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The Effects of Electrostatic Spraying with Organic Acids in the Disintegration of Biofilms Formed by E.coli O157:H7 and Salmonella Typhimurium on Spinach and Cantaloupe

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ABSTRACT

Outbreaks from the consumption of fresh produce are a concern in the United States. The consumptions of fresh produce have increased recently which expose a large segment of society to such outbreaks. Spinach and cantaloupe are minimally heated or processed before consumption which makes them a possible source of foodborne illness. The objective of this research was to investigate the effect of organic acids alone and in combination to reduce attached *Salmonella* Typhimurium (S.T) and *Escherichia coli* O157:H7 (*E.coli*) on spinach and cantaloupe, and to disintegrate biofilm formed by these pathogens by electrostatically spraying with two organic acids. To quantify the attachment, enumeration of attached bacteria was conducted. *E.coli* strains used in the study are ED 14, ED 15, ED 16, MD 46, MD 47, and MD 58. S.T strains used in the study are SD 10, and SD 11. *E.coli* ED 14 demonstrated the highest attachment property. Electrostatic spraying of organic acids showed that in spinach and cantaloupe lactic acid + malic acid at 2.0 % each showed the highest log reductions with 4.1 in spinach and 3.5 in cantaloupe, respectively. Strain dependency was observed in biofilm formation on spinach and cantaloupe homogenate using crystal violet assay. The images by confocal microscope showed the biofilm of *E.coli* and S.T was disintegrated by treatment with organic acids. Quorum sensing activity quantified by autoinducer AI-2 assay bases on the reporter strain *V.harveyi* BB 170 showed inhibition of the autoinducer compound by organic acid treatment.

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DEDICATION

This Master's thesis is dedicated to King Abdullah ibn Abdilaziz whose vision about Saudi Arabia is the sole reason thousands of Saudis are pursuing their education around the world.

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Chapter 1: Introduction

Outbreaks from the consumption of fresh produce are a concern in the United States (Brooks and Flint 2008). The consumption of fresh produce has increased since the last 20 years (Brooks and Flint 2008; Taylor 2009; Olaimat and Holley 2012) because of the healthy trend that consumers around the world are adapting to by eating more fresh produce. Fresh produce are minimally heated or processed before consumption, which makes them a possible source of foodborne illness.

Since human pathogens are found to survive for a long-period of time in water, animal manure, and a variety of agricultural soils (Sivapalasingam and others 2004) contamination of fresh produce by pathogens can occur from the farm to the fork. For instance, fresh produce can be contaminated in the fields, at the time of harvesting, during handling, in processing, at green houses, during distribution while preparing foods at home or in restaurants (Beuchat 1996, 2002; Lynch and others 2009; Jahid and Ha 2012).

Washing with chlorine at the concentrations ranging from 50 to 200 ppm is used frequently for fresh produce decontamination. However, this treatment only reduces bacteria by less than 3 logs (Han and others 2000). Certain types of foods have been linked to certain pathogens outbreaks. For example, melons, tomatoes, and sprouts have been linked with salmonellosis (Hanning and others 2009). Leafy green vegetables have been linked with *E. coli* O157. It is essential to find a way to limit contamination of fresh produce since people consume a lot of fresh produce that have not been thermally treated or effectively washed (Lynch and others 2009).

Washing procedures lack a “kill” step in fresh produce decontamination (Kim and Wei 2012). The purpose of washing is removing soil from the produce (Sapers 2001). As a result,

pathogens survive after washing, causing harmful diseases.

The ability of pathogens to survive washing is attributed to their ability to infiltrate, internalize, and form biofilms and on the plant tissues (Annous and others 2009; Jahid and Ha 2012; Ukuku and others 2005), which makes them a serious problem for the food processing industry (Stier 2005). Biofilms are microbes that attach to surfaces or interfaces that produce exopolymeric substances for protection.

The quest for preventing foodborne illness is essential not only from the point of view of public health organizations but also from an economical perspective since food recalls cost a substantial amount of money (Taylor 2009). Biofilms formed by pathogens are becoming more and more resistant to antimicrobials (Van Houdt and others 2004) (Annous and others 2009) (Agle 2007), biocides (Annous and others 2009). Disinfectants (Rayner and others 2004), and sanitizers (Jahid and Ha 2012) that are used in the food industry (Annous and others 2006). Another problem is that biofilms are more likely to survive severe environmental conditions present in the food industry (Annous and others 2009). Furthermore, bacteria in biofilm are less affected by chemical stresses such as pH, oxygen, and physical stresses like pressure, heat, ultraviolet rays, and freezing (Annous and others 2009; Stier 2005; Agle 2007).

The formation of biofilm is the underlying reason why washing produce surfaces does not remove or inactivate human pathogens (Annous and others 2006; Sapers 2001). It has been reported that cells attached to the biofilm might be 150-3,000 times resistant to hypochlorous acid than cells that are not. For example, attached cells of *Listeria monocytogenes* can survive the exposure to benzalkonium chloride up to 12-20 minutes unlike free cells that only can survive for 30 seconds (Stier 2005).

Biofilm formation occurs in five stages (Tarver 2009). The first step of biofilm formation

is bacterial attachment. The attachment is caused by Van der Waals' forces (Brooks and Flint 2008; Stier 2005; Tarver 2009; Agle 2007). This initial attachment is weak and ease to be removed by rinsing (Stier 2005; Agle 2007). The steps involved in biofilm formation are: irreversible attachment of the bacteria into the surface, formation of microcolonies and biofilm maturation, formation of 3-D structure and water channels, and detachment form the mature biofilm and reattach.

Forming a biofilm in the lab is not an easy task since no standardized approach has been established so far. As a result, every lab that works on biofilms uses different methods to form it, which usually leads to inconsistent results with other laboratories. A standard way to form biofilm needs to be addressed by scientists who work with biofilms (McLean and others 2004).

To observe biofilm matrix, a light microscope with Alcian blue staining has been used (Rayner and others 2004). The use of confocal microscope depicts the three dimensions of biofilm (McLean and others 2004; Agle 2007), and is an effective tool to observe biofilm. Confocal microscope is a better choice than electron microscope in overcoming slides' dehydration (Morris and others 1997; Seo and others 1999), and in identifying different kinds of bacteria rather than a single kind. The roughly estimated composition of a biofilm is 15% cells and 85% matrix (Agle 2007).

Stepanovic (2003) described a procedure for evaluating biofilm formation by modified microtiter plate test. This approach categorized the biofilm formation into four sub-sets: no biofilm formation, weak biofilm, moderate biofilm, strong biofilm formation.

There are different factors that affect the attachment of the biofilm. Surfaces that contain both moisture and nutrients are good environments for biofilm formation. The topography of fresh produce can be a factor that promotes biofilm formation. Leafy green vegetables have

substances such as suberin, cutin, and waxes that enhances the bacterial attachment (Jahid and Ha 2012). The existence of surfactants and exposure to high-temperature of fruits or vegetables may increase infiltration of pathogens into fresh produce (Ukuku and others 2005). Waxy substances on intact fresh produce surfaces suppress the attachment of microbes, which weakens the biofilm formation (Jahid and Ha 2012).

To control biofilm formation many methods have been used. Cleaning and sanitization weaken the persistence of bacterial biofilm (Annous and others 2009; Cramer 2012; Park and others 2012). A novel system; turbulent two-phase flow has been shown to be effective in reducing the level of biofilm by 6 log cycles.

Changing the properties of food contact surfaces is a strategy that can be adapted to reduce the formation of biofilm (Brooks and Flint 2008). For example, altering the stainless steel surface by implanting ions reduces the colonization of microbes. Another approach that can be used to prevent biofilm formation is coating the surface of substrate with an inactive material. This ‘molecular brush’ prevents the attachment.

There are different methods to disintegrate biofilms. Caustic chlorine has been shown to disintegrates the polysaccharide matrix rather than inactivate the microorganisms (Brooks and Flint 2008). It has been stated that in removing biofilm we are in need of substances that can penetrate the polymers surrounding the biofilm. Hydrogen peroxide/peroxyacetic acid-based compounds appear to breach the biofilm and makes it easier to remove (Stier 2005). Enzymes such as proteinase K and trypsin are used in breaking down biofilm that has been formed by *Staphylococcal aureus* (Boles and Horswill 2011). Gas-discharge plasma is a promising approach that can be used in biofilm inactivation (Joaquin and others 2009). However, it has been used on biofilms formed in model system rather than food matrix.

Electrostatic spraying on biofilm on stainless steel has been shown to reduce surface contamination (Cramer 2012). Electrostatic spraying of antimicrobials has been shown to be more effective than conventional spray to control food-borne pathogens on lettuce and spinach (Ganesh and others 2012).

Biofilm formation is a complex process that includes expression of certain genes. Bacterial gene expression in some bacterial species may be regulated by quorum sensing. Quorum sensing is defined as**,** "cell density-dependent signaling systems by which bacteria modulate a number of cellular functions" through signaling compound known as autoinducers or bacterial pheromones. The autoinducers molecules bind to the appropriate transcription regulator(s) when the bacterial population reaches a threshold concentration. Binding of the autoinducers is followed by activation or repression of target genes. Therefore, quorum sensing allows bacteria to show a unified response that the benefits the population (Smith and others 2004). Quorum sensing is a mechanism that bacteria use as a response to harsh environment such as lack of nutrients or severe high/low temperature. Quorum sensing can contribute to the biofilm formation in food-borne bacterial such as *E.coli* O157:H7 and *Salmonella*.

The effect of electrostatic spraying with organic acids on biofilm formed by *E.coli* O157:H7 and *Salmonella* on fresh produce has yet been reported. Our lab studies conclude that electrostatic spraying with organic acids was effective in reducing *E.coli* O157:H7 and *Salmonella* on fresh produce.

The objectives are:

1. To investigate the effectiveness of electrostatic spraying with Malic/ Lactic acids alone and in combination on biofilm formed by *Escherichia coli* O175:H7 on spinach.
2. To investigate the effectiveness of electrostatic spraying with organic acids on biofilm formed by *Salmonella* Typhimurium on cantaloupe.
3. To investigate the effectiveness of organic acids on quorum sensing (pre-step of biofilm formation) on spinach and cantaloupe.

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Chapter 2: Literature Review

- *Salmonella* Typhimurium

Salmonella is a motile, non-spore forming, gram-negative bacterium. *Salmonella* form hydrogen sulfide in media containing glucose. Salmonella cells can utilize citrate as a carbon source. They are mesophilic, with optimum growth temperature between 35 and 37 °C. *Salmonella* is rod shaped, ubiquitous, existing in animals, specifically, in poultry and swine. Water, soil, insects, raw meats and other vehicles are considered to be environmental sources of *Salmonella*. There are two genus of *salmonella* that can cause illness in humans; *S.enterica* and *S.bongori*. (D'Aoust and others 2008).

Salmonella enterica, which is of the greatest public health concern, is comprised of six subspecies: *S.enterica* subsp. enterica, *S.enterica* subsp. salamae, *S.enterica* subsp. arizonae, *S.enterica* subsp. diarizonae, *S.enterica* subsp. houtenae, *S.enterica* subsp. Indica.
Among the six subspecies *Salmonella* enterica, subsp. enterica includes most serotypes frequently associated with foodborne salmonellosis (Ray 2005).
The infective dose of *Salmonella* can be as low as 12-50cells depending upon the age and the health of the host (Hammack and others 2012).

When sufficient amount of *Salmonella* cells is consumed, the pathogens invade mucosa of the small intestine, proliferate in the epithelial cells, and produce a toxin. Patients with salmonellosis may show symptoms of abdominal cramps, diarrhea, nausea, vomiting, chills, and fever. (D'Aoust and others 2008)

Foods associated with *salmonella* include raw meats, poultry, eggs, milk and dairy products, fish, shrimp, cake mixes, cream-filled desserts and toppings, dried gelatin, peanut butter, cocoa, and chocolate.

Salmonellosis outbreaks have been mainly related to poultry and meat products; however,

recent outbreaks have been linked to fresh produce (Tauxe and others 1997; Hanning and others 2009) especially cantaloupe and sprouts (Harris and others 2003).

As an early as 1990, multistate outbreaks from contaminated cantaloupe with *Salmonella* were reported. In 1991, two multistate outbreaks of salmonellosis were associated with cantaloupes. The first included *Salmonella* Chester and affected 245 persons with two cases of deaths in 30 states. The second involved *Salmonella* Poona and occurred in 23 states (Golden and others 1993).

A multistate outbreak of S*almonella* Typhimurium and *salmonella* Newport linked to cantaloupe occurred in 2012. The outbreak was wide spread (among 24 states) and had devastating consequences. Numerous number of people were sickened with 261 persons infected and 3 cases of death were confirmed (CDC 2012 a). In 2011, a multistate outbreak of human *salmonella* enteritidis infections linked to Alfalfa sprouts and spicy sprouts resulted in the infection of 25 persons (CDC 2011). A multistate outbreak of *Salmonella* Litchfield linked to cantaloupe took a place in 2008. The state health department identified 51 ill persons in 16 states as a result of the outbreak consequences (CDC 2008).

Salmonella Typhimurium is able to form biofilm on fresh produce. The forming of the biofilm may be the reason for the ongoing outbreaks related to fresh produce.
Annous and others (2005) reported the ability of *Salmonella* to form biofilm in a short period of time- 2 h at 20 °C on the rind of cantaloupe melons. It has been estimated that every year 2 to 4 million cases of salmonellosis occur in the United States. With the low infective dose and the rapid ability to form a biofilm, outbreaks of salmonella are anticipated to keep increasing.

- *Escherichia coli O157:H7*

Escherichia coli (*E.coli*) is a gram-negative, rod-shaped, highly motile, bacterium that presents naturally in the human gut as part of this flora. *E.coli* can be beneficial to the host in terms of preventing colonization of harmful pathogens in the gut. However, there are six classes of diarrheagenic (cause diarrheal illness) *E.coli:*enteropathogenic *E.coli (EPEC);* enterotoxigenic *E.coli (ETEC);* enteroinvasive *E.coli (EIEC);* diffusely adhering *E.coli(DAEC);* enteroaggregative *E.coli (EAEC); and* enterohmorrhagic *E.coli (EHEC). E.coli* serotype O157:H7 is classified under the EHEC class. The first *E.coli* O157:H7 identified as a pathogen in 1982 with hamburgers as the vehicle (Feng 2012; Doyle 1991). *E.coli* O157:H7 is also associated with a severe form of human disease because of its ability to produce Shiga toxins. The infective does of *E.coli* O157:H7 is very low (10 to 100 cells). One of the largest outbreaks associated with *E.coli* occurred in 1996 in Japan with more than 6,000 cases of *E.coli* infection and 4 cases of deaths. The outbreak affected more than 4000 schools around Sakai city (Buck and others 2003). Infection with E.*coli* O157:H7 causes hemorrhagic colitis (HC). Symptoms of HC include severe abdominal cramp and bloody diarrhea.

Although *E.coli* O157:H7 has been always associated with outbreaks in which the food source is raw meat or meat products, recent outbreaks of *E.coli* have been associated with fresh produce especially leafy greens such as spinach and lettuce.

In 2006, an outbreak of *E.coli* O157:H7 from spinach occurred. The outbreak resulted in the infection of 205 persons and 31 cases of Hemolytic-uremic syndrome (HUS), and 3 cases of death (Grant and others 2008). Another outbreak from the consumption of contaminated spinach and spring mix with *E.coli* O157:H7 took place in 2012. The outbreak resulted in the infection of 33 persons and two cases of HUS (CDC 2012 b).

An outbreak of *E.coli* O157:H7 from lettuce occurred in 2012. 58 persons were infected with the outbreak. The outbreak was spread over 9 states (CDC 2012 c).

Wang and others (2012) reported that both Shiga toxins-producing *E.coli* O157:H7 and non-157 are able to attach and form biofilm on food contact surfaces and food items including meat and vegetables. The ability of *E.coli* O157:H7 to from a biofilm on food contact surfaces poses a high risk of cross contamination. In addition, the ability of *E.coli* O157:H7 to form biofilms on fresh produce can also hinder the effectiveness of washing treatments that have been used.

- *Microbial attachment on fresh produce*

Bacterial attachment to biotic or abiotic surfaces is the first step in biofilm formation. Irreversible attachment in which bacteria are not removed by washing is the most critical step in biofilm formation. Fresh produce can be contaminated with pathogens in different steps in the process from farm to fork. There are many factors that affect the attachment of bacteria to the surface of produce.

- Effect of curli and cellulose on bacterial attachment to fresh produce

Curli are very thin, coiled, extracellular structures on the cell surface of most *E.coli*, and *Salmonella* enterica strains. Curli and cellulose production by bacteria have been shown to have an impact on the attachment. However, the literature showed a variation in the role of curli and cellulose. The differences can stem from variation among the strains of the same pathogen, the surface of produce, and the source from which pathogens were isolated.

