TRANSFER OF *ESCHERICHIA COLI* O157:H7 FROM ICE TO ROMAINE LETTUCE AND EVALUATION OF *E. COLI* ATCC 25922 AS ITS SURROGATE

by

JIN KYUNG KIM

(Under the direction of MARK A. HARRISON)

ABSTRACT

Ice can be used to chill romaine lettuce and maintain relative humidity during transportation. *E. coli* O157:H7 may contaminate water used for ice. Potential contamination of lettuce is of concern since it is usually consumed raw or minimally processed. The potential for *E. coli* O157:H7 contamination of romaine lettuce with either ice contaminated with the pathogen or by transfer from lettuce surfaces via melting ice was determined using a simulated commercial operation. In order to evaluate pathogen transfer by these means in actual commercial facilities, the use of non-pathogenic surrogates is needed. Non-pathogenic *E. coli* strains were compared with *E. coli* O157:H7 based on cryotolerance, cell surface characteristics (hydrophobicity, zeta potential, and morphology) and attachment to lettuce. Survival of *E. coli* O157: H7 in water for extended periods can induce starvation. Starvation may enhance *E. coli* O157:H7 survival under subsequent stresses by changing surface morphology which may affect adherence to produce. After starvation (at 37°C for 4 h, 20°C for 24 h, or 4°C for 7 d),

cryotolerance, cell surface properties and attachment of *E. coli* O157:H7 and a surrogate *E. coli* to lettuce were determined. The selected non-pathogenic surrogate *E. coli* was compared with *E. coli* O157:H7 to determine differences and similarities in attachment to and recovery from romaine lettuce in contact with contaminated ice under simulated commercial operation conditions. *E. coli* O157:H7 distributes onto other produce layers in shipping containers due to melted ice made of contaminated water and transfer from contaminated to uncontaminated surfaces. Based on cryotolerance and cell surface characteristics, *E. coli* ATCC 25922 is a useful surrogate for *E. coli* O157:H7 for studies involving attachment and recovery from chilled produce. *E. coli* ATCC 25922 grown at 37°C showed similar or better cryotolerance regardless of starvation conditions. However, starved cells of both strains attached to lettuce less than non-starved cells. *E. coli* ATCC 25922 showed the same attachment and recovery as *E. coli* O157:H7 from romaine lettuce under icing conditions using a simulated commercial operation condition. Overall *E. coli* ATCC 25922 is a useful surrogate for *E. coli* O157:H7 for produce attachment and recovery studies.

INDEX WORDS: *Escherichia coli* O157:H7, Surrogate microorganism, Ice, Cryotolerance, Bacterial attachment, Bacterial capsule, Curli, Hydrophobicity, Zeta potential, Bacterial outer membrane protein

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JIN KYUNG KIM

B.S., The Kyungpook National University, Rep. of Korea, 1994

M.S., The Kyoungpook National University, Rep. of Korea, 1998

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JIN KYUNG KIM

Major Professor: Mark A. Harrison

Committee: Larry R. Beuchat
Joseph F. Frank
William C. Hurst
Stanley J. Kays

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2007

DEDICATION

This dissertation is first dedicated
To God

To my father and father in law,
Mr. Young Ki Kim and Mr. Jong Rok Cheon
Who have constantly supported me
Thank you for your unchangeable belief and endless love
And may you eternally rest in peace in the arms of God

To my mother and mother in law,
Ms. Gil Jo Oh and Ms. Ok Ran Lee
You always encouraged and prayed for me,
Enabling me to persevere through my studies

To my brothers and sister in law,
Pan Sin Kim, Pan Jun Kim, and Sung Hyun Kim

To my beloved son and daughter,
Jin Cheon and Jin Young Chen
Whose smile has always given me motivation and continuous refreshment

Last but not the least I wish to thank to my beloved husband,
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Thank you for your invaluable sacrifice, constant inspiration, patience and endless love

With you all, I could finish this work
I hope I make you proud

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CHAPTER 1

INTRODUCTION

The production of romaine lettuce in 2004 in the U.S. increased approximately 8 times over that of 1990 (USDA/ERS 2005). Consumption of romaine lettuce has been trending higher over the past two decades. Caesar salad in both the foodservice and retail markets is undoubtedly a major factor behind this increase. In addition, increased nutritional awareness among consumers, the success of prepackaged salads, and a general desire for diversity in foods may facilitate the increase in lettuce production (USDA/ARS 1998). Potential contamination of lettuce with foodborne pathogens is of great concern since the product is usually consumed raw or minimally processed.

Ice is used at different steps in the harvest-handling-marketing chain to maintain quality of fresh produce. It can be used in the field to remove field heat or in hydrocooler systems indirectly to help maintain water temperature (Kays 1997). Since *E. coli* O157:H7 has been found in water and survived for extended periods up to 12 weeks (Wang and Doyle 1998b; Warburton et al. 1998), contamination of ice with this pathogen is a possible hazard.

Surrogates are preferred instead of pathogenic microorganisms to evaluate the efficacy of intervention strategies in food processing operations because of the potential risk posed by pathogens to both the production environment and researchers. Many studies comparing non-pathogenic *E. coli* surrogates with *E. coli* O157:H7 under different environments have been conducted (Duffy et al. 2000; Leenanon and Drake 2001; Pao and Davies 2001; Peri et al. 2002; Thayer and Boyd 1993). Surrogates may not fit all situations to the same degree.

The ultimate goals of this dissertation are to investigate ice as a possible route for the introduction of *E. coli* O157:H7 onto romaine lettuce and to select a non-pathogenic surrogate *E. coli* for *E. coli* O157:H7 based on cryotolerance and cell surface characteristics and to compare behavior of *E. coli* O157:H7 with the surrogate under starvation conditions.

There are four objectives:

1. *Investigate ice as a possible route for introduction of* E. coli *O157:H7 to romaine lettuce*

The possible sources of introduction could be either directly from ice contaminated with *E. coli* O157:H7 or by transfer from contaminated to uncontaminated lettuce surfaces via melting ice. The fate of *E. coli* O157:H7 on the surface of romaine lettuce under icing conditions under simulated commercial operation conditions was determined.

2. *Select non-pathogenic surrogate for* E. coli *O157:H7 based on cryotolerance and attachment to romaine lettuce*

Attachment of microorganisms is influenced by the cell surface characteristics. Cell surface structures (e.g., polysaccharides and fimbriae), hydrophobicity, and surface charge may influence adherence of bacteria to a surface. Non-pathogenic *E. coli* strains were compared with *E. coli* O157:H7 for attachment and recovery traits involving chilled produce based on survival at –18°C and cell surface properties.

3. *Determine attachment properties of* E. coli *O157:H7 and a non-pathogenic surrogate* subjected to starvation

The effect of starvation on *E. coli* O157:H7 and a selected surrogate *E .coli* on their subsequent survival at –18ºC was determined. In addition, capsule and curli production, morphology, hydrophobicity, zeta potential, and outer membrane protein profile were compared and their relationship to attachment and recovery of the microorganisms to produce was determined.

4. *Compare attachment of* E. coli *O157:H7 and a selected, non-pathogenic surrogate to romaine lettuce surfaces in contact with contaminated ice using simulated commercial operation conditions and determine the recovery of the microorganisms from the lettuce surfaces*

E. coli O157:H7 or the selected surrogate *E. coli* containing water was frozen and used to ice lettuce in shipping containers. The containers were held at refrigeration temperature to represent the condition of lettuce transportation. Attachment to romaine lettuce and recovery from the lettuce of the surrogate *E. coli* in contact with contaminated ice was compared with those of *E. coli* O157:H7 under simulated commercial operation conditions.

