume (100 mL) of Dey/Engley neutralizing broth (D/E broth, used only for neutralizing the EW solution, Criterion^{TM}) was poured into the bag of EW-crisped samples or 100 mL of 0.1% peptone water was used for water-crisped and non-crisped samples. Samples then were subjected to homogenization for 1 min (easyMIX^{TM}, BioMerieux). A 1 ml aliquot was removed and serially diluted (1:10) in 0.1% peptone water and aliquots (100 µL) were plated on Palcam agar (Difco) for *L. monocytogenes*; plates were incubated at 37 °C for 20 h. Psychrotrophic bacteria were enumerated by plating on tryptic soy agar (TSA, Difco) and incubated at 7 °C for 7 days. Sample bags were incubated at 37 °C for 20 h for enrichment. The lettuce was sampled for microbiological analysis on Day 0, Day 1, Day 4 and Day 7 to determine the

population of *L. monocytogenes* and psychrotrophic bacteria on romaine lettuce.

4.6 Growth of *L. monocytogenes* in BHI

An aliquot (5 µL) of a *L. monocytogenes* cocktail was transferred into 40 mL BHI and incubated at 5 °C and 15 °C, respectively, for investigating the growth of *L. monocytogenes* in BHI broth. The initial population of *L. monocytogenes* was approximately 5.5 log CFU/mL. *L. monocytogenes* cells were enumerated by plating on Palcam agar on Day 0, Day1, Day 4 and Day 7.

4.7 Statistical analysis

The experiments were replicated two times. The average populations of *L. monocytogenes* and psychrotrophic bacteria were analyzed by SPSS Statistics 22 (IBM) for analysis of variances (ANOVA) test followed by Duncan's post hoc analysis.