Boyer and others (2007) reported that curli-producing *E.coli* O157:H7 cells attached firmly to lettuce whether cut or whole pieces than non-curli-producing *E.coli* O157:H7. However, with

different strains of *E.coli* O157:H7 there was no significant difference between the ones that were able to produce curli and the ones that do not. It can be concluded that the production of curli is not solely responsible for firm attachment and the strain of the bacteria should be taken into account. Ukuku and others (2005) stated that the existence of curli and cellulose *might* able *Salmonella* for better attachment to fresh produce surfaces. A study by Lapidot and Yaron (2009) confirmed that cellulose and curli are essential for enhancing the attachment of *Salmonella* to parsley. However, Macarisin and others (2012) found that curli fibers were important for the attachment of *E.coli* O157:H7 into the spinach while the cellulose was not. A study was conducted to determine the effect of curli expression on cell hydrophobicity and attachment to cut and intact cabbage and lettuce (iceberg and Romaine). The study found that curli-producing strains of *E.coli* O157:H7 cells were more hydrophobic and attached at higher numbers than other weak curli-expressing strains (Patel and others 2011). A part of Patel's results was in disagreement with the Boyer's findings who stated that although curli-producing *E.coli* O157:H7 were significantly more hydrophobic, no association between cell's hydrophobicity and attachment on lettuce was recognized. It can be concluded that the literature varies on the role of curli and cellulose onto attachment. In this research, the attachment was addressed by total plate count method after the fresh produce was washed to remove non-adherent bacteria.

<u>The effect of Cell's hydrophobicity on bacterial attachment to fresh produce</u>

Bacteria cells' hydrophobicity has been known to play a role in the attachment (Palmer and others (2007). However, Boyer and others 2007 reported that there is no association between the cell's hydrophobicity of *E.coli* O157:H7 and the attachment into lettuce. It was reported that hydrophobic cells of *E.coli* O157:H7 have higher number significantly more than non-hydrophobic cells.

Hassan and Frank (2004) reported that cell's hydrophobicity had no role on the attachment of *E.coli* O157:H7 onto lettuce and apple surfaces.

Ukuku and Fett (2002) investigated the effect of hydrophobicity on the attachment of *E.coli* (O157:H7 and non-O157:H7), *Salmonella*, and *Listeria monocytogenes* to cantaloupe rind. The study found that there was linear correlation between bacterial cell surface hydrophobicity and the strength of bacterial attachment to cantaloupe rind.

- The effect of food surface topography on bacterial attachment on fresh produce

The surface topography of fruits and vegetables can be complex. The topography of food surfaces has been shown to play a role in the attachment of bacteria. *E.coli* O157:H7 tend to attach to cut surfaces of cabbage and lettuce (iceberg and Romaine). However, the numbers of *E.coli* O157:H7 cells attached to intact or cut surfaces were not significantly different statistically (Macarisin and others 2012). This finding was in agreement with the findings' of Takeuchi and Frank (2000) and Janes (2001) who stated that both *E.coli* O157:H7 and *Listeria* monocytogenes prefer to attach to the cut edges of chopped lettuce.

Twenty four *Listeria monocytogenes* strains were evaluated based on their ability to attach and colonize on cabbage tissue. All the strains tested were attached to the cut tissue in comparison to the intact leaf surfaces (Ells and Truelstrup2006).

A study was conducted to investigate the effect of the roughness of surfaces of different fruits (Golden Delicious apples, navel oranges, avocadoes, and cantaloupes) on the attachment of *E.coli* O157:H7.The study found that there was a positive liner correlation between the average roughness of fruits surfaces and the rate of adhesion of *E.coli* O157:H7. The confocal microscope was used to determine the roughness of fruit surfaces (Wang and others 2009).

- Foodborne pathogens associated with fresh produce

Fresh produce have been increasingly consumed, efficiently distributed, and amply produced in the past two decades. Contamination of fresh produce by pathogens can occur in the cold chain from the farm to the table including the fields, at the time of harvesting, handling, or processing, and during distribution (Figure 1) (Sivapalasingam and others. 2004; Harris 1997). However, when fresh produce are contaminated, they become less susceptible to sanitization (Olaimat and others 2012). Since fresh produce and fresh-cut produce require minimum processing, they become a vehicle for different microorganisms, some of which are pathogens. The increased consumption of fresh produce can be a result of the healthy trend that consumers around the world are adapting to by eating more fresh produce. Moreover, prepared fresh produce products such as bagged salad better fit more the fast life style. The bagged salad market has been implicated with a rise in food-borne illness cases since the produce is cut or shredded which increases the release of nutrients that aid microbes to grow (Annous and others 2009) (Warriner and others 2009). A way to overcome the problem of bagged salad is the Modified Atmospheric Packaging (MAP). However, MAP may help important pathogens such as *E.coli* to grow, survive, and produce toxin (Chua and others 2008). Fresh produce consummation increased on average 4.5% annually between 1990 and 2004 (Olaimat and others 2012). Simultaneously outbreaks of foodborne illness associated with the consumption of fresh produce have increased (Warriner and others 2009). Chlorine is widely used for washing fresh produce after harvesting. However, bacterial internalization and biofilm formation hinder the effectiveness of chlorine treatment. The biofilm formation and internalization by pathogens may contribute to the increased numbers of outbreaks from fresh produce.

- Biofilm formation on fresh produce

According to Costerton and others (1999) a biofilm is defined, “an assemblage of microorganism adherent to each other or/and to the surface and embedded in a matrix of exopolymers.” The majority of microbes exist as biofilm in the environment and not as planktonic cells (Lemon and others 2008). Many studies concerned about bacterial biofilm on plants were devoted to examine the relationship between the epiphytic microorganisms on the leaf surface. These studies conclude that original biofilms on the plant consists of different bacterial species. In addition, the variation in the biofilm population is affected by the environmental atmosphere such as temperature, humidity, and nutrition availability. Pathogens such as *E.coli* and *Salmonella* are able to form biofilms on fresh produce. Biofilm formation occurs in five stages (Figure 2).

The first step of biofilm formation is attachment (Stier 2005; Brooks and Flint 2008; Agle 2007). The attachment is caused by Van der Waals’ forces (Tarver 2009). This initial attachment is weak and easy to be removed (Stier 2005) by rinsing (Agle 2007). The second step in biofilm formation is irreversible attachment.

Forming a biofilm in the lab is not an easy task since no standardized approach has been acknowledged yet .As a result, every lab that works on biofilms use different methods to form it, which usually leads to inconsistent results with other laboratories. A standard way to form biofilm needs to be addressed by scientists who are involved and work with biofilms (McLean and others 2004).

- Detection of Biofilms in *vitro* and fresh produce

There are many methods to evaluate biofilms in *vitro* and fresh produce. O'Toole (2011) stated that crystal violet assay can be used for biofilm evaluation in microtiter plate. This method is used to observe variation among different strains in the ability to form biofilm, and it is used prior to time-consuming work such as plating and/or observing biofilms under microscopes. However, crystal violet stains both dead/living cells and matrix components that may present in the plate hence crystal violet can provide overestimated results (Djordjevic and others 2002) To solve this problem, direct enumeration of bacteria in biofilms can be used for yielding data that are more accurate (Peters and others 2008; Merritt and others 2005).

Peters and others (2008) used the Syto9 assay, the fluorescein diacetate assay, the resazurin assay, the XTT assay and the dimethyl methylene blue assay to quantify biofilm produced by *Pseudomonasaeruginosa, Burkholderiacenocepacia, Staphylococcus aureus, Propionibacteriumacnes* and *candidaalbicans*in the 96-well microtiter plates. This study reported that some assays such as crystal violet assay was not the best fit to quantify biofilm formed by specific microorganism such as *Pseudomonasaeruginosa.*

Biofilms can be visualized by cryostage scanning electron microscopy. Rayner and others (2004) were able to visualize biofilms that formed naturally on fresh produce surfaces such as carrots, tomatoes, and mushrooms by cryostage scanning electron microscopy.

Scanning electron microscopy was used to observe biofilms that formed naturally on roots of alfalfa, broccoli, clover, sunflower sprouts, sprouts of alfalfa that were grown in the lab (Fett 2000) and mung bean sprouts (Fett and Cooke 2003).

Light microscopy with Alcian blue staining can be used to confirm the presence of the

biofilm matrix. Exopolymeric contains acidic polysaccharides that can be stained by Alcian blue stains and observed under the light microscope (Rayner and others 2004).

Elhariry (2011) used scanning electron microscopy to observed biofilm formed by *Bacillus cereus* on cabbage and lettuce.

- Using Organic Acids as an Antimicrobial

Organic acids such as acetic, lactic, malic, and citric, are present naturally in of foods naturally. Organic acids have been used as food preservatives because of their antimicrobial effects. Although the mechanism(s) by which organic acids act as antimicrobials is not fully understood, reducing the intracellular pH is considered to be the main factor in inhibiting the microbial growth. It has been previously stated that undissociated organic acids are able to penetrate the lipid membrane of the bacterial cell, and upon internalization into the cell cytoplasm it dissociates into anions and protons. The presence of these molecules challenges the bacteria cell to keep its intercellular pH to a point at which the function of its organelles is not affected, which can be done by exporting the excess protons. Exporting protons uses cellular adenosine triphosphate (ATP) and might deplete the cellular energy (Rick 2003; Davidson and others 2013).

The effect of organic acids as antimicrobials has been extensively studied in the literature. Park and others (2011) used different organic acids (propionic, acetic, lactic, malic, and citric acid) with different concentrations 1% and 2% to inactivate *E.coli* O157H7, *Salmonella* Typhimurium, and *Listeria* monocytogenes on red apples and lettuce. The results of Park's study on apples showed that, lactic, malic, and citric had the highest log reduction > 3.42 against the three foodborne pathogens. However, on lettuce, malic and citric acids had the

highest log reduction with 2.98 and 2.86. Although many scientists have investigated the effect of organic acids on fresh produce products, park's study is considered the first that investigated the effect of organic acids on organic produce.

Aerosolized malic acid was used to inhibit foodborne pathogens *E.coli* O157H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on spinach and lettuce. The study revealed that using aerosolized 2% malic acid for 100 min resulted in the highest log reduction of the three pathogens on both spinach and lettuce. Moreover, the study concluded that using aerosolized malic did not adversely alter the quality of spinach or lettuce (Choi and other 2012).

Malic acid (2.6%)-incorporated soy protein film has shown to reduce the log reduction of *E.coli* O157H7, *Listeria monocytogenes*, and *Salmonella gaminara* by 2.1, 2.8, and 6.0 respectively (Eswaranandam and others 2004)

Over and others (2009) investigated the effect of organic acids in both broth culture model and chicken meat systems. The study concluded that at the concentration of 150.0 mM citric, malic, and tartaric had the highest log reduction in the chicken system against *E.coli* O157H7, *Listeria* monocytogenes, and *Salmonella* Typhimurium by > 5, >2, and 4-6 log CFU/g, respectively. However, in the broth culture acetic, citric, lactic, malic, and tartaric showed efficiency at 75mM.

Dipping produce with organic acids have been used to inactivate *Escherichia coli* and *Listeria monocytogenes* on lettuce. Dipping lettuce in different organic acids; lactic, citric, acetic, and ascorbic at the concentration 0.5% for 2 min reduced the number of *Listeria* monocytogenes by 1.5, 1.0, 0.8, < 1.0 $\log_{10}$ CFU g^{-1} respectively. Dipping lettuce in different organic acids; lactic, citric, acetic, and ascorbic at the concentration 0.5% for 2 min reduced the population of *Escherichia coli* by 1.9, 2.0, 1.3, and 1.0 $\log_{10}$ CFU g^{-1} respectively (Akbas and others 2007).

Washing spinach leaves with 2% $_{L}$-lactic acid at 55 °C was shown to reduce bacterial growth of *E.coli* O157:H7 and *salmonella* by 2.7 and 2.3 log CFU/g respectively. However, an adverse effect of color bleaching was reported (Neal and others 2012).

Spraying inoculated cantaloupes and bell peppers with *Salmonella* Typhimurium and *E.coli* O157:H7 with 2% $_{L}$-lactic acid resulted in the lowest log counts on both produces. The log reductions for pathogens were 3 and 3.6 log CFU on cantaloupes and bell peppers respectively. The study concluded that 2% $_{L}$-lactic acid treatment did not cause any adverse effect on the organoleptic characterizations of the products (Alvarado-Caillas and others 2007).

The synergistic effect of organic acids and heat to decontaminate pathogens on fresh produce was investigated. Huang and Chen (2001) found that washing baby spinach with 2% lactic acid to inactivate *E.coli* O157:H7 at 40 °C for 5 min resulted in the highest reduction of *E.coli* O157:H7 (2.7 log CFU/g).

Ganesh and others (2010) stated that malic acid in combination with grape seed extract/lactic acid solution applied by electrostatic spraying exhibited higher inhibition of pathogens that conventional spraying and can be used for commercial applications to enhance food safety.

Ganesh and others (2012) used electrostatic spraying of food-grade organic acids and inorganic acids and plant extracts to decontaminate *E.coli* O157:H7 on spinach and iceberg lettuce. The study findings were that combined lactic acid 3.0% , malic acid 3.0% showed 2.1 to 4.0 log CFU/g reduction of E.coli between the days 1 and 12 on spinach and 1.1 to 2.25 log CFU/g reduction on lettuce.

Massey and others (2013) reported that malic acid (4%) alone and in combination with lactic acid (2%) resulted in a log reduction of 4.6 when the organic acids were spayed electrostatically into cantaloupe inoculated with *E.coli* O157:H7.

- Approaches used to control biofilms on food contact surfaces

Different methods have been used to control biofilm on food contact surfaces. It has been reported that a way to control biofilms is to frequently disinfecting even before the formation of the biofilm. Nevertheless, the time course of the pre-step for the biofilm formation, which is essentially attachment, is rapidly occurs in hours. This fact makes it even harder to decontaminate biofilm in food industries in which the frequency for biofilm formation is much higher than the disinfection regularity. Moreover, cleaning and sanitization weaken the persistence of bacterial biofilm (Annous and others 2009; Park and others 2011; Cramer 2012). A novel system turbulent two-phase flow has been used. This system has been shown to be an effective in reducing the level of biofilm by 6 log cycles (Brooks and Flint 2008).

Changing the properties of food contact surfaces is a strategy that can be adapted to reduce the formation of biofilms. For example, altering the stainless steel surface by implanting ions reduces the colonization of microbes. Another approach that can be used to prevent biofilm formation is by coating the surface of substrate with an inactive material. This ‘molecular brush’ prevents the attachment of microbes (Brooks and Flint 2008). Moreover, modified stainless steel surfaces with poly (ethylene glycol) were smoother and less hydrophobic than the unmodified ones. Attachment and biofilm formation by *Listeria monocytogenes* on modified surfaces were reduced by 90% in comparison with the unmodified stainless steel (Wang and others 2003).

To reduce the attachment (a pre step of biofilm formation) of *Listeria monocytogenes* on stainless steel, a cocktail of lactic acid bacteria was used. In this study, *Pediococcus acidilactic*, *Lactobacillus amylovorus*, and *Lactobacillus* animalis were able to reduce the attachment of *Listeria* which gives the potential of using lactic acid bacteria as a biosanitizer in food contact surfaces (Ndahetuye 2012)

- Approaches used to decontaminate biofilms on food contact surfaces

Biofilms formation on food contact surfaces presents a serious hazard since biofilms are more resistance to disinfections that usually used in the food industry. In addition, biofilms can result in cross contamination since cells detach from the formed biofilms and become planktonic cells that transfer to foods. Many studies were conducted to decontaminate biofilms formed on food contact surfaces.

Trisodium phosphate (TSP) was used to decontaminate biofilms formed by *listeria monocytogenes*, *Salmonella Typhimurium*, *E.coli* O157:H7 and *campylobacter jejuni* on stainless steel. The study showed that TSP was an effective method to inactivate biofilms formed by the previously mentioned pathogens. The study showed *E.coli* O157:H7 to be most susceptible to the treatment (only 1% TSP with contact time of 30 s was sufficient to reduce bacteria in biofilms by 10^5 CFU/cm^2). On the other hand, *Listeria monocytogenes* was the most resistant to the treatment (even 8% TSP with contact time of 2 min could not reduce the bacterial log by 1).
TSP can be used to disinfect pathogens on food contact surfaces or/and poultry products, yet the effectiveness of TSP on fresh produce products has not been evaluated (Somers and others 1994).

Cetylpyridinium chloride (CPC) was investigated to decontaminate biofilms formed by *E.coli* O157:H7 on stainless steel. The study revealed that < 1.0% CPC was sufficient to inactivate *E.coli* O157:H7 biofilms formed on stainless steel (Plauché 2006).

Essential oils were used to reduce biofilms formed by different *Listeria* strains on stainless steel. The study found that thyme, oregano, and carvacrol oils were the most effective against *Listeria* monocytogenes biofilms on stainless steel surfaces (Desai 2012).