CHAPTER 2

LITERATURE REVIEW

Produce

Over the past decade, there has been an increase in per capita consumption of fresh produce because of an increased awareness in a healthy diet. In addition, the consumption of bagged salads and other minimally processed vegetables has been increased due to convenience, ready-to-eat vegetables with fresh-like quality, availability of small quantities and mixture of different vegetables (Ahvenainen 1996; Ragaert et al. 2004). With an increase in consumption, the number of foodborne illnesses linked to the consumption of fresh produce has increased in recent years (Beuchat 2002; Beuchat et al. 1998; Tauxe et al. 1997). The changes in dietary habits, changes in agronomic practices, and increased international trade and distribution have also been suggested for reasons of increasing outbreak occurrence. Pathogen contamination of fresh produce can occur at many points during production, harvest, transport, and market. Contaminated manure, irrigation water, wash water, equipment, or workers have been reported as vehicles of the pathogen transmission to fresh produce (Beuchat 2002). While the probability of contracting a foodborne illness via consumption of fresh produce is very low, a small probability does exist. Today potential contamination of fresh produce is of great concern since the product is usually consumed raw or seldom treated in a manner that can consistently reduce or eliminate foodborne pathogens from the product (FDA 2000). Several studies involving postharvest handling of *E. coli* O157:H7 contaminated lettuce and the effectiveness of various methods of sanitation in reducing the level of the pathogen on lettuce have been conducted (Abdul-Raouf et al. 1993; Beuchat et al. 1998; Seo and Frank 1999; Takeuchi and Frank 2000).

Romaine lettuce Production Practices

Romaine (*Lactuca sativa* var. *longifolia*) is a cool-season crop with distinct temperature requirements. The optimal growing temperature is 10-20°C and cool nights are essential for good-quality lettuce (Deshpande and Salunkhe 1998). Lettuce should be cooled as soon as possible after harvesting because it is highly perishable. For precooling, vacuum cooling, hydrocooling or ice cooling is used (Boyhan et al. 2004). In addition, lettuce has high respiration rates and needs high relative humidity during transportation to maintain quality. A shelf-life of lettuce is around 21 days at 0°C with high relative humidity. Since most refrigeration units on trailers and containers cannot control relative humidity and actually remove moisture from products as a natural part of the cooling process, top-icing can be used for romaine lettuce (Welby and McGregor 2004). Romaine lettuce is hand-cut, harvested by ground pack crews and packed 12- or 24-bunches to a waxed fiberboard box weighing approximately 40 pounds (Ashby 1995). There are three layers of lettuce heads per box. Lettuce production occurs year-round throughout the United States. California and Arizona are responsible for nearly all lettuce production, with California accounting for 73 percent and Arizona 26 percent of U.S. production in 2004 (USDA/ERS 2005). The production of romaine lettuce in 2004 in the U.S. increased approximately 8 times over that of 1990. Per capita consumption of all lettuce varieties has been increasing since 1960. In 2004, total lettuce consumption reached a record high of 34.5 pounds per capita. Although iceberg lettuce remains the most popular, per capita consumption of iceberg lettuce has been declining in more recent years while consumption of romaine lettuce has been increasing. Consumption of iceberg lettuce peaked in 1989 at 28.7 pounds and by 2004 had decreased to 22.5 pounds, still slightly above consumption in 1960. Meanwhile, in 1985 when reporting by ERS began, per capita

consumption of romaine and leaf lettuce was 0.7 and 2.5 pounds per capita, respectively. By 2004 consumption had increased to 8.1 pounds for romaine lettuce, nearly doubling between 2001 and 2004 (USDA/ERS 2005). This shift is partly due to the increased popularity of Caesar salad in the food service and retail markets. In addition, increased nutritional awareness among consumers, the success of prepackaged salads, and a general desire for diversity in foods may facilitate the increase in lettuce production (USDA/ERS 1998).

Use of Ice for Lettuce

Ice can be used in the field to remove field heat or in hydrocooler systems indirectly to maintain water temperature (Kays 1997). Romaine lettuce can be top-iced by placement of crushed ice on top of the container prior to closure to prevent moisture loss from the surface of lettuce in a refrigeration unit during transportation. Ice can be manufactured on-site with an icemaker and ice storage bin, or block ice can be purchased and crushed. High relative humidity (>95% RH) with 0°C is required to optimize the postharvest life of romaine lettuce (Welby and McGregor 2004). Since *E. coli* O157:H7 have been found in water and can survive for extended periods up to 12 weeks (Wang and Doyle 1998b; Warburton et al. 1998), contamination of ice with these pathogens is a hazard. As ice made of water contaminated with *E. coli* O17:H7 melts, the pathogen might be distributed onto other lettuce layers by the melt water. In addition, ice may contact with contaminated produce, resulting in transfer of pathogen from contaminated to uncontaminated lettuce surfaces. Access to some of the settings where ice is used may not be tightly controlled from a food security standpoint. It is possible for an individual to intentionally contaminate the water used in ice production with foodborne pathogens.

Escherichia coli O157:H7

E. coli O157:H7 is an enteric pathogen which was first identified in two independent outbreaks of hemorrhagic colitis in 1982 (Riley et al. 1983). *E. coli* O157:H7 colonizes the intestine and can cause a diarrheal syndrome characterized by a copious bloody discharge. In addition to bloody diarrhea, intestinal infection can lead to potentially fatal sequelae, hemolytic uremic syndromes, in children, elderly patients and other susceptible groups of individuals (Nataro and Kaper 1998). The infectious dose of this organism is less than 100 cells (Meng et al. 2001) and estimates of approximately 73,000 *E. coli* O157:H7 infections and 61 associated deaths occur annually in the United States (CDC 2001). Cattle are known to be a reservoir of *E. coli* O157:H7 (Zhao et al. 1995) and outbreaks of *E. coli* O157:H7 infections have been primarily associated with consumption of contaminated undercooked beef (Doyle 2001). Fresh vegetables, including lettuce, have also been implicated as unexpected vehicles of transmission (Ackers et al. 1998; Beuchat 1999; Breuer et al. 2001; Hillborn et al. 1999; Itoh et al. 1998). The presence of *E. coli* O157:H7 in a variety of food products suggests that this pathogen has the ability to express one or more adhesive structures which allow the pathogen to bind to surfaces of many different types of food (Torres et al. 2005).

Stress Response of *E. coli* O157:H7

Sublethal exposure to various stresses may enhance the survival of bacteria under the subsequent stress conditions and this enhancement may offer cross-protection against other stresses (Abee and Wouters 1999). Many researchers have demonstrated the stress response of *E. coli* O157:H7 to sublethal environmental stresses. Acid stress enhanced subsequent acid tolerance, heat tolerance, and freeze-thaw tolerance (Leenanon and Drake 2001; Williams and

Ingham 1998). Nutrient starvation enhanced heat tolerance (Jenkins et al. 1988; Rowe and Kirk 2000). In addition, Wang and Doyle (1998a) reported heat shock enhanced heat tolerance and acid tolerance. Other studies have reported that cold stress enhanced freeze-thaw resistance in *E. coli* (Goldstein et al. 1990; Leenanon and Drake 2001). Under stress conditions, *E. coli* O157:H7 may produce several stress proteins (Gawanda and Griffiths 2005a; Zhang and Griffiths 2003) and alter membrane lipid composition to survive in these environments (Arneborg et al. 1993; Carty et al. 1999). Sainz et al. (2005) showed survival of pathogenic *E. coli* strains to different acid challenges might be related to the changes in outer membrane protein profiles. Other studies demonstrated that a stress response may affect subsequent virulence (Buncic and Avery 1998; Elhanafi et al. 2004; Leenanon et al. 2003; Weinstein et al. 1988; Yuk and Marshall 2003).

Starvation studies for *E. coli* O157:H7

Water is widely used material in fresh produce production and processing. Starvation can occur under the conditions which microorganisms exist in water for extended periods (Harakudo et al. 2000; Rockabrand et al.1995). Several studies showed *E. coli* O157:H7 can survive in water for extended periods under starvation conditions (LeJeune et al. 2001; Maule 2000; Rice and Johnson 2000; Wang and Doyle 1998b; Warburton et al. 1998). Previous work has shown that the stress imposed by starvation changed the ability of *E. coli* O157:H7 to survive heat treatments and that this thermal tolerance correlated with induction of the heat shock proteins (Rowe and Kirk 2000). Kim and Kim (2004) showed curli expression affected adherence to host epithelial cells using curli producing *E. coli* O157:H7 at 37ºC. Özanca and Flint (2002) demonstrated prolonged starvation affects the relative amounts of outer membrane

porins and may affect survival of this microorganism in lake water environments.

Few studies have addressed the impact of starvation (held in water for extended periods) of *E. coli* O157:H7 on cell surface characteristics including surface structure, hydrophobicity, surface charge, and outer membrane proteins and the relationship between the change of cell surface characteristics and attachment to produce.

