FOODBORNE PATHOGENIC DECONTAMINATION AND QUALITY ENHANCEMENT OF FRESH PRODUCE USING PULSED LIGHT (PL) AND SANITIZER WASHING

by

Wenqing Xu

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal and Food Sciences

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Imported green onions have been associated with three large hepatitis A outbreaks in the United States. Contamination has been found on both domestic and imported green onions. Raspberries have been associated with outbreaks caused by *C. cayetanensis*, Calicivirus, hepatitis A and norovirus. *C. cayetanensis*, hepatitis A as well as norovirus contamination happened through a fecal-oral route which indicates that foodborne pathogens that share this transfer route, such as *Escherichia coli* O157:H7 and *Salmonella* spp. may also contaminate green onions or raspberries leading to outbreak. The objective of our study was to evaluate the potential of using pulsed light (PL) technology alone or in combination with other hurdles to decontaminate green onions and raspberries from *Escherichia coli* O157:H7 and/or *Salmonella* spp.

The first study on green onions was conducted to evaluate decontamination of *Salmonella* Typhimurium using new formula sanitizer washing, PL, as well as synergy between the sanitizer wash and PL. The results showed that for spot inoculated green onions, 0.4 mg/mL thymol individually and the five new formula sanitizers all achieved higher log reduction of *Salmonella* (4.5-5.3 $\log_{10}$ CFU/g reduction) than 200ppm chlorine washing. The 5 second dry PL (4.6 $\log_{10}$ CFU/g) or 60 second wet PL treatment (3.6 $\log_{10}$ CFU/g) was better or comparable to chlorine washing. For dip inoculated green onions, none of our treatments provided >0.8 $\log_{10}$ CFU/g (0.6 - 0.8

log $_{10}$ CFU/g) reduction of *Salmonella*. In the second study, green onions were cut into two segments, stems and leaves, to represent two different matrixes. Stems were more difficult to be decontaminated. Spot and dip inoculation method were compared as well. Similar to the first study, dipped inoculated green onions were found to be more difficult to be decontaminated. To further increase the degree of microbial inactivation, combined treatments were applied. PL combined with surfactant (Sodium dodecyl sulfate, SDS) was found to be more effective. The third study we investigated whether PL-surfactant combination would provide similar inactivation efficacy of *Salmonella* on green onions. Different surfactants (SDS and Tween 80) as well as different concentrations (10, 100 and 1000 ppm) of each surfactant combined with PL were tested. Survival populations of *Salmonella* and quality of green onions (color and texture) were evaluated after treatments as well as during storage (15 days) at 4 ℃. The results showed that PL-SDS and PL-Tween 80 combinations at various surfactant concentrations provided similar additive inactivation efficacy on green onion leaves, but not on the stems. PL-1000 ppm SDS combination negatively impacted color and texture of green onions during 15 day storage at 4 ℃. To consider both safety and quality of green onions, PL combined with lower concentration of surfactant is recommended.

For fresh raspberries, we investigated *Salmonella* and *E. coli* O157:H7 inactivation efficacy of dry PL. Raspberries were spot inoculated with pathogens and treated by PL for 5, 15 or 30 s. PL 30 s provided 4.5 and 3.9 log $_{10}$ CFU/g on

Salmonella and *E. coli* O157:H7, respectively. All the PL treatments maintained lower pathogen survival population during 10 day storage at 4 ℃ compared with the control. We also evaluated the quality of raspberries after PL treatment and during 10 days storage. The results showed that color and texture of raspberries treated by PL 30s changed significantly in 10 days storage. PL 30s did not have a negative impact on total phenolic content (TPC) but increased total anthocyanin content (TAC) significantly. PL 30s provided the lowest total bacterial count (TBC) and total yeast and mold counts (TYMC) at day 0, but failed to maintain its advantage during storage. To consider both safety and quality of fresh raspberries, 5 or 15s PL treatment is recommended.

In the end we observed the bacterial attachment and inactivation results under microscope using scanning electron microscopy (SEM). The results showed that dry PL, wet PL and PL-SDS treatments led to different cell morphology and viability of *E. coli* O157:H7. Different attachment behaviors have been observed on different food matrixes.

Taken together, we can ascertain that PL is an intervention strategy that can potentially deliver > 3 log reduction and elimination of *E. coli* O157:H7 and *Salmonella* on green onions and raspberries. PL can be applied in both dry and wet conditions which make it readily to adapt into the green onion or raspberry industries. PL-surfactant combinations provided significantly better decontamination efficacy on green onions. Dry PL for <15 s provided a decent decontamination efficacy on raspberries without compromising the quality during storage.

Chapter 1

INTRODUCTION

Fresh and fresh-cut fruits and vegetables contain rich sources of nutrients and provide numerous health benefits. The consumption of fresh produce in the U.S. has grown rapidly these years. However, numbers of foodborne outbreaks associated with fresh produce have caused illnesses or even death of outbreaks related individuals as well as negative economic impact on the fresh produce industry (Mishen, 1996; Maki, 2009). In 1998, FDA issued a "Guide to Minimize Microbial Food Safety Hazards for Fruits and Vegetables" that recommended good agricultural practices and GMPs, but foodborne outbreaks related to fresh produce continued. Large outbreaks have been associated with widely consumed commodities such as cantaloupes, raspberries, bagged lettuce and spinach, tomatoes, green onions and sprouts (FAO, 2008).

Green onions are now widely used in the United States as seasonings or as minor components in Asian recipes. Mexico is the dominant force in North American green onion production. The summer productions are usually located in the cooler western coastal areas with poorer infrastructure, which may be more challenging from a food safety perspective (Calvin et al., 2004). The relatively high number of handlers potentially increases the probability of microbial contamination. For both raw and value added green onions, washing is the only approach now used in industry to improve the microbial safety of the products. Contaminated equipment and ice/water

used for washing may also serve as potential source for bacterial or virus contamination (Sharps et al., 2012). Water is a useful tool for reducing potential contamination but it can also transfer pathogenic microorganisms (Gil et al., 2009). Sanitizer washing plays a critical role in removing soil and dirt, pesticide residues and most importantly, reducing contamination, thus maintain safety and extend shelf-life of the fresh produces. Chlorine based washing techniques have been applied by the majority (67 %) of fresh produce manufacturers for decontamination purposes (Gündüz et al., 2010). The limitation for chlorine application may be due to safety concern. Chlorine can react with organic matter to form undesirable by-products such as chloroform ($CHCl_3$) which have suspected carcinogenic or mutagenic effects.

Raspberries are the third most popular berry in the United States for fresh use, after strawberries and blueberries. During 1983 to 2013, there were 11 outbreaks related to raspberries with a total 4, 637 cases. Outbreaks have been associated with *C. cayetanensis* (CDC, 1996; CDC, 1997; CDC, 1998; Ho et al., 2002), Calicivirus (Pönkä et al., 1999), hepatitis A (Reid and Robinson, 1987) and norovirus (Falkenhorst et al., 2005; Cotterelle et al., 2005; Hjertqvist et al., 2006; Maunula et al., 2009). *C. cayetanensis*, hepatitis A as well as norovirus contaminated raspberries through fecal-oral route. Fresh as well as frozen raspberries have both been reportedly associated with outbreaks. Unlike green onions, fresh market raspberries are not washed. Thus, there is no way to use liquid sanitizers to limit the potential microbial risk.

In response to consumer's needs for fresh and healthy, novel techniques have been developed in order to reduce health risk and preserve quality and nutritional values of our food. These techniques can be used as alternatives or complements to traditional chlorine washing which has limited decontamination capacity as well as health concern. In the last decade, several emerging techniques have been proposed to inactivate *Salmonella* and/or *E. coli* O157:H7 in green onion and/or raspberry. These techniques included ozone (Xu and Wu, 2014), high hydrostatic pressure (Neetoo et al., 2011), electrolyzed water (Park et al., 2008) and irradiation (Murugesan et al., 2011; Fan et al., 2003). PL is a non-thermal method for food preservation that involves the use of intense, short duration pulses of a broad spectrum to ensure microbial decontamination on the surface of either foods or packaging materials (Elmnasser et al., 2007). PL treatment of foods has been approved by the FDA (1996) under the code 21CFR179.41. The lamp can generate light from UV to near-infrared (100 to 1100 nm) and UV region is critical to efficiency of pulsed-light treatment (Takeshita et al., 2002). PL has been reported to inactivate microorganisms naturally present (Hoornstra et al., 2002; Gómez-López et al., 2005) as well as inoculated (Fine and Gervais, 2004) on food surfaces. Sharma and Demirci (2003) inoculated alfalfa seeds with *E. coli* O157:H7 and treated the seeds with PL at a different distance for 75 s. They achieved 4.89 log reduction at a distance of 8 cm, but only1.42 log reduction at 13 cm. Little research about PL inactivating *Salmonella* and *E. coli* O157:H7 on green onions and raspberries has been observed.

The aim of the current project is to systematically investigate various non-chlorine sanitizers as well as the novel technique-PL to decontaminate *E. coli* O157:H7 and *Salmonella* on green onions and raspberries. Ultimately, the goal is to recommend a technique that can be feasibly applied to decontaminate green onions and fresh raspberries without compromising the quality of the produce.

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Chapter 2

LITERATURE REVIEW

2.1 Foodborne Pathogens

Foodborne illness (sometimes called foodborne disease, foodborne infection, or food poisoning) is a common, costly-yet preventable-public health problem. The Centers for Disease Control and Prevention (CDC) estimates that each year roughly 1 in 6 Americans (or 48 million people) get sick, 128, 000 are hospitalized, and 3,000 die of foodborne diseases (CDC, 2011a). The spectrum of foodborne pathogens includes a variety of enteric bacteria, aerobes and anaerobes, viral pathogens, parasites, marine dinoflagellate bacteria that produce biotoxins in fish and shellfish, and the self-inducing prions of transmissible encephalopathies (Tauxe, 2002). Of the many pathogens that can contaminate food some of them cause food infection such as *Salmonella spp*., *Campylobacter spp*. *Listeria monocytogenes*, hepatitis A, and norovirus etc. They are carried by food or water and cause illnesses by growing in the human body. Some pathogens, such as *Staphylococcus aureus* and *Clostridium botulinum*, cause food intoxications by producing different types of toxins. Some pathogens can be consumed by human and produce toxins in the intestinal tract which leads to illnesses. *Shigella spp*. and Shiga toxin-producing *Escherichia coli* are in this category.

2.1.1 *Salmonella*

Every year, *Salmonella* is estimated to cause about 1.2 million illnesses in the United States, with about 23,000 hospitalizations and 450 deaths (Scallan et al., 2011). Underreporting exists because that many cases of salmonellosis are not diagnosed and reported to the state health department; this may occur because the ill person does not seek medical care, the health care provider does not obtain a stool culture, or the culture results are not reported to public health officials (CDC, 2013a). It is estimated that 29.3 cases of salmonellosis occur for every one that is laboratory-confirmed and reported (Scallan, et. al, 2011).

Salmonella is a genus of gram-negative, facultative anaerobic, rod-shaped, non-spore forming, motile bacteria. It belongs to the *Enterobacteriacea* family which usually known as enteric bacteria, and was identified in 1885 by D.E. Salmon, an American bacteriologist (Tauxe, 1991). The nomenclature of *Salmonella* has been controversial since the original taxonomy of the genus was based on clinical considerations rather than DNA relatedness. For example, *Salmonella* typhi was named because it is the causative agent of typhoid fever. After the serological analysis was adopted into the Kauffmann-white scheme, the *Salmonella* genus was classified into different serovars. At first, host-specificity was suggested to name serovars, such as *Salmonella* Typhimurium. Then, because some of the serovars named by host-specificity were later proved to be ubiquitous, names derived from the geographical origin of the first isolated strain of the newly discovered serovars were chosen, e.g., *Salmonella Newport*. Now the *Salmonella* genus is classified into two species,

Salmonella enterica and *Salmonella bongori* (Wang, 2011). *Salmonella enterica* species has over 2,500 serovars under six subspecies and most of the human pathogenic *Salmonella* serovars belong to the *S. enterica* subsp. *enterica* subspecies, e.g., *Salmonella enterica* subsp. *enterica* serovar Typhimurium or shorter, *S.* Typhimurium.

Salmonella exists in the intestinal tracts of warm and cold blooded animals. Some serovars are ubiquitous while others are found in one particular host. The serovars commonly isolated from humans, agricultural products, and foods belong to the subspecies *enterica*. The infection caused by *Salmonella* is called Salmonellosis and there are two types of Salmonellosis in humans: typhoid and acute gastroenteritis. Typhoid or enteric fever is caused by bacteriasuch as *S.* Typhi or *S.* Paratyphi A, B or C (Tauxe, 1991) invasion of the bloodstream which leads to watery diarrhea, prolonged and spiking fever, nausea and abdominal cramps. Gastroenteritis is mostly related with foodborne illnesses. The majority of persons infected with *Salmonella* develop diarrhea, fever, and abdominal cramps 12 to 72 hours after infection. The illness usually lasts 4 to 7 days, and most persons recover without treatment. The mortality rate of human salmonellosis depends on the age of the subject, with an average of 4.1% (Wang, 2006). However, the elderly, infants, and those with impaired immune systems are more likely to have a severe illness.

It has been estimated that more than 200 virulence factors are responsible for the pathogenic properties of *Salmonella*. Intracellular survival and replication are critical for *Salmonella* to cause diarrhea of the host. Invasion of host cells and

intracellular survival are dependent on two type III secretion systems, T3SS1 and T3SS2 as well as a type I secretion system and other factors such as fimbriae, flagella and ion transporters (Ibarra and Steele-Mortimer, 2009). Following internalization *Salmonella* survive and replicate within a modified phagosome known as the *Salmonella*-containing vacuole (SCV), which can protect *Salmonella* by avoiding lyzosomal fusion and proliferate in host cells (Swart and Hensel, 2012). In addition, toxins play a notable role in the illness. Most serotypes of *S. enterica* can produce a thermolabile polypeptide enterotoxin that causes diarrhea.

All serovars of *Salmonella* are potentially pathogenic to humans. Serovars as Enteritidis, Typhimurium, and Newport account for about half of culture-confirmed *Salmonella* isolates reported by public health laboratories to the National *Salmonella* Surveillance System (CDC, 2013). Although it is widely known that meats and eggs are the major source of *Salmonella*, from 2006 to 2011 only 7 outbreaks were definitively linked to poultry meat or eggs, while other 17 outbreaks were associated with fresh produce including tomatoes in 2006, cantaloupes in 2008 and 2011, alfalfa sprouts in 2009, 2010 and 2011, papayas in 2011 and so on.

2.1.2 *Escherichia coli* O157:H7

It has been estimated that *E. coli* O157:H7 causes about 73, 500 cases of illness and 60 deaths annually in the U.S. (Brooks et al., 2005). Undoubtedly, other outbreaks have occurred but details have not been published in accessible journals. Of 45 VTEC outbreaks reported to National Surveillance Program, only 7 were described

in the literature (O'Brien et al., 2006). Also, FoodNet data indicate that *E. coli* O157:H7 causes significantly more cases of sporadic infections than cases linked to an outbreak (CDC, 2006). For example, in 2004, only 9 % of 402 confirmed cases of infection with *E. coli* O157:H7 were associated with outbreaks (CDC, 2006).

E. coli O157:H7 is a gram-negative, facultative anaerobic (i.e., possesses both respiratory and fermentative metabolic pathways) rod and is defined by its O (somatic lipopolysaccharide) and H (flagellar) surface antigens. *E. coli* O157:H7 is a foodborne zoonotic agent associated with outbreaks worldwide that poses a serious public health concern (Nguyen and Sperandio, 2012). It was first identified as a possible human pathogen in 1975 in a California patient with bloody diarrhea, and was first associated with a ground beef outbreak in 1982 (Riley et al., 1983). Most *E. coli* O157:H7 strains do not ferment sorbitol and do not contain the enzyme β-glucuronidase (Holt et al., 1994). The optimal growth temperature for *E. coli* O157:H7 is 37 °C (Holt et al., 1994), but it can survive as low as 7 °C and as high as 46 °C. *E. coli* O157:H7 prefers a neutral pH (6.0-7.0), but it can survive in food at a pH range of 3.7-4.4 (Weagant and Bound, 2001). The unique characteristics of *E. coli* O157:H7, such as acid and cold tolerance, make monitoring this foodborne pathogen critical within the food industry.

Ruminants have been identified as the major reservoir of *E. coli* O157:H7, with cattle as the most important source of human infections. Other ruminants known to harbor these bacteria include sheep (Barlow et al., 2006), goats (Beutin et al., 1995), and deer (Cody et al., 1999). *E. coli* O157:H7 can colonize the peri-rectal glands of ruminants and transfer from hides and feces to meat during the slaughter process. *E. coli* O157:H7 has the ability to cause attaching and effacing (A/E) lesions on human

intestinal epithelium which are characterized by close bacterial attachment to the epithelial cell membrane and the destruction of microvilli at the site of adherence. It colonizes the large intestine of humans (Phillips et al., 2000) and produces shiga toxin that binds to endothelial cells expressing globotriaosylceramide-3, allowing absorption into the bloodstream and dissemination of the toxin to other organs (Sandvig, 2001). Some of the genes that are involved in producing A/E lesions and shiga-like toxins can be used to identify the presence of *E. coli* O157:H7. Some infections caused by this pathogen are very mild, but some could be severe or even life-threatening. Very young children and the elderly are more likely to develop severe illness and hemolytic uremic syndrome (HUS) than others, but even healthy older children and young adults can become seriously ill (CDC, 2011b).

E. coli O157:H7 is the most commonly identified Shiga-toxin producing *E. coli* (STEC) in North America. STEC is a group of *E. coli* serotypes that produce shiga-like toxins because of their similarity to toxins produced by *Shigella dysenteriae*. STEC includes both *E. coli* O157:H7 and some non-O157 serotypes. There are over 380 different STEC serotypes isolated from humans and animals, but only a small number of serotypes are linked to human disease. *E. coli* O157:H7 also belongs to Enterohemorrhagic *Escherichia coli* (EHEC) which is a subset of pathogenic *E. coli* that can cause diarrhea or hemorrhagic colitis in humans. Enterohemorrhagic *E. coli* are STEC that possess additional virulence factors, giving them the ability to cause hemorrhagic colitis and hemolytic uremic syndrome (HUS) in humans. Currently no treatment is available for EHEC infections (Goldwater and Bettelheim, 2012). Conventional antibiotic treatments were proven to increase chances of developing hemolytic uremic syndrome (HUS) of patients infected by EHEC

(Safdar et al., 2002; Tarr et al., 2005). The exacerbating of Shiga toxin-mediated cytotoxicity maybe because antibiotics enhance the replication and expression of *stx* genes which encode shiga toxins.

E. coli O157:H7 infections occur worldwide; infections have been reported on every continent except Antarctica. According to CDC investigation (CDC, 2013b), 12 out of 14 outbreaks in the United States related with *E. coli* were caused by *E. coli* O157:H7. The most recent one happened on November 2011, sixty people from 10 states have been infected and romaine lettuce was reported as the source. Animal products such as beef (2007, 2008, 2009 and 2010) and Lebanon bologna (2011) as well as non-animal products such as spinach (2006), pizza (2006), prepackaged cookie dough (2009), cheese (2010) and in-shell hazelnuts (2011) have all served as vehicle foods in *E. coli* O157:H7 outbreaks from 2006 to 2011.

2.2 Microbial Safety of Fresh Produce

Fresh and fresh-cut fruits and vegetables contain rich sources of nutrients and provide numerous health benefits. Since people have become more and more health conscious over the years, the consumption of fresh produce in the U.S. has grown rapidly. In the United States, the weekly sales of prepared fruits and vegetables increased from $1298 and $567 per store in 2005, respectively, to $1587 and $804 in 2010 (Padera, 2010). The annual per capita consumption of fruits and vegetables increased by 8.4 % from 1976-2009, reaching 675 lbs (Cook, 2011). Minimally processed, bagged produce is a relatively recent new product to help meet the growing demand for fresh produce (USDA-ERS, 2001). However, numbers of foodborne outbreaks associated with fresh produce have caused illness, hospitalizations and death

of the consumers, serious economic loss of industries, as well as increasing public concern about microbial safety of fresh produce.

Many foodborne pathogens have animal reservoirs and normally contaminate animal product, for example, *E. coli* O157:H7 has been associated with outbreaks in ground beef, and *Salmonella* has caused outbreaks related with poultry and eggs. There are also many new food vehicles that are plant derived, including plant-derived processed foods, like peanut butter, spices such as black and white pepper, tree nuts, and fresh produce. In 1998, FDA issued a "Guide to Minimize Microbial Food Safety Hazards for Fruits and Vegetables" that recommended good agricultural practices and GMPs, however foodborne outbreaks related to fresh produce continued. Large outbreaks have been associated with widely consumed commodities such as cantaloupes, raspberries, bagged lettuce and spinach, tomatoes, green onions and sprouts (FAO, 2008).

The consequence of produce-associated outbreaks including illnesses or even death of outbreaks related individuals as well as negative economic impact on the fresh produce industry. The estimated cost to tomato growers from the 2008 multi-states *Salmonella* Saintpaul outbreak was approximately $200 million (Maki, 2009) even though it has been proven that jalapeño peppers were the major vehicle, with tomatoes just as a possible vehicle (CDC, 2008). Another good example would be the cyclosporiasis illness outbreak related with raspberries in 1996. Initially, investigators linked this outbreak to California strawberries, the California Strawberry Commission estimated that this false alarm led to $16 million in lost revenue to growers in the central coast of California during the month of June (Mishen, 1996). After Guatemalan raspberries were finally found out to be the responsible source, the FDA,

not yet convinced the problem was resolved, issued an import alert, denying all Guatemalan raspberries entry into the United States. Beginning in the spring 1999 season, the United States allowed entry of raspberries produced under the Model Plan of Excellence (MPE), which was a costly program resulting in a reduction of raspberry growers in Guatemala from 85 in 1996 to 3 in 2002(Calvin et al., 2003). During 2003 to 2010, norovirus, *Salmonella*, and *E. coli* O157:H7 were responsible for most of the outbreaks (Funt and Hall, 2013).

2.3 Contamination of Fresh Produce from Farm to Fork

Produce alone does not normally harbor pathogenic microorganisms, however zoonotic bacterial pathogens such as *Salmonella* and *E. coli* O157:H7 are easily transferred from other sources.

Potential sources of human pathogens include fecal contamination from animals (Yan et al., 2007), composted manure (Islam et al., 2004), soil (Johannessen et al., 2005), irrigation water (Steele et al., 2005), unhygienic workers (Espino-Medina et al., 2006), packing equipment (Duffy et al., 2005), process water used for cooling and washing (Gagliardi et al., 2003), browning inhibitors for fresh cut produce (Wang et al, 2007), etc.

The feces of domesticated animals, wildlife, or humans are usually the ultimate source of enteric pathogens. Studies have shown than up to 30% of all cattle are asymptomatic carriers of *E. coli* O157:H7 (Callaway et al., 2006). At least 80 million dry tons of solid manure is generated annually by the beef, dairy, swine, and poultry industries in the United States (Power et al., 2000). Fresh, non-composted manure will generally have a higher soluble N content than composted manure, which can lead to salt build-up and leaching losses if over applied. Also fresh manure may contain high

amounts of viable weed seeds as well as various microorganisms. People compost the manure to inactivate the weed seeds and microorganisms. Composted manure, however, alone may not be able to supply adequate available nutrients, particularly N, during rapid growth phases of crops with high nutrient demands because some of the ammonia-N will be lost as a gas (Rosen and Bierman, 2005). Although the majority of the microorganisms contained in manure are not pathogenic to humans, zoonotic pathogens have the potential to be transported from the manure to water, soil, food and other areas of environment (Ziemer et al., 2010). Although the prevalence of *E. coli* O157:H7 and *Salmonella* in feces is not clear, the large amount of feces is able to provide a significant source of pathogenic bacteria (Matthews, et al., 2006).

To be able to attach to the plant and cause contamination, pathogens first have to survive in the animal manure as well as soil or water that contact with the manure. Pathogen appears to be well adapted to survive in animal feces and can persist for extended periods ranging from several weeks to many months (Duffy, 2003). Because of this persistence, after manure has been applied on farm, Ziemer (2010) reported longer survival periods in soil and water. Pathogens in manure may transfer to water either directly or through runoff. Heavy rains (Cooley et al., 2007) or flooding (Doyle et al., 2006) is able to increase the levels of bacterial pathogens in water sources. Contaminated water has been implicated in several outbreaks arising from produce, such as tomato (2005-2006) and shredded lettuce (2006).

It has been proven that enteric pathogens have the ability to transfer from contaminated water, soil or manure to plants (Mootian et al., 2009). Recent development of molecular-based methods reduced the detection limit, and indicated that even irrigation with water containing as little as 300 CFU/mL results in

persistence of *S*. Typhimurium on the plants for at least 2 days (Kisluk and Yaron, 2012).

2.4 Bacterial Attachment and Survival on Produce

To contaminate fresh produce and transfer in the food producing chain, foodborne pathogens have to attach themselves on the plant surface to survive or proliferate. The aerial surfaces of vascular plants are covered with a continuous extracellular layer called the cuticle that overlays the cell wall of epidermal cells (Nawrath, 2002). The combination of cutin, waxes and possibly polysaccharides, forms the cuticle (Jeffree, 1996). The cuticle prevents plant dehydration and also protects the plant from infiltration by microorganisms. However, breaks in the cuticle may expose hydrophilic structures from within (Patel and Sharma, 2010). Pathogens use wounds and natural openings like stomata, hydathodes, and lenticels as natural gateways for passage into internal plant tissues where they grow and begin to cause disease (Hirano and Upper, 2000; Melotto et al., 2006; Saldaña et al., 2011) investigated the ability of STEC to reside within the stomata internal cavity and internal tissues of spinach leaves. The scanning electron micrograph (SEM) analysis of cross sections of infected leaves confirmed the presence of STEC bacteria within stomata internal cavities, intercellular spaces of the spongy mesophyll, and notably in the vascular tissue (xylem and phloem). The surface roughness also affects pathogen attachment. The surface topography of fruits and vegetables can be quite complex, because there are multiple scales of topography that contribute to the overall topography (Gómez-López, 2012).Wang et al. (2009) found that an increase in fruit surface roughness would introduce protection to microbes entrapped on fruit surface resulting in reduced washing efficacy.

Postharvest contamination may result in higher levels of contamination because cut leaves serve a better niche for *E. coli* and *Salmonella* (Patel and Sharma, 2010). Liao and Sapers (2000) investigated the attachment and growth of *Salmonella* Chester on fresh-cut apple disks and *in vivo* response of attached bacteria to sanitizer treatments. They suggested that firm attachment of bacteria on calyx, stem, and injured tissue, and partial resistance of attached bacteria to sanitizer treatments, are two major obstacles to be considered when developing methods for cleaning and decontaminating apple fruits destined for juice production and fresh consumption. Attachment depends on plant and bacterial factors as well as on environment conditions. The levels of attachment usually differentiate between intact and damaged plant tissues. Attachment to plant tissues is the initial step of biofilm formation or internalization.

2.5 Biofilm and Internalization

Biofilms are defined as structured communities of bacterial cells enclosed in a self-produced polymeric matrix adherent to inert or living surfaces (Hall-Stoodley et al., 2006; Homoe et al., 2009). It is the predominant mode of bacterial growth, reflected in the observation that approximately 80% of all bacterial infections are related to biofilms (National Institutes of Health (USA)) (Hall-Stoodley and Stoodley, 2009). Increasing evidence shows that microorganisms attached to surfaces are more resistant to sanitizers than their planktonic counterparts (Peta et al., 2003). Although plants are not traditionally considered as hosts for human enteric pathogens, numerous recent outbreaks have associated *Salmonella* and *E. coli* O157:H7 with contaminated fresh produce. It has been reported that *Salmonella enterica* is able to form biofilm on a variety of plants including tomato (Barak and Liang, 2008); basil (Berger et al.,

2009), sprouts (Barak et al., 2002) and lettuce (Kroupitski et al., 2009). Niemira and Cooke (2010) indicated that *E. coli* O157: H7 biofilm formation on romaine lettuce and spinach leaf surfaces reduced efficacy of irradiation and sodium hypochlorite washes.

Several reports demonstrated that enteric pathogens associated with plants cannot be easily inactivated by various surface sterilization methods (Proctor et al., 2001; Weissinger et al., 2000).This not only indicates the impact of epiphytic aggregation and biofilm formation, but also stresses the importance of endophytic growth which is known as internalization. Entrance of *E. coli* O157:H7 cells into stomata of cut lettuce and penetration into damaged tissue has been reported to provide protection against chlorine inactivation (Takeuchi and Frank, 2001). *Salmonella* Typhimurium has also been reported be capable of penetrating the epidermis of iceberg lettuce leaves (Kroupitski et al., 2009). Internalized bacteria are refractory to disinfection which potentially poses a safety hazard to consumers. Attached pathogens that form biofilm or internalize into plant tissue are extremely difficult to remove with current washing or agitation regimens.

2.6 Conventional Method for Decontamination of *Salmonella* and *E. coli* O157:H7 on Fresh Produce

It is well known that fresh and fresh-cut processors usually rely on wash water sanitizers to reduce microbial counts in order to maintain quality and extend shelf-life of the end product. Water is a useful tool for reducing potential contamination but it can also transfer pathogenic microorganisms (Gil et al., 2009). Sanitizer washing plays a critical role in removing soil and dirt, pesticide residues and most importantly,

reducing contamination, thus maintain safety and extend shelf-life of the fresh produces.

2.6.1 Chlorine

Chlorine (Cl) is a very potent disinfectant with powerful oxidizing properties. It is soluble in water, either by injection of chlorine gas or by the addition of hypochlorite salts. Chlorine water consists of a mixture of chlorine gas (Cl_2), hypochlorous acid (HOCl), and hypochlorite ions (OCl^-) in amounts that vary with the pH of water. Hypochlorous acid, the most important form of chlorine in water, is also called "free chlorine" and is the primary agent responsible for microorganism inactivation. However, HOCl is a weak acid in water, meaning that it partially dissociates into the hypochlorite anion (OCl^-). There is always an active equilibrium going on in water as chlorine switches back and forth from the HOCl form to the OCl^- form. At a pH of 5-7, free chlorine is most effective since chlorine is mainly in HOCl form. When pH of the water is above 7, the OCl^- form predominates and the inactivation capacity decreases (Kelley, 2004). Chlorine based washing techniques have been applied by the majority (67%) of fresh produce manufacturers for decontamination purposes (Gündöz et al., 2010). Chlorine attaches very easily to double bonds of biomolecules and causes a lethal effect on the metabolism of the microorganism. It also destroys cell membrane and reacts with DNA. Martínez-Téllez et al. (2009) has investigated the efficacy of chlorine (200 and 250 ppm), hydrogen peroxide (1.5% and 2%), and lactic acid (1.5% and 2%) sanitizers during different exposure times (40, 60, and 90 s) on the reduction of *Salmonella* Typhimurium in inoculated fresh green asparagus and green onions. Chlorine sanitation showed a better efficacy at a higher concentration (inhibition of *Salmonella* at 1.36-1.74 $\log_{10}$

CFU/g). However, no significant difference ($p > 0.05$) between 200 ppm and 250 ppm chlorine was observed. The limitation for chlorine application may be due to safety concern. Chlorine can react with organic matter to form undesirable by-products such as chloroform ($CHCl_3$) which have suspected carcinogenic or mutagenic effects. As a result, lower level of chlorine washing or alternative chlorine-based sanitizers is needed.

2.6.2 Organic acids

Organic acids occur throughout nature and are used extensively in food systems. Citric, malic, tartaric and acetic acids are most commonly used organic acids in food industry. The mechanisms for organic acids inactivating pathogens depend on the undissociated form, as well as their ability to donate hydrogen ions in aqueous system. They can lower the environmental pH, interfere with the cell membrane transport system and permeability, then reduce internal pH and disrupt cellular functions (i.e. enzyme stability) (Lück and Jager, 1997). In Martínez-Téllez's research (2009) results showed that the most effective sanitizer evaluated for green onion disinfection was 2% lactic acid reducing *Salmonella* growth close to 3 $\log_{10}$ CFU/g.

Although limited information about acid washing green onion and raspberry could be obtained, organic acid showed promising antimicrobial effects on other fresh produce. In the study of Sagong et al. (2011), for example, a 1.8 $\log_{10}$ CFU/g reduction of *S.* Typhimurium on organic fresh lettuce washed by 20 mg/ml of citric acid solution for 5 min was observed. Ruiz-Cruz et al. (2007) inoculated shredded carrots with *Escherichia coli* O157:H7, *Salmonella* or *Listeria monocytogenes* and washed them for 1 or 2 min with chlorine (Cl; 200 ppm), peroxyacetic acid (PA; 40 ppm) or acidified sodium chlorite (ASC; 100, 200, 500 ppm) under simulated process

water or tap water. The results showed that PA was more effective than chlorine under simulated process water conditions with its most significant effect observed on the reduction of *Salmonella* (2.1 log $_{10}$ CFU/g); however, PA only reduced *E. coli* O157:H7 by 1.24 log $_{10}$ CFU/g, respectively, under tap and simulated process water conditions.

