INFLUENCE OF CELL ENVELOPE COMPONENTS ON THE ATTACHMENT OF *ESCHERICHIA COLI* O157:H7 TO LETTUCE AND SPINACH UNDER DIFFERENT IONIC ENVIRONMENTS: EFFECT ON THEIR RESISTANCE TO ENVIRONMENTAL STRESS

by

Chi-Ching Lee

(Under the Direction of Joseph F. Frank)

ABSTRACT

Produce-related outbreaks of *Escherichia coli* O157:H7 are the second most common foodborne outbreaks since it was first recognized an enteric pathogen in 1982. The mechanism of attachment of *E. coli* O157: H7 on leafy greens is complex and dependent on a variety of factors including properties of the cell surface, substratum and bulk fluid. The objective of this study was to determinate the role of cell envelope components on attachment of *E. coli* O157:H7 to lettuce and spinach leaf surface and cut edge under different environments. Cell surface charge, hydrophobicity, and capsule characteristics were investigated. In addition, cellulose-deficient derivatives of Shiga toxin–producing *Escherichia coli* (STEC) have been used. The lower surface hydrophobicity, less negative charge, as well as capsule containing more D-Mannose and α-Fucose of cells grown in tryptic soy broth combined to increase attachment to spinach leaves. Cellulose-producing STEC cells attached significantly 0.5 log greater on lettuce leaf surface than cellulose-deficient cells. However, cellulose-deficient cells attached significantly 0.7 log greater to cut edge of lettuce than parental cells. In high water

hardness environment, attachment of wild type cells to leafy greens surface also could be enhanced. In addition, chlorine treatment reduced the population of cellulose-deficient cells at 1.2 log units more than the wild type in 150ppm of chlorine on spinach leaves surface. However, the population of cellulose-producing cells was reduced significantly by 1.5 log units more than its mutant when cells also produced colanic acid. Extra-cellular cellulose production protects STEC cells attached to leafy greens from the effects of chlorine on spinach leave surface.

INDEX WORDS: *Escherichia coli* O157:H7; STEC; Attachment; Lettuce; Spinach; Capsule; Cellulose; Colanic Acid; Water hardness; Chlorine; Surface Charge; Hydrophobicity

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DEDICATION

To my parents, brother and major adviser, Dr. Joseph Frank

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CHAPTER 1

INTRODUCTION

Fresh produce is the second most common transmission vehicle for *Escherichia coli* O157:H7 in 38 (21%) of 183 foodborne outbreaks and 34% of 5269 foodborne outbreak-related cases. It has been associated with apples cider or juice, salad, alfalfa, clover spout, coleslaw, cantaloupes, radish, spinach and leaf lettuce (Beuchat, 2002; Hillborn *et al*., 1999; Rangel *et al*., 2005). Lettuce-related outbreaks account for 34% of produce-related *E. coli* O157:H7 outbreaks which is significantly more frequent than other produce.

Cell attachment is dependent on the hydrodynamics of the fluid, composition of the aqueous medium, ionic and chemical properties of the cell surface and physical properties of the substratum (Donlan, 2002). Many factors have been identified to have an influence on attachment and detachment of *E. coli* O157:H7 on lettuce, such as hydrophobicity, capsule production and surface charge (Hassan *et al.,* 2003; Hassan *et al.,* 2004). The hydrophobic and electrokinetic properties of cells are crucial influences for cell attachment. The hydrophobicity is affected by bacterial exopolysaccharides (EPS) components (Gross and Logan, 1995). Besides, many viable *E. coli* O157:H7 cells can be observed on the cut edges and stomata of lettuce leaves after chlorine treatment (Seo and Frank, 1999).

Furthermore, extracellular structures also influence cell attachment and the resistance of embedded cells to many biocide treatments. These structures are protective matrices at the cell surface, such as cellulose, colanic acid, fimbriae, flagella and EPS. The properties of cell surface, the media of attachment and other environmental factors play important roles on bacterial attachment (Costerton *et al*., 1987; Frank, 2000; Kumar, 1998). Determination of the mechanism of cellulose and colanic acid in *E. coli* O157:H7 attachment to foods and food contact surfaces, and cell protection against sanitizers will provide essential information for developing associated strategies to eliminate or control pathogens in food processing and foodservice environments (Ryu and Beuchat, 2005). The defense mechanism of microorganisms induced by environmental stress is complex. The several stress response and modification of cell structure are performed by *E. coli* which can produce the bacterial EPS including celloluse and colanic acid on the cell surfaces. Cellulose composition with a linear long D-glucose units chain could protect Shiga toxin-producing *E. coli* O157:H7 (STEC) strains against oxidative and acidic treatments (Yoo and Chen, 2012). Colanic acid has been observed to offer *E. coli* O157:H7 protection against acidic, heat, osmotic and oxidative stress (Chen et al., 2004).

This study investigated the effect of three growth media, tryptic soy broth (TSB), Luria broth base, Miller (LB) and nutrient broth (NB) under different ionic environments on the attachment of *E. coli* O157:H7 to lettuce and spinach leaves surface and cut edge. Surface charge, hydrophobicity, and capsule characteristics were determined. In addition, water hardness is an important factor in food safety and processing. In households and manufacturing facilities it can influence the quality of a product and sanitation. In addition, chlorine solution is the most commonly used sanitizer in food processing plants

due to low-cost, safe and easy to use. Therefore, this study also investigated the influence of cellulose on attachment of STEC to iceberg lettuce and baby spinach leaves under different water hardness environments and cell survival under chlorine solution treatment. Mutants lacking cellulose production were used. The hypothesis for this study is that the specific cell envelope components and certain environment conditions will have significant effects on attachment and survival of *E. coli* O157:H7.

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CHAPTER 2

LITERATURE REVIEW

I. The foodborne outbreak of *E. coli* O157:H7 associated with leafy greens

In United States, *E. coli* O157:H7 causes 73,000 illnesses annually and has been characterized as causing thrombotic thrombocytopenic purpura, hemorrhagic colitis, hemolytic-uremic syndrome (HUS) in susceptible people and children (Griffin *et al*., 1991). It is Gram-negative, rod-shaped and predominant facultative anaerobic bacterium in the gastrointestinal tracts of mammals, especially cattle. They have been implicated as the most important a reservoir of *E. coli* O157:H7 and enable be asymptomatic carriers due to lack of Shiga toxin receptor, globotriaosylceramide (Pruimboom-Brees *et al*., 2000). The prevalence of *E. coli* O157:H7 in feedlot cattle herds of North American ranges from 0 to 61% (Jeon *et al*., 2013).

E. coli O157:H7 was first isolated from ground beef and recognized as a pathogen in 1982. Ground beef is the most common vehicle among foodborne outbreaks (75 [41%] of 183 outbreak) and 33% of 5,269 foodborne-related cases between 1982 and 2002. Produce was the transmission vehicle for *E. coli* O157:H7 in 38 (21%) of 183 foodborne outbreaks and 34% of 5,269 foodborne outbreak-related cases from 1982 to 2002 with the 1st recorded incident occurring in 1991. In addition, a total of 350 outbreaks in this period were reported from 49 states, accounting for 8,598 cases of *E. coli* O157 infection, representing 1493 (17%) hospitalizations, 354 (4%) HUS cases, and 40 (0.5%) deaths. Food remained the predominant transmission route and is associated with 52% of 350

outbreaks and 61% of 8,598 outbreak-related cases. Produce-associated outbreaks most commonly occurred in restaurants for 15 outbreaks (39%) (Rangel *et al*., 2005).

According to 2011 Centers for Disease Control and Prevention (CDC) report, the 31 known major pathogens of foodborne outbreak in Unite States caused 9.4 million illnesses, 55,961 hospitalizations and 1,351 deaths each year. Norovirus caused the most illnesses (58%), and nontyphoidal *Salmonella spp.* (11%), *Clostridium perfringens* (10%), *Campylobacter spp.* (9%). Primary causes of hospitalization were nontyphoidal *Salmonella spp.* (35%), norovirus (26%), *Campylobacter spp.* (15%), and *Toxoplasma gondii* (8%). Most death were caused by nontyphoidal *Salmonella spp.* (28%), followed by *T.gondii* (24%), *Listeria monocytogenes* (19%), and norovirus (11%). STEC O157 were estimated to cause 63,153 illnesses, 2,138 hospitalizations and 20 (1%) deaths every year (Scallan *et al*., 2011).

The means by which *E. coli* O157: H7 is introduced into the lettuce and spinach is not well understood. Nevertheless, one hypothesis states that the lettuce plant is contaminated when grown in fields fertilized with improperly treated manure (Ethan *et al*., 2002). *E. coli* O157:H7 has been isolated from fresh produce including apples cider or juice, bean sprouts, salad, cantaloupes and leaf lettuce (Hillforn et al., 1999; Ackers et al., 1998). Produce-related outbreaks were usually the 2nd most common identified outbreaks of *E. coli* O157 and were associated with apple cider, lettuce, radish, alfalfa sprouts, and other mixed salads since 1991 (Beuchat, 2002). As of March 21, 2012, 58 persons infected with the outbreak strain of *E. coli* O157:H7 linked to romaine lettuce were reported to Centers for Disease Control and Prevention (CDC) from 9 states. The causes of an *E. coli* O157:H7 outbreak in 2006 that was associated with contaminated

Dole brand bagged spinach and resulted in 205 confirmed illnesses and three deaths. Among the ill persons, 102 (51%) were hospitalized and 31 (16%) developed a type of kidney failure called hemolytic-uremic syndrome (HUS) (USDA / CDC, 2007). As of December 10 2012, a total of 33 persons infected with the outbreak of STEC O157:H7 related to organic spinach and spring mix blend were reported from five states.

In 2012, 19,531 laboratory-confirmed cases of infection were identified by FoodNet (Table 2.1). The number of STEC O157 for infections and incidence per 100,000 populations are 531 and 1.12. However, outbreaks of *E. coli* O157:H7 are not only in US but also in European countries. In 2007, an international outbreak was contaminated lettuce in the Netherlands and Iceland (Friesema *et al*., 2008). In addition, lettuce-linked outbreaks of pathogenic *E. coli* O157 were been reported during 2005 Sweden and Enterotoxigenic *E. coli* in Denmark 2010 (Ethelberg *et al*., 2010; Soderstrom, *et al*., 2008). Furthermore, produce-associated outbreaks peaked in summer and fall; 74% occurred from July to October. 34% produce-associated outbreaks were from lettuce, 7 (18%) from apple cider or apple juice, 6 (16%) from salad, 4 (11%) from coleslaw, 4 (11%) from melons, 3 (8%) from sprouts, and 1 (3%) from grapes (Rangel *et al*., 2005).

II. The development of biofilm

Biofilms can be defined as structured communities of microorganisms enclosed in a self-produced polymeric matrix and attached to an inert or living surface in any environment (Costerton *et al.*, 1999). The transition of cell biofilm formation undergoes profound change from planktonic (free-swimming) lifestyle to develop a complex, surface-attached community. A common bacterial model of biofilm development has

been proposed and characterized into five stages: reversible attachment, irreversible attachment, microcolony formation, maturation and dispersal (Davey *et al.*, 2000; Donlan, 2002; Dunne *et al.*, 2002; Stoodley *et al.*, 2002).

1. Reversible attachment:

Planktonic cells approach the solid surface by motility or flowing bulk fluid. Once moving across the surface, they overcome the hydrodynamic boundary layer and repulsive forces at the surface. In this stage, several cell envelope structures are involved, such as pili, capsule, flagella, S layer proteins and extracellular polymeric substances.

2. Irreversible attachment:

Aggregated cells on the surface begin to lose their flagella and synthesize curli as well as colanic acid to integrate the biofilm structure. The increasing expression of extracellular polymeric substances, induction of quorum sensing and induction of c-di-GMP modulation can be observed.

3. Microcoloy formation:

The biofilm keeps developing by recruiting planktonic cells from the surrounding fluid to aggregate attached cells.

4. Maturation:

Mature biofilms structure channels by cell columns for transport nutrients and remove waste products to maintain viability of cell communities. EPS also provide adhesive matrix and trap nutrients from surroundings. Several complex architectures and gene expression occur, such as pedestal-like structures, water channels and regulation of Bap protein. Quorum sensing facilitates inter-and intra-cells communication and organizes biofilm growth.

5. Dispersal

Cell dispersion from the biofilms into environments is related with either auto-induction or external-induction. Auto-induction for biofilm dispersing occurs by gene regulation when architectures have matured or cells respond to environmental signals. Fluid sheer force, disinfection of cleaning chemicals and physical washing can cause desperation by these external induction factors. Cells dispersed from biofilms return to planktonic state in natural environment. However, biofilm dispersal in food processing plants preferably is disrupted by cleaning and sanitation processes.

III. Cell Attachment

Attachment is a complex process regulated by various characteristics of the cell surface, medium and substratum. The solid-liquid interface between aqueous medium, such as water or blood, and cell surface provides an ideal environment for the attachment and growth of microorganisms. In Table 2.2, the cell attachment is dependent on the hydrodynamics of the aqueous medium, composition of the medium, ionic and chemical properties of the cell surface, physical properties of the substratum, and conditioning films forming on the substratum (Donlan, 2002).

In aqueous media, ionic strength, temperature, pH, nutrient levels may play a role in the mechanism of microbial attachment to a substratum. Some studies have shown a seasonal effect on bacterial attachment and biofilm formation in different aqueous systems (Donlan *et al*., 1994; Frank, 2000; Fera *et al*., 1989). Water temperature or other seasonally unmeasured parameters may be the factors. Cowan and his colleagues in 1991 showed that an increase in the number of attached bacterial cells was correlated with an

increase in nutrient concentration. Fletcher and other researchers in 1988 found that the attachment of *Pseudomonas fluorescens* to glass surfaces was affected by an increase in the concentration of several cations (calcium, ferric iron, lanthanum, sodium), seemingly by reducing the repulsive forces between the negatively charged bacterial cells and the glass surfaces. In the previous study it was found that *E.coli* O157:H7 can colonize the interior of alfalfa (*Medicago sativa*) seedlings better than *E.coli* K-12. (Dong et al., 2003) In addition, internalized pathogens in produce would be more difficult to be removed during post-harvesting handling than surface contaminants.

Potential foodborne pathogens and the corresponding attachment factors to their animal hosts (Table 2.3). Multiple types of fimbriae, curli, pili and flagella are the major known attachment factors. Besides, Gram-negative bacteria in Table 2.3: *E.coli*, *Salmonella*, *Shigella*, *Vibrio* can express LPS and CPS. Both are major surface glycoconjugates (plant lectins receptor) and similar to the mechanisms of plant nitrogen-fixing, epiphytic, and pathogenic bacteria. (Gerald *et al*., 2006)

Fimbriae

At the cell surface, many factors influence the rate and extent of attachment of microbial cells, such as presence of fimbriae and flagella, hydrophobicity and electrostatic charge (surface free energy) and production of EPS (Donlan, 2002). Most enteric bacteria are negatively charged (i.e. *E. coli* O157:H7) and also contain hydrophobic surface components (Rosenberg and Kjelleberg, 1986). The hydrophobicity in the cell surface is very crucial during adhesion because hydrophobic interactions can be enhanced with an increasing nonpolar nature between the cell and substratum surface.

Previous study indicated the role of fimbriae in the mechanism of bacterial attachment to surfaces (Rosenburg *et al*., 1982; Bullitt and Makowski, 1995). Fimbriae, such as the nonflagellar appendages involved in transferring bacterial plasmids (called pili), contribute to greater hydrophobicity of the cell surface. A number of aquatic bacteria possess fimbriae, which have also been shown to be involved in bacterial attachment to animal cells. Fimbriae play an important role in attachment and hydrophobicity of cell surfaces, presumably by helping to overcome the initial electrostatic repulsion barrier that exists between the substratum and cell surface (Corpe, 1980). Most fimbriae that have been examined have a high proportion of hydrophobic amino acid residues (Rosenberg and Kjelleberg, 1986).

Curli

A variety of mechanisms are involved in attachment of bacteria to complex biological surfaces such as the edible leaves of fresh produce. *E. coli* and other enteric bacteria assemble highly aggregative fibers on their cell surfaces called curli, a thin, coiled fimbriae-like extracellular structure. Gram-negative bacteria assemble functional amyloid surface fibers. Curli fibers are not only potent inducers of the host immune response, but also play a critical role in attachment during biofilm formation (Elisabeth *et al*., 2009). Curli fibers are adhesive surface fibers and have an important role in pathogenesis. However unlike nonpathogenic *E. coli*, *E. coli* O157:H7 producing curli is unusual and can occur in relation with *csgD* promoter point mutations (Uhlich *et al.,* 2001). They increase the attachment of cells on the surface of polystyrene (Prigent-Combaret *et al.,* 2000). They are expressed by *E. coli* and *Salmonella enterica* that

contact phase proteins and bind several host extracellular matrixes. In addition, they also promote bacterial internalization, which has a role in pathogenesis (Gophna *et al*., 2001).