Surrogates

A surrogate is defined as a non-pathogenic species and strain responding to a particular treatment in a manner equivalent to a pathogenic species and strain (FDA 2000). Since the production of fresh produce does not use a processing kill step to eliminate pathogens but may have a decontamination treatment, surrogates may be inoculated onto produce or equipment surfaces as test organisms to determine the efficacy of intervention strategies in food processing operations without the potential risk posed by pathogens to both the production environment and researchers. However, using surrogates has inherent limitations if they do not behave in a same manner to that of the pathogen of interest. Potential surrogates must exhibit characteristics like the target pathogens. An ideal surrogate is a strain of the target pathogen that retains all other characteristics except its virulence. However, due to possible reversion to pathogenicity, such an approach to select surrogate is not usually followed. Generally, surrogates are selected from the population of well-known organisms that have well-defined characteristics and a long history of being non-pathogenic. When selecting surrogates, the following characteristics are desirable: (a) non-pathogenic, (b) inactivation characteristics and kinetics that can be used to predict those of the target organism, (c) behavior similar to target microorganism when exposed to conditions similar to the processing parameters (pH, temperature, oxygen), (d) easy preparation, (e) genetically stable, (f) susceptibility to injury similar to the target pathogen (Busta

et al. 2003). In addition to these characteristics of surrogates, they need to differentiate easily from other microflora. Antibiotic resistance can be used as selective marker (Peri 2003).

Previous studies of surrogates for *E. coli* O157:H7

The behavior of non-pathogenic *E. coli* strains has been compared to *E. coli* O157:H7 under specific process conditions. *E. coli* K-12 MG 1655 was a suitable surrogate on efficacy of electron beam irradiation treatment for fruits (Rodriguez et al. 2005). Peri et al. (2002) suggested *E. coli* K-12 LMM 1010 is a suitable surrogate for *E. coli* O157:H7 after evaluating their survival at acid/alkaline pH levels and reduced water activities. Salter et al. (1998) used *E. coli* M23 as a surrogate of a pathogenic strain of *E. coli* including O157:H7 to develop the temperature dependence of growth rate. Eblen et al. (2005) compared growth and survival characteristics (level of attachment and survival on apple surfaces, resistance to hydrogen peroxide decontamination treatments, and thermal resistance at 60°C) of non-pathogenic *E. coli* strains with *E. coli* O157:H7 and *Salmonella* and reported non-pathogenic *E. coli* strains showed few differences in generation time, lag phase duration, maximum population attained, and pH at stationary phase with those of *E. coli* O157:H7 and *Salmonella*, while considerably more separation among strains was seen in survival characteristics. They suggested *E. coli* ATCC 11775, ATCC 25253, and ATCC 25922 may be useful for evaluating the efficacy of intervention steps in reducing populations of selected strains of *E. coli* O157:H7 and *Salmonella* in processing environments where these pathogens cannot be introduced. Pao and Davies (2001) demonstrated *E .coli* ATCC 11229 and *E. coli* ATCC 25922 showed the resistance to alkaline pH and high temperature and may be utilized as surrogates to conduct research in fresh fruits. *E. coli* ATCC 25922 was found to be a suitable surrogate for evaluating the efficacy of using

hydrogen peroxide in decontaminating apples and cantaloupe melons (Sapers and Sites 2003). Leenanon and Drake (2001) compared *E. coli* O157:H7 (ATCC 43895) with this strain on poststress heat resistance and freeze-thaw resistance after exposing to acid adaptation, acid shock, starvation, and cold stress. They observed that heat and freeze-thaw resistance of *E. coli* O157:H7 and *E. coli* ATCC 25922 was enhanced after acid adaptation and starvation. They, also, reported, heat resistance of both cultures was decreased after cold stress, while freeze-thaw resistance was increased. Thayer and Boyd (1993) successfully determined this strain has the same sensitivity to gamma radiation in meats. In addition, Duffy et al. (2000) used *E. coli* ATCC 25922 as a surrogate for *E. coli* O157:H7 to validate the efficacy of UV inactivation in apple cider processing. However, Mak et al. (2001) demonstrated strain ATCC 25922 was the least thermo-tolerant among all the *E. coli* strains tested before and after acid stress and should not be used as a surrogate for *E. coli* O157:H7 in terms of thermotolerance. The results of these studies suggested that a particular non-pathogenic strain behaves differently under specific process conditions. Thus, surrogates may not fit all challenged situations to the same degree.

Assay used for selection of surrogate

The ability of microorganisms to attach to fresh produce is of great concern since the products are to be sold as raw or minimally processed. Attachment of microorganisms is influenced by the cell surface characteristics (Frank 2001). Cell surface structures (e.g., polysaccharides, fimbriae, outer membrane proteins), hydrophobicity, and surface charge may explain adherence of bacteria to a surface. A limited numbers of reports on the relationship between *E. coli* O157:H7 and food surfaces have been published. Cell hydrophobicity (Dickson and Koohmaraie 1989), cell surface charge (Ukuku and Fett 2002), fimbriae

(Fratamico et al. 1996), and exocellular polysaccharides (Han et al. 2000) have been investigated. There were conflicting results as some reports suggest strong interaction with attachment and others do not. This may be due to the heterogeneous nature of various food surfaces and differences in cell surface composition between the studies (Solomon and Matthews 2006).

Capsules

Capsules are polysaccharides surrounding the bacterial cell wall. In *E. coli*, its composition may be a K antigen or an O antigen (Whitfield and Roberts 1999). Besides these polysaccharides, many strains of *E. coli* can produce an exopolysaccharide called colanic acid, major composition of slime polysaccharides. Generally capsule formation of *E. coli* strains can protect cells from osmotic and oxidative stress, low temperature, and desiccation (Chen et al. 2004; Sledjeski and Gottesman 1996; Whitfield and Roberts 1999). Capsules can be either adhesive or antiadhesive, depending on the attachment surface and medium. By adhering the cells to solid surfaces, capsules prevent cells from washing away and provide a protective environment for them. On the other hand, capsules may antiadhesive because hydrophilic capsule can mask hydrophobic components of the cell envelope, preventing adhesion of the cell to hydrophobic surfaces (Ofek and Doyle 1994). Valle et al. (2006) demonstrated that the treatment of abiotic surfaces with capsular polysaccharides of *E. coli* drastically reduced both initial adhesion and biofilm development by broad-spectrum pathogens due to physiochemical surface alterations. Hydrophobic cells are generally more adherent than hydrophilic ones and most bacteria preferentially adhere to hydrophobic surfaces.

Curli expression

E. coli has ability to express thin aggregative fimbriae, known as curli, on the cell surface. Curli are typically expressed under ambient temperature, low osmotic pressure, deficient nutrients, and stationary phase (Olsén et al. 1989, 1993). Curli consists of a highly insoluble polymer which is resistant to heat (Olsén et al. 1993). Curli are involved in attachment and biofilm formation by non-pathogenic *E. coli* (Prigent-Combaret et al. 2000). Vidal et al. (1998) reported curli produced by non-pathogenic *E. coli* enhanced the attachment of cells on the surface of polystyrene. Gophna et al. (2001) reported curli facilitated internalization of *E. coli* by epithelial and HEp-2 cell lines due to fibronectin binding. Curli expression in serogroup O157:H7 of enterohemorrhagic *E. coli* (EHEC) has been reported (Cookson et al. 2002; Uhlich et al. 2001, 2002; Zogaj et al. 2003). Curli expression by *E. coli* O157:H7 was very rare but when it occurs the organism is more virulent and plays a role in autoaggregation and development of biofilm on inert surfaces (Ryu et al. 2004; Uhlich et al. 2001, 2002). Expression of curli is regulated through both environmental and genetic interactions. Altering environmental conditions such as NaCl content or temperature will cause some curli-producing bacteria to revert to the non-curli-producing white phenotype (Olsén et al. 1993). However, Uhlich et al. (2001) suggested that curli production can occur in association with *csg* promoter point mutations with metabolic flexibility, which means curli expressing is independent of temperature or other environmental factors. Little is known about curli expression by *E. coli* O157:H7 in attachment to produce surface. Jeter and Matthysse (2005) reported the production of curli appears to be sufficient to allow K-12 strains to bind to plant surfaces but curli are not necessary for the binding of pathogenic strains, suggesting that pathogenic *E. coli* strains may have more than one mechanism for binding to plant surfaces.