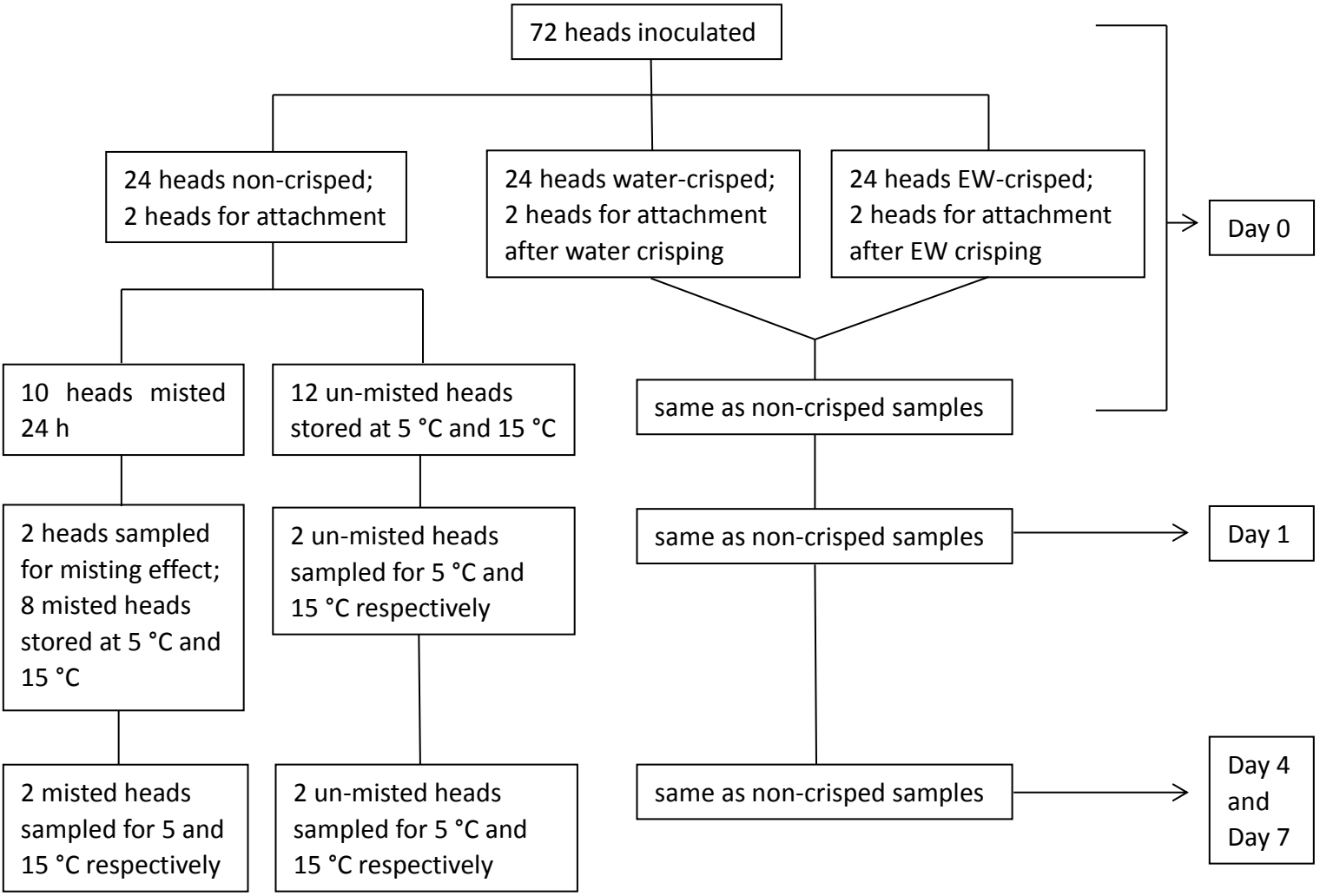


Figure 3. Treatment scheme for romaine lettuce

5. RESULTS

5.1 Growth of *L. monocytogenes* in BHI

Representative data on the growth of *L. monocytogenes* in brain heart infusion (BHI, Difco) broth at 5 °C and 15 °C for 7 days are show in Figure 4. BHI is widely used for isolation and cultivation of *Listeria* and the pH is 7.4 ± 0.2. The initial population of inoculated *L. monocytogenes* was approximately 5 log CFU/mL of BHI which corresponded with the initial pollution inoculated on romaine lettuce.

After 24 h incubation at both 5 °C and 15 °C, there was significant difference ($p<0.05$) on the population of *L. monocytogenes*. During 7 days incubation time at 15 °C the population of bacteria reached 8.9 log CFU/mL. At refrigeration temperature, the growth rate of bacteria was significantly reduced and the lag time increased as the incubation temperature was reduced. Regardless, an increase of $\sim$ 1 log occurred after 7 days at refrigeration temperature. The observation aligns with other studies, where lowered temperature significantly reduced the growth rate of *L. monocytogenes* (Cole *et al.*, 1990; Walker et al., 1990). 'Cold shock' was indicated by Walker where the bacteria population reduced the growth rated when experienced a sudden decrease in temperature especially when the strains were grown at the optimum growth temperature before transferring to the lower temperature . 'Cold shock' could partially explain the delayed growth of *L. monocytogenes* at refrigeration temperature (Walker et al., 1990).

5.2 Change of water/solution quality during crisping

Temperature, pH and free chlorine of tap water and EW solution were measured before and after lettuce crisping (Table 4). The pH value of commercial EW solution used in the study was 6.96 ± 0.06, or near neutral EW (pH ~ 6.5). During one time crisping procedure, 12 heads romaine lettuce were submerged in 100 L of tap water and commercial EW solution. There were no noticeable changes of pH and temperature of tap water and EW were observed after one time crisping. The color and turbidity of water and EW were changed as the soil and debris on the romaine lettuce were washed.

There was approximately 5 ppm free chlorine drop of EW after 5 min crisping. The free chlorine loss was studied by Beuchat, which demonstrated that the rate of reduction in free chlorine was increased as the lettuce/solution ratio was decreased. For instance, there was approximately 10 μg/mL chlorine loss in 5 min treatment when the lettuce/solution was 1:100 compared to a 25 μg/mL loss when the ratio was 1:10 (The initial free chlorine concentration was 112 ± 11 μg/mL). The lettuce/solution in present study was 3:50. Besides, a more rapid reduction in free chlorine concentration in solution was observed when used to wash shredded lettuce because the release of tissue juice reacting with chlorine (Beuchat et al., 2004).