Two *csgDEFG* and *csgABC* operons, which are two divergently transcribed units, operate the biosynthesis of curli fimbriae that is composed of CsgA, the structural protein subunit. CsgD is required for the biosynthesis of curli and cellulose (Bokranz *et al.*, 2005). It is also a transcriptional response regulator of the LuxR superfamily. The *Salmonella* rdar morphotype is adhesive extracellular matrix adhesive components cellulose and curli fimbriae. The genetic expression of rdar morphotype has been investigated primarily in *Salmonella enterica* serovar *Typhimurium* (ATCC 1402). Regulation of curli expression was also studied in *E. coli* K-12 strains (Hammar *et al*., 1995; Römling *et al*., 1998a, 2000; Zogaj *et al*., 2001).

Flagella

Most *E. coli* are capable of motion by multiple peritrichous flagella, which have a role in cell attachment and colonization of plant tissue. Flagellar filaments are important in motility for chemotaxis of biofilm formation (Brisset *et al.*, 1991). *Pseudomonas putida* in soil promotes plant growth promoter and suppresses fungal pathogens. Motility was related with efficiency of *P. putida* attachment to sterile wheat roots in a simplified model system, indicating flagellin as a potential attachment factor (Turnbull *et al*., 2001). *P. putida* colonized roots are used for biocontrol of plant pathogens, serving as important competitors (Rainey *et al*., 1999) In *P. putida*, flagellin acts as an essential factor for chemotaxis and colonization of potato roots (De Weger *et al*., 1987). Non-piliated

variants of *P. putida* strains are not able to bind to roots of corn seedlings very well in comparison with piliated/fimbriated strains (Vesper, 1987).

Cellulose

Cellulose is a polysaccharide consisting of a linear chain of β (1→4) linked D-glucose units. It can be found in primary cell wall of plants. Cellulose production is important for biofilm formation on *S. enteritidis* (Solano *et al*., 2002). Studies by Zogaj and his colleagues in 2003 showed a fecal isolate of *Enterobacter sakazakii* to produce cellulose but not curli and fimbriae. The presence of cellulose synthase, the catalytic subunit of which is encoded by *bcsA*, was confirmed and expressed constitutively by *E. sakazakii*. Structural genes (*csgBA*) for curli fimbriae and a transcriptional activator (*csgD)* were present and intact even though the fecal isolate of *E. sakazakii* did not produce curli fimbria (Zogaj *et al.*, 2003). In the cellulose biosynthesis, the *bcsABZC* operon encodes structural genes with the catalytic subunit of the cellulose synthase encoded by *bcsA* (Zogaj *et al.*, 2001). The other genes in the bacterial cellulose synthase (*bcsABCZ*) operon were intact, including a regulatory subunit (*bcsB*), an oxidoreductase (*bcsC*), and an endoglucanase (*bcsZ*) (Solano *et al*., 2002).

Capsule

The capsule is a layer of surface-associated polysaccharide, covering most stains of *E. coli*. It is demonstrated in the high amount of variation in the cell surface polysaccharides produced by different strains. The primary cell surface polysaccharides with recognized virulence determinants are serotype-specific, containing the LPS O

antigens and capsular K antigens. In a previous study, the capsules of some pathogenic strains of *E. coli* produced K1 antigens (polysialic acid) related with septicemia, urinary tract infections, and meningitis (Whitfield *et al.*, 1999). Several studies have indicated that biofilm formation was inhibited by capsule expression. Schembri et al. (2004) found that capsule formation suppressed the function of bacterial adhesion in *E. coli* K12 and *Klebsiella pneumoniae*. Joseph and Wright (2004) reported that expression of capsular polysaccharide in *Vibrio vulnificus* inhibits attachment and biofilm formation. Capsule expression also blocks the regulation of CF29K adhesion (Favre-Bonte et al., 1999). In addition, there is an inverse relationship between expression of capsule and type 1 fimbriae in *K. pneumoniae* (Matatov et al., 1999). All of these studies showed that expression of capsular polysaccharide inhibits bacterial adhesins. However, capsule production influences attachment of *E. coli* O157:H7 to lettuce leaves (Hassan *et al.,* 2004).

Exopolysaccharides

Exopolysaccharide (EPS) production is correlated with cell adhesion and biofilm formation (Weiner *et al*., 1995) and EPS act as a conditioning film on inert surfaces (Allison *et al*., 1987). Moreover, cell attachment and formation of three-dimensional biofilm structures have an influence by functioning as an adhesive or anti-adhesive (Ofek *et al*., 1994; Danese *et al*., 2000). EPS produced by *E. coli* O157:H7 can also prevent cells from environmental stresses, serving as a physical barrier (Junkins *et al*, 1992). In a nutrient-limited environment, the EPS-overproducing mutant in biofilm had increased

resistance against stresses in lettuce juice when compared to a strain that did notoverproduce EPS (Ryu *et al*., 2004).

Ryu and Beuchat in 2005 investigated the effect of EPS and curli production of *E. coli* O157:H7 on the surface of stainless steel. The study indicated that EPS produced by *E. coli* O157:H7 is like an anti-adhesive and inhibits the initial attachment of *E. coli* O157:H7 cells. However, EPS did not inhibit the development and maturation of biofilm. On the other hand, even though curli production was not involved in the initial attachment of cells to stainless steel coupons, it increased biofilm development. Biofilm formation and EPS as well as curli production enhanced the resistance of *E. coli* O157:H7 to chlorine.

IV. Chlorinated Water

Chlorinated water is widely used in food industry for sanitizing purpose because of its availability, low-cost, and acceptable level of efficacy. Its most potent bactericidal form is hypochlorous acid (HOCl) which is produced from the active compound, sodium hypochlorite (NaClO), in water. However, HClO also partially dissociates to hypochlorite ion (ClO-) and hydrogen ion (H+) in aqueous solution under basic pH. The concentration of chlorine commonly used in food processing plants ranges from 50 to 200 ppm, based on practicality and federal or state regulations.

E. coli exposed to HOCl lose cell viability due to inactivation of many vital mechanisms (Rakita *et al*, 1989; Rakita *et al*, 1990; Rosen *et al*, 1985; Rosen *et al*, 1987). The mode of HOCl bactericidal activity includes inhibition of DNA replication and glucose oxidation (Barrette *et al*, 1987; Chesney *et al*, 1996; Mcfeters *et al*, 1983; Rosen

et al, 1998), protein inactivation and aggregation (Winter *et al*, 2008), as well as cell membrane destruction (Camper *et al*, 1979). In addition, chlorine resistance is related to cellulose production in *Salmonella enteritidis*. After exposure to 30 ppm NaClO for 20 min, only 0.3% of cellulose-deficient *S. enteritidis* mutants survived in comparison to 75% of wild-type cellulose producing *S. enteritidis* (Solano *et al*., 2002). A similar increase in chlorine resistance may be observed if *Enterobacter sakazakii* biofilms primarily consist of cellulose (Heredia *et al*., 2009).

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TABLE 2.1. Number of cases of bacterial and parasitic infections, hospitalizations, and deaths by pathogen from 1996 to 2012.

	Cases		Hospitalizations		Deaths	
Pathogen	**Number**	**Incidence[a]**	**Number**	**%**	**Number**	**%**
Bacteria						
Salmonella	7,800	16.42	2,284	29	33	0.42
Campylobacter	6,793	14.30	1,044	15	6	0.09
Shigella	2,138	4.5	491	23	2	0.09
STEC[b] non-O157	551	1.16	88	16	1	0.18
STEC O157	531	1.12	187	35	1	0.19
Vibrio	193	0.41	55	29	6	3.11
Yersinia	155	0.33	59	38	0	0
Listeria	121	0.25	116	96	13	10.74
Parasites						
Cryptosporidium	1234	2.6	236	19	6	0.49
Cyclospora	15	0.03	3	20	0	0
Total	**19,531**		**4,563**		**68**	

a. Per 100,000 population.

b. STEC : Shiga toxin–producing *Escherichia coli*

From: Incidence and Trends of Infection with Pathogens Transmitted Commonly Through Food – Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 1996–2012. April 19, 2013, Morbidity and Mortality Weekly Report, CDC. 62(15);283-287

TABLE 2.2. Variables important in cell attachment and biofilm formation.

	Properties
Substratum	Texture
	Roughness
	Hydrophobicity
	Conditioning Film
Bulk Fluid	Flow velocity
	pH
	Temperature
	Cation Presence
Cell surface	Hydrophobicity
	Fimbriae
	Flagella
	Extracellular polymeric substances (EPS)

From: Donlan, R.M., 2002. Biofilms: Microbial life on surfaces, Emerg. Inf. Dis. 8: 881–890.

TABLE 2.3. Potential attachment factors of human pathogens

Bacteria	**Attachment Factors**
Escherichia coli	G-fimbriae, Flagellin
Enterohemorrhagic E.coli	Fimbriae
Enteropathogenic E.coli	Fimbriae, Pili
Enterotoxigenic E.coli	Fimbriae, Pili
Listeria monocytogenes	Flagellin
Salmonella enterica	Fimbriae, Pili, Curli
Shigella flexneri	Pili, Fimbriae
Vibrio cholerae	Fimbriae

From: Sapers, G.M., Gorny, J.R., Youse, A.E., 2006. Microbiology of Fruits and Vegetable, p49-50

CHAPTER 3

INFLUENCE OF CELL SURFACE PROPERTIES ON ATTACHMENT OF *ESCHERICHIA COLI* O157:H7 TO SPINACH AND LETTUCE LEAVES

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ABSTRACT

The attachment of bacteria on leafy greens surfaces is a complex process influenced by the cell surface, growth medium and substratum. The mechanisms by which *E. coli* O157:H7 attaches to leafy greens are not well understood. This study investigated the effect of growth media (tryptic soy broth (TSB), Luria broth base, miller (LB) and nutrient broth (NB) and ionic environments on the ability of *Escherichia coli* O157:H7 to attach to spinach and lettuce. Surface charge, hydrophobicity, and capsule characteristics were determined. Spinach and lettuce leaves were inoculated with 9 log CFU/ml cells for 2 h at 4°C. Cells on the cut edge and surface were enumerated using plate counts. Addition of calcium ions to the attachment medium increased attachment of cells grown in TSB and NB on lettuce surfaces and cut edges as compared to those grown in TSB. However, there was no effect on cell attachment to spinach in the presence of calcium.Cells preferentially attached to the cut edges of spinach leaves in comparison with intact surfaces. Cells grown in TSB, LB, or NB and attached in distilled water exhibited 1, 0.2 and 0.5 log greater attachment to spinach than lettuce, respectively. The capsule of cells grown in TSB exhibited well-defined capsules, but not those grown in LB and NB. Lectin characterization indicated that capsules from cells grown in TSB contained more D-mannose and α-Fucose than the capsules of cells grown in LB and NB. Cells grown in LB were more negatively charged than those grown in TSB and NB. Encapsulated TSB-cells attach significantly better to spinach surfaces in the presence of EDTA than non-encapsulated LB/NB-grown cells. Capsule production seems to insignificantly affect cell attachment in ionic environments. These findings indicate that

hydrophobic and electrostatic interactions and ions in the media play more important roles in attachment than does capsule production by cells.

INTRODUCTION

Escherichia coli O157:H7 is estimated to cause 73,000 illnesses In United States annually. It causes thrombotic thrombocytopenic purpura, hemorrhagic colitis, hemolytic-uremic syndrome (HUS) in susceptible people and children (GRIFFIN and TAUXE, 1991). Produce was the transmission vehicle for *E. coli* O157:H7 in 38 (21%) of 183 recent foodborne outbreaks. Food remained the predominant transmission route from 1982 to 2002, accounting for 52% of 350 outbreaks and 61% of 8,598 outbreak-related cases. Produce-associated outbreaks most commonly occurred in restaurants (15 [39%]) and 34% of produce-associated outbreaks were from lettuce (7 [18%]) (RANGEL et al., 2005). Since 1990, *E. coli* O157:H7 has been isolated from fresh produce including apples cider and juice, bean sprouts, salad, cantaloupes and leaf lettuce (ACKERS et al., 1998; HILBORN et al., 1999). Produce-related outbreaks are the 2nd most often identified outbreaks of *E. coli* O157 since 1991 and are often associated with apple cider, lettuce, and radish (BEUCHAT, 2002).

Attachment is a complex process regulated by various characteristics of the cell surface, growth medium and substratum. The solid-liquid interface between aqueous medium and cell surface provides an environment for the attachment and growth of microorganisms. Cell attachment is dependent on the hydrodynamics of the aqueous medium, composition of the medium, ionic and chemical properties of the cell surface,

physical properties of the substratum, and conditioning films formied on the substratum (DONLAN, 2002). In an aqueous medium, ionic strength, temperature, pH, and nutrient levels may play a role in microbial attachment to a substratum. Fletcher and other researchers in 1988 found that the attachment of *Pseudomona fluorescens* to glass surfaces was influenced by increasing the concentration of some cations (calcium, ferric iron, lanthanum, sodium) and it appeared that the repulsive forces between the negatively charged bacterial cells and the glass surfaces were reduced (FLETCHER, 1988).

Various cell surface factors influence the rate and extent of attachment of microbial cells, such as presence of fimbriae and flagella, hydrophobicity (surface free energy) and production of extracellular polymeric substances (EPS) (DONLAN, 2002). Most bacteria are negatively charged (i.e. *E. coli* O157:H7) but still contain hydrophobic surface components (ROSENBERG et al., 1982). Hydrophobic interactions between the cell surface and the substratum surface enhance the nonpolar character on either one or both surfaces, resulting in greater attachment strength.

The capsule is a layer of surface-associated polysaccharide, produced by most strains of *E. coli*. The diversity of *E. coli* is demonstrated in the high amount of variation in the cell-surface polysaccharides produced by different strains. The primary cell surface polysaccharides provide virulence determination are serotype specific and includethe lipopolysaccharide (LPS) O antigens and capsular K antigens. Capsule formation suppresses the function of bacterial adhesions in *E. coli* K12 and *Klebsiella pneumoniae* (SCHEMBRI et al., 2004). Joseph and Wright in 2004 reported that expression of capsular polysaccharide in *Vibrio vulnificus* inhibits attachment and biofilm formation (JOSEPH and WRIGHT, 2004). The attachment ability and envelop properties of cells are influenced

by growth environment. TSB supported capsule production by *E. coli* O157:H7 and also increased its attachment to lettuce and apple surfaces (HASSAN and FRANK, 2004). The aim of this study was to determine the effect of an ionic environment and cell surface properties on attachment of *E. coli* O157:H7 to spinach and lettuce. The cells were grown in different media to produce cells of differing surface characteristics (hydrophobicity, surface charge, and capsule presence).

MATERIALS AND METHODS

Bacterial strain and culture conditions

E. coli O157:H7 994 (beef jerky isolate supplied from the Center for Food Safety, University of Georgia, Griffin, GA) cells were grown individually in tryptic soy broth (TSB), Luria broth base, miller (LB), or nutrient broth (NB) at 37 °C by two successive 24 ± 2 h transfers. Cultures were centrifuged at 4400 x g (6,500 rpm) and 4°C for 15 min. The supernatant was discarded. The pellet was washed twice with sterile de-ionized water and suspended in the appropriate amount of sterile de-ionized water to contain approximately 8 log CFU/ml for the sample inoculum.

Quantification of cell attachment to lettuce and spinach

Iceberg lettuce and baby spinach were purchased at local grocery stores. Undamaged leaves were selected for use. Inner leaves of iceberg lettuce were cut into designated sizes (2 × 2 cm) after aseptically removing the outer leaves and core by using a sterile knife and surgical scalpel. In addition, the petioles of the baby spinach leaves

were removed and the leaves cut into 2 × 2 cm pieces using a sterile surgical scalpel. All leaf pieces were rinsed five times with excess sterile de-ionized water (SDW). For attachment experiments, a square leaf section was submerged in 10mL bacterial suspension at 4°C for 2h. Leaves were taken from the cell suspension using sterile tweezers and rinsed twice with excess SDW. The outermost tissue on each side of the square leaf was cut (0.15 cm from the edge) and used as the cut edge sample. The cut edge tissue and inner portions of the leaves (intact surface) were separately combined with 10 ml of sterile 0.1% peptone water in polypropylene tubes and the cells were released from the tissue using a homogenizer (Omni mixer, model 17105) set at energy level 5 for 30 seconds which is sufficient to disrupt the leaf tissue. Samples were immersed in an ice bath during homogenization. Homogenized samples were serially diluted in sterile 0.1% peptone water. The number of *E. coli* 157:H7 attached to each leaf surface and cut edge was determined by plating on sorbitol-MacConkey (SMAC) agar with incubation of plates at 35°C for 24h.