Hydrophobicity

Bacterial adhesion may be influenced by hydrophobicity, which can vary between species, serotypes or strains. It can change with variation in growth conditions, physiological states of cells, and composition of suspension media (Doyle 1991; Hassan and Frank 2004). Relative hydrophobicity of bacterial cells has been determined by several methods, such as bacterial adhesion to hydrocarbon (BATH; Rosenberg and Kjelleberg 1986; Pelletier et al. 1997), hydrophobic interaction chromatography (HIC; Jonsson and Wadstrom 1983; Ukuku and Fett 2006), aggregation in the presence of different salt solutions (SAT; Lindhal et al. 1981), adhesion to nitrocellulose filters (NCF; Lachica and Zink 1984; Balebona et al. 2001) and contact angle measurement (CAM; Van Loosdrecht et al. 1987b). Each method has specific advantages and shortcomings; large variations have been observed among different measurement methods (Rosenberg and Kjelleberg 1986). BATH assay has been mostly used in literatures for relative hydrophobicity. Xylene or hexadecane among the hydrocarbons tested for hydrophobicity appeared the most marked response. Benito et al. (1997) reported bacterial cell surface hydrophobicity showed an apparent linear relationship with attachment to beef muscle. Dickson and Koohmaraie (1989) found hydrophobicity of *E. coli* was linearly correlated with bacterial adhesion to fat tissue but not to muscle tissue. Marin et al. (1997) also found a strong relationship between strength of attachment to beef muscle and hydrophobicity of lactic acid bacteria. On the other hand, Li and McLandsborough (1999) reported no correlation was observed between surface hydrophobicity of *E. coli* strains and adhesion to beef muscle. Similar results were reported by Bouttier et al.(1997). This apparent conflict may be due to the variations of systems used and bacterial strains tested. For example, different mixing conditions (e.g., temperature, time, amount of hydrocarbon added) may produce different results

(Van Loosdrecht et al. 1987a). In addition, the amount of surface area created during mixing of the two liquid phases and the size and number of hydrocarbon droplets obtained in the aqueous phase can be different from strain to strain (Van Loosdrecht et al. 1987a).

Surface charge

Surface charges can occur due to the formation of an electrostatic double layer on each surface, depending on the ionic strength and pH of the surrounding medium (Frank 2001). Elevated concentrations of ions reduce the electrostatic double layer and the repulsive forces, leading to less effect of variations of the cell surface charges in high ionic strength such as marine environments. In low-ionic strength environments, the microbial charges are of greater importance (Stenström 1989). Bacterial adhesion may be influenced by the cellular surface charge and its interaction with the charge on solid surfaces (Li and McLandsborough 1999; Van Loosdrecht et al. 1987b). Sharma et al. (1985) reported that bacterial surface charge is influenced by carboxyl, amino, sulfate and phosphate groups within the cell envelop. Bacterial cells have a net negative charge on the cell wall, although the magnitude of this charge varies from strain to strain (Li and McLandsborough 1999). They revealed no correlation between surface charge of *E. coli* strains and strength of adhesion to beef muscles. Kim et al. (1996) also showed no correlation between surface charge and the adhesion of *Salmonella* to meat. On the other hand, Dickson and Koohmaraie (1989) found linear relationships between the negative charges of bacterial strains including *E. coli* O157:H7 and adhesion to adipose or muscle tissues.

Outer membrane proteins

Outer membrane proteins (Omps) of Gram-negative bacteria are important molecules that interface the cell with the environment. *Escherichia coli* possess a number of outer

membrane proteins (Omps) some of which play an important role in the specific or nonspecific membrane transport and permeability of nutrients. The major Omps include the porins (e.g., OmpC and OmpF) and the OmpA protein in the approximately 35,000-dalton range (Nikaido and Vaara 1985). The porins play an important role in membrane transport, through forming a passive hydrophilic channel, allowing nonspecific diffusion of small molecules (less than 600 Da) across the outer membrane barrier (Molloy et al. 2000). OmpA is associated with the peptidoglycan layer and has a function of stabilizing the outer membrane and retaining the rod shape of the bacterial cell (Havekes and Hoekstra 1976; Sonntag et al. 1978). Expression of outer membrane proteins is affected by medium pH, osmolarity, starvation, and different nutrient sources (Nikaido and Vaara 1985; Özkanca and Flint 2002; Sato et al. 2000; Zhang and Ferenci 1999). For example, the level of OmpC increases with increased osmolarity when cells are growing in neutral and alkaline media, whereas the level of OmpF decreases at high osmolarity (Sato et al. 2000). Özkanca and Flint (2002) reported an increase in the expression of OmpF may be essential for the survival of enteric bacteria in an environment with low osmolarity by increasing the permeability of the outer membrane. This survival strategy could allow bacteria to survive longer periods of starvation (Nikaido and Vaara 1985). Zhang and Ferenci (1999) reported OmpF changed when *E. coli* was exposed to prolonged lactose limitation and outer membrane permeability was affected. They revealed non-specific pathways are the primary target for selection to improve outer membrane permeability whereas, Notley-McRobb and Ferenci (1999) reported *E. coli* under glucose limitation increased outer membrane permeability for glucose through overexpression of the LamB glycoporin. Sainz et al. (2005) found Omp profiles in non-adapted acid challenged cells were changed when compared with non-challenged cells and these changes resulted in significantly enhancing their survival in acid conditions.

Visualization of bacterial cells by electron microscopy

The cell surface including outer membrane of gram-negative bacteria is considered to play an important part in effective physical barrier to various environments (Raetz and Whitfield 2002). Direct microscopic observation is potentially a useful and simple approach for obtaining information that would help in understanding the function of cell surface structures. Scanning electron microscopy and transmission electron microscopy have been used to study the ultrastructural features of cultured-cell surfaces under different treatments including starvation conditions (Mendoca et al. 1994; Solomon et al. 2002; Yoon et al. 2005). Due to artifacts which may be produced during sample preparation for electron microscopy and sensitivity of bacterial cells to chemical fixation agent, changes in cell surface structures under specific treatments may not accurately represent the true native structures. However, electron microscopy images may demonstrate relative visual evidence with other subsequent analysis. In addition, advances in the electron microscopy including ability to observe fully hydrated samples at room or body temperatures could help eliminate many artifacts of sample preparation and allow routine and reproducible imaging.

References

Abee, T. and J.A. Wouters. 1999. Microbial stress response in minimal processing. *Int. J. Food Microbiol.* 50:65-91.

Abdul-Raouf, U.M., L.R. Beuchat, and M.S. Ammar. 1993. Survival and growth of *Escherichia coli* O157:H7 on salad vegetables. *Appl. Environ. Microbiol.* 57:1999-2006.

Ackers, M.L., B.E. Mahon, E. Leahy, B. Goode, T. Damrow, P.S. Hayes, W.F. Bibb, D.H. Rice, T.J. Barrett, L. Hutwagner, P.M. Griffin, and L. Slutsker. 1998. An outbreak of *Escherichia coli* O157:H7 infections associated with leaf lettuce consumption. *J. Infect. Dis*. 177:1588-1593.

Ahvenainen, R. 1996. New approaches in improving the shelf life of minimally processed fruit and vegetables. *Trends Food Sci. Technol*. 7:179-187.

Arneborg, N., A.S. Salskov-Iversen, and T.E. Mathiasen. 1993. The effect of growth rate and other growth conditions on the lipid composition of *Escherichia coli*. *Appl. Microbiol. Biotech*. 39:353-357.

Ashby, B.H. 1995. Protecting perishable foods during transport by truck. USDA/AMS Available at http://www.ams.usda.gov/tmd/TSB/Protecting_Perishable_Foods_2006.pdf. Accessed 4 January 2007.

Balebona, M.C., M.A. Moriñigo, and J.J. Borrego. 2001. Hydrophobicity and adhesion to fish cells and mucus of *Vibrio* strains isolated from infected fish. *Int. Microbiol*. 4:21-26.

Benito, Y., C. Pin, M.L. Marin, M.L. Garcia, M.D. Selgas, and C. Casas. 1997. Cell surface hydrophobicity and attachment of pathogenic and spoilage bacteria to meat surfaces. *Meat Sci.* 45:419-425.

Beuchat, L.R. 1999. Survival of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces applied to lettuce and the effectiveness of chlorinated water as a disinfectant. *J. Food Prot.* 62:845-849.

Beuchat, L.R. 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microbes Infect.* 4:413-423.

Beuchat, L.R., B.V. Nail, B.B. Adler, and M.R.S. Clavero. 1998. Efficacy of spray application of chlorinated water in killing pathogenic bacteria on raw apples, tomatoes, and lettuce. *J. Food Prot.* 61:1305-1311.