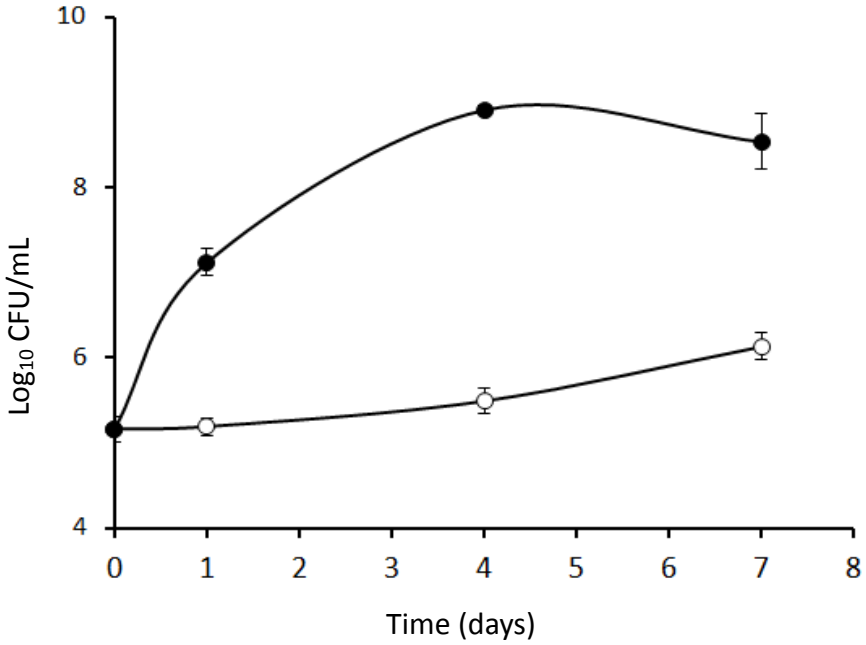


Figure 4. Growth of *L. monocytogenes* cocktail when cultured in BHI incubated at 5 °C and 15 °C (○, ●)

Table 4. The measurements of temperature, pH and free chlorine of tap water and EW before and after crisping procedure

		Before crisping	After crisping
Tap water	Temperature (°C)	17.38 ± 0.39*	17.2 ± 0.50
	pH	7.70 ± 0.08	7.62 ± 0.07
	Free chlorine (ppm)	0	0
EW	Temperature (°C)	17.25 ± 0.42	16.78 ± 0.41
	pH	6.96 ± 0.06	6.97 ± 0.08
	Free chlorine (ppm)	55.25 ± 2.06	49.5 ± 1.92

*Temperature, pH, and free chlorine values are the means of four measurements ± standard deviation.

5.3 Behavior of *L. monocytogenes* on romaine lettuce

The population of *L. monocytogenes* on romaine lettuce under each treatment is shown in Figure 5. The initial population of inoculated *L. monocytogenes* cocktail on romaine lettuce control samples in two experiments was 5.55 ± 0.21 log CFU/g. Spot inoculation was applied to simulate contamination through exposure to droplets of contaminated water or soil in the field (Koseki *et al.*, 2003). Crisping with tap water and EW sanitizer for 5 min significantly reduced ($p<0.05$) the population of *L. monocytogenes*, achieving a reduction of 1.32 and 2.93 log, respectively.

Following the crisping procedure (including 2 h chilling), half of the heads of lettuce was misted in a commercial display cabinet connected to tap water for 24 h. Misting treatment resulted in an approximately 1 log additional reduction in the population of *L. monocytogenes* on each crisping treatment group. But misting treatment had no significant effect on the population of *L. monocytogenes* on each treated lettuce group. The reduction of bacteria population maybe attributed to the washing effect of the misting treatment (Mohd-Som *et al.*, 1994). Misting had no significant effect on each crisping treatment group, but did for the non-crisped group ($p<0.05$). The misting effect probably varied with the display location as non-crisped lettuce heads were placed at the top layer of misting cabinet where subjected to more misting treatment.

By day 4 and day 7 post-treatment, *L. monocytogenes* populations on most treatment and storage groups remained constant or even declined, with the

exception of the water-crisped lettuce stored at 15 °C which had significant increase on the *L. monocytogenes* population. Compared to control lettuce samples which were not crisped and misted, crisped samples had significant difference ($p<0.05$) in the population of *L. monocytogenes,* especially crisping with EW sanitizer, which had 2.87, 3.04, 1.88 and 2.23 log reduction compared to non-crisped sample. Misting treatment for 24 h had no significant effect ($p>0.05$) in reducing bacterial load of EW-crisped lettuce on both day 4 and day 7 while it had significant effect on non-crisped samples. Storage temperature (5 °C and 15 °C) didn't show a significant impact on the growth of *L. monocytogenes* based on ANOVA analysis, which is in contrast to some previous investigations. For example, the growth of *L. monocytogenes* at 13 °C was greater than at 5 °C (Carrasco *et al.*, 2008). A similar growth trend of *L. monocytogenes* occurred on iceberg lettuce held at 5 °C and 10 °C (Beuchat and Brackett, 1990).