Citric acid was investigated for reducing bacterial biofilm formed on polyvinyl chloride pipes. The investigation found that 10.000 mg/L citric acid concentration and 60 min contact time were the optimal conditions for bacterial biofilm inactivation (Tsai and others 2003).

Lactic acid and steam treatments were studied for their ability to inactivate biofilms formed by *E.coli* O157:H7, *Salmonella* Typhimurium, and *Listeria* monocytogenes on polyvinyl chloride and stainless steel. Inoculated coupons (polyvinyl chloride/stainless steel) with the above pathogens were left 6 days at 25 °C to allow biofilms formation. Coupons then were treated with 2% lactic for 15 s or 30 s followed by exposure to steam for 20 s. After the use of combined treatment biofilm cells were reduced to below the detection limit (1.48 log) (Ban and others 2012).

A study investigated the effectiveness of irradiation and sodium hypochlorite to decontaminate biofilm formed by *Pseudomonas aeruginosa*, *Listeriainnocua*, and *Escherichia coli* on polypropylene, polyethylene, and polycarbonate. The study found that gamma irradiation was effective for reducing the biofilms formed by different pathogens. On the other hand, sodium hypochlorite had a moderate effect (Byun and other 2007).

Peracetic acid and peroctanoic acid sanitizers were investigated to their ability to decontaminate mixed-culture biofilms formed by *Listeria* monocytogenes and *Pseudomonas* on

stainless less. The study revealed that peracetic acid sanitizer was more effective than peroctanoic acid in biofilms decontaminations (Fatemi and Frank 1999).

A study investigated the effectiveness of different disinfectants to decontaminate biofilm formed by gram positive bacteria such as *Bacillus* subtilis and *Staphylococcus*. Gram negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella T*yphimurium on stainless steel slides (1.5 cm in width, 2 cm in length, and 3-mm in depth). The slides were left 6 days after inoculation with the above microorganisms to allow biofilm formation. The study found that among eight different disinfectants, hydrogen peroxide was the most effective on all tested bacteria (Rushdy and Othman 2011).

Nisin and lysozyme were investigated of their ability to decontaminate biofilm formed by 25 different strains of *Staphylococcus aureus*. The study revealed that the use of 25 µg/ml nisin was sufficient to inhibit biofilm formation of all the strains used in the study. On the other hand, the lysozyme used was not effective against all the tested strains (only six strains were affected).

The effect of the above biopreservatives (nisin and lysozyme) was studied in a 96-well plate made of polystyrene (Sudagidan and Yemenicioglu 2012).

Ethylenediaminetetraacetic acid (EDTA) was studied to inhibit biofilm formed by *Listeria* monocytogenes. The study revealed that adding EDTA at the beginning of biofilm formation was the most effective in reducing biofilm formation. However, EDTA was not effective against 8-h-old biofilm. The effect of EDTA against biofilm formed was studied in a 96-well plate made of polyvinyl chloride (Chang and others 2012).

The effect of alkaline and acidic electrolyzed water to decontaminate biofilm formed by *Listeria monocytogenes* on stainless steel was investigated. Stainless steel coupons were immersed in not-rich media for 48 h at 24 °C. The study found that the use of alkaline

electrolyzed water followed by acidic electrolyzed water was statistically significantly higher inactivation of L*isteria* monocytogenes than the use of acidic water solely (Ayebah and others 2005).

Quaternary ammonium compound-based (QAC) and acid-based (AB) sanitizers were investigated for their ability to decontaminate one-week-old biofilm formed by *E.coli* O157 and *Salmonella* Typhimurium on stainless steel. The study found that both sanitizers were not effective to reduce the formed biofilm (Fouladkhah and others 2013).

Scallop shell powder was used to remove biofilm formed by *Listeria monocytogenes*, *Staphylococcus aureus*, and *E.coli* O157:H7 on stainless steel. The study revealed that the use of scallop shell powder was effective to reduce two-day-old biofilm on stainless steel (Bodur and Cagri-Mehmetoglu 2012).

The use of aerosolized sanitizers showed an important effectiveness on biofilm formed on stainless steel and Polyvinyl chloride (PVC) (Park and others 2012).

- **Approaches used to reduce biofilms on food and fresh produce**

For biofilm decontamination, the biocide or the antimicrobial has to penetrate the extracellular polymeric substance (EPS) to reach into the bacteria that are protected by the EPS (Meyer 2003).

The composition of EPS differs from a biofilm to another biofilm. For example, polysaccharides are always thought to be the most predominant component in the biofilm matrix; however, there were some situations when proteins and humic substances were the major components in the EPS matrix (Starkey and others 2004). The variation in the composition of a biofilm can be the result of the environmental conditions and the strain of bacteria. Chlorine can be used in biofilm decontamination. However, the concentration of Chlorine needed for

significantly decontaminate biofilm was 1,000 ppm in comparison with 10 ppm needed for decontaminate planktonic cells (Jahid and Ha 2012). Studies that were conducted to eliminate biofilms formed on fresh produce scarce in comparison with studies investigated the elimination of biofilms formed on polystyrene or food contact surfaces. A study that used ozone (2 mg/L), chlorine (100 mg/L) and organic acid (0.25g/100 g citric acid plus 0.50 g/100 ascorbic acid) treatments at 10 °C for 2 min to remove biofilm formed by *E.coli* O157:H7, *Listeria monocytogenes* on green leaf lettuce (*Lactuca sativa*) found that none of the used treatments was sufficient to reduce the cells that were embedded in biofilms (O¨ lmez and others 2010).

Irradiation was used to decontaminate biofilm formed by *E.coli* O157:H7 on spinach and lettuce leaves. In the experiment spinach and lettuce leaves were dip inoculated and stored at 4 °C at (0, 24, 48, and 72 h) to allow biofilm formation. Leaves were treated with different doses of irradiation (0, 0.25, 0.5, 0.75, or 1 kGy). It was found that the D 10, which is the dose required for 1 log reduction, increased significantly when the storage time increased. It increased from 0.19 kGy on o h to 0.52 to 0.54 kGy on spinach, and from 0.19 on 0 h to 0.40 to 0.43 kGy on lettuce (Niemira and Cooke 2010).

Washing with sodium hypochlorite at different concentrations (0, 300, or 600 ppm) with a contact time of 3 min to reduce biofilm formed by *E.coli* O157:H7 on leaves of spinach and lettuce had a moderate effect. The log reductions of baby spinach, lettuce were 1.3 and 1.8 log CFU/g respectively (Niemira and Cooke 2010).

Since Irradiation is not preferable by many consumers, there is a need for approaches that are more appealing. The use of organic acids (natural compound in foods) and the use of electrostatic spraying can help in disintegrating biofilms on fresh produce by the use of hurdle technology.

- Quorum Sensing

Quorum sensing is “cell density-dependent signaling systems by which bacteria modulate a number of cellular functions” through signaling compound known as autoinducers or bacterial pheromones (Smith and others 2004). Quorum sensing is a strategy that bacteria uses as a response to environmental stresses such as lack of nutrients or sever temperature. Quorum sensing can contribute to the biofilm formation in food-borne pathogens bacteria such as *E.coli* O157:H7 and *Salmonella*. Biofilm is considered to be a layer of mainly polysaccharides that protect the bacteria against external factors. Gram- negative bacteria such as *E.coli* O157:H7 and *Salmonella T*yphimurium produce two types of autoinducers: acylated homoserine lactones (AI-1) and furanosyl borate diester (AI-2) (Lu and others 2005). Bacteria producing the above mentioned autoinducers have to possess a luxS gene which code the Luxs proteins that biosynthesis AI-2.

Various investigations have been conducted to inhibit AI-2 activity. Pillai and others (2006) reported that some food extracts such as turkey patties, chicken breast, mozzarella cheeses, beefsteak, beef patties, and goat milk cheese were able to inhibit AI-2 activity, which was determined by using *V.harveyi* reporter strain BB170. The inhibition of AI-2 activity percentages by food extracts were 99.8% for turkey patties, 97.5% for chicken breast, 93.7% for mozzarella cheeses, 90.6% for beefsteak, 84.4% for beef patties, and 65.3% for goat milk cheese. Lu and others (2004) stated that food additives such as sodium propionate, sodium benzoate, sodium acetate inhibited the activity of AI-2 activity. These authors used the additives at FDA-recommended concentrations. Sodium propionate virtually inhibited AI-2 activity. Sodium benzoate and sodium acetate inhibited the AI-2 activity by 93.3% and 75% respectively. However, sodium nitrate did not have any significant effect even when used at high

concentration (200 ppm). *V.harveyi* reporter strain BB170 assay was used to determine the effect of the additives on AI-2 like activity. The authors also determined the effect of the additives on the reporter strain to confirm that the reduction in the AI-2 activity was not due to the growth inhibition of the reporter strain but to the effect of the additives.

Widmer and others (2007) reported that using fatty acids isolated from poultry meat reduced the activity of AI-2. Since all the fatty acids share similar structure, their inhibitory activity might stem from the chemical structure. However, more studies are needed to determine the mechanism by which fatty acids inhibit AI-2 activity. Soni and others (2008) identified the fatty acids that Widmer reported and found that palmitic acid, steric acid, oleic acid, and linoleic acid were able to inhibit AI-2 activity. The fatty acids were used at various concentrations (1, 5, and 10 mM). The level of inhibition correlates with the increase in the concentration of the fatty acids. Oleic acid was the most effective fatty acids among palmitic acid, steric acid, and linoleic acid *V.harveyi* reporter strain BB170 assay was used to determine the effect of the fatty acids on AI-2 like activity. Bodini and others (2009) indicated that *p*-coumaric acid was able to hinder the activity of quorum sensing in *Chromobacterium*, *Agrobacterium*, and *Pseudomonas*. Further studies are needed to understand *p*-coumaric acid role in the inhibition of quorum sensing.

Choo and others (2006) found that vanilla extracts have been shown to have quorum sensing inhibition property. Vanilla extract contains vanillin, 4-hydroxybenzaldehyde, 4-hydroxybenzyl methyl ether, esters, phenols, and hydrocarbons. All of the above mentioned compounds do not have a similar structure to the natural autoinducer which enable them to stack to the autoinducer molecule and block it. Hence, the compound that inhibits quorum sensing in vanilla has not been discovered yet. Investigations are needed to determine the compounds in vanilla that inhibits the quorum sensing activity and the mechanism by which it does.

Koh and Tham (2009) disclosed that traditional Chinese medicinal plants were effective against quorum sensing activity. In Koh's study 10 traditional Chinese medicinal plants were investigated for their ability to inhibit quorum sensing activity. Eight plants of the traditional Chinese medicinal plants inhibited quorum sensing Prunus armeniaca, Prunella vulgaris, Nelumbo nucifera, Panax notoginseng (root and flower), Punica granatum, Areca catechu, and Imperata cylindrica. The results from the study indicate that traditional Chinese medicinal plants have compounds inhibit the quorum sensing activity. However, more investigations are needed to identify and isolate the compounds that inhibit quorum sensing activity.

Quave and others (2011) recorded that Italian medicinal plants were effective against quorum sensing activity. In Quave's study, three medicinal plants (Ballota nigra, Castanea sative, and Sambucus ebulus) were able to inhibit quorum sensing activity in methicillin-resistant *Staphylococcus aureus* (MRSA). However, the active compounds in the plants are not known yet. More studies can be conducted to identify and isolate the active compounds.

Lee and others (2013) screened 498 plant extracts for their ability to inhibit *E.coli* O157:H7. Only 16 plants inhibited the biofilm formation of *E.coli* O157:H7 without having an effect on the planktonic cells, and 14 plants extracts hinder the motility of the *E.coli* O157:H7. The most active plant carex dimorpholepis was able to inactivate AI-2 quorum sensing genes. However, the carex dimorpholepis did not show a bactericidal effect.

The effect of organic acid on auotinducer-2 activity has not been investigated hence the study investigated the effect of organic acid on autoinduce-2 (quorum sensing molecule) on fresh produce samples (spinach and cantaloupes).

Cell-to-cell signaling known as quorum sensing has been shown to have a role in biofilm formation in foodborne pathogens. Bacterial gene expression in some bacterial species may be

regulated by quorum sensing, a cell density-dependent signaling system mediated by chemical auotinducer molecules produce by bacteria. The autoinducer bind to the appropriate transcription regulator(s) when the bacteria population reaches a threshold concentration sufficient to facilitate binding to the receptor. As a result, a method to interpret quorum sensing can be used as a preventative measure to hinder biofilm formation on fresh produce.

In most of the reported studies, investigation are lacking isolating and identifying of the active compound that inhibit quorum sensing. More research is needed to identify other compounds that can interpret the quorum sensing process such as organic acids. This study investigated the effect of organic acid on quorum sensing in fresh produce homogenate. Since AI-2 is the quorum sensing compound, Vibrio *harveyi* BB170 was used since it produces bioluminescence as a response to AI-2.

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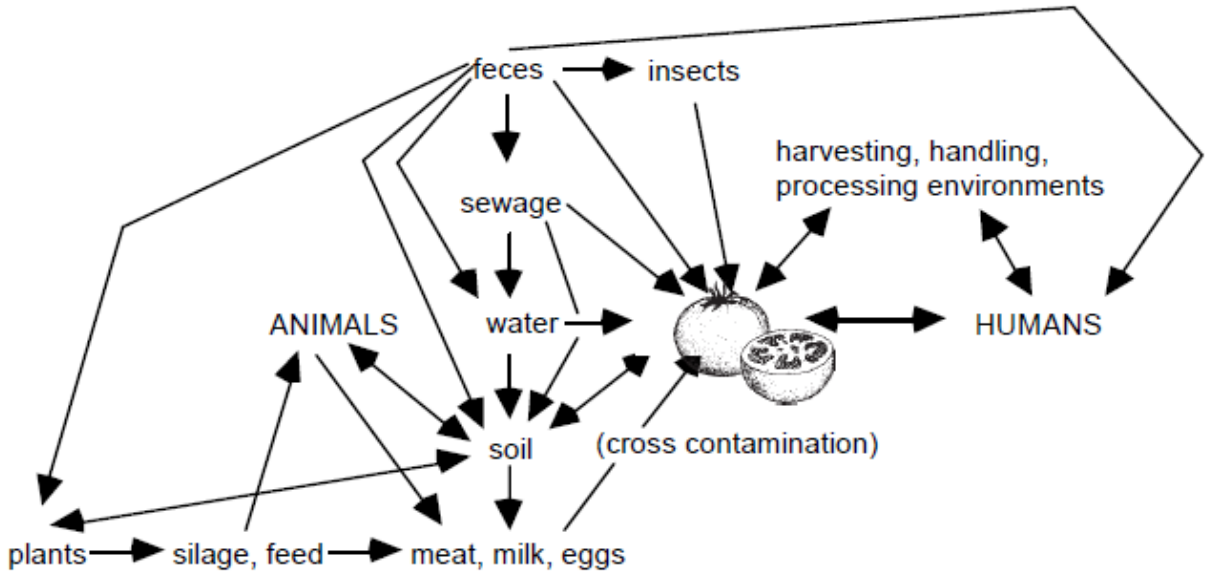

Figure 1-Mechanisms by which produce can become contaminated with pathogenic microorganisms adapted from (Harris 1997) with permission.

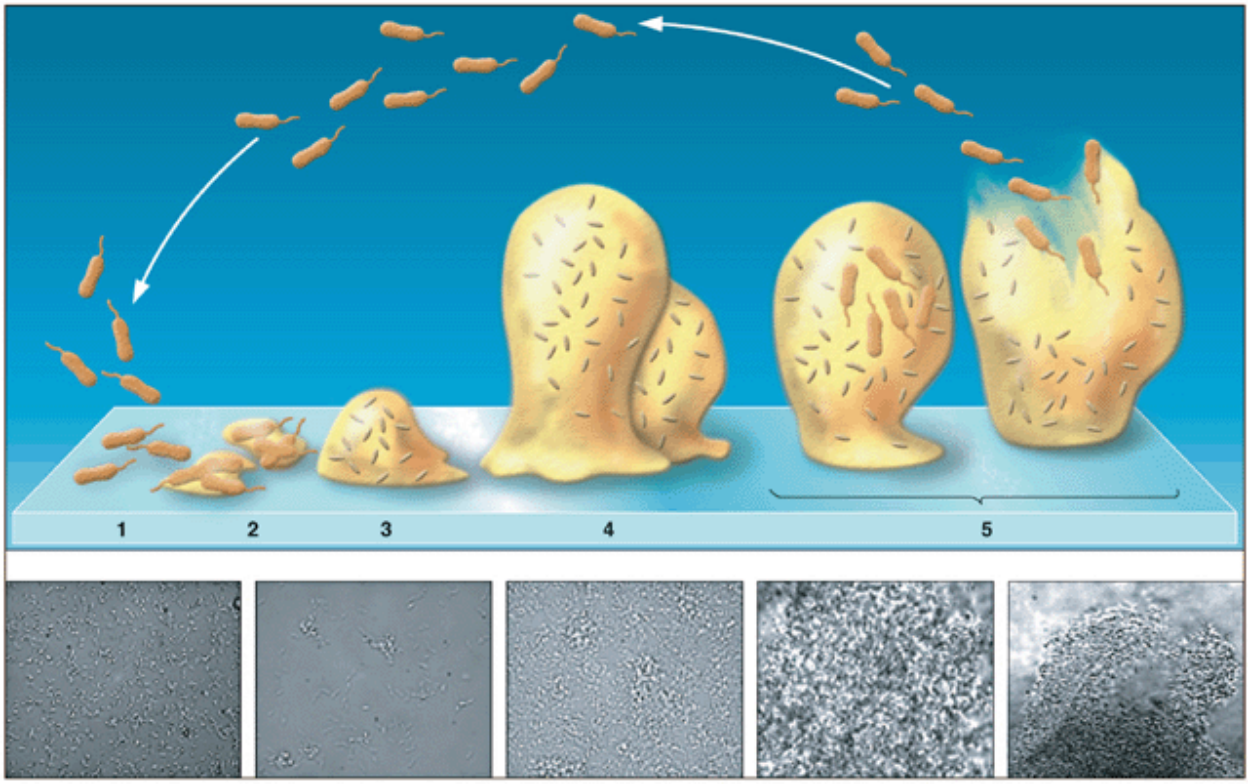

Figure 2-Diagram showing the development of a biofilm as a five-stage process adapted from (Stoodley and others 2002) with permission.