Capsule visualization and carbohydrate characterization

After cells were prepared as previously described, washed twice with sterile de-ionized water, and suspended in sterile de-ionized water, capsules were visualized. A small portion of the cell suspension was placed on a microscope slide and mixed with a loop full of India ink. A clean cover slip was placed over the preparation avoiding air bubbles. The slide was pressed down and blotted gently with a filter paper strip. Capsule was examined with oil immersion under phase contrast microscopy (Nikon Eclipse E6000, Nikon Canada, Inc.). The capsule presence was identified as unstained halos

(clear zones) around cells in the dark background.

Characterizataion of capsule carbohydrate content through lectin binding

E. coli O157:H7 capsules were characterized using fluorescein isothiocyanate (FITC)-conjugated lectins with different carbohydrate-binding specificities. Purified lectins were purchased from Vector Laboratories Inc., Burlington Ontario, Canada. Lectins from the following plant sources were used in this study: *Canavalia ensiformis* (concanavalin A), *Lotus tetragonolobus*, *Lens culinaris* (common lentil) *and Bauhinia purpurea*.

Carbohydrate binding competition experiments were performed by mixing the corresponding inhibition sugar with each lectin before addition of the cell suspension. The inhibition sugars (Sigma Chemical Co., Saint Louis, MO) were D-Mannose (500mM) for FITC-Concanavalin A, α-Fucose (200mM) for FITC-lectin *Lotus tetragonolobus, N*-Acetylgalactosamine (GalNAc, 200mM) for lectin *Bauhinia purpurea*. , *N*-Acetylglutamic acid (GluNAc,200mM) for FITC-lectin *Lens culinaris.*

The assay for carbohydrate-ectin binding was similar to that of Robitaille et al. 2006. Cells were prepared as previously described, washed twice with PBS (pH 7.2) and suspended at an OD_{600} of 0.5 in PBS. Fluorescein isothiocyanate (FITC)-conjugated lectins were combined with cells at final concentration 20μg/mL. Cells were incubated at 37°C for 2h, centrifuged, washed twice with PBS and resuspended in PBS. Cells were visualized using epifluorescence microscopy (Nikon Eclipse E6000, Nikon Canada, Inc., excitation wavelength 470nm, Emission wavelength 515nm).

Capsular polysaccharides were quantified using a LS-50B luminescence spectrometer (Perkin Elmer products) to determine fluorescence intensity (excitation wavelength 485nm and emission wavelength 530nm). The fluorescence of untreated cells served as the autofluorescence control. To normalize the fluorescence intensity, the amount of specific carbohydrate on the capsule of cells was estimated by relative fluorescence (Rf), which is the ratio of the response of FITC-lectin bound to cells and unstained cells with an arbitrary maximum estimated by the fluorescence intensity of FITC only binding at 530nm. The values reported are the means four replicates with standard deviation (SD).

Hydrophobicity assay

Hydrophobicity was estimated semi-quantitatively using bacterial adhesion to hydrocarbon. Cells were assayed as described by (LI and MCLANDSBOROUGH, 1999a; SWEET et al., 1987). Cultures were grown as previously described. Cells were suspended in phosphate buffered saline (PBS, pH 6.4) after centrifugation, and the pellet was washed twice as previously described. Bacterial suspensions (4 ml) from TSB, LB and NB were added to each of four 10-mm diameter glass test tubes for each bacterium tested, representing three tests and one control. One ml of xylene was added to each bacterial assay tube except for the control. Tubes were mixed by using a mini-vortexer (VWR Scientific products) for 40s and placed in 37°C water bath for 30 mins to allow the xylene and aqueous phases to separate. The aqueous lower layer (3 ml portion) in each tube was transferred to clean tubes by using Pasteur pipettes. Excess hydrocarbon was removed from aqueous lower layer by bubbling air through longer vortex for 40s.

The absorbance at 600 nm of aqueous layer was determined using a Beckman DU-530 spectrophotometer. The percentage reduction in absorbance of the bacterial assay tubes compared to the control (without xylene) was calculated using the following formula:

Adhesion to Hydrocarbon (%) = 100×(Ac - Ab) / Ac

Ab: absorbance of bacterial assay tubes

Ac: absorbance of the control tubes

Cell surface charge measurement

Cells were prepared as described above and suspended individually in sterile de-ionized water, 1mM ethylenediamine-tetraacetic acid (EDTA), 2 mM sodium chloride (NaCl), 2mM calcium chloride ($CaCl_2$) and 2 mM ferric chloride ($FeCl_3$) solutions EDTA is able to chelate metal ions in solutions. Zeta potential for cells in each suspension was measured using Brookhaven's Zeta Plus instrument (Brookhoven Instruments, Holtsville, New York).

Statistical analysis

All of *E. coli* O157:H7 data were analyzed with Statistical Analysis System (SAS) Software version 9.1.3 (SAS Institute Inc., Cary, NC) using the general liner model (GLM) procedure. Analysis of variance (ANOVA) was calculated using the overall means of four replications obtained from cells grown in three media using a significance level of P value <0.05. Difference among sample means was determined using Duncan's multiple-range test (alpha = 0.05).

RESULTS

Effect of growth media and ionic environment on attachment of *E.coli* O157:H7 to lettuce leaves

Cells grown in TSB, LB and NB were suspended in sterile de-ionized water (SDW), EDTA, sodium (Na^+), calcium (Ca^{2+}) and ferric (Fe^{3+}) solution. The attachment population on the surface and cut edge are given as log CFU per surface area (mm^2) and per length of cut edge (mm) (Table 3.1 and Table 3.2). In three growth media, the attachment population of LB-cells to lettuce leaf surfaces in the presence of EDTA is significantly less than TSB-cells and NB-cells (Table 3.1). There were no significant differences in the attachment between the TSB- LB- and NB-cells in the other ionic environments. LB-cells showed 0.4 log greater attachment than TSB-cells in SDW, but this difference was not significant. In addition, LB-grown cells attached more than the other two types of cells in the absence of supplementary ions in SDW. Moreover, TSB-cells showed 0.4 log greater attachment than NB-cells in sodium solution; NB-cells showed 0.5 log greater attachment than LB-cells in calcium solution. The addition of Ca^{2+} to attachment solution enhanced attachment of TSB-cells and NB-cells, but addition of Na^+ enhanced attachment of TSB-cells only. Neither Na^+ nor Ca^{2+} affected attachment of LB-cells. The attachment population of LB-cells significantly decreased in EDTA solution instead. When EDTA chelated all the ions in SDW, the attachment population of LB-cells is significantly less. This may indicate that LB-cells required metal ions in SDW for enhanced attachment, rather than Na^+ and Ca^{2+}. In addition, this result demonstrates that EDTA inhibited the ability of attachment via binding to ions only for LB-cells.

Attachment of LB- grown cells to the cut edge in SDW was significantly greater than for TSB-cells and NB-cells (Table 3.2). In addition, LB-cells attached significantly more than other two types of cells in the other ionic environments. Moreover, Ca^{2+} significantly increased the attachment population of TSB-cells and NB-cells to cut edge, not LB-cells. Addition of Na^{+} to the attachment solution did not affect attachment to the cut edge of any of the cells. For LB-cells, neither Na^{+} nor Ca^{+2} enhanced attachment to cut edge. The pattern of attachment of cells to the cut edge is similar to that observed for the leaf surface. While cells were suspended in the Fe^{3+} environment, there was obviously a reduced attachment population no matter what on surface and cut edge of lettuce. It showed there is less cell attachment in more acidic Fe^{3+} solution (pH=3) than other aqueous solutions (pH=5.8). As far the attachment of leaf surface and cut edge, cells grown in the three different media attach to cut edge significantly more than leaf surface (data not shown).

Effect of growth media and ionic environment on attachment of *E .coli* O157:H7 to spinach leaves

Data similar to that obtained on attachment to lettuce was obtained for attachment to spinach leaves. The attachment population of all types of cells was similar in all types of ionic environments except in the presence of EDTA (Table 3.3). Numbers of TSB-cells in EDTA media attached more significantly to spinach surface than other two type of cell. In addition, the attached population of NB- and LB-cells decreased when all the metal ions in suspension solution were chelated by EDTA. Addition of Na^{+} or Ca^{2} to attachment solution did not influence any type of cell to attach leaf surface. It showed

capsule production and hydrophobic interaction affected attachment of cells only in the absence of metal ions.

When cells attached to cut edge, there are similar numbers of all types of cells in all type of ionic environments except in Ca^2 medium (Table 3.4). Neither Na^+ nor Ca^2 enhanced attachment of all types of cells. In addition, there was more attached population of TSB- and NB-cells in SDW compared to the attached populations in nearly all other ionic environments. This indicates that metal ions have a minimal influence on attachment of cells to spinach surface and cut edge. In addition, the attached populations of three types of cells to the cut edge of spinach was also significantly greater than the attached populations on the spinach leaf surfaces (data not shown). This indicates that cells attached preferentially to the cut edges of spinach leaves. Similar to the results for lettuce leaves, all types of cells generally attached significantly less in Fe^{3+} media than in other attachment media. This indicates attachment of cells decreased in Fe^{3+} media possibly due to the high acidic environment.

Presence of capsules and cell morphology

Cells grown in the various media exhibited different morphology and capsule production (Figure 3.1). The cells grown in LB and NB were rod shaped, whereas the TSB-grown cells appeared less elongated and more coccoid-like. These cells also exhibited a well-defined capsule, unlike cells grown in LB and NB.

Capsular polysaccharides

Carbohydrate-binding specificities of the fluorescent lectins used in this study are shown in Table 3.5 (MANGIA et al., 1999).The binding ability of four FITC-conjugated lectins to the capsular polysaccharides of *E.coli* O157:H7 was determined (Table 3.6). The data indicate that the surfaces of cells grown in the various media contain different levels of mannose. In addition, the TSB-grown cells exhibit higher level of mannose, α-fucose and *N*-acetylglutamic acid (GluNAc), but not *N*-acetylgalactosamine (GalNAc). Besides, Rf values of FITC-BP binding cell agglutination were as low as autofluorescence and not significantly differenent. Moreover, Rf values also were not significantly different among FITC-ConA, FITC-LT and FITC-LC binding to LB- and NB-cells. The results also showed that levels of GalNAc were very low in the capsule of these cells and the capsules of LB- and NB-cells contain similar level of D-Mannose, α-Fucose and GluNAc.

When the sugar was added to the reaction mix along with FITC-lectin to determine competitive inhibition, the Rf value associated with cells grown in three media greatly decreased in comparison with the Rf value without the competitive sugar. The percentage of inhibitory D-mannose added in TSB-, LB- and NB- cells with FITC-ConA were 85.1%, 92.3% and 71.7%, respectively. In addition, when α-Fucose was added along with FITC-LT and D-GluNAc was added along with FITC-LC, the percentage of inhibition due to the sugar was 82.2% and 43.1%, respectively. The Rf values without inhibitory sugar in FITC-BP binding to TSB-, LB-, and NB-cells were low, such that only autofluorescence alone was detected whenfree GalNAc was added. The results confirmed the binding specificity of each lectin toward various targeted sugar haptens.

The observation of epifluorescence microscopy is also in accord with quantitative

measurements of the relative fluorescence (Figure 3.2). The stronger fluorescence signal was observed in FITC-ConA binding TSB- andLB-cells and FITC-LT binding TSB-cells than other images. However, there is no obvious fluorescence signal observed in the all images of FITC-BP binding cells. This demonstrates that the qualitative measurement of capsule composition in TSB/LB/NB-cells correspond with the value level of D-Mannose, α-Fucose, GalNAc and GluNAc in capsule of cells as in the previous description.

Hydrophobicity of *E. coli* O157:H7 grown in different media and Effect of suspension conditions on cell surface charge

The surface hydrophobicity of cells grown in TSB, LB and NB was determined (Table 3.7). The hydrophobicity on TSB-grown cells was significantly less than that of LB- and NB-grown cells as estimated by affinity to xylene.

The zeta potentials of cells grown in different media and suspended in various aqueous solutions are shown in Table 3.7. The purpose of cells suspended in EDTA solution is to chelate metal ions in the cell environment solution so that the surface charges could be determined without the influence of adsorbed cations. The surface charge of cells grown in various media and suspended in EDTA solution were similar, as indicated by zeta potential. The zeta potential of cells suspended in SDW was influenced by the growth medium. In addition, the zeta potential value of TSB-cells suspended in SDW, EDTA and Na^+ solution were mostly positive compared to LB-cells and NB-cells. By contrast, values were mostly negative when LB-cells were suspended in SDW, EDTA, Na^+ and Ca^{2+}. The zeta potential of cell surfaces in ferric chloride solution were positive, unlike the zeta potentials of cell surfaces in other ionic environments. This may result

from the pH of ferric chloride solution (pH =3) which is more acidic than the other aqueous solution (pH=5.8). In summary, the zeta potential of cells in these five ionic environment from positive to negative proceeded in the following order: Fe > Ca > EDTA > Na > SDW. The result of hydrophobicity and cell surface charge provided apparent explanation to support the result of attachment population of TSB/LB/NB-cells in different ionic environments.

DISCUSSION

The attachment of TSB/NB cells to the cut edges of lettuce leaves significantly increased in the presence of calcium ions and attachment of these cells to the surfaces of lettuce leaves slightly increased under these conditions, indicating divalent cations are important for attachment of *E. coli* O157:H7 to lettuce. Divalent cations have been found to facilitate the attachment of *Acinetobacter* sp. to the stainless steel (LEWIS et al., 1989). In a previous study, the population of *E. coli* O157:H7 in presence of calcium ions attached to lettuce surface significantly increased, but there was no effect for attaching to the cut edge of the lettuce leaves after 24hrs inoculation at 4°C (HASSAN and FRANK, 2003). Nevertheless, the current study indicates calcium ions affected attachment of TSB-cells to lettuce cut edge more than the surface after 2 hrs inoculation at 4°C. This indicates the importance of attachment time. *E.coli* attachment during initial biofilm formation displayed periodic density patterns continuing during biofilm development (AGLADZE et al., 2003). Initial rates of cell attachment are dependent on electrostatic barriers to formation of close cell-substrate contact (VOGER and BUSSIAN, 1987). There

are two different steps, which are called as reversible and irreversible attachment stages (AGLADZE et al., 2003; MERRITT et al., 2007; O'TOOLE and KOLTER, 1998; SAUER et al., 2002). In the presence of calcium, the population attachment of cells to leaf surface may increase with attachment time and the number of cells attached to cut edge may decrease with inoculation time. This study demonstrates that calcium enhances cell attachment to cut edge in the initial attachment phase shown reversible attachment stages, and to leaf surface in late attachment period which become irreversible attachment stages.

Attachment of LB-cells to lettuce cut edges, but not to spinach cut edges, was higher, and the zeta potential of LB-cells was significantly more negative than the other two types of cells. The electrokinetic (zeta) potential of cells is correlated with electrostatic interactions (VAN LOOSDRECHT et al., 1987).

Electrostatic interactions between the cell surface and substratum are at least partially responsible for differences in attachment. Among these three culture media, there is only glucose in the formula of TSB, unlike the formulasof LB and NB. Lack of sugar enables cells to produce capsules with different compositions. The glucose deficient environment affected the ability of capsular production of LB/NB cells to adapt this environmental stress. In this study, cells grown in TSB were more hydrophilic than cells grown in LB and NB, which were only moderately hydrophilic. Loss of capsule may increase hydrophobicity (CHARLAND et al., 1998). The more hydrophilic TSB-grown cells attached better to spinach cut edges than the less hydrophilic- cells in SDW because the cut edges may be more hydrophilic, as they lack the waxy cuticle that covers the leaf surface. However, hydrophilicity does not appear to be important in the attachment *E. coli* O157:H7 to spinach leaf surfaces. This may be due to the high hydrophobicity of the

spinach leaf surfaces. Even though LB/NB cells are significantly more hydrophobic than TSB-cells, LB/NB cells are still moderately hydrophilic and still have less affinity for hydrophobic surfaces on spinach leaf surfaces as compared to the lettuce surfaces.