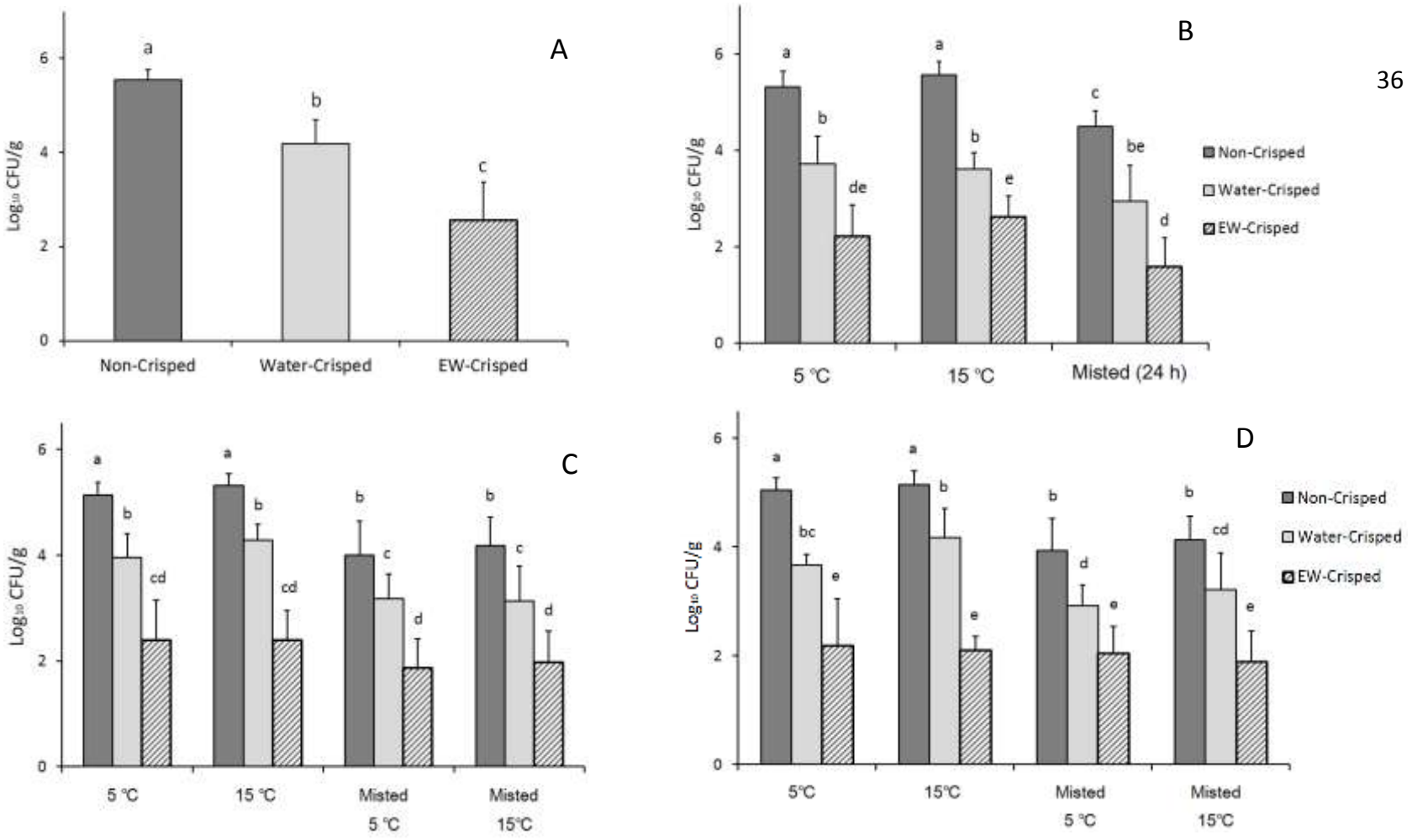


Figure 5. Populations of *L. monocytogenes* on romaine lettuce stored at 5 °C and 15 °C as influenced by crisping and misting on Day 0 (A), Day 1 (B), Day 4(C) and Day 7(D)

*Different letters on the graph indicted the means were significantly different ($p<0.05$)

5.4 Behavior of psychrotrophic bacteria on romaine lettuce

The population of psychrotrophic bacteria on romaine lettuce under each treatment is shown in Figure 6. The population of natural microflora on the romaine lettuce untreated was approximately 5.2-5.7 log CFU/g. A large population of Gram-negative psychrotrophic bacteria, especially *Pseudomonas* species are commonly found on minimally processed vegetables. Other psychrotrophic bacteria include pathogens such as *Yersinia* and *Aeromonas* (Szabo et al., 2000; Magnnuson et al., 1990).