Chapter 3: Effects of electrostatic spraying with organic acids in the disintegration of biofilms formed by *E.coli* O157:H7 on spinach.

Introduction

Outbreaks from the consumption of fresh produce are a concern in the United States (Brooks and Flint 2008). In 2013, a multistate outbreak of *E.coli* O157:H7 infection linked to ready-to-eat salads occurred. The outbreaks resulted in the infection of 33 persons and hospitalization of seven and two persons developed hemolytic uremic syndrome. No deaths cases were reported as a consequence of the outbreak.

Another multistate outbreak took place in 2012 linked *E.coli* O157:H7 to organic spinach and spring mix blend. 33 persons were infected and 13 were hospitalized. Two ill persons developed hemolytic uremic syndrome.

The consumption of fresh produce has increased since the last twenty years (Brooks and Flint 2008; Taylor 2009)) because of consumer demand.

Contamination of fresh produce by pathogen from the farm to the fork. For instance, fresh produce can be contaminated in the fields, at the time of harvesting, handling, or processing, and during distribution (Sivapalasingam and others 2004; Beuchat 1996). Certain types of foods have been linked to certain pathogen outbreaks; for example, salmonellosis with melons, tomatoes, and some selections of sprouts. *E. coli* O157:H7 has been associated with leafy green vegetables since only a few cells are required for *E.coli* O157:H7 is able to survive for extended periods in water, soil, and can tolerate refrigerated temperatures. *E.coli* O157:H7 can be destroyed by high temperatures such as cooking (Kendall and Davis 2014). Fresh produce are not thermally treated, hence makes them a possible source of foodborne illness. The primary purpose of washing produce is not to remove bacteria, but to remove soil from the produce (Sapers 2001). As a result, dependence on conventional washing will not remedy the problems of

pathogen outbreaks in fresh produce. Other approaches are needed to alleviate the burden of fresh produce contamination.

It is recognized that human pathogens have the ability to form biofilm on fresh produce (Annous and others 2009; Jahid and Ha 2012; Morris and others 1997; Kim and Wei 2012; Rayner and others 2004). Biofilm formation occurs in five steps 1. Reversible attachment 2.iireversiable attachment 3.formation of microcolonies and biofilm maturation 4. Formation of three-D structure 5. Detachment from the mature biofilm and reattach. Biofilms can be resistant to antimicrobials (Van Houdt and others 2004; Annous and others 2009a; Agle 2007), biocides (Annous and others 2009), disinfectants (Rayner and others 2004; Jahid and Ha 2012) and sanitizers (Jahid and Ha 2012) that are used in food industry (Annous and others 2006), which makes biofilm a serious problem to this industry (Stier 2005). In addition, Biofilms are more likely than planktonic bacteria to survive in harsh environmental conditions (Simões and others 2010).

Various methods have been used to disintegrate biofilms. Horswill and Boles (2011) reported that enzymes such as proteinase K and trypsin can be used in disintegrating biofilms that has been formed by *Staphylococcal aureus*. Another approach has been in washing with caustic chlorine in disintegrating the polysaccharide matrix of the biofilm rather than inactivation of the microorganisms (Brooks and Flint 2008). Irradiation has been reported to reduce pathogens that exist in biofilm and/or internalized within the tissue of fresh produce (Annous and others 2009a). However, Niemira and Cooke (2010) reported that irradiation was not effective against biofilm formed by *E.coli* O157:H7 in spinach.

Various Investigations have been conducted to study the anti-microbial activity of organic acids.

Malic acid (2.6%) incorporated soy protein films reduced the log number CFU/mL from 8.3, 9.0, and 8.9 of *L. monocytogenes, S. gaminara, and E. coli* to 5.5, 3.0, and 6.8, respectively (Eswaranandam and others 2004). López-Malo and others (2012) concluded that organic acids are effective antimicrobial against salmonella. Organic acids are GRAS (Generally recognized as safe), inexpensive, slightly affect the taste of the product, and have unlimited acceptable daily intake (López-Malo and others 2012).

Several studies have been conducted using electrostatic spraying as a technique to reduce bacterial growth. For example, electrostatic spraying with 200 ppm of ala-quaternary ammonium significantly reduced biofilm formed by *Listeria monocytogenes* on ceramic tile, FRP (plastic wall board), polypropylene conveyor belt mesh tops (24% open mesh), and stainless steel (Dow 2008). This study found that the use of electrostatic sprayer was very effective as a pre-treatment in controlling biofilm formation in all surfaces used in the study.

Russell (2003) reported that electrostatic spraying was an effective method to reduce pathogens from eggshell surfaces. It was stated that a combination of malic acid with a solution of grape seed extract and lactic acid by electrostatic spraying showed significant inhibition of pathogens in comparison to conventional spraying (Ganesh and others 2010). The U.S Department of Agriculture Research Service (ARS) reported that electrostatic spraying on biofilm formed on stainless steel might reduce surface contamination (Cramer 2012).

The objective of the study was to investigate the effectiveness of electrostatic spraying with organic acids on attachment and biofilm formation of *E.coli* O157H7 on spinach leaves.

Material and Methods

1. Culture preparation

Agar slant cultures of green fluorescent protein GFP-labeled ED 14 E.*coli* O157:H7 (CV267); ED15 *E.coli* O157:H7 (6980-2); ED16 *E.coli* O157:H7 (6982-2); MD58 *E.coli* O157:H7 (CV261), MD46 *E.coli* O157:H7 (F4546); and MD47 *E.coli* O157:H7 (K4492) were obtained from the University of Georgia, Center for Food Safety, Griffin, GA. Frozen stock cultures were made with a single colony of each microorganism. Frozen stock cultures at -70 °C of the above were thawed, transferred to 10 mL of brain heart infusion homogenate (BHI) (Becton Dickinson Microbiology Systems, Becton Dickinson and company, Sparks, MD, U.S.A.) and incubated at 37 °C for 24 h with 200-rpm using a New Brunswick Scientific (Edison NJ, U.S.A.) agitating incubator. Second-day inoculum was prepared by inoculating 10 µl of first-day culture into fresh 10 mL of BHI and incubated in a shaker maintained at 37 °C for 24 h. The second-day culture (10^7 log CFU/mL) was used to inoculate spinach in the studies. Fresh spinach leaves were purchased from a local grocery store

2. Determination of the *E.coli O157:H7* bacterial strain adhesion

Spinach leaves were washed with water to remove any debri, disinfected with sodium hypochlorite solution (6.25 mL/ L deionized water) to reduce microbial background, and rewashed with sterile deionized water to remove any chlorine residual. Disinfected spinach leaves were randomly cut with sterilized stainless steel cork-borer to produce leaf disks of 1 cm diameter. These Spinach leaf disks were transferred into a 24-well plate that contained 1.9 mL of 0.1% peptone water. One hundred microliters of peptone and bacterial culture (1:1) was also added to 24-well plate. The 24-well plate was incubated for 24 h at 37 °C. Spinach disks were rinsed with sterile water to remove non-attached cells, transferred to Whirlpak bag that contained

2 mL of PBS and stomached for 3 min. Stomached samples were serial diluted using PBS and spread-plated on MacConkey agar media. The plates were incubated at 37 °C for 24 h and colony counts were taken after incubation period. The number of counted colonies represents attached bacteria.

3. Investigation the effect of electrostatic spraying with organic acids on attached bacteria

Disinfected spinach leaves were randomly cut with sterilized stainless steel cork-borer to produce leaf disks of 1 cm in diameter. The leaf disks were transferred into 24-well plate that contained 1.9 mL of 0.1% peptone water. One hundred microliters of peptone and *E.coli* O157:H7 (1:1) was also added and incubated for 24 h at 37 °C. The spinach leaf disks were rinsed with sterile water to remove non-attached cells, transferred to Whirlpak bag for spraying electrostatically with malic and lactic acid solutions alone (1.0, 2.0, 3.0, 4.0 %) and in combination (0.5+0.5, 1.0+1.0, 1.5+1.5, 2.0+2.0%). After spraying, spinach disks were transferred to Whirlpak bag that contained 2 mL of PBS and stomached for 3 min. Stomached samples were serial diluted using PBS and spread-plated on MacConkey agar media. The plates were incubated at 37 °C for 24 h. Colony counts were taken after incubation period to enumerate the amount of *E.coli* O157:H7 on spinach leaves. Log reductions were calculated by subtracting the log counts obtained by spraying electrostatically with malic and lactic acid solutions alone (1.0, 2.0, 3.0,4.0 %) and in combination (0.5+0.5, 1.0+1.0, 1.5+1.5, 2.0+2.0%) from the log counts obtained from the control.

4. Crystal violet binding assay

Crystal violet binding assay described by Kim and others (2009) was used to study the biofilm-forming ability of the *E. coli*. Crystal violet binds to polysaccharides of biofilm matrix. Through the binding to adhering biofilm, the amount of biofilm produced can be measured. The

bound crystal violet can be mobilized (separates from the biofilm) with ethanol and the absorbance at 595 nm can be determined. Spinach leaves were disinfected with sodium hypochlorite solution. A 96-well plate was prepared by adding the bacterial culture 10^7 along with spinach homogenate and incubated for 24 h at 37 °C to allow attachment and biofilm formation (optimum temperature for *E.coli*). This work was done to investigate the effect of organic acids on the biofilm formed by various *E.coli* O157:H7 strains (ED 14, ED 15, ED 16, MD 46, MD 47, MD 47, and MD 58).

4.1 Antimicrobials preparation

A stock solution was prepared by dissolving 1.0 g of malic acid powder into 10 mL of sterile water. The stock was diluted to prepare 1.0, 2.0, 3.0, and 4.0% of malic acid concentrations. Lactic acid stock was prepared by dissolving 8.35 mL of lactic into 10 mL of sterile water. The stock was diluted to prepare 1.0, 2.0, 3.0, and 4.0% of lactic acid concentrations. The above antimicrobial test solutions (100 µl) were added to rows of wells in a 96-well plate containing spinach homogenate and bacterial culture and the plate was incubated for 24 h at 37 °C. Non-inoculated wells were used as control.

4.2 Homogenate preparation

Fresh spinach was purchased from a local grocery store on the day of the experiment. Spinach leaves were disinfected using as sodium hypochlorite solution discussed earlier. Leaves were ground and stomached with sterile water. Homogenate of the spinach (90 µl) was dispensed into each wells of 96-well plate for the bacteria to grow and form biofilm by E.coli O157:H7.

4.3 Measuring the absorbance

Biofilm formation and reduction of *E.coli* O157:H7 were indirectly assessed by staining with crystal violet and measuring crystal violet absorbance, using detaining solution. The

incubated plate containing spinach homogenate, *E.coli* O157:H7 culture and antimicrobial was washed three times with deionized water and left to dry for 30 min under laminar hood. After drying, the plate was stained with 125 µl of 0.2% crystal violet for 30 min at room temperature. The wells were rinsed three times with deionized water and left to dry under laminar hood for 15 min. To solubilize the crystal violet 200 µl of 95% ethanol was added. Absorbance was measured by microplate reader at 595 nm.

5. Preparation and determination of biofilm and its disruption with organic acids

A Confocal microscope was used to observe biofilm on fresh produce. Fresh spinach was purchased from a local grocery store on the day of the experiment. Leaves were rinsed with water, and submerged in sodium hypochlorite solution (6.25 mL/liter) for 3 min to reduce microbial background. The leaves were then submerged in sterile water for 3 min to wash off any chlorine residual. The leaves were placed in petri dish, inoculated with 25 µl of ED 14 *E.coli* O157:H7 second day culture, and stored at 8 °C for 72 h to allow biofilm formation. After storing, spinach leaves were washed with 2 ml of sterile water to remove un-attached bacteria. Glutaraldehyde 1.0% was added to fix the slides. Gum Arabic (25 µl) was added to preserve the 3D structure of the formed biofilm. After 24 h, 50 µl of glycerol was added to prevent dehydration of the inoculated spot.

The inoculated spot was removed by using sterile scalpel and placed to microscopic slides. Slides were sprayed with organic acids using the electrostatic sprayer and water was used as control. The sprayed spinach leaves were left to dry under the safety cabinet. Upon drying, the leaves were observed under confocal microscope to observe biofilm disruption.

Statistical analysis

Completely Randomized Design was used in the study. One-way Analysis of variance (ANOVA) was performed using JMP 11.0. Significant difference was determined at ($P < 0.05$). Tukey HSD multiple comparison was used to compare means. The experiments were repeated three times with triplicate replications.

Results and Discussion

1. Determination of the bacterial strain adhesion

Total plate count of attached bacteria was used to determine the strain that showed the highest level of attachment on the spinach leaf disks before determining the effect of electrostatic spraying with malic acid, lactic acid alone and in combination on attached *E.coli* O157:H7. The number of bacteria was counted after rinsing the spinach disk with water to remove non-adherent bacteria the total plate counts of different strains of bacteria are given in Table.1. The *E.coli* strain ED 14 had the highest count of cells, 74 CFU/cm which showed its stronger adherence to the spinach in comparison to other strains. Some strains such as ED 16 did not show any adherence. Strains ED 15 and MD 46 attachments were not significantly different from each other (5.0 and 9.0 CFU/cm respectively). MD 58 strain showed a high number of CFU/cm). Hence, the *E.coli* strain ED 14 was chosen to investigate the effect of electrostatic spraying on the reduction of attached bacterial cells on spinach disk and the disruption of biofilm formation.

2. Evaluation of electrostatic spraying with organic acids on attached bacterial

ED 14 (the strain that showed the best attachment attribute into spinach disk) was investigated to determine the effect of electrostatic spraying with lactic, malic acids alone and in combination on spinach. Log reductions (CFU per disk) were obtained by subtracting the number

of bacterial colonies on the antimicrobial (lactic/malic) treated disk from the number of colonies on the inoculated disk treated with pH-adjusted to 2.4 (similar to pH as that of other treatments) to eliminate pH effects contributing to decontamination water (control).

The results of the enumeration of attached *E.coli* can be found in Table 2. The combination treatment of LA 2.0 + MA 2.0 demonstrated the highest log reduction of 4.14. The data indicated that lactic acid was more effective than malic acid in reducing the attached *E.coli* O157:H7 on spinach leaf. The highest concentration of lactic acid (4.0 %) reduced the attached *E.coli* O157:H7 by 3.17 logs. Malic acid (4.0%) resulted in a log reduction of 2.03.

Our study demonstrated that the effect of combined treatment is synergistic and not additive. When LA 2.0% and MA 2.0% were applied separately they resulted in log reductions of 1.46 and 0.74, respectively. However, the combined treatment of LA 2.0% + MA 2.0% resulted in a log reduction of 4.1. Kroupitskiet and others (2009) reported 2.5, 1.7 log reductions on intact and cut edges of lettuce leaves respectively ($P< 0.05$), with 200 ppm free chlorine treatment. The lettuce was inoculated with *Salmonella* Typhimurium for 2 h to allow attachment In our study, *E.coli* O157:H7 was given 24 h to allow attachment to give the bacteria sufficient time to attach. Park and others (2011) used other organic acids (propionic, acetic, lactic, malic, and citric acid) with different concentrations 1% and 2% to inactivate *E.coli* O157H7, *Salmonella Typhimurium*, and *Listeria* monocytogenes in lettuce**.** The results by Park and others (2011) study showed that malic and citric acids had the highest log reduction of 2.98 and 2.86, respectively. After 10 min treatment. In our study, the treatment time did not exceed 10 sec and the reduction was higher than Park and others (2011) study Liao and Cooke (2001) reported that trisodium phosphate (3% to 12%) used to decontaminate *Salmonella* Chester attached on pepper disks, resulted in log reductions of 1 to 2. Tamblyn and Conner (1997) reported that malic acid

applied by dipping for 15 sec at 23 °C at various concentrations (0.5, 1.0, 2.0, 4.0, and 6.0%) had 0 log reduction ($P< 0.05$) on *Salmonella* typhimurium firmly attached to broiler chicken.