2.6.3 Essential oils

Thyme oil and its major antibacterial components thymol and carvacrol with GRAS status (FDA, 2013a), have been found to possess antimicrobial activity *in vitro* against a broad spectrum of bacteria (Di Pasqua et al., 2006; Gill and Holley, 2006). The antimicrobial effect of thymol may be due to its ability to partition out of the water phase and into the lipid membrane of the microbial cell (Dorman and Deans, 2000). Singh et al. (2002) studied thyme oil as a washing solution against foodborne *E. coli* O157:H7 on lettuce and baby carrots. Results showed that thyme oil followed by aqueous ClO_2/ozonated water, or ozonated water/aqueous ClO_2 were significantly (p<0.05) more effective in reducing *E. coli* O157:H7 (3.75 and 3.99 log; 3.83 and 4.34 log reduction) on lettuce and baby carrots, respectively. But limited research is available on their antimicrobial application on green onion and raspberry.

2.6.4 Hydrogen peroxide

The bactericidal efficacy of hydrogen peroxide has been demonstrated in both water and food systems (Liao and Sapers, 2000). The antimicrobial mechanism of hydrogen peroxide is to form a reactive oxygen species such as the hydroxyl radical and singlet oxygen, which can damage DNA and membrane constituents (Juven and Pierson, 1996). Hydrogen peroxide has GRAS status (21 CFR 178.1005) (FDA,

2013b), which can be used in both vapor and liquid phase to control the bacteria and extend the shelf life of minimally processed fruits and vegetables (Sapers and Simmons, 1998). On apple disks, 6% hydrogen peroxide gave a greater reduction of *Salmonella* Chester than trisodium phosphate, calcium hypochlorite, or sodium hypochlorite (Liao and Sapers, 2000). In Martínez-Téllez et al. (2009), hydrogen peroxide was the least effective agent for *Salmonella* Typhimurium reduction on green onion when compared with lactic acid and chlorine. No effect was observed of the exposure time of inoculated product with sanitizer up to 90 seconds. Although, limited application on inactivation of *Salmonella* and *E. coli* O157:H7 in green onion and raspberry has been observed, hydrogen peroxide has been used on other fresh produce for decontamination purpose. In Wei's research (Wei et al., 2005) salad was inoculated with *Salmonella* Typhimurium. After washing with warm water (45-50 °C) containing H_2O_2 (1-4%) for 60-210 s, 0.79-3.05 $\log_{10}$ CFU/g reduction was observed immediately after treatment, and 1.1-2.63 $\log_{10}$ CFU/g reduction was observed after storage.

2.6.5 Sodium dodecyl sulfate (SDS)

SDS is an anionic surfactant and an FDA-approved food additive (FDA 1978) (21 CFR 172.822) used as emulsifier (not exceeding 1000ppm for egg white solids; 125ppm for frozen and liquid egg white, whipping agent (not exceeding 0.5% by weight of gelatine used in the preparation of marshmallows), surfactant (not exceeding 25ppm in beverage and fruit juice) as well as wetting agent (not exceeding 10ppm in vegetable oil and animal fats). Surfactants need to be considered as one of the choices in sanitizer combination for two reasons. First, it allows the release of tightly bound contaminations from the surface which may enhance the removal of foodborne

pathogens from fresh produce. Zhao et al. (2010) reported that inoculated alfalfa seeds dried for 4 h then treated for 5 min at 21°C with 0.5% levulinic acid and 0.05% SDS reduced the population of *E. coli* O157:H7 and *Salmonella* Typhimurium by 5.6 and 6.4 log $_{10}$ CFU/g, respectively. On seeds dried for 72 h, the same treatment reduced *E. coli* O157:H7 and *Salmonella* Typhimurium populations by 4 log $_{10}$ CFU/g. FIT Fruit and Vegetable Wash™ (Procter and Gamble Co., Cincinnati, Ohio, U.S.A.) is marketed as an effective antimicrobial (Oner and Walker, 2010). It consists of GRAS status substances levulinic acid (FDA 2004) (21 CFR 172.515) and sodium dodecyl sulfate (SDS). Predmore and Li (2011) indicated that while tap water alone and chlorine solution (200 ppm) gave only <1.2 log reductions in virus titer in all fresh produce, a solution containing 50 ppm of surfactant was able to achieve a 3 log reduction in virus titer in strawberries and an approximately 2 log reduction in virus titer in lettuce, cabbage, and raspberries. Second, most disinfectants require direct contact. Water films that form on very small contours on plant surface, however, may prevent the sanitizers from directly contacting target microorganisms. Adding surfactant such as SDS can reduce water surface tension and may enhance the efficiency of other disinfectants.

2.7 Novel Techniques for Decontamination of *Salmonella* and *E. coli* O157:H7 on Fresh Produce

In response to consumer's needs for fresh and healthy, novel techniques have been developed in order to reduce health risk and preserve quality and nutritional values of our food. These techniques can be used as alternatives or complements to traditional chlorine washing which has limited decontamination capacity as well as health concern. In the last decade, several emerging techniques have been proposed to

inactivate *Salmonella* and/or *E. coli* O157:H7 in green onion and/or raspberry. These techniques included ozone (Bialka and Demirci, 2007), high hydrostatic pressure (Neetoo et al., 2011), electrolyzed water (Park et al., 2008) and irradiation (Murugesan et al., 2011; Fan et al., 2003).

2.7.1 Ozone

Ozone, one of the most powerful oxidizing agents used in food industry, is generated by a high energy input that splits the oxygen molecule in the air. Promising results of its microbial inactivation (Oztekin et al., 2005, Bialka and Demirci, 2007) have been reported since it was approved as GRAS (generally recognized as safe) in 1997. Bialka and Demirci (2007) treated pathogen-inoculated fruits with aqueous ozone (concentrations of 1.7 to 8.9 mg/L) at 20℃ for 2 to 64 min. The results showed that maximum pathogen reductions on raspberries were 5.6 and 4.5 $\log_{10}$ CFU/g for *E. coli* O157:H7 and *Salmonella*, respectively, at 4℃.

2.7.2 High hydrostatic pressure (HHP)

High Hydrostatic Pressure (HHP), High Pressure Processing (HPP) and Ultra High Pressure (UHP) are all names for the same process which is one of the emerging techniques as an alternative to thermal processing. HHP can inactivate vegetative microbial cells by breaking non-covalent bonds and causing damage to the cell membrane. It has been demonstrated to effectively inactivate foodborne pathogens and spoilage flora on a wide range of fruits and vegetables (Yaldagard et al., 2008). Studies have shown that after pressure treatment at 400-450 MPa (soaked) or 450-500 MPa (wetted) for a retention time of 2 min at 20-40 ℃, wild-type and antibiotic-resistant mutant strains of *Salmonella* and *E. coli* O157:H7 inoculated on green onions

were undetectable immediately after treatment and throughout the 15 days storage at 4℃ (Neetoo et al., 2011).

2.7.3 Electrolyzed water

Electrolyzed water (EW or EO, also known as electrolyzed oxidizing water) is produced by the electrolysis of ordinary tap water containing dissolved sodium chloride. Studies investigated the antimicrobial efficacy of electrolyzed water and acidic electrolyzed water against *E. coli* O157:H7, *Salmonella* Typhimurium and *Listeria monocytogenes* on green onions showed that acidic EW treatment was able to significantly reduce populations of the three tested pathogens from the surfaces of green onions with increasing exposure time (Park et al., 2008).

2.7.4 Irradiation

Irradiation is a non-thermal process capable of destroying foodborne pathogens. Murugesan et al. (2011) studied the effect of irradiation on *Salmonella* survival and quality of 2 varieties of whole green onions. Results showed that a 5-log reduction of *Salmonella* can be achieved at a dose of 1.6 kGy. No significant difference was observed in color and texture between irradiated samples and control. Fan et al. (2003) studied the effect of irradiation at doses of 1, 2, and 3 kGy on the background microflora of fresh-cut green onion leaves. The authors observed that doses > 2 kGy were required in order to improve the microbiological quality and safety of green onions with such treatments, however it did result in an increased loss of aroma and deterioration of visual quality.

2.7.5 Pulsed UV light (PL)

PL is a non-thermal method for food preservation that involves the use of intense, short duration pulses of a broad spectrum to ensure microbial decontamination on the surface of either foods or packaging materials (Elmnasser et al., 2007). PL treatment of foods has been approved by the FDA (1996) under the code 21CFR179.41. The lamp can generate light from UV to near-infrared (100 to 1100nm) and UV region is critical to efficiency of pulsed-light treatment (Takeshita et al., 2002). The UV part of the spectrum in PL has been proven to have photochemical and/or photo-thermal effect. The primary target of the PL is DNA which is the same as UV light. PL and UV are both attributed primarily to a photochemical transformation of pyrimidine bases in the DNA to form dimers (Giese and Darby, 2000) which will cause a problem for DNA replication. However, UV light treatment was not as severe as PL and in some experimental conditions can be reversible because of the DNA repair system, while PL can inactivate the DNA repair system itself to cause irreversible damage of the DNA and death of the bacteria (Smith et al., 2002). Besides photochemical effects, the microorganism inactivation action of PL may also be due to photo-thermal mechanisms. Absorption of the UV light will cause overheating of cellular tissue which leads to intracellular water vaporization (Wekhof, 2000), cell membrane disruption (Takeshita et al., 2003) and finally death of the microorganism. PL has been reported to inactivate microorganisms naturally present (Hoornstra et al., 2002; Gómez-López et al., 2005a) as well as inoculated (Fine and Gervais, 2004) on food surfaces. Sharma and Demirci (2003) inoculated alfalfa seeds with *E. coli* O157:H7 and treated the seeds with PL at a different distance for 75s. They achieved 4.89 log reduction at a distance of 8 cm, but only1.42 log reduction at 13 cm. Little

research about PL inactivating *Salmonella* and *E. coli* O157:H7 on green onions and raspberries has been observed.

2.8 Green Onion Industry

Because of their milder onion-like flavor, green onions are now widely used in the USA as seasonings or as minor components in Asian recipes. Mexico is the dominant force in North American green onion production because 1) green onion is a labor-intensive crop that is cheaper to grow in Mexico than in the United States, and 2) it helps meet the year-round demand of the consumers. Green onions are generally harvested by hand, and can be packed in the field, in a packinghouse or in a value added processing plant. As shown in Figure 2.1(FDA, 2010), raw green onions need to go through the packinghouse for washing, trimming, banding, packing in ice and then cold storage. Value added green onions are different from raw green onions since they are packed in a processing facility where they are cleaned, trimmed, sometimes cut and packed in some form of plastic, protective packing and then cold storage.

2.9 Microbial Safety of Green Onions

Mexico is the major exporter of green onions to the United States. The summer productions are usually located in the cooler western coastal areas with poorer infrastructure, which may be more challenging from a food safety perspective (Calvin et al., 2004). During pre-harvest, pathogens in animal manure or irrigation water may contaminate plants surface or even internalize and transfer to other parts of the plants (Solomon et al., 2002). Quality preservation approaches, if not applied correctly, may cause a microbial safety issue. Soil kept on the root before any trimming or washing occurs to control the dehydration, water sprayed during harvest or in the field

container just after harvest to reduce water loss, and washing with water in a packinghouse to reduce the microbial loads as well as ice and/or ice slurries used to cool green onions may all serve as contamination source as long as contaminated soil or water is used. From harvesting to packing, green onion requires as many as nine manual labor steps (Calvin et al., 2004). The relatively high number of handlers potentially increases the probability of microbial contamination. For both raw and value added green onions, washing is the only approach now used in industry to improve the microbial safety of the products. Contaminated equipment and ice/water used for washing may also serve as potential source for bacterial or virus contamination (Sharps et al., 2012).

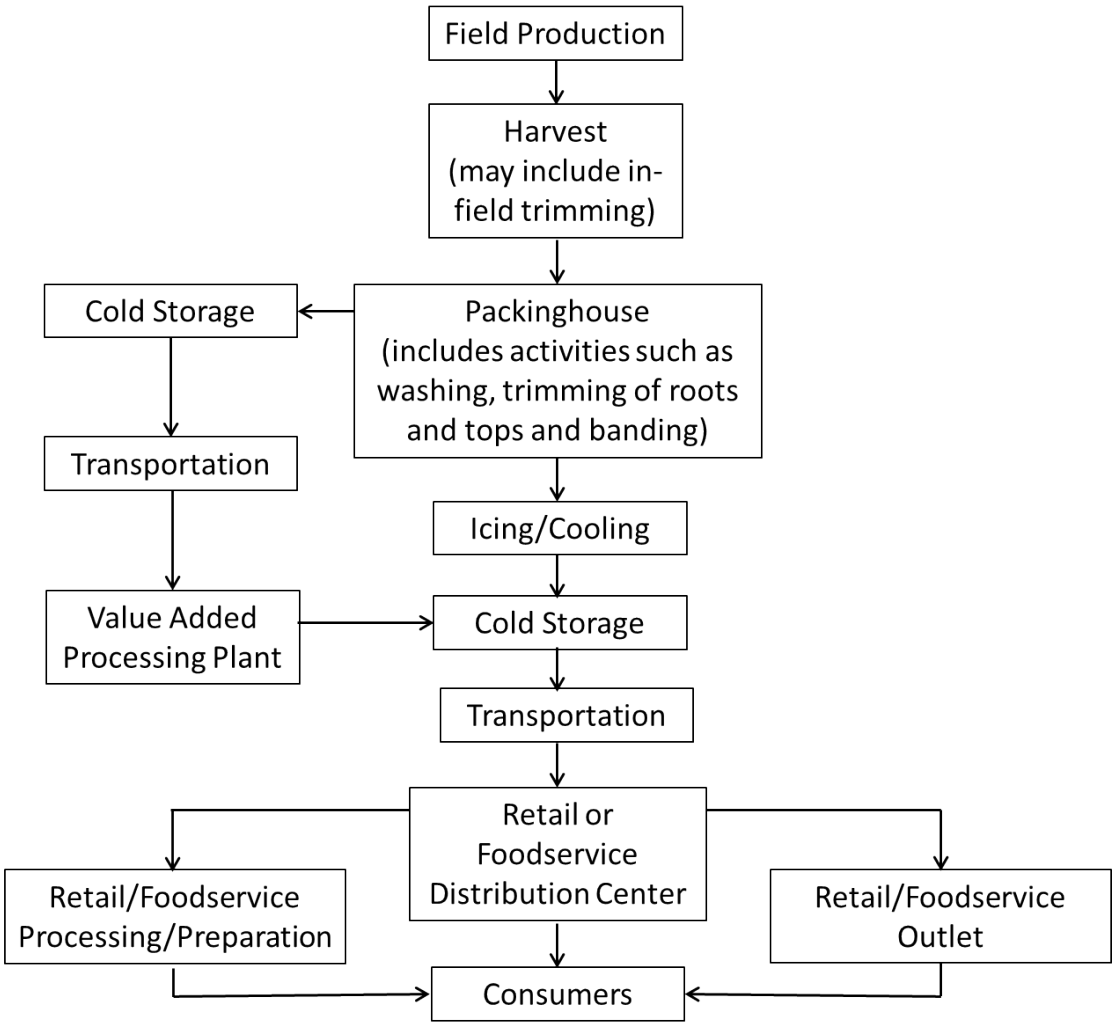

Figure 2.1: General supply chain flow of green onions

2.10 Raspberry Industry

Raspberries are the third most popular berry in the United States for fresh use, after strawberries and blueberries. As the world's third-largest producer, most of raspberries growers in the U.S. are located in Washington, California and Oregon. Washington State leads the nation in red raspberry (Rubus idaeus) production. In 2010, Washington raised 61 million pounds of red raspberries valued at $50 million.

Oregon leads the United States in black raspberry (Rubus occidentalis) production with 1.8 million pounds grown in 2011, valued at $2.3 million. California raised 81 million pounds of total raspberries valued at $200 million (Geisler, 2012). However, only 15% of the domestic demand for raspberry fruit is met by the production in the United States. Most imports are arriving from Canada in July and August, and Mexico and Chile from November through May. In 2010, the United States imported 13,927 metric tons (MT) of fresh Mexican raspberries valued at $118 million, and a total of 442 MT of Canadian raspberries valued at $658,000 (Geisler, 2012).

Raspberries designated for fresh market are picked at pink to light red stage, when fruit can be removed from receptacles by hand without tearing the berry. For commercial operations in the U. S., raspberries are gently tugged from the receptacle and placed quickly into plastic clamshells. Clamshells are then placed in master containers which are cardboard cartons with holes in the sides for forced air cooling and reinforced corners for stacking. Cartons are taken to cooling facilities, either into refrigerated trucks in place in the field, or to nearby refrigerated storage (Funt and Hall, 2013). Timing of the refrigerator has great impacts on the shelf-life of raspberries.

2.11 Microbial Safety of Raspberries

During 1983 to 2013, there were 11 outbreaks related to raspberries with a total 4, 637 cases. In earlier time, raspberries outbreaks have been associated with *C. cayetanensis* (CDC, 1996; CDC, 1997; CDC, 1998; Ho et al., 2002), Calicivirus (Pönkä et al., 1999) and hepatitis A (Reid and Robinson, 1987). Most recently, there were four raspberry outbreaks related with norovirus (Falkenhorst et al., 2005; Cotterelle et al., 2005; Hjertqvist et al., 2006; Maunula et al., 2009). *C. cayetanensis*,

hepatitis A as well as norovirus contaminated raspberries through fecal-oral route. Fresh as well as frozen raspberries have both been reportedly associated with outbreaks. Imported berries were often implicated, indicating that this problem extends beyond those countries where there have been outbreaks (FAO, 2008). In 1996, the FDA used epidemiological and trace back evidence to associate a large foodborne cyclosporiasis illness outbreak with Guatemalan raspberries which infected 1,465 people in the United States and Canada (Calvin et al., 2004).

Berries are highly susceptible to microbial contamination because (1) berries may be harvested from the wild as well as cultivated, thus, the diversity of the production chain and industry can be extensive; (2) harvesting is primarily undertaken by a large number of people which is considered the primary source of berry contamination; and (3) animal entrance into fields and packing houses, irrigation water, picking berries close to the soil line as well as the use of manure as fertilizer all increases the probability of microbial contamination via the fecal-oral route. The challenge for raspberry decontamination exists because, (1) raspberries are made up of many individual fruits (drupelets) held together by hairs (trichomes) and waxes (Mackenzie, 1979) which provides a harbor for foodborne pathogens, and (2) unlike tree fruit, fresh market raspberries are not washed. Thus, there is no way to use liquid sanitizers to limit the potential microbial risk.

2.12 Effects of Sanitizer Washing and PL on Quality of Fresh Produce

Over the last decade, consumers have increasingly required processed foods to have a more natural flavor, color and a sufficient shelf-life. Safety and quality, including apparent quality and bioactivities are both critical for the fresh produce

industry. Researches were trying to develop techniques with satisfying antimicrobial efficacy without compromising quality.

Researchers reported that sanitizer washing would not affect the quality of fresh produce, including texture, color and sensory (Lu and Wu, 2010; Sagong et al., 2011). However, there was a publication that reported that strawberries and raspberries showed great sensitivity to anthocyanin bleaching at bactericidal peroxide levels (Sapers and Simmons, 1998). For PL treatment, Sharma and Demirci (2003) found that pulsed light did not significantly affect the percentage germination of alfalfa seeds. Lammertyn et al. (2003) reported that PL treatment did not significantly reduce strawberry sepal quality decay rate during storage for 10 days at 12 ℃. However, the inactivation of microorganisms present on food surfaces does not necessarily result in an extension of shelf-life (Gómez-López et al., 2007). Research done by this reviewer in 2005 showed that PL treatment failed to prolong the shelf-life of shredded iceberg lettuce and shredded white cabbage, stored under modified atmosphere packaging at 7℃ (Gómez-López et al., 2005b).

Regarding bioactivities, Kim (Kim et al., 2007) has observed that dipping fresh-cut tomato into H_2O_2 resulted in reduced phenolic and antioxidant levels after 7 d in storage by at least 5% and 20%, respectively, and produced an initial decline in vitamin C and lycopene. There has been research that showed that UV-C treatments on fresh-cut green onions enhanced their antioxidant activity (Kasim and Kasim, 2010).

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Chapter 3

POTENTIAL APPLICATION OF SANITIZERS AND PULSED LIGHT (PL) TO ELIMINATE *SALMONELLA ENTERICA* TYPHIMURIUM ON GREEN ONIONS

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ABSTRACT

Pathogenic bacteria such as *Salmonella* and *Shigella flexneri* have been linked to green onion contamination. This study was conducted to evaluate decontamination of *Salmonella* Typhimurium using new formula sanitizers washing (0.4 mg/mL thymol and five new formula sanitizers including 300ppm H_2O_2 + 4% SDS, 2mg/mL citric acid + 4% SDS, 0.2mg/mL thymol + 4% SDS, 0.2mg/mL thymol + 2mg/mL citric acid and 0.2mg/mL thymol + 2mg/mL acetic acid), pulsed UV light (PL) as well as synergy between the sanitizer wash and PL. New formula sanitizers based on decontamination efficacy of single washing solutions (organic acids, hydrogen peroxide (H_2O_2), essential oil or surfactant) were applied to decontaminate spot inoculated green onions. PL, the novel technique, alone has been applied to inactivate *Salmonella* on both dip and spot inoculated green onions. *Salmonella* inactivation of PL-new formula sanitizer combinations on dip inoculated green onions has been investigated as well. As a result, for spot inoculated green onions, 0.4 mg/mL thymol individually and the five new formula sanitizers all achieved higher log reduction of

Salmonella (4.5-5.3 log $_{10}$ CFU/g reduction) than 200ppm chlorine washing. These new formulas of sanitizer would be potential alternatives to chlorine. The 5s dry PL (4.6 log $_{10}$ CFU/g) or 60s wet PL treatment (3.6 log $_{10}$ CFU/g) was better or comparable as chlorine washing. The sanitizer combinations did not provide significantly higher log reduction than PL, and PL has the potential of being used in green onion industry for decontamination purpose. For dip inoculated green onions, none of our treatment provided >0.8 log $_{10}$ CFU/g (0.6-0.8 log $_{10}$ CFU/g) reduction of *Salmonella*. As a result, PL-new formula sanitizer combinations had no or minimal enhanced efficacy to inactivate *Salmonella* dip inoculated on green onions.
KEY WORDS: *Salmonella*; green onions; new formula sanitizer; pulsed light (PL)

3.1 Introduction

Pathogenic contamination has been found on both domestic and imported green onions (FDA, 2001a and FDA, 2001b). Green onion is highly susceptible to contamination for three reasons: (1) green onion surfaces are particularly complex or adherent to viral or fecal particles (Dato et al., 2003); (2) it is differ in morphology from other leafy green vegetables such as lettuces, spinach, cabbages, chicory and leafy fresh herbs, which is easier for pathogens to hide in its hollow tube leaf (FAO and WHO, 2008) as well as multi-layered structural stem and (3) like radishes and other crops that are hand bunched, green onion involve more hand labor in the harvesting and packing process than most fruits and vegetables, which increases the probability of microbial contamination (Calvin et al., 2004). Pathogenic bacteria such as *Salmonella* and *Shigella flexneri* as well as viruses such as hepatitis A virus have been linked to green onion contamination (California Department of Food and Agriculture, 2010).

Raw green onions need to go through the packinghouse for washing, trimming, banding, packing on ice and then cold storage (FDA, 2010). Value added green onions are packed in a processing facility where they are cleaned, trimmed, sometimes cut and packed in some form of plastic, protective packing and then cold storage as well. For both raw and value added green onions, washing is an essential step to lower the microbial load. Chlorine based washing technique has been applied by majority (67%) of fresh produce manufactories for decontamination purposes (Gündüz et al., 2010). However, chlorine based disinfectants have its limited efficacy to reduce foodborne pathogens (Beuchat, 1998) on fresh produce and the rising concern to its highly suspected carcinogenic by-products. Researchers are trying to find alternative sanitizers that are more effective, safer to use and more environment friendly.

The study of formulation rather than using single component sanitizer has drawn a great attention. Chlorine with lower concentration, quaternary ammonium compounds, organic acids, essential oils, hydrogen peroxide, bubbling ozone as well as surfactant have all been studied as candidates for sanitizer combination on variety of fresh produce. Lu and Wu (2010) has reported that thymol (0.2 mg/ml) combined with 5% sodium dodecyl sulfate (SDS) and acetic acid (2 mg/ml) can reduce more than 5 $\log_{10}$ CFU/g of *Salmonella* on grape tomatoes. Singh et al. (2002) reported that among the sequential washing of chlorine dioxide (ClO_2), ozone, and thyme essential oil, thyme oil followed by aqueous ClO_2/ozonated water, or ozonated water/aqueous ClO_2 were significantly ($p<0.05$) more effective in reducing *E. coli* O157:H7 (3.75 and 3.99 log; 3.83 and 4.34 log reduction) on lettuce and baby carrots, respectively.

In the last decade, several emerging techniques have been proposed to inactivate *Salmonella* on green onions. These techniques included ozone (Long III et

al., 2011), high hydrostatic pressure (Neetoo et al., 2011), electrolyzed water (Park et al., 2008) and irradiation (Murugesan et al., 2011). As a novel non-thermal technique, pulsed light (PL) has been approved by the FDA for treatment of foods (FDA, 1996) with the maximum energy dose of 12 J/cm^2. Intense and short duration pulses of broad spectrum (100 to 1100 nm) in PL can inactivate microorganisms on the food surfaces (Elmnasser et al., 2007). PL targets on DNA of the microorganism similar as UV light (Giese and Darby, 2000), but it can inactivate DNA repair system itself to cause irreversible damage of the DNA and death of the bacteria, a benefit that might not be achieved by UV light treatment in some experimental conditions (Smith et al. 2002) . To our knowledge, however, no research has been conducted on inactivation of *Salmonella* on green onions using PL as well as PL-sanitizer combination. *Salmonella* Typhimurium T43 was used in our study, as *Salmonella* serotype Typhimurium is one of the two most common cause of Salmonellosis in the United States which account for about half of culture-confirmed *Salmonella* isolates in the National *Salmonella* Surveillance System (CDC, 2013). In this study, *Salmonella* Typhimurium inactivation efficacy of new formula sanitizer washing, dry PL and wet PL treatment were investigated on spot inoculated green onions. Since dip inoculated pathogens was reported more difficult to be decontaminated (Durak et al., 2012), PL-new formula sanitizer combinations has been applied on dip inoculated green onions to improve *Salmonella* inactivation.

3.2 Materials and Methods

3.2.1 Bacterial strain and inoculum preparation

Single wild-type *Salmonella enterica* Typhimurium strain T43 was used in our study. The strain was grown on tryptic soy agar (TSA, Difco Laboratories, Sparks, MD, USA) for 2-3 days at 35℃. Single colonies were transferred to 10 mL of tryptic soy broth (TSB, Difco). The culture was incubated at 35℃ for overnight and second-transferred to 10 mL of fresh TSB to yield an approximate population of 10^9CFU/mL after 24h incubation at 35℃. The culture was diluted to 10^8 and 10^7 CFU/mL using sterile 0.1% peptone water (Difco) for use in the studies below.

3.2.2 Green onion preparation and inoculation

Green onions were purchased from a local supermarket and stored at 4±2 °C for a maximum of 24 h before use. As described by Neetoo et al. (2011), the full-length green onions were cut into segments weighing ~5 g and these portions partly included the compressed stem and the foliage cluster. To spot inoculate the green onions, 50 μL of 10^8 CFU/mL inoculum was deposited on the lateral surface of stems as five droplets. Inner surface was not inoculated. This inoculation was performed based on our previous findings (Xu et al., 2013). To dip inoculate the green onions, stems were fully submersed in 10^7 CFU/mL inoculum in a sterile stomach bag in the ratio of 1:5 (w:v). Bags were heat-sealed immediately and slight shaking was applied for 1 min. Green onions were taken out by sterile tweezers and placed in petri dishes. Spot or dip inoculated green onions were air dried in the biosafety hood for 2 or 4 h, respectively, before the decontamination treatments.

3.2.3 Efficacy of new formula of washing inactivation of spot inoculated *Salmonella enterica* Typhimurium on green onions

Six single sanitizers were applied to decontaminate *Salmonella enterica* Typhimurium spot inoculated on green onions, including hydrogen peroxide (H_2O_2, 300, 600 and 800ppm); sodium dodecyl sulfate (SDS, 0.01, 1 and 4%); thymol dissolved in 4% ethanol (0.2 and 0.4mg/mL); acetic acid (0.5, 1 and 2mg/mL); malic acid (0.5, 1 and 2 mg/mL) and citric acid (0.5, 1 and 2 mg/mL). The test concentrations were selected based on the previous studies in our lab or published work (Huang and Chen, 2011; Lu and Wu, 2010; Lu et al., 2013) but lower concentrations were used in current study. Distilled water (DI water) and 200ppm chlorine washing were also included as comparison. Chlorine was made from sodium hypochlorite to achieve 200ppm free chlorine solution with adjusted pH to 6.5. One green onion (5g) was submerged in 100mL washing solution in a beaker with continuous agitation for 1 min at room temperature. The single sanitizers with different antimicrobial mechanisms were combined and applied to spot inoculated green onion washing as described above. Combinations included 300ppm H_2O_2 + 4% SDS, 2mg/mL citric acid + 4% SDS, 0.2mg/mL thymol + 4% SDS, 0.2mg/mL thymol + 2mg/mL citric acid and 0.2mg/mL thymol + 2mg/mL acetic acid.

3.2.4 Efficacy of PL treatment inactivation of spot inoculated *Salmonella enterica* Typhimurium on green onions

PL was produced by a bench-top pulsed light system (SteriPulse-XL, Model RS-3000C, Xenon Corp., Wilmington, Mass., U.S.). The 16 inch linear clear fused quartz PL lamp (LH840) delivered 505 joules/pulse (1.27 J/cm^2) energy with 3 pulses/sec pulse rate.

Dry PL treatment was applied by directly placing green onions in the PL chamber. Since serious quality loss such as softer and shrunk texture, and darker color of green onions, especially leaves, was observed when dry PL was applied for more than 15s, 5s and 15s were chosen as our treatments times. The energy received by green onions during dry PL treatments was measured by Vega - Color Screen Handheld Laser Power/Energy Meter (OphirOptronics Ltd., Wilmington, Mass., U.S.A.). For wet PL treatment, a segment of green onions stem (~5g) was placed in a beaker with 100mL DI water and a stir bar. An ultra-thin magnetic stirrer (Lab Disc, Fisher Scientific) was placed under the PL chamber to create turbulent flow inside the beaker through the stirrer so that green onions could rotate freely. Spot inoculated green onions were treated by dry or wet PL for different length of time, and the ones without any treatment were served as control.

3.2.5 Efficacy of PL and PL-sanitizer combination treatment inactivation of dip inoculated *Salmonella enterica* Typhimurium on green onions

Dip inoculated green onions have been treated with PL and PL-sanitizer combinations, including 300ppm H_2O_2 + 4% SDS, 2mg/mL citric acid + 4% SDS, 0.2mg/mL thymol + 4% SDS,0.2mg/mL thymol + 2mg/mL citric acid and 0.2mg/mL thymol + 2mg/mL acetic acid. Wet PL treatment was applied as described above. PL-sanitizer combination was applied by replacing DI water with the combinations of sanitizers.

3.2.6 Microbial analyses

Control and treated green onions were placed into sterile stomach bags and phosphate-buffered saline (PBS, pH 7.4±0.2) was added into the bags with 1:9 ratio (w:v) to neutralize the residual from sanitizers such as organic acids. Green onions

with PBS were homogenized by stomacher (400 Circulator, Seward Co., West Sussex, UK) at 300 rpm for 2min. The homogenate was serially diluted in 0.1% peptone water and plating on xylose lysine deoxycholate (XLD) agar. Black or black-centered colonies typical of *Salmonella enterica* Typhimurium colonies were enumerated after incubation for 48h at 37℃. Survivor populations were calculated in CFU/g. Inactivation efficacy was reported as the $\log_{10}$ CFU/g reduction which was the difference between log survivor population of control and each log survivor population of treated sample.

Enrichment was carried out when we got zero plate count. One hundred and thirty five milliliters of TSB were added into the stomach bag containing sample and PBS solution directly and streaked on XLD agar after incubated for 48 hours. Zero survivors were reported when no typical colony presented. Otherwise, detection limit was reported.

3.2.7 Statistical analyses

All experiments were replicated 3 times and results were reported as Mean±SD. JMP (SAS Cary, NC, USA) software was used for statistical analyses. Dunnett's method was used to determine difference between each treatment with control (DI water washing). Significant difference was reported at alpha level of 0.05.