Lectin agglutination tests were performed on the cell surfaces of five *Escherichia coli* serotypes (MANGIA et al., 1999). In the results of that study, the lectins that specifically bind to mannose on cells surface reacted with all five *E. coli* serotypes. Only one of the five stains, O125:H9, reacted with most of the lectins, including lectins isolated from *Lotus tetragonolobus* and *Bauhinia purpure.* Therefore, FITC-conjugated lectins isolated from four sources were determined in this study. They were assumed to react with *Escherichia coli* O157:H7. Furthermore, the fluorescence-based lectin-bind assay was performed successfully in the accumulation of capsule of *Streptococcus thermophilus* grown in milk (ROBITAILLE et al., 2006). Mannose exists in the capsule of all types of cells and there is the most quantity of mannose on TSB-cells capsule under in the same concentration of FITC-lectins for agglutination and very low level of *N*-acetylgalactosamine was found on the capsule of all types of cells. The capsule of TSB-cells contains higher proportion of sugar than LB-cells and NB-cells. .

E. coli O157:H7 cells grown in media that support high capsular EPS production have higher attachment potential to human intestine cell line INT407 monolayers (JUNKINS and DOYLE, 1992). However, there is no significant difference in cell attachment to spinach leaf surface whatever cells grown under conditions that favor high capsule production or repress capsule production. So hydrophobic and electrostatic interactions play a more important role in attachment of cells to spinach leaf surface than capsule production of cells. The electrokinetic potential of cells increases with

decreasing of cell hydrophobicity (VAN LOOSDRECHT et al., 1987). It was confirmed that encapsulated TSB-cells have a more positive surface charge and are highly hydrophilic in this study. Hydrophobicity and surface charge of cells may be influenced by growth medium. In addition, the zeta potential of TSB/LB/NB-cells were significantly different in these ionic environments. A possible explanation for the difference of surface charge in encapsulated TSB-cells and unencapsulated LB/NB-cells may be that the charged groups leading to surface charge are located in the capsule. The composition of capsular EPS on the cell surface is related to bacterial surface charge such as phosphate groups, carboxyl groups and proteins. When encapsulated TSB-cells and unencapsulated NB-cells in SDW have a more negative charge on cell surface, the population attachment to spinach surface in SDW and cut edge also increased. It may be noted that the influence of electrokinetic potential to attachment on spinach leaf surface and cut edge wasthan capsule production and hydrophobicity on the cell surface.

The influence of pH and ionic strength of the cellular surface of *E.coli* has been confirmed and most *E.coli* strains, including O157:H7 strains, are moderately hydrophobic. For *E.coli* O157:H7 it has been observed that a higher pH environment imparts a negative surface charge and that cell adhesion to beef muscle increases under lower ionic strength environments (LI and MCLANDSBOROUGH, 1999b). In this study, the positive zeta potential value on cell surface was observed in the acidic ferric chloride solution condition. The attachment of *E. coli* O157:H7 to lettuce and spinach significantly decreased in this highly acidic environment, which was also associated with less negative cellular surface charge as compared to other conditions studied. It may be likely due to the influence of the acidic environment to leafy green substratum.

E.coli O157:H7 attached better to the cut edge lettuce leaf than the surface in sterile distilled water (TAKEUCHI et al., 2000b). In this study, attachment numbers of all type of cells on leaf surface of lettuce and spinach were also significantly more than on cut edge. The surface of lettuce and spinach leaves is covered by hydrophobic cuticle (ANDREWS, 1991). All type of cells expressed low hydrophobicity on the surface, which may be responsible for its lower attachment to the lettuce and spinach surface cuticle in comparison with cut edge. In addition, cells attached better to spinach then to lettuce in SDW. Scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) show attachment of bacteria on the leaf surface at the stomata, epidermal cell margins, broken cuticles, trichromes and on leaf veins (MONIER and LINDOW, 2004; YADAV et al., 2005). *Salmonella* were found nearly completely in the intercellular margins, epidermal cells as well as around stomata on the lower surface of a spinach leaf (WARNER et al., 2008). The possible of explanation of more cells attaching to spinach could be that structural and topographical features and hydrophobic interactions of leaves surface affected cell attachment.

CONCLUSION

The current study showed the complex nature of relationship between the ionic environment, cell surface properties and cell attachment to leafy greens. Presence of capsule and decreased hydrophobicity increased attachment to intact spinach surface but not that of lettuce. Addition of calcium ions and a decreased negative charge on cells surface enhanced attachment to lettuce cut edge, but decreased attachment to spinach cut

edge. *E.coli* O157:H7 attached more to cut edge than the intact leaf surface and also more to spinach than lettuce. Moreover, attachment to leaf surfaces decreased when divalent cations were chelated. This study clarifies the mechanisms of influential factors of cell attachment to fresh produce.

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TABLE 3.1. Attachment of *E.coli* O157:H7 grown in three media to the surface of lettuce in the presence of different ionic environments.[c,d]

	Mean ± SD[a] (log CFU/mm^2)				
Growth Medium	SDW	EDTA	Na	Ca	Fe
TSB[b]	$2.44\pm0.39^{\alpha,1}$	$2.72\pm0.27^{\alpha,1}$	$2.92\pm0.24^{\alpha,1}$	$2.87\pm0.16^{\alpha,1}$	$1.74\pm0.24^{\alpha,2}$
LB	$2.85\pm0.30^{\alpha,1}$	$2.04\pm0.16^{\beta,2}$	$2.77\pm0.39^{\alpha,1}$	$2.62\pm0.15^{\alpha,1}$	$1.63\pm0.37^{\alpha,3}$
NB	$2.68\pm0.15^{\alpha,2}$	$2.79\pm0.35^{\alpha,12}$	$2.51\pm0.30^{\alpha,2}$	$3.16\pm0.37^{\alpha,1}$	$1.87\pm0.37^{\alpha,3}$

a. SD: standard deviations of four independent experiments

b. TSB, Tryptic soy broth; LB, Luria broth base Miller; NB, Nutrient broth; SDW, Sterile deionized water; EDTA: Ethylenediamine-tetraacetic acid; Na: Sodium chloride, Ca: Calcium chloride, Fe: Ferric chloride.

c. *E.coli* O157:H7 was not detected on control samples in which leaves had not been submerged with bacterial inoculum. Detection limit is 40 CFU/ per sample.

d. Means within each column followed by same letter and within the each row followed by the same superscript numbers are not significantly different ($P = 0.05$) .

TABLE 3.2. Attachment of *E.coli* O157:H7 grown in three media to the cut edge of lettuce in the presence of different ionic environments.[c,d]

	Mean ± SD[a] (log CFU/mm)				
Growth Medium	SDW	EDTA	Na	Ca	Fe
TSB[b]	$2.84\pm0.26^{\beta,3}$	$3.31\pm0.54^{\beta,2}$	$3.16\pm0.33^{\alpha,2}$	$3.90\pm0.04^{\alpha,1}$	$2.55\pm0.30^{\alpha,3}$
LB	$4.41\pm0.37^{\alpha,1}$	$3.80\pm0.48^{\alpha,2}$	$3.75\pm0.46^{\alpha,2}$	$4.40\pm0.10^{\alpha,1}$	$2.40\pm0.34^{\alpha,3}$
NB	$3.09\pm0.55^{\beta,2}$	$2.50\pm0.60^{\gamma,3}$	$3.41\pm0.20^{\alpha,12}$	$3.90\pm0.52^{\alpha,1}$	$2.37\pm0.37^{\alpha,4}$

a. SD: standard deviations of four independent experiments

b. TSB, Tryptic soy broth; LB, Luria broth base Miller; NB, Nutrient broth; SDW, Sterile deionized water; EDTA: Ethylenediamine-tetraacetic acid; Na: Sodium chloride, Ca: Calcium chloride, Fe: Ferric chloride.

c. *E.coli* O157:H7 was not detected on control samples in which leaves had not been submerged with bacterial inoculum. Detection limit is 40 CFU/ per sample.

d. Means with the same column followed by same letter and the same superscript numbers in the row are not significantly different ($P = 0.05$) based on Duncan's multiple range tests.

TABLE 3.3. Attachment of *E.coli* O157:H7 grown in three media to the surface of spinach in the presence of different ionic environments.[c,d]

	Mean ± SD[a] (log CFU/mm^2)				
Growth Medium	SDW	EDTA	Na	Ca	Fe
TSB[b]	$3.43\pm0.22^{\alpha,1}$	$3.19\pm0.20^{\alpha,2}$	$3.05\pm0.26^{\alpha,2}$	$3.24\pm0.07^{\alpha,2}$	$2.85\pm0.35^{\alpha,3}$
LB	$3.09\pm0.29^{\alpha,1}$	$2.15\pm0.04^{\beta,2}$	$2.80\pm0.38^{\alpha,1}$	$3.21\pm0.17^{\alpha,1}$	$2.75\pm0.30^{\alpha,2}$
NB	$3.21\pm0.22^{\alpha,1}$	$2.56\pm0.48^{\alpha\beta,2}$	$2.91\pm0.34^{\alpha,1}$	$2.93\pm0.29^{\alpha,1}$	$2.50\pm0.10^{\alpha,2}$

a. SD: standard deviations of four independent experiments

b. TSB, Tryptic soy broth; LB, Luria broth base Miller; NB, Nutrient broth; SDW, Sterile deionized water; EDTA: Ethylenediamine-tetraacetic acid; Na: Sodium chloride, Ca: Calcium chloride, Fe: Ferric chloride.

c. *E.coli* O157:H7 was not detected on control samples in which leaves had not been submerged with bacterial inoculum. Detection limit is 40 CFU/ per sample.

d. Means with the same column followed by same letter and the same superscript numbers in the row are not significantly different ($P = 0.05$) based on Duncan's multiple range tests.

TABLE 3.4. Attachment of *E.coli* O157:H7 grown in three media to the cut edge of spinach in the presence of different ionic environments.[c,d]

	Mean ± SD[a] (log CFU/ or mm)				
Growth Medium	SDW	EDTA	Na	Ca	Fe
TSB[b]	$4.17\pm0.46^{\alpha,1}$	$3.27\pm0.18^{\alpha,3}$	$3.80\pm0.25^{\alpha,2}$	$3.72\pm0.05^{\alpha,2}$	$3.20\pm0.57^{\alpha,3}$
LB	$3.59\pm0.40^{\alpha,1}$	$3.61\pm0.45^{\alpha,1}$	$3.67\pm0.37^{\alpha,1}$	$3.66\pm0.22^{\alpha,1}$	$2.76\pm0.34^{\alpha,2}$
NB	$3.81\pm0.22^{\alpha,1}$	$3.29\pm0.28^{\alpha,2}$	$3.64\pm0.22^{\alpha,1}$	$3.17\pm0.02^{\beta,2}$	$2.66\pm0.48^{\alpha,3}$

a. SD: standard deviations of four independent experiments

b. TSB, Tryptic soy broth; LB, Luria broth base Miller; NB, Nutrient broth; SDW, Sterile deionized water; EDTA: Ethylenediamine-tetraacetic acid; Na: Sodium chloride, Ca: Calcium chloride, Fe: Ferric chloride.

c. *E.coli* O157:H7 was not detected on control samples in which leaves had not been submerged with bacterial inoculum. Detection limit is 40 CFU/ per sample.

d. Means with the same column followed by same letter and the same superscript numbers in the row are not significantly different ($P = 0.05$) based on Duncan's multiple range tests.

TABLE 3.5. Carbohydrate-binding specificities of fluorescein isothiocyanate (FITC) and FITC-conjugated lectins(MANGIA et al., 1999).

Probes	Specificity
FITC only	Amine and sulfhydryl groups on proteins
FITC-*Canaualia ensqormis* (ConA)	D-Mannose; D-Glucose
FITC-*Lotus tetragonolobus* (LT)	α-Fucose
FITC-*Bauhinia purpurea* (BP)	*N*-Acetylgalactosamine(GalNAc)
FITC-*Lens culinaris* (LC)	*N*-Acetylglutamic acid (GluNAc); D-Mannose; α-Gliadin

TABLE 3.6. The relative fluorescence of *E .coli* O157:H7 resulting from FITC-lectin binding in the presence of competitive sugars.[e]

Growth medium	Mean ± SD[b] (Rf)[a]								
	FITC[d]	FITC-ConA	FITC-ConA + D-Mannose	FITC-LT	FITC-LT + α-Fucose	FITC-BP	FITC-BP + GalNAc	FITC-LC	FITC-LC-GluNAc
TSB	100	29.6±5.2$^{\alpha}$	4.4 ±0.4 $^{\alpha}$	10.7±1.8$^{\alpha}$	1.9±0.7$^{\alpha}$	A[c]	A	6.4±0.6$^{\alpha}$	3.6±0.2
LB	100	18.3±1.1$^{\beta}$	1.4±0.5$^{\beta}$	3.5±0.6$^{\beta}$	3.1±0.1$^{\alpha}$	0.1±0.0$^{\alpha}$	A	2.1±0.2$^{\beta}$	A
NB	100	4.6±0.8$^{\gamma}$	1.3±0.5$^{\beta}$	1.9±0.6$^{\beta}$	A	0.2±0.0$^{\alpha}$	A	1.3±0.4$^{\beta}$	A

a. Relative fluorescence (Rf) = 100(a-b)/(c-b), where a is the response of lectin bound to the cell, b is the response of an unstained cell and c is the response of an arbitrary maximum.

b. Values are the means of four experiments ± standard deviation (SD).

c. A: autofluorescence alone detected.

d. FITC: Fluoresecein isothiocyanate, FITC-ConA: Fluoresecein isothiocyanate-conjugated Concanavalin A. FITC-LT: Fluoresecein isothiocyanate-conjugated *Lotus tetragonolobus.* FITC-BP: Fluoresecein isothiocyanate-conjugated *Bauhinia purpurea*. FITC-LC: Fluoresecein isothiocyanate-conjugated *Len culinaris*. *N*-Acetylgalactosamine(GalNAc). *N*-Acetylglutamic acid (GluNAc).

e. Means in the same column followed by the same letter are not significantly different (*P* = 0.05) based on Duncan's multiple range tests.

TABLE 3.7. Hydrophobicity of *E. coli* O157:H7 determined by adherence to hydrocarbons (Xylene) and Zeta potential (mV) of cell surface charge of *E. coli* O157:H7 grown in tryptic soy broth (TSB), Luria broth base Millar (LB) and nutrient broth (NB) in different ionic environments.

Growth Medium	Hydrophobicity (%)[b]	Zeta potential (mV)[c]				
		SDW[a]	EDTA	Na	Ca	Fe
TSB	4.0±0.9$^{\alpha}$	-10.3±0.5$^{\alpha,4}$	-3.2±0.7$^{\alpha,3}$	-3.9±1.0$^{\alpha,3}$	-1.4±1.0$^{\beta,2}$	5.9±0.9$^{\beta,1}$
LB	22.4±4.6$^{\beta}$	-24.1±1.0$^{\gamma,4}$	-6.0±0.9$^{\beta,3}$	-6.6±1.5$^{\beta,3}$	-4.7±0.4$^{\gamma,2}$	12.1±1.1$^{\alpha,1}$
NB	27.0±2.8$^{\beta}$	-18.9±0.4$^{\beta,4}$	-4.5±1.1$^{\alpha,3}$	-5.1±0.7$^{\alpha,3}$	0.1±0.7$^{\alpha,2}$	7.0±0.3$^{\beta,1}$

a. SDW: Sterile deionized water, EDTA: 1mM ethylenediaminetetraacetic acid, Na: 2 mM sodium chloride (NaCl), Ca: 2mM calcium chloride ($CaCl_2$). Fe: 2 mM ferric chloride ($FeCl_3$).

b. Values are the means ± standard deviation (SD) of four replicates.

c. Means followed by the different letter are significantly different ($P < 0.05$) in hydrophobicity and zeta potential individually.

Figure 3.1 Comparison of *E. coli* O157:H7 cells grown in TSB, LB and NB for 24hrs. The cells were stained by India ink and observed with phase contrast microscopy. Image (a)(b)(c) of objects were magnified 600 times and image (d)(e)(f) of objects were magnified 1000 times. The clear zones surrounding cells present the capsule. Images are representative of at least four experiments.