3. Crystal violet binding assay

Crystal violet stains the biofilm formed and this method was used to study/evaluate biofilm in the 96-well plate. The method was conducted as explained earlier. Absorbance of the mobilized crystal violet gives indirect assessment of the biofilm formed and any biofilm reduction from organic acids. Absorbance of ED 14, ED 15, ED 16, MD 46, MD 47, and MD 58 were 1.56, 1.30, 1.18, 1.00, 1.31, and 1.12 respectively. The results in Table. 3 shows that the effect of organic acids on *E.coli* O157:H7 was strain-dependent. The variation in biofilm formation among the strains might be due the different sources of the strains. ED 15, ED 16, and MD 58 were isolated from beef. MD 46, MD 47, and ED 14 were isolated from alfalfa sprout, lettuce, cattle respectively 14 strain had the highest biofilm formation (1.56). However, it is not significantly different ED 15, ED 16 and MD 47 with 1.30, 1.18, and, 1.31 respectively. The strain that had the lowest biofilm formation was MD 46 with an absorbance of 1.0. Therefore, it was the strain most vulnerable to organic acids. The combined treatments of MA + LA 3.0% resulted in a biofilm reduction of 0.13. However, the combined treatment of MA + LA 3.0% resulted in the highest biofilm reduction in ED 15, ED 16, MD 46, MD 47, and MD 58 with absorbance values of 0.23, 0.21, 0.13, 0.18 and 0.27 respectively. It is noted that the combined treatment was not significantly different from lactic acid 4.0% ($P <0.05$). In ED 14, the combined treatment of MA + LA 3.0% resulted in the highest biofilm reduction and was significantly from other treatments Lactic acid (1.0, 2.0, 3.0, and 4.0%) and malic acid (1.0, 2.0, 3.0, and 4.0%) ($P <0.05$). Kim and others (2009) studied the biofilm formation ability of *E.coli* O157:H7 on spinach homogenate. The highest absorbance of biofilm formation was (1.0) on

spinach homogenate. However, when the same strain was used in meat homogenate such as beef, pork, chicken, and turkey, the biofilm formed was higher than fresh produce. The biofilm formation in beef, pork, chicken, and turkey homogenate was 1.2, 1.5, 1.3, and 1.09, respectively. The strain used in Kim's study was isolated from cattle feces. Therefore, the strain might be more prone to develop better biofilm on meat homogenate than fresh produce homogenate. From this data the strain used in our study had better biofilm-capability than Kim's study in spinach. (ED 14 absorbance is 1.56 while Kim's strain is 1.0 on spinach) by comparing the values of absorbance of the strain used in our study and in Kim's study. The strain used in our study resulted in higher absorbance values meaning more biofilm. The studies were done under the same conditions. The only variable was the strains used.

Although crystal violet is a rapid method to determine biofilm formation, results can be an overestimation since crystal violet stains matrix components that may be present in the 96-well plate and does not allow visual observation of the formed biofilm. The crystal violet assay could only allow to study biofilm on spinach homogenate on 96-well plate. Therefore, to study biofilm on spinach leave, confocal microscope was used.

4. Biofilm observation under confocal microscopy

Confocal microscopy was used for its ability to visualize the 3D structure of the biofilm and to observe the slide without any dehydration treatment that might affect the formed biofilm. Figure 1 shows that the pH-adjusted water treatment (control) had a little effect on the under the microscope. Figure 2shows that most bacterial cells were present as a biofilm and proves that the biofilm was not fully disrupted. Nevertheless, Figure 3 shows that most bacteria are individually present as single cells and not protected by layer of biofilm. The result demonstrate that malic acid treatment at 4.0% concentration was effective in disrupting biofilm formed by *E.coli* on

spinach. The thickness of the biofilm formed by *E.coli* O157:H7 on spinach could not be measured by the confocal microscope.

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Table 1-Number of attached colonies of various *E.coli* O157:H7 strains on spinach disk

Strain*	No. of attached colonies (CFU/disk)
ED 14	74 ± 2.1A
ED 15	5± 1.9DE
ED 16	0.0 ± 0.0E
MD 46	9.0 ± 1.4D
MD 47	27± 1.6C
MD 58	33±2.7B

***Strains of *E.*coli O157:H7. Values provided are means ± standard deviations (P<0.05). Strains connected by the same letters are not significantly different from each other**

Table 2-Log reductions of attached *E.coli* strain ED 14 on spinach disk after electrostatically spraying with different organic acids.

Treatments (%)*	Log reductions
Control	0H
LA 1.0	1.13 ±0.37E
LA 2.0	1.48 ± 0.07D
LA 3.0	2.14 ± 0.04C
LA 4.0	3.17±0.42B
MA 1.0	0.52±0.03G
MA 2.0	0.74± 0.05FH
MA 3.0	1.72±0.03D
MA 4.0	2.03±0.05C
LA 0.5+ MA 0.5	0.91±0.06EF
LA 1.0+MA 1.0	1.03±0.09E
LA 1.5+ MA 1.5	2.04±0.05C
LA 2.0 + MA 2.0	4.14±0.06A

***Treatments: control- spraying with water; LA-Lactic acid; MA- Malic acid.**
Values connected by same letters are not significantly different (P < 0.05).
Values are expressed as means ± standard deviations

Table 3-Crystal violet assay of *E.coli* O157:H7 strains after malic and lactic treatments alone and in combinations in spinach homogenate incubated for 24h.

Treatment%*	ED 14**	ED 15**	ED 16**	MD 46**	MD 47**
Control	1.56±0.16A	1.30±0.30A	1.18±0.23A	1.00±0.26A	1.31±0.46A
LA 1.0	0.93±0.04BC	0.90±0.23B	0.33±0.12B	0.28±0.17C	0.53±0.18B
LA 2.0	0.91±0.03BCD	0.84±0.27BC	0.50±0.4BC	0.22±0.02C	0.50±0.30BC
LA 3.0	0.85±0.04CDE	0.73±0.40BC	0.40±0.2BC	0.21±0.06C	0.40±018BCD
LA 4.0	0.83±0.08DE	0.70±0.28BC	0.38±0.2BC	0.17±0.09C	0.34±0.14BCD
MA 1.0	0.96±0.04B	0.65±0.24BC	0.55±0.4BC	0.71±0.32B	0.35±0.18BCD
MA 2.0	0.94±0.04BC	0.60±0.24BC	0.33±0.12C	0.25±0.08C	0.31±0.13BCD
MA 3.0	0.87±0.05BC	0.55±0.10C	0.29±0.09C	0.24±0.04C	023±0.03CD
MA 4.0	0.79±0.05E	0.53±0.23CD	0.26±0.07C	0.15±0.03C	0.22±0.04CD
MA LA 3.0	0.64±0.02A	0.23±0.08D	0.21±0.09C	0.13±0.02C	0.18±0.10D

***Treatments: control- no treatment; LA - Lactic acid; MA- Malic acid.**

**** Strains of *E.coli* O157:H7**

Values are expressed as means ± standard deviations.

Values connected by same letters are not significantly different (P < 0.05).

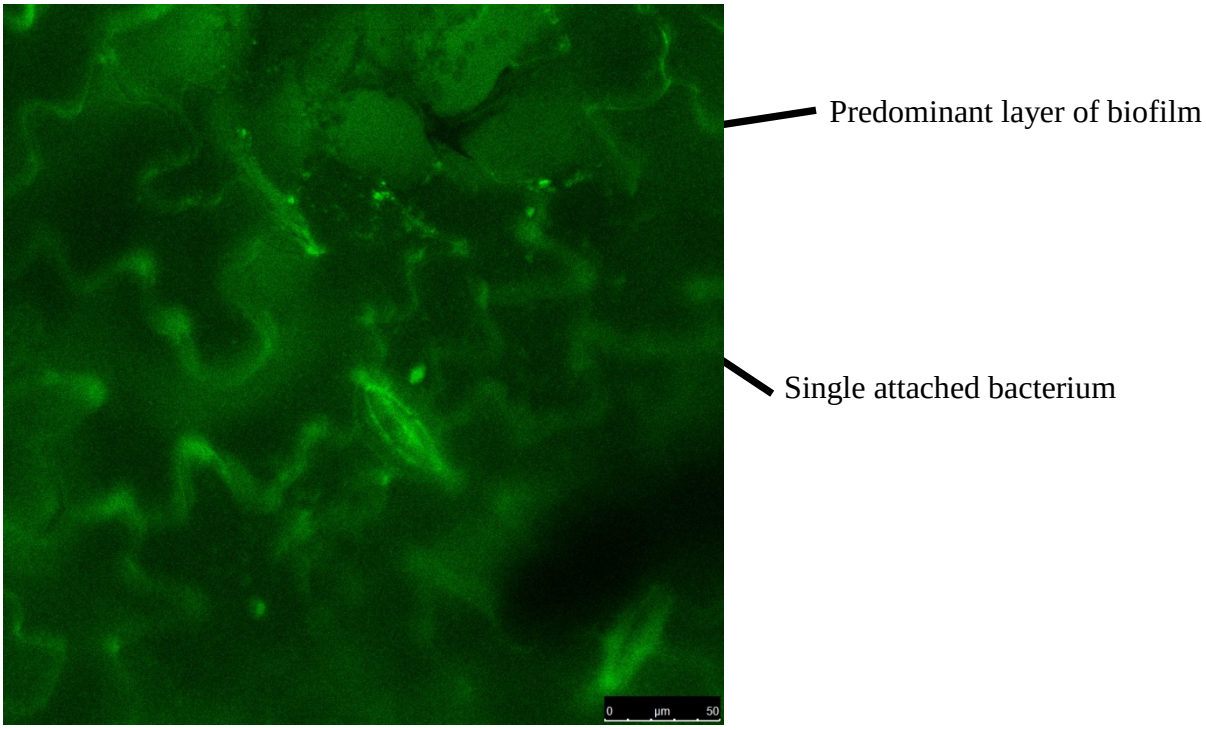

Figure 1-Micrograph showing attachment and biofilm formation of GFP-labeled *E.coli* O157:H7 strain ED 14 on spinach leave. (Magnification – 50x)

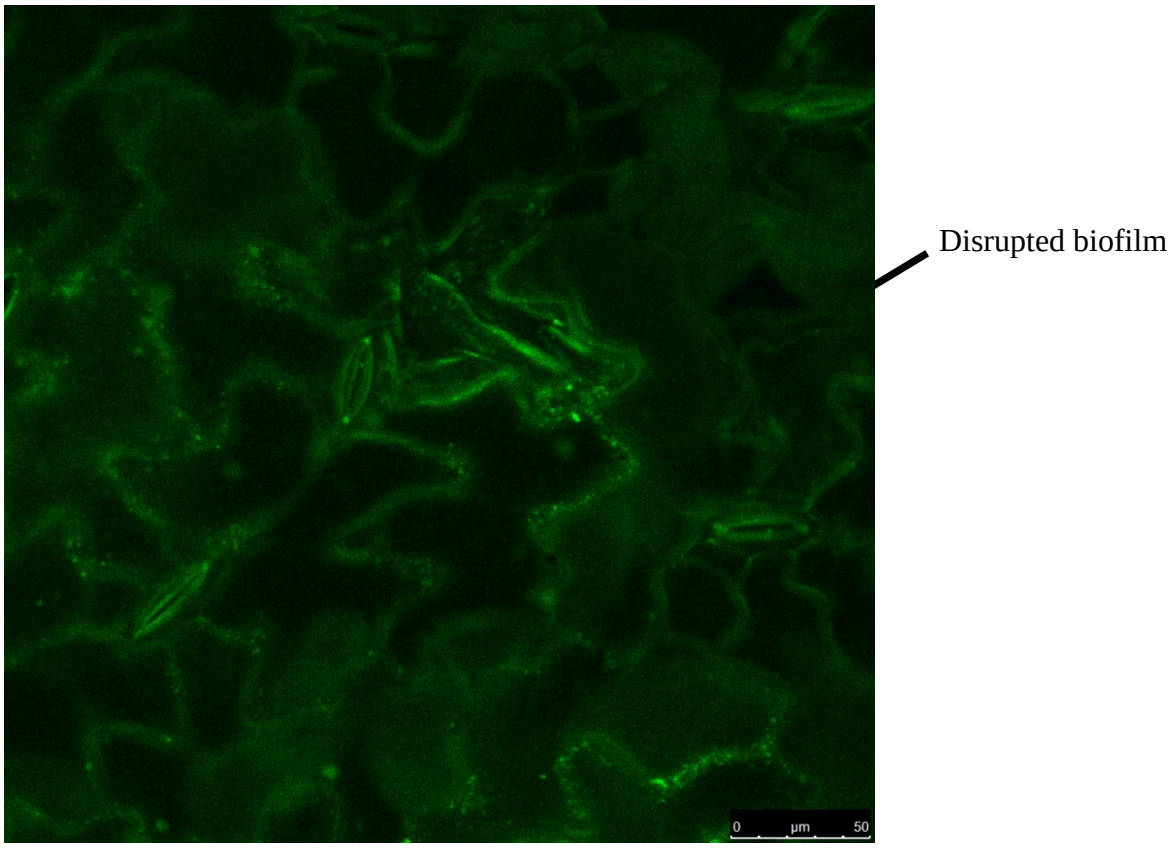

Figure 2-Micrograph showing the effect of electrostatic spraying with malic 2.0 % on attachment and biofilm formation of GFP-labeled *E.coli* O157:H7 strain ED 14 on spinach leave. (Magnification–50x).

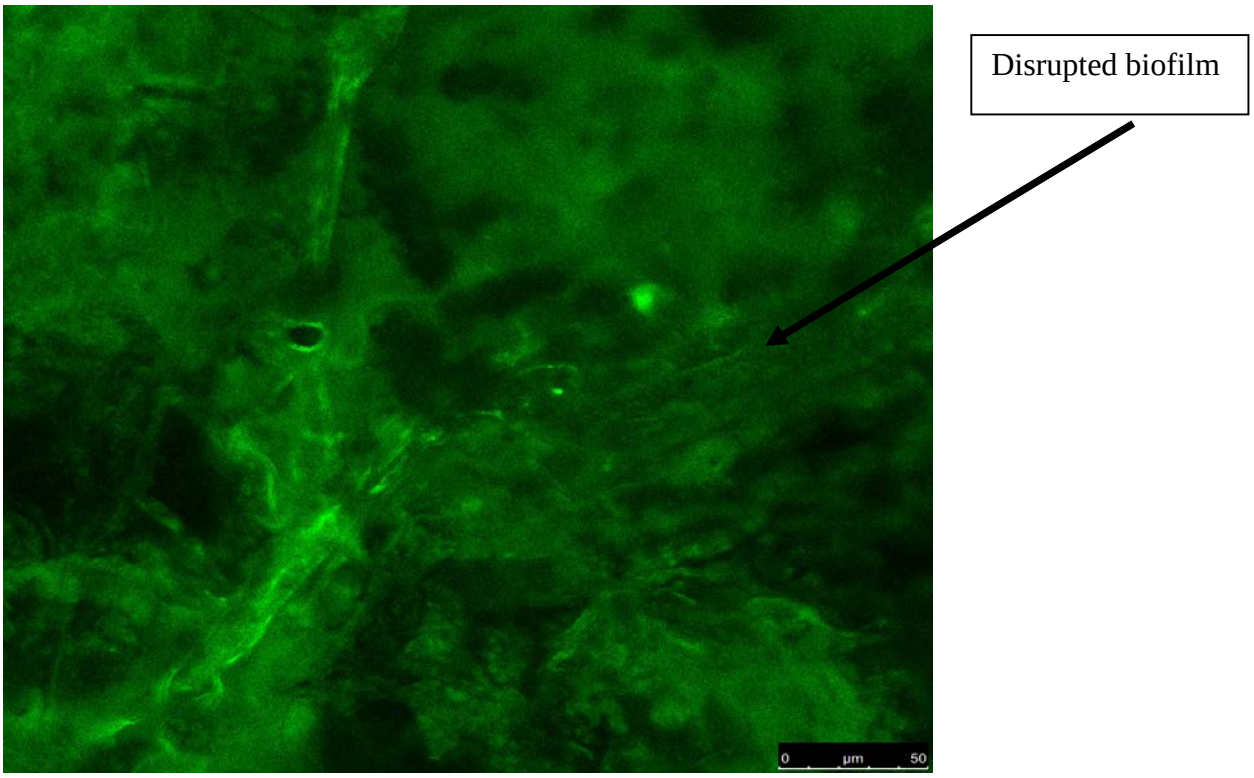

Figure 3-Micrograph showing the effect of electrostatic spraying with malic 4.0 % on attachment and biofilm formation of GFP-labeled *E.coli* O157:H7 strain ED 14 on spinach leave. (Magnification – 50x).