3.3 Results and Discussion

3.3.1 Efficacy of single washing solution inactivation of spot inoculated *Salmonella enterica* Typhimurium on green onions

The reductions of spot inoculated *Salmonella* on green onions after washing with chlorine, H_2O_2, thymol, SDS, and organic acids were shown in Table 3.1. Results

showed that 200ppm chlorine washing achieved 3.2 log $_{10}$ CFU/g reduction of *Salmonella*, which was significantly more effective than water washing (1.1 log $_{10}$ CFU/g) (p<0.05). Decontamination efficacy of 10ppm chlorine has also been tested and only 1.2 log $_{10}$ CFU/g reduction was observed, which is no significantly better than water washing. Martínez-Téllez et al. (2009) investigate the efficacy of 200 and 250ppm of chlorine during different exposure times (40, 60, and 90 s) on the reduction of *Salmonella* Typhimurium in inoculated fresh green onions. Their results showed that 250 ppm chlorine washing provided 1.36-1.74 log $_{10}$ CFU/g reduction of *Salmonella* but there was no significant difference between two concentrations (200 and 250ppm) and three washing times (40, 60 and 90s). Comparing with our study, the difference may mainly be due to the different inoculation method. Dip inoculation was applied in Martínez-Téllez's study (2009) and it has been reported that dip inoculated bacteria was more difficult than the spot inoculated ones to be decontaminated by surface treatment methods such as washing.

The bactericidal efficacy of H_2O_2 has been demonstrated in both water and food systems (Liao and Sapers, 2000). H_2O_2 has GRAS status (21 CFR 181.1366, FDA 2001c), which can be used in both vapor and liquid phase to control the bacteria and extend the shelf life of minimally processed fruits and vegetables (Sapers and Simmons, 1998). Our results showed that 300ppm H_2O_2 (2.2log $_{10}$ CFU/g) washing had significantly better decontamination efficacy than water washing (1.1 log $_{10}$ CFU/g). No concentration dependent improvement has been observed when we used higher concentrations, and 800ppm H_2O_2 provided only 1.7 log $_{10}$ CFU/g of *Salmonella* reduction. Antimicrobial mechanism of H_2O_2 may be its ability to form

reactive oxygen species such as the hydroxyl radical and singlet oxygen, which can damage DNA and membrane constituents (Juven and Pierson, 1996).

SDS is an anionic surfactant and an FDA-approved food additive such as a wetting agent or an emulsifier (125-1000 ppm, 21 CFR 172.822, FDA, 2012). Use of SDS in the washing might facilitate the release of tightly bound contaminations from the surface to enhance the removal of foodborne pathogens and virus from fresh produce (Predmore and Li; 2011), or reduce water surface tension and enhance the direct contact of sanitizers with pathogens. The results showed that SDS itself did not have any higher decontamination efficacy than water washing (1.08 $\log_{10}$ CFU/g). The *Salmonella* log reduction of 0.01, 1 and 4% SDS were 1.0, 1.3 and 1.9 $\log_{10}$ CFU/g, respectively. Our finding was in agreement with Zhao's study (Zhao et al., 2009), whose results revealed that 0.05 % SDS alone at pH 6.0 had very limited *Salmonella* inactivation activity (~0.1 $\log_{10}$ CFU/g reductions). Researchers such as Rajagopal et al. (2002) as well as Williams and Payne (1964) provided the explanation that Enterobacteriaceae are normally tolerance to SDS and many bacteria in this family can grow in the presence of 5% SDS.

Thyme oil and its major antibacterial components thymol and carvacrol (Burt, 2005), with GRAS status (FDA, 2009), have been found to possess antimicrobial activity against a broad spectrum of bacteria (Di Pasqua et al., 2006; Gill and Holley, 2006). The antimicrobial effect of thymol may due to its ability to partition out of the water phase and into the lipid membrane of the microbial cell (Dorman and Deans, 2000). In this study, the thymol concentrations were chosen based on its MIC of 0.2mg/mL and MBC of 0.4mg/mL to inactivate *Salmonella* Typhimurium according to the previous studies in our lab (Lu and Wu, 2010). Our results showed that the

lower concentration of thymol (0.2 mg/mL) provided only 1.9 log $_{10}$ CFU/g reduction of *Salmonella* on green onions, which was no significant difference from water washing (1.1 log $_{10}$ CFU/g), while 0.4mg/mL of thymol has significantly higher log reduction (4.8 log $_{10}$ CFU/g) than that of water washing. This finding was comparable with Lu and Wu's study (2010), which indicated that thymol (especially at the concentration of 0.4 mg/ml) was the most effective (>4.1-log reductions, $p < 0.05$) among the three natural antimicrobial agents (thymol, thyme oil and carvacrol) to inactivate *Salmonella* Typhimurium on grape tomatoes.

Organic acids such as citric acid, acetic acid, lactic acid, malic acid etc. occur throughout nature and are used extensively in food systems. The mechanisms for organic acids inactivating pathogens depend on the undissociated form, as well as their ability to donate hydrogen ions in aqueous system. Organic acids can lower the environmental pH, interfere cell membrane transport system and permeability, then reduce internal pH and disrupting cellular functions (i.e. enzyme stability) (Lück and Jager, 1997). Among the three organic acids studied, 2mg/mL citric acid washing achieved significantly higher *Salmonella* log reduction (2.8 log $_{10}$ CFU/mL reduction) than the water washing (1.1 log $_{10}$ CFU/g) for green onions. Citric acid showed to be the most effective decontamination agents compared with acetic and malic acids at the concentration of 2mg/mL.

3.3.2 Efficacy of new formula of washing inactivation of spot inoculated *Salmonella enterica* Typhimurium on green onions

For all individual sanitizer washing treatments, 300ppm H_2O_2, 2 mg/mL of malic acid and 2mg/mL of citric acid achieved >2 log $_{10}$ CFU/g and 0.4 mg/mL of thymol achieved >4 log $_{10}$ CFU/g reduction of *Salmonella* Typhimurium spot

inoculated on green onions. To increase decontamination efficacy and further maintain the microbial safety of green onion, new formula of sanitizers have been studied by combining sanitizer-sanitizer or sanitizer-surfactant. Formulation was designed based on mechanisms of different washing solution categories. Surfactant (SDS) has been combined with oxidant (H_2O_2), organic acid (citric acid) or thymol to investigate whether surfactant would improve decontamination efficacy of various sanitizers. Thymol at 0.2 mg/mL was combined with acetic acid and citric acid to investigate whether its antimicrobial efficacy would be improved by acidic agents. The concentrations of other sanitizers or surfactant were selected based on the individual washing results with highest log reduction of *Salmonella*. Three new formula sanitizers have been investigated including 300ppm H_2O_2 + 4% SDS, 2mg/mL citric acid + 4% SDS and 0.2mg/mL thymol + 4% SDS. The results showed that 4% SDS significantly ($p<0.0001$) improved the decontamination efficacy of 300ppm H_2O_2, 2mg/mL citric acid and 0.2% thymol. The spot inoculated *Salmonella* reductions were 5.3, 5.3 and 4.5 log $_{10}$ CFU/mL, respectively. Similar as our findings, the combination of SDS + levulinic acid has been investigated by Zhao et al. (2009) to decontaminate *Salmonella* and *E. coli* O157:H7 on lettuce. Their results showed that the combination of 3% levulinic acid plus 1% SDS for 20 s reduced both *Salmonella* and *E. coli* O157:H7 populations by 6.7 log $_{10}$ CFU/g. The decontamination efficacy improvement provided by SDS may due to two reasons: (1) surfactant allows the release of tightly bound contaminations from the surface, which may enhance the removal of foodborne pathogens from fresh produce and (2) most sanitizers require direct contact to be effective. Water films on plant surface, however, may prevent the sanitizers from directly contacting target microorganisms. Adding surfactant such as

SDS can reduce water surface tension and may enhance the efficiency of other sanitizers (Predmore and Li, 2011).

The combination of essential oil (thymol) and organic acid (acetic acid or citric acid) has also been studied to inactivate spot inoculated *Salmonella* on green onions. Two new formula sanitizers have been investigated including 0.2mg/mL thymol + 2mg/mL citric acid and 0.2mg/mL thymol + 2mg/mL acetic acid. The results showed that when combined with 2mg/mL citric acid or 2mg/mL acetic acid, the spot inoculated *Salmonella* decontamination efficacy of 0.2 mg/mL thymol increased significantly from 1.9 to 5.0 or 5.3 $\log_{10}$ CFU/g reduction, respectively, which was comparable as 0.4 mg/mL thymol washing (4.8 $\log_{10}$ CFU/g). Similarly as our findings, additive or synergic antimicrobial effect of organic acid and essential oil has been reported by some researchers. de Barros et al. (2012) reported that a synergic effect of *Origanum vulgare* L. essential oil and lactic acid inactivate *Staphylococcus aureus* on the base of Fractional Inhibitory Concentration (FIC) index.

Table 3.1: Efficacy of new formula of washing inactivation of spot inoculated *Salmonella enterica* Typhimurium on green onions

	Salmonella Log reduction (Log10 CFU/g)
water	1.1±0.2
Chlorine 200ppm	3.2±1.0 *
Oxidants	
H_2O_2 300 ppm	2.2±1.2
H_2O_2 600 ppm	2.1±0.5
H_2O_2 800 ppm	1.7±1.2
Surfactant	
SDS 0.01%	1.0±0.5
SDS 1%	1.3±0.7
SDS 4%	1.9±0.8
Essential oil	
0.2mg/mL Thymol	1.9±0.7
0.4mg/mL Thymol	4.8±0.6 *
Organic acids	
Acetic acid 0.5mg/mL	1.0±0.5
Acetic acid 1.0mg/mL	1.6±0.6
Acetic acid 2.0mg/mL	2.2±0.6
Malic acid 0.5mg/mL	1.0±0.7
Malic acid 1.0mg/mL	1.2±0.7
Malic acid 2.0mg/mL	2.3±0.5
Citric acid 0.5mg/mL	1.3±0.8
Citric acid 1.0mg/mL	1.8±0.7
Citric acid 2.0mg/mL	2.8±0.6 *
Combinations	
300ppm H_2O_2+4% SDS	5.3±0.6 *
2mg/mLcitric acid+4% SDS	5.3±0.0 *
0.2mg/mL thymol+4% SDS	4.5±0.4 *
0.2mg/mL thymol+2mg/mL citric acid	5.0±0.6 *
0.2mg/mL thymol+2mg/mL acetic acid	5.3±0.6 *

Data represented *Salmonella* Typhimurium mean population reduction (log_{10} CFU/g) ±standard deviation. The initial populations of *Salmonella* for spot inoculated stems 5.3 log_{10} CFU/g. Values marked by * means they were significantly different with the control (water washing) at alpha level of 0.05

3.3.3 Efficacy of PL and PL-sanitizer combination treatment inactivation of spot or dip inoculated *Salmonella enterica* Typhimurium on green onions

Green onions were initially treated by dry PL for 5, 15, 30 and 60s. The energy dose received by the green onion was measured as the fluence of 5.0, 14.3, 28.2 or 56.1 J/cm^2, respectively. However, severe quality degradation such as soft tissue and burned appearance has been observed after 30 or 60s. Thus, only 5 and 15s dry PL treatments were applied. As shown in Fig 3.1, 5s dry PL treatment was sufficient for *Salmonella* decontamination which provided the reduction of 4.6 log $_{10}$ CFU/g. There was no significant improvement when we used longer time (15s). To better protect qualities and avoid shadow effect of the surface treatment, wet PL treatment was applied for 5, 15, 30 and 60s since no significant quality loss was observed as long as 60s. During the wet PL treatment, green onions were submerged in water, which absorbed part of the energy. Continuous rotation of green onions was generated easily by stirring bar which helped every surface of green onions to receive evenly energy. The results showed that spot inoculated *Salmonella* reduction increased from 1.0 to 3.6 log $_{10}$ CFU/g when the time extended from 5 to 60s. Dry PL treatments at 5 and 15s were significantly more effective than water washing. The efficacy of wet PL treatment was increased with the increase of treatment time from 5s to 60s. They all have the potential of being used as alternative for 200ppm chlorine washing (3.2 log $_{10}$ CFU/g reduction).

According to previous results and discussion, for spot inoculated green onions, sanitizer combinations (4.5-5.3 log $_{10}$ CFU/g reduction) or 5s dry PL treatment (4.6 log $_{10}$ CFU/g reduction) was effective to inactivate *Salmonella*, which were close to the 5 log reduction of pathogen level (FDA, 2001). Thus, the combination of sanitizer and PL treatment was not further investigated on spot inoculated green onions. Spot

inoculation was to mimic contamination caused by unhygienic touch from hands of workers or equipment during harvest, packing or value-added processing. However, in the practical situation, there is a possibility that green onions may be contaminated by washing water since washing is usually the first step in the packing house to remove dirt and soil (FDA, 2010). Thus, we simulated this contamination by applying dip inoculation. Dip inoculated green onions were treated by 60s wet PL, five sanitizer combinations described in section 3.3.1 as well as PL-sanitizer combination. Dry PL was not applied on decontamination of dip inoculated green onions in this study, because wet PL treatment may be easier to be included into the current washing processing of green onions in industry. Also, it is easier for wet PL to distribute energy evenly than dry PL which may better protect the quality of green onions. As shown in Fig 3.2, dip inoculated green onions were more difficult to be decontaminated than spot inoculated ones. The overall log reductions were less than 0.8 $\log_{10}$ CFU/g. Combining PL with the most effective sanitizer combinations didn't provide significant improvement. This was similar as the findings of Xu et al. (2013) about decontamination of *E. coli* O157:H7 on green onions using PL. For spot inoculation, bacteria might be placed on the surface of green onion which may benefit inactivation efficacy by surface treatments such as PL and sanitizer washing. While the dip inoculation method allowed *Salmonella* to access freely into the hollow leaves of green onion leaves and compressed layer of stems, which provided a harbor for those cells and became more difficult to be decontaminated. As suggested by Durak et al. (2012), dip inoculation facilitated the infiltration of *E. coli* O157:H7 (GFP) into fresh produce through the stomata, cuts, damaged leaves, and other open areas on the leaf surfaces. Once pathogens attached and/or localized in protected areas such as stomata

and within the tissue, washing with deionized water and chlorine may have no or very little effect on their removal (Durak et al., 2012). More work is thus needed to improve the decontamination efficacy on dip inoculated green onions, which will shed lights on decreasing risk of cross-contamination due to the use of contaminated washing water. Future work is also important to understand *Salmonella* behavior such as its attachment and internalization on green onions during the sanitizer wash or PL treatments. In addition, the impacts of new washing or PL on green onions' quality during the shelf life need to be thoroughly studied to ensure the treated green onions are safe and high quality.

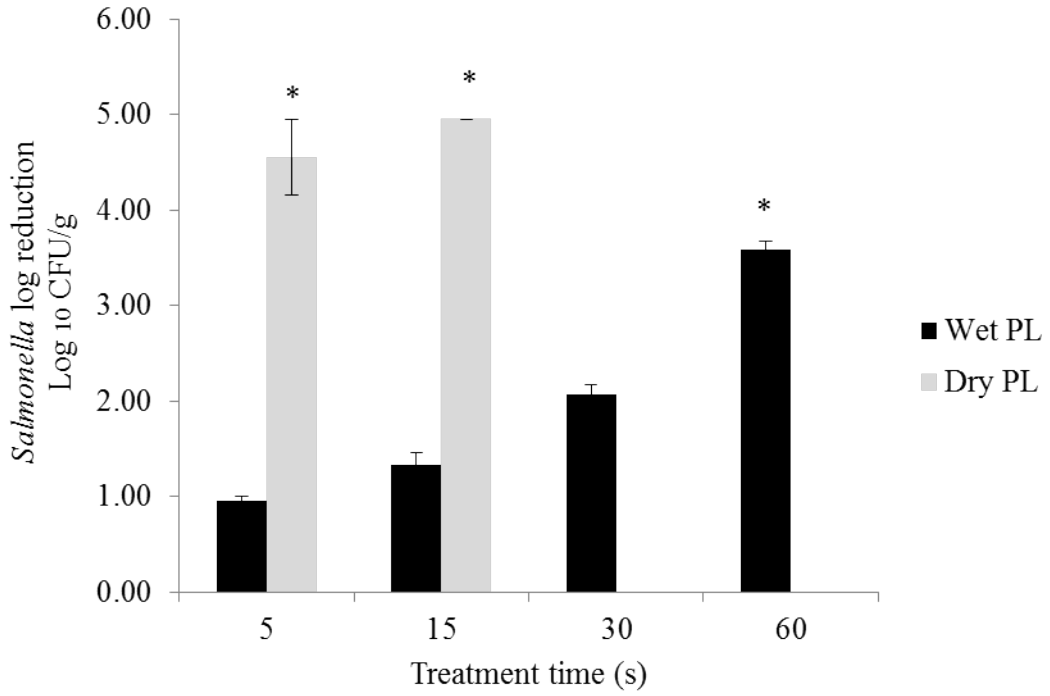

Figure 3.1: Efficacy of PL treatment inactivation of spot inoculated *Salmonella enterica* Typhimurium on green onions.

Spot inoculated green onions were treated using dry PL (5 and 15s) and wet PL (5, 15, 30 and 60s). The initial populations of Salmonella Typhimurium on spot inoculated green onions were 5.3 log $_{10}$ CFU/g. Values marked by * means they were significantly different with the control (water washing) at alpha level of 0.05.

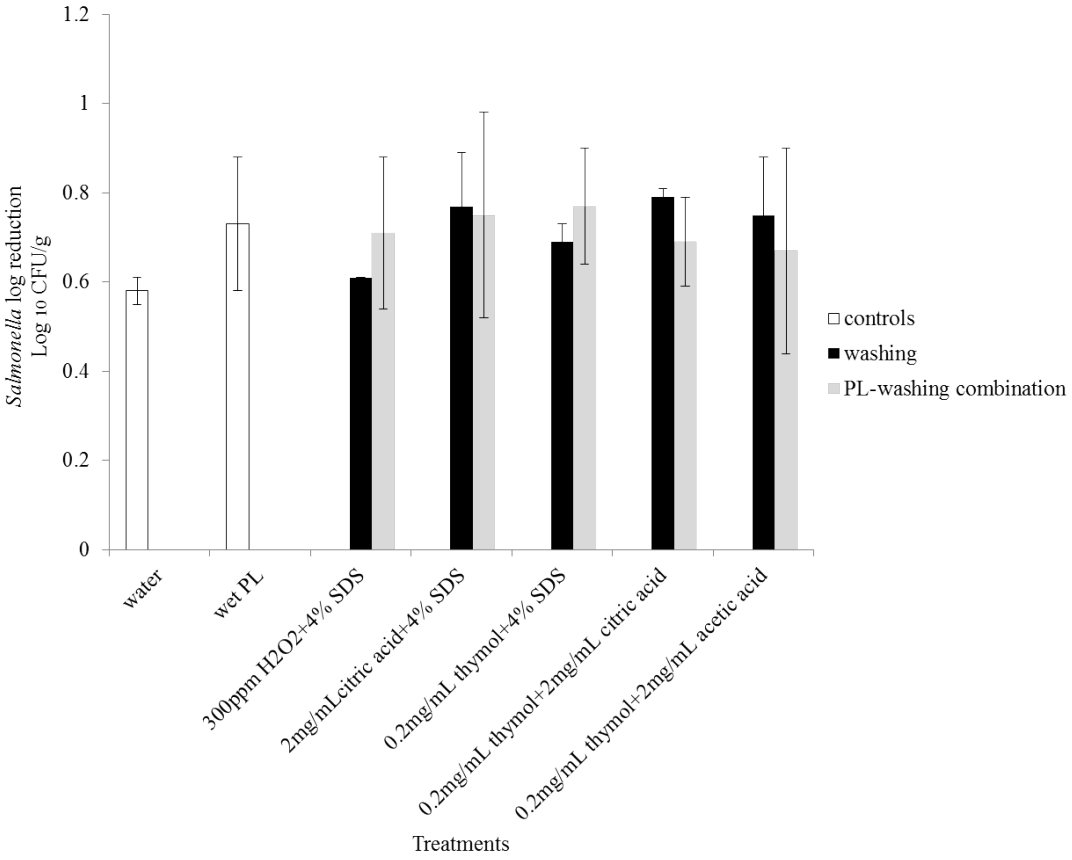

Figure 3.2: Efficacy of PL and PL-sanitizer combination treatment inactivation of dip inoculated *Salmonella enterica* Typhimurium on green onions.

Dip inoculated green onions were treated using wet PL (60s) and PL-sanitizer combinations (60s), including 300ppm H_2O_2 + 4% SDS, 2mg/mL citric acid + 4%

SDS, 0.2mg/mL thymol + 4% SDS, 0.2mg/mL thymol + 2mg/mL citric acid and 0.2mg/mL thymol + 2mg/mL acetic acid. The initial populations of Salmonella Typhimurium on dip inoculated green onions were 4.0 log $_{10}$ CFU/g. Values marked by * means they were significantly different with the control (water washing) at alpha level of 0.05.

3.4 Conclusion

For spot inoculated green onions, four washing combinations including 300ppm H_2O_2 + 4% SDS, 2mg/mL citric acid + 4% SDS, 0.2mg/mL thymol + 4% SDS, 0.2mg/mL thymol + 2mg/mL citric acid and 0.2mg/mL thymol + 2mg/mL acetic acid all achieved significantly higher log reduction of *Salmonella* than 200ppm chlorine washing, which may be developed as new formula of sanitizer to alternate chlorine. As the novel technique, 5s dry PL treatment provided higher log reduction than chlorine washing. The 60s wet PL was comparable as chlorine washing, which means PL has the potential of being used in green onion industry for decontamination purpose. For dip inoculated green onions, none of our treatment provided promising effectiveness (0.6-0.8 log $_{10}$ CFU/g reduction of *Salmonella*). Combining PL with sanitizer combinations in the presented study did not improve the decontamination efficacy for dip inoculated green onion decontamination. More work will be done to better understand and improve the PL alone and possible combination with sanitizer for the dip inoculation.

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Chapter 4

DECONTAMINATION OF *ESCHERICHIA COLI* O157:H7 ON GREEN ONIONS USING PULSED LIGHT (PL) AND PL-SURFACTANT-SANITIZER COMBINATIONS

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ABSTRACT

Imported green onion has been associated with three large outbreaks in the USA. Contamination has been found on both domestic and imported green onions. The objective of our study was to investigate *E. coli* O157:H7 inactivation efficacy of pulsed light (PL) as well as its combination with surfactant and/or sanitizers on green onions. Green onions were cut into two segments, stems and leaves, to represent two different matrixes. Stems were more difficult to be decontaminated. Spot and dip inoculation method were compared and dipped inoculated green onions were found to be more difficult to be decontaminated. Results showed that 5s dry PL (samples were not immersed in water during PL treatment) and 60s wet PL (samples were immersed in water and stirred during PL treatment) treatments provided promising inactivation efficacy (>4 $\log_{10}$ CFU/g) for spot inoculated stems and leaves. For dip inoculated green onions, 60s wet PL treatment was comparable with 100 ppm chlorine washing, demonstrating that PL could be used as an alternative to chlorine. To further increase the degree of microbial inactivation, combined treatments were applied. PL combined

with surfactant (SDS) was found to be more effective than single treatments of PL, SDS, chlorine, citric acid, thymol, and hydrogen peroxide and binary combined treatments of PL with one of those chemicals. Addition of chlorine or hydrogen peroxide to the PL-SDS combination did not further enhanced its microbial inactivation efficacy. The combination of PL and 1000 ppm of SDS reduced the *E. coli* O157:H7 populations dipped inoculated on the stems and leaves of green onions by 1.4 and 3.1 $\log_{10}$ CFU/g, respectively. Our findings suggest that PL could potentially be used for decontamination of *E. coli*. O157:H7 on green onions, with wet PL added with SDS being the most effective PL treatment.

KEY WORDS: *E. coli* O157:H7; Green onions, Pulsed Light (PL), SDS

4.1 Introduction

Because of their milder onion-like flavor, green onions are now widely used in the USA as seasonings or minor components in Asian recipe. Mexico is the dominant force in North American green onion production because green onion is a labor-intensive crop that is cheaper to grow in Mexico than in the United States. Imported green onion has been associated with three large hepatitis A outbreaks in USA, including four deaths and 1028 illnesses (Calvin et al., 2004). Contamination has been found on both domestic and imported green onions (FDA, 2001a and FDA, 2001b). Green onions are generally harvested by hand, and can be packed in the field, in a packinghouse or in a value added processing plant (FDA, 2010). There are numerous points which can cause pathogen contamination. Green onions may be contaminated in the field before harvest due to soil, water or animals. Every step in post-harvest and value added processing may introduce pathogen contamination as well.

E. coli O157:H7 is the most commonly identified Shiga toxin-producing" *E. coli* (STEC) in North America (CDC, 2011). Some infections caused by this pathogen are very mild, but some could be severe or even life-threatening. Very young children and the elderly are more likely to develop severe illness and hemolytic uremic syndrome (HUS) than others, but even healthy older children and young adults can become seriously ill (CDC, 2011). Although according to CDC there has not been any *E. coli* O157:H7 outbreak related with green onions, pathogenic bacteria such as *Salmonella* and *Shigella flexneri* as well as viruses such as hepatitis A virus that are often transmitted via the fecal-oral route have all been linked to green onion contamination (California Department of Food and Agriculture, 2010), which implicate that *E. coli* O157:H7 may also become potential contamination source for green onion because this pathogen also cause problem via fecal-oral route.

For both raw and value added green onions, however, washing is the only approach now used in industry to improve the microbial safety of the products (FDA, 2010). Water is the basic tool used to remove soil and debris and to reduce microbial populations during washing process. However, water may also serve as vehicle for cross-contamination. As a result, chlorine based disinfectants are usually added in the wash water to increase the rate of microbial reduction and to prevent the potential cross-contamination of human pathogens during washing. Currently, chlorine based disinfectants have been questioned because of its limited efficacy to reduce foodborne pathogens on fresh produce as well as its highly suspected carcinogenic by-products. Researchers are trying to identify alternative sanitizers or develop novel techniques that are more effective, safer to use and more friendly to the environment.

In response to consumer's needs for fresh and healthy foods, novel techniques have been developed in order to reduce health risk and preserve quality and nutritional values of our food. These techniques can be used as alternatives or complements to traditional chlorine washing which has limited decontamination capacity as well as health concern. In the last decade, several emerging techniques have been proposed to inactivate *Salmonella* and/or *E. coli* O157:H7 in green onion. These techniques included ozone (Bialka and Demirci, 2007a), high hydrostatic pressure (Neetoo et al., 2011), electrolyzed water (Park et al., 2008) and irradiation (Murugesan et al., 2011; Fan et al., 2003).

Pulsed light (PL), a novel non-thermal technique using high-power electrical energy to generate intense pulses of light, can potentially be used to decontaminate green onions. Its intense, short duration pulses of broad spectrum can inactivate microorganisms on the surface of either foods or packaging materials (Elmnasser et al., 2007). PL has been approved by the FDA for treatment of foods with the total cumulative treatment not exceeding 12.0 J/cm^2 (FDA, 1996).The lamp can generate light from UV to near-infrared (100 to 1100 nm) and the UV region is mainly responsible for microbial inactivation since no killing effect has been observed after removing the UV wavelength lower than 320nm (Takeshita et al. 2002). UV part of the spectrum in PL has been proved having photochemical and/or photo-thermal effect. PL has been reported to inactivate microorganisms naturally present (Hoornstra et al., 2002; Gómez-López et al. 2005) as well as inoculated (Fine and Gervais, 2004) on food surfaces. To our knowledge, no research has been conducted on inactivation of *E. coli* O157:H7 on green onions using PL. Thus, the objective of our study was to

determine the inactivation efficacy for *E. coli* O157:H7 on green onions by PL, PL-surfactant/sanitizer, and PL-surfactant-sanitizer combinations.

4.2 Materials and Methods

4.2.1 Bacterial strain and inoculum preparation

Single wild-type *E. coli* O157:H7 strain (250, sprout outbreak isolate) was used in our study. The strain was adapted to grow in the presence of 100 μg/mL of nalidixic acid (Fisher Scientific, Hampton, NH, USA) and 100 μg/mL of streptomycin (Sigma, St. Louis, MO, USA). The strain was grown on tryptic soy agar ((Difco Laboratories, Sparks, MD, USA) plus 0.6% yeast extract (Difco) supplemented with 100 μg/mL of nalidixic acid and 100μg/mL of streptomycin (TSAYE-NS) for 2-3days at 35℃. Single colonies were picked and transferred to 10 mL of tryptic soy broth (Difco) plus 0.6% yeast extract (Fisher) supplemented with same antibiotics (TSBYE-NS). The culture was incubated at 35℃ for overnight and second-transferred to 10 mL of fresh TSBYE-NS to yield an approximate population of 10^9CFU/mL after 24h incubation at 35℃. The culture was diluted to 10^8 and 10^7 CFU/mL using sterile 0.1% peptone water (Difco) for use in the studies below.

4.2.2 Green onions preparation and inoculation

Green onions were purchased from a local supermarket and stored at 4±2 °C for a maximum of 24 h before use. Full-length green onions was cut into segments, compressed stems (~5 g) and hollow leaves (~1 g), and air dried in a biosafety hood.

To spot inoculate the green onions, 50 μL or 10 μL of 10^8 CFU/mL inoculum was deposited on the outside surface of stems and leaves, respectively, as five droplets. The initial population on stems and leaves were 4.9 and 5.2 $\log_{10}$ CFU/g,

respectively. To dip inoculate the green onions, stems or leaves were fully submersed in 10^7 CFU/mL inoculum in a sterile stomach bag in the ratio of 1:5 or 1:10 (weight of green onions: volume of the inoculum), respectively. The initial population was 4.5 and 5.5 $\log_{10}$ CFU/g on stems and leaves, respectively. Bags were heat-sealed immediately and slight shaking was applied for 1 min. Green onions were taken out by sterile tweezers and placed in petri dishes. Spot or dip inoculated green onions were air dried in the biosafety hood for 2 or 4 h, respectively, before the decontamination treatments.

4.2.3 Dry PL or wet PL treatment inactivation of *E. coli* O157:H7 on spot or dip inoculated green onions

PL was produced by a bench-top pulsed light system (SteriPulse-XL, Model RS-3000C, Xenon Corp., Wilmington, Mass., U.S.). The 16 inch linear clear fused quartz PL lamp (LH840) delivered 505 joules/pulse (1.27 J/cm^2) energy with 3 pulses/sec pulse rate.

For dry PL treatment, green onions, including stems and leaves, were placed in PL chamber in sterile petri dishes without covers. The energy received by green onions during dry PL treatments was measured by Vega - Color Screen Handheld Laser Power/Energy Meter (Ophir Optronics Ltd., Wilmington, Mass., U.S.A.). Fluence of PL for 5, 15, 30 or 60s dry treatments was 5.0, 14.3, 28.2 or 56.1 J/cm^2, respectively. Since serious quality loss of green onions, especially leaves, was observed when PL was applied for more than 15s, we chose 5s and 15s as our treatments times. Since PL is a surface treatment, inactivation efficacy of treatment with or without turning was compared. Single side spot inoculated green onions were treated for 3s or 8s with the inoculated side facing the lamp. Then they were turned to

the opposite side and treated for 2s or 7s, which provided the total treatment time of 5s or 15s. Similar to spot inoculated samples, dip inoculated green onions were treated for one side for half of the treatment time and turned to the opposite side for treatment. PL treatments without turning were applied continuously for 5s or 15s for both spot and dip inoculated green onions. Green onions inoculated with either method but without PL treatment served as control.

For wet PL treatment, a segment of green onions stems or leaves was placed in a beaker with 200mL DI water and a stir bar. An ultra-thin magnetic stirrer (Lab Disc, Fisher Scientific) was placed under the PL chamber to create turbulent flow inside the beaker through the stirrer so that green onions could rotate freely. The size of the stirring bar was Dia. x L: 0.37 x 1 in. (0.9 x 2.5cm) and the number of the turns was around 700 rpm. The energy dose of PL received by green onions in the water was lower than direct measurement, since no significant quality loss was observed after wet PL treatment for up to 60s. Spot or dip inoculated green onions were treated by PL for different length of time and the ones without any treatment were served as control.

4.2.4 Combination of PL and chlorine on the inactivation of *E. coli* O157:H7 on dip inoculated green onions

Sodium hypochlorite was appropriately diluted to make chlorine solution with 10 or 100ppm of free chlorine. Free chlorine micro check test strips (HF Scientific, Ft. Myer, FL) was used to quantify free chlorine level. To stimulate industry conditions, working chlorine solution was prepared by adjusting pH to 6.5-7.0 and temperature to 4℃. In order to compare the inactivation efficacy of single and combination treatments, chlorine washing, PL or PL-chlorine combination was each applied for 60s. For chorine washing, a segment of dip inoculated green onions stem or leaf was

placed in a beaker with 200mL of working chlorine solution (10ppm or 100ppm) without PL treatment. For PL treatment alone, inoculated green onions were immersed in 200mL DI and treated by PL with stirring the same way as described in Section 3.2.3. For PL-chlorine combination, dip inoculated green onions were placed in 200mL working chlorine solution (10ppm or 100ppm) and immediately treated for 60 s by PL with stirring.