Bouttier, S., C. Linxe, C. Ntsama, G. Morgant, M.N. Bellon-Fontaine, and J. Founiat. 1997. Attachment of *Salmonella choleraesuis* to beef muscle and adipose tissues. *J. Food Prot*. 60:16-22.

Boyhan, G.E., W.C. Hurst, W.T. Kelly, G..W. Krewer and K.C. Taylor. 2004. Postharvest handling and transportation of fruits and vegetables. Available at http://pubs.caes.uga.edu/caespubs/pubs/PDF/FS-100.pdf. Accessed 4 January 2007.

Breuer, T., D.H. Benkel, R.L. Shapiro, W.N. Hall, M.M. Winnett, M.J. Linn, J. Neimann, T.J. Barrett, S. Dietrich, F.P. Downes, D.M. Toney, J.L. Pearson, H. Rolka, L. Slutsker, P.M. Griffin, and I. Team. 2001. A multistate outbreak of *Escherichia coli* O157:H7 infections linked to alfalfa sprouts grown from contaminated seeds. *Emerg. Infect. Dis*. 7:977-982.

Buncic, S. and S.M. Avery. 1998. Effects of cold storage and heat-acid shocks on growth and verotoxin 2 production of *Escherichia coli* O157:H7. *Food Microbiol.* 15:319-328.

Busta, F.F., T.V. Suslow, M.E. Parish, L.R. Beuchat, J.N. Farber, E.H. Garrett, and L.J. Harris. 2003. Comprehensive reviews in food science and food safety - The use of indicators and surrogate microorganisms for the evaluation of pathogens in fresh and fresh-cut produce. Vol. 2:179-185.

Carty, S.M., K.R. Sreekumar, and C.R. Raetz. 1999. Effect of cold shock on lipid A biosynthesis in *Escherichia coli*: Induction at 12°C of an acyltransferase specific for palmitoleoyl-acyl carrier protein. *J. Biol. Chem*. 274:9677-9685.

Centers for Disease Control and Prevention, National Center for Infectious Diseases, Division of Bacterial and Mycotic Diseases. 2001. *Escherichia coli* O157:H7: general information. Available at: http://www.cdc.gov/ncidod/dbmd/diseaseinfo/escherichiacolipg.htm. Accessed 7 February 2007.

Chen, J., S.M. Lee, and Y. Mao. 2004. Protective effect of exopolysaccharide colanic acid of *Escherichia coli* O157:H7 to osmotic and oxidative stress. *Int. J. Food Microbiol*. 93:281-286.

Cookson, A.L., W.A. Cooley, and M.J. Woodward. 2002. The role of type I and curli fimbriae of shiga toxin-producing *Escherichia coli* in adherence to abiotic surfaces. *Int. J. Med. Microbiol*. 292:195-205.

Deshpande, S,S, and D.K. Salunkhe. 1998. Lettuce. p. 493-509. *In* Salunkhe, D.K. and S.S. Kadam (Eds.), Handbook of vegetable science and technology: production, composition, storage and processing. Marcel Dekker, Inc., New York. Available at http://www.netlibrary.com/Reader/ Accessed 5 February 2007.

Dickson, J.S. and M. Koohmaraie. 1989. Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces. *Appl. Environ. Microbiol*. 55:832-836.

Doyle, M.P. 2001. *Escherichia coli* O157:H7 and its significance in foods. *Int. J. Food Microbiol.* 12:289-301.

Doyle, R.J. 1991. Strategies in experimental microbial adhesion research. p. 291-316. *In*: Mozes, N., P.S. Handley, H.J. Busscher, and P.G. Rouxhet (Eds.), Microbial cell surface analysis – Structural and physicochemical methods. VCH Publishers, New York.

Duffy, S., J. Churey, R.W. Worobo, and D.W. Schaffner. 2000. Analysis and modeling of the variability associated with UV inactivation of *Escherichia coli* in apple cider. *J. Food Prot*. 63:1587-1590.

Eblen, D.R., B.A. Annous, and G.M. Sapers. 2005. Studies to select appropriate nonpathogenic surrogate *Escherichia coli* strains for potential use in place of *Escherichia coli* O157:H7 and *Salmonella* in pilot plant studies. *J. Food Prot*. 68:282-291.

Elhanafi, D., B. Leenanon, W. Bang, M.A. Drake. 2004. Impact of cold and cold-acid stress on poststress tolerance and virulence factor expression of *Escherichia coli* O157:H7. *J. Food Prot.* 67:19-26.

Food and Drug Administration. 2000. Kinetics of microbial inactivation for alternative food processing technologies. Available at: http://vm.cfsan.fda.gov/~comm/ift-glos.html. Accessed 12 December 2006.

Frank, J.F., 2001. Microbial attachment to food and food contact surfaces. *Adv. Food Nutr. Res*. 43:320-370.

Fratamico, P.M., F.J. Schultz, R.C. Benedict, R.L. Buchanan, and P.H. Cooke. 1996. Factors influencing attachment of *Escherichia coli O157:H7* to beef tissues and removal using selected sanitizing rinses. *J. Food Prot*. 59:453-459.

Gawande, P.V. and M.W. Griffiths. 2005. Effects of environmental stresses on the activities of the *uspA, grpE* and *rpoS* promoters of *Escherichia coli* O157:H7. *Int. J. Food Microbiol.* 99:91-98.

Goldstein, J.N., S. Pollitt, and M. Inouye. 1990. Major cold shock protein of *Escherichia coli. Proc. Natl. Acad. Sci. USA* 87:283-287.

Gophna, U., M. Barlev, R. Seijffers, T.A. Oelschlager, J. Hacker, and E.Z. Ron. 2001. Curli fibers mediate internalization of *Escherichia coli* by eukaryotic cells. *Infect. Immun*. 69:2659-2665.

Han, Y., D.M. Sherman, R.H. Linton, S.S. Nielsen, and P.E. Nelson. 2000. The effects of washing and chlorine dioxide gas on survival and attachment of *Escherichia coli* O157:H7 to green pepper surfaces. *Food Microbiol*. 17:521-533.

Harakudo, Y., M. Miyaha, and A. Kumadai. 2000. Loss of O157 O antigenicity of verotoxin-producing *Escherichia coli* O157:H7 surviving under starvation conditions. *Appl. Environ. Microbiol.* 66:5540-5543.

Hassan, A.N. and J.F. Frank. 2004. Attachment of *Escherichia coli* O157:H7 grown in tryptic soy broth and nutrient broth to apple and lettuce surfaces as related to cell hydrophobicity, surface charge, and capsule production. *Int. J. Food Microbiol*. 96:103-109.

Havekes, L.M. and W.P.M. Hoekstra. 1976. Characterization of an *Escherichia coli* K-12. *J. Bacteriol*. 126:593-600.

Hillborn, E.D., J.H. Mermin, P.A. Mshar, J.L. Hadler, A. Voetsch, C. Wojtkunski, M. Swartz, R. Mshar, M.A. Lambert-Fair, J.A. Farrar, M.K. Glynn, and L. Slutsker. 1999. A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. *Arch. Intern. Med*. 159:1758-1764.

Itoh, Y., Y. Sugita-Konishi, F. Kasuga, M. Iwaki, Y. Hara-Kudo, N. Saito, Y. Noguchi, H. Konuma, and S. Kumagai. 1998. Enterohemorrhagic *Escherichia coli* O157:H7 present in radish sprouts. *Appl. Environ. Microbiol*. 64:1532-1535.

Jenkins, D.E., J.E. Schultz, and A. Matin. 1988. Starvation-induced cross protection against heat or H_2O_2 challenge in *Escherichia coli. J. Bacteriol.* 170:3910-3914.

Jeter, C. and A.G. Matthysse. 2005. Characterization of the binding of diarrheagenic strains of *E. coli* to plant surfaces and the role of curli in the interaction of the bacteria with alfalfa sprouts. *Mol. Plant Microbe Interact*. 18:1235-1242.

Jonsson, P. and T. Wadstrom. 1983. High surface hydrophobicity of *Staphylococcus* as revealed by hydrophobic interaction chromatography. *Curr. Microbiol*. 8:347-353.