Chapter 4: Effect of electrostatic spraying with organic acids in the disintegration of biofilm formed by *Salmonella* Typhimurium on cantaloupe melon.

Introduction

Outbreaks associated with the consumption of fresh produce have increased in the last two decades. The increase in the outbreaks coincides with the increase of the consummation of fresh produce in the last 20 years because of the healthy trend that consumers around the world are adapting to by eating more fresh produce (Olaimat and Holley 2012).

Salmonella outbreaks from fresh produce have been on the rise specifically outbreaks related to tainted cantaloupe melons (Annous and others 2005). A multistate outbreak of *salmonella* associated with cantaloupe melon occurred in 2011(CDC 2011). The outbreak resulted in the infection of 20 persons. A more severe multistate outbreak of *Salmonella* Typhimurium and *Salmonella* Newport occurred in the following year. The outbreak resulted in the infection of 261people (228 persons with outbreak strain *Salmonella* Typhimurium and 33 with *Salmonella* Newport) in 24 states. This outbreak resulted in the hospitalization of 94 persons and three cases of death were confirmed as a result of the outbreak (CDC 2012). The outbreak's consequences did not only harm consumers but also affected the producer. The Food and Drug Administration announced a recall of cantaloupes originating from Chamberlain Farms of Owensville in Indiana (FDA 2012). Cantaloupes can be contaminated on the farm with *Salmonella* either directly through seeds, manure in fertilizer and irrigation water or indirectly through wild animals and insect vectors. Processing practices such as washing, icing, and hydro cooling presently pose a potential risk for the cross contamination of melons (Oria 2001). However, according to a survey of farm and processing facilities, the main source of melon contamination with *Salmonella* is the submergence of cantaloupes in tainted water in the post-harvest facilities (Gagliardi and others 2003).

Washing treatments have been shown to be ineffective in reducing pathogens on cantaloupe. Ukuku and Sapers (2001a) reported that the effectiveness of washing cantaloupes with chlorine or hydrogen peroxide was sufficient in reducing *Salmonella* Stanley up to 3 log CFU/cm^2 when the treatments immediately followed the inoculation. However, both of the treatments were ineffective in reducing inoculated cantaloupes with *Salmonella* that were stored at 4 or 20 °C for 3 days.

Washing with chlorine or hydrogen peroxide was ineffective in removing *E.coli* ATCC 25922 on cantaloupe surfaces (Ukuku and others 2001b). Washing artificially-inoculated apples with *E.coli* O157:H7 and treating with 1% H_2O_2 was effective in reducing the population of the pathogen; however, the treatment was less effective on the population of *E.coli* O157:H7 inoculated on cantaloupe (Sapers and Sites 2003). The use of warm water (50 °C) was insufficient to reduce the population of microbes on the rind of cantaloupes. Adding some surfactants such as sodium dioctyl sulfosuccinate, sodium 2-ethylhexyl sulfate, or sodium dodecyl did not enhance the effectiveness of water even when the solutions were applied at 50 °C (Sapers and others 2001).

Washing with chlorine is widely used to decontaminate fresh produce. Chlorine at concentrations of 50 to 200 ppm with a contact time of 1 to 2 min is routinely used as a produce sanitizer in wash, spray, and flume waters by the food industries (Beuchat and others., 1998; Cherry 1999; Pirovani and others., 2004). Chlorine dioxide (3 ppm) and acidified sodium chlorite (500 to 1200 ppm), peracetic acid (40 and 80 ppm), hydrogen peroxide (2%), ozone (20 ppm) and lactic acid are other alternative chemical sanitizers for produce. However, washing with chlorine is both inconsistent and unpredictable (Rodgers and others 2004). Moreover,

chlorine may interact with organic matter causing formation of harmful byproducts like trihalomethanes that have been linked to miscarriages and cancer (Huang and Batterman 2009).

The synergistic effect of chlorine washing and irradiation to decontaminate aerobic microbes was investigated. Palekar and others (2004) first washed cantaloupe melons with chlorine then treated the cantaloupe's slices with low dose of irradiation. This study found that the combined treatment was effective in reducing the microbial load. However, more work is needed to target specific pathogens linked to cantaloupes' outbreaks such as *Salmonella*.

It is recognized that human pathogens have the ability to form biofilms on fresh produce (Morris and others 1997; Rayner and others 2004; Annous and others 2009; Jahid and Ha 2012; Kim and Wei 2012).*Salmonella* was able to form biofilm on cucumber, mango, guava, parsley, and cantaloupe melons (Annous and others 2005; Lapidot and others 2006; Tang and others 2012). Biofilms can be resistant to antimicrobials (Van Houdt and others 2004; Agle 2007; Annous and others 2009a ;), biocides (Annous and others 2009b), disinfectants (Rayner and others 2004; Jahid and Ha 2012) and sanitizers (Jahid and Ha 2012) that are used in the food industry (Annous and others 2006), which makes biofilms a serious problem to this industry (Stier2005). In addition, Biofilms are more likely than planktonic bacteria to survive severe environment conditions (Simões and others 2010)

Studies conducted to eliminate biofilms formed on fresh produce are scarce. In addition, the nature of the cantaloupes' surfaces facilities the attachment and biofilm formation by microorganisms (Cherry 1999). The main aim of this study therefore was to investigate the effect of electrostatic spraying with organic acids to disintegrate biofilms formed by *Salmonella* Typhimurium on cantaloupes.

Material and Methods

1. Culture preparation

Agar slant cultures of green fluorescent protein GFP-labeled SD 10 and SD 11 *Salmonella* Typhimurium were obtained from the University of Georgia, Center for Food Safety, Griffin, GA. Frozen stock cultures were made with a single colony of each microorganism. Frozen stock cultures at -70 °C of the above were thawed, transferred to 10 mL of brain heart infusion (BHI) (Becton Dickinson Microbiology Systems, Becton Dickinson and company, Sparks, MD, U.S.A.) and incubated at 37 °C for 24 h with 200-rpm agitation using a New Brunswick Scientific (Edison NJ, U.S.A.) agitating incubator. Second-day inoculum was prepared by inoculating 10 µl of first-day culture into fresh 10 mL of BHI and incubated in the shaker maintained at 37 °C for 24 h. The second-day culture (10^7 log CFU/mL) was used to inoculate cantaloupes rinds in the studies. Fresh cantaloupes were purchased from a local grocery store.

2. Determination of the *Salmonella* Typhimurium bacterial strain adhesion

Cantaloupe rinds were selected because it's the first part that bacteria attach to and therefore internalize into the edible part. Rinds can aid the attachment of bacteria because of its surface properties such as roughness, crevices, and pits. Cantaloupes rinds were washed with water to remove any debris, disinfected with sodium hypochlorite solution (6.25 mL/ L deionized water), and rewashed with sterile deionized water. Disinfected cantaloupes rinds were randomly cut with sterilized stainless steel cork-borer to produce rind disks of 1 cm diameter. Cantaloupes rind disks were transferred into a 24-well plate that contained 1.9 mL of 0.1% peptone water and 100 µl of peptone and bacterial culture (1:1). The 24-well plate was incubated for 24 h at 37 °C. Cantaloupe rinds were rinsed with sterile water to remove non-attached cells, transferred to Whirlpak bag that contained 2 mL of PBS and stomached for 3 min. Stomached

samples were serial diluted using PBS and spread-plated on XLT4 (Xylose Lysine Tergitol 4) agar media. The plates were incubated at 37 °C for 24 h and colony counts were taken after incubation period to enumerate the number of *Salmonella* Typhimurium Cantaloupe rinds.

3. Evaluation of electrostatic spraying with organic acids on attached bacterial

Cantaloupe rinds were randomly cut with sterilized stainless steel cork-borer to produce cantaloupe disks of 1 cm in diameter. The rind disks were transferred into 24-well plate that contained 1.9 mL of 0.1% peptone water and 100 µl of 1:1 peptone + Salmonella *Typhimurium* culture and incubated for 24 h at 37 °C to allow bacterial attachment. The cantaloupe rind disks were rinsed with sterile water to remove non-attached cells, transferred to Whirlpak bag for spraying electrostatically with malic and lactic acid solutions alone (1.0/ 2.0/ 3.0/ 4.0 %) and in combination (0.5+0.5/ 1.0+1.0/ 1.5+1.5/ 2.0+2.0%). After spraying, rind disks were transferred to Whirlpak bag that contained 2 mL of PBS and stomached for 3 min. Stomached samples were serially diluted using PBS and spread-plated on XLT4agar media. The plates were incubated at 37 °C for 24 h. Colony counts were taken after incubation period to enumerate the amount of *Salmonella* Typhimurium on cantaloupe rinds. Log reductions were calculated by subtracting the log counts obtained by spraying electrostatically with malic and lactic acid solutions alone (1.0/ 2.0/ 3.0/ 4.0 %) and in combination (0.5+0.5/ 1.0+1.0/ 1.5+1.5/ 2.0+2.0%) from the log counts obtained for the control.

4. Crystal violet binding assay.

Crystal violet binding assays described by Kim and others (2009) with was used to study the biofilm-forming ability of the *Salmonella* Typhimurium. Cantaloupe rinds were disinfected with sodium hypochlorite solution. A 96-well plate was prepared by adding the bacterial culture

along with cantaloupe homogenate and incubate for 24 h at 37 °C to allow attachment and biofilm formation. This work was done to investigate the effect of organic acids on the biofilm formed by *Salmonella* Typhimurium strains SD 10 and SD 11.

4.1 Antimicrobials preparation

A stock solution was prepared by dissolving 1.0 g of malic acid powder into 10 mL of sterile water. The stock was diluted to prepare 1.0, 2.0, 3.0, and 4.0% of malic acid concentrations. Lactic acid stock was prepared by dissolving 8.35 mL of lactic into 10 mL of sterile water. The stock was diluted to prepare 1.0, 2.0, 3.0, and 4.0% of lactic acid concentrations. Combined treatment of 3.0% of lactic and malice acid was also prepared as it showed to be effective based on our lab work (Ganesh and others 2010).

The above antimicrobial test solutions (100 µl) were added to rows of wells in a 96-well plate containing rind homogenate and bacterial culture and the plate was incubated for 24 h. Non-inoculated wells were used as a control.

4.2 Homogenate preparation

Fresh cantaloupes were purchased from a local grocery store on the day of the experiment. Cantaloupe rinds were disinfected using as sodium hypochlorite solution discussed earlier. Rinds were ground and stomached with sterile water. Homogenate of the cantaloupe (90 µl) was dispensed into each wells of 96-well plate for the bacteria to grow and form biofilm.

4.3 Measuring the absorbance

Biofilm absorbance, measured as intensity reduction of a light beam transmitted through the film, should correlate with its mass. The incubated plate containing cantaloupe homogenate, *Salmonella* Typhimurium culture and antimicrobial was washed three times with deionized water and left to dry for 30 min. After drying, the plate was stained with 125 µl of 0.2% crystal violet

for 30 min at room temperature. After removing the staining solution, the wells were rinsed three times with deionized water and let to dry. To solubilize the crystal violet 200 µl of 95% ethanol was added. Absorbance was measured by microplate reader at 595 nm.

5. Biofilm observation under confocal microscopy.

A Confocal microscope was used to observe biofilm on cantaloupe. Fresh cantaloupe was purchased from a local grocery store on the day of the experiment. Rinds were rinsed with water, and submerged in sodium hypochlorite solution (6.25 mL/liter) for 3 min to reduce microbial background. The rinds were then submerged in sterile water for 3 min to wash off any chlorine residual. The rinds were placed in petri dish, inoculated with 25 µl of SD 10 *Salmonella* Typhimurium second day culture, and stored at 8 °C for 72 h to allow biofilm formation. After storing, cantaloupe rinds were washed with 2 ml of sterile water to remove un-attached bacteria. The inoculated spot was removed by using sterile scalpel and place on microscopic slides. Slides were sprayed with organic acids using the electrostatic sprayer and water was used as control. The sprayed cantaloupe rinds were left to dry under the safety cabinet. Upon drying, the rinds were observed under confocal microscope to observe biofilm disruption.

Statistical analysis

Completely Randomized Design was used in the study. One-way Analysis of variance (ANOVA) was performed using JMP 11.0. Significant difference was determined at ($P < 0.05$). Tukey HSD multiple comparison test was used to compare means. The experiments were repeated three times with triplicate replications.

Result and discussion

1. Determination of the *Salmonella* Typhimurium bacterial strain adhesion.

Finding the strain that has better attachment quality is essential to my work since the first step in biofilm formation is the irreversible attachment. There are many factors such as topography of food surface, contact time, flagella and fimbriae, cell surface charge, cell surface hydrophobicity, and curli expression that contribute to the attachment of pathogens into plant surface. Also, the literature is elusive in stating the factors that can aid in the attachment. The total plate count is straightforward method to determine the attachment based on the number of the recovered colonies after rinse the cantaloupe. Therefore, the total plate count to compare the attachment properties between the two Salmonella stains (SD 10 and SD 11) was used. Table 1, shows that SD 10 has better attachment since the recovered colonies after washing with water to remove non-adherent bacteria is 70 colonies. SD 11 was poorly attached to the cantaloupe disk. Only three colonies were counted after rinsing the cantaloupe rind disk with water. SD 10 was chosen to evaluate the effect of electrostatic spraying with organic acid on cantaloupe since it showed better attachment properties than SD 11.

2. Enumeration of attached *salmonella* after spraying with organic acids

The enumeration of attached *Salmonella* strain SD 10 on cantaloupe disk after spraying with organic acids is given in Table 2. The combined treatment of 2.0% Lactic acid+ 2.0 % malic acid had the highest log reduction (3.58). Lactic acid proved to be more effective than malic acid in reducing the attached Salmonella. The highest concentration of lactic acid used resulted in 3.36 log reduction while malic acid had a log reduction of 1.97. The difference between log reduction caused by spraying with the combined treatment of LA 2.0**%** +MA 2.0**%**

(10^7) is not significant from that of LA 4.0% (P >0.05). From an economical prospective, it is recommended that the industry could use lactic acid at 4.0% concentration instead of the combined treatment.

Lactic acid at 4.0% can have its adverse effect on flavor. However, the inedible rinds of cantaloupes was treated and not the edible part. Therefore, flavor attributes should not be affected. The combined treatment of LA 2.0%+ MA 2.0 % had log reduction of 3.58. When solely used, lactic acid at 2.0% and malic acid at 2.0% resulted in 1.55 and 1.05 log reductions, respectively. The highest log reduction observed in spinach was 4.14 in comparison with 3.58 in cantaloupes. This can be explained by the morphology characteristics of cantaloupe rinds such as roughness, crevices, and pits that aid the attachment of microorganisms.

Few studies focused on decontaminating cantaloupes rinds. Annous and others **(**2013**)** used hot water (at 90 °C) to decontaminate cantaloupe rinds inoculated with *Salmonella* Poona. The hot water resulted in a log reduction > 5.0 log CFU/g of rind (10^9). Sapers and others (2001) reported that washing cantaloupe rinds with mild-heat water treatment (at 50 °C) was not effective in reducing the microbial load on the cantaloupes. Ukuku and Sapers (2001a) reported that the effectiveness of washing cantaloupes with chlorine or hydrogen peroxide was sufficient in reducing *Salmonella* Stanley up to 3 log CFU/cm^2 when the treatments immediately followed the inoculation (the pathogen was not given time for attachment). In this work, *Salmonella* was given 24 h to attach to cantaloupe disk and log reduction achieved was higher 3.58 CFU/cm. Alvarado-Caillas and others **(**2007**)** reported that spraying 2% $_L$-lactic acid on cantaloupes inoculated with *Salmonella* Typhimurium and *E.coli* O157:H7 resulted in log reduction of 3.0 for both pathogens. In this work, higher concentration of lactic was used (4.0%) to obtain a log reduction of 3.58. The use of hot water seems to be the most effective measure to decontaminate

Salmonella. However, recontamination of cantaloupe can occur if the same water is used to decontaminate cantaloupe.

3. Crystal violet binding assay

Crystal violet assay was conducted to determine the biofilm formation and the effect of organic acids on biofilm formed by *salmonella* in cantaloupe homogenate. Overall, the biofilm formation is strain dependent. The SD 11 strain developed slightly higher biofilm than SD 10 with absorbance readings of 0.65 and 0.60 respectively ($P>0.05$). However, it can be shown from Table 3 that biofilm formed by SD 11 is a more susceptible to organic acids. In both strains, the combined treatment resulted in the lowest absorbance (lower biofilm formation) with 0.24, 025 to SD 10 and SD 11, respectively. The different concentration of organic acids used did not significantly differ from each other ($P>0.05$).The combined treatment of LA+MA 3.0% reduced the biofilm formed by *Salmonella* strains (SD 10 and SD 11) significantly ($P<0.05$). Almost all the studies utilize crystal violet to evaluate biofilm formation in media (model) and not in foods matrix. However, Kim and others (2009) reported the use of crystal violet to evaluate biofilm formation by *E.coli* O157:H7 in different foods homogenates (Beef, pork, chicken, turkey, cantaloupe, lettuce, alfalfa, and spinach). Kim and others (2009) found that *E.coli* O157:H7 formed the highest biofilm after 24 h of incubation with cantaloupe homogenate. However, Kim's study did not utilize crystal violet to evaluate biofilm formation by *salmonella* strains on fresh produce. In this work, the ability of *Salmonella* strains to form biofilm on cantaloupe homogenate was investigated.

