Effect of Environmental Factors and Residual Sanitizers on Survival and Attachment of Indicator Organism and Human Pathogens on Fresh Produce

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EFFECT OF ENVIRONMENTAL FACTORS AND RESIDUAL SANITIZERS ON SURVIVAL AND ATTACHMENT OF INDICATOR ORGANISM AND HUMAN PATHOGENS ON FRESH PRODUCE

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The School of Nutrition and Food Sciences

by

Vijay Singh Chhetri
M.Sc. Tribhuvan University, 2009
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Dedicated to my parents, Narayan and Ganga, and my wife, Nirodha.
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ABSTRACT

A comprehensive understanding of the behavior of microbial contaminants on produce surfaces in agricultural farms and during post-harvest activities is essential for the development of produce safety risk management strategies. Our studies investigated the survival and the attachment of *E. coli* on watermelon surfaces in agricultural settings with different levels of vegetation, and the effect of residual sanitizers on the survival and the attachment of *E. coli* O157: H7 and *L. monocytogenes* on spinach leaves. The attachment strength ($S_R$) of the *E. coli* cells on watermelon surfaces significantly increased ($P<0.05$) from 0.04 to 0.99 in the first 24 h, which was primarily due to the decrease in loosely attached population, given that the population of strongly attached cells was constant. The daily die-off rate of *E. coli* ranged from -0.12 to 1.3 log CFU/cm$^2$. Chlorine treatment reduced the *E. coli* level by 4.2 log CFU/cm$^2$ (initial level 5.6 log CFU/cm$^2$) and 0.62 log CFU/cm$^2$ (initial level 1.8 log CFU/cm$^2$), on the watermelons that had an attachment time of 30 min and 120 h respectively. The survival rate of generic *E. coli* inoculated on watermelon rind discs was variable with the level of vegetation. The discs placed at low vegetation level had the highest die-off (3 log CFU/cm$^2$) compared to medium and high vegetation levels. On spinach leaves treated with chlorine, significant reductions ($P < 0.05$) in *E. coli* O157:H7 and *L. monocytogenes* populations were observed within 15 min, with total reductions of 2.64 and 3.15 log CFU/cm$^2$ respectively after 48 h. On 0.5% lactic acid treated leaves, the reductions in *E. coli* O157:H7 and *L. monocytogenes* populations were 3.07 log CFU/cm$^2$ (24 h) and 1.40 log CFU/cm$^2$ (48 h), respectively. The effect of residual sanitizers was significantly greater on loosely attached populations compared to strongly attached populations. The results reported in these studies may be useful while developing pre-harvest and post-harvest risk management strategies.
CHAPTER 1
INTRODUCTION

Fresh produce is a commonly implicated food commodity for foodborne outbreaks in the United States (Heiman, Mody, Johnson, Griffin, & Gould, 2015; Nguyen et al., 2015). Although there are significant improvements in food safety, the number of outbreaks associated with produce has increased in the last few decades (Bennett et al., 2018; Nguyen et al., 2015). In the period between 1998 and 2013, raw produce was associated with a total of 972 outbreaks resulting in 34,674 illnesses, 2,315 hospitalizations, and 72 deaths in the USA (Bennett et al., 2018). Norovirus, Salmonella and shiga toxin–producing E. coli were the most frequently reported etiological agents (Crowe, Mahon, Vieira, & Gould, 2015; Bennett et al., 2018).

Pre-harvest and post-harvest activities are reported to be important events of microbial contamination (Ackers et al., 1998, Gelting, Baloch, Zarate-Bermudez, & Selman, 2011). Pre-harvest contamination of produce is commonly originated from the soil, inadequately composted manure, contaminated irrigation water, and improper human handling of produce (Annous, Solomon, Cooke, & Burke, 2005; Tomas-Callejas et al., 2011). Wild animals, birds, reptiles, rodents and insects may act as a source and a vector for transferring various pathogens to fresh produce (Brandl, 2006). To reduce the food safety risks, the produce industries use sanitizer wash of fresh produce as a key event in post-harvest activities (Banach, Sampers, Van Haute, & Van der Fels-Klerx, 2015; López-Gálvez, Gil, Truchado, Selma, & Allende, 2010). However, the water rinsing step following sanitizer wash and other post sanitizing activities such as chopping, shredding, handling, storage, and packaging also have been reported to be the causes of cross-contamination (Danyluk & Schaffner, 2011; Francis et al. 2017; Pérez-Rodríguez et al., 2014; Murray, Wu, Shi, Jun Xue, & Warriner, 2017). L. monocytogenes has been frequently recovered from fresh produce processing operations (Nguyen-the & Carlin, 1994; Zhang & Farber, 1996).
Contact surfaces such as conveyor belts, knives, and reusable crates have been demonstrated as potential sources of contamination between produce batches (Zilelidou, Tsourou, Poimenidou, Loukou, & Skandamis, 2015).

After contamination events, microbial contaminants adapt to the intrinsic and extrinsic environment of fresh produce for attachment and survival (Fontaine, Mariotti, & Abbadie, 2003). Studies have shown that the survival of bacterial pathogens in agricultural field settings is influenced by environmental conditions such as solar radiation, relative humidity, temperature, availability of nutrients and interaction with other natural microflora (Brandl, 2006; Nyeleti, Cogan, & Humphrey, 2004; Tomás-Callejas et al., 2012; Weller et al., 2017). In the agricultural field, microbial population declines with time due to natural die-off (Barker-Reid et al., 2009; Wood, Bezanson, Gordon, & Jamieson, 2010). Food Safety Modernization Act (FSMA) Produce Safety Rule (PSR) has considered this natural phenomenon as a corrective measure to reduce risks associated with agricultural water (FDA 2015; Gradl, & Worosz, 2017). According to the FSMA PSR, producers unable to meet the microbial water quality criteria could use a die-off rate of 0.5 log per day while calculating the waiting period between the last irrigation and harvest.

Microbial attachment and biofilm formation is another big issue in produce safety. The ability of bacterial cells to attach and form biofilm can be influenced by environmental factors such as temperature, relative humidity, and contact time (Ells, & Hansen, 2006; Toyofuku et al., 2016). Biofilm formation is a common defensive mechanism bacteria employed to protect themselves from environmental stress (Morris, & Monier, 2003). Bacterial attachment is a key factor influencing the efficacy of post-harvest treatments (Lee, Wahman, Bishop, & Pressman, 2011; Ukuku, & Sapers, 2001). Studies demonstrated a lower efficacy of sanitizer treatment against pathogens in biofilm or in strongly attached form (Ukuku, & Sapers, 2001; Ryu, &
Beuchat, 2005). Thus, an understanding of the behavior of microorganisms on produce in terms of attachment and survival is essential to develop produce safety risk management strategies.

Chlorine is one of the commonly used sanitizers in produce wash operations. Studies have shown that chlorine treatment can leave a residue at detectable levels on produce surfaces for hours (Cho et al., 2010). Formation of carcinogenic halogenated disinfection by-products (DBP) like trihalomethanes (THMs) during chlorine treatment is an issue. However, the amount of the THMs on fresh produce has been reported to be negligible (Gómez-López, Marín, Medina-Martínez, Gil, & Allende, 2013; Klaiber, Baur, Wolf, Hammes, & Carle, 2005; López-Gálvez et al., 2010). Organic acids are also used in produce wash operations (Akbas, & Ölmez, 2007). Organic acids are generally recognized as safe (GRAS) (Bell, Cutter, & Sumner, 1997). They are stable compounds (De Villiers, Wurster, & Narsai, 1997), thus, they may persist on produce surfaces for a long period. On the commercial scale, produce is stored for hours to days before being delivered to consumers. During that period, the residues may exert antimicrobial activities on produce surfaces and the change in surface-characteristics of the produce by the sanitizers may influence bacterial attachment (Jahid & Ha, 2012).

This study was conducted to see if natural bacterial die-off in pre-harvest conditions and if residual sanitizer after washing could have a role in reducing produce safety risks. The first part of this study examined the die-off rate and attachment behavior of *E. coli* on watermelon surfaces in an agricultural field setting in the south-central part of the U.S.A. The second part investigated the effect of surrounding vegetation on microbial die-off on watermelon surfaces. Our third and fourth parts evaluated the effect of residual sanitizers on the survival and attachment of *E. coli* O157: H7 and *L. monocytogenes* on baby spinach during refrigerated storage (4 °C).
References


CHAPTER 2
LITERATURE REVIEW

2.1. Fresh produce safety issues

Fresh produce are an essential part of the human diet. They are rich in vitamins and minerals, and supply fiber (Rickman, Barrett & Bruhn, 2007). Studies reported that the consumption of fruit and vegetables could protect from chronic diseases such as cancers and coronary heart disease (Qadri, Yousuf, & Srivastava, 2015; Rickman et al 2007). Because of their health benefits, the consumption of fruits and vegetables has increased over past few decades (Warriner et al., 2009). Concomitantly, an increasing number of outbreaks of illnesses associated with fresh produce has been reported (Huang & Chen, 2011; Olaimat & Holley, 2012).

The Majority of fresh produce are typically consumed raw or minimally processed and are regarded as high-risk foods. Produce is estimated to be responsible for 20 million illnesses, resulting in the loss of $38.6 billion annually in the US (Scharff, 2018). Between 1973–2014, fruit and vegetable crops were the most commonly implicated commodities for several foodborne illness outbreaks (Crowe, Mahon, Vieira, & Gould, 2015; Nguyen et al., 2015). The common etiological agents have been viruses (norovirus and hepatitis A); protozoa; shiga toxin–producing Escherichia coli, Listeria monocytogenes, Vibrio cholera,; Aeromans hydrophila, Salmonella, Bacillus cereus and Campylobacter (Crowe et al 2015; Danyluk, Goodrich-Schneider, Schneider, Harris, Worobo, 2012; Herman, Hall, & Gould, 2015; Keskinen, Burke, & Annous, 2009). Salmonella and E. coli O157:H7 were the etiological agents associated with most of the outbreaks illnesses (Buck, Walcott & Beuchat, 2003; Namvar, Fan, & Dunfield, 2009; Warriner, Huber,).
2.2. Pre-harvest contamination

Several studies have traced pre-harvest events as important causes of microbial contamination (Partk et al., 2012). Fruit and vegetable crops have the potential to be contaminated with pathogenic microorganisms in the field. Pre-harvest contamination of produce is commonly originate from the soil, inadequately composted manure, contaminated irrigation water, and improper human handling of produce (Annous, Solomon, Cooke, & Burke, 2005; Tomas-Callejas et al., 2011). The intrusion of crops by wild animals, birds, reptiles, and rodents, as well as insects and nematodes, act as vectors for transferring various pathogens (Brandl, 2006). Contamination sources and pathways are discussed below:

Manure is an important source of pre-harvest contamination of fresh produce. The manure obtained from livestock may be contaminated with enteric pathogens such as \textit{E. coli} O157 and \textit{Salmonella} spp. (Doyle and Erickson, 2008). \textit{C. jejuni} is a normal member of the gastrointestinal microflora of poultry, pigs and cattle (Warriner et al., 2009). In the agricultural field, foodborne pathogens can survive in animal manure for extended periods resulting in fresh produce contamination. \textit{E. coli} O157:H7 survived in bovine manure for over 70 days at 5°C (Semenov, Van Bruggen, Van Overbeek, Termorshuizen & Semenov, 2007). At 22 or 30°C, this pathogen survived up to 49 days (Semenov et al., 2007). Nicholson, Groves & Chambers, (2005) observed that, following manure spreading to land, \textit{E. coli} O157, \textit{Salmonella} and \textit{Campylobacter} survived in the soil for up to one month, and \textit{Listeria} survived for more than one month. The contamination of fresh produce may occur by subsurface runoff, splash dispersal during rain events and irrigation, dust particles transfer from soil onto produce surfaces and during pre-harvest activities (Liu, Hofstra & Franz, 2013).
Soil is a source of several human pathogens including *B. cereus*, *Cl. perfringens*, *Cl. botulinum*, *L. monocytogenes* and *Aeromonas* (Beuchat, 1996). The types of pathogens present in the soil may be dependent on the manure type, management during stockpiling, method of application, application rate, frequency of application, and time between application and planting or harvesting (Whipps, Hand, Pink & Bending, 2008). In addition, contaminated irrigation water may result in soil contamination (Liu et al., 2013). The level of contamination of fresh produce is influenced by factors such as proximity of the edible portion of the plant to the soil, concentration of pathogen in contaminated soil and the type of crop grown in the soil (Doyle and Erickson, 2008). Root crops are more likely to be contaminated than crops which grow above the ground. While greater microbial populations in soil pose greater risk of contamination of crops (Liu et al., 2013).

Studies have reported internalization of pathogens in plants from soil by root uptake (Franz et al., 2007). Significant populations of both *S. entericaservar Typhimurium* and *E. coli* O157:H7 was observed in sterilized leaf samples from plants grown in contaminated soil (Franzet al., 2007). The common route of internalization of human pathogens has been proposed to be penetrations at cracks in seed coats, invasion at lateral root junctions in seedlings, and aerial tissues (Doyle and Erickson, 2008).

Irrigation water is a potential vehicle of human pathogens for pre-harvest contamination of produce. The level of produce contamination is greatly influenced by the quality of irrigation water and type of irrigation system (Aruscavage, Lee, Miller & LeJeune, 2006; Brackett, 1999; Warriner et al., 2009). The risk associated with irrigation water is highlighted in FSMA PSR and it’s the first time we have a federal regulation specific for farm food safety. According to the rule, for water applied to produce that are consumed raw with a direct application method the geometric mean (GM) and the statistical threshold (STV) of generic *E. coli* should not exceed
126 CFU/100 mL and 410 CFU/100 mL of water, respectively (FDA, 2015). Although all kinds of irrigation systems pose risks of produce contamination, flood and spray irrigation poses a greater risk of disseminating pathogens on produce surfaces (Olaimat & Holley, 2012). They can deliver microbial pathogens onto the edible portion of the crop indicating a greater risk of contamination (FDA, 1998). Solomon, Potenski & Matthews, (2002) reported that spray irrigation resulted in the contamination of 90% of lettuce plants with *E. coli* O157 compared to 19% of lettuce plants contaminated by surface irrigation. Some studies revealed that sprinkling systems increased the likelihood of internalizing pathogens in produce (Alegbeleye, Singleton & Sant’Ana, 2018). Although numerous studies have demonstrated the potential correlation between microbiological quality of irrigation water and incidence of human pathogens on fruits and vegetables, there is limited evidence of the outbreaks associated with irrigation water (Harris et al., 2012).