4.2.5 Combination of PL and surfactant on the inactivation of *E. coli* O157:H7 on dip inoculated green onions

Dip inoculated green onion stems or leaves were placed in sodium dodecyl sulfate (SDS) solution with concentration from 10, 100 to 1000ppm at 4℃. To compare efficacy of single and combination treatments, surfactant washing, PL and PL-surfactant combination were all applied for 60s with stirring.

4.2.6 Combination of PL and non-chlorine based sanitizer on the inactivation of *E. coli* O157:H7 on dip inoculated green onions

Based on previous study in our lab, citric acid (1mg/mL), thymol (0.2mg/mL) and hydrogen peroxide (H_2O_2, 300ppm) was selected from the categories of organic acid, essential oil and oxidant and kept at 4℃ before used. Single and combinations of PL and sanitizers were studied. Treatment time was 60s and stirring was also applied.

4.2.7 Combination of PL-surfactant-sanitizer on the inactivation of *E. coli* O157:H7 on dip inoculated green onions

Based on the results from Sections 3.2.4-3.2.6, the following combinations were studied, SDS + chlorine, and SDS+ H_2O_2, PL + SDS + chlorine, PL + SDS + H_2O_2. SDS (1000ppm), chlorine (10ppm) and H_2O_2 (300ppm) were kept at 4℃. Treatment time was 60s and stirring was also applied.

4.2.8 Microbial analyses

Control and treated green onions were placed into sterile stomach bags and D/E neutralized broth (Difco) was added into the bags with 1:9 ratio (w:v). Samples with D/E broth were homogenized by stomacher (400 Circulator, Seward Co., West Sussex, UK) at 260rpm for 2min. The homogenate was serially diluted in 0.1% peptone water and plating on TSAYE-NS plates. *E. coli* O157:H7 colonies were enumerated after incubation for 48h at 37℃. Survivor populations were calculated in CFU/g. Inactivation efficacy was reported as $\log_{10}$ CFU/g reduction which was difference between log survivor population of control and each log survivor population of treated sample.

4.2.9 Statistical analyses

All experiments were replicated 3 times and results were reported as Mean±SD. JMP (SAS Cary, NC, USA) software was used for statistical analyses. Tukey's test was used to determine difference between each treatment. Significant difference was reported when $p<0.05$.

4.3 Results and Discussion

4.3.1 Dry PL or wet PL treatment inactivation of *E. coli* O157:H7 on spot or dip inoculated green onions

Green onions were initially treated by dry PL for 5, 15, 30 and 60s. However, since significant quality loss was observed for 30 and 60s treated green onions, including softer and shrunk tissue, darker color and cooked smell, only 5 and 15s were chosen for further evaluation of dry PL treatment. Because PL is a surface treatment and does not have a good penetration depth, we turned the samples in the middle of the treatments. *E. coli* inactivation efficacy of dry PL with or without turning was

compared. Two inoculation methods were applied. Spot inoculation was to mimic contamination caused by unhygienic touch from hands of workers or equipment during harvest, packing or value-added processing, while dip inoculation was to simulate contamination during washing, which is usually the first step in the packing house (FDA, 2010). As shown in Table 4.1, for spot inoculated green onions, dry PL was very effective for *E. coli* inactivation on both leaves and stems. The log reduction ranged from 4.1 to 5.2 $\log_{10}$ CFU/g. There was no significant difference between 5s PL with and without turning as well as between 5s and 15s treatments, which indicated that for spot inoculated (10^6 CFU/g) green onions, 5s dry PL treatments would be sufficient to inactivate *E. coli* on stems and leaves. Bialka and Demirci (2007b) used dry PL to decontaminate *E. coli* and *Salmonella* on blueberries. From their findings, PL treatment (22.6 J/cm^2) provided 4.3 log reduction on *E. coli* O157:H7 without affecting the quality of blueberries, which was less effective than the application on green onions.

Dip inoculated green onions were more difficult to be decontaminated than spot inoculated ones. For spot inoculation, bacteria might be placed on the surface of green onion which may benefit inactivation efficacy by surface treatments such as PL and sanitizer washing. While during dip inoculation bacteria could flow freely with the fluid and get into hollow space of the green onion leaves and compresses layers on stems, which protect them from surface inactivation treatments. As suggested by Durak et al. (2012), dip inoculation of produce facilitated the infiltration of *E. coli* O157:H7(GFP) into green onions through the stomata, cuts, damaged leaves, and other open areas on the leaf surfaces. Once pathogens become attached and/or localized in protected areas such as stomata and within the tissue, washing with

deionized water and chlorine may have no or very little effect on their removal (Durak et al., 2012). This phenomenon might also be used to explain our observation that it was more difficult to inactivate dip inoculated *E. coli* than spot inoculated cells by PL.

Overall log reduction was lower than 1.2 log $_{10}$ CFU/g for dip inoculated green onions. The most effective treatment was 15s treated stems with and without turning (1.1 log $_{10}$ CFU/g). While for leaves, no significant difference has been observed between 5 and 15s PL treatments as well as between turning and not turning during each treatment.

In order to monitor the temperature change of the green onions during the dry PL treatment, thermocouple was used and the sensor was placed in the subsurface of green onions (data not shown). The temperature of leaves and stems reached 52.0±1.0℃ and 43.5±0.5℃, respectively. To better protect qualities and avoid shadow effect, wet PL treatment was applied for 5, 15, 30 and 60s since no significant quality loss was observed after 60s. Green onions were submerged in water which absorbed part of the energy. Unlike the manual and one-time turning in dry PL, random rotation was generated more easily by stirring bar. Two inoculation methods were applied before wet PL treatment as well. Inactivation efficacy of wet PL treatment was compared with 60s water washing with same inoculation and rotation speed. As shown in Table 4.2, for spot inoculated green onions, as time increased from 5 to 60s, *E. coli* log reductions on leaves increased from 2.1 to 4.6 log $_{10}$ CFU/g. All PL treatments were significant better than 60s water washing (0.7 log $_{10}$ CFU/g). Inactivation efficacy on green onion stems was also time-dependent which increased from 0.5 to 4.1 log $_{10}$ CFU/g when treatment time prolonged from 5 to 60s. At the end of 60s wet PL treatment, similar results (>4 log reduction) were achieved for both leaves and

stems. For dip inoculation, no more than 1.2 log reductions were observed when wet PL was applied for dip inoculated leaves and stems from 5 to 60s.

Comparing results between dry PL and wet PL treatment, it could be concluded that if green onions contaminated in situations as similar as spot inoculation, 5s dry PL and 60s wet PL can reach promising *E. coli* inactivation efficacy (>4 $\log_{10}$ CFU/g reduction). The reason that the wet PL treatment was not as effective as the dry PL treatment is not clear. It is possible that during wet PL treatment, the bacterial cells inoculated on the surface were carried inside the green onions by water, which made them less exposed to PL. In wet PL treatments the overheating of bacteria is also less important and damage effects on their DNA structures are major, while in dry PL treatment, both overheating of bacteria and damage of their DNA structures could be possible. However, if green onions were contaminated by pathogens in unclean wash water as simulated by dip inoculation, neither dry nor wet PL was able (<1.5 $\log_{10}$ CFU/g) to keep the microbial safety of green onions. In order to increase inactivation efficacy and maintain qualities of green onions at the same time, hurdle techniques are needed to be considered. According to FDA guideline (FDA, 2010) for both raw and value added green onions, washing is an essential step to lower the microbial load. Combination of PL and sanitizer washing may be the easiest and more effective way to apply the hurdle technique since PL lamp could be placed on the top of a washing tank in a closed chamber and no additional processing step is needed.

Table 4.1: Dry PL treatment inactivated *E. coli* O157:H7 on spot or dip inoculated green onions.

		spot inoculation		dip inoculation	
	time(s)	stems	leaves	stems	leaves
Turned	5	4.8±0.4A	4.1±0.1A	0.2±0.1B	0.6±0.1A
	15	4.9±0.2A	4.5±0.1AB	1.1±0.2A	0.9±0.1A
Unturned	5	4.5±0.3A	4.9±0.5A	0.2±0.1B	0.6±0.1A
	15	4.9±0.2A	5.2±0.3A	1.1±0.2A	0.8±0.1A

Data represented *E. coli* O157:H7 mean population reduction ($\log_{10}$ CFU/g) ±standard deviation. The initial populations of *E. coli* O157:H7 for spot inoculated stems and leaves were 4.9 and 5.2 $\log_{10}$ CFU/g, respectively. And the initial populations on dip inoculated stems and leaves were 4.5 and 5.5 $\log_{10}$ CFU/g, respectively. Turned meant turning involved in the middle of PL treatment. Unturned meant there was no turning involved in the middle of PL treatment. Data in the same column followed by the same capital letter were not significantly different (p >0.05).

Table 4.2: Wet PL treatment inactivated *E. coli* O157:H7 on spot or dip inoculated green onions

		spot inoculation		dip inoculation	
	time(s)	stems	leaves	stems	leaves
water	60	0.5±0.2C	0.7±0.0D	0.4±0.2C	0.0±0.1B
PL	5	0.5±0.2C	2.1±0.1C	0.4±0.1BC	0.3±0.1B
	15	0.8±0.3C	3.9±0.2B	1.1±0.2A	0.3±0.0B
	30	2.4±0.3B	4.2±0.4AB	0.8±0.1AB	1.2±0.1A
	60	4.1±0.1A	4.6±0.4A	0.9±0.2AB	1.2±0.1A

Data represented *E. coli* O157:H7 mean population reduction ($\log_{10}$ CFU/g) ±standard deviation. The initial populations of *E. coli* O157:H7 for spot inoculated stems and leaves were 4.9 and 5.2 $\log_{10}$ CFU/g, respectively. And the initial populations on dip inoculated stems and leaves were 4.5 and 5.5 $\log_{10}$ CFU/g, respectively. Data in the

same column followed by the same capital letter were not significantly different (p>0.05).

4.3.2 Combination of PL and chlorine on the inactivation of *E. coli* O157:H7 on dip inoculated green onions

Currently, researchers are trying to find alternatives to chlorine or lower chlorine working solution concentration by using hurdle techniques. Sanitizer-sanitizer (Huang and Chen, 2011) or sanitizer-novel techniques combination (Singh et al. 2002) studies have both drawn great attention. As discussed in 3.1, PL treatment was not as effective when we used dip inoculation instead spot inoculation to contaminate green onions. Also because wet PL was better for the quality maintenance of green onions, we focused on evaluation of the inactivation efficacy of wet PL treatment on dip inoculated green onions.

As shown in Fig 4.1, there was no significant difference between *E. coli* reduction of 10 and 100ppm chlorine washing for both leaves and stems, ranging from 0.9 to 1.2 $\log_{10}$ CFU/g. For PL single treatments, both 30 and 60s were tested and 60s PL achieved comparable inactivation efficacy as chlorine. The *E. coli* reductions were 1.2 and 0.9 $\log_{10}$ CFU/g on leaves and stems, respectively. Also because of the lacking of residual compounds and the absence of applying chemicals with health concern (Gómez-López et al., 2007), PL could be used as alternative to chlorine based washing.

In order to achieve higher *E. coli* inactivation efficacy, combination of chlorine and PL were applied. The 10 or 100ppm chlorine was combined with 30 or 60s PL treatment, which could be easily set up by changing of water in wet PL into chlorine solution without any additional treatment step. For all four combination treatments,

overall *E. coli* reduction was higher on leaves than stems. There was no significant difference between each combination treatment applied on stems, ranging from 0.8 to 1.0 log $_{10}$ CFU/g, while additive effect could be observed on leaves. For leaves, the 10ppm chlorine combined with 60s PL, 100ppm chlorine combined with 30s PL, and 100ppm chlorine combined with 60s PL were significantly more effective than chlorine washing or PL alone, resulting log reduction of 2.3, 2.2 and 2.4 log $_{10}$ CFU/g, respectively. Since there was no significant difference between 10 or 100ppm chlorine when combined with 60s PL, lower concentration was recommended due to both cost and health concern. Different inactivation efficacy has been observed on stems and leaves. This may due to their different structures. Green onion stems have compressed layers and it was difficult for surface treatments such as PL or sanitizers to reach pathogens that got into the layers during dip inoculation. Leaves, however, have one layer and are hollow in the middle. Therefore it was easier for inactivation treatments to reach the pathogens hiding inside.

Combination of chlorine and UV, which is the main antimicrobial contributor of PL (Takeshita et al. 2002), has been utilized in disinfection installations, since UV can inactivate protozoa which are difficult to be destroyed by chlorination (Clancy et al., 2000). The interactions between free chlorine and UV light may affect the inactivation efficacy (Feng et al, 2007). Some studies suggested that UV light may cause the degradation of chlorine. Zheng et al, (1999) reported that the higher the UV dose applied the greater the chlorine dose was required to keep a fixed concentration in the effluent water. However, Örmeci et al. (2005) pointed out that the decay of chlorine and monochloramine at typical UV disinfection doses (<100 mJ/cm^2) was

negligible. The additive effect of PL and chlorine for inactivation of *E. coli* O157:H7 observed in our study suggests that chlorine was not completely degraded.

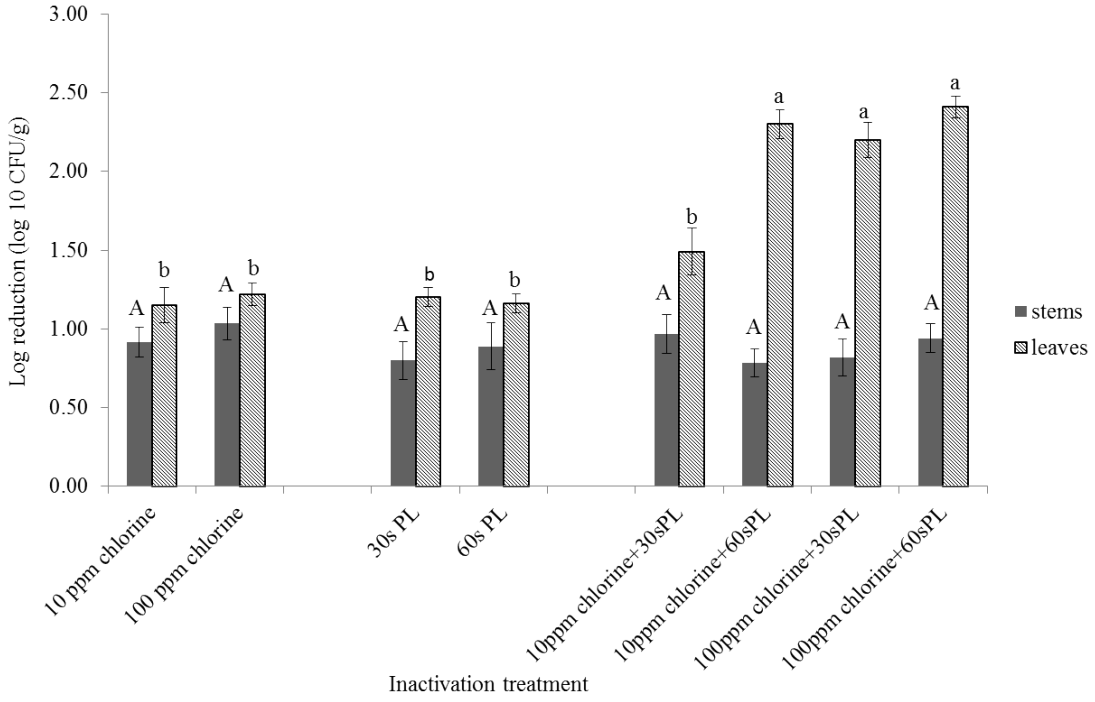

Figure 4.1: Combination of PL and chlorine on the inactivation of *E. coli* O157:H7 on dip inoculated green onions.

Dip inoculated green onions were treated using chlorine washing (10 and 100ppm), PL (30 and 60s) and PL-chlorine combinations. Chlorine washing was applied for 60s. The initial populations of *E. coli* O157:H7 for dip inoculated stems and leaves were 4.5 and 5.5 log $_{10}$ CFU/g, respectively. Values in stems group marked by the same capital letter were not significantly different (p >0.05). Values in leaves group marked by the same lowercase letter were not significantly different (p >0.05).

4.3.3 Combination of PL and surfactant on the inactivation of *E. coli* O157:H7 on dip inoculated green onions

Sodium dodecyl sulfate (SDS) is an anionic surfactant and an FDA-approved food additive (FDA, 1978). Surfactant allows the release of tightly bound contaminations from the surface which may enhance the removal of foodborne pathogens from fresh produce. Also adding surfactant can reduce surface tension of the water film formed on plant surface resulting higher inactivation efficacy. In our study, different concentrations of SDS (10, 100 and 1000ppm) were used to wash green onions for 60s alone or combine with 60s PL treatments. As shown in Fig.4.2, there was no significant difference between each concentration of SDS washing for stems and leaves, ranging from 0.2 to 0.6 log $_{10}$ CFU/g. SDS washing was similar as water washing which achieved only 0.4 log $_{10}$ CFU/g on stems and almost no effect on leaves (0.0 log $_{10}$ CFU/g). When combined with PL, however, *E. coli* log reduction increased significantly on leaves. Highest reduction (3.1 $\log_{10}$ CFU/g) was achieved by combination of 1000ppm SDS and 60s PL treatment. For stems, 1000ppm SDS combined with PL (1.4 log $_{10}$ CFU/g) was significantly more effective than 1000ppm SDS washing.

Comparing with PL and chlorine combination results, PL combined with SDS was considered as better option because the enhancement effect was not only observed on leaves but also on stems which were more difficult to be decontaminated. The compressed layers of stems might provide a better shelter for *E. coli*, making it difficult to be removed and inactivated by PL. Unlike chlorine washing alone which achieved around 1.0 log reduction, SDS itself didn't show any significant inactivation efficacy. However, the inactivation efficacy of PL and SDS combination was significantly higher than PL and chlorine combination on leaves, and comparable with

each other on stems, which showed that the former one provided more obvious synergistic effect. This may due to the fact that SDS could enhance the removal of foodborne pathogens from fresh produce. Thus, PL and SDS combination could be used as alternative to chlorine washing since they can achieve more than 3 log $_{10}$ CFU/g reductions on dip inoculated green onion leaves and they avoided the health issue or environmental concerns that may be caused by chlorine.

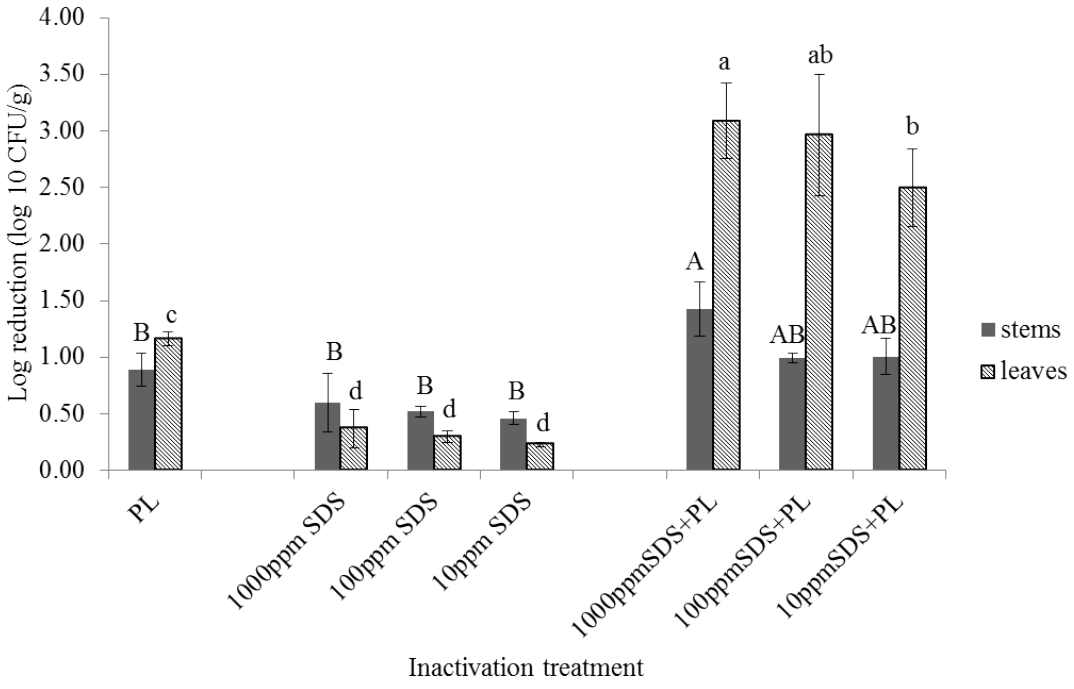

Figure 4.2: Combination of PL and surfactant on the inactivation of *E. coli* O157:H7 on dip inoculated green onions.

Dip inoculated green onions were treated using SDS washing (10, 100 and 1000ppm), PL (60s) and PL-SDS combinations. SDS washing was applied for 60s. The initial populations of *E. coli* O157:H7 for dip inoculated stems and leaves were 4.5 and 5.5 log $_{10}$ CFU/g, respectively. Values in stems group marked by the same

capital letter were not significantly different (p >0.05). Values in leaves group marked by the same lowercase letter were not significantly different (p >0.05).

4.3.4 Combination of PL and non-chlorine based sanitizer on the inactivation of *E. coli* O157:H7 on dip inoculated green onions

The most common non-chlorine based sanitizers that have been studied by researchers and also in our previous research, include essential oil, organic acids and oxidants. Thyme oil and its major antibacterial components thymol and carvacrol, with Generally Recognized as Safe (GRAS) status (FDA, 2009), have been found to possess antimicrobial activity in vitro against a broad spectrum of bacteria (Di Pasqua et al., 2006; Gill and Holley, 2006). As shown in Fig.4.3, when combined 0.2mg/mL thymol with 60s PL treatment, there was no significant enhancement on green onion stems. It seems 1.2 log higher reduction has been achieved on leaves when compared with thymol washing alone. There, however, was no significant difference when compared with 60s PL treatment (1.2 log $_{10}$ CFU/g), which showed that no additive effect has been formed by this combination.

Organic acids occur throughout nature and are used extensively in food systems. Citric, malic, tartaric and acetic acids are most commonly used organic acids in food industry. As shown in Fig.4.3, 1mg/mL citric acid could hardly achieve more than 0.3-log reduction. In PL and citric acid combination, there was also no significant enhancement since the combination provided reduction of 0.8 and 1.2 log $_{10}$ CFU/g on stems and leaves, respectively, and 60s PL treatment alone has already achieved reduction of 0.9 and 1.2 log $_{10}$ CFU/g on stems and leaves, respectively.

The bactericidal efficacy of hydrogen peroxide has been demonstrated in both water and food systems (Liao and Sapers, 2000). Hydrogen peroxide has GRAS status

(FDA, 2001c), which can be used in both vapor and liquid phase to control the bacteria and extend the shelf life of minimally processed fruits and vegetables (Sapers and Simmons, 1998). In our study (shown in Fig.4.3), 300ppm hydrogen peroxide was slightly more effective than thymol and citric acid which achieved reduction of 0.6 and 0.4 log $_{10}$ CFU/g on stems and leaves, respectively. When combined with 60s PL treatment, significant increase of inactivation efficacy has been observed on leaves (2.6 log $_{10}$ CFU/g) but not on stems (0.7 log $_{10}$ CFU/g).

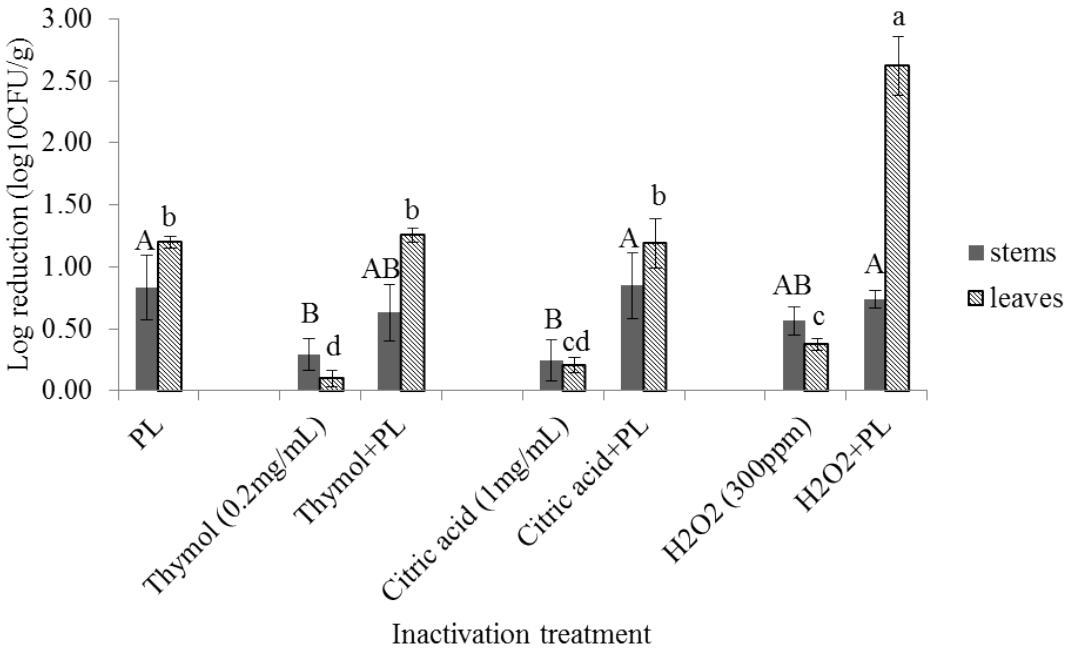

Figure 4.3: Combination of PL and non-chlorine based sanitizer on the inactivation of *E. coli* O157:H7 on dip inoculated green onions.

Dip inoculated green onions were treated using thymol (0.2mg/mL), citric acid (1mg/mL) and H_2O_2 (300ppm) washing, PL (60s) and PL-non-chlorine based sanitizer

combinations. Single washing treatments were applied for 60s. The initial populations of *E. coli* O157:H7 for dip inoculated stems and leaves were 4.5 and 5.5 $\log_{10}$ CFU/g, respectively. Values in stems group marked by the same capital letter were not significantly different (p>0.05). Values in leaves group marked by the same lowercase letter were not significantly different (p >0.05).

4.3.5 Combination of PL-surfactant-sanitizer on the inactivation of *E. coli* O157:H7 on dip inoculated green onions

PL-surfactant-sanitizer combinations were designed according to the results aforementioned. Ten ppm chlorine, 1000ppm SDS and 300ppm H_2O_2 were chosen since they significantly increased *E. coli* inactivation efficacy on green onions when they were combined with PL treatment for 60s. It was possible that the surfactant removed pathogens from the surface of green onions which made it easier to inactivate, SDS was thus applied in both combinations. Surfactant like SDS can reduce surface tension of the water film formed on plant surface and allow the release of tightly bound contaminations from the produce surface, which may enhance the removal of foodborne pathogens from fresh produce. *E. coli* inactivation efficacy of PL + 10ppm chlorine + 1000ppm SDS, PL + 300ppm H_2O_2 + 1000ppm SDS and both treatments without PL were evaluated. As shown in Fig.4.4, for green onion leaves, chlorine + SDS or H_2O_2 + SDS was not significantly more effective than chlorine, SDS or H_2O_2 alone at the same concentration. When chlorine + SDS or H_2O_2 + SDS combined with PL, the inactivation efficacy increased significantly, resulting 3.2 (PL+ chlorine + SDS) and 3.1 $\log_{10}$ CFU/g (PL + H_2O_2 + SDS) reductions (p <0.05). Same results were observed from stems. When combined with PL, the log reductions reached 1.6 (PL + chlorine + SDS) and 1.5 $\log_{10}$ CFU/g (PL + H_2O_2 + SDS), which

were significantly higher than chlorine + SDS (0.4 log $_{10}$ CFU/g) and H_2O_2 + SDS washing (0.6 log $_{10}$ CFU/g).

However, when comparing PL-surfactant-sanitizer with PL-surfactant combination, there was no significant difference on both stems and leaves. Sanitizers did not play major role in the additive effect. Therefore, PL-surfactant combination was recommended to inactivate *E. coli* O157:H7 on green onions.

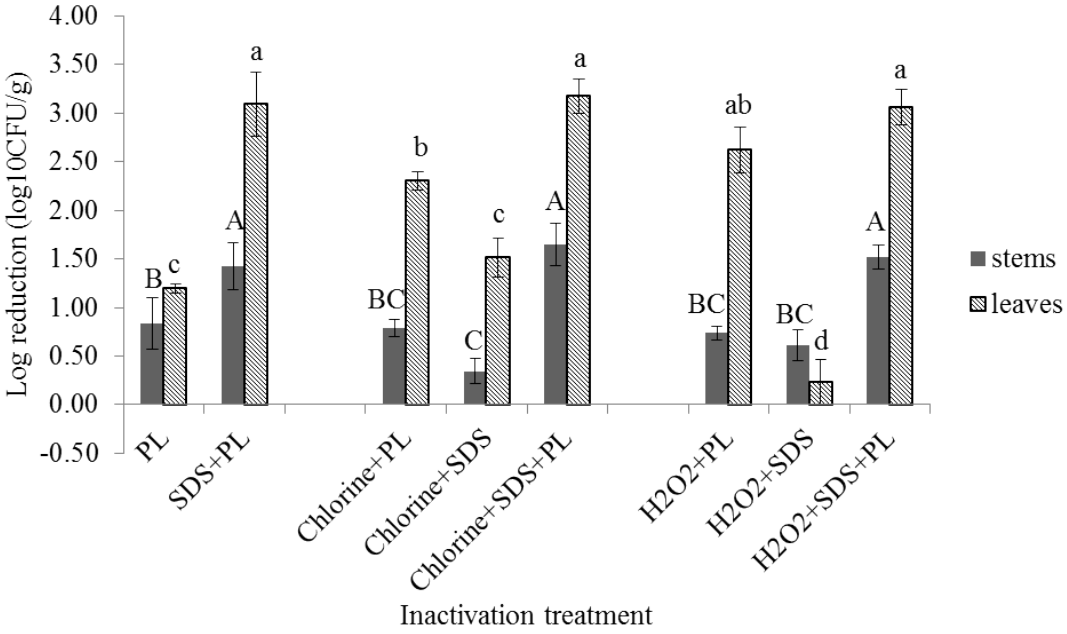

Figure 4.4: Combination of PL-surfactant-sanitizer on the inactivation of *E. coli* O157:H7 on dip inoculated green onions.

PL-chlorine, PL-SDS and PL- H_2O_2 combinations were also included for comparison. Dip inoculated green onions were treated using PL (60s), surfactant-sanitizer combination (10ppmchlorine + 1000ppmSDS and 300ppmH_2O_2 +

1000ppmSDS) washing as well as PL-surfactant-sanitizer combination (10ppmChlorine + 1000ppmSDS + 60sPL and 300ppmH_2O_2 + 1000ppmSDS + 60s PL). The initial populations of *E. coli* O157:H7 for dip inoculated stems and leaves were 4.5 and 5.5 log $_{10}$ CFU/g, respectively. Values in stems group marked by the same capital letter were not significantly different (p >0.05). Values in leaves group marked by the same lowercase letter were not significantly different (p >0.05).

4.4 Conclusion

PL could potentially be used for decontamination of *E. coli*. O157:H7 on green onions. Wet PL treatment preserved quality better than dry PL. The 5s dry PL and 60s wet PL treatment provided promising inactivation efficacy (>4 log $_{10}$ CFU/g) for spot inoculated stems and leaves. For dip inoculated green onions, 60s wet PL treatment was comparable with 100ppm chlorine washing, the former one could be considered as alternative to chlorine. To further increase the microbial safety of green onion contaminated by dip inoculation, hurdle technique was applied since it was difficult to reach more than 1.5 log $_{10}$ CFU/g by dry or wet PL treatment alone. PL combined with surfactant (SDS) was found to be the most effective treatment.