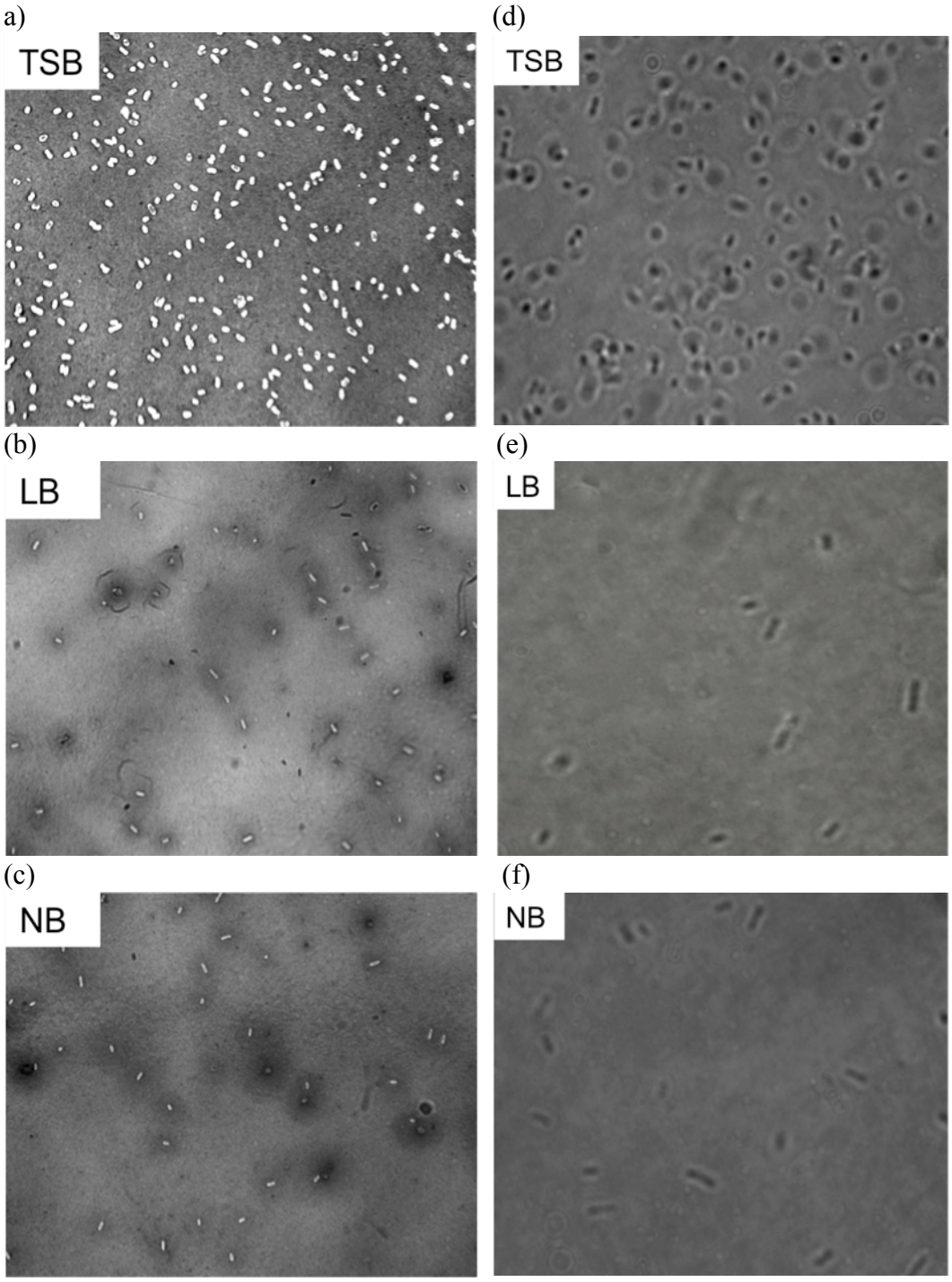
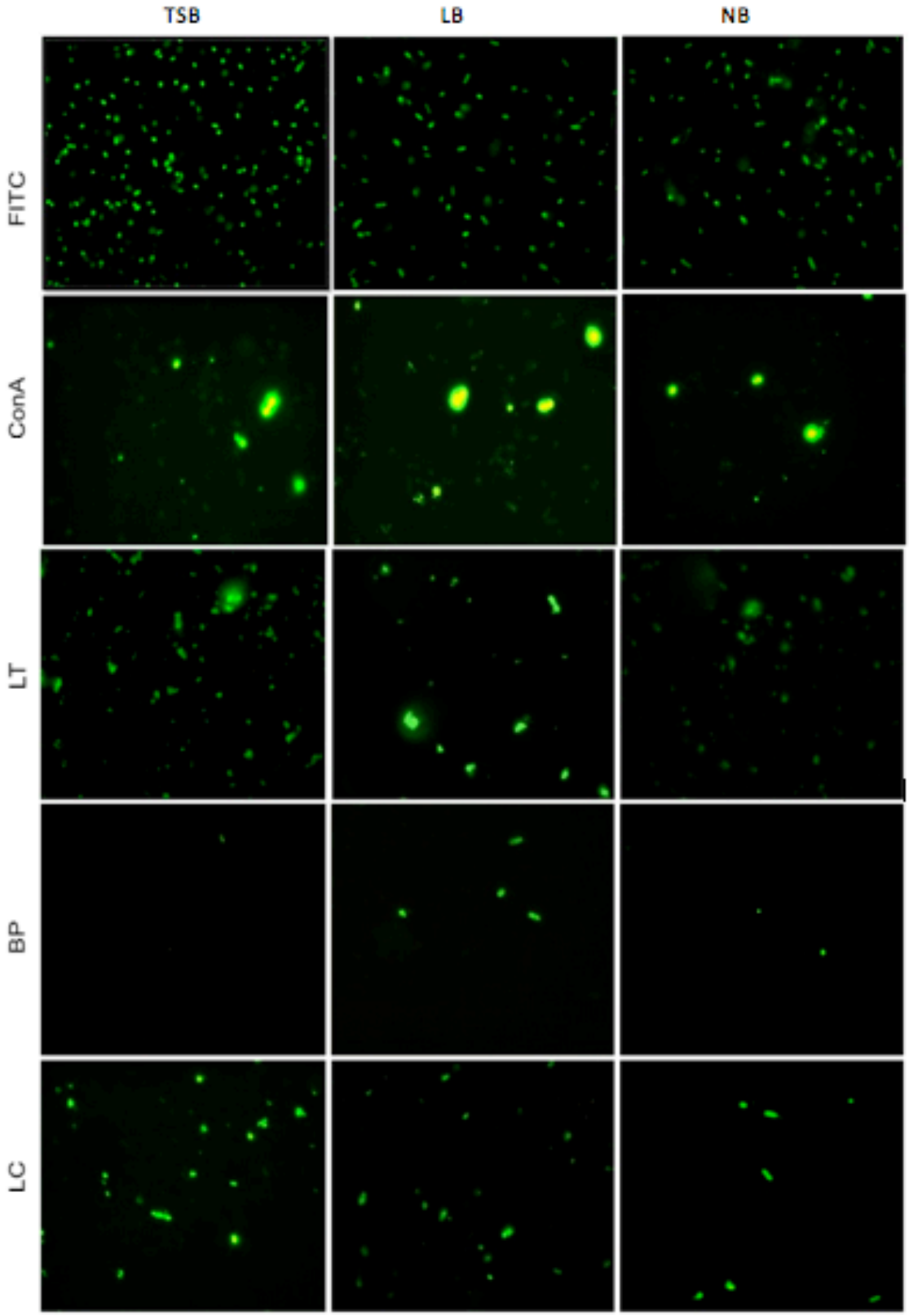


Figure 3.2 Epifluorescence microscopic images of *E.coli* O157:H7 using fluorescein isothiocyanate (FITC), FITC-labeled Concanavalin A agglutinin (ConA), FITC-labeled lectin extracted from *Lotus tetragonolobus* agglutinin (LT), FITC- labeled lectin extracted from *Bauhinia purpurea* agglutinin(BP) and FITC-labeled lectin extracted from *Lens culinaris* (LC). *E.coli* O157:H7 was grown grown in tryptic soy (TSB), nutrient broth (NB) and Luria broth base Miller (LB). Images are representative of at least four experiments.


TSB
LB
NB
FITC
ConA
LT
BP
LC

CHAPTER 4

THE ROLE OF CELLULOSE IN ATTACHMENT OF SHIGA TOXIN–PRODUCING *ESCHERICHIA COLI* TO LETTUCE AND SPINACH IN DIFFERENT WATER HARDNESS ENVIRONMENTS

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ABSTRACT

Cellulose is a linear polysaccharide polymer composed of D-glucose with β (1→4) glycosidic bond and usually exists in bacterial exopolysaccharide (EPS). The role of cellulose produced by Shiga toxin–producing *Escherichia coli* (STEC) on attachment to leafy greens is unclear. This study was undertaken to investigate the attachment of cellulose-deficient derivatives of STEC to lettuce and spinach in different water hardness environments. Two cellulose-producing wild-type STEC strains 19 and 49 and their cellulose- deficient derivatives were used. Strain 49 also produces EPS comprised of colanic acid. Viability of cells was determined by plate counts on the surface and cut edge after leaves were inoculated with 10^8 CFU/ml cells at 4°C for 2 hours. Hydrophobicity and surface charge were also determined. Strain 49 attached significantly 0.3 and 0.6 log greater to surface and 0.9 and 0.4 log greater cut edge of spinach compared to strain 19 in wild-type and cellulose-deficient cells. In addition, there was significantly greater attachment for cellulose-proficient cells on lettuce surface than cellulose-deficient cells, but not on spinach surface. Contrary to the leaf surface results, more cellulose-deficient cells attached (0.66 and 0.3 log greater) in strain 19 and 49 to cut edge of lettuce than cellulose-proficient cells. Strain 19 was more hydrophobic than strain 49. In addition, there was a decreasing level of attachment for strain 49 when water hardness increased from 200 to 1000pm on lettuce and spinach leaf surfaces. The zeta potential of strain 49 was more negative than strain 19. Cells containing colanic acid,which were less hydrophobic, had a greater potential to attach surface and cut edge of spinach when compared to cellulose-producing cells which were more hydrophobic.

Cell attachment to leafy green surfaces was also enhanced under higher water hardness environments.

INTRODUCTION

Foodborne outbreaks of Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 associated with consumption of leafy greens have been reported for decades (ARUSCAVAGE et al., 2008; COOLEY et al., 2007; SODHA et al., 2011). In the United States, STEC O157 is linked to 3,200 hospitalizations and more than 9,6000 episodes of diarrheal illness every year (SCALLAN et al., 2011). In addition, outbreaks of STEC O157 are not only in US but also in European countries. In 2007, an international outbreak was contaminated lettuce in the Netherlands and Iceland (FRIESEMA et al., 2008). In addition, lettuce-linked outbreaks of pathogenic *E. coli* O157 were been reported in 2005 Sweden and Enterotoxigenic *E. coli* in 2010 Denmark (ETHELBERG et al., 2010; SODERSTROM et al., 2008).

Cell attachment is a complex process regulated by various characteristics of the cell surface, growth medium and substratum (DONLAN, 2002). The hydrophobic and electrokinetic properties of cells are crucial influences for cell attachment. The hydrophobicity is affected by bacterial exopolysaccharides (EPS) components (GROSS and LOGAN, 1995). The electrokinetic potential of cell surface charge can be linked to the attachment of three *E. coli* strains on polymethacrylate surfaces (HARKES et al., 1991). In addition, the components of bacterial EPS such as cellulose and colanic acid are associated with cell attachment and biofilm formation. Cellulose was not required for *E. coli* O157:H7 attachment to spinach leaves (MACARISIN et al., 2012) even though

cellulose plays a crucial role in biofilm formation of *E. coli* 1094 on glass slides (DA RE and GHIGO, 2006). Both cellulose and curli favor attachment of *E. coli* O127:H6 and O157:H7 to cultured mammalian cells, cell colonization, biofilm development and survival (SALDANA et al., 2009). The colanic acid production is significantly correlated with adhesion forces of *E. coli* (RAZATOS et al., 1998) but biofilm formation of *E. coli* BW25113 cells is inhibited due to colanic acid overproduction (ZHANG et al., 2008).

Hard water contains high amounts of minerals such as calcium bicarbonate and magnesium bicarbonate. Water hardness of river and stream varies and is affected by regional climate and geological system. The source of irrigation water in the field for fresh produce is mainly from local stream and underground water. Therefore, we hypothesized that the influence of water hardness would significantly affect attachment of pathogenic *E.coli* to leafy greens. This study investigated the role of cellulose on attachment of *E .coli* to iceberg lettuce and baby spinach leaves by using mutants lacking cellulose production. The effect on cell attachment under different water hardness environments, hydrophobicity and electronegativity of the cell surface was determined.

MATERIALS AND METHODS

Bacterial strains and culture conditions

E. coli O157:H7 994 (beef jerky isolate) was supplied by the Center for Food Safety, University of Georgia, Griffin, GA. Cells were grown in tryptic soy broth (TSB) at 35 °C for two successive 24 ± 2h transfers. The four STEC strains were obtained from the laboratory of Dr. Jinru Chen, Department of Food Science and Technology,

University of Georgia, Griffin. Two wild type cellulose-producing strains 19B (O5:H-) and 49B (O103:H2) were used. In addition, two mutants 19D (O5:H-) and 49D (O103:H2) which were their cellulose deficient derivates by spontaneous selection were also used(YOO et al., 2010).The four strains were grown in Luria-Bertani no salt (LBNS) broth at 28 °C by two successive 24 ± 2h transfers. Cultures were centrifuged at 4400g (6,500 rpm) 4°C for 15 min. The supernatant fluid was discarded. Pellet was washed twice with sterile de-ionized water and suspended in the appropriate amount of sterile de-ionized water to contain approximately 8 log CFU/ml for sample inoculation.

Leafy green preparation

Iceberg lettuce (*Lactuca sativa*) and baby spinach (*Spinacia oleracea*) were purchased in local grocery stores. Undamaged leaves were selected for use. Inner leaves of iceberg lettuce were cut into designated sizes (square piece 2 × 2 cm) after aseptically removing outer leaves and core by using a sterile knife and surgical scalpel. In addition, the petioles of the baby spinach leaves were removed and the leaves cut into 2 × 2 cm squares using a sterile surgical scalpel. All pieces of leaves were rinsed five times with excess sterile de-ionized water (SDW).

Quantification of cell attachment

Synthetic hard water was prepared according to Association of Official Analytical Chemists (AOAC) official methods 960.09(E). For attachment experiments, a square leaf section was submerged in 10mL bacterial suspension with 0, 50, 100, 150, 200, 400, 700 and 1000ppm water hardness at 4°C for 2h. Leaves were taken from the cell suspension

using sterile tweezers and rinsed twice with excess SDW. The outermost tissue in each side of square leave piece was removed with a sterile surgical scalpel as cut edge 0.15cm. The cut edge leave tissue and inner portions of the leaves (intact surface) were separately combined with 10 ml of sterile 0.1% peptone water solution in polypropylene tubes and the cells released from the tissue by disruption using a homogenizer (Omni mixer, model 17105) set at energy level 5 for 30 seconds, sufficient to disrupt the leaf tissue. Samples were immersed in an ice bath during homogenization. Homogenized samples were serially diluted in sterile 0.1% peptone water. The number of viable bacteria attached to each leave surface and cut edge were determined by plating on sorbitol-MacConkey (SMAC) agar with incubation at 35°C for 24h.

Hydrophobicity assay

Hydrophobicity was estimated semi-quantitatively using bacterial adhesion to hydrocarbon as described by(LI and MCLANDSBOROUGH, 1999b). Cultures were grown as previously described. Cells were suspended in phosphate buffered saline (PBS) after centrifugation, and the pellet was washed twice as previously described. Bacterial suspensions (4 ml) were added to each of four 10-mm diameter glass test tubes for each bacterium tested, representing three tests and one control. One ml of xylene was added to each bacterial assay tube except for the control. Tubes were vortex-mixed by using a mini-vortexer (VWR Scientific products) for 40s and placed in 37°C water bath for 30 mins to allow the immiscible xylene and aqueous phases to separate. The aqueous lower layer (3 ml portion) in each tube was transferred to clean tubes by using Pasteur pipettes. Excess hydrocarbon was removed by bubbling air through longer vortex for 40s. The

absorbance at 600 nm of the aqueous layer was determined using a Beckman DU-530 spectrophotometer. The percentage reduction in absorbance of the bacterial assay tubes compared to the control (without xylene) was calculated.