Wild animals and their activities in the agricultural field have been reported to be the causes of produce contamination (Kwan et al., 2014; Laidler et al., 2013; Weller et al., 2017). Wildlife may defecate on agricultural land resulting in contamination of the growing fresh produce (Atwill et al., 2015). The respiratory systems, skin, hooves and hair or feathers of animals are the sources of human pathogens (Ray and Bhunia, 2008). Their intrusion into the agricultural farm may cause the transfer of pathogens from these parts to the edible portion of produce. Moreover, they might damage the leaves or other parts of fresh produce providing an entry points for foodborne pathogens into the plant (Orozco et al., 2008).
2.3. Microbial survival on produce surfaces

Several studies reported the survival behavior of bacterial cells on produce surfaces. *E. coli* and *Salmonella* survived on parsley in the field for several days (Islam, Morgan, Doyle & Jiang, 2004). Stine, Song, Choi & Gerba, (2005) observed that human pathogens were able to survive for 14 days on cantaloupe, lettuce and pepper. However, in phyllosphere, human pathogens are required to adapt to a number of extreme and fluctuating environmental conditions combined with unique physio-chemical characteristics to survive and grow (Berger et al., 2010). For example, *Pseudomonas* spp. protects itself from UV by producing pigments (Heaton and Jones, 2008). The environmental stress in agricultural field include solar radiation, relative humidity, temperature, availability of nutrients and interaction with other natural microflora (Brandl, 2006; Weller et al., 2017). If the bacterial cells are unable to adapt to environmental stress, their die-off occurs over time. Sunlight of tropical latitudes (Davies & Evison, 1991; Nyeleti, Cogan, & Humphrey, 2004; Obiri-Danso, Paul, & Jones, 2001) and concomitant increase in the surface temperature of produce (Tomas-Callejas et al., 2011) have an inhibitory effect against various microbial pathogens. The combined effect of greater temperatures and drying conditions could efficiently reduce the microbial load (Van Donsel, Geldreich & Clarke, 1967). It was observed that sunlight reduced *Salmonella* levels in fresh water sources (Davies & Evison., 1991). Lack of nutrients on phyllosphere could be another stress factor for the microbial inhibition (Fontaine, Mariotti, & Abbadie, 2003).

FDA Food Safety Modernization Act (FSMA) produce safety rule (PSR) has considered the natural die-off of bacterial cells as a corrective measure to reduce risks associated with agricultural water and biological soil amendments (FDA 2015; Gradl & Worosz, 2017). The growers unable to meet microbial water quality criteria could use a die-off rate of 0.5 log per day while calculating
the waiting period between the last irrigation and harvest. However, the die-off rate may be influenced by several factors such as surface characteristics of produce and the geographical location (Allende, Singleton, & Sant’Ana, 2018; Grad & Worosz, 2017; Weller et al., 2017).

2.4. Attachment/biofilms formation of bacterial cells on produce surfaces and the effect of sanitizers treatments

Attachment is the first step of bacterial colonization on produce surfaces (Alegbeleye et al., 2018). Once attached, conventional washing strategies are unable to remove them from produce surfaces (Beuchat and Scouten, 2002). Studies have reported several mechanisms for bacterial attachment (Berger et al., 2010). The expression of pilus curli helped in adhesion of *Salmonella Enteridis* and *S. Newport* to alfalfa sprouts (Barak, Jahn, Gibson & Charkowski, 2007). *Salmonella* express curli, cellulose and capsule which contribute to environmental fitness (Gibson et al., 2006). Curli has been found to be associated with the attachment and biofilm formation in *E. coli* (Macarisin, Patel, Bauchan, Giron & Sharma, 2012; Patel, Sharma & Ravishakar, 2011). Curli expression is one of the common characteristics of *Enterobacteriaceae* family (Zogaj, Bokranz, Nimtz, & Römling, 2003). There was a greater level of attachment of curli-positive *E. coli* O157:H7 strains on spinach surfaces compared with curli-deficient strains (Macarisin et al., 2012). However, the curli expression could be dependent on environmental factors such as temperature, oxygen level and osmolality (Boyer et al., 2007, Evans & Chapman, 2014). Studies found that the ability of *Listeria* to attach to surfaces is affected by flagellar motility (Gründling, Burrack, Bouwer & Higgins, 2004; Kamp and Higgins, 2011). Bacterial attachment can be influenced by factors such as temperature, pH, bacteria features, produce surface properties, and exposure time (Reina, Fleming & Breidt, 2002; Kuan et al., 2017).
Bacterial attachment may lead to biofilm formation (Ryu J-H, and Beuchat, 2005; Ukuku and Sapers, 2001). A biofilm is an aggregation of microorganisms entrapped in a matrix of highly hydrated extracellular polymeric substances (EPS) (Feng et al., 2015). Biofilm formation comprises of five stages: (i) An initial reversible attachment of planktonic bacteria to the solid surface (ii) transition from reversible to irreversible attachment by production of extracellular polymers (iii) early development of biofilm architecture (iv) development of microcolonies into a mature biofilm (v) dispersion of cells from the biofilm into the surrounding environment and return to the planktonic state (Houdt, & Michiels, 2005). EPS consists of a mixture of polymeric compounds, primarily polysaccharides (Vu, Chen, Crawford, & Ivanova, 2009). Bacteria can develop biofilms on biotic as well as abiotic surfaces including living tissues (Donlan, 2002).

Bacterial attachment and biofilm formation is a key factor influencing the efficacy of post-harvest treatments (Lee, Wahman, Bishop & Pressman, 2011; Ukuku and Sapers, 2001). The bacteria in biofilms are 100-1,000 times more resistant to antimicrobials than their planktonic siblings (Davies, 2003; Donlan & Costerton, 2002; Jahid & Ha, 2012). As the biofilm offers protection to bacterial cells, chemical sanitizers become unable to eliminate most biofilm-associated bacteria (Lapidot, Romling, Yaron, 2006). Studies indicated that chlorine and other antimicrobial treatments are ineffective against pathogens firmly attached on produce (Ryu J-H & Beuchat, 2005; Ukuku &Sapers, 2001). Salmonella strains that can form extensive biofilm had stronger attachment to Romaine lettuce leaves compared to weak biofilm-producing strains (Kroupitski, Pinto, Brandl, Belausov & Sela, 2009). E. coli O157:H7 in biofilm increased resistance to chlorine treatment compared to its planktonic form (Ryu and Beuchat, 2005). This may be attributed to the limited penetration ability of this sanitizer into exopolymeric substances or biofilm matrix. In addition, bacterial cells embedded within the crevices and fissures of the
watermelon rind may be another factor (Gautam, Dobhal, Payton, Fletcher & Ma, 2014). This phenomenon may limit the access of bacterial cells to the applied sanitizer solutions.

2.5. Chlorine and lactic acid wash as a post-harvest intervention strategy

Sanitizing produce surfaces with chemical sanitizers is a common post-harvest treatment strategy employed by most of the producers (Keskinen et al., 2009). Chlorine is a widely used sanitizer for fresh fruit and vegetable (Beuchat & Ryu, 1997). For leafy vegetables, it is used at a concentration of 50 to 200 ppm (Parish et al., 2003). Organic acid wash is another easy and convenient technique used by some produce industries. Organic acids are naturally present in food and are generally recognized as safe (GRAS) (Dickson 1992).

During chlorine treatment, free chlorine in the form of hypochlorous acid (HOCl) and hypochlorous ion (OCl−) are considered as active compounds against microorganism (Parish et al., 2003). Exposure to chlorine results in the alterations in cellular metabolism, phospholipid destruction and oxidative action with irreversible enzymatic inactivation (Knox Stumpf, Green & Auerbach, 1948). Among active compounds formed in the solution, hypochlorous acid has the greatest bactericidal activity; however, it reacts quickly with inorganic and organic matter in solution forming combined chlorine -monochloramines and dichloramines (Delaquis, Fukumoto, Toivonen & Cliff, 2004). The amount of monochloramines increases with increase in pH, whereas the amount of dichloramines increases with decrease in pH (Kelly & Sanderson, 1960). The monochloramine has been reported to have antimicrobial activity (Haas and Karra 1984: Kouame & Haas, 1991). Free chlorine in combination with monochloramine showed more powerful disinfectant activity than when they were acting separately (Kouame & Haas, 1991). Organic acids, primarily citric, lactic and acetic acid have been successfully used on several food matrices (Bell, Cutter & Sumner, 1997; Parish et al., 2003). Organic acids inhibit bacteria by
reducing pH of the food environment, disrupting membrane transport and/or permeability of bacterial cells, accumulating toxic anion inside the cell, or reducing internal cellular pH by the accumulation of hydrogen ions inside the cell (Booth et al., 2003). It was reported that the treatment with lactic acid or citric acid (0.5%) for 2 minutes reduced \textit{E. coli} up to 2.0 log 10CFU/g and \textit{L. monocytogenes} up to 1.5 log10 CFU on iceberg lettuce (Akbas & Ölmez, 2007). In another study, citric acid solution (1.0%) reduced mesophilic population by about 1.5 log CFU/g in 5 min on lettuce (Francis & O'Beirne, 2002). A treatment with 2% acetic acid solution for 15 min reduced the populations of \textit{Yersinia enterocolitica} by more than 7 log in parsley (Karapinar & Gonul, 1992).

\textbf{2.6. Post-sanitizing cross contamination}

After sanitizer wash operations, produce may go through several steps such as chopping, shredding, handling and storage and packaging. Inclusion of a water-rinsing step the following sanitizer wash may help spreading pathogens throughout the produce (Murray, Wu, Shi, Jun Xue, & Warriner, 2017; Pérez-Rodríguez et al., 2014). Other post sanitizing activities including chopping, shredding, handling and storage and packaging have also been reported to be the causes of cross contamination (Francis, Thomas, & O'beirne, 1999). Contact surfaces such as conveyer belts, knives, and reusable crates have also been demonstrated as potential sources of contamination between produce batches (Zilelidou, Tsourou, Poimenidou, Loukou, & Skandamis, 2015). \textit{L. monocytogenes} have been frequently recovered from processing operations used to prepare ready to eat vegetables (Nguyen-the & Carlin, 1994). Thus, it is essential to have an appropriate strategy in place that can prevent or control cross-contamination during post sanitizing activities.
2.7. Viability of bacterial cells by PMA-PCR

In response to the environment stress and sanitizers, bacteria may convert into a viable but non-culturable state (VBNC) (Orta de Velasquez et al., 2017; Oliver, 2010). In this state, cells may be unable to grow on culture media but retain viability posing greater risk to public health (Yuan, Zheng, Lin & Mustapha, 2018). When the microbial quality of produce or any food is monitored, it is very important to determine the viability of cells in addition to their abundance. Studies demonstrated induction of VBNC state in Listeria monocytogenes and E. coli O157:H7 under stress conditions on the plant surfaces (Dinu & Bach, 2013; Dreux et al., 2007). A small portion of E. coli and S. typhimurium population (<0.4%) entered into VBNC state in the presence of free and combined chlorine in waste water (Oliver, Dagher & Linden, 2005). Chlorine disinfection resulted in the development of VBNC Helicobacter pylori (Orta de Velasquez et al., 2017). A study found that when L. monocytogenes cells were treated with potassium sorbate, the cells converted into VBNC state for several hours (Cunningham, O’Byrne, & Oliver, 2009). An approach, called viability PCR has been introduced for the detection of viable cells. This technique has been successfully used for bacteria, viruses, fungi and protozoa (Elizaquve, Aznar, & Sánchez, 2013). The viability of a bacterial cells is determined based on the integrity of bacterial cells (Yuan et al., 2018). The technique uses PCR or qPCR with nucleic acid intercalating dyes such as propidium monoazide (PMA) or ethidium monoazide (EMA) (Nocker, Cheung, & Camper, 2006; Nogva, Dromtorp, Nissen & Rudi, 2003). The dyes penetrate the compromised membranes of dead cells and its azide groups covalently bind to cellular DNA to form irreversible nitrogen-carbon bonds under photolysis (Yuan et al., 2018). As a result, bound DNA cannot be amplified by a subsequent PCR assay excluding the dead cells (Fittipaldi, Nocker & Codony, 2012). The viable cells that have intact cell membranes will not be
affected by the dyes (Dinu & Bach, 2013).

2.8 References


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CHAPTER 3
ATTACHMENT STRENGTH AND ON-FARM DIE-OFF RATE OF 
ESCHERICHIA COLI ON WATERMELON SURFACES

3.1. Introduction

Fresh produce is highly implicated in foodborne disease outbreaks (Crowe, Mahon, Vieira, & Gould, 2015; Graça, Esteves, Nunes, Abadias, & Quintas, 2017; Herman, Hall, & Gould, 2015). Produce is estimated to be responsible for 20 million illnesses, resulting in the loss of $38.6 billion annually in the US (Scharff, 2010). During 2010–2014, produce was on the top list of food categories responsible for Salmonella and shiga toxin–producing E. coli associated outbreaks (Crowe et al., 2015). Several studies have traced pre-harvest events as important causes of microbial contamination (Ackers et al., 1998; Leff & Fierer, 2013; Park et al., 2012). Produce is typically consumed raw or minimally processed. Therefore, appropriate risk management strategies should be in place in the field production stage to minimize food safety risks.

The transfer of microbial cells from sources to produce surfaces and their attachment are the key events in pre-harvest contamination. Common sources of pre-harvest contamination are soil, inadequately composted manure, irrigation water, dust, insects, an intrusion of wild animals, and human handling (Atwill et al., 2015; Olaimat & Holley, 2012; Sela, Nestel, Pinto, Nemny-Lavy, & Bar-Joseph, 2005; Weller et al., 2017). Environmental factors such as temperature, relative humidity, and contact time influence bacterial attachment and biofilm formation (Ells & Hansen, 2006; Iturriaga, Escartin, Beuchat, & Martinez-Penicche, 2003; Jitendra Patel & Sharma, 2010; Toyofuku et al., 2016). Once attached to fruit surfaces, bacterial cells initiate biofilm formation.

(Alegbeleye, Singleton, & Sant’Ana, 2018; Van Gerven, Klein, Hultgren, & Remaut, 2015), which is a common defensive mechanism bacteria employ to protect themselves from environmental stress (Morris & Monier, 2003). Bacterial attachment is a key factor influencing the efficacy of post-harvest treatments (Lee, Wahman, Bishop, & Pressman, 2011; Ukuku & Sapers, 2001). Studies indicated that chlorine and other antimicrobial treatments could become ineffective against pathogens firmly attached on produce (Kondo, Murata, & Isshiki, 2006; Ryu & Beuchat, 2005; Ukuku & Sapers, 2001). Thus, an understanding of the behavior of microorganisms on produce in terms of attachment and survival is essential to develop produce safety risk management strategies.