Kays, J.S. 1997. Heat, heat transfer, and cooling. p. 457-507. *In* Postharvest Physiology of Perishable Plant Products. Exon Press. GA

Kim, K.Y., H.S. Lillard, J.F. Frank, and S.E. Craven. 1996. Attachment of *Salmonella typhimurium* to poultry skin as related to cell viability. *J. Food Sci*. 61:439-448.

Kim, S.H. and Y.H. Kim. 2004. *Escherichia coli* O157:H7 adherence to HEp-2 cells is implicated with curli expression and outer membrane integrity. *J. Vet. Sci*. 5:119-124.

Lachica, R.V. and D.L. Zink. 1984. Plasmid-associated cell surface charge and hydrophobicity of *Yersinia enterocolitica*. *Infect. Immun*. 44:540-543.

Leenanon, B., D. Elhana., and M.A. Drake. 2003. Acid adaptation and starvation effects on Shiga toxin production of *Escherichia coli* O157:H7. *J. Food Prot.* 66:970-977.

Leenanon, B. and M.A. Drake. 2001. Acid stress, starvation, and cold stress affect post stress behavior of *Escherichia coli* O157:H7 and nonpathogenic *E. coli. J. Food Prot.* 64:970-974.

LeJeune, J.T., T.E. Besser, and D. Hancock. 2001. Cattle water troughs as reservoirs of *Escherichia coli* O157. *App. Environ. Microbiol*. 67:3053-3057.

Li, J. and L.A. McLandsborough. 1999. The effects of the surface charge and hydrophobicity of *Escherichia coli* on its adhesion to beef muscle. *Int. J. Food Microbiol.* 53:185-193.

Lindhal, M., A. Faris, T. Wadstrom, and S. Hjerten. 1981. A new test based on salting out to measure relative surface hydrophobicity of bacterial cells. *Biochim. Biophys. Acta*. 677:471-476.

Mak, P.P., B.H. Ingham, and S.C. Ingham. 2001. Validation of apple cider pasteurization treatments against *Escherichia coli* O157:H7, *Salmonella*, and *L. monocytogenes*. *J. Food Prot*. 64:1679-1689.

Marin, M.L., Y. Benito, C. Pin, M.F. Fernandez, M.L. Garcia, M.D. Selgas, and C. Casas. 1997. Lactic acid bacteria: hydrophobicity and strength of attachment to meat surfaces. *Lett. Appl. Microbiol.* 24:14-18.

Maule, A. 2000. Survival of verocytotoxigenic *Escherichia coli* O157 in soil, water and on surfaces. *Symp. Ser. Soc. Appl. Microbiol*. 29:71S-78S.

Mendonca, A.F., T.L. Amoroso, and S.J. Knabel. 1994. Destruction of Gram-negative food-borne pathogens by high pH involves disruption of the cytoplasmic membrane. *Appl. Environ. Microbiol*. 60:4009-4014.

Meng, J., M.P. Doyle, T. Zhao, and S. Zhao. 2001. Enterohemorrhagic *Escherichia coli.* p.193-213. *In* M. P. Doyle (ed.), Food microbiology: fundamentals and frontiers, 2nd ed. ASM Press, Washington, D.C.

Molloy, M.P., B.R. Herbert, M.B. Slade, T. Rabilloud, A. Nouwens, K.L. Williams, and A.A. Gooley. 2000. Proteomic analysis of the *Escherichia coli* outer membrane. *Eur. J. Biochem*. 267:2871- 2881.

Nataro, J.P. and J.B. Kaper. 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev*. 11:142-201.

Nikaido, H. and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev*. 49:1-32.

Notley-McRobb, L. and T. Ferenci. 1999. The generation of multiple coexisting mal-regulatory mutations through polygenic evolution in glucose-limited populations of *E. coli*. *Environ. Microbiol.* 1:45-52.

Ofek, I. and R.J. Doyle. 1994. Bacterial Adhesion to Cells and Tissues. Chapman and Hall, New York.

Olsén, A., A. Arnqvist, M. Hammar, S. Sukupolvi, and S. Normark. 1993. The RpoS sigma factor relieves H-NS-mediated transcriptional repression of *csgA*, the subunit gene of fibronectin-binding curli in *Escherichia coli*. *Mol. Microbiol*. 7:523-536.

Olsén, A., A. Jonsson, and S. Normark. 1989. Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. *Nature* 338:652-655.

Özkanca, R. and K.P. Flint. 2002. The effect of starvation stress on the porin protein expression of *Escherichia coli* in lake water. *Lett. Appl. Microbiol*. 35:533-537.

Pao, S. and G. Davies. 2001. Comparing attachment, heat tolerance and alkali resistance of pathogenic and non-pathogenic bacterial cultures on orange surfaces. *J. Rapid Methods and Automation in Microbiol.* 9:271-278.

Pelletier, C., C. Bouley, C. Cayuela, S. Bouttier, P. Bourlioux, and M.N. Bellon-Fontaine. 1997. Cell surface characteristics of *Lactobacillus casei* subsp. *casei, Lactobacillus paracasei*, subsp. *paracasei*, and *Lactobacillus rhamnous* strains. *Appl. Environ. Microbiol*. 63:1725-1731.

Peri, A. 2003. Screening surrogates for food-borne pathogens: pH, water activities and high temperatures. M.S. Thesis. Illinois Institute of Technology. Chicago, IL.

Peri, A., S. Keller, S. Ravishankar, C. Rodriguez, and P.J. Slade. 2002. Bacterial pathogen surrogate evaluation - tolerance of candidates to acid and alkaline pH and reduced water activity. Session 61C-10. IFT Annual Meeting. Anaheim, CA.

Prigent-Combaret, C., G. Prensier, T.T. Le Thi, O. Vidal, P. Lejeune, and C. Dorel. 2000. Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains, role of flagella, curli and colanic acid. *Environ. Microbiol*. 2:450-464.

Ragaert, P., W. Verbeke, F. Devlieghere, and J. Debevere. 2004. Consumer perception and choice of minimally processed vegetables and packaged fruits. *Food Qual. Prefer*. 15:259-270.

Raetz, C.R. and C. Whitfield. 2002. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* 71: 635-700.

Rice, E.W. and C.H. Johnson. 2000. Short communication: survival of *Escherichia coli* O157:H7 in dairy cattle drinking water. *J. Dairy Sci*. 83:2021-2023.

Riley, L.W., R.S. Remis, S.D. Helgerson, H.B.McGee, J.G.Wells, B.R. Davis, R.J. Herbert, E.S. Olcott, L.M. Johnson, N.T. Hargrett, P.A. Blake, and M.L. Cohen. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N. Engl. J. Med.* 308:681-685.

Rockabrand, D., T. Arthur, G. Korinek, K. Livers, and P. Blum. 1995. An essential role for the *Escherichia coli* DnaK protein in starvation induced thermotolerance, H_2O_2 resistance, and reductive division. *J. Bacteriol.* 177:3695-3703.

Rodriguez, O., M.E. Castell-Perez, and R.G. Moreira. 2005. Effect of fruit maturity stage (sugar content) on efficacy of electron beam irradiation treatments. Session 54F-22. IFT Annual Meeting. New Orleans, Lou.

Rosenberg, M. and S. Kjelleberg. 1986. Hydrophobic interactions: role in bacterial adhesion. *Adv. Microb. Ecol*. 9:353-393.

Rowe, M.T. and R.B. Kirk. 2000. Effect of nutrient starvation on the resistance of *Escherichia coli* O157:H7 to subsequent heat stress. *J. Food Prot.* 63:1745-1748.

Ryu, J.-H., H. Kim, J.F. Frank, and L.R. Beuchat. 2004. Attachment and biofilm formation on stainless steel by *Escherichia coli* O157:H7 as affected by curli production. *Lett. Appl. Microbiol*. 39:359-362.

Sainz, T., J. Pérez, J. Villaseca, U. Hernández, C. Eslava, G.Mendoza, and C. Wacher. 2005. Survival to different acid challenges and outer membrane protein profiles of pathogenic *Escherichia coli* strains isolated from pozol, a Mexican typical maize fermented food. *Int. J. Food Microbiol.* 105:357-367.

Salter, M.A., Ross, T. and T. McKeein. 1998. Applicability of a model for non-pathogenic *Escherichia coli* for predicting the growth of pathogenic *Escherichia coli*. *J. Appl. Microbiol.* 85:357-364.

Sapers, G.M. and J.E. Sites. 2003. Efficacy of 1% hydrogen peroxide wash in decontaminating apples and cantaloupe melons. *J. Food Sci*. 68:1793-1797.