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Chapter 5

APPLICATION OF PULSED LIGHT (PL)-SURFACTANT COMBINATION ON INACTIVATION OF *SALMONELLA* AND APPARENT QUALITY OF GREEN ONIONS IN SHELF-LIFE

Under review of LWT- Food Science and Technology

ABSTRACT

Decontamination of *E. coli* O157:H7 on green onions using pulsed light (PL) and PL-sanitizer/surfactant combinations has shown that the combination of PL and 1000 ppm of surfactant (sodium dodecyl sulfate, SDS) was more effective than single treatments. In this study, we investigated whether PL-surfactant combination would provide similar inactivation efficacy of *Salmonella* on green onions. Different surfactants (SDS and Tween 80) as well as different concentrations (10, 100 and 1000ppm) of each surfactant combined with PL were tested. Survival populations of *Salmonella* and quality of green onions (color and texture) were evaluated after treatments as well as during storage (15 days) at 4 ℃. The results showed that PL-SDS and PL-Tween 80 combinations at various surfactant concentrations provided additive inactivation efficacy on green onion leaves (2.6-2.9 $\log_{10}$ CFU/g and 2.4-2.7 $\log_{10}$ CFU/g, respectively), but not on their stems (0.4-1.0 $\log_{10}$ CFU/g and 0.3-0.6 $\log_{10}$ CFU/g, respectively). PL-1000ppm SDS combination negatively impacted color and texture of green onions during 15 day storage at 4 ℃. To consider both safety and

quality of green onions, PL-10ppm SDS was selected since it showed similar *Salmonella* inactivation on green onions as PL-1000ppm SDS but did not affect quality of green onions during shelf-life.

KEY WORDS: Green onions; *Salmonella* inactivation; pulsed light (PL); surfactant

5.1 Introduction

Green onions (*Allium fistulosum* L.) have been widely used in Asian and Mexico cuisines such as soup, salsa, dips etc. The United States began importing green onions from Mexico in 1980 and importation has been increasing since then. In 2007, 91 percent of U.S. green onions consumed were supplied by Mexico (Calvin and Martin, 2010). Green onions from Mexico have caused three hepatitis A outbreaks in 2003 and resulted in four deaths and 1028 illnesses (Calvin et al., 2004). However, contamination has been found on both domestic and imported green onions (FDA, 2001a, 2001b). Not just in the U.S., an outbreak of *Salmonella* Oranienberg in Ontario, Canada was linked to the consumption of green onions which were sold unwrapped and held together with a rubber band in supermarkets (CTV News, 2010).

Green onions were grown in Mexico year round to meet the consumer needs, making them the major exporter. The summer productions are usually located in the cooler western coastal areas with poorer infrastructure, which may be more challenging from a food safety perspective (Calvin et al., 2004). Green onion requires as many as nine manual labor steps from harvesting to packing (Calvin et al., 2004). The relatively high number of handlers potentially increases the probability of microbial contamination. During pre-harvest, pathogens in animal manure or irrigation water may contaminate plants surface or even internalize and transfer to other parts of the plants (Solomon et al., 2002; IsIam et al., 2004). From harvesting to packinghouse

as well as value added processing, contaminated equipment, unhygienic handlers and ice/water used for washing may also serve as potential source for bacterial or virus contamination (Sharps et al., 2012). Furthermore, the hollow leaves of green onions may aid the attachment of microorganisms by providing them easy access to the larger surface area (FAO and WHO, 2008) and the compressed stems protect microorganisms from various decontamination treatments (Xu et al., 2013).

Chlorine has been applied by 67% of fresh produce manufacturers (Gündüz et al., 2010) because of its cross-contamination prevention, but chlorine based disinfectants have been questioned because of its limited efficacy to reduce foodborne pathogens (Beuchat, 1998) on fresh produce as well as its highly suspected carcinogenic by-products (Hidaka et al., 1992). There is a need to find an alternative decontamination strategy that is more effective, safer to use and more environmental friendly. In the last decade, several emerging techniques including ozonated water (Xu and Wu, 2014), high hydrostatic pressure (Neetoo et al., 2011), electrolyzed water (Park et al., 2008) and irradiation (Fan et al., 2003; Murugesan et al., 2011) have been studied to inactivate *Salmonella* and/or *E. coli* O157:H7 in green onions. Studies of hurdle techniques, especially combinations of emerging technique and traditional sanitizer washing have drawn great attention. Ge et al. (2013) induced internalization of *Salmonella* Typhimurium in green onions and treated the contaminated green onions with chlorine, peroxyacetic acid (PPA) and ultraviolet C irradiation (UV-C)-chlorine/PPA combinations. The results showed that no significant reduction was observed when chlorine (0.06-0.17 $\log_{10}$ CFU/g) or PAA (0.15-0.63 $\log_{10}$ CFU/g) was used alone. However, a significant reduction (1.00-1.49 $\log_{10}$ CFU/g) of *Salmonella* was observed in green onions treated with UV-C-chlorine/PPA

combinations. This combination achieved the comparable decontamination efficacy as the UV-C alone with higher fluencies.

In our previous study (Xu et al., 2013), dip inoculated green onions were more difficult to be decontaminated than spot inoculated ones. *E. coli* O157:H7 decontamination efficacy of single treatments of PL, sanitizers (chlorine, citric acid, thymol and hydrogen peroxide), surfactant (sodium dodecyl sulfate, SDS) as well as PL-sanitizer/surfactant combinations were investigated. The combination of PL and 1000 ppm of SDS was found to be more effective than other treatments which reduced the *E. coli* O157:H7 populations dip inoculated on the stems and leaves of green onions by 1.4 and 3.1 $\log_{10}$ CFU/g, respectively.

Based on our previous results, it would be interesting to further study whether PL-1000ppm SDS combination has similar decontamination efficacy on *Salmonella* and whether the type and concentration of surfactants would affect the efficacy. The objective of this study was thus to decontaminate *Salmonella* on green onions using not only PL combined with single concentration of SDS, but PL-Tween 80 and PL-SDS with concentrations from 10ppm to 1000ppm. Furthermore, in order to meet the consumers' needs of fresh and safe food, *Salmonella* survival population and quality of green onions right after treatment and during 15 days storage at 4 ℃ were evaluated.

5.2 Materials and Methods

5.2.1 Bacterial strains and inoculum preparation

A wild-type *Salmonella* strain Newport H1275 that was adapted to grow in the presence of 100 μg/mL of nalidixic acid (Fisher Scientific, Hampton, NH, USA), was

used in our study. The pathogenic strain was grown on tryptic soy agar (Difco Laboratories, Sparks, MD, USA) plus 0.6% yeast extract (Difco) supplemented with 100 μg/mL of nalidixic acid (TSAYE-N) for 2 days at 35℃. Single colonies were transferred to 10 mL of tryptic soy broth (Difco) plus 0.6% yeast extract (Fisher) supplemented with 100 μg/mL of nalidixic acid (TSBYE-N). The culture was incubated at 35℃ for overnight and second-transferred to 10 mL of fresh TSBYE-N to yield an approximate population of 10^9 CFU/mL after 24h incubation at 35℃. The culture was diluted to 10^7 CFU/mL using sterile 0.1% peptone water (Difco) for the dip inoculation described below.

5.2.2 Green onions preparation and inoculation

Green onions were purchased from a local supermarket and stored at 4±2 °C for a maximum of 24h before use. Full-length green onions were cut into either compressed stems (~5g) or hollow leaf (~1g) segments, and then air dried in a biosafety hood.

To dip inoculate the green onions, stems or leaves were fully submersed in 10^7 CFU/mL inoculum in a sterile stomach bag in the ratio of 1:5 or 1:10 (weight of green onions: volume of the inoculum), respectively. Bags were heat-sealed immediately and slight shaking was applied for 1 min. Green onions were taken out by sterile tweezers and placed in sterilized petri dishes. Dip inoculated green onions were air dried in the biosafety hood for 2 or 4 h, respectively, before the decontamination treatments. The initial population was 4.5 and 5.5 $\log_{10}$ CFU/g on stems and leaves, respectively.

5.2.3 Reduction of *Salmonella* on green onions using PL-surfactant combination treatment and survival population during storage

As described in our previous publication (Xu et al., 2013), PL was produced by a bench-top pulsed light system (SteriPulse-XL, Model RS-3000C, Xenon Corp., Wilmington, Mass., U.S.). The 16 inch linear clear fused quartz PL lamp (LH840) delivered 505 joules/pulse (1.27 J/cm2) energy with 3 pulses/sec pulse rate. Dip inoculated green onion stems or leaves were placed in a beaker with DI water (PL alone), 10, 100 or 1000 ppm of SDS or Tween 80 solution (PL-surfactant combination) and a stir bar. Surfactant washing was applied for 60s without PL. Water and 10ppm chlorine washing for 60s served as a comparison. Green onions without any treatment served as a control.

The treated and untreated green onion stems or leaves were then placed into sterile bags and stored at 4±1 ℃ for 15 days. Samples were microbiologically analyzed at day 0, day 5, day 10 and day 15 by transferring them into stomacher bags with D/E neutralized broth (Difco) at 1:9 ratio (w:v). Samples with D/E broth were homogenized by stomacher (400 Circulator, Seward Co., West Sussex, UK) at 260 rpm for 2min. The homogenate was serially diluted in 0.1% peptone water and plating on TSAYE-N plates. *Salmonella* colonies were enumerated after incubation for 48h at 37℃. Survivors were calculated in CFU/g. Inactivation efficacy was reported as $\log_{10}$ CFU/g reduction which was the difference between log survivor population of control and each log survivor population of treated sample.

5.2.4 Effect of PL-surfactant combination treatment on the quality of green onions

Un-inoculated green onion stems and leaves were treated (PL-surfactant combination, DI water or 100ppm chlorine washing) as described above. The quality

of treated and untreated green onions were analyzed immediately after treatment (day 0) and stored at 4 ℃ for 15 days. Pictures were taken to show the overall appearance on day 5, day 10 and day 15. Color evaluation of treated and untreated green onion stems and leaves were independently performed with the chromameter (Minolta CR-400, Minolta, Osaka, Japan). Color parameters will be quantified in the Hunter L, a, and b color space where "L" refers to lightness/darkness, "a" refers to redness/greenness, and "b" refers to yellowness/blueness.

Texture of treated and untreated green onion stems and leaves were measured as shear strength using a texture analyzer (TA. XT2i, Texture Technologies, New York, USA). Samples were placed on the TA-91 platform and compression tests were carried out. The maximum force (Fmax) needed to compress the samples will be recorded using the Texture Expert Exceed software (Texture Technologies).

5.2.5 Statistical analyses

All experiments were replicated 3 times and results were reported as Mean±SD. JMP (SAS Cary, NC, USA) software was used for statistical analyses. For inactivation efficacy, results were reported as *Salmonella* log reduction ($\log_{10}$ CFU/g) and student's t test was used to determine difference between each treatment. For survival population, results were reported as *Salmonella* log survival population ($\log_{10}$ CFU/g). For survival population of *Salmonella* and shelf-life quality of green onions, Dunnett's test was used to determine difference between treated and untreated (control) samples on day 0, day 5, day 10 and day 15. Significant difference was reported when $p<0.05$.

5.3 Results and Discussion

5.3.1 Reduction of *Salmonella* on green onions using PL-surfactant combination treatment

Green onion stems were washed by water, 10ppm chlorine, SDS (10-1000ppm) and Tween 80 (10-1000ppm). As shown in Figure 5.1, there was no significant reduction in *Salmonella* when surfactants were used alone (0.0-0.2 $\log_{10}$ CFU/g). To investigate whether there is any additive effects between surfactants and PL, green onion stems were treated with PL-SDS (10, 100 and 1000ppm) and PL-Tween 80 (10, 100 and 1000ppm) with 60s PL treatment alone as control. Although PL-1000ppm SDS had significantly higher *Salmonella* reduction (1.0 $\log_{10}$ CFU/g) than 1000ppm SDS (0.0 $\log_{10}$ CFU/g) and water washing (0.2 $\log_{10}$ CFU/g), there was no significant difference between this combination and 60s PL treatment alone (0.5 $\log_{10}$ CFU/g). When comparing all PL-surfactant combinations with PL alone using Duneett's test, none of the combination was better than PL treatment itself, no matter what type of surfactant we applied from concentration 10ppm to 1000ppm. Thus, no additive effect on *Salmonella* inactivation has been observed when PL-surfactant combinations were applied on green onions stems decontamination. Stems with compressed layers were more difficult to decontaminate than the hollow leaves, especially for dip inoculated green onions, which has been noticed in our previous study (Xu et al., 2013). The overall log reduction of *Salmonella* (<1.0 $\log_{10}$ CFU/g) on stems were significantly lower than that on green onion leaves. Tween 80 did not perform better than SDS when used alone or in the PL-surfactant combiation.

For green onion leaves, similar as stems, there was no significant difference between water and surfactants (SDS and Tween 80) washing (0.1-0.4 $\log_{10}$ CFU/g). However, comparing PL-surfactant combinations with single treatments, PL-10ppm

SDS(2.6 log $_{10}$ CFU/g), PL-100ppm SDS (2.5 log $_{10}$ CFU/g), PL-1000ppm SDS (2.9 log $_{10}$ CFU/g), PL-10ppm Tween 80 (2.7 log $_{10}$ CFU/g) and PL-1000ppm Tween 80 (2.4 log $_{10}$ CFU/g) had significantly higher *Salmonella* inactication efficacy than PL (1.5 log $_{10}$ CFU/g) as well as SDS or Tween 80 alone at the same concentration. PL-100ppm Tween 80 (2.4 log $_{10}$ CFU/g) provided similar *Salmonella* log redution as PL-1000ppm Tween 80 but because of larger standerd diviation, this combination was not significantly different from PL treatment alone. No significant difference has been observed among three different concentrations of SDS or Tween 80 as well as between two surfactants, which means SDS and Tween 80 provided similar additive effects on *Salmonella* inactivation efficacy when combined with PL.

Surfactants themselves did not provide siginificanly higher inactivation efficacy than water washing at the concentration up to 1000ppm (0.1%). This results agreed with Zhao et al. (2009) who treated the *Salmonella* Enteritidis in pure culture with different concentrations of SDS. No significant inactivation efficacy has been observed until they increased the SDS concentration to 1.5% which was 15 times higher than the concentration we used in our study. The limited antimicrobial of SDS on *Salmonella* may due to the tolerance of *Enterobacteriaceae* family to SDS (Rajagopal et al., 2002). When combined with other sanitizers or novel inactivation techniques, surfactant was reported having additive effects. Zhao et al. (2009) reported the significant increase of *Salmonella* inactivation efficacy when combine SDS with organic acid. Their explanation for this additive effect was that SDS showed increased denature protein surfaces and damage cell membranes, when the pH is reduced to 1.5-3.0 by organic acid. Our previous study (Xu et al., 2013) also reported additive effect of SDS against *E.coli* when combined with PL, which could be related with the

possibility that surfactant allows the release of tightly bound contaminations from the surface and thus enhance the removal of foodborne pathogens from fresh produce.

Based on their chemical structure, surfactants can be classified as either anionic or nonionic compounds. Tween 80 was the example of non-ionic surfactant, while SDS was an anionic surfactant (Predmore, 2011). Like SDS, there is no regulation on using polysorbates sanitizers (Tweens) in food processing (FDA 21CFR172.840). However, the application of Tween 80 was more difficult than that of SDS because of the stickiness of the Tween 80. The gel-like feature of Tween 80 increases time and agitation required for distribution. SDS, on the contrast, was easier to apply with its dry powder form. In our study Tween 80 did not provide significantly higher *Salmonella* inactivation efficacy than SDS on both stems and leaves of green onions. Thus, SDS was selected for further experiments.

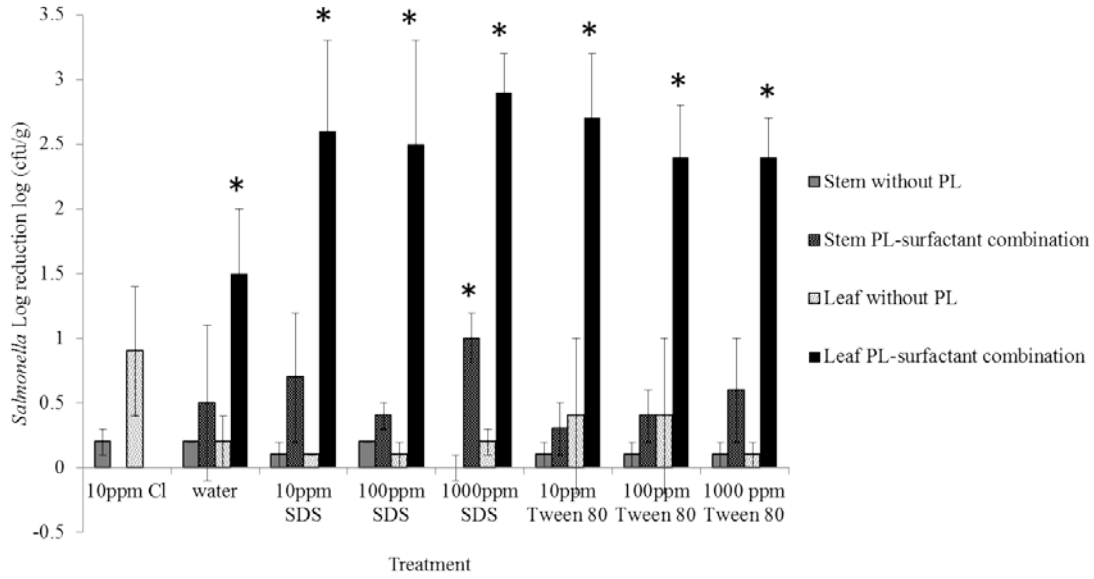

Figure 5.1: Combination of PL and different concentration of surfactants on the inactivation of *Salmonella* on dip inoculated green onions.

Dip inoculated green onions were treated using PL-SDS and PL-Tween 80 combinations for 60s. Three concentrations of SDS and Tween 80 (10, 100 and 1000ppm) were combined with PL. Water, 10ppm chlorine as well as PL alone had also been applied for 60s as comparison. Values marked by * for stem or leaf group were significantly different (p >0.05) from water washing for green onion stem or leaf, respectively.

5.3.2 Survival population of *Salmonella* on green onions during storage

For green onion stems, as shown in Fig.5.2A, *Salmonella* survivors on all treated samples had no significant change during 15 days storage time, including water (from 3.2 to 3.3 log $_{10}$ CFU/g) and 10ppm chlorine washing (from 3.2 to 3.2 log $_{10}$ CFU/g) as well as three PL-surfactant combinations. The unwashed control (from 3.4 to 3.8 log $_{10}$ CFU/g) had slightly higher *Salmonella* population after 10 days. Similar results have been reported by Strawn and Danyluk (2010) who studied the fate of *E. coli* O157:H7 and *Salmonella* on fresh cut papayas. Their results showed that there was no significant change of *Salmonella* population on papayas stored at 4℃ for 28 days. In our study, at day 0, *Salmonella* population on water (3.2 log $_{10}$ CFU/g) and 10ppm chlorine (3.2 log $_{10}$ CFU/g) washed green onion stems had no significant difference with control (3.4 log $_{10}$ CFU/g), while PL-10ppm (2.6 log $_{10}$ CFU/g), 100ppm (2.9 log $_{10}$ CFU/g) or 1000ppm SDS (2.5 log $_{10}$ CFU/g) had significantly lower *Salmonella* population. There was no significant difference among those three PL-surfactant combinations. Although no significant decrease of *Salmonella* population has been observed on 10ppm chlorine washed green onion stems (3.2 log $_{10}$ CFU/g), at day 15, *Salmonella* population was significantly lower than the control group (3.8 log $_{10}$ CFU/g). PL-10ppm (3.0 log $_{10}$ CFU/g), 100ppm (3.0 log $_{10}$ CFU/g) or

1000ppm (2.8 log $_{10}$ CFU/g) SDS kept the significantly lower *Salmonella* survival population when compared with control group after 15 days storage. No significant difference has been observed among those PL-surfactant combinations.

As shown in Fig.5.2B, for green onion leaves, *Salmonella* survival population on control (from 5.4 to 5.2 log $_{10}$ CFU/g), water washing (from 5.1 to 5.2 log $_{10}$ CFU/g) and 10ppm chlorine (from 4.4 to 4.5 log $_{10}$ CFU/g) had no significant change during 15 days storage. Among three PL-surfactant combinations, *Salmonella* population on PL-1000ppm SDS (from 2.4 to 2.6 log $_{10}$ CFU/g) treated samples had no significant change, population on PL-100ppm (from 2.8 to 3.9 log $_{10}$ CFU/g) and PL-10ppm SDS (from 2.6 to 3.4 log $_{10}$ CFU/g) treated green onion leaves, however, increased significantly during 15 days storage. At day 0, although all PL-surfactant combination had significantly lower *Salmonella* population comparing with control, water or 10ppm chlorine washed green onion leaves, there was no significant difference between each PL-surfactant combination. After 15 day storage, PL-surfactant combinations still maintained significantly lower *Salmonella* population than other samples. At the end of storage, *Salmonella* population was ranked as PL-1000ppm SDS (2.6 log $_{10}$ CFU/g) <PL-10ppm SDS (3.4 log $_{10}$ CFU/g) <PL-100ppm SDS (3.9 log $_{10}$ CFU/g) with significant difference in between (p<0.05).

To sum up, PL-10ppm, 100ppm and 1000ppm SDS all significantly lowered the *Salmonella* population on green onion stems and maintained the significant difference during the storage. For green onion leaves, three PL-surfactant combinations significantly lowered the *Salmonella* population at day 0. During 15 day storage, all three combinations still maintained the *Salmonella* survival population significantly lower than control, water and 10ppm chlorine treatments. Similar results

have been published by Williams et al. (2012) on inactivation of *Salmonella* spp. on tomato surface by PL and selected sanitizers. The 60s PL treatments alone achieved less than 2 log reductions, but when it was combined with 10 mL/L hydrogen peroxide, the bacterial reduction significantly increased to over 4 log during the 8-day storage ($p < 0.05$) which indicated that sanitizers combined with PL could generate a significant and lasting inactivation of *Salmonella* spp. on the surface of tomatoes.

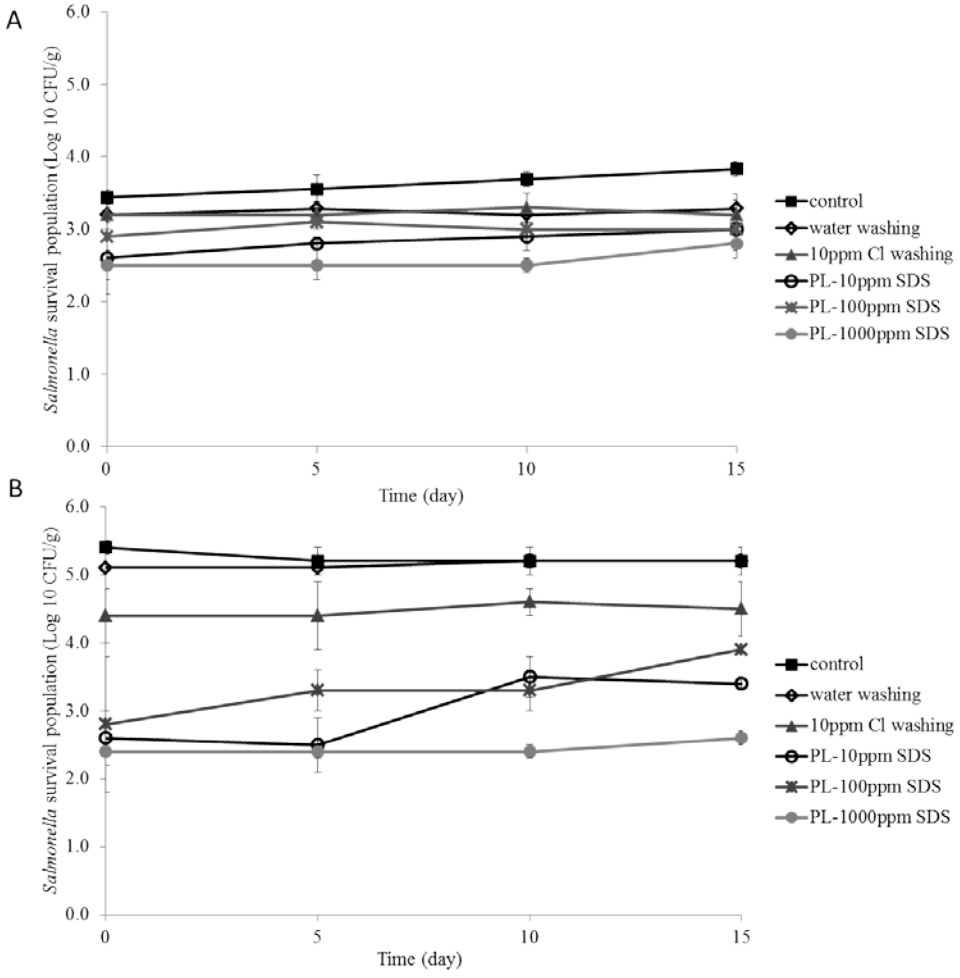

Figure 5.2: Combination of PL-SDS on *Salmonella* survival population on dip inoculated green onions during 15 days storage.

Dip inoculated green onions stems (A) and leaves (B) were treated using PL-10ppm, 100ppm or 1000ppm SDS combinations for 60s. *Salmonella* survival population was count at day 0, 5, 10 and 15. Water and 10ppm chlorine washing had also been applied for 60s as comparison. Green onions without treatment served as control.

5.3.3 Effect of PL-SDS combination treatment on the color and texture of green onions during 15 days shelf-life at 4 ℃

Over the last decade, consumers have increasingly required produce to have a more natural flavor, color and a sufficient shelf-life. Influence of decontamination on quality of fresh produce varies depending on the type of treatment, contact time, types of produce etc. It has been reported that sanitizer washing would not affect quality of some fresh produce, including texture, color and sensory (Lu and Wu, 2010; Sagong et al., 2011). However, strawberries and raspberries showed great sensitivity to anthocyanin bleaching at bactericidal peroxide levels (Sapers and Simmons, 1998).

In addition, quality defects may not happen right after treatment, but negative impact on quality during shelf-life could be the major concern. Yellowing of the stems (Kasim, 2009) and/or loss of green pigmentation of the leaves (Cantwell et al., 2001) after treatment or during shelf-life are considered color defects of green onions. Thus, the b* values (yellowness) of green onion stems and a* values (greenness) of leaf have been investigated in our study. As shown in Table 5.1, during 15 days storage, the b* value of control and chlorine did not show significant change. But out of our expectation, the b* value of the water washing and all PL-SDS combinations significantly decreased which indicated less yellowing of all these samples. However,

among all the stem samples, there was no visible color change has been observed. The color change were more obvious on green onion leaves

For green onion leaves, overall loss of green pigmentation has been observed during 15 day storage for all samples. The increase of a* was not significant for control and water washed leaves. The 10ppm chlorine treatment showed a significant loss of green color during the first 5 days, but had no significant difference from the control after a 15 day shelf-life. It seems the rate of color loss was faster than other groups at the beginning of storage then slowed down during the rest of shelf-life time. PL-1000ppm SDS caused significant color change at day 10 and was significantly different from all other samples at the end of 15 days. PL-100ppm and PL-10ppm SDS, on the other hand, had no significant impact on color of green onion leaves compared with control.

Table 5.1: Effect of PL-SDS combination treatment on the color of green onions during 15 days shelf-life at 4 ℃.

	Day 0	Day 5	Day 10	Day 15
Stems (b)*				
control	18.9±0.8 a	19.4±0.4 a	19.1±0.2 a	17.1±0.2 a
water	18.9±1.1 a	19.1±1.2 a	18.8±0.9 a	15.6±0.4 b
10ppm CL	18.7±0.7 a	20.4±1.1 a	18.6±0.5 a	16.6±0.5 a
PL+1000ppm SDS	19.3±0.1 a	17.8±0.1 a	15.6±0.7 b	10.7±1.2 b
PL+100ppm SDS	18.8±0.1 a	18.7±0.5 a	17.6±0.7 b	14.9±0.8 b
PL+10ppm SDS	19.6±0.6 a	19.4±0.8 a	18.6±1.1 a	15.8±0.5 b
Leaves (a)*				
control	-3.6±0.4 A	-3.2±0.4 A	-3.4±0.6 A	-2.6±0.4 A
water	-3.7±0.1 A	-3.7±0.6 A	-3.4±0.3 A	-2.6±0.5 A
10ppm CL	-4.2±0.4 A	-4.4±0.4 B	-3.9±0.6 A	-1.9±0.3 A
PL+1000ppm SDS	-3.9±0.1 A	-3.3±0.1 A	-1.9±0.2 B	-0.9±0.2 B
PL+100ppm SDS	-4.2±0.3 A	-3.8±0.2 A	-3.4±0.3 A	-1.8±0.1 A
PL+10ppm SDS	-3.5±0.9 A	-3.6±0.6 A	-3.8±0.3 A	-2.1±0.7 A

Data representing mean firmness values ± standard deviation. For the same green onion section, values in the same column followed by the same small letter are not significantly different (p> 0.05) and values in the same column followed by the same capital letter are not significantly different (p> 0.05).

Although there was a general trend towards softer tissue (decrease of firmness) with a 15 day shelf-life, treatments did not have as much impact on texture of green onion stems. As shown in Table 5.2 no significant difference has been observed on stems for control and all treated samples. For green onion leaves, however, PL-1000ppm SDS has caused significant softening since day 10. Our results were slightly different from Hierro's study (2012) about efficacy of PL treatment on the inactivation of selected pathogens and the shelf-life extension of beef and tuna carpaccio. Their results showed that PL showed a greater impact on the sensory quality of tuna

carpaccio compared to beef. None of the fluences assayed extended the shelf-life of either product. In our study, however, combination of PL with low concentration (10 or 100ppm) SDS did not show any significant color and texture impact.

Table 5.2: Effect of PL-SDS combination treatment on texture of green onions during 15 days shelf-life at 4 ℃

	Day 0	Day 5	Day 10	Day15
Stems				
control	11.3±0.8 a	11.3±1.6 a	10.6±1.9 a	10.6±0.8 a
water	11.9±1.5 a	10.8±1.3 a	9.9±0.9 a	9.1±0.9 a
10ppm CL	10.9±0.3 a	10.7±0.3 a	9.7±0.8 a	10.2±0.6 a
PL+1000ppm SDS	12.6±0.6 a	11.6±0.3 a	10.4±0.1 a	9.9±1.2 a
PL+100ppm SDS	13.1±0.3 a	11.5±2.5 a	10.9±2.6 a	10.3±1.2 a
PL+10ppm SDS	12.3±2.1 a	10.9±1.9 a	9.6±0.6 a	9.1±0.3 a
Leaves				
control	6.4±0.5 A	6.7±0.4 A	6.4±0.8 A	4.7±0.3 A
water	6.7±0.2 A	6.2±0.3 A	5.9±0.1 A	4.5±0.7 A
10ppm CL	6.6±0.7 A	6.6±0.4 A	6.7±1.5 A	4.8±1.5 A
PL+1000ppm SDS	6.1±0.7 A	5.9±0.8 A	4.3±0.4 B	2.8±0.6 B
PL+100ppm SDS	6.4±0.6 A	6.1±0.7 A	5.4±0.5 A	4.2±0.3 A
PL+10ppm SDS	6.4±0.5 A	6.2±0.7 A	6.3±0.6 A	4.8±0.6 A

Data representing mean firmness values ± standard deviation. For the same green onion section, values in the same column followed by the same small letter are not significantly different ($p > 0.05$) and values in the same column followed by the same capital letter are not significantly different ($p > 0.05$).

5.4 Conclusion

PL-surfactant combination could potentially be used for decontamination of *Salmonella* on green onions. PL-SDS and PL-Tween 80 had similar inactivation efficacy on *Salmonella*, while SDS was chosen for further study because of its easier application. PL-1000ppm SDS combination showed highest log reduction of *Salmonella* on both stems and leaves and maintained the lowest survival population on leaves during 15 day storage at 4 ℃, however, the color and texture of green onions treated by this combination changed significantly in 15 day shelf-life. To consider both safety and quality of green onions, PL-10ppm SDS was selected since it showed similar *Salmonella* inactivation on green onions as PL-1000ppm SDS but did not affect quality of green onions during shelf-life.

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Chapter 6

THE IMPACT OF PULSED LIGHT (PL) ON MICROBIAL DECONTAMINATION AND QUALITY OF FRESH RASPBERRIES

ABSTRACT

Raspberries have served as vehicles for transmission of foodborne pathogens through fecal-oral route and have resulted in 11 outbreaks in the United States. However, because of its dedicated structures and perishability, water based sanitizer washing cannot be used for raspberry decontamination. As a non-thermal technique, pulsed light (PL) may have the potential to maintain both safety and quality of fresh raspberries. The first objective of our study was to investigate *Salmonella* and *E. coli* O157:H7 inactivation efficacy of pulsed light (PL) on fresh red raspberries. Raspberries were spot inoculated with foodborne pathogens and treated by PL for 5, 15 or 30 s. PL treatment for 30 s inactivated *Salmonella* and *E. coli* O157:H7 by 4.5 and 3.9 log $_{10}$ CFU/g, respectively. All the PL treatments maintained lower pathogen survival population during 10 days storage at 4 ℃ compared with the control. The qualities of raspberries after PL treatment, including color, texture, total phenolic content(TPC), total anthocyanin content (TAC), total bacteria count (TBC) as well as total yeast and mold count (TYMC), have also been evaluated during the 10 days storage. Color and texture of raspberries treated by PL 30 s changed negatively in 10 days storage. PL 30 s provided the lowest TBC and TYMC at day 0, but failed to

maintain its advantage during storage. To consider both safety and quality of fresh raspberries, 5 or 15 s PL treatment was recommended for decontamination.