The survival of bacterial pathogens in agricultural field settings is influenced by environmental conditions such as solar radiation, relative humidity, temperature, availability of nutrients and interaction with other natural microflora (Bezanson et al., 2012; Brandl, 2006; Moyne et al., 2011; Nyeleti, Cogan, & Humphrey, 2004; Tomás-Callejas et al., 2011; Weller et al., 2017). United States Food and Drug Administration has included natural die-off as a corrective measure to reduce risks associated with agricultural water and biological soil amendments, in the Food Safety Modernization Act (FSMA) produce safety rule (PSR) (FDA, 2015; Gradl & Worosz, 2017). According to the FSMA PSR, producers that could not meet the microbial water quality criteria could use a die-off rate of 0.5 log per day while calculating the waiting period between the last irrigation and harvest. However, variable results are reported for the die-off rate of *E. coli*, and some studies indicated that the die-off rate could be affected by the surface characteristics of produce and the geographical location (Walsh, Bennett, Mahovic, & Gould, 2014; Weller et al., 2017). Watermelons have been frequently associated with multi-state and multi-country outbreaks (Byrne et al., 2014; Walsh et al., 2014). Since watermelon grows on the ground, this produce is
highly susceptible to microbial contamination during pre-harvest activities. The microbial die-off study on watermelons in the south-central part of the U.S.A may provide a commodity and geographical location specific microbial survival data for predictive die-off rate calculations. The present study examined the die-off rate and attachment behavior of *E. coli* on watermelon surfaces in an agricultural field setting in a south central part of the U.S.A.

### 3.2. Materials and Methods

**Experimental overview**

A watermelon field (22 x 28 m²) at the Louisiana State University Agriculture Center (LSU AgCenter) Botanic Gardens in Baton Rouge, Louisiana, in summer 2016 was used in this study. Watermelon variety ‘Legacy’ was sourced from Reimer Seeds (MD, USA), and planted into jumbo 6-0-6 plastic inserts and trays. The medium used was Sunshine Mix 1. The medium, inserts, and trays were purchased from BWI Companies Inc. (Nash, TX). To achieve uniform germination, seeds planted into the trays were placed on heat mats set at 29°C for 24 hours. The trays were removed off the mats but kept in the greenhouse for 3 weeks. After the first true leaf emerged, seedlings were fertilized with Peter’s 20-20-20 (ICL Specialty Fertilizers, the Netherlands) weekly at 200 ppm N. Five hundred pounds (65 lbs actual N per acre rate) of Expert Gardener 13-13-13 (Schultz Company St. Louis, MO) was incorporated into the field 7 days prior to planting. After transferring the plants to the field, overhead irrigation was used to establish plants, until the vines began to run. Irrigation was not applied within the last month of growth prior to the harvest. In this study, only municipal water was used for irrigation, and no biological soil amendments of animal origin were added to the seedlings or to the field thus minimizing microbial contamination.
Testing natural coliform and *E. coli* levels on the watermelon surfaces

After 80 days, watermelon samples (n=25) were randomly harvested from different locations of the field using sterile disposable gloves. Watermelon length, width (L x W cm²) and fresh weight (g) were measured. The visible soil was removed using sterile tissue paper. Each watermelon was cut horizontally into halves, upper (facing to sunlight) and lower (facing to the ground) and the edible portion was removed using sterile stainless-steel knives. The area of each watermelon surfaces was measured \( \text{Area} = \pi \times \text{Semi-Major Axis} \times \text{Semi-Minor Axis} \). The areas of the half watermelon surfaces ranged from 187 to 366 cm². Watermelon rinds from each halves were then aseptically transferred into a sterile polythene bags and immediately transported to the laboratory maintaining 4°C. After receiving the samples in the lab, 0.1% peptone water (100 mL) was added into each bag and the rind was hand massaged with peptone water for 5 min to dislodge the microorganisms from the melon surfaces. The supernatant after massaging was used for the microbial analysis. Petrifilm™ EC plates (3M™ Microbiology Products Co., St. Paul, MN) were used to enumerate coliform and *E. coli* levels on the watermelon surfaces.

*E. coli* inoculum preparation

This study used a cocktail of three *E. coli* strains (ATTC 23716, 25922 & 11775). These strains are among the few well-characterized potential surrogates for *E. coli* O157:H7 for use in field trials (Abberton, Bereschenko, van der Wielen, & Smith, 2016; Harris et al., 2012). Frozen cultures were activated in three successive passes and harvested to an initial inoculum size of 7-8 log CFU/mL by following the procedure described by Adhikari et al (Adhikari et al., 2016).

Curli expression of *E. coli*

The level of curli expression of *E. coli* strains was determined using the congo red binding assay (Macarisin, Patel, Bauchan, Giron, & Sharma, 2012; J Patel, Sharma, & Ravishakar, 2011).
Each culture was streaked on Congo red indicator (CRI) agar (0·1% tryptone, 0·05% yeast extract, 1·5% agar, 0·004% Congo red and 0·002% Coomassie brilliant blue) and incubated at 22°C and 32°C for 48 h. We recorded *E. coli* producing red colonies as curli producers, while those producing colorless colonies were recorded as curli negative.

**Watermelon inoculation with *E. coli* **

Watermelons (n=160) in the field were selected at random, and a circle (50 cm²) was drawn on the upper surface of each watermelon fruits using a permanent marker pen. Prior to inoculation, the *E. coli* cocktail was agitated 25 times in a 30 cm arc to ensure thorough mixing. Each marked surface was then spot inoculated with 200 µL inoculum distributed into 15 small droplets and spread using a sterilized cotton swab.

**Recovery of strongly and loosely attached *E. coli* from watermelon surfaces**

At 24 h time intervals (0, 24, 48, 72, 96 & 120 h), six watermelon samples were harvested randomly each time (n=36). The inoculated rind area of each watermelon was cut as a disk (50 cm², 42 g) and separated from the edible portion using a sterile stainless steel knife. We placed the disks in sterilized plastic bags and transported to the laboratory maintaining 4°C. After that, we separated the outer green peel (8 g) using a sterilized vegetable peeler and placed in a sterile Security-Snap Bottle (Fisher Scientific, USA). The attachment assay was performed using the method described by Ells & Hansen (2006) with a slight modification. Briefly, 25 ml of PBS (pH 7.2) containing 0.1% Tween 20 was added to the peel and vortexed for 20 s to remove loosely attached bacterial cells. We repeated the washing process in a new bottle with fresh PBS containing 0.1% Tween 20. We collected and mixed spent wash solutions for the enumeration of loosely attached *E. coli* cells. The washed rinds were transferred to 50 ml Falcon® tubes containing 25 mL of PBS and homogenized for 30 s at high speed using a Fisher Scientific™ 150 Hand Held...
Homogenizer (Fisher Scientific, USA). Between each sample, we sanitized the homogenizer with 70% ethanol and rinsed three times with sterile distilled water to remove residual alcohol. The homogenate was used to enumerate strongly attached *E. coli*. Enumeration of *E. coli* was done by plating the homogenates and their respective wash solutions on 3M™ Petrifilm *E. coli*/Coliform count plates and incubating the plates at 37 °C for 48 h. The attachment strength (S<sub>R</sub> value) was calculated as the ratio of bacterial population recovered from homogenate (strongly attached cells) to the total bacterial cells (strongly attached + loosely attached) (Jitu Patel, Singh, Macarisin, Sharma, & Shelton, 2013). The weather data for the study period was retrieved from https://www.wunderground.com.

**Scanning Electron Microscopy analysis**

Watermelon rinds obtained after double PBS-tween 20 buffer wash were cut off (about 2x2 mm area and 0.5 mm thickness) using a sterile blade. The cut pieces were fixed overnight in an FAA solution (95% Ethanol (50 mL), Glacial Acetic Acid (5 mL), 40% Formaldehyde (10 mL), distilled water (35 mL). The fixed samples were dehydrated in a graded ethanol series (50%, 70%, and 100%). The samples were then dried in a Denton DCP Critical Point Dryer (Denton Vacuum, LLC, USA) with CO<sub>2</sub> as the transition gas. We mounted the samples on aluminum SEM stubs and coated with platinum in an EMS 550X sputter coater (Electron Microscopy Sciences, USA). The samples were examined with a JSM-6610 scanning electron microscope (JEOL USA Inc., USA) at an accelerating voltage of 10 KV in the high vacuum mode.

**Chlorine treatment**

Watermelons (n=18, 9 each at 30 min and 120 h post-inoculation) were harvested using sterilized disposable gloves. Each watermelon was completely dipped into 10 liters of aqueous 150 ppm chlorine (available) solution (25°C, pH 7.3) (Clorox®, Oakland, CA, USA) for 3 min.
Chlorine concentration was measured by Clorox Smart Strips (Clorox®, Oakland, CA, USA). After the treatment, we removed the inoculated disks from the fruits as previously mentioned, and separated the outer green peel using a vegetable peeler. The peel was homogenized in 100 mL of Dey-Engley (D/E) Neutralizing Broth using a Fisher Scientific™ 150 Hand Held Homogenizer (Fisher Scientific, USA). We enumerated \textit{E. coli} populations in the homogenate using 3M™ Petrifilm \textit{E. coli}/Coliform count plates.

**Statistical analysis**

Coliforms and generic \textit{E. coli} populations recovered from the watermelon samples by direct plating on the 3M petrifilms were converted to log CFU/cm$^2$. The bacterial die-off and attachment strength over the time was analyzed using ANOVA with Proc mixed feature of SAS 9.4 (SAS Institute, Cary, NC, USA.). The level of statistical significance was $p<0.05$ in all cases.

3.3. Results

**Levels of natural coliforms and \textit{E. coli} on upper and lower half surfaces of watermelons**

All the tested samples were positive for coliforms. The coliforms levels were not significantly different ($P>0.05$) with an average population of 2.20±0.18 log CFU/cm$^2$ and 2.65±0.17 log CFU/cm$^2$ on the upper and the lower half samples, respectively. We observed a low prevalence of natural \textit{E. coli} on the surfaces of the watermelon fruits (Appendix). Out of 25 watermelons, 10 were positive for \textit{E. coli} with five upper half and six lower half positive samples. Only five watermelons had \textit{E. coli} levels more than 1 log CFU/cm$^2$. Although some lower half surfaces had higher count (up to 2.64 log CFU/cm$^2$) compared to upper half surfaces ($< 1$ log CFU/cm$^2$), overall \textit{E. coli} prevalence or level was not significantly different between the surfaces.
Die-off rate and attachment strength of *E. coli* on watermelon surfaces

The die-off rate of *E. coli* inoculated on the watermelon surfaces is shown in Fig 3.1. A significant reduction (P<0.05) in *E. coli* population (total) was observed within 24 hours (Day 2). In the period between 24 to 96 h, there was no significant (P>0.05) reduction in *E. coli* levels. At 120 h, the total reduction was 1.94 log CFU/cm² (from 3.65 log CFU/cm² to 1.71 log CFU/cm²). The daily die-off rate was variable during the study period, and the daily die-off pattern was more likely multiphasic up to 120 h. The highest daily die-off was 1.30 log CFU/cm² in day 2 (after 24 h) followed by 0.63 log CFU/cm² in day 6, which were greater than the FSMA produce safety rule predicted die-off rate (0.5 log CFU/day) on produce surfaces. However, in other days, the die-off rates were lower than the predicted value.
Fig. 3.1. Die-off and attachment of *E. coli* on watermelon surfaces. Average *E. coli* count on the watermelon surfaces and FSMA-PSR predicted count on produce surfaces based on the die-off rate of 0.5 log CFU/day, up to 120 h (Day 6). The population of loosely attached and strongly attached cells is also shown for up to 120 h. $S_R$ Value (Attachment Strength) = Strongly Attached Cells/ Total Cells (strongly attached + loosely attached). Data are shown as mean values ± standard deviation.
All the tested *E. coli* strains (ATCC 23716, ATCC 25922 and 11775) produced intense red colored colonies on CRI agar at 32°C indicating a strong curli expression. However, at 22°C, only *E. coli* ATCC 25922 showed curli expression. Other tested strains (ATTC 23716 and 11775) produced curli negative (colorless) colonies at that temperature.

The level of *E. coli* attachment on the watermelon surfaces is shown in Fig 3.1. The attachment strength \( S_R \) of *E. coli* on the watermelon surfaces increased significantly \( (P<0.05) \) within 24 h. The initial \( S_R \) value at 0 h (i.e., after 30 min of inoculation) was 0.04, which increased to 0.99 after 24 h, and this level maintained up to 120 h. There was a significant reduction \( (P<0.05) \) in loosely attached population (from 3.6 log CFU/cm\(^2\) to 0.20 log CFU/cm\(^2\)) in 24 h. Afterward, the lower level of loosely attached population maintained up to 120 h. However, there was no significant change in the strongly attached population up to five days (the graph overlapped by total count). Thus, the increase in \( S_R \) over time was mainly attributed to the decrease in the loosely attached population, given that the population of strongly attached cells was constant.

**Scanning Electron Microscopy (SEM) analysis**

The SEM micrographs showed a strong colonization of small rod-shaped bacterial cells on the surface of watermelons after 24 h (Fig. 3.2). Since these samples were inoculated with *E. coli* cells (~ 4 log/cm\(^2\)) and there was a trend of attachment over time, the majority of those cells were possibly *E. coli*. At 0 h, individual cells were predominant on the watermelon surfaces (Fig 2 A). After 24 h, the cells were smaller and clustered together. As time progressed, the cells became overlaid with a sheath of substances resembling an extracellular matrix (Fig. 3.2 C). After 120 h, it was hard to observe bacterial cells on the watermelon surfaces.
Fig. 3.2. Scanning electron micrographs of *E. coli* inoculated watermelons surfaces. SEM images (SEI 10 KV, 5,000x & 10,000x) of watermelon surfaces after inoculating with *E. coli* and leaving them in an agriculture field for 0 h (A), 24 h (B), 48 h (C), 72 h (D), 96 h (E) and 120 h (F).
Efficacy of chlorine treatment

The efficacy of aqueous chlorine (150 ppm) against *E. coli* on the watermelon surfaces at two different attachment times (30 min and 120 h) is shown in Fig 3.3. The efficacy of chlorine treatment in reducing the *E. coli* level was significantly (P< 0.05) higher on the freshly inoculated watermelons than on those watermelons inoculated 120 h before the treatment. The average reduction due to the chlorine treatment was 4.2 log CFU/cm$^2$ and 0.62 log CFU/cm$^2$ on 30 min and 120 h post-inoculation watermelon samples, respectively.

3.4. Discussion

Coliforms and *E. coli* were uniformly distributed on the watermelon surfaces.