Crisping treatment with tap water and EW sanitizer for 5 min significantly reduced ($p<0.05$) the population of natural microflora on the romaine lettuce by 0.7 and 1.3 log CFU/g, respectively. EW sanitizer had significantly better efficacy in reducing the psychrotrophic bacteria compared to the crisping treatment with tap water. The reduction of established natural microbiota was found less than the reduction of *L. monocytogenes* inoculated on the lettuce. After 24 h misting, there was no significant effect of misting treatment observed on the population of psychrotrophic bacteria. And the population of psychrotrophic bacteria on the EW-crisped lettuce recovered to a similar level found on the water-crisped lettuce since there was no difference found between the water and EW-crisped samples. The efficacy of sanitizers in reducing the populations of established natural microbiota of vegetables was less than for artificially inoculated bacteria. Natural microflora may form biofilms either on the outer and inner layers of vegetables. The biofilm could

protect the microflora from many chemical sanitizers especially these trapped in the inner layers of vegetables (Abadias *et al.*, 2008).

By day 4 and day 7 post-treatment, populations of psychrotrophic bacteria increased significantly ($p<0.05$), particularly for lettuce stored at 15 °C, which the population of psychrotrophic bacteria increased significantly between day 1 and day 4. The populations of psychrotrophic bacteria reached 6.26-6.77 log CFU/g kept at 5 °C and 6.99-7.54 log CFU/g kept at 15 °C. Moreover, the natural microbiota on crisped or misted samples recovered and reached a similar population level as control samples. There was no difference on the bacterial populations by plating on TSA observed between treated lettuce and untreated lettuce. In contrast to the investigation on the growth of inoculated *L. monocytogenes* on the lettuce, the storage temperature had a significant effect ($p<0.05$) on the population of psychrotrophic bacteria on both day 4 and day7. It was found that the natural microflora had more resistance to the crisping and misting treatment and recovered more rapid than the artificially inoculated bacteria.