KEY WORDS: Raspberries; PL; *Salmonella*; *E. coli* O157:H7; quality

6.1 Introduction

Raspberries are the third most popular berry in the United States for fresh use, after strawberries and blueberries. As the world's third-largest producer, most of raspberries growers in the U.S. located in Washington, California and Oregon. However, only 15% of the domestic demand for raspberry fruit is met by production in the U.S. Most imports are arriving from Canada in July and August, and Mexico and Chile from November through May. Berries have been associated with outbreaks in both North America and Europe, and have caused numerous serious illnesses. Imported berries were often implicated, indicating that this problem extends beyond those countries where there have been outbreaks (FAO, 2008).

During 1983 to 2013, there were 11 outbreaks related with raspberries with total 4, 637 cases. In earlier time, raspberries outbreaks have been associated with *Cyclospora cayetanensis* (CDC, 1996, 1997, 1998; Ho et al., 2002), Calicivirus (Pönkä et al., 1999) and Hepatitis A (Reid and Robinson, 1987). Most recently, there were four raspberry outbreaks related with Norovirus (Cotterelle et al., 2005; Falkenhorst et al., 2005; Hjertqvist et al., 2006; Maunula et al., 2009). Fresh as well as frozen raspberries have both been reportedly associated with outbreaks. Outbreaks of foodborne illness in the United States associated with imported raspberries affects not only consumers and the growers of the contaminated product, but also frequently other suppliers to the U.S. market, including U.S. producers (Calvin, 2004). Take the cyclosporiasis illness outbreak in 1996 for example, initially, investigators linked this

outbreak to California strawberries, the California Strawberry Commission estimated that this false alarm led to $16 million in lost revenue to growers in the central coast of California during the month of June (Mishen, 1996). After Guatemalan raspberries were finally found out to be the responsible source, the FDA, not yet convinced the problem was resolved, issued an import alert, denying all Guatemalan raspberries entry into the United States. Beginning in the spring 1999 season, the United States allowed entry of raspberries produced under the Model Plan of Excellence (MPE), which was a costly program resulting in a reduction of raspberry growers in Guatemala from 85 in 1996 to 3 in 2002(Calvin et al., 2003).

C. cayetanensis, hepatitis A as well as norovirus contaminate raspberries through fecal-oral route. It indicates that other foodborne pathogens such as *Salmonella* and *E. coli* O157:H7 which share the same transmission route all have the potential risk. Contamination of fresh raspberries may come from animal entrance into fields and packing houses, irrigation water, picking berries close to the soil line as well as unhygienic workers. The challenge for raspberry decontamination exists because, (1) raspberries are made up of many individual fruits (drupelets) held together by hairs (trichomes) and waxes (Mackenzie, 1979) which provides harbor for foodborne pathogens, and (2) unlike tree fruit, fresh market raspberries are not washed. Thus, there is no way to use liquid sanitizers to limit the potential microbial risk.

Pulsed light (PL) is a non-thermal method for food preservation that involves the use of intense, short duration pulses of a broad spectrum to ensure microbial decontamination on the surface of either foods or packaging materials (Elmnasser et al., 2007). PL treatment of foods has been approved by the FDA (1996) under the code 21CFR179.41 with the total cumulative treatment not exceeding 12.0 J/cm^2. The lamp

can generate light from UV to near-infrared (100 to 1100nm) and UV region was reported to be critical to efficiency of PL treatment (Takeshita et al., 2002). To our best knowledge, research about PL inactivation of *Salmonella* or *E. coli* O157:H7 on raspberries is limited.

Raspberries are delicate fruits and are highly perishable for many reasons. Aesthetic quality of raspberries is mainly determined by color, firmness and mold content. Color change from pink to dark red leads to the loss of attractiveness to consumers. Raspberries grown for fresh market have to be able to retain firmness during harvest, handling and storage and have a shelf-life of 10 days or more (Toivenon et al., 1999). From prospective of health benefit, berries are a good source of polyphenols, especially anthocyanins, micronutrients, and fiber. In epidemiological and clinical studies, these constituents have been associated with improved cardiovascular risk profiles (Basu et al., 2010). Ripe raspberries are also highly susceptible to gray mold (*Botrytis cinerea*), which causes serious losses to some greenhouse growers of raspberries. Molds can also develop in harvested fruit if it is held too long before processing (FDA, 2013). Thus, in this study we not only investigated the decontamination efficacy of *Salmonella* and *E. coli* O157:H7 on fresh raspberries by using PL treatment (right after the treatment and during storage), potential quality change caused by PL has also been evaluated during 10 days storage, including color, texture, total phenolic content (TPC), total anthocyanin content (TAC), total bacteria count (TBC) as well as total yeast and mold count (TYMC).

6.2 Materials and Methods

6.2.1 Bacterial strain and inoculum preparation

Single wild-type strains including *Salmonella* (*S.* Newport H1275, sprout outbreak isolate) and *E. coli* O157:H7 (250, sprout outbreak isolate) were used in our study. Wild strains were obtained from culture collection in Department of Animal and Food Sciences at University of Delaware. The *Salmonella* strain was adapted to grow in the presence of nalidixic acid (50 μg/mL, Fisher Scientific, Hampton, NH, USA) alone to create a single antibiotic resistance strain, while the *E. coli* O157:H7 strain was adapted to grow in the presence of nalidixic acid (100 μg/mL) plus streptomycin (100 μg/mL, Sigma, St. Louis, MO, USA) to create a double antibiotics resistance strain. Both resistance strains were grown on tryptic soy agar (TSA, Difco Laboratories, Sparks, MD, USA) plus 0.6 % yeast extract (YE, Difco) supplemented with nalidixic acid only (TSAYE-N for *Salmonella*) or nalidixic acid and streptomycin (TSAYE-NS for *E. coli* O157:H7) for 2-3 days at 35 ℃. Single colonies were picked and transferred to 10 mL of tryptic soy broth (TSB, Difco) plus 0.6 % yeast extract (Fisher) supplemented with same single or double antibiotics (TSBYE-N for *Salmonella* or TSBYE-NS for *E. coli* O157:H7). The culture was incubated at 35 ℃ overnight and second-transferred to 10 mL of fresh TSBYE-N or TSBYE-NS to yield an approximate population of 10^9 CFU/mL after 24 h incubation at 35 ℃. The culture was diluted to 10^8 CFU/mL using sterile 0.1 % peptone water (Difco) and used as inoculum.

6.2.2 Raspberries preparation and inoculation

Fresh raspberries were purchased from a local supermarket and stored at 4±2 °C for a maximum of 4 h before use. Medium size (~5 g) raspberries with pink color

and ideal firmness were selected. The selected raspberries were intact and had no noticeable physical injury. To spot inoculate raspberries, 50 μL of 10^8 CFU/mL inoculum was deposited on the outside surface of raspberries as five droplets. Spot inoculation was applied to simulate the contamination caused by animal feces drops, irrigation water splashing or unhygienic touch. Inoculated raspberries were dried in the biosafety hood for 2h before future use.

6.2.3 Inactivation of *Salmonella* and *E. coli* O157:H7 using PL treatment and pathogen survivor population during storage

PL was generated by a bench-top pulse light system (SteriPulse-XL, Model RS-3000C, Xenon Corp., Wilmington, Mass., U.S.). The 16 inch linear clear fused quartz PL lamp (LH840) delivered 505 joules/pulse (1.27 joules/cm^2) energy with 3 pulses/sec pulse rate. For PL treatment, raspberries were placed in a PL chamber in sterile petri dishes without covers. Since serious quality loss of raspberries was observed when PL was applied for more than 30s, we chose 5, 15 and 30 s as our treatments times. The quality loss included shrunk tissue, darker color and cooked smell. Fluence for our system was 5.0, 14.3 or 28.2 J/cm^2 for 5, 15 or 30 s PL treatments, respectively. Treated and untreated raspberries (control) were then tested immediately or placed into sterile bags with openings for breathing and then stored at 4±1 ℃ for 10 days.

6.2.4 Microbial analyses

Raspberries were microbiologically analyzed at day 0, day 5 and day 10. They were transferred into sterile stomacher bags with D/E neutralized broth (Difco) at 1:9 ratio (w:v) and homogenized by stomacher (400 Circulator, Seward Co., West Sussex, UK) at 260 rpm for 1min to help releasing and evenly distributing pathogens. The

homogenate was serially diluted using 0.1 % peptone water and plated on TSAYE-N (for *Salmonella*) or TSAYE-NS (for *E.coli* O157:H7) plates using sterile spreader. Colonies were enumerated after incubation for 48 h at 37 ℃. Pathogen survivor population was reported as log $_{10}$ CFU/g.

6.2.5 Effect of PL on color and texture of raspberries

Raspberries without artificial inoculation were either treated by PL as described above or served as control (without PL) and then stored in clean bags with openings at 4±1 ℃ for 10 days. The color and texture of raspberries were analyzed at day 0, day 5 and day 10. Color was tested by using chromameter (Minolta CR-10, Minolta, Osaka, Japan). Color parameters will be quantified in the Hunter L* (lightness/darkness), a* (redness/greenness), and b* (yellowness/blueness) color space. Texture of raspberries was measured as shear strength using a texture analyzer (TA. XT2i, Texture Technologies, New York, USA). Samples were placed on the TA-91 platform and compression tests were carried out. The maximum force (Fmax) needed to compress the samples were reported.

6.2.6 Effect of PL on TPC and TAC of raspberries

TPC of raspberries with or without PL treatment was determined as described by Xu et al. (2010) with slight modification. Raspberries were homogenized in stomacher bags with 25 mL of ethanol with the ratio of 1:5 (w/v). The bags were heat sealed and let sit for 30 min with gentle massaging to extract the polyphenol compounds. Extractions were centrifuged at 4000rpm for 10min (Beckman Coulter, Inc. Microfuge®, Indianapolis, IN, U.S.) and supernatants were collected. 30 μL of supernatants (dilute if necessary) was added into clear 96-well plate with a clear

bottom followed by adding 60 μL of the Folin–Ciocalteau reagent (1×10^{-4} M) and 120 μL of sodium carbonate solution (20 %, w:v). The plate was covered with clear lid, shook horizontally to mix and incubated for 30 min at 35 ℃. A serial of known concentration of gallic acid was used as standard. Absorbance was measured at 765 nm using the Synergy $^{TM\ 2}$ multi-mode microplate reader (BioTek Instruments, Inc. Winooski, VT, U.S.). The results were expressed as micrograms of gallic acid equivalents (GAEs) per 100 grams of fresh weight (FW).

TAC of raspberries was measured using pH differential method reported by Bunea et al. (2011) with slight modification. Raspberries with or without PL treatments were placed in 50 mL Conical centrifuge tubes with 30 mL of methanol-water solution (80:20, v:v) containing 0.1 mL/L acetic acid. The mixture was homogenized by ultra-turrax (IKA Works, Inc., Wilmington, NC) for 2 min, incubated in the dark for 1 h and then ultrasonicated for 15 min to aid anthocyanin extraction. After centrifugation at 5000 rpm for 30 min at room temperature, the volume of the supernatants, which was raspberry anthocyanin extract, was recorded. The anthocyanin extract was mixed with a 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5) with a dilution factor at 6.0. The absorbance of each mixture was measured at 510 nm and 700 nm for the correct haze. The absorbance (A) of anthocyanin extract was calculated by the following formula:

A = (A510- A700) pH 1.0-(A510 - A700) pH 4.5

where A was the absorbance of the anthocyanin extract. A510 means the absorbance measured at 510nm, and A700 means the absorbance measured at 700nm.

The monomeric anthocyanin pigment concentration in the original raspberry sample was expressed in equivalence of cyaniding-3-glucoside using the following formula:

anthocyanin content =(A ×MW× DF×1000)/ε×1

where MW (449.2 g/mol) was the molecular weight of cyanidin-3-glucoside; DF was the dilution factor (6 in our study); and ε was the molar absorptivity, which equal to 26, 900 for cyanidin-3-glucoside.

6.2.7 Effect of PL on TBC and TYMC of raspberries

Raspberries without artificial inoculation were used for TBC and TYMC evaluation. At day 0, day 5 and day 10, raspberries with or without PL treatment were placed in sterile stomacher bags with 45mL of 0.1% peptone water (1:9 ratio, w:v) after the PL treatment and homogenized by stomacher at 260 rpm for 1min. The homogenate was either plated on TSA for TBC enumeration or on Potato Dextrose Agar (PDA, Difco) for TYMC enumeration. Before counting colonies, TSA plates were incubated at 35°C for 48 h while PDA plates were incubated at 25 °C for 3-5 days (Tournas et al., 2001).

6.2.8 Statistical analyses

All experiments were replicated 3 times and results were reported as Mean±SD. JMP 10 (SAS Cary, NC, USA) software was used for statistical analyses. Survivor population of *Salmonella* and *E. coli* O157:H7 were reported as log survival population ($\log_{10}$ CFU/g). For survival population of pathogens and quality of raspberries (including color, texture, TBC, TYMC, TPC and TAC) during storage, Dunnett's test was used to determine differences between treated and untreated

(control) samples on day 0, day 5 and day 10 and Student's t test was used to determine the difference between each treated samples and same treatment on different days. Significant difference was reported when $p<0.05$.

6.3 Results and Discussion

6.3.1 Inactivation of *Salmonella* and *E. coli* O157:H7 using PL treatment and pathogen survivor population during storage

As shown in Fig. 6.1, PL inactivated *Salmonella* in a time-dependent manner (from 5 s to 30 s) which was also a fluence-dependent manner (from 5.0 to 28.2 J/cm^2). The 5s PL treatment significantly lowered the *Salmonella* population from 5.5 (control) to 2.1 log $_{10}$ CFU/g. *Salmonella* population was lowered to 1.5 or 1.0 log $_{10}$ CFU/g after 15 or 30s PL treatment, respectively. The population decrease was significant at the alpha level of 0.05. The highest log reduction (4.5 log $_{10}$ CFU/g reduction) of *Salmonella* was provided by 30 s PL treatment.

During 10 days storage, there was no significant difference for the control ranging from 5.5 to 5.0 log $_{10}$ CFU/g. PL 5s treated raspberries showed a slight population increase in the first 5 days and a slight decrease in the last 5 days. Raspberries treated by 15s PL, however, had a significantly increased *Salmonella* population overall. During the first 5 days of storage, the population increased significantly from 1.5 to 2.2 log $_{10}$ CFU/g and there was no significant increase after day 5 through the end of the storage. The 30s PL showed basically the same trend as PL 15s. There was a significant increase in *Salmonella* population during first half of the storage, from 1.0 to 2.3 log $_{10}$ CFU/g, and no significant change has been observed from day 5 to day 10 even though the population did slightly decrease to 2.0 log $_{10}$ CFU/g. At day 10, end of storage, *Salmonella* survivor population for PL 15s (2.3 log

$_{10}$ CFU/g) and PL 30s (2.0 log $_{10}$ CFU/g) treatments were still significantly lower than the control (5.0 log $_{10}$ CFU/g) but there was no more significant difference compared with PL 5s (1.8 log $_{10}$ CFU/g), which indicated that a longer exposure time of PL provides high inactivation efficacy on *Salmonella* right after the treatment, however the advantange is lost after 10 days storage.

Inactivation of *E. coli* O157:H7 using PL was shown in the same figure (Fig.6.1). Population of *E. coli* O157:H7 decreased significantly from 5.7 (control) to 2.1 log $_{10}$ CFU/g after 5s of PL treatment, which showed that PL has siginificant *E. coli* O157:H7 inactivation efficacy (3.6 log $_{10}$ CFU/g reduction). Different from *Salmonella*, with increase of PL treatment time from 5s to 30 s, there was no significant further improvement of pathogen inactivation. *E. coli* O157:H7 population on 5s PL treated raspberries stayed low during the first 5 days and lowered significantly from day 5 (2.1 log $_{10}$ CFU/g) to day 10 (1.2 log $_{10}$ CFU/g). The 15s PL led to a significantly lower pathogen survivor population on day 10 (1.5 log $_{10}$ CFU/g) compared with day 0 (2.3 log $_{10}$ CFU/g). For 30s PL treatment, *E. coli* O157:H7 survivor population did not change significantly during storage. At the end of 10 days, there was no significant difference among three PL treatments, which was also observed for *Salmonella* population.

Our results showed that PL was an efficient technique to inactivate *Salmonella* and *E. coli* O157:H7 on fresh raspberries and maintained the lower pathogen survivor population during the 10 days storage. PL has been reported to inactivate microorganisms naturally present (Gómez-López et al., 2005; Hoornstra et al., 2002) as well as inoculated (Fine and Gervais, 2004) on food surfaces. Our previous study showed that PL provided 4.9 and 5.2 log reduction on *E. coli* O157:H7 spot inoculated

on green onion stems and leaves, respectively (Xu et al., 2013). Sharma and Demirci (2003) inoculated alfalfa seeds with *E. coli* O157:H7 and treated with PL at different distance (8 and 13 cm) for 75s. They achieved 4.89 log reduction at distance of 8 cm, but only 1.42 log reduction at 13 cm, which indicated the PL decontamination efficacy is dependent on the distance. It also seems that fresh produce with smoother surface and simpler structure was easier to decontaminate by using PL. Reported in Bialka's study (2007), PL was applied to different berries to inactivate artificially inoculated *E. coli* O157:H7 and *Salmonella*. Maximum reductions of 4.3 and 2.9 $\log_{10}$ CFU/g were achieved on blueberries after a UV dose of 22.6 J/cm2 for *E. coli* O157:H7 and *Salmonella*, respectively. On raspberries, however, the maximum reductions of *E. coli* O157:H7 and *Salmonella* were 3.9 and 3.4 $\log_{10}$ CFU/g at much higher fluence (72 and 59.2 J/cm2, respectively).

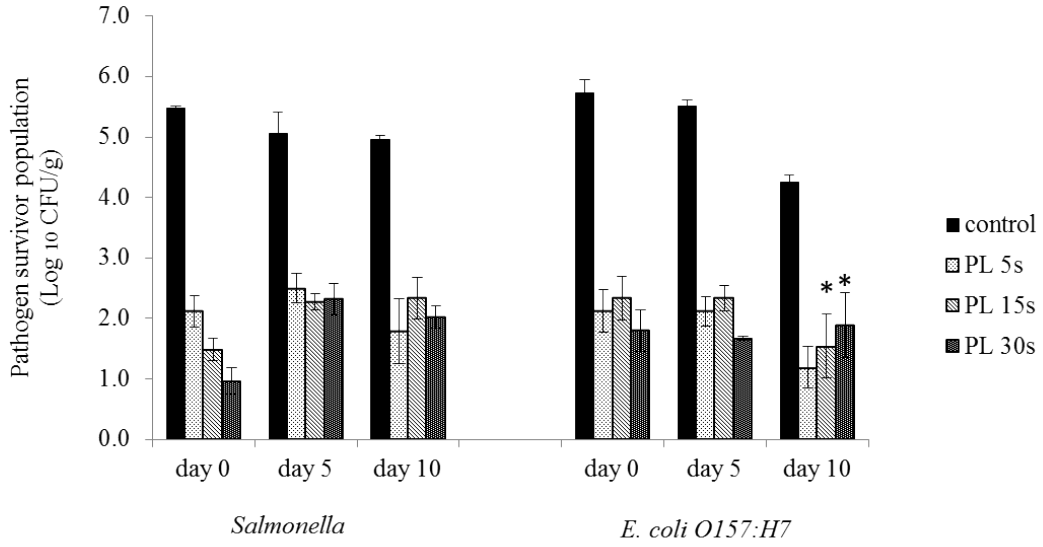

Figure 6.1: Inactivation of *Salmonella* and *E. coli* O157:H7 using PL treatment and pathogen survivor population during storage.

Fresh raspberries were spot inoculated with *Salmonella* or *E. coli* O157:H7 and then treated using PL for 5, 15 or 30s. Untreated raspberries served as control. Pathogen survival populations were evaluated at day 0, day 5 and day 10 during storage at 4 ℃. Values marked by * were significantly different (p <0.05) from their respective controls on that same day.

6.3.2 Effect of PL on color and texture of raspberries

Attractive color is one of the main sensory characteristics of berry fruits and products (Rein and Heinonen, 2004). Color changing from pink to red to dark red may not be attractive in some markets. In this study we monitored the L*, a* and b* values, but only reported L* and a* which indicated the change of brightness/darkness and redness/greenness, respectively. As shown in Table 6.1, there was no significant change for the brightness (L*) after the PL treatment at day 0 ranging from 31.6 to 32.3. However, the redness (a*) of raspberries changed significantly after 15s PL treatment, which might be due to color variation of individual fruits since PL treatment with longer time (30s) did not cause significant color change. During 10 days storage, brightness of raspberries without PL treatment decreased significantly from 32.3 to 27.1 while the redness did not change significantly. The 5s PL treated raspberries showed the same results with decrease of brightness and no significantly change of redness. After being treated for 15 s or 30 s, raspberries became darker red including significantly decrease of L* value and increase of a* value after 10 days storage.

Firmness is another quality indicator for raspberries not only for fresh market but also for processing. Texture of the raspberries showed the similar trend as color, as shown in Table 6.1. When texture analysis was conducted immediately after PL

treatment, those have been treated for 5s showed the significantly softer texture which may because of the individual variation. During 10 days storage, the control and all PL treated berries resulted in significantly softer texture at day 10 comparing with day 0. PL 30s caused the significant softening during 10 days of storage.

Over the last decade, consumers have an increasing requirement for natural and fresh food quality. Quality and safety are both critical for the fresh produce industry. People want to develop techniques with satisfying antimicrobial capacity without compromising quality. In this study, 30s PL treatments did not affect the quality of raspberries right after treatment, however, the influence on quality became significantly greater during first 5 days of storage. In Bialka and Demirci's study (2007), PL was applied at three distances (3, 8 and 13 cm) for 60s with UV-dose of 32.4, 22.6, and 12.8 J/cm^2, respectively for the decontamination of blueberries. There was no significant color difference between treated and untreated blueberries. However, no quality during storage was monitored. Charles et al. (2013) has evaluated the impact of PL on firmness and color of fresh-cut mangoes. Mangoes were treated by PL with fluence of 8 J/cm^2 and placed in glass jar and stored at 6°C for 7 days. The results showed that PL preserved the tissue firmness and color of mangoes during storage. This difference may be because of higher fluence used in our study (28.2 J/cm2 for 30s PL treatment) as well as tougher texture of mangoes compared with raspberries used in our study.

Table 6.1: Effect of PL treatment on the color (L* and a*), texture, TPC and TAC of raspberries during 10 days storage at 4 ℃.

	color (L*)	color (a*)	Texture (N)	TPC (mg GAE/100 g FW)	TAC (mg cyanidin-3-glucoside equivalents /100 g FW)
Day 0					
Control	32.3±1.2	22.8±2.1	2.6±0.8	39.1±6.0	42.6±2.8
PL 5s	32.2±0.3	20.9±1.2	1.9±0.5*	38.3±7.3	38.7±6.8
PL 15s	31.6±0.3	18.7±1.2*	2.4±0.6	43.0±3.9	44.8±3.0
PL 30s	32.2±1.2	21.6±1.1	2.5±0.8	32.1±5.9	48.6±2.7*
Day 5					
Control	31.5±0.6	20.6±2.0	2.5±0.5	33.1±6.9	35.3±1.5
PL 5s	30.9±0.2	20.1±1.3	2.0±0.5	30.5±5.3	33.7±4.9
PL 15s	31.2±2.1	21.4±2.1	2.1±0.2	27.3±3.7*	44.0±0.2*
PL 30s	30.6±1.3	22.0±2.3	1.5±0.4*	25.4±2.6*	42.4±4.4*
Day 10					
Control	27.1±0.4	21.3±0.6	1.9±0.1	29.6±8.4	24.6±4.0
PL 5s	26.6±1.5	20.6±0.9	1.8±0.1	31.3±1.1	27.0±2.4
PL 15s	30.2±0.6*	23.0±0.4*	2.2±0.3	27.5±2.1	30.0±2.4*
PL 30s	22.0±2.3*	25.4±0.8*	0.6±0.3*	25.5±3.7	25.6±4.2

Data representing mean values ± standard deviation. For individual testing, Values marked by * were significantly different (p <0.05) from their respective controls on that same day.

6.3.3 Effect of PL on TPC and TAC of raspberries

Although polyphenols are widely distributed in fruits and vegetables, not all foods are equally good sources, and soft fruits such as strawberries, raspberries and blueberries are now recognized as especially rich sources (Beattie et al., 2005). Berries contain polyphenols including phenolic acids, flavonoids, tannins and stilbenes. As shown in Table 6.1, TPC was not affected by PL right after the treatment. This is

similar as findings from Luksiene et al. (2013) who treated strawberries with high-power pulsed light (3.6–10.8 J/cm^2) and no TPC decrease was observed after the treatment. Other researches, on the other hand, have reported conflicted results. Murugesan (2012) has studied the changes in total polyphenolics in elderberry (*Sambucus nigra*) following treatment with various doses of pulsed light. The results showed that PL treatments enhanced the antioxidant properties of elderberry fruits by increasing their TPC. They also found that the increase of energy from 4500J/m^2/pulse to 6000J/m^2/pulse brought a decrease in the TPC. Guner (2012) treated blueberries with PL and concluded that 60 s or 90 s of PL significantly increased the total phenolic contents of blueberries. These previous findings suggested that TPC varied among different energy and treatment duration used in PL.

In our study, during 10 days storage, TPC of the control decreased significantly from 39.1 to 29.6 mg Gallic acid equivalents per 100 g FW. Similar results have been reported by Verde et al. (2013). In their study, fresh raspberries have been stored at 4℃ for 14 days and the results of TPC indicated a decrease with the storage. In our study, TPC in PL treated raspberries also decreased during 10 days storage. However, significant drop of TPC in the first 5 days has only been observed in raspberries treated by 15 or 30s PL. At the end of storage, there was no significant difference on TPC among the untreated and PL treated raspberries, which indicated that although PL did not improve TPC of raspberries, neither did it affect the TPC compared with untreated raspberries.

TAC was recorded since anthocyanins contribute to the red color as well as antioxidant abilities of raspberries. Raspberries contain a distinct spectrum of 11 different anthocyanins, the most abundant being cyanidin-3-sophoroside, cyanidin-3-

(2G-glucosylrutinoside), and cyanidin-3-glucoside (Mullen et al., 2002). As shown in Table 6.1, PL 5s (38.7 mg cyanidin-3-glucoside equivalents /100 g FW) and 15s (44.8 mg cyanidin-3-glucoside equivalents /100 g FW) did not affect the TAC of raspberries compared with untreated berries (42.6 mg cyanidin-3-glucoside equivalents /100 g FW) right after treatment. PL 30s, however, significantly increased the TAC to 48.6 mg cyanidin-3-glucoside equivalents /100 g FW. It has been reported (Rodov et al., 2012) that brief postharvest exposure to pulsed light (10-90s) stimulates coloration and anthocyanin accumulation in fig fruit (*Ficus carica* L.).

In our study, during 10 days storage, TAC in the control and treated samples significantly decreased while all PL treated berries showed higher TAC at the end of storage. TAC has been reportedly affected by various factors, such as fruit maturity, storage temperature and pulsed light treatment. Wang et al. (2009) showed that cyanindin-based anthocyanins of raspberries increased during the postharvest period, and red raspberries harvested at different developmental stages continued their anthocyanins development during storage even under the dark conditions. Kalt et al. (1999) reported that temperature above 0 ℃ has been shown to increase the anthocyanin contents of red raspberries on storage. Rodov et al. (2012) who studied the influence of PL on fig fruit also reported that after 5 days of storage in the dark at 20 ℃, the anthocyanin content of the skin of fruit exposed to 90-300 s PL was 20 times higher than that observed for the untreated control. In our study we didn't observe significant increase of TAC for the untreated raspberries, but we did agree with other researchers on the positive effect of PL on TAC during storage.

6.3.4 Effect of PL on TBC and TYMC of raspberries

Raspberries are usually rotten by softening and growth of mold and/or bacteria. Initial population of total bacteria of raspberries was 2.5 log $_{10}$ CFU/g. PL treatment decreased significantly populations of aerobic bacteria in raspberries compared with the control. As shown in Fig. 6.2, after PL treatment, TBC in raspberries were 1.8, 1.5 and 1.0 log $_{10}$ CFU/g for 5, 15 and 30s of PL treatment, respectively. After 10 days of storage at 4°C, the control had 4.2 log $_{10}$ CFU/g of TBC, while raspberries treated with 5, 15 and 30s of PL had the TBC of 2.5, 2.3 and 3.3 log $_{10}$ CFU/g, respectively which were significantly lower than that of control. PL 30s showed the highest (1.5 log $_{10}$ CFU/g reduction) of TBC at day 0. At the end of storage, although still significant lower than the control, TBC of PL 30s was significantly higher than PL treatment with shorter time (5 and 15s). This may because the longer duration PL treatment affected the structure of raspberry fruits which benefits bacteria growth, which might be related with soften texture observed in PL30s treated raspberries.

Same inactivation efficacy of PL has been found on yeasts and molds (Fig.6.3). The initial TYMC of raspberries was 3.4 log $_{10}$ CFU/g while after treatment, population of yeasts and molds for raspberries were 2.4, 2.0 and 1.8 log $_{10}$ CFU/g with 5, 15 and 30s of PL treatment, respectively. PL 30s provided 1.6 log $_{10}$ CFU/g reduction of TYMC in raspberries at day 0 which was comparable with Guner's (2012) result. In her research, blueberries were treated with 120s PL and 1.27 log $_{10}$ CFU/g reduction of total yeasts and molds has been observed. During our 10 days storage, TYMC of the control reached 4.2 log $_{10}$ CFU/g while treated raspberries with 5, 15 or 30s of PL had 2.5, 2.2 and 2.8 log $_{10}$ CFU/g, respectively. At day 10, PL treated raspberries maintained significantly lower TYMC than the control. There was

no significant difference between each PL treatment although raspberries treated with PL 30s had slightly higher TYMC.

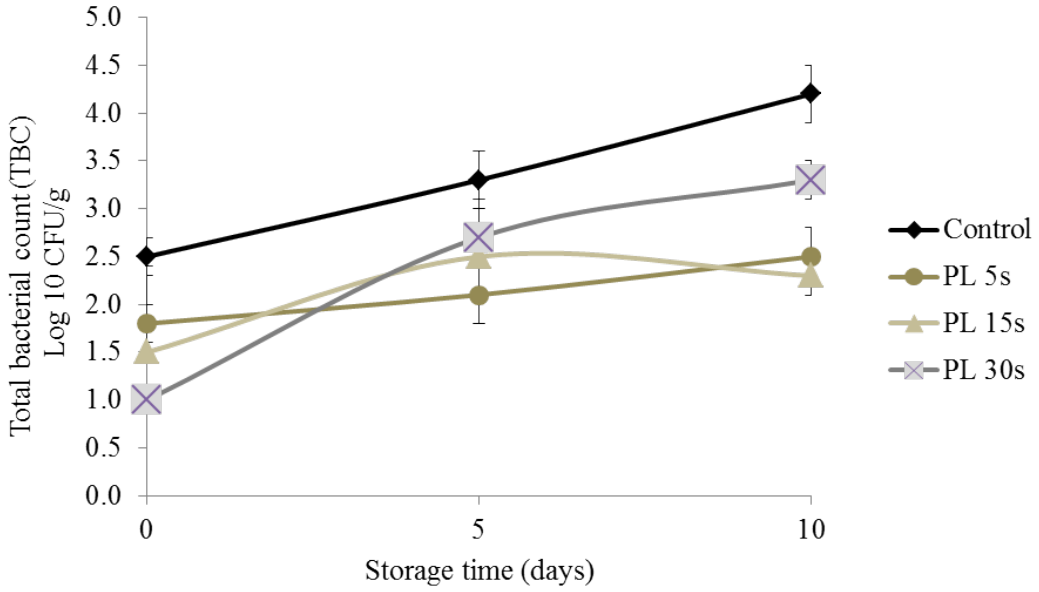

Figure 6.2: Effects of PL on TBC of raspberries during storage.

Raspberries without inoculation were used in total bacterial count (TBC) test. Fresh raspberries were treated with PL for 5, 15 or 30s. TBC were evaluated were evaluated at day 0, day 5 and day 10 during storage at 4 ℃.