The lower part of watermelon surfaces which sits on soil is generally assumed to have a higher microbial level than the upper part of the watermelon. Studies on melon-rinds indicated a higher level of microbial contamination on ground spots as compared to the non-ground spot areas of melon rinds (Fleming, Pool, & Fruit, 2005; Parnell, Harris, & Suslow, 2005). In addition, the upper surfaces may have a greater chance of exposure to sunlight than the lower surfaces. However, we did not observe a significant difference in the levels of coliforms between upper and lower half surfaces of the watermelon. This result agrees with our previous study, which observed a similar level of coliform and generic *E. coli* counts on watermelons with different levels of sunlight exposure caused by the surrounding vegetation (Chhetri et al., 2018). The top part of watermelon surfaces may be equally prone to the bacterial contamination as the bottom part in the agriculture field. Results were observed in the condition where municipal water was used for irrigation without the addition of biological soil amendments of animal origin. Therefore, further investigation to
Fig. 3.3. Efficacy of chlorine (150 ppm for 3 min) on *E. coli* inoculated watermelon surfaces. 30 min: Inoculated 30 min prior to the treatment; 120 h: Inoculated 120 h prior to the treatment. The bar diagrams with different lowercase letters on the top are significantly different (P < 0.05) from each other. The detection limit of the test was 0.28 log CFU/cm².
establish an association between the distribution of microbial cells on produce surfaces and sources of contamination is needed.

**Majority of E. coli die-off was within 24 h of contamination.**

Our study demonstrated that die-off of *E. coli* does occur on watermelon rind surfaces in an agricultural field environment. The reduction in culturable *E. coli* population on the surface of watermelon fruits was significant at 24 h. However, the reduction rate did not follow the same pattern over time. The rapid decline in *E. coli* population in the first 24 h was evident in most of the studies (Yu, Newman, Archbold, & Hamilton-Kemp, 2001), which was likely due to the sudden change in the environmental conditions. Limitation of nutrients, sunlight, desiccation, and competition with surface microflora might have been the predominant stress factors (Yu et al., 2001). Several studies have reported an average die-off rate of around half a log on produce matrices, such as lettuce and spinach (Barker-Reid et al., 2009; Wood, Bezanson, Gordon, & Jamieson, 2010). For instance, Weller et al. (2017) demonstrated a mean *E. coli* die-off rate of 0.52 log MPN on lettuce heads under field condition in Freeville, New York. Similarly, Barker-Reid et al. (2009) observed a total reduction of *E. coli* level on lettuce by 2.2-log in 5 days (the average daily die-off rate of 0.44 log) in southeastern Australia. Wood et al. (2010) observed an exponential decline in *E. coli* population on spinach after irrigation over the time under field condition in Canada. In contrast, we did not observe any significant change in the *E. coli* levels between 48 to 96 h. This result may be attributed to the bacterial ability to adapt to a new environment and/or to develop resistance to the environmental stresses, by altering their cellular physiology (Annous, Solomon, Cooke, & Burke, 2005; Beuchat, 2002; Brooks, Turkarslan, Beer, Lo, & Baliga, 2011; De Carvalho, 2007; Phaiboun, Zhang, Park, & Kim, 2015). However,
further studies are needed to better understand the survival mechanisms of bacterial cells, specifically on the surface of watermelon fruits.

We compared the daily die-off rate with the rate recommended by FSMA produce safety rule (0.5 log per day) to see if it followed a similar pattern. Out of 6 days, two days (day 2 and day 6) had higher daily die-off rate than the recommended rate. However, for other days the population did not change significantly. In other studies, daily die-off rate of *E. coli* on produce varied from 0.4 to 1.64 log MPN/day (Chhetri et al., 2018; Hutchison, Avery, & Monaghan, 2008; Moyne et al., 2011; Xu, Buchanan, & Micallef, 2016). As the geographical locations where the studies were conducted were different among studies, the variation in die-off rate might be due to the differences in environmental factors. We recorded the temperature, relative humidity and the precipitation for 120 h (Table 3.1). There was not much change in temperature and the relative humidity values during the study period. However, these parameters may vary every hour during day and night. Thus, further study is needed to determine the association between environmental factors and the survival of bacterial cells on produce surfaces. The bacterial strains used in the studies could have been another factor (Stine, Song, Choi, & Gerba, 2005). Weller et al. (2017) observed a significantly lower die-off rate on the lettuce head samples harvested after a rain event. We did not have sufficient data to establish an association between precipitation and die-off. Our study showed that waiting days between contamination and harvest could be a way to reduce the bacterial load on produce surfaces. However, the die-off rate may be dependent on multiple factors.
Table 3.1. Weather conditions during the study period

<table>
<thead>
<tr>
<th>Day (Hour)</th>
<th>Day light length (H:Min:S)</th>
<th>Temperature (°C)</th>
<th>Relative humidity (%)</th>
<th>Average Precipitation (Inches)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Low</td>
<td>High</td>
<td>Day Average</td>
</tr>
<tr>
<td>Day 1 (0 h)</td>
<td>NA</td>
<td>24.4</td>
<td>34.4</td>
<td>29.4</td>
</tr>
<tr>
<td>Day 2 (24 h)</td>
<td>13:49:50</td>
<td>24.4</td>
<td>35.5</td>
<td>30.0</td>
</tr>
<tr>
<td>Day 3 (48 h)</td>
<td>13:48:45</td>
<td>25.0</td>
<td>35.5</td>
<td>30.5</td>
</tr>
<tr>
<td>Day 4 (72 h)</td>
<td>13:47:37</td>
<td>23.8</td>
<td>33.3</td>
<td>28.8</td>
</tr>
<tr>
<td>Day 5 (96 h)</td>
<td>13:46:28</td>
<td>23.8</td>
<td>35.0</td>
<td>29.4</td>
</tr>
<tr>
<td>Day 6 (120 h)</td>
<td>13:45:18</td>
<td>23.5</td>
<td>32.7</td>
<td>28.8</td>
</tr>
</tbody>
</table>

The temperature values in Fahrenheit were converted to centigrade. ND: Not detected. Temperature: low, high, and day average. Relative humidity: low and high. These weather data were retrieved from https://www.wunderground.com.
Attachment strength of *E. coli* on watermelon surface increased over time.

The ability of bacterial pathogens to attach and form biofilm to produce surfaces is one of the biggest concerns in produce safety. Microorganisms are highly tolerant of various disinfectants when they are in a strongly attached form or in biofilms (Jahid & Ha, 2012). In our study, the attachment strength of *E. coli* on watermelon surfaces increased with time. The proportion of strongly attached cells to loosely attached cells reversed after 24 h. The majority of survivors or culturable *E. coli* cells were in strongly attached form, and the increase in $S_R$ value over time was mainly attributed to the decrease in loosely attached population. Therefore, further study is needed to understand the fate of *E. coli* cells on produce surfaces, especially die-off and the possibility of transforming into strongly attached cells. Our $S_R$ results agree with the findings of other studies, which demonstrated the increasing attachment strength of pathogens on produce (vegetables) surfaces over time (Ells & Hansen, 2006; Jahid & Ha, 2012; J Patel et al., 2011). The attachment strength ($S_R$ value) of *E. coli* O157: H7 increased from 0.09 to 0.45 on intact cabbage surface after 24 h (Patel et al., 2011). In another study, Ells and Hansen (2006) reported that the initial $S_R$ of *L. monocytogenes* to intact cabbage surfaces was 0.66, which increased to 0.82 after 24 h of storage at 37°C.

The initial step of the bacterial colonization to produce surfaces is the reversible attachment (Jahid & Ha, 2012). In our study, the proportion of loosely attached cells was significantly higher than strongly attached cells at 0 h, after inoculation. Other studies also reported similar results (Patel et al., 2013). This result may be attributed to the initial reversible and weak attachment of bacterial cells. One of the subsequent steps of the colonization involves the production of the exopolymeric substances (EPS) which leads to an irreversible attachment (Jahid & Ha, 2012). Bacteria use this mechanism to protect themselves from the harsh environment of phyllosphere.
and to buffer environmental changes such as nutrient stress, desiccation and UV irradiation (Annous et al., 2005; Elasri & Miller, 1999; Monier & Lindow, 2005). Scanning electron micrograph showed a different bacterial arrangement after 24 h, which may be associated with EPS production. The bacterial cells were enclosed in a structure similar to the extracellular polymeric matrix demonstrated by other studies (Annous et al., 2005; Ells & Hansen, 2006). Annous et al. (2005) observed a fibrillary material after 2 h, and salmonella cells embedded in an extracellular polymeric substance in 24 h at 10 & 20°C. Ells and Hansen (2006) observed a greater number of clusters of Listeria spp with an extrapolymeric coating (biofilm) on cabbage surfaces after 24 h. These results indicate that the higher level of bacterial attachment might be associated with the production of the exopolymeric substance. If the bacterial cells are embedded in an exopolymeric substance, even surfactants-based washing becomes ineffective in removing them from produce surfaces.

The bacterial attachment and biofilm formation to produce surface are complex processes which may alter physicochemical properties of both bacteria and plant surface (Hirano & Upper, 2000; Ukuku & Fett, 2002). In E. coli, curli has been found to be associated with the attachment and biofilm formation on biotic and abiotic surfaces (Macarisin et al., 2012; Patel et al., 2011). Curli-expressing E. coli O157:H7 strains showed a higher level of attachment to spinach surfaces compared to the curli-deficient mutants (Macarisin et al., 2012). The curli expression of E. coli is dependent on environmental factors such as temperature, oxygen level and osmolarity (Boyer et al., 2007; Evans & Chapman, 2014). In our study, all three E. coli strains exhibited a better curli expression ability at 32°C. While the field temperature during the study period ranged from 23.5°C to 35.5°C. The favorable environmental temperature might have contributed to the attachment of E. coli cells to watermelon surfaces. However, bacterial cell surface charge and hydrophobicity
could be other potential factors associated with the attachment. Patel et al. (2010) reported that strong curli-expressing *E. coli* O157:H7 showed a higher surface hydrophobicity and a higher level of attachment to the cabbage and iceberg lettuce surfaces than other weak curli expressing strains. **The efficacy of the chlorine wash reduces with increase in bacterial attachment level**

This study showed that the chlorine treatment was effective on watermelon surfaces immediately after the contamination with *E. coli*. However, the efficacy of the treatment decreased significantly when treated after 120 h of contamination. Our results concurred with the findings of other studies (Boyer et al., 2007; Elasri & Miller, 1999; Evans & Chapman, 2014; Hirano & Upper, 2000; Monier & Lindow, 2005; Ukuku & Fett, 2002). Ölmez & Temur (2010) found that the sanitizer treatments (including chlorine treatment) reduced 99% of total *E.coli* and *L. monocytogenes* population on lettuce leaves after 6 h of inoculation. However, after 48 h, the treatment reduced only up to 90% of the bacterial population. In an another study, chlorine treatment was less effective when applied after 60 min of the inoculation than 20 to 40 min of the inoculation on the cantaloupe rinds (Ukuku & Fett, 2002). The results indicate that the efficacy of the chlorine treatment is dependent on the level of bacterial attachment. The reduced efficacy of chlorine against strongly attached cells or cells enclosed in biofilms may be attributed to the limited penetration ability of this sanitizer into exopolymeric substances or biofilm matrix (De Beer, Stoodley, Roe, & Lewandowski, 1994). Bacterial cells embedded within the crevices and fissures of the watermelon rind may be another factor. Cracks and fissures on melon rind surfaces may allow bacterial cells to enter interior tissues (Gautam, Dobhal, Payton, Fletcher, & Ma, 2014); this phenomenon may limit the access of bacterial cells to the applied sanitizer solutions. *E. coli* levels on the watermelons harvested at 0 h and at 120 h, before chlorine treatment, was different. Further
study is needed to see if there is an effect of the initial bacterial load on the effectiveness of chlorine treatment on watermelon surfaces.

Overall, we calculated an average daily die-off rate of *E. coli* on watermelon surfaces in an agriculture setting in a south central location of the United States. The differences in die-off rate from other studies could be due to a number factors, including environmental conditions (Louisiana versus other locations, summer versus other seasons), level of vegetation in the field, type of produce, type of bacterial strains and length of the study. Further studies, therefore, are needed to identify associated factors and their level of influence on the die-off rate, specifically on human pathogens. We observed an increase in attachment level of *E. coli* with time in field condition and a lower efficacy of chlorine treatment on the watermelon surfaces 6 days after the inoculation. The results indicated that the efficacy of the chlorine might be dependent on the attachment level of bacterial cells. The results reported in this study may be useful while developing pre-harvest and post-harvest risk management strategies.

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CHAPTER 4
EFFECT OF SURROUNDING VEGETATION ON MICROBIAL SURVIVAL OR DIE-OFF ON WATERMELON SURFACES IN AN AGRICULTURE SETTING

4.1. Introduction

Fruit and vegetable crops have the potential to be contaminated with pathogenic microorganisms in the field. Since fresh produce is often consumed raw, a higher risk of foodborne illness is posed with this food group. The number of foodborne illness outbreaks associated with the consumption of fresh fruits and vegetables has noticeably increased in the last decade (Huang & Chen, 2011; Olaimat & Holley, 2012). During 1973–2014, fruit and vegetable crops were the most commonly implicated commodities for several foodborne illness outbreaks (Crowe, Mahon, Vieira, & Gould, 2015; Herman, Hall, & Gould, 2015; Nguyen et al., 2015). Pre-harvest contamination of produce is commonly originated from soil, inadequately composted manure, contaminated irrigation water and improper human handling of produce (Annous, Solomon, Cooke, & Burke, 2005; Tomas-Callejas et al., 2011). The intrusion of crops by wild animals, birds, reptiles and rodents, as well as insects and nematodes acts as a vector for transferring various pathogens (Brandl, 2006). The survival and growth of microorganisms is influenced by several environmental factors and agricultural practices such as exposure to solar UV radiation, temperature changes, humidity and poor fertilizer regimes (Bezanson et al., 2012; Brandl, 2006; Nyeleti, Cogan, & Humphrey, 2004; Tomas-Callejas et al., 2011; Weller et al., 2017).

Several studies reported that the sunlight of tropical latitudes (Davies & Evison, 1991; Nyeleti, Cogan, & Humphrey, 2004; Obiri-Danso, Paul, & Jones, 2001) and concomitant increase

in the surface temperature of produce (Tomas-Callejas et al., 2011) have an inhibitory effect against various microbial pathogens. Moreover, sunlight is found to reduce Salmonella levels in fresh water sources (Davies & Evison, 1991) and on food contact surfaces such as stainless steel (Nyeleti et al., 2004). Furthermore, microbial populations decline with decreasing nutrient availability because of failing to lower their metabolic rate to adopt the starvation condition (Fontaine, Mariotti, & Abbadie, 2003). All these factors contribute to the natural decline of microbial populations on produce surfaces in the field and should be considered for microbial die-off rate calculations (Davies & Evison, 1991; Reddy, Khaleel, & Overcash, 1981).