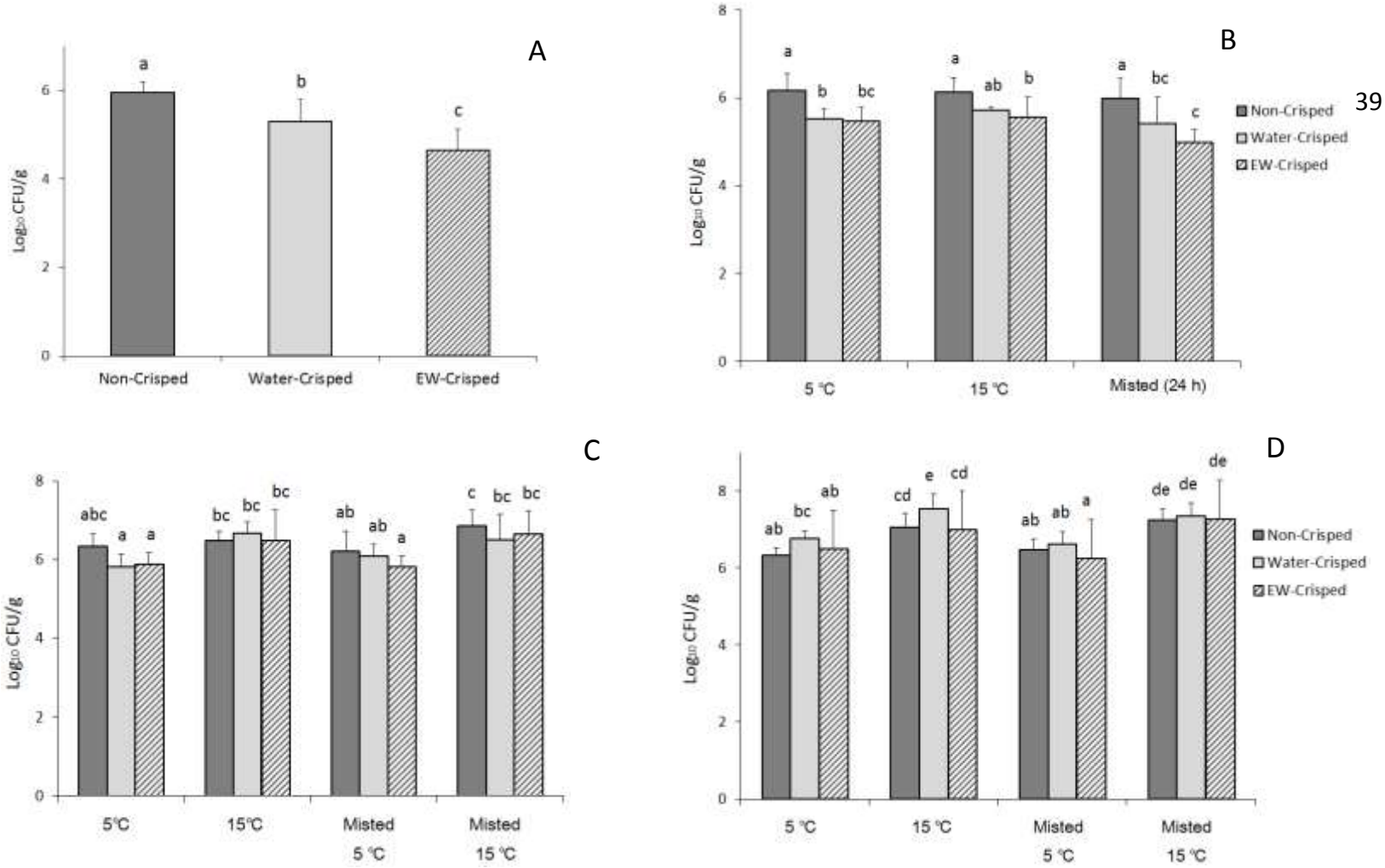


Figure 6. Populations of psychrotrophic bacteria on romaine lettuce stored at 5 °C and 15 °C as influenced by crisping and misting on Day 0 (A), Day 1 (B), Day 4(C) and Day 7(D)

*Different letters on the graph indicted the means were significantly different (p<0.0

5.5 Evaluation of lettuce appearance

General appearance is one of the most important quality attributes that consumer use to evaluate the quality of vegetables. Photos of outer leaves and butt-end of each lettuce samples were taken before microbiological analysis for the visual evaluation of the lettuce appearance change. There was no noticeable appearance difference found by visual inspection among non-crisped lettuce, water-crisped lettuce and EW-crisped lettuce. But the appearance changes of the lettuce along with the storage time were observed. The discoloration of butt-ends and decay of lettuce leaves were found on both day 4 and day7, particularly for the lettuce stored at 15 °C. Investigation by Park found no significant influences by EW treatment on the taste, color, and appearance of lettuce by a sensory panel (Park *et al.,* 2008).

6. DISCUSSION

EW sanitizer was applied in the crisping procedure to mitigate cross-contamination and reduce the bacterial population on of romaine lettuce. The free chlorine concentration and pH of EW sanitizer used in the present study were approximately 55 ppm and 6.96, respectively, which would not cause skin irritation on hands. There was approximately 5 ppm free chlorine depletion during 5 min crisping.

Crisping with commercial EW sanitizer resulted in a significant ($p<0.05$) reduction of the population of *L. monocytogenes* inoculated on romaine lettuce compared to tap water crisping. Misting treatment for 24 h didn't have a significant effect on the population of *L. monocytogenes* on each group. The efficacy of EW was in agreement with the study on the effectiveness of neutral EW sanitizers by Guentzel, which found an approximately 2.5 log reduction of the *L. monocytogenes* population on lettuce leaves after dipping for 10 min with EW containing 50 mg/L total chlorine (Guentzel e*t al.*, 2008). Other investigations have also observed the effectiveness of EW on inactivating pathogens on vegetables (Deza, Araujo and Garrido, 2003; Keoseki *et al.*, 2001). The effectiveness of sanitizers depends on various factors, which can be attributable to the contact time, strains used, structural characteristics of the vegetable surface, inoculation methods, etc. For instance, *L. monocytogenes* was suggested to be more resistant to the EW than *E. coli* O157: H7 speculatively because of the different cell wall structure between Gram-negative and

Gram-positive bacteria (Kim et al., 2000). Pathogen reduction depends on the inoculation method as well, less bacterial reduction was found on dip-inoculated lettuce than spot-inoculation because more bacterial cells were spread more widely in stomata, trichomes and damaged tissue. Sanitizers had reduced effects inactivating bacteria cells attached to these locations (Keskinen et al., 2009; Singh *et al.*, 2002). Therefore, the apparent disinfectant efficacy of sanitizers varies depending on the experiment design.