Adhesion to Hydrocarbon (%) = 100×(Ac - Ab) / Ac.

Ab: absorbence of bacterial assay tubes,

Ac: absorbence of the control tubes

Cell surface charge measurement

Cells were prepared as described above and suspended individually in 0, 50, 100, 150, 200, 400, 700 and100ppm water hardness solution. Zeta potential in each suspension was measured using Brookhaven's Zeta Plus instrument (Brookhoven Instruments, Holtsville, New York).

Statistical analysis

All data were analyzed with Statistical Analysis System (SAS) Software version 9.1.3 (SAS Institute Inc., Cary, NC) using the general liner model (GLM) procedure. Analysis of variance (ANOVA) was calculated using the overall means of four replications obtained from cells grown in three media using a significance level of *P* value <0.05. Difference among sample means was determined using Duncan's multiple-range test (alpha = 5%).

RESULTS

Attachment of STEC on the surface and cut edge of lettuce and spinach

The attachment of STEC population to lettuce and spinach leaves was investigated by using wild-type cells and cellulose-deficient mutants in SDW. On the leaf surface of iceberg lettuce, wild-type strains 19B and 49B attached at significantly greater levels (0.5 and 0.9 log units) than cellulose-deficient strains 19D and 49D (Table 4.1). These data indicate that cellulose on the cell surface enhances the ability of the cells to attach to lettuce leaf. However, there is less attachment of wild type (19B and 49B) cells to cut edge of lettuce than their cellulose-deficient mutant. More cellulose-deficient cells attached (0.7 and 0.3 log greater) for strain 19 and 49 to the cut edge of lettuce than cellulose-producing cells. These data indicate that cellulose-production may decrease cell attachment to leaf cut edge.

For spinach leaves, strain 49B and 49D attached 0.3 and 0.6 log greater to surface and 0.9 and 0.4 log greater to cut edge compared to strain 19B and 19D. There was no significant difference in attachment between wild-type cell and cellulose-deficient mutant. These data demonstrate that colanic acid in the cell envelope may play a more important role than extra-cellular cellulose to increase attachment ability to spinach leave surface and cut edge (Table 4.1).

The examination of bacterial hydrophobicity

Four strains grown in LBNS broth were evaluated for their bacterial hydrophobicity towards hydrocarbons (xylene) on cell surface. Strain 19 cells were significantly different with strain 49 cells. Strain 19B and 19D had relatively more

affinity to xylene (55.57% and 46.20% respectively) followed by strain 49B and 49D for which the percent hydrophobicity values were 20.22% and 28.82%, respectively. Strain 19B and 19D cells were significantly more hydrophobic than colanic acid–producing strain 49B and 49D cells (Table 4.2). These results imply that the presence of colanic acid may be associated with a more hydrophilic cell surface.

Attachment of STEC to lettuce in different water hardness environments

The influences of cellulose-production and a water hardness environment on STEC attachment to lettuce and spinach leaves were determined. Data presented in Figure 4.1 indicate that there was a progressive ordering with increasing attachment of strain 49B/49D cells with water hardness levels of 50ppm/100ppm to 1000ppm on lettuce leaves surfaces. However, this result was not observed in one wild type strain (19). In addition, strain 49 (wild type) cells attached to lettuce more than strain 19 (wild type) in hard water. This indicates that the influence of colanic acid to cell attachment in different water hardness environments is more important than cellulose.

At the cut edge of lettuce leaves, the attachment population of strain 49 cells in SDW is significantly higher than in hard water. In addition, there was no significantly different attachment population of strain 19 and 49 (wild type) cells among all levels of water hardness. These results indicate that the ability of cells to attach to cut edges is not enhanced in the presence of hard water. The result when using strain 994 was similar to that obtained with strain 49. These results demonstrate that the attachment ability of *E.coli* O157 increases with increasing water hardness.

Fewer 19D (cellulose-deficient, colonic deficient) cells attached to lettuce leafsurfaces in SDW when compared to attachment in hard water. These results indicate that water hardness may play an important role on cell attachment in the absence of cellulose and colanic acid, likely due to increased electrostatic interactions. The influence of cellulose to cell attachment was clearly observed only in 50ppm water hardness for strain 19 and 49 cells on surface. The number of cellulose-deficient mutant was significantly more than the wild-type parent cells. It indicated that the presence of cellulose might reduce cell attachment in low water hardness environment. Therefore, it appears that the presence of minerals in water may compensate for the lack of EPS production in cell attachment.

Attachment of STEC to spinach in different water hardness environments

The influences of cellulose and water hardness for STEC attachment to spinach leaves was also investigated (Figure 4.2). There was an ordering of decreasing attachment of strain 49 with increase in water hardness from 0 to 150ppm, and afterwards from 200 to 1000 ppm in order progressively increasing attachment of cells on spinach leaves surface. But this result of was not found for strain 19.

No influence of water hardness on cell attachment on cut edge was observed. However, the result of 19D on spinach was similar to that obtained for lettuce, as cells in hard water were able to attached more than in SDW. As with lettuce, the ions in hard water may enhance cell attachment to spinach leaf surfaces and cut edges where there is an absence of cellulose and colanic acid. There was no significant difference among all

levels of water hardness and between wild-type parents 19 cell and mutant on leaf surfaces.

The measurement of cell surface charge

The zeta potential of cell surface was measured in 0 ppm to 1000 ppm water hardness environments. A difference of electrokinetic properties on cell surface was observed. In Figure 4.3, the zeta potential of strain 49 was significantly more negative than that of strain 19 at all water hardness levels. In addition, the surface charge of cells in SDW was also significantly more negative than in any level of hard water.

DISCUSSION

The cellulose-deficient mutants had extensively lower attachment ability to lettuce leave than wild-type parent cells. However, cellulose did not play a significant role in attachment to spinach leave surface and cut edge; rather the role of colanic acid was significantly more influential instead. In previous studies, the importance of cellulose and colanic acid for attachment of *E. coli* to other surfaces has been addressed in different results. Colanic acid and cellulose were associated with the biofilm formation of *E.coli* K-12 on plastic surface but not required for *E. coli* O157:H7 attaching to mammalian cells. In addition, there was reduction of *E. coli* O157:H7 cellulose synthase deficient mutants in binding to alfalfa sprouts and colanic acid deficient mutants also exhibited a large reduction in attachment (MATTHYSSE et al., 2008). On the other hand, *E. coli* O157:H7 did not produce cellulose as a part of the extracellular matrix

during its own biofilm formation on glass and polystyrene surface (UHLICH et al., 2006). Cellulose was not found to be a necessary factor for developing strong attachment to spinach leaves (MACARISIN et al., 2012). Colanic acid has been showed to be required for developing biofilm of *E. coli,* but not for attachment (DANESE et al., 2000).

Leaf surfaces resist the attachment of microorganisms by the presence of a hydrophobic waxy cuticle layer (ANDREWS and HIRANO, 1991). *E. coli* O157:H7 prefer to attach on the cracked cuticle or damaged part of leaves which lacks the waxy tissue because the surface of *E. coli* O157:H7 is highly hydrophilic (DEWANTI and WONG, 1995; MAFU et al., 1991) and wax is hydrophobic. Previous research found that viable *E. coli* O157:H7 cells preferentially attached to the cut edge of lettuce leaves instead of to intact leaf surface as visualized by confocal scanning laser microscopy (TAKEUCHI et al., 2000b). Strain 49 with colanic acid production was less hydrophobic and its surface charge was more negative than strain 19. This surface characteristic may explain why strain 49 attached preferentially to spinach leaf surfaces and cut edges compared to strain 19. On the other hand, the influence of hydrophobic and electrostatic interactions was not observed in cell attachment to lettuce surfaces. It seems that cellulose has a more crucial role in *E. coli* attachment to lettuce leaf.

In this study, synthetic hard water was prepared based on Association of Official Analytical Chemists official methods. The hard water contained calcium and magnesium cations (Ca^{2+},Mg^{2+}). In previous research, the influence of divalent cations on cell attachment has not been consistent. Magnesium cations are more effective than calcium cations in helping the attachment of chicken fibroblasts to protein-coated plastic surfaces. However, increasing the concentration of divalent cations did not enhance cell

attachment and the initiation of cell attachment seemed be inhibited by high calcium concentration (TAKEICHI and OKADA, 1972). The attachment of *Pseudomonas fluorescens* to glass surfaces was also affected by an increase in the concentration of several cations such as calcium, ferric iron, lanthanum and sodium (FLETCHER, 1988).

When strain 49 (wild-type) cells were suspended in the higher water hardness, attachment to lettuce and spinach leaves also increased. *E. coli* O157:H7 also gave similar results when attaching to lettuce leave, except for strain 19. According to the U.S. Department of the Interior and U.S. Geological Survey (USGS) in 2013, high hard waters were measured in streams in Kansas, Texas, New Mexico, Arizona and southern California. Irrigation water from streams in these areas may enhance cells attachment to iceberg lettuce and baby spinach leaves,increasing the risk of *E.coli* O157:H7 outbreak on leafy greens.

CONCLUSION

Cellulose-proficient cells exhibited a significantly greater level of attachment on lettuce surfaces than cellulose-deficient cells, but the same was not found for spinach surface. STEC strain 49B and 49D cells producing colanic acid were less hydrophobic and had a higher negative zeta potential. These cells also demonstrated significantly greater potentials to attach to the surfaces and cut edges of spinach in comparison with the more hydrophobic and less negative charges of the cells of STEC strains 19B and 19D. In high water hardness environments, attachment of strain 49 (wild type) cells to leafy green surfaces was also enhanced.

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TABLE 4.1. Attachment of four Shiga toxin–producing *E. coli* (STEC) strains to lettuce and spinach on surface and cut edge.[γ]

	Lettuce				Spinach			
	Surface[α]		Cut edge		Surface		Cut edge	
Strain	Mean[β]	SD[δ]	Mean	SD	Mean	SD	Mean	SD
19B	2.40[a]	0.46	2.35[c]	0.27	1.96[a]	0.20	1.97[b]	0.31
19D	1.86[b]	0.32	3.04[b]	0.21	1.90[a]	0.16	2.20[b]	0.32
49B	2.17[a]	0.19	3.19[b]	0.53	2.26[b]	0.17	2.86[a]	0.28
49D	1.27[c]	0.34	3.48[a]	0.53	2.50[b]	0.08	2.59[a]	0.34

[α] Surface (log CFU/mm^2); Cut edge(log CFU/mm)

[β] Means of surface and cut edge followed by the different lowercase letter in the same column are significantly different ($P > 0.05$)

[γ] Cellulose-producing wild-type STEC strains 19B and 49B and their cellulose-deficient derivative 19D and 49D. Strain 49 also can produce colanic acid.

[δ] SD: standard deviations of four independent experiments

TABLE 4.2. Hydrophobicity of four Shiga toxin–producing *E. coli* (STEC) strains determined by adherence to hydrocarbons (Xylene).

	Hydrophobicity (%)	
Strain	Mean[α]	SD[β]
19B	55.57[a]	6.48
19D	46.20[a]	3.89
49B	20.22[b]	0.45
49D	28.82[b]	2.97

[α] Means of surface and cut edge followed by the different lowercase letter in the same column are significantly different ($P > 0.05$).

[β] SD: standard deviations of four independent experiments

FIGURE 4.1. Attachment of *E. coli* O157:H7 strain 994 (T) and cellulose-producing wild-type STEC strains 19B and 49B as well as their cellulose-deficient derivative 19D and 49D to iceberg lettuce (a) surface and (b) cut edge in the different water hardness environments. Error bars indicate standard deviations of four independent experiments.

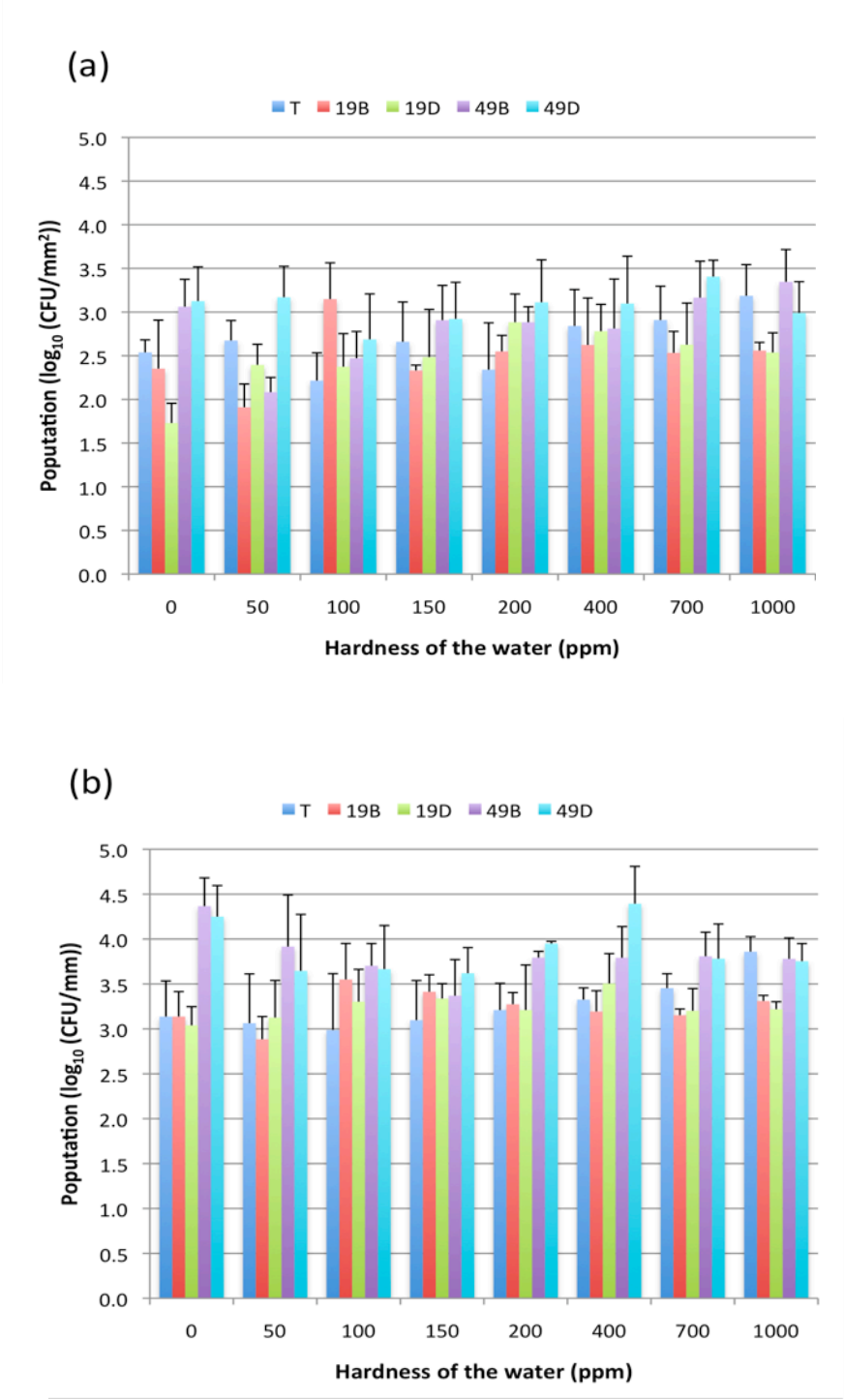
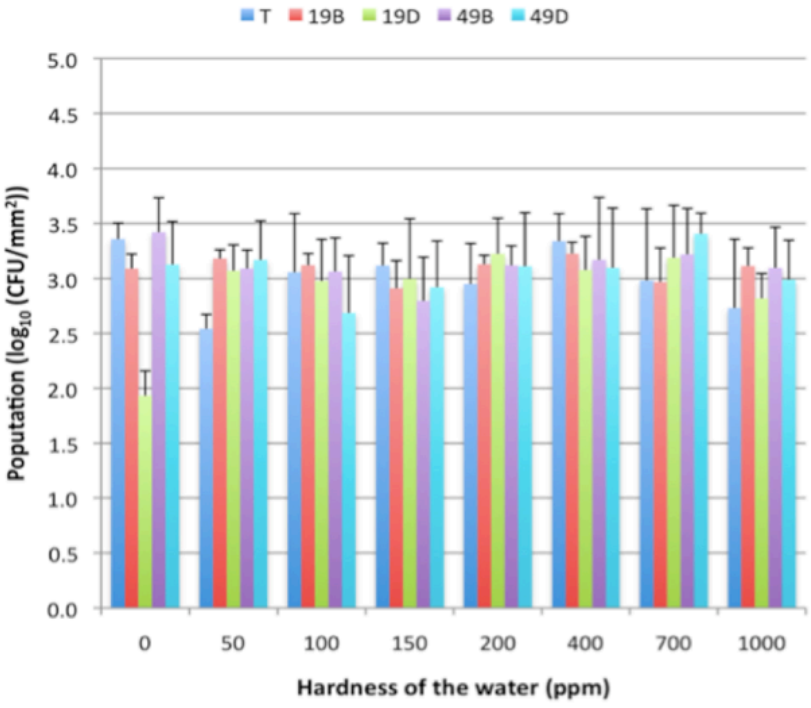


FIGURE 4.2. Attachment of *E. coli* O157:H7 strain 994 (T) and cellulose-producing wild-type STEC strains 19B and 49B as well as their cellulose-deficient derivative 19D and 49D to baby spinach (a) surface and (b) cut edge in the different water hardness environments. Error bars indicate standard deviations of four independent experiments.