To minimize pre-harvest microbial food safety risk originated from contaminated irrigation water, the FDA Food Safety Modernization Act (FSMA) Produce Safety Rule requires agricultural water used for covered produce must be safe and of adequate sanitary quality (U.S.FDA, 2015). It should also meet the generic E. coli requirements as proposed in the U.S. Environmental Protection Agency’s 2012 Recreational Water Quality Criteria. However, the rule provides flexibility to growers that are not able to meet the microbial water quality criteria initially by extending the time interval between the last irrigation and first harvest to allow for microbes to naturally die (U.S.FDA, 2015). Research on surface of produce highlights the effect of environmental factors on the die-off rate of microorganisms (Wood, Bezanson, Gordon, & Jamieson, 2010). However, the level of exposure to environmental stressors, in particular solar UV radiation on surface of produce may be influenced by growing practices. Presence of weed and/or the surrounding vegetation from the plant may cover fruit surfaces, thus preventing the exposure of contaminated surface to the natural sunlight. As per our knowledge, the effects of surrounding vegetation have not been considered while calculating microbial die-off rates on produce such as melons. This is
especially imperative as melons grow in direct contact with the soil with surrounding vegetation making them at a higher risk of microbial contamination. Several recent outbreaks have been attributed to microbial contamination of melon crops (McCollum et al., 2013). During 1973–2011, watermelon, cantaloupe and honeydew melons were responsible for 34 food borne disease outbreaks (Walsh, Bennett, Mahovic, & Gould, 2014). Investigating the effect of surrounding vegetation on the microbial die-off rate would help generate data for on-farm food safety risk assessments. Thus, the objective of this study was to determine the effect of surrounding vegetation (weeds and vines) on the survival or die-off rate of generic E. coli on watermelon rind surfaces in an agricultural setting.

4.2. Materials and Methods

Experimental overview

The present study was conducted on July and August at the Louisiana State University Agriculture Center (LSU AgCenter) Botanic Gardens in Baton Rouge, Louisiana. The test field was divided into three blocks and each block contained 6 plots (12 x 30 ft²). Eighteen total plots were tested. Plots were initially treated with one of the following five pre-emergent herbicides or an untreated control. The herbicide treatments were applied 24 hours prior to transplanting. Preemergent herbicide treatments included Strategy 5 pts/acre (Ethalfluralin & Clomazone), Command 3ME 0.67 pts/acre (Clomazone), Strategy 5 pts/acre plus Sinbar 4 oz/acre, Valor 1 oz/acre (Flumioxazin), Sinbar 4 oz/acre (Terbacil) and an untreated control. Preemergent herbicides were applied using a CO₂ backpack sprayer delivering 15 gallons per acre. Twenty-four hours after herbicide application, ‘Legacy’ watermelon seedlings were transplanted. Additional herbicides, Sandea (0.67 oz/acre) and Prowl (1 qt/acre), were applied to the row middles as a lay-by application 14 d after planting. Overhead irrigation was applied to establish plants, until flowering
period. Eighty days after initial preemergent herbicide application, multiple types of vegetation (weeds) were observed in each plot. Dominant vegetation (weeds) included Yellow nutsedge (Cyperus esculentus), Goosegrass (Eleusine indica), Large Crabgrass (Digitari sanguinalis) and Barn yard grass (Echinochloa crus-galli). The surrounding vegetation (weeds plus vine) in each plot was evaluated using a 0 to 10 scale, where “0” represented “no vegetation”, “5” represented 50% plot coverage in vegetation and “10” represented complete or 100% coverage of the plot with vegetation (Fig. 4). Using this scale, we developed three categories of surrounding vegetation: (i) low level (0-3) (ii) medium level (4-6), and (iii) high level (7-10). Some plots had fewer numbers of fruits, so the sample collection was based on the surrounding vegetation rather than the plot it was grown. Our preliminary studies revealed that there was no significant effect of herbicides on the level of naturally coliform and generic *E. coli* on watermelon surface.

A total of 80 watermelons, 30 each from low and medium surrounding vegetation and 20 from high surrounding vegetation (Strategy, Valor, Strategy plus Sinbar, Sinbar & Command: 14 each and Untreated: 10), were harvested from the test plots. We were unable to gather 30 melons from the high vegetation (weed) test areas because the overwhelming presence of weeds made it difficult for watermelon vines to flower and fruit. Each melon was carefully removed from the field using disposable gloves. Gloves were changed with each harvested melon. Melons were labeled with the plot number and the level of surrounding vegetation. The dimensions of each watermelon (*i.e.* length and width) and fresh weight were recorded to calculate the bacterial count per cm$^2$. The harvested melons were then aseptically transferred into a sterile polythene bags (48.3 x 58.4 cm$^2$) and immediately transported to the food safety laboratory at 4ºC. After receiving the samples at the lab, a 200 mL of 0.1% peptone water was added to each watermelon bag and was hand massaged intensively for 5 min to dislodge the microorganisms from the melon surface.
our preliminary study, we observed low number of generic *E. coli* on watermelon surface. Thus, to reduce the dilution level, we used only 200 mL 0.1% peptone water but initiated a longer massaging time. The eluent was then used for the microbial analysis.

**Testing natural coliform, generic *E. coli* levels and the bacterial pathogens on the watermelon surfaces**

Quanti-Tray 2000-Colilert® (IDEXX Laboratories, Portland, ME) and Petrifilm™ EC plates (3M™ Microbiology Products Co., St. Paul, MN) were used to enumerate generic *E. coli* and coliform levels on the watermelon rind surface, respectively. The Quanti-Tray® method was used to enumerate the generic *E. coli* levels because of its lower detection limit (0.30 log MPN/sample) than petrifilm and VRBA plating methods. Briefly, each eluent sample (100 mL) obtained from hand massaging was poured into a sterile plastic container containing Quanti-Tray® reagent powder. Contents were thoroughly mixed by gentle agitation and then poured into Quanti-Trays. The trays were sealed using a heat sealer (Quanti-Tray-2X, IDEXX Laboratories, Inc. USA) and incubated at 35 ± 0.2°C for 24 h. The colors of the wells were compared with a comparator provided by IDEXX laboratories, and the number of wells showing fluorescence under a UV lamp (WL200, Hanovia LTD, Aquionics, UK) was recorded as generic *E. coli* positive samples. The results were expressed in terms of MPN using a chart provided by IDEXX Laboratories. The enumeration of coliforms on the watermelon rind was done by using 3M™ Petrifilm and the results were expressed in CFU. This is because coliforms were present at higher numbers and the dilution used to detect generic *E. coli* resulted in all wells positive for coliforms. Each sample was also analyzed for bacterial pathogens (*E. coli* O157:H7 and *Salmonella* spp) using immunomagnetic separation (BeadRetriever™, Thermo Fisher Scientific, USA) technique followed by spread plating on selective media. However, we did not detect the presence of *E. coli* O157:H7 or *Salmonella* spp on the watermelon surface.
**Watermelon disc preparation and inoculation with generic \(E.\) coli**

To bring uniform light exposure on the surface of watermelons and better understand the effect of surrounding vegetation on the die-off rate of bacteria, studies were also conducted by artificially inoculating watermelon discs with generic \(E.\) coli. Subsequently, these inoculated discs were exposed to natural sunlight in the field under different levels of surrounding vegetation. Briefly, fresh watermelons grown in our test plots were first washed with sterile deionized water and air-dried inside a biological safety cabinet for 1 h at room temperature. A total of 63 watermelon discs (surface area of 20 cm\(^2\) and thickness of 0.5 cm) were prepared using sterile stainless-steel knives by coring off the edible portion. The watermelon discs were placed on sterile petri-dishes with the outer epidermal surface facing up.

A cocktail of three generic \(E.\) coli strains (ATTC 23716, 25922 & 11775) were used in this study. These strains are among the few well-characterized surrogates for use in field trials (Harris et al., 2012). Frozen cultures were activated in three successive passes by following the procedure described by Adhikari, Syamaladevi, Killinger, and Sablani (2015). The final inoculum size was \(10^9\) CFU/mL. The bacterial cocktail was agitated 25 times in a 30 cm arc to ensure thorough mixing. The surface of the discs placed in sterile petri-dishes was spot inoculated with 50 µL inoculum distributed into 15 small droplets. The inoculated discs were dried inside the biological safety cabinet for 12 h.

**Enumeration of inoculated generic \(E.\) coli on watermelon rind disc**

After harvesting all the watermelons, three plots were selected representing one for each level of surrounding vegetation (low, medium, and high). Inoculated watermelon discs kept on sterile petri plates (n=21) were placed randomly around in each level of surrounding vegetation plot (Fig. 4).
Samples of three watermelon discs from each plot were collected at 0, 12, 36, 60, 84 and 108 h (at 6 pm). The discs were stored in an ice chest and transported to the laboratory maintaining 4°C. Each disc was placed into a sterile stomacher bag containing 90 ml of 0.1% peptone water and hand-massaged for one minute followed by blending in a stomacher (BagMixer® 400S, Interscience, Woburn, MA, USA) for 5 minutes. The eluent was used for the enumeration of the generic *E. coli* by plating on Violet Red Bile Agar (Criterion™, Bio-Rad Laboratories, Inc., USA) (with detection limit: 1.65 log CFU/cm²) and incubated at 37°C for 24 h.

**Statistical analysis**

Data were analyzed using PROC MIXED feature of SAS 9.4 (SAS Institute, Cary, NC, USA). Significant difference (*P* ≤ 0.05) among the mean values of the bacterial count from different vegetation levels was determined using Fisher's LSD test.

**4.3. Results and Discussion**

**Level of natural coliforms and generic *E. coli* on watermelons surrounded by different levels of vegetation**

The generic *E. coli* count on the watermelon collected from three different vegetation levels, that is, low (1 log MPN/sample), medium (1.46 log MPN/sample), and high (1.23 log MPN/sample) is shown in Figure 1. The results did not show a significant difference (*p* > .05) in generic *E. coli* count among three different levels of vegetation. Similar results were observed for total coliforms. The coliform counts were 3.7, 3.66, and 3.91 log CFU/cm² on low, medium, and high vegetation levels, respectively (Figure 2). Under the tested conditions, the results of the current study indicate that generic *E. coli* and total coliforms were naturally prevalent (although at low levels) on the surface of watermelons. However, the level of vegetation surrounding the watermelons did not show a significant effect (*p* > .05) on the bacterial count.
Fig. 4.1. Generic *E. coli* levels on watermelon surface harvested from low, medium, and high level of surrounding vegetation plots. The level of surrounding vegetation was converted to numeric value (0–10); 0 meant for no vegetation and 10 meant for highest amount of vegetation. Categorically, those plots with 0–3 were considered as low vegetation plots, 4–6 as medium level of vegetation plots, and 7–10 as high level of plots. The detection limit was 0.30 log MPN/sample. Data are presented as means ± standard deviation. Means with same small case letters are not significantly different.
Fig. 4.2. Coliform levels on watermelon surface harvested from low, medium, and high level of surrounding vegetation plots. The level of surrounding vegetation was converted to numeric value (0–10); 0 meant for no vegetation and 10 meant for highest amount of vegetation. Categorically, those plots with 0–3 were considered as low level of vegetation plots, 4–6 as medium level of vegetation plots, and 7–10 as high level of vegetation plots. The detectable limit was 0.30 log MPN/sample
Several studies reported a higher level (>5 log CFU/g) of microbial count on fresh produce such as cantaloupe, lettuce and broccoli (Johnston et al., 2005; Liu, Tan, Yang, & Wang, 2017; Zhang & Yang, 2017). The aerobic mesophilic count was more than 3 log CFU/g in the fresh cut-honeydew melons (Chong, Lai, & Yang, 2015). The total coliform level on the cantaloupe rinds collected from the field was 2.4 log CFU/g (Gagliardi, Millner, Lester, & Ingram, 2003). The level of E. coli varied with the types of the fresh produce, with highest count (geometric mean of 1.5 log CFU/g) in cantaloupe (Johnston et al., 2005). Pre-harvest activities are the important determinants for microbial contamination in produce. Studies reported that biological soil amendments of animal origin and untreated surface water are important sources of generic E.coli contamination in the farm (Annous et al., 2005; Tomas-Callejas et al., 2011). In our study, the low prevalence of generic E. coli on the watermelons at the time of harvest can be attributed to the factors such as following effective weed management strategies, avoiding use of untreated soil amendments as fertilizers, and untreated surface water for irrigation. The municipal water of sound microbiological quality used in the current study for the irrigation of watermelon plots helped to minimize irrigation water related contamination. Furthermore, the watermelons on the test plots were harvested 80 days after initial transplant. The prolonged exposure to natural sunlight and other environmental stress conditions may be associated with the lower levels of naturally present coliforms and generic E. coli on watermelon surface. These results emphasize the importance of following good agricultural practices (GAPs) to mitigate the risk associated with the microbial contamination of produce in farm environment. However, due to low prevalence of naturally present generic E. coli on the surface of watermelons under the tested conditions, the effect of surrounding vegetation on the microbial die-off rate is not fully understood. This information is critical when the grower does not meet the water quality requirements of FSMA produce safety
rule and solely relies upon on the farm microbial die-off to ensure safety of harvestable produce.

To understand the effect of surrounding vegetation on the microbial die-off rate at high microbial loads, a simulated on-farm study was conducted by artificially inoculating watermelon rind.