Sato, M., K. Machida, E. Arikado, H. Saito, T. Kagekawa, and H. Kobayashi. 2000. Expression of outer membrane proteins in *Escherichia coli* growing at acid pH. *Appl. Environ. Microbiol.* 66:943-947.

Seo, K.H. and J.F. Frank. 1999. Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. *J. Food Prot.* 62:3-9.

Sharma, M.M., Y.I. Chang, and T.F. Yen. 1985. Reversible and irreversible surface charge modification of bacteria for facilitating transport through porous media. *Colloids Surf.* 16:193- 206.

Sledjeski, D.D. and S. Gottesman. 1996. Osmotic shock induction of capsule synthesis in *Escherichia coli* K-12. *J. Bacteriol*. 178:1204-1206.

Solomon, E.B., S. Yaron, and K R. Matthews. 2002. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Appl. Environ. Microbiol.* 68:397-400.

Solomon, E.B. and K.R. Matthews. 2006. Interaction of live and dead *Escherichia coli* O157:H7 and fluorescent microspheres with lettuce tissue suggests bacterial processes do not mediate adherence. *Lett. Appl. Microbiol*. 42:88-93.

Sonntag, I., H. Scharz, and U. Henning. 1978. Cell envelope and shape of *Escherichia coli*: multiple mutants missing the outer membrane lipoprotein and the major outer membrane proteins. *J. Bacteriol*. 136:280-285.

Stenström, T.X. 1989. Bacterial hydrophobicity, an overall parameter for the measurement of adhesion potential to soil particles. *Appl. Environ. Microbiol*. 55:142-147.

Takeuchi, K. and J.F. Frank. 2000. Penetration of *Escherichia coli* O157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. *J. Food Prot.* 63:434-440.

Tauxe, R., H. Kruse, C. Hedberg, M. Potter, J. Madden, and K. Wachsmuth. 1997. Microbial hazards and emerging issues associated with produce; a preliminary report to the National Advisory Committee on Microbiologic Criteria for Foods. *J. Food Prot.* 60:1400-1408.

Thayer, D.W. and G. Boyd. 1993. Elimination of *Escherichia coli* O157:H7 in meats by gamma irradiation. *Appl. Environ. Microbiol.* 59:1030-1034.

Torres, A.G., C. Jeter, W. Langley, and A.G. Matthysse. 2005. Differential binding of *Escherichia coli* O157:H7 to alfalfa, human epithelial cells, and plastic is mediated by a variety of surface structures. *Appl. Environ. Microbiol*. 71:8008-8015.

Uhlich, G.A., J.E. Keen, and R.O. Elder. 2001. Mutations in the *csgD* promoter associated with variations in curli expression in certain strains of *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 67:2367-2370.

Uhlich, G.A., J.E. Keen, and R.O. Elder. 2002. Variations in the *csgD* promoter of *Escherichia coli* O157:H7 associated with increased virulence in mice and increased invasion of HEp-2 Cells. *Infect. Immun.* 70:395-399.

Ukuku, D.O. and W.F. Fett. 2002. Relationship of cell surface charge and hydrophobicity to strength of attachment of bacteria to cantaloupe rind. *J. Food Prot.* 65:1093-1099.

Ukuku, D.O. and W.F. Fett. 2006. Effects of Cell Surface Charge and Hydrophobicity on Attachment of 16 *Salmonella* Serovars to Cantaloupe Rind and Decontamination with Sanitizers. *J. Food Prot.* 69:1835-1843.

USDA/ERS 1998. Leafy greens: foundation of the vegetable industry. Agricultural outlook/ February. Available at http://www.ers.usda.gov/publications/agoutlook/jan1998/ao248b.pdf. Accessed 4 January 2007.

USDA/ERS 2005. U.S. Lettuce statistics. Availablt at http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1576. Accessed 3 February 2007.

Valle, J., S. Da Re, N. Henry, T. Fontaine, D. Balestrino, P. Latour-Lambert, and J.-M. Ghigo. 2006. Broad-spectrum biofilm inhibition by a secreted bacterial polysaccharide. *Proc. Natl. Acad. Sci.* 103:12558-12563.

Van Loosdrecht, M.C.M., J. Lyklema, W. Norde, G. Schraa, and A.J.B. Zehnder. 1987a. The role of bacterial cell wall hydrophobicity in adhesion. *Appl. Environ. Microbiol*. 53:1893-1897.

Van Loosdrecht, M.C.M., J. Lyklema, W. Norde, G. Schraa, and A.J.B. Zehnder. 1987b. Electrophoretic mobility and hydrophobicity as a measure to predict the initial steps of bacterial adhesion. *Appl. Environ. Microbiol*. 53:1898-1901.

Vidal, O., R. Longin, C. Prigent-Combaret, C. Dorel, M. Hooreman, and P. Lejeune. 1998. Isolation of an *Escherichia coli* K-12 mutant strain able to form biofilms on inert surfaces: Involvement of a new *ompR* allele that increases curli expression. *J. Bacteriol*. 180:2442-2449.

Wang, G. and M.P. Doyle. 1998a. Heat shock response enhances acid tolerance of *Escherichia coli* O157:H7. *Lett. Appl. Microbiol*. 26:31-34.

Wang, G., and M.P. Doyle. 1998b. Survival of enterohemorrhargic *Escherichia coli* O157:H7 in water. *J. Food Prot*. 61:662-667.

Warburton, D.W., J.W. Austin, B.H. Harrison, and G. Sanders. 1998. Survival and recovery of *Escherichia coli* O157:H7 in inoculated bottled water. *J. Food Prot*. 61:948-952.

Weinstein, D.L., R.K. Holmes, and A.D. O'Brien. 1988. Effect of iron and temperature on Shiga-like toxin 1 production by *Escherichia coli*. *Infect. Immun*. 56:106-111.

Welby, E.M. and B. McGregor. 2004. Maintaining product quality during transportation. p. 53-104. *In* Agricultural Exprot Transportation Handbook. USDA. Available at http://www.ams.usda.gov/tmd/export/maintaing_product_quality.htm. Accesed 10 January 2007.

Whitfield, C. and I.S. Roberts. 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. *Mol. Microbiol*. 31:1307-1319.

Williams, N.C. and S.C. Ingham. 1998. Thermotolerance of *E. coli* O157:H7 ATCC 43895 following exposure to 1.5% acetic acid. *J. Food Prot*. 61:1184-1186.

Yoon, J.W., J.Y. Lim, Y.H. Park, and C.J. Hovde. 2005. Involvement of the *Escherichia coli* O157:H7(pO157) *ecf* operon and lipid A myristoyl transferase activity in bacterial survival in the bovine gastrointestinal tract and bacterial persistence in farm water troughs. *Infect. Immun*. 73:2367-2378.

Yuk, H.G. and D.L. Marshall. 2003. Heat adaptation alters *Escherichia coli* O157:H7 membrane lipid composition and verotoxin production. *Appl. Environ. Microbiol.* 69:5115-5119.

Zhang, E. and T. Ferenci. 1999. OmpF changes and complexity of *Escherichia coli* adaptation to prolonged lactose limitation. FEMS *Microbiol. Lett*. 176:395-401.

Zhang, Y. and M.W. Griffiths. 2003. Induced expression of the heat shock protein genes, *uspA* and *grpE,* during starvation at low temperature and their influence on thermal resistance of *Escherichia coli* O157:H7. *J. Food Prot.* 66:2045-2050.

Zhao, T., M.P. Doyle, J. Shere, and L. Garber. 1995. Prevalence of enterohemorrhagic *Escherichia coli* O157:H7 in a survey of dairy herds. *Appl. Environ. Microbiol*. 61:1290-1293.

Zogaj, X., W. Bokranz, M. Nimtz, and U. Römling. 2003. Production of cellulose and curli fimbriae by members of the Family *Enterobacteriaceae* isolated from the human gastrointestinal tract. *Infect. Immun*. 71:4151-4158.

CHAPTER 3

TRANSFER OF *ESCHERICHIA COLI* O157:H7 TO ROMAINE LETTUCE DUE TO CONTACT WATER FROM MELTING ICE[1]

[1] Jin Kyung Kim and Mark A. Harrison. To be submitted to *Journal of Food Protection*, 2007.