4. **Biofilm observation under confocal microscopy**

Confocal microscopy was used to detect disruption on biofilm formed by *Salmonella* on cantaloupe rinds. The rinds were sprayed with combined treatment since it is this treatment that resulted in the highest log reduction (3.58) on attached Salmonella on cantaloupe rind.

Figure.1 shows the surface of non-inoculated cantaloupe surface. The surface of cantaloupe rind can aid the attachment of bacteria for its roughness and crevices. It shows that the bacteria can be protected from the washing treatment. In the electrostatic spray, charged particulates are smaller than 100 um. Therefore, it can internalize the surface of cantaloupe rinds where the bacteria can be hidden.

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Table 1-Number of Attached colonies of *Salmonella* strains on cantaloupe rind

Strain*	No. of attached colonies (CFU/disk)
SD 10	70.5 ± 4.7A
SD 11	3.5 ± 1.4B

Strains of *Salmonella
Values are expressed as means± standard deviations.
Values connected by the same letters are not significantly different (P<0.05)

Table 2-Log reductions of *Salmonella* strain SD 10 on cantaloupe disk after electrostatic spraying with different organic acids

Treatments (%)*	Log reductions
Control	0H
LA 1.0	1.05 ±0.44EF
LA 2.0	1.55 ± 0.01DE
LA 3.0	2.45 ± 0.09BC
LA 4.0	3.36±0.10A
MA 1.0	0.44±0.05GH
MA 2.0	1.05± 0.16EF
MA 3.0	1.90±0.12D
MA 4.0	1.97±0.08CD
LA 0.5+ MA 0.5	0.90±0.03FG
LA 1.0+MA 1.0	2.00±0.06EF
LA 1.5+ MA 1.5	2.65±0.06B
LA 2.0 + MA 2.0	3.58±0.04A

***LA: Lactic acid; MA: Malic acid**
Values are expressed as means± standard deviations.
Values with the same letters are not significantly different (P<0.05).

Table 3-Crystal violet assay of two strains of *Salmonella* after malic acid and lactic acid treatments alone and in combination in cantaloupe homogenate incubated for 24 h at 37 °C

Treatment (%)*	SD 10**	SD 11**
Control	0.60±0.10A	0.65±0.02A
LA 1.0	0.54±0.11AB	0.54±0.01B
LA 2.0	0.45±0.12BC	0.43±0.04C
LA 3.0	0.41±0.06CD	0.38±0.01D
LA 4.0	0.30±0.06DE	0.26±0.02E
MA 1.0	0.49±0.08ABC	0.53±0.03B
MA 2.0	0.44±0.10BC	0.44±0.03C
MA 3.0	0.30±0.02DE	0.35±0.01D
MA 4.0	0.30±0.02DE	0.35±0.01D
MA + LA 3.0	0.24±0.08E	0.25±0.14E

***LA: Lactic acid; MA: Malic acid**
**** SD 10 and SD 11 are strains of *Salmonella* Typhimurium**
Values are expressed as means± standard deviations
Values with the same letters are not significantly different (P<0.05).

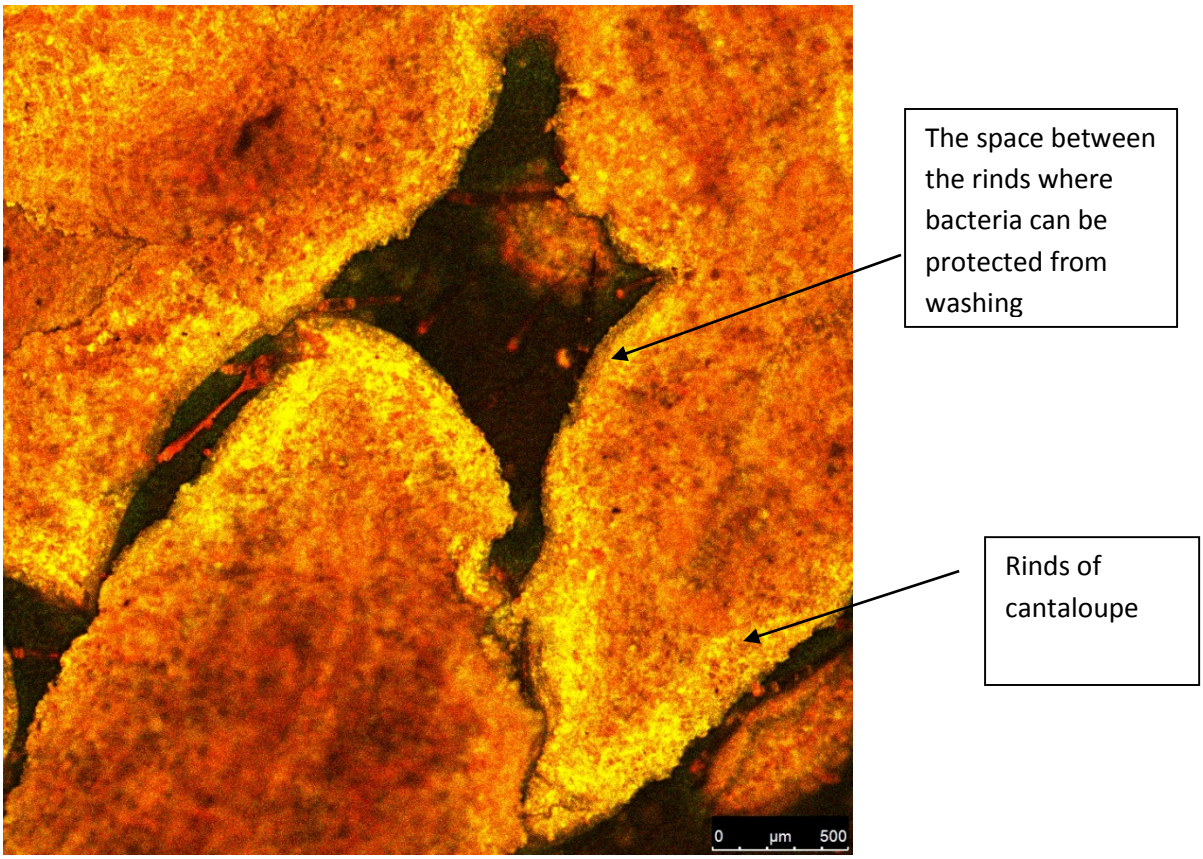

Figure 1-Non-inoculated surface of cantaloupe rind showing the crevices and pits between which the bacteria can entrap, attach, and form biofilm.

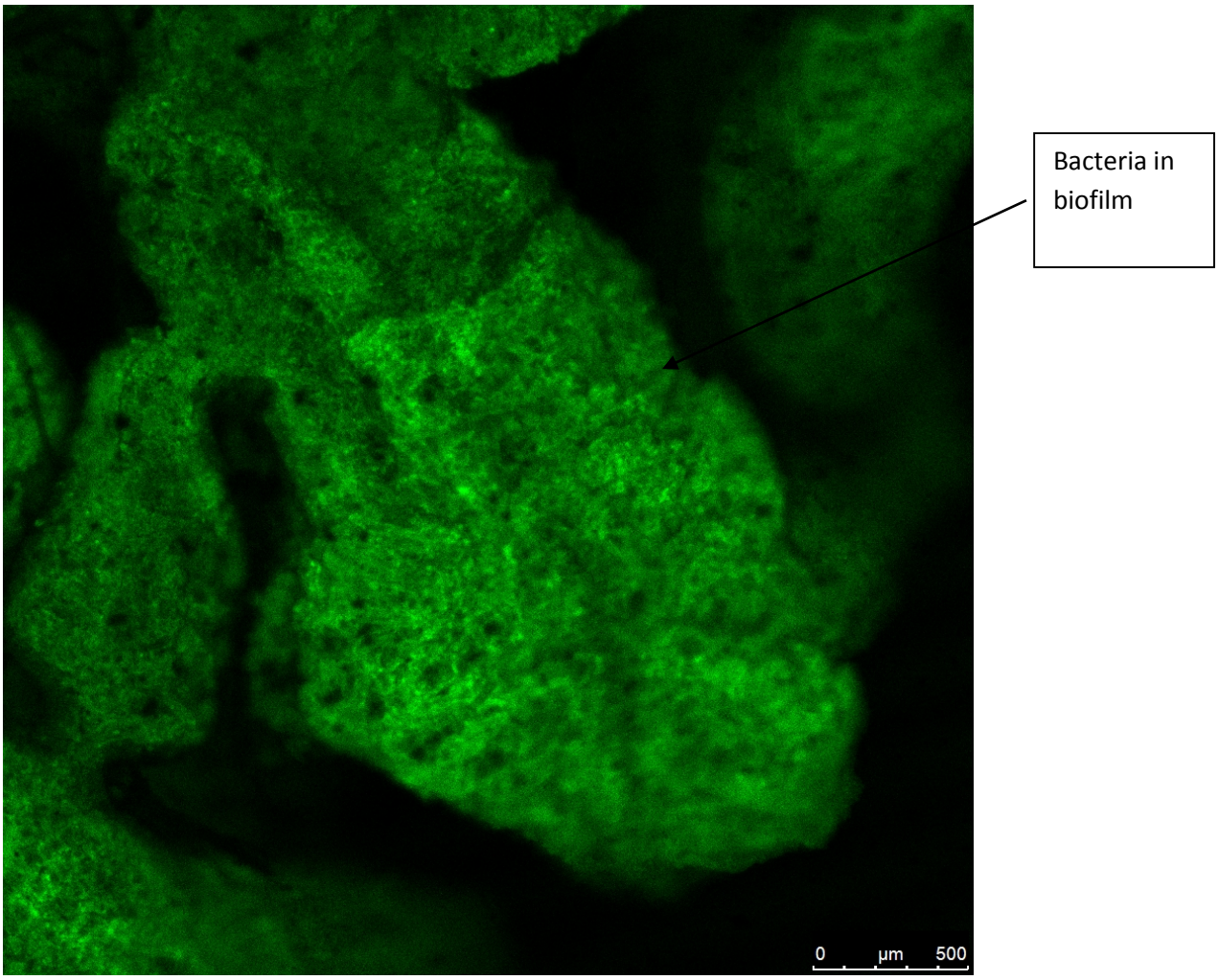

Figure 2-Biofilm formed by *Salmonella* on cantaloupe rinds after electrostatic spraying with water treatment (control).

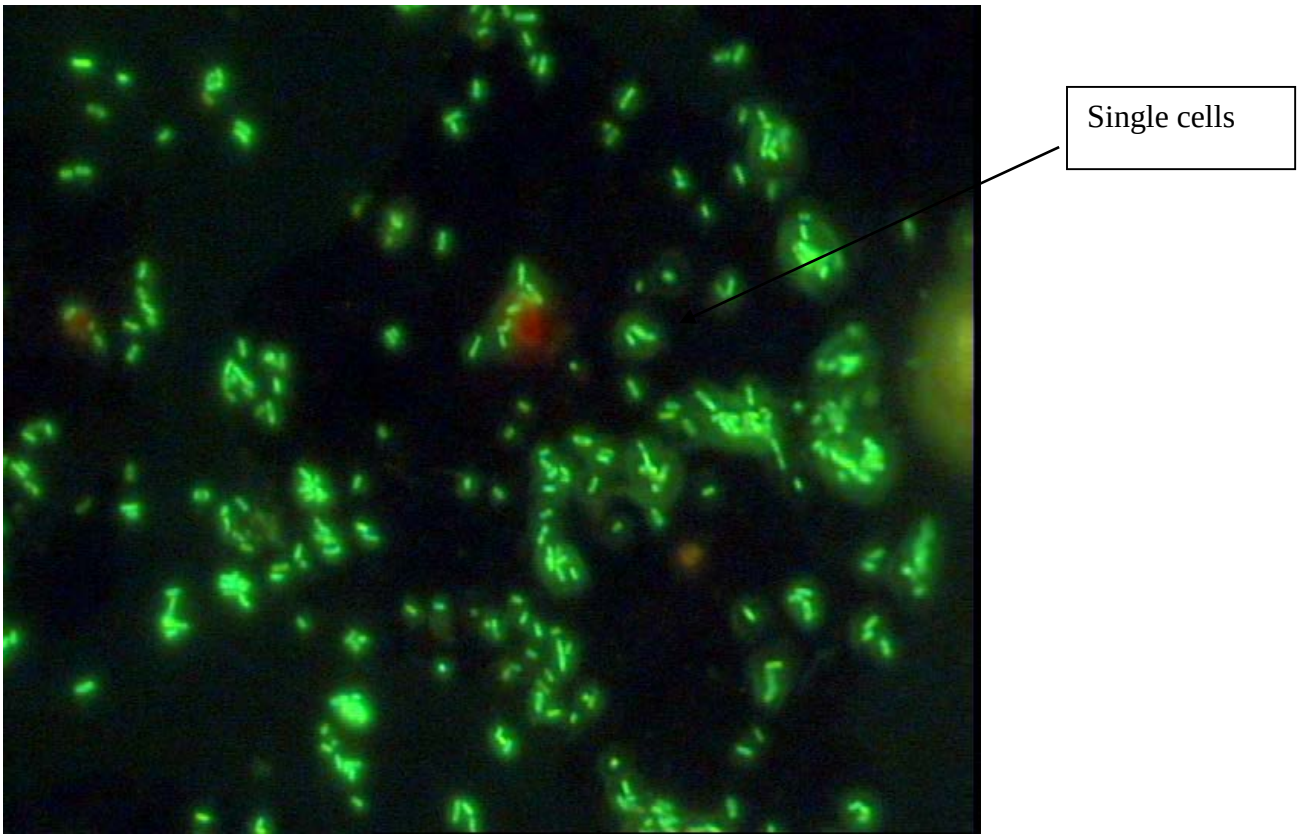

Figure 3-Biofilm disruption on cantaloupe rinds after electrostatic spraying with LA 2.0%+ MA 2.0%.

Chapter 5: Effect of organic acids on quorum sensing in *E.coli* O157:H7 and *Salmonella* Typhimurium on spinach and cantaloupe.

Introduction

Quorum sensing is defined as "cell density-dependent signaling system by which bacteria modulate a number of cellular functions" through signaling compound known as autoinducers or bacterial pheromones (Smith and others 2004).Quorum sensing is a strategy that bacteria use as a response to environmental stresses such as lack of nutrients or sever temperature. Autoinducer is a term given to the signal molecules compounds that bacteria**l** cells utilize to control the expression of specific genes.

Gram- negative bacteria such as *E.coli* O157:H7 and *Salmonella* Typhimurium produce two types of autoinducers: acylated homoserine lactones (AI-1) and furanosyl borate diester (AI-2) (Lu and others 2005). Bacteria that produce AI-1 and AI-2 autoinducers have to possess a luxS gene which code the Luxs proteins that biosynthesize AI-2. (Figure 1). The autoinducer molecules affect various gene expression such as virulence, toxicity, sporulation, plasmid transformation, antibiotic production, and biofilm formation (Bainton and others 1992; Bassler and Greenberg 1997; Davies and others 1998; Luo and Farrand 2001; Oger and Farrand 2002; Sperandio and others 2001).

Quorum sensing is a type of regulatory process that ensures that there is sufficient cell density before a specific gene product is made. This process allows bacteria to increase in numbers before starting to produce a particular gene produce such as biofilm.

When the concentration of AI-1 and AI-2 exceed a certain threshold within the cells, it binds and activates a regulatory protein which then binds to a specific site on the DNA. The binding of this regulatory protein transcription activator results in the production of biofilm (Bassler 1999).

The AI -2 activity inhibition has been reported by other investigators. Widmer and others (2007) used fatty acids isolated from poultry meat and shown reduction of AI-2 activity. Bodini and others (2009) demonstrated that *p*-coumaric acid was able to hinder the activity of quorum sensing. Choo and others (2006) work showed that vanilla extracts quorum sensing inhibition property. Koh and Tham (2009) used traditional Chinese medicinal plants and published the effectiveness of traditional Chinese medicinal against quorum sensing activity. However, the effect of organic acid on auotinducer-2 activity has not been investigated hence the study investigated the effect of organic acid on autoinduce-2 (quorum sensing molecule) on fresh produce samples (spinach and cantaloupes).

Quorum sensing has been shown to have a role in biofilm formation in foodborne pathogen such as *E.coli* O157:H7 and *Salmonella* Typhimurium. The role of quorums sensing in biofilm formation is to ensure that there is enough number of bacteria before biofilm formation. As a result, a method to interpret quorum sensing can be used as a preventative measure to hinder biofilm formation on fresh produce. To detect the presence of AI-2 in foods samples, *Vibrio harveyi* BB170 was be used since it produces bioluminescence as a response to AI-2 existence.