**Effect of surrounding vegetation on the die-off rate of generic *E. coli* on watermelon rind disc**

The die-off rate of generic *E. coli* on the watermelon discs under three different levels of vegetation is shown in Fig 3. At all three levels of surrounding vegetation, a significant reduction in the generic *E. coli* was observed from 0 to 12 h. The highest reduction was observed in the low vegetation level (2.58 log) followed by medium (1.14 log) or high (0.95 log) vegetation level. However, the difference in the reduction was statistically not significant (*P* > 0.05) between medium and high vegetation levels. A relatively high reduction of generic *E. coli* on the surface of watermelon was expected in the first few hours of exposure to on-farm daylight conditions. Brandl (2006) reported that the direct exposure of produce surface to the sunlight has detrimental effect on the survival of enteric bacteria. In our study, the watermelon discs at low surrounding vegetation were found to be fully exposed to sunlight. While the surface of watermelon was not fully exposed to sunlight at medium and high surrounding vegetation. This explains the possible cause for the high rate of initial reduction of generic *E. coli* at low vegetation level compared to others.
Fig. 4.3. Die-off of generic *E. coli* on watermelon discs under three level of surrounding vegetation plots. The level of surrounding vegetation was converted to numeric value (0–10); 0 meant for no vegetation and 10 meant for highest amount of vegetation. Categorically, those plots with 0–3 were considered as low level of vegetation plots, 4–6 as medium level of vegetation plots, and 7–10 as high level of vegetation plots. The detection limit was 1.65 log CFU/cm$^2$. 
Fig. 4.4. Inoculated watermelon discs kept on sterile petri plates placed randomly around in each level of surrounding vegetation plot.
After 12 h, a gradual increase in the levels of generic *E. coli* was observed in all vegetation levels (Fig. 4.3) indicating that bacterial cells might have adapted to the surrounding environment. In adverse environmental conditions, bacterial cells could maintain structural and genetic integrity by altering their cellular physiology (Brooks, Turkarslan, Beer, Lo, & Baliga, 2011). Berney et al. (2006) reported that *E. coli* could express adaptive response to UVA radiation, and resume their growth when UVA light is very scarce or when the UVA irradiation is stopped. In our study, the night time (i.e. between 12 h (6 pm) to 24 h (6 am) may have allowed adequate time for the bacterial cells to recover from damage and resume their growth. Studies reported that relative humidity and temperature could affect microbial survival or die-off rate on produce surface (Del Rosario & Beuchat, 1995; Stine, Song, Choi, & Gerba, 2005; Weller et al., 2017). During the current study, the relative humidity ranged from 62 to 74% and the average temperature ranged from 25 to 28.56°C (Table 4.1). We calculated correlation coefficient to see if the relative humidity and environmental temperature were associated with the bacterial survival. There was positive correlation (r=0.76) between the relative humidity and the bacterial survival in the medium vegetation level; however, the correlation was not significant in low and high vegetation levels (data not shown). We observed negative correlation (-0.93) between environmental temperature and the bacterial survival in high vegetation level, however, there was no significant correlation in other vegetation levels. The results indicated that the surrounding vegetation could alter environmental factors such as relative humidity and temperature which affect microbial survival.
Table 4.1. Data of on-farm environmental conditions over a period of 108 h for die-off rate measurement

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Day light length (h)</th>
<th>Average Temperature (°C)</th>
<th>Average Relative humidity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>NA</td>
<td>25</td>
<td>71</td>
</tr>
<tr>
<td>12</td>
<td>11:52:23</td>
<td>28.56</td>
<td>64</td>
</tr>
<tr>
<td>36</td>
<td>12:15:56</td>
<td>25.55</td>
<td>69</td>
</tr>
<tr>
<td>60</td>
<td>12:14:06</td>
<td>25.55</td>
<td>65</td>
</tr>
<tr>
<td>84</td>
<td>12:12:17</td>
<td>25.55</td>
<td>62</td>
</tr>
<tr>
<td>108</td>
<td>12:10:27</td>
<td>25</td>
<td>74</td>
</tr>
</tbody>
</table>
Several studies reported that the sunlight and its ultraviolet component is a significant factor in inhibiting pathogens (Davies & Evison, 1991; Heaton & Jones, 2008; Nyeleti et al., 2004). Nyeleti et al. (2004) found that simulated sunlight decontaminated *Salmonella* on the stainless-steel surface under field conditions. In addition, longer sunlight exposure may result in increased surface temperature of fruit and desiccation rate, which ultimately may cause additional stress to the bacterial cells on produce surface (Tomas-Callejas et al., 2011). In another study, Stine et al. (2005) reported that the cantaloupe surface under shading favors longer bacterial survival than those fully exposed to simulated sunlight. In contrast, Erickson et al. (2010) reported that physical shading condition did not significantly contribute to the *E. coli* survival on lettuce leaves than sunny condition. The effect of direct sunlight on microbial survival on produce surface cannot be underestimated. During initial stage of bacterial contamination in agricultural field, the surrounding vegetation could be a critical factor determining the bacterial growth on produce surface.

After 108 h, the highest reduction was observed in low vegetation level (3 log CFU/cm\(^2\)) (Fig. 3) with an average die-off rate of 0.66 log per day. This result was consistent with the results reported by other studies, especially on lettuce surface matrix. In field conditions, Weller et al. (2017) observed that the average daily die-off rate of *E. coli* on lettuce head was 0.52 log from their 10 days of die-off study. In a similar study, Bezanson et al. (2012) observed an average daily die-off rate of *E. coli* O157:H7 on lettuce by 0.56 log. It was interesting to notice that, in low vegetation level, the generic *E. coli* counts at 108 h were similar to those of 12 h. However, in medium and high vegetation level, the counts recovered to the initial level (~6 log CFU/cm\(^2\)). This indicates that the on-farm microbial die-off rate estimates based on environmental conditions such as exposure to sunlight, UV fraction of sunlight light, temperature and relative humidity variations...
and bacterial recovery mechanisms seems plausible in low vegetation conditions. Whereas, generic
\emph{E. coli} exhibited high survival especially when the melon surface was surrounded with medium
and high vegetation.

Overall, this study investigated the effect of surrounding vegetation on the survival of
generic \emph{E. coli} on the surface of watermelon. Controlling the common contamination sources such
as soil and irrigation water, the levels of naturally present generic \emph{E. coli} were very low (<1.5 log
MPN/watermelon), and the effect of surrounding vegetation was found to be insignificant.
However, there was a significant effect of surrounding vegetation on microbial survival when
watermelon rind discs were artificially contaminated with high levels of laboratory strains of
generic \emph{E. coli}. The findings of this study emphasize the importance of considering the effect of
surrounding vegetation while making decisions based on microbial persistence on produce surface.
Future studies should focus on combining contaminated irrigation water or composted and
manure-based soil amendments in conjunction with weed control to estimate generic \emph{E. coli} die-off rates on watermelon rind surfaces.

4.4. References

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CHAPTER 5
EFFECT OF RESIDUAL CHLORINE AND ORGANIC ACIDS ON SURVIVAL AND ATTACHMENT OF ESCHERICHIA COLI O157: H7 AND LISTERIA MONOCYTOGENES ON SPINACH LEAVES DURING STORAGE

5.1. Introduction

Produce has been one of the most commonly implicated food categories for foodborne outbreaks in the United States (Heiman, Mody, Johnson, Griffin, & Gould, 2015; Nguyen et al., 2015). During 1998–2013, the total number of outbreaks associated with raw produce was 972, which resulted in 34,674 illnesses, 2,315 hospitalizations, and 72 deaths (Bennett et al., 2018). Despite significant improvements in food safety, the number of outbreaks associated with produce has increased in the last few decades (Bennett et al., 2018). Norovirus, Shiga toxin-producing Escherichia coli (STEC), Salmonella and Listeria monocytogenes are the most common etiological agents associated with the outbreaks (Beuchat, 1996; Heiman et al., 2015).

Use of sanitizers in produce wash operation is a common practice in preventing cross-contamination between clean and contaminated products (Banach, Sampers, Van Haute, & Van der Fels-Klerx, 2015). However, after wash operations, there remains the chance of cross-contamination of produce. The water-rinsing step following sanitizing may spread pathogens throughout the produce (Murray, Wu, Shi, Jun Xue, & Warriner, 2017; Pérez-Rodríguez et al., 2014). Other post sanitizing activities such as chopping, shredding, handling and storage, before packaging, also have been reported to be the causes of cross contamination (Francis, Thomas, & O’beirne, 1999). Studies have frequently recovered L. monocytogenes from processing operations used to prepare ready to eat vegetables (Nguyen-the & Carlin, 1994). Contact surfaces such as

conveyor belts, knives, and reusable crates have also been demonstrated as potential sources of contamination between produce batches (Zilelidou, Tsourou, Poimenidou, Loukou, & Skandamis, 2015). Thus, it is essential to have an appropriate strategy in place that can prevent or control cross-contamination during post sanitizing activities.

Chlorine has a short shelf life in the presence of organic matter (Len et al., 2002). However, studies have shown that chlorine treatment can leave a residue at detectable levels on produce surfaces for hours (Cho et al., 2010). Organic acids, including lactic acid, are stable compounds (De Villiers, Wurster, & Narsai, 1997), that may persist on produce surfaces for a long period. Although the formation of carcinogenic halogenated disinfection by-products (DBP) like trihalomethanes (THMs) during chlorine treatment is an issue, the amount of the THMs on fresh produce has been reported to be negligible (Gómez-López, Marín, Medina-Martínez, Gil, & Allende, 2013; Klaiber, Baur, Wolf, Hammes, & Carle, 2005). Organic acids are generally recognized as safe (GRAS) (Bell, Cutter, & Sumner, 1997; Ölmmez & Kretzschmar, 2009). In the commercial scale, produce are stored for hours to days before being delivered to consumers. During that period, the residues may exert antimicrobial activities on produce surfaces. The change in surface-characteristics of the produce by the sanitizers may have an influence on the bacterial attachment (Jahid & Ha, 2012). The present study evaluated the effect of residual sanitizers on the survival and attachment of *E. coli* O157: H7 and *L. monocytogenes* on baby spinach during refrigerated storage (4°C).
5.2. Material and methods

Inoculum preparation

A cocktail of three *E. coli* O157: H7 strains (EC 4042, H1730 & ATCC 43895) and three *L. monocytogenes* strains (V7, LCDC 81-861 & 101M) were used in this study. Frozen cultures were activated in three successive passes and harvested to an initial inoculum of 8-9 log CFU/mL by following the procedure described by Adhikari et al. (2016). The broths used for the activation of *E. coli* O157: H7 and *L. monocytogenes* were tryptic soy broth (TSB) and TSB with 0.6% yeast extract, respectively.

Selecting spinach leaves and sanitizer treatment

Pre-bagged baby spinach leaves (Organic Marketside) were purchased from the local market on the day of the experiment. Intact leaves of similar size (~1g each leaf) were selected. Chlorine solution (free chlorine: 100 ppm, total chlorine 123 ppm, pH 8.4) was prepared following manufacturer’s instruction (Regular 8.25%, Clorox®, Oakland, CA, USA) immediately before use. The concentration of the chlorine was measured by colorimetric reaction with diethyl-p-phenylenediamine (DPD) using a Hach DR 900 Multiparameter Colorimeter (Hach CO, USA). 0.5% (pH 2.09) lactic acid (Alfa Aesar, MA, USA) and 0.5% (pH 2.83) acetic acid (BDH, PA, USA) solutions were prepared with sterilized deionized water. The spinach leaves were dipped (separately) into the chlorine solution (20°C) and organic acid solutions (20°C) for 5 min and 3 min respectively. Excess liquid on the spinach leaves was drained (30 secs) using a salad spinner (OXO®, PA, USA), and the leaves were packed in vegetable storage (LDPE) bags (Royal, PA, USA) and stored in a refrigerator (4°C).
Determination of residual chlorine

Chlorine solutions (250 ml) were adjusted to pHs 5.5 and 7.3 using 0.1N citric acid. The unadjusted chlorine solution had the pH of 8.4. The solutions were stored in glass bottles uncapped at room temperature (25°C). The concentration of free and total chlorine in the solutions was measured up to 6 h using a Hatch DR 900 Multiparameter Colorimeter. For the measurement of residual chlorine on the spinach leaves, a total of 5 g treated leaves were soaked in ten times (w/v; 50 ml) the amount of deionized water for 5 min with continuous stirring in a beaker covered with aluminum foil (Cho et al., 2010). The residual chlorine concentration in the rinsed water solution was measured up to 120 min by the method described above, and the residual chlorine concentration per kg leaves was calculated.

Bacterial inoculation

The spinach leaves were collected right after the sanitizer treatments (Chlorine, Lactic Acid and Acetic Acid) and after 30 min (chlorine only) of refrigerated storage following the treatment. A stainless steel ring (area-10 cm²) was placed on the center of the upper leaf surfaces, and the inside circular area was inoculated with 10µl inoculum of culture and spread with a glass hockey rod. Between each sample, the ring was sanitized with 70% ethanol and rinsed three times with sterile distilled water. The inoculated leaves were packed in vegetable storage bags and stored up to 48 h at 4°C.

Survival and Attachment Assay

The attachment characteristic of each tested *E. coli* O157: H7 strain was determined based on the curli expression using the congo red binding assay (Patel, Sharma, & Ravishakar, 2011). Each strain activated in TSB overnight were streaked on Tryptic Soy Agar (Criterion™, CA, USA) supplemented with 0.004% Congo red and 0.002% Coomassie brilliant blue and incubated at
13°C, 22°C, 28°C and 37°C up to 72 h. *E. coli* producing red colonies were recorded as curli producers, while those producing colorless colonies were recorded as curli negative. The attachment characteristics of *E. coli* O157: H7 and *L. monocytogenes* was investigated using attachment assay (Patel et al., 2011). Briefly, each pre-inoculated leaf (treated and control) was collected in a sterile water collection bottle (100 mL, Security-Snap™, Thermo Scientific™, USA) at 0 min, 15 min, 24 h and 48 h of storage. Then, 25 ml of PBS (pH 7.2) containing 0.1% Tween-20 was added to each bottle and vortexed for 20 sec. The leaves were finally rinsed with 10 mL PBS (1X) to remove the foam from the leaf surfaces. The washed solutions were collected in a sterile water sampling bottle and used to enumerate loosely attached cells. Each washed leaf was transferred into a 50 ml Falcon® tube containing 10 mL of PBS and homogenized for 30 sec at a high speed using a Fisherbrand™ 150 Hand Held Homogenizer (Fisher Scientific, USA). Between each sample, the homogenizer was sanitized with 70% ethanol and rinsed three times with sterile distilled water to remove residual alcohol. The homogenate was used to enumerate strongly attached cells. Adequate dilutions of each wash solution and homogenate were plated on MacConkey Agar with Sorbitol supplemented with Cefixime and Tellurite, Criterion™ (CA, USA) and Oxford Listeria Agar (Oxiod, UK) for the enumeration of *E. coli* O157: H7 and *L. monocytogenes*, respectively. The plates were incubated at 37 °C for 24 to 48 h. The total bacterial count was the sum of loosely attached (wash solution) and strongly attached (homogenate) cells, and the attachment strength (S₆ value) was calculated as the ratio of bacterial population recovered from homogenate (strongly attached cells) to the total bacterial cells (strongly attached + loosely attached).
Statistical analysis

*E. coli* O157: H7 & *L. monocytogenes* populations recovered from the spinach surfaces were converted to log CFU/cm². The reduction and attachment strength over time was analyzed by ANOVA using Proc mixed feature of SAS 9.4 (SAS Institute, Cary, NC, USA.). The level of statistical significance was P<0.05 in all cases.