(a)



(b)

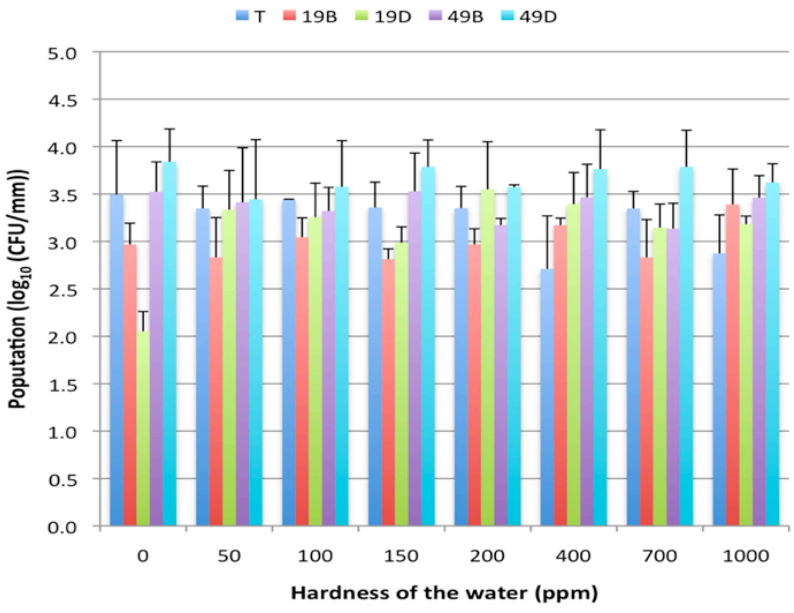
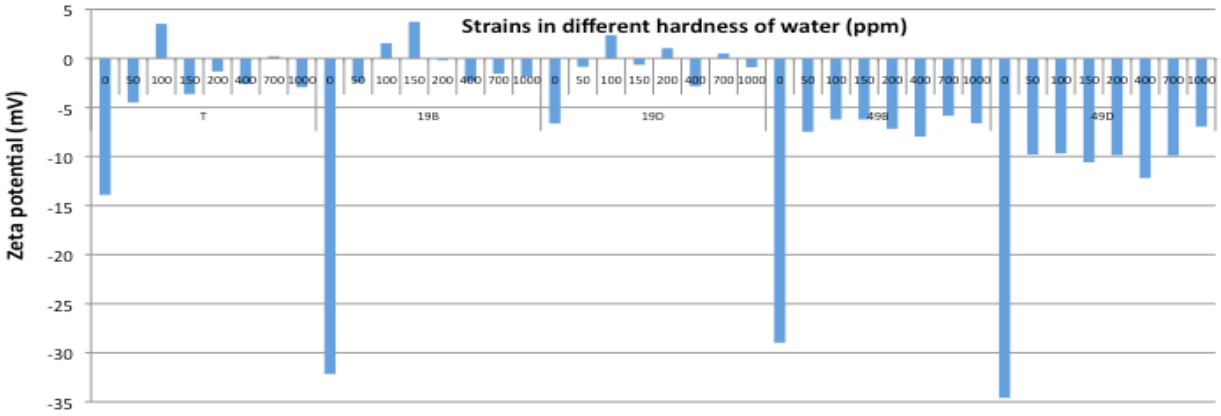


FIGURE 4.3. Zeta potential (mV) of cell surface charge of *E. coli* O157:H7 strain 994 (T) and cellulose-producing wild-type STEC strains 19B and 49B as well as their cellulose-deficient derivative 19D and 19D in the different water hardness environments.



CHAPTER 5

INFLUENCE OF EXTRA-CELLULAR CELLULOSE PRODUCTION ON THE SURVIVAL OF SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* ON SPINACH AND LETTUCE AFTER CHLORINE TREATMENT

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ABSTRACT

Chlorinated water with the maximum allowable level 200 ppm free chlorine is widely used to sanitize water used in the fresh-cut produce industry. Shiga toxin–producing *Escherichia coli* (STEC) produce extra-cellular cellulose which may influence stress tolerance. This study was undertaken to investigate the role of extra-cellular cellulose produced by STEC on the survival of STEC after attachment to lettuce and spinach followed by chlorine treatment. Four STEC strains, two wild-type cellulose-producing and their cellulose-deficient derivatives, were used. One strain produced colanic acid in addition to cellulose. Leaves of spinach and lettuce were treated with chlorinated water (50 and 150 ppm) after cells were attached to the surfaces and cut edges of lettuce at 4 °C for 2 hours. Chlorine treatment reduced populations of cellulose-deficient cells 1.2 log CFU/ mm^2 more than the wild type in 150ppm of chlorine on spinach leaf surfaces. However, populations of cellulose-producing cells were reduced by 1.5 log CFU/mm^2 more than its mutant counterparts when the cells can also produce colanic acid. There were no significant differences in survival between four strains attached at the cut edge. Extra-cellular cellulose production protects STEC cells attached to leafy greens from the effects of chlorine on spinach leaf surfaces.

INTRODUCTION

For decades, a variety of Shiga toxin-producing *Escherichia coli* (STEC) serotypes have been associated with foodborne outbreaks in the United States (BROOKS et al., 2005; PATON and PATON, 2003; RANGEL et al., 2005). They cause severe gastroenteritis in human including mild to bloody diarrhea and hemolytic-uremic syndrome (HUS) (GOULD et al., 2013; GRIFFIN and TAUXE, 1991). Multistate outbreaks of STEC O157 and non-O157 infections linked to fresh produce have been reported by Centers for Diseases Control and Prevention (CDC). These include STEC O26 in raw clover sprouts at restaurants in 2012, STEC O157:H7 in organic spinach and spring mix blend in 2012 and STEC O121 in frozen food products in 2013.

Microorganisms prefer to attach, aggregate, grow in community-based sedentary microcolonies, and produce biofilms on surfaces in various environments including freshwater, marine water, food, and medical and industrial systems (CARPENTIER and CERF, 1993; GIBSON et al., 1999; MOSTELLER and BISHOP, 1993). For the food industry, the formation of biofilms is of concern because they can be found on food contact surfaces, equipment and water distribution systems and they contributes to product contamination and foodborne illness outbreaks, especially those associated with pathogenic and spoilage microorganisms (CHMIELEWSKI and FRANK, 2003). Therefore, effective sanitizing in food industry and restaurants play an important role to decreasing the risk of food contamination.

Attachment of pathogens to food contact surfaces has been investigated by many researchers (FRANK and KOFFI, 1990; KIM and FRANK, 1994; TAKEUCHI et al., 2000a). The efficiency of disinfectants against a variety of pathogenic biofilms has been

determined (DHALIWAL et al., 1992; RONNER and WONG, 1993). Chlorine is the most commonly applied sanitizer for water used in the food industry because of its low-cost, accessibility and acceptable disinfecting effect. Its most potent bactericidal form is hypochlorous acid (HOCl) from anion hypochlorite (OCl^-). Although chlorine washing is widely applied on fresh produce, it is not sufficient to inactivate and remove microorganisms from vegetables and fruits (BEUCHAT, 1998; BRACKETT, 1992; FISHBURN et al., 2012). Its main function is to prevent cross-contamination. Chlorine solution at permitted concentration (< 200ppm) can typically reduce the population of bacteria on fresh-cut produce surface by 1 to 2 log units (BEUCHAT et al., 1998; KESKINEN et al., 2009; SAPERS et al., 1999; ZHUANG et al., 1995). Many viable *E. coli* O157:H7 cells can be observed on the cut edges and stomata of lettuce leaves after chlorine treatment (SEO and FRANK, 1999a).

Survival and growth of *E. coli* in foods is associated with numerous environmental and food-related factors such as temperature, pH, and water activity. Pathogenic and nonpathogenic *E. coli* have various physiological responses to sublethal intrinsic and extrinsic stress produced by heat, acidity, osmosis, oxidation, and sanitizer exposure (CHUNG et al., 2006). When microorganisms encounter environmental stress, multiple defense mechanisms are triggered and cell envelope structure is modified. *E. coli* is able to secrete the bacterial exopolysaccharides (EPS) including celloluse and colanic acid that are associated with the cell surface. EPS consists of long chain polymers and is involved with formation of biofilm as well as stress defenses. Colanic acid is encoded by *wca* gene cluster and provides *E. coli* O157:H7 protection against acidic, heat, osmotic and oxidative stresses (CHEN et al., 2004; MAO et al., 2001). Cellulose

consists of a linear long chain of β(1→4) linked D-glucose units and may protect STEC against oxidative and acidic treatments (YOO and CHEN, 2012). The hypothesis of this study is that the protective effect of cellulose and calonic acid of STEC against chlorine solution could be observed on leafy green surfaces and cut edges. The objective was to determine the influence of cellulose on survival of *E. coli* O157 attached to iceberg lettuce and baby spinach after chlorine treatment. Two cellulose-producing wild-type STEC cells and their cellulose deficient derivatives were utilized in this study and two levels of chlorine concentration were employed in the treatment.

MATERIALS AND METHODS

Spinach and Lettuce

Baby spinach(*Spinacia oleracea*) and iceberg lettuce (*Lactuca sativa*) were purchased from retail grocery stores in Athens, Georgia. The petioles of the spinach leaves were removed and the selected intact leaves were cut into 2 by 2 cm squares without including midrib parts by using a sterile surgical scalpel. The core and outer leaves of lettuce were discarded by using a sterile knife and surgical scalpel. Undamaged inner leaves of lettuce were cut into designated pieces (2 by 2 mm). All pieces of leaves were rinsed five times with sterile de-ionized water (SDW) to remove soil on the leaf surfaces before following inoculation experiments.

Preparation of STEC strains and their cellulose deficient derivates

The four STEC strains were obtained from the laboratory of Dr. Jinru Chen, Department of Food Science and Technology, University of Georgia, Griffin. Two wild type cellulose-producing strains 19B (O5:H-) and 49B (O103:H2) were used. In addition, two mutants 19D (O5:H-) and 49D (O103:H2) which were their cellulose deficient derivatives by spontaneous selection were also used (YOO et al., 2010). The four strains were grown in Luria-Bertani no salt (LBNS) broth at 28 °C by two successive 24 ± 2h transfers. *E. coli* O157:H7 994 (beef jerky isolate) was supplied from the Center for Food Safety, University of Georgia, Griffin, GA. Cells were grown in tryptic soy broth (TSB) at 35 °C for two successive 24 ± 2h transfers. Cells were collected by centrifuging at 4400g (6,500 rpm) 4°C for 15 min. After the supernatant fluid was discarded, the pellet was washed twice with SDW and suspended in the appropriate amount of SDW to contain approximately 8 log CFU/ml for sample inoculation.

Chlorine treatment after cell attachment

A square piece of spinach or lettuce leaf was submerged in 10mL bacterial inoculum at 4°C for 2hrs to allow cells to attach to surfaces and cut edges. Leaves were taken from the cell suspension with sterile tweezers and rinsed twice with excess SDW to remove unattached cells. Leaves were then submerged with 50 and 150ppm chlorinated water for 2 minutes. Leaves were taken from the chlorinated solution with sterile tweezers and rinsed three times with excess SDW to remove chlorine solution.

Determination of STEC Survival

To enumerate survival of STEC after treatment, the outermost leaf tissue in each side of the square piece was removed by using a sterile surgical scalpel to provide a 1.5mm wide strip of the cut edge and total four cut edge strips from one square piece of leaf. The cut edge tissue and inner portions of the leaves (intact surface) were separately combined with 10 ml of sterile 0.1% peptone water solution and placed in homogenizer blender bags. Cells were released by disrupting tissue using hand massaging to squeeze the bag for 1min. The leaf juice was serially diluted with 0.1% sterile peptone water. The population of viable cells on each leaf surface and cut edge were determined by plating using tryptic soy agar (TSA) and sorbitol-MacConkey (SMAC) agar incubated at 35°C for 24hrs.

Statistical analysis

Each experiment was utilized duplicate leaves and was repeated four times. Data were obtained as CFU per sample of leaf or cut edge and were analyzed with Statistical Analysis System (SAS) Software version 9.1.3 (SAS Institute Inc., Cary, NC) using the general liner model (GLM) procedure. Analysis of variance (ANOVA) was calculated with overall means of four replications based on a 95% confidence level. Multiple comparisons of sample means was obtained by using Duncan's multiple-range test (alpha = 5%).

RESULTS

Survival of STEC on lettuce surface and cut edge after chlorine treatment

Lettuce leaf pieces were treated with 50ppm and 150ppm chlorine solution after cell attachment, and surviving cells determined. The data (Figure 5.1) indicated that there were significantly less viable number of 49B and 49D strains remaining on the lettuce surface cells after treatment at 50ppm and 150ppm chlorine leaf surface of iceberg lettuce in comparison with the cell number before chlorine treatment. For cellulose-deficient mutants, chlorine treatment reduced the population of colanic acid-producing cells at 1 log unit more than colanic acid-deficient cells in 50 and 150ppm of chlorine. This result indicates that cells that produce colanic acid (but not cellulose) have decreased resistance chlorine when on the lettuce surface. However, the inactivation of the cellulose-deficient 49D strain is 0.8 log greater than its wild type counterpart after 150 ppm chlorine treatment. This result indicates that cellulose when combined with colonic acid may have a role in protecting cells from chlorine stress in on lettuce surface.

The influence of colonic acid and cellulose production on survival on the cut edge is similar to that observed on leaf surface. In addition, the population of all strains after chlorine treatment is significantly lower on the cut edge than cell number before treatment. However, this result was not observed on the leaf surface. Cellulose could also provide cell protection in the presence of colanic acid. In addition, survival of strain 994 after treatment was greater than other STEC strains on both surface and cut edge, indicating variation in strain responses to chlorine treatment.

Survival of STEC on spinach surface and cut edge after chlorine treatment

Survival of cellulose and colanic acid deficient mutants of STEC was also determined using cells attached to spinach followed by treatment with chlorine. Chlorine treatment reduce the number of 19D and 49B cells more than 19B and 49D cells on spinach surface and cut edge (Figure 5.2). Chlorine treatment reduced the number of cellulose-deficient 19D cells 1 log unit more compared with wild-type 19B cells. However, the chlorine treatment reduced the number of wild-type 49B cells 1.5 log units more than the cellulose-deficient mutant 49D cells (colanic acid-producing) cells Similar reductions were not observed for cells attached to the cut edge. These data indicate that extra-cellular cellulose may protect STEC cells attached to spinach leave surface from inactivation by chlorine. However, these cells were not be protected by the cellulose when they produced colanic acid. The presence of colanic acid may influence the protective effect of cellulose.

Sublethally-injured cells on surface and cut edge of lettuce and spinach after chlorine treatment

The numbers of sublethally-injured cells remaining of the leaf surface and cut edge after chlorine treatment were determined by subtracting the CFU obtained using selective media (Sorbitol MacConkey agar) from those obtained using non-selective media (tryptic soy agar).

There were no injured cells detected for strain 49D cells on spinach and only the wild-type 19B cells and their mutant 19D cells were detected on spinach surface at 150 ppm chlorine treatment. There were significantly more injured cells for wild-type strain

19B at 150ppm. This result indicates that cellulose production may reduce sublethal damage resulting from the sanitizing ability of chlorine. Such sublethal injury may be temporary, as it can lead to complete inactivation. In addition, significantly more injured cells for strain 994 were observed than other four strains. However, more injured 19D mutant cells were found on lettuce compared with wild-type. In addition, there were significantly more injured cells on cut edge than leaf surface. These data also indicate that chlorine is less effective on the cut edge than on surface and also that chlorine inactivation being stronger on leaf surface possibly causes greater lethal damage to cells thereby leaving fewer injured cells.