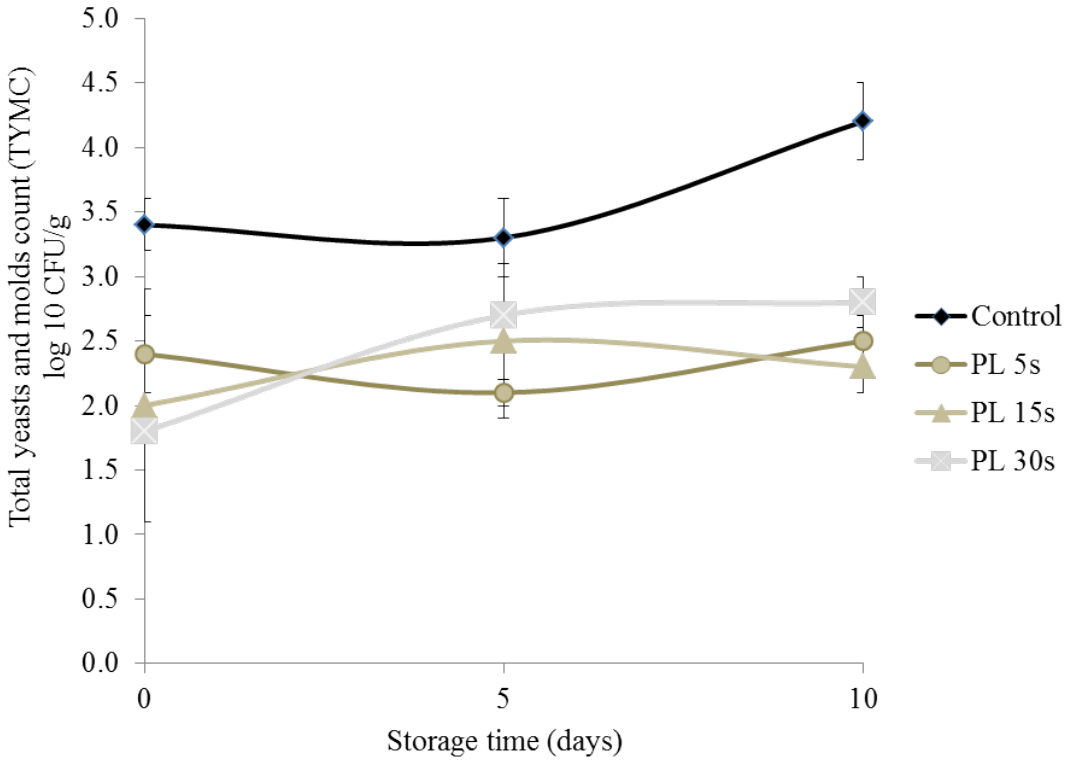

Figure 6.3: Effects of PL on TYMC of raspberries during storage.

Raspberries without inoculation were used in total yeasts and molds count (TYMC) test. Fresh raspberries were treated with PL for 5, 15 or 30s. TYMC were evaluated were evaluated at day 0, day 5 and day 10 during storage at 4 ℃.

6.4 Conclusion

PL could potentially be used for decontamination of *Salmonella* and *E. coli* O157:H7 on raspberries. *Salmonella* reduction by PL showed a fluence dependent manner with PL 30s providing highest log reduction of *Salmonella* (4.5 $\log_{10}$ CFU/g). *E. coli* O157:H7 reduction, however, was not significantly affected by fluence. PL 30s provided slightly higher *E. coli* reduction (3.9 $\log_{10}$ CFU/g) on fresh raspberries. All the PL treatments maintained lower pathogen survival populations during 10 days storage at 4 ℃ compared with the control. Color and texture of raspberries treated by PL 30s changed significantly in 10 days storage. PL 30s did not have a negative impact on TPC and TAC. PL 30s provided the lowest TBC and TYMC at day 0, but failed to maintain its advantage during storage. To consider both safety and quality of fresh raspberries, 5 or 15s PL treatment was recommended. Under SEM, PL showed severe damage to the cell membrane on smooth surface. Surface structure of raspberries affected the attachment of bacterial cells and the surface roughness provided protection for pathogenic bacteria.

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Chapter 7

SURVIVAL POPULATION OF *SALMONELLA* AND *E. COLI* O157:H7 AS WELL AS QUALITY OF RASPBERRIES DURING FROZEN STORAGE AFTER DECONTAMINATION BY USING SANITIZER WASHING OR PULSED LIGHT (PL)

ABSTRACT

Frozen raspberries have caused several foodborne outbreaks. Since washing before freezing is normally conducted in industry, sanitizer combinations as well as pulsed light (PL) were studied to inactivate foodborne pathogens in raspberries. Raspberries were artificially inoculated with *Salmonella* or *E. coli* O157:H7 and then treated by sanitizer washing including citric acid + sodium dodecyl sulfate (CA+SDS), citric acid + thymol (CA+THY) or PL (dry PL, wet PL and PL-SDS combination). Survival populations of two pathogens were determined right after treatments and during frozen storage at -20℃ for three months. Quality of raspberries evaluated during frozen storage included color, total phenolic content (TPC), total anthocyanin content (TAC), total bacterial count (TBC) as well as total yeast and mold counts (TYMC). Washing with CA+SDS or CA+THY, reduced the *Salmonella* population from 4.9 to 1.3, or 1.7 log $_{10}$ CFU/g, respectively; and reduced *E. coli* O157:H7 population from 5.1 to 1.0 and 1.4 log $_{10}$ CFU/g. Dry PL, wet PL as well as PL-surfactant (SDS) combination all showed promising decontamination efficacy on raspberries (1.5 - 3.0 log $_{10}$ CFU/g for *Salmonella* and 1.8 - 3.3 log $_{10}$ CFU/g for *E.*

coli O157:H7). All treated raspberries maintained the low pathogen populations (0.7 - 1.8 log $_{10}$ CFU/g for *Salmonella* and 0.9 - 1.8 log $_{10}$ CFU/g for *E. coli* O157:H7) during three months storage at -20 ℃ compared with the control (1.6 log $_{10}$ CFU/g for *Salmonella* and 1.5 log $_{10}$ CFU/g for *E. coli* O157:H7). Concerning the quality of raspberries, the redness increased in PL treated raspberries, which did not show any unattractiveness. No negative change of TPC or TAC has been observed after the treatments or during storage. Sanitizer washing and PL decreased the TBC and TYMC on raspberries and maintained the low natural microorganism population (0.4 - 0.8 log $_{10}$ CFU/g for TBC and 0.6 - 0.9 log $_{10}$ CFU/g for TYMC) during the storage.
KEY WORDS: Frozen raspberries; sanitizer washing; PL; *Salmonella*; *E. coli* O157:H7; quality

7.1 Introduction

Fresh and frozen common berries (i.e., blackberries, blueberries, raspberries, and strawberries) are popular and healthy foods. When berries are picked for fresh consumption, they are either placed directly in retail containers in the field or packed in a packinghouse without washing because they are highly perishable. Berries are usually washed before freezing, but they are not usually blanched or heat treated unless they are used in preserves or other processed products.

During 1983 to 2013, there were 11 outbreaks related with raspberries with a total of 4, 637 cases, 7 of which were associated with frozen raspberries. In the 1990s, there were an outbreak caused by Calicivirus (Pönkä et al., 1999) and two outbreaks caused by Hepatitis A (Reid and Robinson, 1987; Ramsay and Upton, 1989). Most

recently, there were four outbreaks caused by norovirus (Falkenhorst et al., 2005; Cotterelle et al., 2005; Hjertqvist et al., 2006; Maunula et al., 2009). For two hepatitis A outbreaks, pickers were believed to be the source of the contaminations. While for norovirus outbreaks, source of contamination was unknown. Foodborne pathogens including bacteria such as *Salmonella* and *E. coli* O157:H7 and virus such as hepatitis A and norovirus all share the same contamination route (fecal-oral route), which indicates that there is a possibility that *Salmonella* and *E. coli* O157:H7 may cause an outbreak on frozen raspberries. Based on a previous study, Knudsen et al. (2001) reported that *E. coli* O157:H7 and *Salmonella* can survive in frozen strawberries for periods of greater than 1 month.

Unlike the fresh raspberries which has no washing involved in postharvest, frozen berries are washed before frozen. The washing process could enhance safety of frozen berries. Based on our previous studies (Xu and Wu, 2014), new formula sanitizers washing such as 2mg/mL citric acid + 4% SDS (CA+SDS), 0.2mg/mL thymol + 4% SDS (CA+THY) achieved higher log reduction of *Salmonella* (4.5 - 5.3 $\log_{10}$ CFU/g reduction) than 200 ppm chlorine washing (3.2 $\log_{10}$ CFU/g) on spot inoculated green onions. In another study of ours (Xu et al., 2013), pulsed light (PL), and its combination with surfactant (sodium dodecyl sulfate, SDS) could potentially be used for decontamination of *E. coli* O157:H7 on green onions, with PL - SDS being the most effective PL treatment. It is thus interested to determine if these effective treatments could have comparable decontamination efficacy on raspberries used for frozen processing. The objective of this study was to investigate whether our new formula sanitizers, PL, PL - SDS combination would enhance the microbial safety of

frozen raspberries without compromising the quality of the berries. Quality of raspberries evaluated during frozen storage included color, total phenolic content (TPC), total anthocyanin content (TAC), total bacterial count (TBC) as well as total yeast and mold counts (TYMC).

7.2 Martials and Methods

7.2.1 Bacterial strain and inoculum preparation

Single wild-type strains including *Salmonella* (*S.* Newport H1275, sprout outbreak isolate) and *E. coli* O157:H7 (250, sprout outbreak isolate) were used in our study. Wild strains were obtained from culture collection in Department of Animal and Food Sciences at University of Delaware. The *Salmonella* strain was adapted to grow in the presence of nalidixic acid (100 μg/mL, Fisher Scientific, Hampton, NH, USA) alone to create a single antibiotic resistance strain, while the *E. coli* O157:H7 strain was adapted to grow in the presence of nalidixic acid (100 μg/mL) plus streptomycin (100 μg/mL, Sigma, St. Louis, MO, USA) to create a double antibiotics resistance strain. Both resistance strains were grown on tryptic soy agar (TSA, Difco Laboratories, Sparks, MD, USA) plus 0.6% yeast extract (YE, Difco) supplemented with nalidixic acid only (TSAYE-N for *Salmonella*) or nalidixic acid and streptomycin (TSAYE-NS for *E. coli* O157:H7) for 2 - 3 days at 35 ℃. Single colonies were picked and transferred to 10 mL of tryptic soy broth (TSB, Difco) plus 0.6 % yeast extract (Fisher) supplemented with same single or double antibiotics (TSBYE-N for *Salmonella* or TSBYE-NS for *E. coli* O157:H7). The culture was incubated at 35 ℃

overnight and second-transferred to 10 mL of fresh TSBYE-N or TSBYE-NS to yield an approximate population of 10^9 CFU/mL after 24 h incubation at 35 ℃. The culture was diluted to 10^8 CFU/mL using sterile 0.1 % peptone water (Difco) and used as inoculum.

7.2.2 Raspberries preparation and inoculation

Fresh raspberries were purchased from a local supermarket and stored at 4±2 °C for a maximum of 4 h before use. Medium size (~5 g) raspberries with pink color and ideal firmness were selected. The selected raspberries were intact and had no noticeable physical injury. To spot inoculate raspberries, 50 μL of 10^8 CFU/mL inoculum was deposited on the outside surface of raspberries as five droplets. Spot inoculation was applied to simulate the contamination caused by animal feces drops, irrigation water splashing or unhygienic touch. Inoculated raspberries were dried in the biosafety hood for 2 h before future use.

7.2.3 Inactivation of *Salmonella* and *E. coli* O157:H7 using sanitizer washing

Based on our previous studies (Xu and Wu, 2014), two sanitizer combinations were applied to decontaminate *Salmonella and E. coli* O157:H7 spot inoculated on raspberries, including 2 mg/mL citric acid + 4 % SDS (CA+SDS), and 2 mg/mL citric acid + 0.2 mg/mL thymol (CA+THY). Distilled water (DI water) was also included as comparison. One raspberry (5 g) was submerged in 100 mL washing solution in a beaker with continuous agitation for 1 min at room temperature.

7.2.4 Inactivation of *Salmonella* and *E. coli* O157:H7 using PL treatment

Based on our previous studies (Xu et al., 2013), three PL treatments were applied. Dry PL 15s, wet PL 60s and PL - 100ppmSDS combinations. PL was generated by a bench-top pulse light system (SteriPulse-XL, Model RS-3000C, Xenon Corp., Wilmington, Mass., U.S.). The 16 inch linear clear fused quartz PL lamp (LH840) delivered 505 joules/pulse (1.27 joules/cm^2) energy with 3 pulses/sec pulse rate. For dry PL treatment, raspberries were placed in a PL chamber in sterile petri dishes without covers. The inoculated side was facing the lamp. Two berries were treated each time. The 15s was chosen as treatment time according to our previous study (Xu et al., 2013). Fluence for our system was 14.3 J/cm^2.

For wet PL and PL-SDS combination treatment, two raspberries were placed in a beaker with 200 mL DI water or 100 ppm SDS and a stir bar. An ultra-thin magnetic stirrer (Lab Disc, Fisher Scientific) was placed under the PL chamber to create turbulent flow inside the beaker through the stirrer so that raspberries could rotate freely. The size of the stirring bar was Dia. × L: 0.37 × 1 in. (0.9 × 2.5cm) and the speed of the turns was around 700 rpm.

7.2.5 Individual quick frozen (IQF) of the raspberries

Treated and untreated raspberries (control) were then placed separately on a tray and frozen at -20±1 ℃. After frozen for 3 h, raspberries were bagged in zip-lock bags and stored in freezer until tested. Samples were analyzed at month 0 (test immediately after IQF), month 1, month 2 and month 3.

7.2.6 Microbial analyses

Raspberries were microbiologically analyzed at month 0, month 1, month 2 and month 3. They were transferred into sterile stomacher bags with D/E neutralized broth (Difco) at 1:9 ratio (w:v) and homogenized by stomacher (400 Circulator, Seward Co., West Sussex, UK) at 260 rpm for 1min to help releasing and evenly distributing pathogens. The homogenate was serially diluted using 0.1 % peptone water and plated on TSAYE-N (for *Salmonella*) or TSAYE-NS (for *E.coli* O157:H7) plates using sterile spreader. Colonies were enumerated after incubation for 48 h at 37℃. Pathogen survivor population was reported as $\log_{10}$ CFU/g.

7.2.7 Effect of sanitizer washing and PL on color of raspberries

Raspberries without artificial inoculation were either treated by sanitizer washing, PL as described above or served as control and then stored in freezer for 3 months. The color and texture of raspberries were analyzed at month 0, month 1, month 2 and month 3. Color was tested by using chromameter (Minolta CR-10, Minolta, Osaka, Japan). Color parameters were quantified in the Hunter L* (lightness/darkness), a* (redness/greenness), and b* (yellowness/blueness) color space.

7.2.8 Effect of sanitizer washing and PL on TPC and TAC of raspberries

TPC of raspberries with or without PL treatment was determined as described by Xu et al. (2010) with slight modification. Raspberries were homogenized in stomacher bags with 25 mL of ethanol with the ratio of 1:5 (w/v). The bags were heat sealed and let sit for 30 min with gentle massaging to extract the polyphenol compounds. Extractions were centrifuged at 4000 rpm for 10 min (Beckman Coulter,

Inc. Microfuge®, Indianapolis, IN, U.S.) and supernatants were collected. 30 μL of supernatants (dilute if necessary) was added into clear 96-well plate with a clear bottom followed by adding 60 μL of the Folin-Ciocalteau reagent (1×10^{-4} M) and 120 μL of sodium carbonate solution (20 %, w:v). The plate was covered with clear lid, shook horizontally to mix and incubated for 30 min at 35 ℃. A serial of known concentration of gallic acid was used as standard. Absorbance was measured at 765 nm using the Synergy™ 2 multi-mode microplate reader (BioTek Instruments, Inc. Winooski, VT, U.S.). The results were expressed as micrograms of gallic acid equivalents (GAEs) per 100 grams of fresh weight (FW).

TAC of raspberries was measured using pH differential method reported by Bunea et al. (2011) with slight modification. Raspberries with or without PL treatments were placed in 50 mL Conical centrifuge tubes with 30 mL of methanol-water solution (80:20, v:v) containing 0.1 mL/L acetic acid. The mixture was homogenized by ultra-turrax (IKA Works, Inc., Wilmington, NC) for 2 min, incubated in the dark for 1 h and then ultrasonicated for 15 min to aid anthocyanin extraction. After centrifugation at 5000 rpm for 30 min at room temperature, the volume of the supernatants, which was raspberry anthocyanin extract, was recorded. The anthocyanin extract was mixed with a 0.025 M potassium chloride buffer (pH 1.0) and 0.4 M sodium acetate buffer (pH 4.5) with a dilution factor at 5.0. The absorbance of each mixture was measured at 510 nm and 700 nm for the correct haze. The absorbance (A) of anthocyanin extract was calculated by the following formula:

A = (A510- A700) pH 1.0-(A510 - A700) pH 4.5

where A was the absorbance of the anthocyanin extract. A510 means the absorbance measured at 510 nm, and A700 means the absorbance measured at 700 nm.
The monomeric anthocyanin pigment concentration in the original raspberry sample was expressed in equivalence of cyaniding-3-glucoside using the following formula:
anthocyanin content =(A ×MW× DF×1000)/ε×1

where MW (449.2 g/mol) was the molecular weight of cyanidin-3-glucoside; DF was the dilution factor (5 in our study); and ε was the molar absorptivity, which equal to 26, 900 for cyanidin-3-glucoside.

7.2.9 Effect of new formula sanitizer washing and PL on TBC and TYMC of raspberries

For TBC and TYMC evaluation, no artificial inoculation was involved. At month 0, month 1, month 2 and month 3, raspberries with or without PL treatment were placed in sterile stomacher bags with 45 mL of 0.1 % peptone water (1:9 ratio, w:v) after the PL treatment and homogenized by stomacher at 260 rpm for 1 min. The homogenate was either plated on TSA for TBC enumeration or on Potato Dextrose Agar (PDA, Difco) for TYMC enumeration. Before counting colonies, TSA plates were incubated at 35 °C for 48 h while PDA plates were incubated at 25 °C for 3-5 days (Tournas et al., 2001).

7.2.10 Statistical analyses

All experiments were replicated 3 times and results were reported as Mean±SD. JMP (SAS Cary, NC, USA) software was used for statistical analyses. Survivor population of *Salmonella* and *E. coli* O157:H7 were reported as log survival population ($\log_{10}$ CFU/g). For survival population of pathogens and quality of

raspberries (including color, texture, TBC, TYC, TMC, TPC and TAC) during storage, Dunnett's test was used to determine differences between treated and untreated (control) samples on month 0, month 1, month 2 and month 3. Student's t test was used to determine the difference between each treated samples and same treatment on different days. Significant difference was reported when $p<0.05$.

7.3 Results and Discussion

7.3.1 Inactivation of *Salmonella* and *E. coli* O157:H7 using sanitizer washing during frozen storage

Fig.7.1 showed the survival population of *Salmonella* and *E. coli* O157:H7 during 3 months frozen storage after washed by sanitizer combinations. The initial *Salmonella* and *E. coli* O157:H7 on the raspberries were 4.9 and 5.1 $\log_{10}$ CFU/g, respectively. Washing the raspberries with sanitizer combinations, CA+SDS or CA+THY, reduced the *Salmonella* population to 1.3 and 1.7 $\log_{10}$ CFU/g, respectively and reduced *E. coli* O157:H7 population from 5.1 to 1.0 and 1.4 $\log_{10}$ CFU/g, respectively. All the reductions of the two pathogens were significant while no significant differences were detected between two combinations. During three months of frozen storage, the *Salmonella* and *E. coli* O157:H7 population (4.9 and 5.1 $\log_{10}$ CFU/g, respectively) in the untreated raspberries decreased to 1.6 and 1.5 $\log_{10}$ CFU/g, respectively. Significant decrease happened during the first two months, while in the last month, no significant change has been observed. Although the optimal temperature for enteric pathogens is around 37 ℃, *Salmonella* and *E. coli* O157:H7 were proved to survive under frozen conditions (Knudsen et al., 2001).

In our study, all the raspberries treated by sanitizer combinations maintained low pathogen population during storage. After three months, raspberries treaded by

CA+SDS had survival population of 0.6 (*Salmonella*) or 0.5 log $_{10}$ CFU/g (*E. coli* O157:H7). Raspberries treated by CA+THY had survival population of 0.9 (*Salmonella*) or 0.5 log $_{10}$ CFU/g (*E. coli* O157:H7). The control groups had survival population of 1.6 (*Salmonella*) or 1.5 log $_{10}$ CFU/g (*E. coli* O157:H7). At the end of three months, population of *Salmonella* and *E. coli* O157:H7 in washed raspberries were significantly lower than the untreated one (p<0.5) which indicated sanitizer washing prior to freezing enhanced the pathogenic microbial inactivation in the frozen berries.

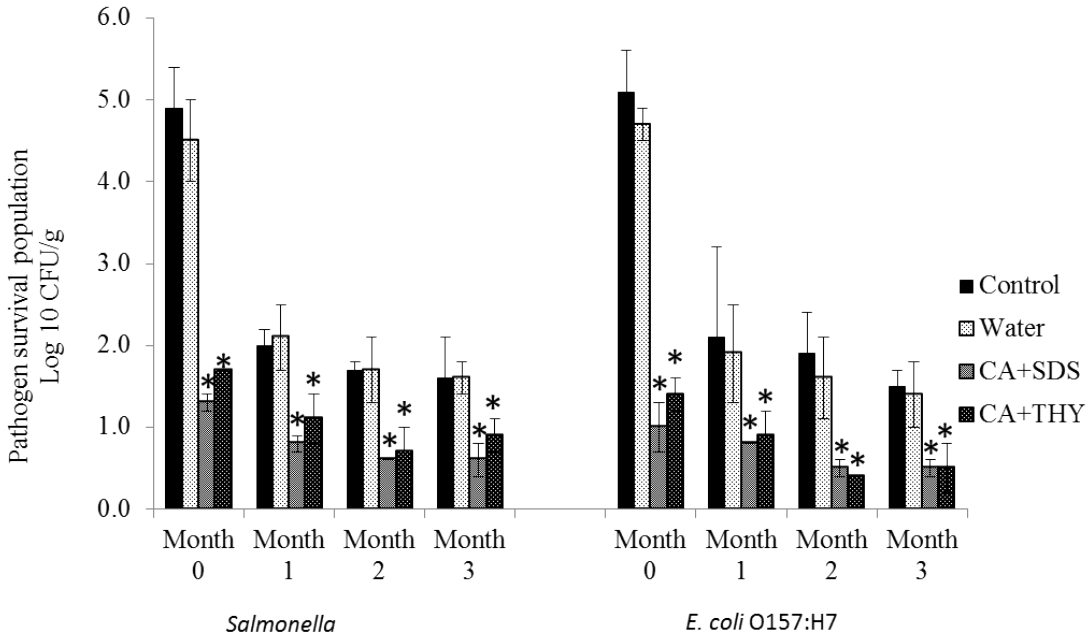

Figure 7.1: Inactivation of *Salmonella* and *E. coli* O157:H7 using sanitizer washing and pathogen survivor population during storage.

Fresh raspberries were spot inoculated with *Salmonella* or *E. coli* O157:H7 and then treated using two sanitizer combinations. Untreated raspberries served as control. Pathogen survival populations were evaluated at Month 0, Month 1, Month 2 and Month 3 during storage at -20 ℃. Values marked by * were significantly different (P <.05) from their respective controls on that same time point.

7.3.2 Inactivation of *Salmonella* and *E. coli* O157:H7 using PL during frozen storage

Unlike fresh raspberries which are usually unwashed, frozen raspberries require washing before frozen. In this case, not only the dry PL, but also the wet PL as well as PL-surfactant combinations have the potential being applied in frozen raspberry industry. Based on our previous findings (Xu et al., 2013; Xu and Wu, 2014), dry PL 15 s, wet PL 60 s or PL-100 ppmSDS combination were selected to inactivate *Salmonella* and *E. coli* O157:H7 on raspberries. As shown in Fig. 7.2, after 15s of dry PL treatment, the *Salmonella* and *E. coli* O157:H7 population decreased to 1.5 and 1.8 $\log_{10}$ CFU/g, respectively. Wet PL 60s had lower inactivation efficacy comparing with dry PL treatment, which only decreased the population of *Salmonella* and *E. coli* O157:H7 to 3.0 and 3.3 $\log_{10}$ CFU/g, respectively. However, after combined with surfactant (SDS), the pathogen survival population was further lower to 1.7 and 2.0 $\log_{10}$ CFU/g, respectively. To compare three PL treatment, the inactivation efficacy were dry PL 15s >PL-SDS combination>wet PL 60s. Significant difference was found between each treatment on both pathogenic bacteria. The control, however, had significantly higher population of *Salmonella* (4.9 $\log_{10}$ CFU/g) or *E. coli* O157:H7 (5.1 $\log_{10}$ CFU/g).

During storage at -20 ℃ for three months, further decrease of the survival population of *Salmonella* and *E. coli* O157:H7 has been observed on all treated raspberries, including the control which had 1.6 and 1.5 $\log_{10}$ CFU/g for *Salmonella* and *E. coli* O157:H7, respectively. *Salmonella* and *E. coli* O157:H7 population on dry PL treated raspberries decreased to 1.2 and 1.5 $\log_{10}$ CFU/g, respectively and this was not a significant drop. Raspberries treated by wet PL and PL-SDS combinations showed significant decrease on both pathogenic bacteria after three months, although there was no significant difference between each month. At the end of the frozen storage, the *Salmonella* population decreased to 1.8 and 0.7 $\log_{10}$ CFU/g for wet PL and PL-SDS combination, respectively. The survival population of *E. coli* O157:H7 decreased to 1.8 and 0.9 $\log_{10}$ CFU/g for wet PL and PL-SDS combination, respectively. This may be because the injury of pathogenic bacteria cells induced by wet PL and PL-SDS led to their even lower survival capacity under frozen conditions. It was possible that dry PL treatment was a more severe treatment which caused direct death of the pathogens, thus no further decrease of the population has been observed during frozen storage.

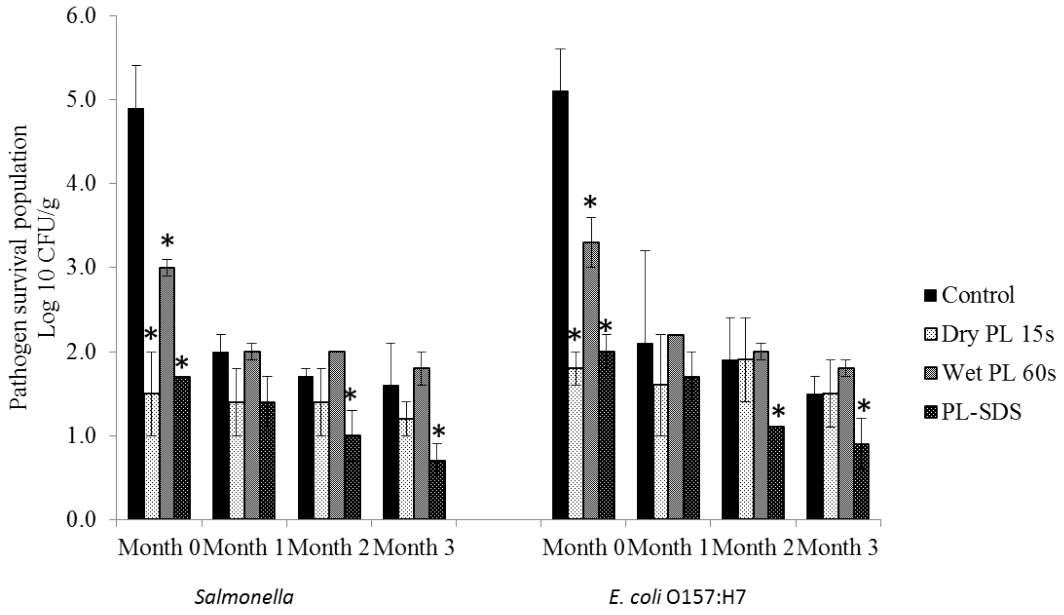

Figure 7.2: Inactivation of *Salmonella* and *E. coli* O157:H7 using PL and pathogen survivor population during storage.

Fresh raspberries were spot inoculated with *Salmonella* or *E. coli* O157:H7 and then treated using dry PL for 15s, wet PL for 60s or PL-SDS combination for 60s. Untreated raspberries served as control. Pathogen survival populations were evaluated at Month 0, Month 1, Month 2 and Month 3 during storage at -20 ℃. Values marked by * were significantly different (p <0.05) from their respective controls on that same time point.

7.3.3 Effect of sanitizer washing and PL on color, TPC and TAC of raspberries during frozen storage

Although, three color parameters were quantified in L* (brightness/darkness), a* (redness/ greenness), and b* (yellowness/blueness), only L* and a* values were

reported in this study since the unattractive color change of the raspberries were mainly from light pink to dark red. As shown in Table 7.1, there was no significant color change right after the treatments. L* values were ranging from 30.9 to 32.3 and a* values were ranging from 19.8 to 22.8.

During storage, there was no significant change of the brightness (L*) of untreated and treated raspberries. The redness of the untreated raspberries slightly increased from 22.8 to 24.9 after three months, which however was not a significant change. Raspberries washed by sanitizer combinations also showed slightly increase in redness, while raspberries treated by PL had significant higher of a* value (redness). At Month 3, a* values for dry PL, wet PL and PL-SDS combinations were 27.9, 28.2 and 28.8, respectively. The results indicated that neither sanitizer washing nor PL treatments had significantly impact on the color of raspberries. PL treatment increased the redness of raspberries without causing the darkening, which was not considered as unattractiveness in this study.

Ripe raspberries have a high antioxidant capacity and total phenolic content compared with most other berries (Freeman et al., 2011). In the present study, the TPC of untreated raspberries (Table 7.1) did not change significantly during 3 months of storage (38.7 to 33.8 mg GAE/100g FW). Similar results have been reported by de Ancos et al. (2000a) who investigated the ellagic acid, vitamin C, and total phenolic contents and radical scavenging capacity affected by freezing and frozen storage in raspberry fruit. Their results showed that at the end of long-term frozen storage (12 months), no significant change of total phenolic content extracted was tested. In our study, for the raspberries treated with sanitizer combinations as well as PL treatments, there was no significant difference between control and treated berries at Month 0,

ranging from 35.4 - 42.6 mg GAE/100g FW. Similar results have been reported by Luksiene et al. (2013) who evaluated the impact of high-power pulsed light on microbial control and nutritional properties of strawberries. In their study, no significantly differences were observed in total phenolic content before and after the pulsed light treatment After 3 months, however, berries treated by dry PL 15 s (40.5 mg GAE/100g FW) and Wet PL 60 s (38.0 mg GAE/100g FW) showed significantly higher TPC than the control (33.8 mg GAE/100g FW).

Anthocyanins belong to flavonoid groups and are responsible for the attractive colors-ranging from red to blue-of flowers and fruits. As shown in Table 7.1, there was no significant change of the TAC right after decontamination treatments. TAC of the untreated berries was 38.7 cyanidin-3-glucoside equivalents /100 g FW while TAC of the treated raspberries was ranging from 35.4 to 42.6 mg cyanidin-3-glucoside equivalents /100 g FW. Sanitizer washing as well as PL treatments did not affect TAC of raspberries significantly. During three months of frozen storage, however, the TAC decreased in all samples including the control. TAC of control decreased from 31.1 to 21.4 mg cyanidin-3-glucoside equivalents /100 g FW. TAC of sanitizer washed raspberries also decreased from 33.5 (CA+THY) or 33.0 (CA+SDS) to 23.6 (CA+THY) or 24.8 (CA+SDS), and there was no significant difference among control and sanitizer washed ones at the end of three months. However, at the end of storage, TAC of dry PL 15s (30.4 cyanidin-3-glucoside equivalents /100 g FW), wet PL 60s (29.7 cyanidin-3-glucoside equivalents /100 g FW) and PL-SDS (31.4 cyanidin-3-glucoside equivalents /100 g FW) were significantly higher than that of the control (21.4 cyanidin-3-glucoside equivalents /100 g FW). This suggested that PL was able to enhance the TAC content. Besides potential decontamination processing, the

stability of anthocyanins to freezing and frozen storage also depends on the seasonal period of harvest. The profile change of anthocyanins compounds also showed different degradation rate. Cyanidin 3-glucoside was reported to be the most easily suffered the degradation reactions that take place during processing and the storage period (de Ancos et al., 2000b).