5.3. Results and Discussion

Residual chlorine in the wash solutions and on the treated spinach surfaces

The loss of residual chlorine in the chlorine wash solutions with different pHs is shown in Fig. 5.1. Free chlorine (FC) is the measure of hypochlorous acid (HOCl), hypochlorite ion (OCl⁻) and dissolved chlorine gas (White, 2009). Total chlorine (TC) is the sum of free chlorine and combined chloramines (Zhou, Luo, Nou, Lyu, & Wang, 2015). At pH 8.4 (without pH adjustment) the initial (0 h) level of residual chlorine (FC: 85.5±2.0 & TC: 107.5±1.5 mg/L) was greater compared to pH 7.3 (FC: 81.5±2.5 & TC: 102.5±2.2) and 5.5 (FC: 80±1.6 & TC: 103.5±2.8 mg/L). The chlorine loss was greatest (P<0.05) at pH 5.5; FC and TC decreased to 17.75± 6.0 mg/L, and 53.75±0.4 mg/L respectively after 6 h. The greatest level of residues was maintained in the solution with the pH of 8.4. After 6 h, the level of FC and TC was 67±7.8 mg/L and 101.5±1.4 mg/L respectively. Our study concurred with other study results. Len et al (2002) observed that there was a decrease in chlorine loss with an increase in pH in electrolyzed oxidizing water. Almost no change in chlorine concentration was observed at pH 9.0 up to 240 min. Waters and Hung (2014) observed similar results.
Fig. 5.1. The residual chlorine (total and free) in the wash solutions with different pH (5.5, 7.3 and 8.4) during storage at 25°C. FC: free chlorine, TC: total chlorine. The data are presented as mean ± standard deviation.
The level of residual chlorine on spinach leaves treated with chlorine (free chlorine 100 ppm, pH 8.4, unadjusted) and refrigerated is shown in Fig. 5.2. Immediately after treatment, the spinach leaves had free (FC) and total residual chlorine (TC) concentrations of 7.2±0.8 and 10.6±0.9 mg/kg, respectively (Fig. 2). The concentration depleted continuously with time. After 30 min, the concentration of FC and TC was 6±2.0 mg/kg and 7±0.7 mg/kg respectively. The FC level was detectable only up to 90 min (0.3±0.1 mg/kg). Total chlorine concentration was 1 mg/kg at 120 min.

In the presence of organic matter, the loss of chlorine accelerates through the decomposition reaction (Len et al., 2002). Studies have demonstrated that plant exudates led to the decomposition reaction during chlorine-based wash operations (Delaquis, Fukumoto, Toivonen, & Cliff, 2004; Gil, Selma, López-Gálvez, & Allende, 2009). In our study, the invisible leaf exudates might have contributed to the rapid loss of residual chlorine on the spinach surfaces. A similar study observed a significant depletion of residual chlorine on fresh-cut lettuce after 3 min (Cho et al., 2010). However, a detectable amount of chlorine was present until 7 h of storage at 4°C. The presence of residual chlorine on spinach for more than an hour indicated that the residue could have a role in antimicrobial activity during storage.
Fig. 5.2. Residual chlorine (total and free) on spinach surfaces during storage (4°C). The data are presented as mean ± standard deviation.
Effect of residual sanitizers on the survival of *E. coli* O157: H7 and *L. monocytogenes*

i) Residual chlorine

The influence of residual chlorine on the survival of *E. coli* O157: H7 and *Listeria monocytogenes* on spinach leaf surfaces is shown in Fig. 5.3. The chlorine residue resulted in a significantly greater level of reduction (P<0.05) in *E. coli* O157: H7 and *L. monocytogenes* populations compared to the control (distilled water), during refrigerated storage. After 15 min, the population of *E. coli* O157:H7 and *L. monocytogenes* on chlorine sanitized spinach surfaces decreased by 1.78 log and 2.14 log CFU/cm$^2$, respectively. After 48h, the total reductions were 2.64 log CFU/cm$^2$ and 3.15 log CFU/cm$^2$, respectively.

In this study, chlorine (30 min) samples represent leaves contaminated in 30 min after sanitizer wash. Although post-sanitizing/washing activities may vary with the operation system, we assumed that the first 30 min is the most critical time in terms of cross-contamination. As the chlorine in the permissible limit has been found to be unable to eliminate pathogens completely from fresh produce (Behrsing, Winkler, Franz, & Premier, 2000), post sanitizing activities such as transportation, handling, cutting, shredding and storage may contribute the transfer of pathogens from contaminated to clean leaves. Sometimes, the processing facility itself becomes a source of cross-contamination (Nguyen-the & Carlin, 1994). Our study showed that the residues present on the spinach leaves within 30 min after the treatment reduced the pathogens load significantly (P<0.05) in 15 min of exposure. After 48 h, the total reductions in *E. coli* O157: H7 and *L. monocytogenes* population were by 2.88 log CFU/cm$^2$ and 1.32 log CFU /cm$^2$ respectively.
Fig. 5.3. Effect of residual chlorine on the survival of *E. coli* O157: H7 (A) and *L. monocytogenes* (B) on spinach leaves during refrigerated storage. The data are presented as average counts ± Standard Error. Different lowercase letters on the top of the bar diagrams indicate significant difference (P<0.05) in between the treatments.
In this study, chlorine (30 min) samples represent leaves contaminated in 30 min after sanitizer wash. Although post-sanitizing/washing activities may vary with the operation system, we assumed that the first 30 min is the most critical time in terms of cross-contamination. As the chlorine in the permissible limit has been found to be unable to eliminate pathogens completely from fresh produce (Behrsing, Winkler, Franz, & Premier, 2000), post-sanitizing activities such as transportation, handling, cutting, shredding and storage may contribute the transfer of pathogens from contaminated to clean leaves. Sometimes, the processing facility itself becomes a source of cross-contamination (Nguyen-the & Carlin, 1994). Our study showed that the residues present on the spinach leaves within 30 min after the treatment reduced the pathogens load significantly (P<0.05) in 15 min of exposure. After 48 h, the total reductions in E. coli O157: H7 and L. monocytogenes population were by 2.88 log CFU/cm² and 1.32 log CFU/cm² respectively.

Although considerable research has been carried out on the disinfection efficacy of chlorine on pre-inoculated produce surfaces, to the authors’ knowledge, there are limited reported studies on the influence of residual chlorine against pathogens on produce surfaces during subsequent storage. Studies have reported that the hypochlorite in commonly used concentration could reduce bacterial pathogens by 1 to 2 log (Parish et al., 2003). Li et al. (2001) observed similar results against E. coli O157: H7 population on lettuce right after chlorine treatment. However, the population on the treated lettuce significantly decreased compared to the untreated controls during refrigerated storage (5°C). The higher level of reduction of the bacterial population on sanitizers treated leaves may be attributed to the pre-existence of chlorine on the leaf surfaces, which might have created an unfavorable condition for the colonization and survival of the bacteria. Refrigerated storage temperature might have further contributed for the overall reductions (Abdul-Raouf, Beuchat, & Ammar, 1993). A study reported a synergistic effect of chlorine and
refrigerated temperature for the higher level of reductions in *E. coli* O157: H7 population on chlorine treated lettuce (Li, Brackett, Chen, & Beuchat, 2001). The results indicated that the residual chlorine exerted antimicrobial activity steadily during the storage.

**ii) Residual organic acids**

The survival of *E. coli* O157: H7 and *L. monocytogenes* on organic acid- treated spinach surfaces during storage is shown in Fig. 5.4. There were no significant changes in *E. coli* O157: H7 populations within 15 min of storage at 4° C, however, after 24 h, the population decreased from 5.0 ± 0.5 log CFU/cm² to 2±0.8 log CFU/cm² on lactic acid treated leaves (Fig. 4 A). The *L. monocytogenes* population on lactic acid treated leaves decreased from 5.8± 0.4 log CFU/cm² to 5.09 ±0.3 log CFU/cm² in 15 min of storage (Fig. 4 B). After 48 h, the population reduced to 4.42 ±0.3 log CFU/cm². For acetic acid (0.5%) treated leaves, there was no significant effect for both pathogens.
Fig. 5.4. Effect of residual organic acids on the survival of *E. coli* O157: H7 (A) and *L. monocytogenes* (B) on spinach leaves during refrigerated storage. The data are presented as average counts ± Standard Error. Different lowercase letters on the top of the bar diagrams indicate a significant difference (P<0.05) in between the treatments.
Variable results have been reported on the effectiveness of lactic acid and acetic acid in inhibiting pathogens on pre-inoculated produce. It was reported that lactic acid (0.5%) wash for 2 min was as effective as aqueous chlorine (100 ppm) wash against *E. coli* and *L. monocytogenes* on fresh-cut iceberg (Akbas & Ölmez, 2007). Lactic acid (1%) treatment resulted in a 2.70 log reduction of *E. coli* O157:H7 population on baby spinach (Huang & Chen, 2011). While another study reported that 0.5% lactic acid could reduce the population of the pathogen on lettuce by less than 1 log_{10} CFU/g (Sagong et al., 2011). The level of reduction in 15 min of exposure was similar to the findings of the latter study. However, continuous decline of *E. coli* O157: H7 population (by 3 log CFU/g) after 24 h of storage indicated that lactic acid residue can exert continuous antimicrobial activity during storage. The residual lactic acid was more effective against *E. coli* O157: H7 compared to *L. monocytogenes*.

In our study, we did not observe a significant effect of acetic acid (0.5%) residue on the bacterial survival up to 48 h of storage. Another study reported less than 1 log CFU/g reduction in *E. coli* O157: H7 population on pre-inoculated iceberg lettuce when treated with 0.5% acetic acid (Chang & Fang, 2007). The effectiveness of the acid increased with increase in the concentration (Chang & Fang, 2007; Sagong et al., 2011). We tried to increase the concentration of acids in the wash water. However, the increase in concentration greatly affected the physical appearance of the spinach leaves after 48 h. At 0.5%, there was almost no effect on the color and texture (Fig. 5.5). At 1% lactic acid, some yellowish-brown spots were observed, and at 1% acetic acid, the petiole (base) part of the leaves withered losing the surface glossiness.
Fig. 5.5. Physical appearance of spinach leaves treated with organic acids. A) 0.5% lactic acid B) 1.0% lactic acid, C) 0.5% acetic acid D) 1% acetic Acid. The arrow in B is showing brownish yellow spots on the spinach leaf, and the arrow in D is showing welting of petiole and the base.
Effect on the bacterial attachment

Each of the bacterial strains used in the study was assessed for their attachment characteristics through curli expression (*E. coli*) and attachment assay (*L. monocytogenes*). The curli expression has been found to be an important mechanism in *E. coli* for the attachment and biofilm formation (Patel et al., 2011). We observed that all three *E. coli* O157: H7 strains had similar curli expression characteristics. They produced curli negative colonies (colorless) at 13°C, 22°C and 28°C and curli positive colonies at 37°C, after 72 h of incubation. The attachment strengths of tested *L. monocytogenes* strains at 0 h ranged from 0.13 to 0.23, which increased with the storage time. There was no significant difference (P<0.05) in attachment strength among the tested strains (data not shown). The ability of bacterial pathogens to attach on produce surfaces is a big challenge to produce safety. The efficacy of the antimicrobials has been found to be dependent on the level of the bacterial attachment or biofilm formation (Jahid & Ha, 2012; Ryu & Beuchat, 2005). Our study evaluated the changes in the loosely attached and strongly attached populations of the pathogens on the sanitizer treated spinach surfaces during refrigerated storage (Fig. 5.6 and 5.7). After 15 min of exposure (0 h), all except chlorine treated leaves had a greater level of the loosely attached population of *E. coli* O157: H7 compared to the strongly attached population (Sₐ<0.5). After 24 h, the loosely attached population decreased below the strongly attached population on the chlorine and lactic acid treated leaves. After 48 h, the level of reduction in strongly attached population and loosely attached population ranged from 0.68 (chlorine) to 1.10 log CFU/cm² (lactic acid) and 1.85 log CFU/cm² (chlorine) to 3.07 log CFU/cm² (lactic acid) respectively.
Fig. 5.6. Attachment of *E. coli* O157: H7 on sanitizers treated spinach surfaces during refrigerated storage (4°C). The population of loosely attached and strongly attached cells is shown. $S_R$ Value (Attachment Strength) = strongly attached cells/total cells (strongly attached + loosely attached). A) Control (Deionized water) B) Chlorine C) Chlorine (30 min) D) Lactic acid E) Acetic acid. In the figures, 0 h represents the time 15 min after the bacterial inoculation. Immediately after inoculation (0 min), loosely attached population, strongly attached population and $S_R$ values were 6.1 log CFU/cm², 4.9 log CFU/cm² and 0.06 respectively (not shown in the figure), which were not significantly different from the values after 15 min (0 h) on the control samples. The initial inoculum levels on the chlorine treated and organic acid treated samples were different (P < 0.05).
The initial proportion of the loosely attached *L. monocytogenes* cells was greater compared to the strongly attached population on all treatments. The reductions in the loosely attached populations were significantly (P<0.05) higher compared to the strongly attached populations for the chlorine and lactic acid treated leaves during storage. After 48 h, the reduction in the loosely attached population was 2.46 log and 1.07 log CFU/cm² for chlorine and lactic acid treated leaves respectively. While in the strongly attached population, the reduction was by 0.81 log and 0.54 log CFU/cm² respectively. We observed that there were no significant changes in the bacterial populations on acetic acid and deionized water treated leaves.

On the control leaves, there was no significant change in the attachment strength (S_R) of *E. coli* O157: H7 on the spinach surfaces during refrigerated storage (Fig 5.6 A). The results agree with the findings of other studies (Patel et al., 2011; Xicohtencatl-Cortes, Chacón, Saldaña, Freer, & Girón, 2009). It was observed that the majority of the tested *E.coli* O157: H7 strains had lower S_R values (P<0.05) on the vegetable surfaces during storage up to 24 h (10°C) (Patel et al., 2011). There was no significant colonization of *E. coli* O157: H7 on spinach and lettuce leaves up to 24 h at 4°C (Xicohtencatl-Cortes et al., 2009). A lower level of attachment strength of this pathogen may be attributed to its lower activity and the lack of curli expression at refrigerated temperature (Abdul-Raouf et al., 1993). Increase in S_R value of *L. monocytogenes* during storage indicated that this pathogen has the potential to attach to produce at low temperature. Its greater level affinity to produce surface is also demonstrated by other studies. *L. monocytogenes* had a higher level of attachment (80% attached cells) to cabbage surfaces after 24 h of storage at 10°C (Ells & Hansen, 2006). There was an increase in attached *L.*
*L. monocytogenes* population from around 3 log to 5 log CFU/g at 10°C after 24 h (Gorski, Palumbo, & Mandrell, 2003).

Although the $S_R$ values increased after 24 h, the overall populations of the strongly attached cells did not change significantly on the chlorine and lactic acid treated leaves. Studies have demonstrated a negative association between sanitizers and bacterial attachment. It was observed that the sanitizers reduced the attachment level of the *Salmonella* spp. and *L. monocytogenes* on abiotic surfaces (Sinde & Carballo, 2000). In our study, the sanitizer residues might have prevented the attachment of the remaining pathogens during storage. The lower level of efficacy of sanitizers against attached pathogens has been reported by several studies (Ryu & Beuchat, 2005; Ukuku & Fett, 2002), which was evident also in our study.