The spinach leaf surface and cut edge did not produce significantly different numbers of injured cells. On the other hand, significantly more injured cells (strains 19B and 49B) were produced on the lettuce cut edge as compared to the leaf surface when 50 ppm level chlorine was used. Similar results were obtained for the 19B cells when using 150ppm chlorine. Strains 19B and 49B are able to produce cellulose. This result indicates that cellulose production may reduce sublethal damage resulting from the sanitizing ability of chlorine. Such sublethal injury may be temporary, as it can lead to complete inactivation. In addition, this observation was only for injured cells on the lettuce cut edge, not on the leaf surface . These data also indicate that chlorine is less effective on the cut edge than on surfaces or alternately, chlorine inactivation being stronger on leaf surface causes lethal damage to cells thereby leaving fewer injured cells.

DISCUSSION

A protective effect of colanic acid to chlorine was not observed in this study. Rather, cells producing only colanic acid were more sensitive to chlorine treatment. Colanic acid is an extracellular polyanionic acidic hereropolysaccharide which is produced by Enterobacteriaceae, and is composed of L-fucose, D-glucose, D-galactose, D-glucuronate sugars with attached O-acetyl and pyruvic acid side chains (GOEBEL, 1963; LAWSON et al., 1969; SUTHERLAND, 1969). It is a mucoid substance released by cells and also known as the M antigen (GAREGG et al., 1971). In the food industry, the efficacy of chlorine is enhanced by reducing pH to produce more hypochlorous acid. T-128 (a formulation of washing aid associated with the manufacturer Smartwash Solutions, LLC., Salinas, CA) was reported to enhance the reduction of *Pseudomonas* and *Salmonella* population in biofilms on stainless steel when organic material increasing in chlorinated wash solution (LEMONS, 2009; SHEN et al., 2012). The pH value in sodium hypochlorite solution can be controlled at pH 6.5 by adding of T-128 as stabilizer containing generally recognized as safe chemicals mainly composed of propylene glycol and phosphoric acid. Organic material is highly reactive to free chlorine and reduces its capacity of germicidal activity in washing solutions. Extensive vegetable exudates are released from lesion tissues into washing solutions during fresh-cut processing (LUO, 2007). However, bactericidal activity of chlorine is not significantly influenced by sugars and starches (LASMANIS and SPENCER, 1953). Therefore, it is possible that exocellular colonic acid of cells may reduce the pH around the cell resulting in more free hypochlorous acid and increasing bactericidal activity at the cell surface.

Cells of *E. coli* O157:H7 that produce colanic acid were more resistant to oxidative and osmotic stress than their wild-type parent cells in solution (CHEN et al., 2004). Selected cellulose-producing STEC strains were protected from chlorine, NaCl osmotic stress, oxidative treatment of hydrogen peroxide and glacial acetic treatment in aquatic environments (YOO and CHEN, 2010; YOO and CHEN, 2012). Although production of cellulose and colanic acid can provide protection for cells in suspension but such protection may not occur for cells attached to the leaf surface and cut edge. Planktonic bacteria are more susceptible to physical stress and antimicrobial chemicals than attached cells or cells in microcolonies (CHUNG et al., 2006). The protective mechanism of cellulose and colanic acid against environmental stress is still unclear. A variety of cell envelope structures and gene regulators are involved in attachment of bacteria. When cells attach to leaf surfaces, they may trigger a combination of mechanisms to modify the protective effect of cellulose and colanic acid and even cause cells to become more susceptible to chlorine inactivation.

When cellulose is in the presence of strong inorganic acid at the high-temperature region, the acid-catalyzed hydrolysis with cleavage of α-1-4-glycosidic linkage occurs. Although colanic acid is not highly acidic and the environmental temperature was not high, it may still slightly break down the structure of cellulose and cause somehow degradation of the glycosidic linkage. In this moment, cells may be more sensitive to bactericidal ability of chlorine. Moreover, the degradation of both cellulose and colanic acid may cause synergistic effect to enhance chlorine inactivation.

The depth of penetration of *E. coli* O157:H7 on lettuce leaves is related to cell viability after chlorine treatment. Many viable cells were located in cut edge, trichomes,

stomata and cracks of cuticle by observation using confocal scanning microscopy following chlorine treatment (SEO and FRANK, 1999b). More injured cells were observed on lettuce cut edge than leave surface. Chlorine inactivation is more effective on cells exposed on the substratum surface so that significant difference of injured cells could not be found. However, the bactericidal ability of chlorine may be reduced because the internal leaf liquids at the cut edge may provide protection to the cells. Therefore, these cells exhibited sublethal injury rather instead of death.

CONCLUSION

The results of this study showed that extra-cellular cellulose production protects STEC cells attached to leafy greens from the effects of chlorine on spinach leaves surface. While cells also produced colanic acid, cellulose production was not able to provide cells protective effect against chlorine inactivation. Extracellular cellulose and attachment to leaf structures provide cell protection from lethal damage under chlorine treatment.

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TABLE 5.1 The log CFU population of sublethally-injured cells[α] on surface and cut edge of spinach after chlorine treatment.[β]

Strain	50ppm		150ppm	
	Surface	Cut edge	Surface	Cut edge
T	2.15[a]	4.66[b]	N/A	5.29[c]
19B	2.11[a]	5.20[c]	2.52[b]	5.08[b]
19D	2.70[b]	4.02[a]	1.34[a]	4.85[a]
49B	N/A[χ]	4.85[b]	N/A	4.81[a]
49D	N/A	N/A	N/A	4.67[a]

α. The population of sublethally-injured cells = Log [(CFU/ml of tryptic soy agar)–(CFU/ml of sorbitol MacConkey agar)]

β. Means with the same column followed by same superscript letter are not significantly different ($P = 0.05$) based on Duncan's multiple range tests.

χ. No sublethally-injured cells were detected.

TABLE 5.2. The log CFU population of sublethally-injured cells[α] on surface and cut edge of lettuce after chlorine treatment.[β]

Strain	50ppm		150ppm	
	Surface	Cut edge	Surface	Cut edge
T	2.89[d]	5.58[d]	2.61[c]	5.21[b]
19B	1.34[a]	4.30[b]	1.80[b]	4.13[a]
19D	2.28[c]	4.75[c]	N/A[χ]	4.28[a]
49B	1.82[b]	4.02[a]	0.93[a]	3.94[a]
49D	1.98[b]	4.41[b]	0.46[a]	N/A

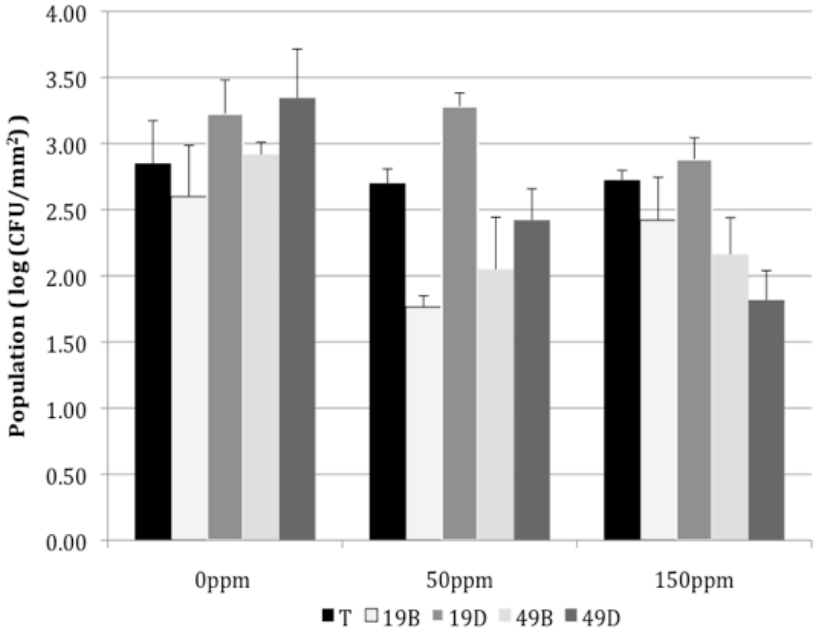
α. The population of sublethally-injured cells = Log [(CFU/ml of tryptic soy agar) – (Log CFU/ml of sorbitol MacConkey agar)]

β. Means with the same column followed by same superscript letter are not significantly different ($P = 0.05$) based on Duncan's multiple range tests.

χ.No sublethally-injured cells were detected.

FIGURE 5.1. Survival of *E. coli* O157:H7 strain 994 (T) and cellulose-producing wild-type STEC strains 19B and 49B as well as their cellulose-deficient derivative 19D and 49D to iceberg lettuce surface (a) and cut edge (b) after 50 and 150ppm chlorine treatment. Error bars indicate standard deviations of four independent experiments.

(a)



(b)

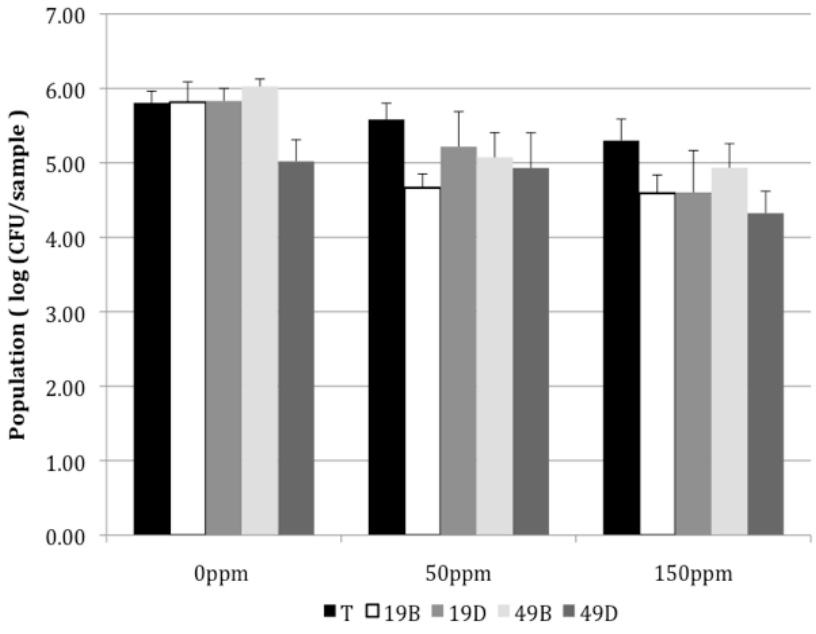
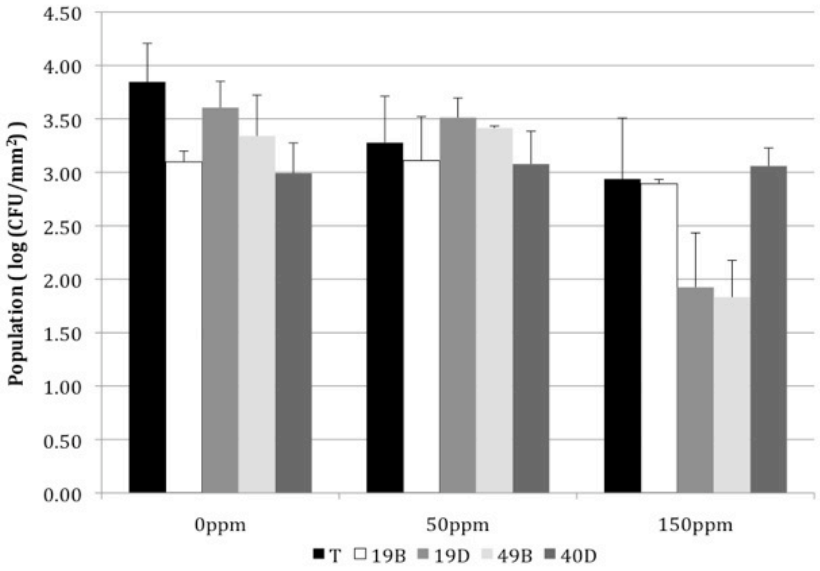
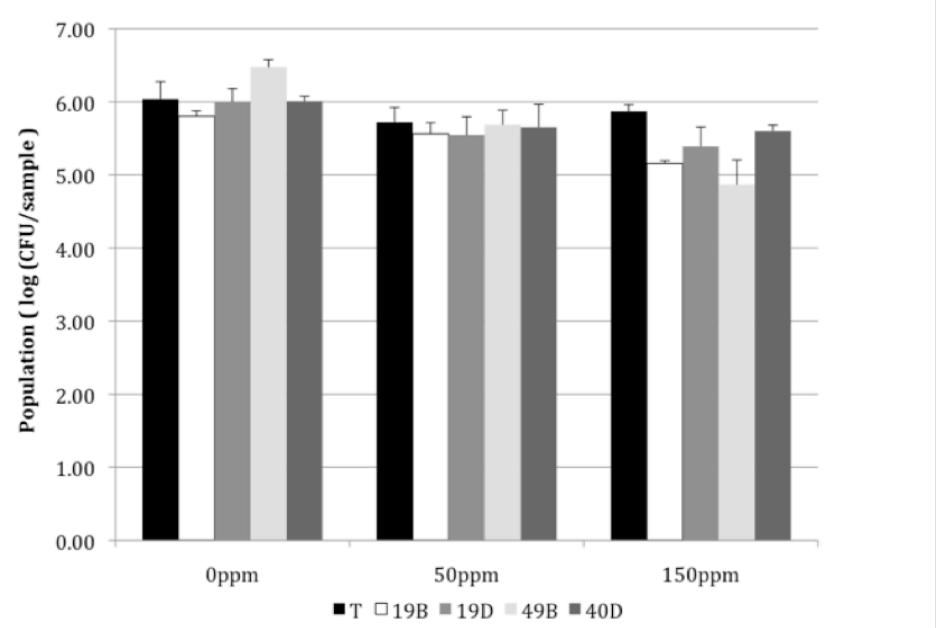


FIGURE 5.2. Survival of *E. coli* O157:H7 strain 994 (T) and cellulose-producing wild-type STEC strains 19B and 49B as well as their cellulose-deficient derivative 19D and 49D to baby spinach surface (a) and cut edge (b) after 50 and 150ppm chlorine treatment. Error bars indicate standard deviations of four independent experiments.

(a)



(b)



CHAPTER 6

CONCLUSION

In this study, we demonstrated that the influence of capsule and cellulose on *E. coli* attachment to leafy greens in ionic environments and different water hardnesses. The protective role of cellulose to chlorine treatment was also determined. When cells encounter different growth media and environments, their cell surface charge, hydrophobicity and composition of cell envelop would be changed.

Attachment of *E. coli* O157:H7 to intact spinach leaf surface was enhanced in presence of capsule and decreased hydrophobicity, but not to lettuce surface. However, cell attachment to lettuce cut edges could be increased with addition of calcium and decreased negative charge, but not to spinach cut edges. Cell attachment to lettuce leaf surfaces decreased when divalent cations were chelated. In addition, the cell population that is attached is more on spinach than on lettuce and also more on cut edges than intact leaf surfaces. Hydrophobicity, electrostatic interactions and ions in the media play a more important role in attachment of than capsule production of cells.

There was significantly greater attachment for cellulose-proficient cells on lettuce surfaces than cellulose-deficient cells, but not on spinach surfaces. STEC strain 49B and 49D cells surface containing colanic acid, less hydrophobicity and more negative in zeta potential have significantly more potential to attach surface and cut edge of spinach in comparison with cellulose-producing, more hydrophobic and less negative on the surface of STEC strain 19B and 19D cells without producing colanic acid. In high water hardness

environment, attachment of cells to leafy green surfaces also could be enhanced. According to 2005 U.S. Geological Survey, high hard waters were measured in streams in Kansas, Texas, New Mexico, Arizona and southern California. Irrigation water from stream in these areas may enhance cells attachment to iceberg lettuce and baby spinach leaves.

Extra-cellular cellulose production protects STEC cells attached to spinach leaves surface from the effects of chlorine. However, the protective role of cellulose was not observed when cells also produced colonic acid in addition to cellulose. Moreover, the sanitizing effectiveness of chlorinated water is significantly stronger at the leaf surface, with higher concentrations of chorine and for spinach leaves at 150ppm chlorine. Extracellular cellulose provided cell protection from lethal damage under chlorine treatment. These findings showed the mechanism of influential factors of cell attachment to leafy greens has been clarified.