Crisped lettuce showed significant differences in *L. monocytogenes* population compared to non-crisped samples during 7 days storage. This suggests that some active compounds in EW may remain on lettuce samples and some injured cells could not recover on selective agar. Besides, the population of *L. monocytogenes* on most treated groups stored at both 5 °C and 15 °C remained constant during 7 days storage, with no significant increase even at ambient temperature. The observation noted in present study was in contrast to the hypothesis and some previous studies which found significant proliferation at higher storage temperatures (Carrasco *et al.*, 2008; Beuchat and Brackett, 1990). However, most research has been conducted on shredded lettuce or lettuce leaves, rarely have whole heads of lettuce been investigated. One possible reason is that crisping or washing of whole heads of lettuce is an entirely different process compared to that used for shredded pieces. Cut edges release exudates and other organic matter, which can react with the chlorine compounds in EW to cause the depletion of the

free chlorine concentration and reduce the effectiveness of the sanitizer. Besides, the cut edges and wounds contribute to the attachments of bacterial cells and excessive nutrients from the cut edges may support the proliferation of *L. monocytogenes* on lettuce (Nou and Luo, 2010; Palma-Salgado *et al.*, 2014). The competitive activities of natural microflora could be another reason to explain the growth trend of *L. monocytogenes*. The indigenous populations from lettuce have been found to impede *Listeria* growth (Beuchat and Brackett, 1990; Francis and Beirne, 1998). High numbers of some pseudomonad strains have been observed to reduce the growth of *L. monocytogenes* on endive as well (Carlin *et al.*, 1995).

The reduction in population of indigenous microbiota was found less than for the inoculated *L. monocytogenes*, but crisping with tap water and EW still showed significant reduction ($p<0.05$) on the population of psychrotrophic bacteria. This observation was in agreement with the investigation by Abadias (Abadias *et al.*, 2008). By day 4 and day 7 post-treatment, populations of psychrotrophic bacteria on crisped samples recovered significantly and reached similar populations as non-crisped samples. *Pseudomonas* is the most common indigenous microbiota found on leafy green vegetables. Temperature had a significant effect on the growth of psychrotrophic bacteria in contrast to *L. monocytogenes.* The reduced effects of crisping and greater proliferation of natural microflora could be ascribable to the formation of biofilms. Natural microflora has been reported to form biofilms on fresh produce and biofilms may constitute up to 80% of the total microbial flora. Sanitizers

were found not be effect in activating cells embedded inside biofilms or those cells attached to some inaccessible sites like stomata (Annous, Fratamico and Smith, 2009; Ölmez and Temur, 2010). Misting treatment didn't have significant effects on the populations of psychrotrophic bacteria probably because the washing effects could not reach the inaccessible sites inside the lettuce head. Compared to the natural microbiota, artificially inoculated pathogens are predominantly attached to the outer leaves of lettuce, which may partially explain a better effectiveness of crisping or misting treatments.

The influence of crisping and misting on the quality of romaine lettuce were rudimentarily evaluated in the present study. There was no substantial difference among treatments and temperatures combination groups by visual evaluation. Some investigations demonstrated that EW washing didn't affect the organoleptic characteristics of fresh produce (Deza, Araujo and Garrido, 2003; Park *et al.*, 2001). A more detailed sensory evaluation may be done in the future study.

7. CONCLUSION

Results of the present study suggest that crisping, particularly crisping with electrolyzed water had a negative effect on the survival and growth of *L. monocytogenes* on lettuce. Therefore, crisping with electrolyzed water sanitizer could improve the microbial safety of lettuce. Misting appeared to only marginally influence bacterial populations on lettuce. Storage temperature also had an influence on the bacterial growth. The treatments applied by retailers and maintenance of appropriate storage temperature should be both ensured for the safety of fresh produce.

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