Overall, we observed that the residual chlorine and lactic acid had a promising effect on reducing *E. coli* O157: H7 and *L. monocytogenes* populations on spinach surfaces during refrigerated storage (4°C). The overall reduction was mainly attributed to the reduction in the loosely attached populations. The results indicated that the use of chlorine and lactic acid in produce wash operations could have a role in reducing the risk associated with post sanitizing cross-contaminations.
Fig. 5.7. Attachment of *L. monocytogenes* on the sanitizers treated spinach surfaces during refrigerated storage (4°C). The population of loosely attached and strongly attached cells is shown. $S_R$ Value (Attachment Strength) = strongly attached cells/ total cells (strongly attached + loosely attached). A) Control (Deionized water) B) Chlorine C) Chlorine (30 min) D) Lactic acid E) Acetic acid. In the figures, 0 h represents the time 15 min after the inoculation. Immediately after inoculation (0 min), loosely attached population, strongly attached population and $S_R$ values were 6.5 log CFU/cm$^2$, 5.4 log CFU/cm$^2$ and 0.07 respectively (not shown in the figure), which were not significantly different from the values after 15 min (0 h) on the control samples. The initial inoculum levels on the chlorine treated and organic acid treated samples were different ($P < 0.05$).
5.4. References


6.1. Introduction

Bacterial pathogens can enter into a viable but non-culturable (VBNC) state due to environmental stress and sanitizers (Orta de Velasquezet, Yanez Noguez, Casasola Rodriguez & Roman Roman, 2017; Oliver, 2010). Oliver, Dagher & Linden, 2005 observed that E. coli and S. typhimurium population (<0.4%) entered into VBNC state in the presence of chlorine in wastewater. Another study showed that chlorine disinfection resulted in induction of VBNC state in Helicobacter pylori (Orta de Velasquezet et al., 2017). When L. monocytogenes cells were treated with potassium sorbate, the cells converted into VBNC state for several hours (Cunningham, O’Byrne, & Oliver, 2009). These findings indicated that microbial assessment requires a technique that can discriminate live pathogens in food.

Conventional culture-based techniques and PCR assay have a limitation to detect VBNC cells (Yuan, Zheng, Lin & Mustapha, 2018). Propidium Monoazide (PMA) PCR was developed to overcome this limitation (Nocker, Sossa & Camoer, 2007). PMA-PCR can discriminate live pathogens in a food matrix within 3 h providing important information from a public health risk perspective (Mustapha & Liu, 2014). This technique has been successfully used for bacteria, viruses, fungi and protozoa (Elizaquive, Aznar, & Sánchez, 2013).

PMA combined quantitative PCR discriminates live and dead microbial cells based on the integrity of the cells (Yuan et al., 2018). PMA penetrates the compromised membranes of dead cells, and its azide groups covalently bind to cellular DNA to form irreversible nitrogen-carbon
bonds under photolysis resulting in bound DNA unable to amplify by a subsequent PCR assay (Fittipaldi, Nocker & Codony, 2012). On the other hand, the viable cells that have intact cell membranes will not be affected by the dyes (Dinu & Bach, 2013). Our previous study showed a significant reduction of *E. coli* O157: H7 & *L. monocytogenes* due to residual chlorine and lactic acid on spinach surface using culture based method (Chhetri, Janes, King, Doererler & Adhikari, 2019). This study investigated the viability proportion of *L. monocytogenes* and *E. coli* O157:H7 on chlorine and lactic acid treated spinach surfaces.

### 6.2. Material and methods

Pre-bagged baby spinach leaves (Organic Marketside) were purchased from the local market on the day of the experiment. Intact leaves of similar size were selected. Chlorine solution (free chlorine: 100 ppm, total chlorine 123 ppm, pH 8.4) was prepared immediately before use. The concentration of the chlorine was measured by colorimetric reaction with diethyl-p-phenylenediamine (DPD) using a Hach DR 900 Multiparameter Colorimeter (Hach CO, USA). 0.5% lactic acid (pH 2.09) (Alfa Aesar, MA, USA) solutions was prepared with sterilized deionized water. The spinach leaves were dipped (separately) into the chlorine solution (20°C) and 0.5% lactic acid (20°C) for 5 min and 3 min, respectively. Excess liquid on the spinach leaves was drained (30 secs) using a salad spinner (OXO®, PA, USA). The leaves were spot inoculated with a cocktail (3 strains) of *E. coli* O157:H7 (EC 4042, H1730 & ATCC 43895) and *L. monocytogenes* (V7, LCDC 81-861 & 101M), and stored up to 48 h maintaining 4°C. The leaf samples were processed for the microbial analysis at 0 h, 24 and 48 h of storage. Briefly, the leaves were homogenized in 20 mL PBS (1X) and filtered in a stomacher filter bags (Control Numero 5, Seward, UK). The supernatant solutions (400 µL each) were placed in 24 well microplate. For the live bacterial count, the samples were treated with PMA dye following manufacturer’s instructions
(Biotium, Inc. USA). For DNA extraction, the samples were suspended in 100 μL of PrepMan® Ultra Sample Preparation Reagent, and heated for 10 min (Applied Biosystems, Foster City, CA, USA). The extracted DNA in the supernatant solution was centrifuged at 13,000 g for 5 min. A total of 2 μL supernatant was used as the template DNA for real-time PCR of assay. For *E. coli* O157: H7, the primer and probe used was targeted for open reading frame (ORF)- Z3276. For *L. monocytogenes*, the primer pairs were specific to “hly” gene, as shown below.

<table>
<thead>
<tr>
<th><em>E. coli</em> O157: H7 “Z3276”</th>
<th><em>L. monocytogenes</em> “hly”</th>
</tr>
</thead>
<tbody>
<tr>
<td>For: 5’-GCACTAAAAGCTTGGAGCAGTTC-3’</td>
<td>For: 5’-GGGAAATCTCTCAGGGATGATG-3’</td>
</tr>
<tr>
<td>Rev: 5’-AACAATGGGTCAGCGTGTAAGGCTA-3’</td>
<td>Rev: 5’-CGATGATTGAACCTCATCTTCTTGC-3’</td>
</tr>
</tbody>
</table>

All qPCR reactions were performed in a Cepheid SmartCycler (Cepheid, USA). The cycling parameters for the quantification of *E. coli* O157: H7 were: 7 min at 95 °C (initial polymerase activation) followed by 40 cycles of 5s at 95 °C (denaturation) and 30s at 52°C (annealing/extension). For *L. monocytogenes*, the parameters were 8 min at 95 °C (initial polymerase activation) followed by 40 cycles of 5 s at 95 °C (denaturation) and 30 s at 63°C (annealing/extension). For the standard curve, the bacterial cultures (~ 10.5 log CFU/mL) were spiked in PBS and diluted to 10 fold. The inoculated solutions were processed in the similar way to the sample supernatants. The difference between the amount of total (Ct\(_{\text{total}}\)) and live-cell-derived DNA (Ct\(_{\text{live}}\)) was assessed by calculating dCt values (dead cells), and the values were converted to corresponding bacterial counts (Fig 6.1).
Fig. 6.1. Standard curve of bacterial pathogens using quantitative PCR. A) *E. coli* O157: H7 b) *L. monocytogenes*. A series of 10 fold dilutions of cells obtained after PMA treatment. Y-axis, Ct : threshold cycle ; X axis, bacterial count –log CFU/mL or g.

Fig. 6.2. Threshold level and Ct value on a real-time PCR amplification curve.
Table 6.1. Viability of *E. coli* O157: H7 and *L. monocytogenes* on sanitizer treated spinach leaves

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Sanitizers</th>
<th>0h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Live</td>
<td>Dead</td>
<td>Live</td>
</tr>
<tr>
<td><em>E. coli</em> O157: H7</td>
<td>Distilled water</td>
<td>7.02</td>
<td>0.48</td>
<td>7.80</td>
</tr>
<tr>
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<td>6.13</td>
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<td>Lactic acid</td>
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<td>Lactic acid</td>
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*Dead cells: Total cells – live cells, count: log CFU/g*
6.3. Results and discussion

The proportion of live and dead population of *L. monocytogenes* and *E. coli* O157:H7 on sanitizer treated leaves during storage using PMA-qPCR is shown in table 6.1. The number of live cells decreased with time on both treated and control spinach leaves. At 24 h of storage, live *E. coli* O157: H7 population slightly increased (from 7.02 to 7.80 log CFU/g) on control samples. However, on chlorine and lactic acid treated leaves, live *E. coli* O157: H7 population decreased from 7.18 and 7.78 log CFU/g to 6.13 and 6.50 log CFU/g, respectively. Total viable count further decreased to 4.32 and 3.36 log/g, respectively. We observed a similar trend on the viability of *L. monocytogenes* on spinach surfaces during refrigerated storage. On the control samples, there was an increase in live count. After 24 h, the live *L. monocytogenes* population increased from 6.41 to 6.56 log CFU/g, which further increased to 7.14 log CFU/cm² after 48 h of storage. However, the live population on chlorine and lactic acid treated leaves decreased from 6.56 log and 7.15 log CFU/g to 5.54 and 6.44, respectively, after 24 h. After 48 h, the population dropped to 3.77 and 3.44, respectively. Although residual chlorine resulted in greatest reduction in the live population of both pathogens at 24 h, after 48 h, the reduction was greatest on lactic acid treated leaves. Like our previous study (Chhetri, Janes, King, Doerrler & Adhikari, 2019), this study showed a promising role of chlorine and lactic acid in reducing viable bacterial pathogens on produce surfaces.

Studies have shown that chlorine resulted in induction of VBNC in bacterial pathogens (Cunningham, O’Byrne, & Oliver, 2009; Oliver, Dagher & Linden, 2005; Orta de Velasquez et al., 2017). In the presence of 0.96 mg/l of free chlorine in water, *Helicobacter pylori* lost its culturability in 5 min (Moreno et al., 2007). *E. coli* and *S. typhimurium* population (<0.4%) entered into VBNC state in the presence of chlorine in wastewater. Similarly, potassium sorbate induced
the VBNC state of \textit{L. monocytogenes} (Cunningham, O’Byrne, & Oliver, 2009). The PMA-qPCR has been used by several studies to examine the effect of sanitizers on the viability of bacterial pathogens (Elizaquível, Aznar, & Sánchez, 2014). Nocker, Sossa, & Camper (2007) found viability PCR suitable for monitoring disinfection efficacy of sanitizers such as hypochlorite and benzalkonium against \textit{S. typhimurium, L. monocytogenes, E. coli} O157:H7 and \textit{Mycobacterium avium}. This method has been suitable for viable bacterial analysis in fresh-cut vegetables (Elizaquível et al., 2012). However, the sensitivity of the method is dependent on the concentration of PMA, length of exposure to photo-activation and type of samples (Elizaquível, Aznar, & Sánchez, 2014; Elizaquível et al., 2012; Li and Chen 2012). Our present results were obtained using a general protocol provided by the manufacturer (Biotium Inc, USA). The protocol used PMAxx of final concentration of 25 µM and 50 µM for \textit{E. coli} O157: H7 and \textit{L. monocytogenes}, respectively, and photo activation time of 15 min. As the type of food matrix may influence on the sensitivity of the method, our further studies will consider the potential influencing factors. Studies used several types of genetic markers to detect \textit{E. coli} O157: H7 (Yuan, Zheng, Lin, & Mustapha, 2018, Zhong, & Zhao, 2018). In this study, we used ORF Z3276 because of its specificity and sensitivity with PMA –qPCR (Li and Chen 2012). Here, we have presented preliminary results. Our future study will present detailed findings on the viability of the pathogens due to residual sanitizers.

6.4. References


CHAPTER 7
CONCLUSIONS

Pre-harvest and post-harvest activities have been reported to be important sources of produce contamination. The survival of microbial contaminants on produce surfaces can be influenced by environmental stress in agricultural field and post-harvest conditions. This study investigated the survival and the attachment of bacteria on produce surfaces in an agricultural environment and in the presence of residual sanitizers. FDA Food Safety Modernization act has considered bacterial die-off under agricultural environments as one of the strategies to reduce produce safety risks. We calculated an average daily die-off rate of \textit{E. coli} on watermelon surfaces in an agriculture setting in a south central location of the United States. Our results indicated that microbial die-off occurs on produce surfaces under agriculture environment conditions. However, the die-off rate may vary from one day to another after a contamination event. The daily die-off rate of \textit{E. coli} ranged from -0.12 to 1.3 log CFU/cm$^2$, with a total reduction by 1.94 log CFU/cm$^2$ in 5 days. There was a significant effect of surrounding vegetation on bacterial die-off rate when watermelon rind discs were artificially contaminated with high levels of laboratory strains of generic \textit{E. coli}. These findings emphasize the importance of considering microbial die-off and the associated factors while developing food safety risk management strategies.

The attachment strength (S$R$ value) of the \textit{E. coli} cells on watermelon surfaces significantly increased (P<0.05) from 0.04 to 0.99 in the first 24 h, which was primarily due to the decrease in loosely attached population, given that the population of strongly attached cells was constant. This indicated that the majority proportion of \textit{E. coli} cells might remain in strongly attached form on produce surfaces under agriculture environment conditions. We observed a lower efficacy of chlorine treatment with increase in attachment level emphasizing the importance of considering
the bacterial attachment level while developing post-harvest risk management strategies.

Use of sanitizers in produce wash operation is a common practice in preventing cross-contamination between clean and contaminated products. After sanitizer wash operations, the produce may go through several steps such as chopping, shredding, handling, storage, and packaging. Our study observed that the residual chlorine and lactic acid could have a promising effect on reducing *E. coli* O157: H7 and *L. monocytogenes* populations on spinach surfaces during refrigerated storage (4°C). Our PMA-PCR study also revealed similar results. The viable population of *E. coli* O157: H7 and *L. monocytogenes* on spinach surfaces due to residual sanitizers decreased over time. In the commercial scale, produce is stored for hours to days before being delivered to consumers. The use of chlorine and lactic acid in produce wash operations could have a role in reducing the risk associated with post sanitizing cross-contaminations.
## APPENDIX. SUPPLEMENTARY MATERIAL

Natural *E. coli* levels on upper half and lower half surfaces of watermelons

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Location on the watermelons surfaces</th>
<th>Total count CFU/sample</th>
<th>Surface Area of the samples (cm²)</th>
<th>Count CFU/cm²</th>
<th>Count log CFU/cm²</th>
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VITA
Vijay Singh Chhetri was born in Arghakhanchi, Nepal. Vijay earned his Bachelor and Master’s degree in Microbiology from Tribhuvan University, Nepal. After receiving his Master’s degree, he worked as a food safety consultant and an instructor of food microbiology for around 5 years. Vijay began his Ph.D. in Food Science and Technology in June 2015 at Louisiana State University. He has worked as a graduate research assistant in the School of Nutrition and Food Sciences (SNFS) from June 2015 to May 2019. As a graduate student, Vijay has been fortunate to be involved in LSU AgCenter Food Safety Extension activities. He is currently a Ph.D. candidate. He anticipates graduating in May 2019.