Table 7.1: Effect of sanitizer washing and PL treatment on the color (L* and a*), TPC and TAC of raspberries during 3 months storage at -20 ℃

	color (L*)	color (a*)	TPC (mg GAE/100 g FW)	TAC (mg cyanidin-3-glucoside equivalents /100 g FW)
Month 0				
Control	32.3±1.2	22.8±2.1	38.7±5.0	31.1±0.2
Water	32.2±0.3	20.9±1.2	36.4±3.8	32.4±1.0
CA+THY	31.6±0.3	22.1±0.8	37.3±1.8	33.5±2.2
CA+SDS	32.2±0.9	21.6±0.2	35.4±2.0	33.0±5.5
Dry PL 15s	31.8±0.8	22.3±1.1	42.6±5.1	34.0±0.1
Wet PL 60s	32.0±1.2	21.9±0.4	41.5±5.0	33.6±1.0
PL+SDS	30.9±1.3	19.8±0.6	39.4±4.0	35.6±1.9
Month 1				
Control	32.8±1.6	22.8±1.3	37.8±2.5	27.8±2.8
Water	31.3±0.8	22.9±0.8	39.7±3.4	30.1±6.7
CA+THY	31.3±1.1	24.3±1.2	38.4±1.0	29.4±2.4
CA+SDS	30.8±0.9	22.8±0.9	36.3±4.2	30.9±3.7
Dry PL 15s	31.8±0.6	25.6±0.7*	37.1±3.5	33.0±1.5*
Wet PL 60s	32.2±0.7	22.9±0.6	38.8±5.7	35.0±2.6*
PL+SDS	30.5±0.5	23.3±0.0	40.5±0.9	34.7±3.8*
Month 2				
Control	33.0±1.4	23.5±1.6	34.1±1.3	25.0±3.5
Water	30.9±0.9	24.0±0.0	34.4±0.7	27.0±1.8
CA+THY	31.7±0.6	26.6±0.3*	34.9±2.2	29.2±1.3
CA+SDS	30.8±0.6	24.2±0.5	35.9±1.5	28.5±3.4
Dry PL 15s	32.9±1.0	26.3±0.5	38.6±1.0	33.5±1.6*
Wet PL 60s	31.4±0.0	25.0±0.4	37.3±4.4	31.8±2.1*
PL+SDS	31.2±0.8	27.9±1.1*	36.5±1.3	35.2±4.2*
Month 3				
Control	33.5±1.6	24.9±1.1	33.8±0.8	21.4±1.6
Water	30.6±0.9	25.6±0.2	37.0±3.8	21.3±5.0
CA+THY	31.9±0.6	25.6±0.9	36.9±3.6	23.6±2.2
CA+SDS	28.3±0.1	25.8±1.2	35.6±0.8	24.8±5.8
Dry PL 15s	32.2±0.7	27.9±0.8*	40.5±5.7*	30.4±2.2*
Wet PL 60s	30.0±0.9	28.2±0.7*	38.0±3.1*	29.7±3.7*
PL+SDS	30.8±0.6	28.8±1.3*	34.7±1.6	31.4±4.3*

Data representing mean values ± standard deviation. For individual testing, Values marked by * were significantly different (p<0.05) from their respective controls on that same day.

7.3.4 Effect of sanitizer washing and PL on TBC and TYMC of raspberries during frozen storage

The initial TBC and TYMC on raspberries were about 1.8 and 2.4 $\log_{10}$ CFU/g, respectively (Fig. 7.3). Sanitizer washing as well as PL treatments resulted in significantly lower TBC and TYMC comparing with untreated raspberries. Right after decontamination treatments, the reduction of TBC was 0.6 - 1.1 $\log_{10}$ CFU/g, and the reduction of TYMC was 1.0 - 1.4 $\log_{10}$ CFU/g. Further decrease in TBC and TYMC has been noticed after frozen storage for a month for all raspberries including untreated ones. However, all treated raspberries showed significantly lower TBC and TYMC values. At the end of frozen storage, the TBC and TYMC of untreated raspberries decreased to 0.6 and 0.8 $\log_{10}$ CFU/g, respectively which were similar as TBC and TYMC in treated raspberries. The results indicated that frozen storage itself had the ability to lower the population of natural microflora (TBC and TYMC). Because lethal effects are difficult to quantify for filamentous molds, the effects of freezing on vegetative fungi have been investigated mostly with yeasts. As with bacteria, the susceptibility of vegetative cells to freezing damage varies widely with the growth phase of the cells, the conditions under which they are cultivated and exposure to other stresses before freezing (Sun, 2011). In our study, sanitizer washing and PL treatment had the ability to lower the natural microorganism population and can maintain the low population during frozen storage, which may because of the stress generated by the decontamination treatment.

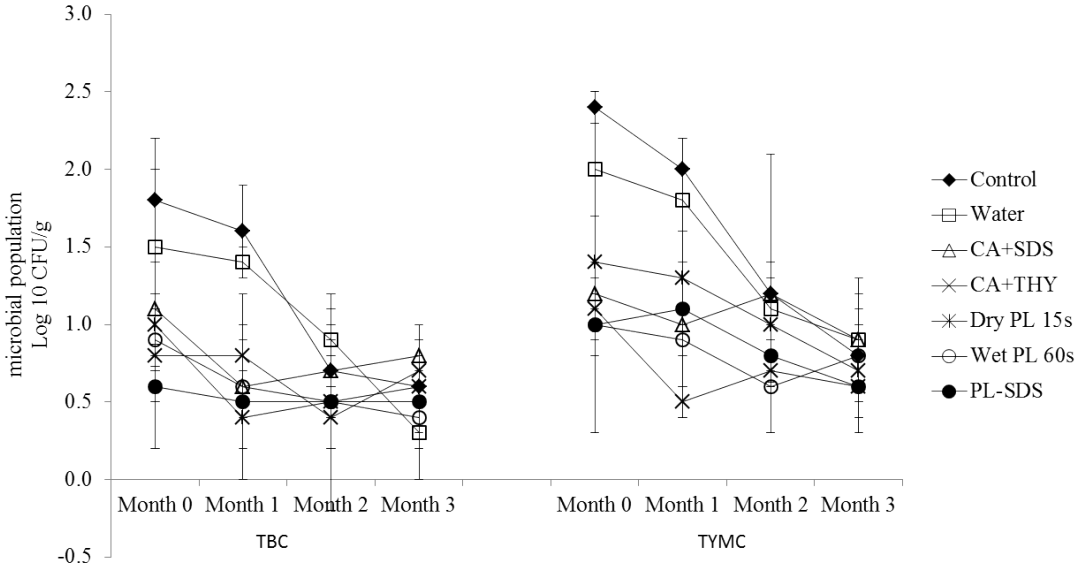

Figure 7.3: Effects of sanitizer washing and PL on TBC and TYMC of raspberries during storage.

Raspberries without inoculation were used in total bacterial count (TBC) and total yeasts and molds count (TYMC) test. Fresh raspberries were treated with two sanitizer combinations and three PL treatments and then stored at -20 ℃ for three months. TBC and TYMC were evaluated were evaluated at Month 0, Month 1, Month 2 and Month 3.

7.4 Conclusion

Sanitizer combinations (citric acid + thymol and citric acid +SDS) as well as PL could potentially be used for decontamination of *Salmonella* and *E. coli* O157:H7 on raspberries before frozen. 2 mg/mL citric acid + 4 % SDS and 2 mg/mL citric acid +0.2mg/mL thymol, reduced the *Salmonella* population from 4.9 to 1.3 and 1.7 $\log_{10}$ CFU/g, respectively and reduced *E. coli* O157:H7 population from 5.1 to 1.0 and 1.4 $\log_{10}$ CFU/g. Dry PL, wet PL as well as PL-surfactant (SDS) combination all showed promising decontamination efficacy on raspberries. Compare three PL treatments, the inactivation efficacy were dry PL 15 s >PL-SDS combination>wet PL 60 s and significant difference was found between each treatment on both pathogenic bacteria. All treated raspberries maintained the low pathogen populations during three month storage at -20 ℃ compared with the control. Brightness of the raspberries was not affected by decontamination treatments. The redness increasing, however, has been observed in PL treated raspberries, which did not show any unattractiveness. No negative impact has been observed on TPC and TAC after the treatments or during the three months storage. Sanitizer washing and PL decreased the TBC and TYMC on raspberries and maintained the low natural microorganism population during the storage.

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Chapter 8

ATTACHMENT OF *E. COLI* O157:H7 ON PRODUCE AND DAMAGE OF BACTERIAL CELL ENVELOPE BY DRY PULSED LIGHT (PL), WET PL AND PL-SURFACTANT COMBINATIONS

ABSTRACT

Pulsed light (PL) could potentially be used for decontamination of *Salmonella* or *E. coli*. O157:H7 on green onions as well as raspberries. However, the mechanism of inactivation PL was not clear. It has also been observed that the decontamination efficacy varied on different food matrixes. In this study we first investigated the inactivation mechanisms of dry PL, wet PL and PL-surfactant on *E. coli* O157:H7 attached on glass coverslips by using the scanning electron microscopy (SEM). The results showed that dry PL caused intracellular liquid evaporation and led to flatten out on the edge of the cells. Wet PL and PL-surfactant treated bacterial cells showed more membrane disruption. By using SEM, we also studied the bacterial attachment and decontaminate by PL on our two fresh produce: green onions and raspberries. The results revealed different topology of the food matrixes as well as the interactions between bacterial cells and microstructure on the surface of green onions and raspberries.

KEY WORDS: Attachment; decontamination; PL; PL-surfactant

8.1 Introduction

In the United States, the weekly sales of fresh produce have increased dramatically these past years (Padera, 2010). However, numbers of foodborne outbreaks associated with fresh produce have caused illness, hospitalizations and death of the consumers, serious economic loss of industries (Maki, 2009), as well as increasing public concern about microbial safety of fresh produce. Large outbreaks have been associated with widely consumed commodities such as cantaloupes, raspberries, tomatoes, green onions, sprouts as well as bagged lettuce and spinach (FAO, 2008). Imported green onion has been associated with three large hepatitis A outbreaks in the United States. Raspberries have been associated with 11 outbreaks caused by *C. cayetanensis*, calicivirus, hepatitis A and norovirus (CDC, 1996, 1997, 1998; Pönkä et al., 1999; Reid and Robinson, 1987; Maunula et al., 2009). Since *Escherichia coli* O157:H7 and *Salmonella* share the fecal-oral transfer route with aforementioned bacterial or viral pathogens, they may be the potential contamination source for green onions or raspberries at any point during pre-harvest, harvesting and post-harvest which may possibly lead to an outbreak.

PL is a non-thermal method for food preservation that involves the use of intense, short duration pulses of a broad spectrum (100-1100 nm) to ensure microbial decontamination on the surface of either foods or packaging materials (Elmnasser et al., 2007). PL treatment of foods has been approved by the FDA (1996) under the code 21CFR179.41. PL has been reported to inactivate microorganisms naturally present (Hoornstra et al., 2002; Gómez-López et al. 2005a) as well as inoculated (Fine and Gervais, 2004) on food surfaces. The mechanism of inactivation PL was not clear. The UV part of the spectrum in PL has been suggested having photochemical and/or photo-thermal effect. In our previous studies, we found that PL could potentially be

used for decontamination of *E. coli*. O157:H7 on green onions (Xu et al., 2013) as well as raspberries. For green onions, dry and wet PL have been studied and the results showed that 5s dry PL and 60s wet PL treatment provided promising inactivation efficacy (> 4 $\log_{10}$ CFU/g) for spot inoculated stems and leaves. For dip inoculated green onions, PL combined with surfactant (sodium dodecyl sulfate, SDS) was found to be the most effective treatment. Fresh raspberries decontaminated using only dry PL was studied, since no washing was involved in post-harvest. The results showed that PL 5 s or 15 s provided antimicrobial efficacy without affecting the quality of the berries during 10 days storage.

To contaminate fresh produce and transfer in the food producing chain, foodborne pathogens have to attach themselves on the plant surface to survive or proliferate. Pathogens use wounds and natural openings like stomata, hydathodes, and lenticels as natural gateways for passage into internal plant tissues where they grow and begin to cause disease (Hirano and Upper, 2000; Melotto et al., 2006). The surface roughness may affects pathogen attachment and the surface topography of fruits and vegetables can be quite complex (Gómez-López, 2012).Wang et al. (2009) found that an increase in fruit surface roughness would introduce protection to microbes entrapped on fruit surface resulting in reduced washing efficacy. Fresh cut surfaces serve an even better niche for *E. coli* and *Salmonella* (Patel and Sharma, 2010). Liao and Sapers (2000) investigated the attachment and growth of *Salmonella* Chester on fresh-cut apple disks and suggested that firm attachment of bacteria on calyx, stem, and injured tissue and partial resistance of attached bacteria to sanitizer treatments are two major obstacles to be considered when developing methods for cleaning and decontaminating apple fruits. Attachment depends on plant and bacterial factors as

well as on environment conditions. In our previous study it also showed that PL had different decontamination efficacy on green onion stems, leaves (Xu et al., 2013) as well as raspberries. We suggested that it may due to surface structure of food matrixes. The aim of this study was to 1) investigate the inactivation mechanisms of PL and PL-surfactant on *E. coli* O157:H7 attached on glass coverslips, and 2) observe the bacterial attachment on green onions (stems and leaves) and decontamination effect of dry PL.

8.2 Materials and Methods

8.2.1 Bacterial strain and inoculum preparation

Single wild-type *E. coli* O157:H7 strain (250, sprout outbreak isolate) was used in our study. *E. coli* O157:H7 strain was adapted to grow in the presence of 100 μg/mL of nalidixic acid (Fisher Scientific, Hampton, NH, USA) and 100 μg/mL of streptomycin (Sigma, St. Louis, MO, USA). The strain was grown on tryptic soy agar ((Difco Laboratories, Sparks, MD, USA) plus 0.6 % yeast extract (Difco) supplemented with 100 μg/mL of nalidixic acid and 100 μg/mL of streptomycin (TSAYE-NS) for 2 - 3 days at 35 ℃. Single colonies were picked and transferred to 10 mL of tryptic soy broth (Difco) plus 0.6 % yeast extract (Fisher) supplemented with the same antibiotics (TSBYE-NS). The cultures were incubated at 35℃ for overnight and second-transferred to 10 mL of fresh TSBYE-NS to yield an approximate population of 10^9 CFU/mL after 24 h incubation at 35 ℃. The culture was centrifuged at 4,629 ×g for 15 min at 4 ℃. Cell pellets were re-suspended in 1 mL sterile 0.1 % peptone water and used as inoculum.

8.2.2 Coverslip samples preparation for Scanning Electron Microscopy (SEM)

Fisherbrand™ microscope coverslips (Circles No. 1; 0.13 to 0.17mm thick; Size: 18mm) were placed in clean petri dishes. 0.1 % of poly-L-lysine was applied in the center of the coverslips and incubated at room temperature for 5 min. Liquid was pipetted out and the coverslips were dried in the biosafety hood at room temperature for 12 h.

For dry PL treatment, 0.3 mL of *E. coli* O157:H7 inoculum was placed on center of coverslips coated with poly-L-lysine and incubated at 37 ℃ for 30min. Extra inoculum was removed and a single layer of bacteria cells was formed on the surface. Inoculated coverslips were placed in sterile petri dishes without covers in a PL chamber and treated for 15 s. PL was produced by a bench-top pulsed light system (SteriPulse-XL, Model RS-3000C, Xenon Corp., Wilmington, Mass., U.S.) as reported in our previous publication (Xu et al., 2013). Fluence of PL for 15 s dry treatment was 14.3 J/cm^2. Inoculated coverslips without PL treatment served as control. Treated and untreated cover glasses were then placed in sterile clear 6-wells cell culture plates and bacterial cells were fixed by 2 % of glutaraldehyde for 1 h before critical points drying (CPD) to stabilize the tissue components and to reduce their extraction in the dehydration and transitional solvents. Fixation was conducted in a flammable fume hood.

For wet PL treatment, *E. coli* O157:H7 inoculum was re-suspended in DI water or 100 ppm SDS solution and pipetted in sterile clear 12-wells cell culture plates. The culture plates were placed in PL chamber and treated for 60 s which represented PL and PL-surfactant (PL-100 ppm SDS) treatments. *E. coli* O157:H7 suspensions without PL treatment served as control. Treated and untreated *E. coli* O157:H7 cultures were centrifuged at 4,629 ×g for 15 min at 4 ℃ and re-suspended in 1 mL

sterile 0.1 % peptone water. Suspensions of *E. coli* O157:H7 were transferred to the center of coverslips coated with 0.1 % poly-L-lysine and incubated at 37 ℃ for 30 min. Extra inoculum was removed and a single layer of bacteria cells was formed on the surface of coverslips. Coverslips inoculated with treated or untreated *E. coli* O157:H7 were then placed in sterile clear 6-wells cell culture plates and cells were fixed by 2 % of glutaraldehyde for 1 h. Fixation was conducted in a flammable fume hood.

8.2.3 Produce samples preparation for Scanning Electron Microscopy (SEM)

Green onions and fresh red raspberries were purchased from a local supermarket and stored at 4±2 °C for a maximum of 24 h before use. Full-length green onions were cut into segments, compressed stems (~5 g) and hollow leaves (~1 g), and air dried in a biosafety hood. Medium size (~5 g) raspberries with pink color and ideal firmness were selected. The selected raspberries were intact and had no noticeable physical injury.

Green onions and raspberries were dip inoculated with *E. coli* O157:H7. Stems or leaves were fully submersed in 10^7 CFU/mL inoculum in a sterile stomach bag in the ratio of 1:5 or 1:10 (weight of green onions: volume of the inoculum), respectively. Raspberries were fully submersed in a sterile stomach bag with the inoculum (10^8 CFU/mL) in the ratio of 1:5 (weight of raspberries: volume of the inoculum). Bags were heat-sealed immediately and slight shaking was applied for 1 min. Green onions and raspberries were taken out by sterile tweezers and placed in clean petri dishes. Produce samples were air dried in the biosafety hood for 4 h before the decontamination treatments. Dry PL treatment (15 s) was applied as described

before. Produce samples for SEM were cut to small pieces and fixed as described in 8.2.2.

8.2.4 Scanning Electron Microscopy (SEM)

After fixation, coverslips as well as produce samples were washed 3 times (30 min/time) with filtered buffer (1×PBS) and went through post-fixation process by incubating with Osmium Tetroxide (OsO4) for 2 h at room temperature. Then samples were rinsed thrice with filtered ultrapure water (30 min/time) and then dehydrated in series of ethanol (25 %, 50 %, 75 %, 95 % and 100 %) with 30 min for each concentration. 100 % ethanol was applied twice to ensure complete dehydration. Samples were transferred to the metal carrier baskets that came with CPD apparatus. The dehydrated samples were dried immediately by autosamdri-815B Critical Point Dryer (Tousimis® Rockville, MD, USA) followed by mounting onto SEM stubs and sputter-coating (Denton Vacuum Bench Top Turbo III, Moorestown, NJ, USA) with a thin layer of gold-palladium. The coated samples were observed under SEM (Hitachi S4700 FESEM; Hitachi Ltd, Tokyo, Japan) at an accelerating voltage of 3 kV. Pictures were acquired at different magnifications to observe the relationship between produce surface and bacteria as well as individual bacterial cell morphology.

8.3 Results and Discussion

8.3.1 Effect of dry PL, wet PL and PL-surfactant combinations on *E. coli* O157:H7 inactivation

Scanning electron microscopy (SEM) was used to examine the ultra-structural changes in bacteria induced by dry PL, wet PL and PL-surfactant combinations. As shown in Fig. 8.1, there was a clear structural difference between untreated and treated

E. coli cells. From A to E, the left sides were images taken under smaller magnification, while the right sides were images taken under bigger magnification which provided more ultrastructure details of the bacterial cells. The untreated *E. coli* cells were about 2.0 μm long and displayed a smooth and intact surface (Fig. 8.1A). Fig. 8.1B showed the *E. coli* cells treated by dry PL for 15 s. Some cells lost their membrane integrity completely which lead to flattening out of the cells from the edges. This may be because dry PL treatment suddenly increased the temperature of intracellular fluid which led to water vaporization in a relatively short time (Wekhof, 2000). This observation was comparable with Cheigh et al., (2012) who used transmission electron microscopy (TEM) analysis to observe the cell damage induced by intense pulsed light. Their results reveal the destruction of cellular structures such as the cell wall and cytoplasmic membrane. As shown in Fig. 8.1C, wet PL treated cells showed less membrane alteration than dry PL treated ones. The surface of the cells looked corrugated, and there were some dimples in these cells, but their average length remained unaltered. For wet PL treatment, water absorbed some part of the energy delivered from PL to the bacteria. After longer treatment time, there was no complete membrane rupture observed. It seems that wet PL treatment was not as intensive as dry PL treatment, which agreed with our previous results (Xu et al., 2013) about inactivation of *E. coli* O157:H7 on green onions, dry PL 5 s and wet PL 60 s provided similar inactivation efficacy (>4 $\log_{10}$ CFU/g). *E. coli* O157:H7 treated by PL-100ppm SDS showed completely distinct signs of damage to the cell envelope (Fig. 8.1E). The cells seemed to burst upon PL-SDS exposure, showing open holes and deep craters in their envelope. It has been reported that surfactants can lower the surface tension of cell membranes and open up the once tight interface; leaving leaky

spots that allow other chemicals to seep into cells and the important ions to seep out (Talaro, 2007). *E. coli* cells treated by SDS alone (Fig. 8.1D) did not show any alteration of the *E. coli* cells which matched with our previous results that SDS alone was proven to have limited inactivation efficacy on *E. coli* O157:H7 (Xu et al., 2013). However, Rahagopal (2003) studied the mechanism of surfactant resistance of gram negative bacteria by using *Escherichia coli* as the model organism and sodium dodecyl sulfate (SDS) as the model detergent. The result showed that the outer membrane as a selective barrier was one of the five compartments of the cell required for resistance. After combined with PL, the cell membrane damage caused by PL may lower the SDS resistance which may further lower the surface tension and damage the cells. Altogether, clear alterations of the cell surface structure, e.g., change from smooth to rough, decreased surface stiffness, and total cell membrane rupture were observed after PL treatment. Outer membrane damage was suggested as the first step, followed by permeabilization of the inner one and finally the total disintegration of both, causing leakage of the cytoplasmic contents (Li et al., 2007). Results from SEM proved the existence of photothermal effects of the PL.

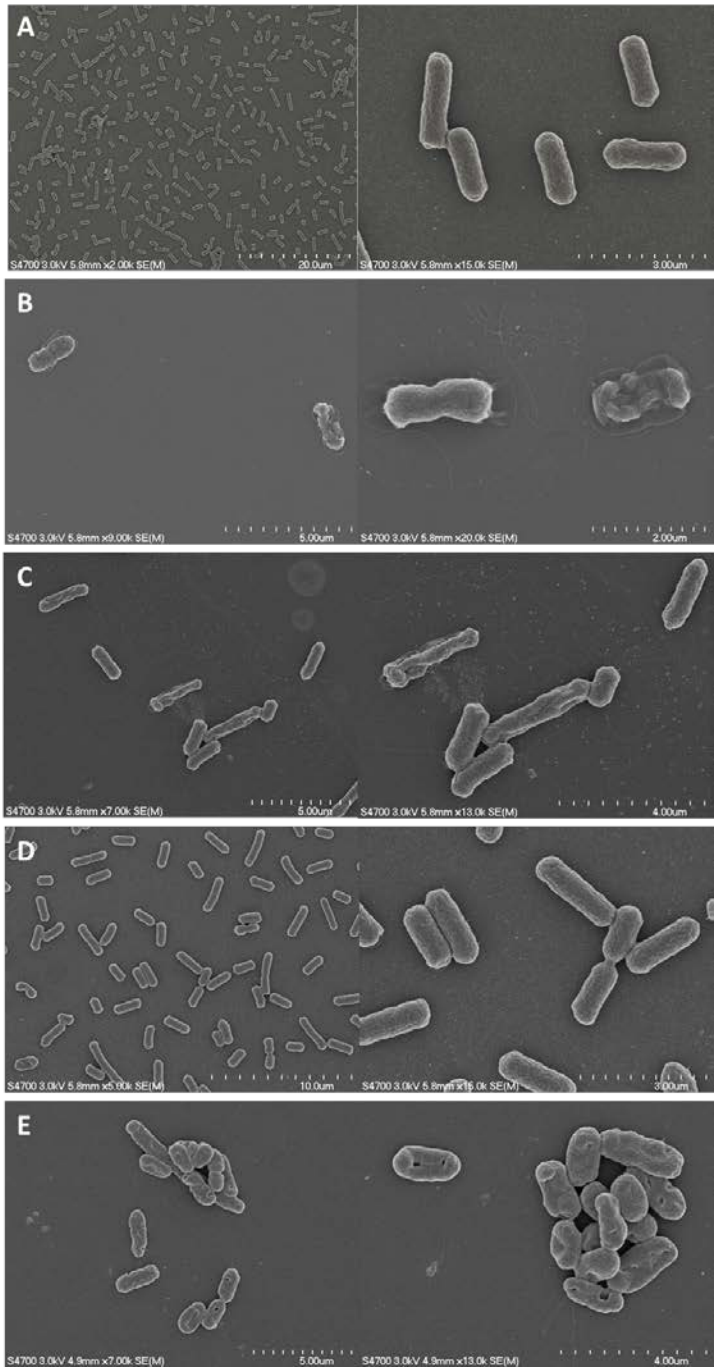

Figure 8.1: SEM micrographs of *E. coli* O157:H7 on coverslips

8.3.2 Attachment *E. coli* O157:H7 on green onions and raspberries and effect of dry PL on *E. coli* O157:H7 inactivation

The ability of *E. coli* O157:H7 attaching to green onions and raspberries has been proven under SEM (Fig. 8.2). Also the bacteria showed specific attachment preference when bacterial cells had free access to the plants and can move freely with the inoculum since dip inoculation was applied. In Fig.8.2, left sides were images of the untreated produce (control) while right sides were images of the produce treated by 15s dry PL. As shown in Fig. 8.2A, *E. coli* O157:H7 attached to green onion stems with most of them gathered along the veins. It seems that grooves along the veins helped to catch the bacteria when the whole surface had equal exposure to bacteria cells. Green onion leaves, however, had totally different surface structure compared with stems (Fig. 8.2B). The leaves were covered with wax which caught bacterial cells within. The surface roughness affected the attachment site of bacteria. In the case of green onion, stems with overall smoother than leaves may be one of the reasons why we observed more uptake of *E. coli* O157:H7 on leaves when dip inoculation was applied in our previous study (Xu et al., 2013).

Raspberries are made up of many individual fruits (drupelets) held together by hairs (trichomes) and waxes (Mackenzie, 1979). It seems that *E. coli* O157:H7 was preferred for the trichomes of the raspberries. Although to our knowledge, no research has been conducted to investigate how the surface structure of raspberries would affect *E. coli* O 157:H7 attachment, Sirinutsomboon (2011) built the surface structures on silicon to mimic stomata, trichomes, and grooves between plant epidermal cells using photolithography as a microfabrication method to determine the effect of microstructure on bacterial attachment. The results showed that the trichome base had attachment levels 3-5 times as large as those of the more distant areas.

After dry PL treatment for 15s, *E. coli* O157:H7 cells on green onion stems showed similar a pattern as the dry PL treated cells on coverslips. Edges of the bacterial cells flatten out and the intracellular contents were shrunk. Bacterial cells on green onion leaves and raspberries, however, showed similar morphology with those on coverslips and treated by wet PL 60 s which was considered as a relatively minor treatment. This may be because green onion stems had the smoothest surface among the three food matrixes we used. It may benefit the access of PL to the bacterial cells. It seems that this hypothesis did not match with our previous results by using the plating method which showed that green onion stems were tougher to decontaminate than leaves. However, since it was difficult for bio-imaging to provide the whole picture of the entire pieces of produce, the fact that bacteria may get into compressed layers of the stems or the hollow leaves and cause variation of decontamination efficacy may be underestimated. The harbor effect of the produce were clearly observed on green onion leaves and raspberries based on the microstructure of the produce surface.

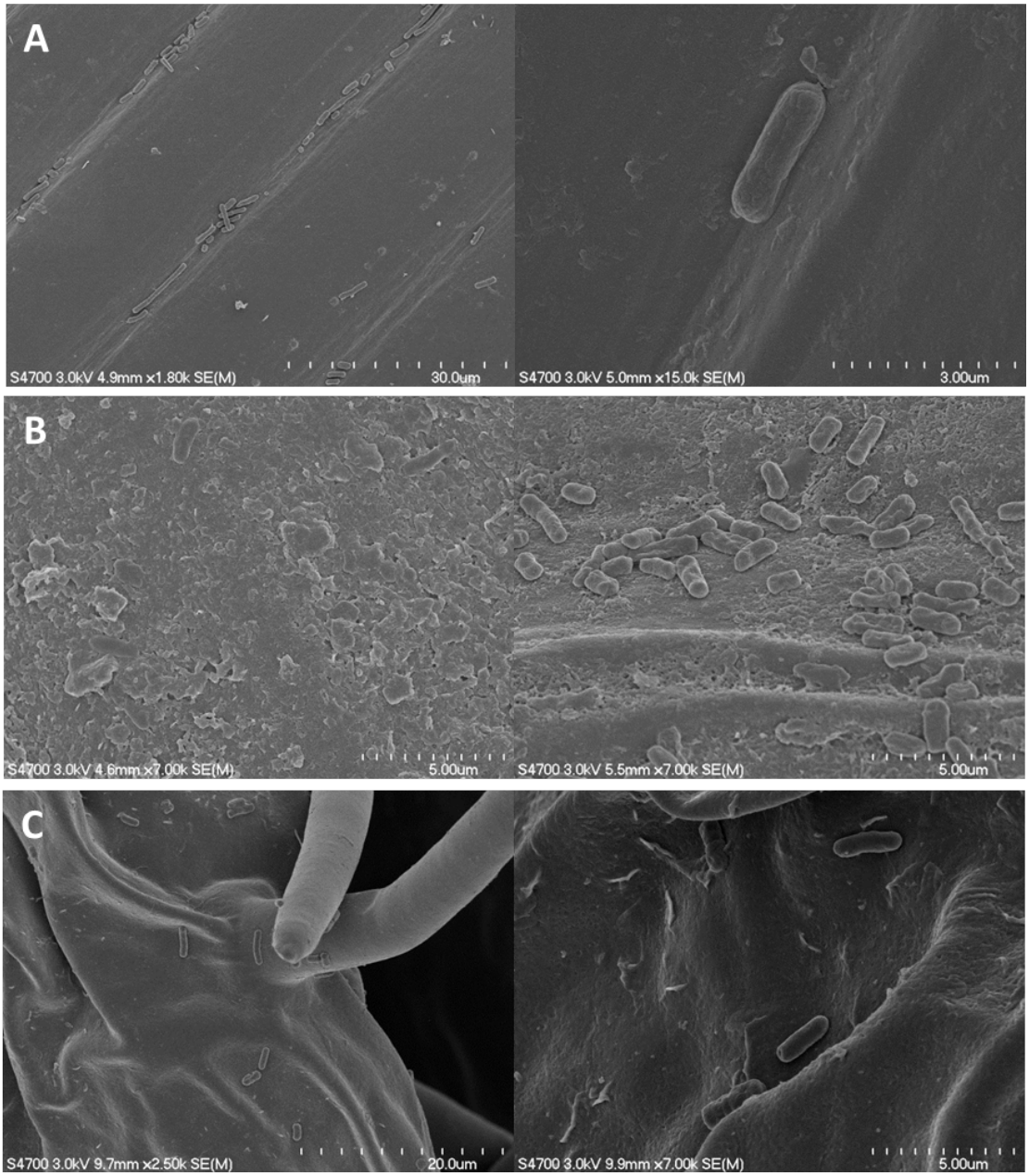

Figure 8.2: SEM micrographs of *E. coli* O157:H7 on green onions and raspberries.

8.4 Conclusions

PL was proved by the SEM image to have photo-thermal effect on *E. coli* O157:H7 which caused a change of cell morphology, membrane disruption, and shrinking of intracellular tissues. Dry PL was the most intense treatment which led to complete membrane disruption. Compared with wet PL alone, PL-SDS showed higher inactivation efficacy and severer damage of the cells. Surface structure of green onions (stems and leaves) as well as raspberries affected the attachment of bacterial cells and the surface roughness related with bacterial cell envelope structure after PL treatment.

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Chapter 9

FUTURE RESEARCH

The findings from the various studies on the use of pulsed light (PL) alone or in combination with surfactant show that these intervention strategies have the ability to deliver greater than 4-log reduction in the levels of *Escherichia coli* O157:H7 and *Salmonella* on green onions and raspberries.

The future research would be conducted in two areas. First, explore the mechanisms of PL and PL-surfactant decontamination by studying the interference with attachment related genes.

A multiplex PCR assay was applied to investigate possible impact of PL on virulence genes of interest of *Salmonella* and *E. coli* O157:H7 such as *eae*, *omp*C, *rpo*S etc. Second, different factors that may affect application of PL will be investigated, such as organic load of the water, produce load, agitation etc.

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