1. Materials and Methods

Autoinducer (AI-2) detection assay in spinach and cantaloupe was conducted as described by Kim and others (2009). For this purpose *E.coli* O157:H7/ Salmonella culture, spinach/cantaloupe homogenate, and vibrio culture were prepared.

1.1 E.coli O157:H7 / Salmonella Typhimurium culture preparation

Agar slant cultures of green fluorescent protein GFP-labeled ED 14 E.*coli* O157:H7 (CV267); ED15 *E.coli* O157:H7 (6980-2); ED16 *E.coli* O157:H7 (6982-2); MD58 *E.coli*

O157:H7 (CV261), MD46 *E.coli* O157:H7 (F4546); MD47 *E.coli* O157:H7 (K4492) ; *Salmonella* Typhimurium SD 10 and *Salmonella* Typhimurium SD 11 were obtained from the University of Georgia, Center for Food Safety, Griffin, GA. Frozen stock cultures were made with a single colony of each microorganism. Frozen stock cultures at -70 °C of the above were thawed, transferred to 10 mL of brain heart infusion homogenate (BHI) (Becton Dickinson Microbiology Systems, Becton Dickinson and company, Sparks, MD, U.S.A.) and incubated at 37 °C for 24 h with 200-rpm agitation using a New Brunswick Scientific (Edison NJ, U.S.A.) agitating- incubator. Second-day inoculum was prepared by transferring 10 µl of first-day culture into fresh 10 mL of BHI and incubated in a shaker maintained at 37 °C for 24 h. The second-day culture (10^7 log CFU/mL) was used to inoculate spinach and cantaloupe in the following studies.

1.*2 Spinach/cantaloupe homogenate preparation for autoinducer assay*

Fresh produce (spinach/cantaloupe) were purchased from a local grocery store on the day of the experiment. Leaves/Rinds were rinsed with water, and submerged in sodium hypochlorite solution (6.25 mL/liter) for 3 min to reduce the microbial background. The leaves/rinds were submerged in sterile water for 3 min and left under the biological safety cabinet for 2 h to dry. Spinach leaves/ cantaloupe rinds were placed in sterile bags weighted and macerated. Sterile water was added at a volume of twice the weight of the sample to each bag and stomached for 3 min at 8.0 strokes/sec. Spinach/cantaloupe homogenate (90 µl) was dispensed into a of 96-well plate (Bacton Dickinson and Co. Franklin lakes, NJ).

Ten microliter of the second day culture of *E.coli* O157:H7/ *Salmonella* Typhimurium **(**10^7**)** was added to the spinach/cantaloupe homogenate respectively. The 96-well plate incubated for 12 h at 25 °C.

The bacterial cultures were then removed and centrifuged at 13,000 rpm at 25 °C for 5 min to separate the cell-free supernatants that contained the autoinducer and the supernatant was stored at – 20 °C to conduct autoinducer activity assay.

1.3 Autoinducer activity assay

The reporter strain *V. harveyi* BB170 (ATCC BBA-1117), only senses the AI-2 molecule, and *V.harveyi* BB152 (ATCC BBA-1119), which produces AI-1 and AI-2 was cultured overnight in autoinducer bioassay (AB).

The AB medium was prepared as follows. A solution consisting of NaCl (17.5 g/L), $Mgso_4$ (12.3 g/L), and vitamin-free casamino acids (2 g/L) was dissolved in 1 L of water and adjusted to pH 7.5 and was sterilized by autoclaving (15 min, 121°C). When the solution was cooled, autoclave-sterilized 1 M potassium phosphate (pH 7.0, 10 mL/L), 50% glycerol (20 mL/L), filter-sterilized 0.1 M $_{L}$-arginine (10 mL/L) were added Overnight culture V. harveyi BB170 was diluted (1:5000) in fresh AB medium. The diluted cells (90 µl) was dispensed into each well of 96-well plate.

For autoinducer activity assay, each cell-free culture of *E.coli* O157:H7/ *Salmonella* Typhimurium (10 µl) grown in spinach/cantaloupe homogenate was added to the reporter strain dispensed into 96-well microplate. Wells contain *V.harveyi* BB152 served as a positive control because it produces both AI-1 and AI-2. The plate was incubated at 30 for 3 h with shaking. Luminescence was measured by using a plate reader. This assay was used to determine which stain among the *E.coli* O157:H7 strains (ED 14, ED 15, ED 16, MD 46, MD 47, and MD 58) and *Salmonella* Typhimurium strains (SD 10 and SD 11) has the highest relative AI-2 activity values.

Determine the effect of organic acids on the AI-2 activity

To determine the effect of organic acids on AI-2 produced by *E.coli* O157:H7/ and *Salmonella Typhimurium*, these were added to the above 96-well plate that contain the reporter strain *V. harveyi* BB170 and the cell-free supernatant of inoculated fresh produce different concentrations of organic acids. Lactic acid (1.0, 2.0, 3.0, 4.0%) and malic acid (1.0, 2.0, 3.0, 4.0%) respectively. Combined concentrations of lactic and malic acids were also investigated. LA+MA1% and LA+MA 4%. The 96-well plate was incubated at 30° C for 3 h with shaking. Luminescences were measured by using a plate reader at 490 nm.

Determine the effect of organic acids on *V.harveyi* strain BB170 bacteria

Overnight AB medium cultures of *V.harveyi* BB170 were diluted (1:5000) with fresh AB medium. The subculture of *V.harveyi* BB170 and organic acids that had the highest AI-2 activity (Lactic acid 4%, Malic acid 4%, and LA+ MA 4%) were added to individual sterilized tubes at the ration of 9:1 (vol/vol, *V.harveyi* BB170: organic acids solution sample), the same as the AI-2 bioassay, and the incubated at 30° C for the same incubation time as the AI-2 bioassay. A serial dilution was made for enumeration viable cells of *V.harveyi* BB170 on Marine agar (Becton Dickinson).

Statistical analysis

Completely Randomized Design was used in the study. One-way Analysis of variance (ANOVA) was performed using JMP 11.0. Significant differences was determined at ($P < 0.05$). Tukey HSD multiple comparison test was used to compare means. The experiments were repeated three times with triplicate replications.

Result and discussion

Relative AI-2 activities of *E.coli* and *Salmonella* on spinach and cantaloupe

The result of Relative AI-2 in spinach homogenate inoculated with various strains of *E.coli* for 12 h is given in Table.1. The relative AI-2 activity was calculated as follows: average value of the sample/ average value of the negative control. From Table.1 it can be shown that ED 14 strain had the highest relative AI-2 activity 55 RLU and it is significantly different from ED 15, ED 16, MD 46, MD 47 and MD 58. Therefore, ED 14 was selected to investigate the effect of various organic acids concentrations; Lactic acid (1.0, 2.0, 3.0, and 4.0%) and malic acid (1.0, 2.0, 3.0, and 4.0%). Lactic +Malic 1.0% and Lactic +Malic 4.0% to inhibit AI-2 compound.

Table.3 shows the relative AI-2 activity by two Salmonella *Typhimurium* strains (SD 10 and SD 11) in cantaloupe homogenate after 12 h incubation. SD 10 strain had much higher relative AI-2 activity of give the activity in comparison to SD 11 (53, 21) respectively. Hence, SD 10 strain was chosen to investigate the effect of various organic acids concentrations; Lactic acid (1.0, 2.0, 3.0, and 4.0%) and malic acid (1.0, 2.0, 3.0, and 4.0%). Lactic +Malic 1% and Lactic +Malic 4% to inhibit AI-2 activity.

Effect of organic acids on the AI-2 activity

Varying concentrations of Lactic acid (1.0, 2.0, 3.0, and 4.0%) and malic acid (1.0, 2.0, 3.0, and 4.0%). Lactic +Malic 1.0% and Lactic +Malic 4.0%.were tested for their ability to inhibit AI-2 activity in spinach and cantaloupe inoculated with *E.coli* O157:H7, ED 14 strain and *Salmonella Typhimurium*, SD 10 strain Inhibition of *E.coli* O157:H7, ED 14 AI-2 activity in spinach homogenate is shown in table.2. Overall, lactic acid was more effective than malic acid. The highest concentration of malic acid 4.0% resulted in 37% inhibition whereas the lactic acid

4.0% inhibited the AI-2 activity by 80%. The combined concentrations of lactic acid and malic acid was also investigated. When the two organic acid were combined, the inhibition of relative AI-2 activity was not significant from lactic acid 4.0%. It was expected that the combined organic acids would result in synergistic effect or additive effect. The combined treatment of LA+MA 1.0% result only in inhibition of 17%. While LA+MA4.0% inhibit the relative activity by 25%. This can be explained by the fact that the inhibitory properties of the organic acids are due to their chemical structure. The use of two organic acids can cause an overlapping of their work to inhibit the AI-2 active compound.

Table. 4 shows the inhibition of Salmonella *Typhimurium,* SD 10 strain by organic acids Lactic acid (1.0, 2.0, 3.0, and 4.0%) and malic acid (1.0, 2.0, 3.0, and 4.0%). Lactic +Malic 1.0% and Lactic +Malic 4.0%. The combined treatment of LA+MA 4% had the highest inhibition by 80 %/. However, it is not significantly different than lactic acid 4% which resulted in inhibition of 76%. Other studies investigated different compounds to inhibit AI-2 activity. Lu and others (2004) investigated the effectiveness of some food extracts and food additives in inhibiting AI-2 activity. Turkey patties showed the highest inhibition of 99.8% among the food additives used, sodium propionate virtually inhibited AI-2 (99.6%). Soni and others (2008) reported that mixture of palmitic acid, steric acid, oleic acid, and linoleic acid resulted in inhibition of AI-2 activity between 52 to 65%.

Widmer and others (2007) reported that using fatty acids isolated from poultry meat reduced the activity of AI-2. Combined solution of steric acid, palmitic acid, oleic acid, and linoleic acids inhibited the AI-2 activity by 59.5%. Choo and others (2006) found that the use of 1.0% and 2.0% vanilla extract reduce the quorum sensing by 87.73 % and 98.41%.

***The* effect of organic acids on *V.harveyi* strain BB170 bacteria**

To confirm that the declined AI-2 actively was not the result of growth inhibition of the reporter strains *V. harveyi* BB170. The influence of organic acids on the multiplication of the reporter strain was studied. A mixture of (1:9) of organic acids in combination with the diluted reporter strain *V. harveyi* BB170 in AB medium was incubated at 30° C as mentioned previously After incubation, the samples were plated on Marine agar. **T**able.5. shows the effect of organic acid on *V.harveyi* BB170 growth. The treatments selected were Lactic acid 4.0%, Malic acid 4.0%, and LA+ MA 4.0% because it was at these concentrations that AI-2 inhibition was the highest with 80%, 46%, and 80%respectively. The growth of *V.harveyi* in AB medium without any added organic acids (control) was 5.36 (log CFU/mL). LA 4.0% treatment resulted in 5.28 (log CFU/mL) LA+MA 4.0% had a *Vibrio harveyi* growth of 5.21 (CFU/mL). It should be noted that there were no significant differences between the control (growth in AB medium) and the various treatments, suggesting that inhibition of reporter strain growth was not the basis of AI-2 activity inhibition by the organic acids.

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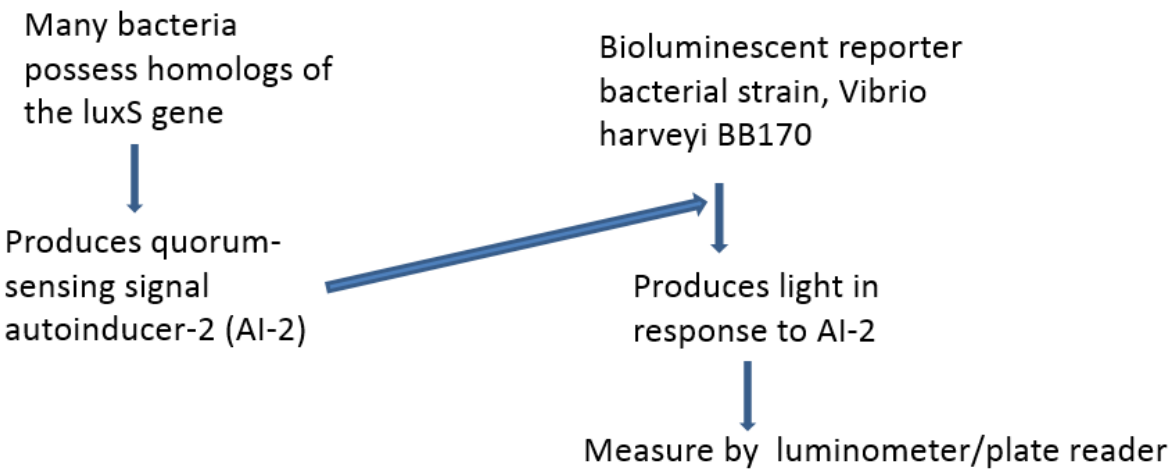

Figure 1-Simplified scheme of quorum sensing principle.

Table 1-Relative AI-2 like in spinach homogenate inoculated with various stains of *E.coli* for 12 h

Strain*	Relative AI-2 like activity**
ED 14	55 ± 2.14A
ED 15	28 ± 2.60B
ED 16	36 ± 2.61C
MD 46	15.2 ± 2.30D
MD 47	15.3 ± 0.94D
MD 58	14.27 ± 0.92D

***Strains of *E.*coli O157:H7. Values provided are means ± standard deviations**
Values conducted by the same letter are not significantly different (P<0.05).
**** Relative AI-2 like activity was calculated as the ration of luminescence of the test sample to the negative control (AB medium)**

Table 2-Inhibition of ED 14 AI-2 activity in spinach by organic acids

Treatments%*	Inhibition %**
LA 1.0	26±0.06E
LA 2.0	33±0.03CD
LA 3.0	49±0.05B
LA 4.0	80±0.02A
MA 1.0	23±0.04F
MA 2.0	26±0.02F
MA 3.0	28±0.03DE
MA 4.0	37±0.04C
LA MA 1	17±0.03E
LA MA 4	25±0.03E

Values provided are means ± standard deviations
Values connected by the same letter are not significantly different (P<0.05).
***LA: lactic acid, MA: Malic acid**
****** calculated based on the positive control values.**

Table 3-Relative AI-2 like in cantaloupe homogenate inoculated with stains of Salmonella for 12 h

Strain*	Relative AI-2 like activity**
SD 10	53 ± 0.92A
SD 11	21 ± 0.71B

*** Strains of Salmonella *typhimurium*. Values provided are means ± standard deviations Values connected by the same letter are not significantly different (P<0.05).**
**** Relative AI-2 like activity was calculated as the ration of luminescence of the test sample to the negative control (AB medium)**

Table 4-Inhibition of *Salmonella typhimurium* SD 10 AI-2 activity in cantaloupe by organic acids

Treatments (%)*	Inhibition (%)**
LA 1.0	27±0.03F
LA 2.0	36±0.02DE
LA 3.0	58±0.01B
LA 4.0	76±0.02A
MA 1.0	16±0.04G
MA 2.0	26±0.02F
MA 3.0	28±0.03DE
MA 4.0	46±0.04C
LA MA 1	39±0.03D
LA MA 4	80±0.01A

Values provided are means ± standard deviations
Values connected by the same letter are not significantly different (P<0.05).
***LA: lactic acid, MA: Malic acid**
****** calculated based on the positive control value.**

Table 5-Effect of organic acids on the growth of the reporter strain *V. harveyi* BB170

Treatment*	Growth (log CFU/mL)
AB medium	5.36 ± 0.14A
LA 4 %	5.28 ± 0.23A
MA 4%	5.25 ± 0.05A
LA MA 4%	5.21 ± 0.21A

Values provided are means ± standard deviations
Values connected by the same letter are not significantly different (P<0.05).
***AB medium: Autoinducer bioassay medium; LA: Lactic acid; MA: Malic acid**

Conclusion

Outbreaks from fresh produce is a continuing problem in the U.S. that has economical and health consequences. The persistence of the problem can be partly attributed to pathogens such as *E.coli* O157:H7 and *Salmonella* forming biofilms. In this work, electrostatic spraying with organic acids was utilized (hurdle-technology) to reduce biofilm formed by *E.coli* O157:H7 and Salmonella on spinach and cantaloupe respectively. Biofilm formation initiates with the irreversible attachment of bacteria onto plant surface. Lactic acid 4.0 % was able to reduce *E.coli* O157:H7 by 4.1 on spinach while combined malic acid 3.0%+ lactic 3.0% reduced Salmonella on cantaloupe by 3.58. The safety of fresh produce industry can be enhanced by the use of natural antimicrobials applied by electrostatic sprayer. Further studies on the effect of electrostatic spraying on other fresh produce and food contact surfaces are recommended. The inhibition of quorum sensing was also studied. The current study suggests that organic acids are effective against quorum sensing.