Ice can be used to chill romaine lettuce and maintain relative humidity during transportation. *E. coli* O157:H7 may contaminate water used for ice. Potential contamination of lettuce is of concern since it is usually consumed raw or minimally processed. The objective was to determine the potential for *E. coli* O157:H7 contamination of romaine lettuce from either ice contaminated with the pathogen or by transfer from lettuce surfaces via melting ice. In experiment 1, lettuce was spot inoculated with *E. coli* O157:H7 and chilled with ice prepared from uncontaminated tap water. In experiment 2, water inoculated with *E. coli* O157:H7 was frozen and used to ice lettuce in shipping containers. Three heads of lettuce were stacked in each container and stored at 4 or 20°C. After ice melted, attachment and recovery of *E. coli* O157:H7 after rinsing with chlorinated water (200 µg free chlorine/ml) from sampling sites were determined. For experiment 1, the population of *E. coli* O157:H7 attached to inoculated sites was 3.8 and 5.5 cfu/cm^2 at 4 and 20°C, respectively. While undetectable, other non-inoculated sampling sites became contaminated with the pathogen due to ice melt. For experiment 2, 3.5-3.8 log cfu *E. coli* O157:H7/cm^2 was attached to the top leaf on the first head. Populations decreased on the other heads with the lowest at the bottom leaf on the third head. After rinsing with chlorinated water, *E. coli* O157:H7 remained on the surface of the top head (1.8-2.0 log cfu/cm^2) and was detectable at other sampling areas. There was no difference in numbers of *E. coli* recovered from each sampling site at 4 and 20°C. Results show that *E. coli* O157:H7 can be transferred onto other produce layers in shipping containers due to melted ice made of contaminated water and from contaminated to uncontaminated leaf surfaces.

INTRODUCTION

Large quantities of ice are used to maintain the quality of some fresh produce at different steps in the harvest-handling-marketing chain. It can be used in the field to remove field heat or in hydrocooler systems indirectly to help maintain water temperature (Kays 1997). In addition, top-icing is required for some produce types such as broccoli, turnips, spinach, green onions, and carrots with tops. These products have tolerance to contact with water and ice, have high respiration rates, and need high relative humidity. Since most refrigeration units on trailers and containers cannot control relative humidity and actually remove moisture from produce as a natural part of the cooling process, top-icing or package-icing is necessary to keep relative humidity during transportation as well as cool produce fast (Welby and McGregor 2004). Ice can be manufactured on-site with an icemaker and ice storage bin, or block ice can be purchased and crushed.

The production of romaine lettuce in 2004 in the U.S. increased approximately 8 times over that of 1990. Consumption of romaine lettuce per capita increased dramatically between 1990 (1.2 lbs) and 2004 (8.1 lbs; USDA/ERS 2005). This shift due to the popularity of Caesar salad in the food service and retail markets, increased nutritional awareness among consumers, the success of prepackaged salads, and a general desire for diversity in foods (USDA/ERS 1998). Today potential contamination of lettuce is of great concern since the product is usually consumed raw or with little intervention to reduce or eliminate foodborne pathogens from the product (FDA 2000).

Romaine lettuce is a cool-season crop with distinct temperature requirements. The optimal growing temperature is 10-20°C and cool nights are essential for good-quality lettuce (Deshpande and Salunkhe 1998). Lettuce is highly perishable and should be cooled as soon as

possible after harvesting. For precooling, vacuum cooling, hydrocooling or ice cooling is used (Boyhan et al. 2004). Romaine lettuce is hand-cut, harvested by ground pack crews and packed 12- or 24-bunches in waxed fiberboard boxes (Ashby 1995). There are three layers of lettuce heads per box. Romaine lettuce can be top-iced by placement of crushed ice on top the container prior to closure.

Since almost all known bacterial enteropathogens of the *Enterobaceriaceae*, including *Escherichia coli*, have been found in water and survive for extended periods (Wang and Doyle 1998; Warburton et al. 1998), contamination of ice with these pathogens could be a hazard. Due to melted ice made of contaminated water, *E. coli* O157:H7 might be distributed onto uncontaminated produce. In addition, ice may contact contaminated produce, resulting in transfer of pathogens from contaminated to uncontaminated surfaces. To date, there are no documented studies on the attachment and recovery of *E. coli* O157:H7 on surfaces of romaine lettuce under icing conditions using simulated packing and distribution of romaine lettuce.

The objective of this study was to investigate ice as a possible route for introduction of foodborne pathogens to romaine lettuce. The possible contamination sources could either be directly from ice contaminated with *E. coli* O157:H7 or by transfer from contaminated to uncontaminated lettuce surfaces via melting ice. The fate of *E. coli* O157:H7 on the surface of romaine lettuce contacted with ice under simulated commercial operation conditions was determined.

MATERIALS AND METHODS

Bacterial strain and inoculum preparation. *E. coli* O157:H7 932 (human isolate) was used. It was determined to be sensitive to a level of 50 μg/ml nalidixic acid (Sigma

Chemical Co., St. Louis, MO). It was made nalidixic acid-adapted through the transfers in tryptic soy broth (TSB, Becton, Dickinson and Co., Sparks, MD) supplemented with increasing concentrations of nalidixic acid (TSBN) from 2.5 to 50 µg /ml for 7 days at 37ºC. Nalidixic acid-adapted cells were surface plated on tryptic soy agar supplemented with 50 µg of nalidixic acid/ml (TSAN) and incubated at 37ºC for 24 h. Stock cultures were stored at –80ºC in culture broth containing 15% glycerol until used. Frozen stock cultures maintained at –80ºC were activated by two successive transfers in 10 ml of TSBN and incubated at 37ºC for 24 h. These active cultures were transferred to 5 L of TSBN and incubated for 24 h at 37°C. Cultures were centrifuged at 4,550 x g for 30 min (Model 5681, Forma Scientific Inc., Milford, MA) at 20ºC and the cells were resuspended into sterile deionized water (SDW) to yield ca. 10^8 cfu/ml.

Lettuce. Romaine lettuce (*Lactuca sativa* var. *longifolia*) was purchased at a local grocery store (Athens, GA) on the shipping day from the warehouse to keep consistent freshness and all experiments were started at the purchasing day. At the start of the experiment, wilted and blemished outer leaves were removed. Tap water and uninoculated lettuce were tested to confirm the absence of nalidixic acid-adapted *E. coli* O157:H7 and *E. coli* using TSAN after incubation at 37ºC for 24 h in enrichment broth.

Container preparation. To simulate packing, we used plastic containers (20x30x30cm) with ventilation holes. Sterile wire grid racks were used on the bottom of each container to prevent lettuce from touching melted ice that accumulated in the container. Sterile wire crown was used on the top of lettuce to hold ice and keep ice from dropping to the bottom of container without touching lettuce (Fig. 3-1).

Ice preparation. For experiment 1 (contamination of lettuce before contact with ice), ice was prepared from uncontaminated tap water using portable ice-maker, placed into Ziploc

bags (approx. 150 g; S.C. Johnson & Son, Inc., Racine, WI), and stored at –20ºC until used. Before making ice, tap water was stored at 4°C overnight. Aquachek total/free chlorine test kits (Hach Company, Loveland, CO) was used to measure the concentration of free chlorine in tap water. Free chlorine was less than 0.1µg/ml. For experiment 2 (ice contamination with pathogen), 100 ml of the inoculum preparation was added to one liter of tap water which had been stored overnight at 4°C in advance, to give an inoculum concentration of approximately 10^7cfu/ml before ice was made.

Experiment 1. *E. coli* O157:H7 transfer from contaminated to uncontaminated lettuce surfaces via melted ice. Lettuce was equilibrated to room temperature (ca. 20°C) or stored at refrigerator (4°C). For lettuce at 20°C, 100 µl of inoculum (10^8 cfu/ml) was spot inoculated on two marked areas (3x3cm) on the abaxial surface of the top leaf on the first head of lettuce and dried for 1 h at 20°C in the laminar flow hood. Inoculated areas were gently rinsed using SDW to remove unattached cells. During rinsing, inoculated lettuce surfaces were faced downside and rinse water was confined to the marked area to prevent unattached cells from contacting the lettuce surface other than the inoculated site. Then, three heads of lettuce were stacked in each container with the top head with the inoculated site on top. Lettuce was chilled with ice prepared from uncontaminated tap water for 1 h at 20°C, followed by storage at 4°C. Temperature on the top leaf contacting ice was recorded using a continuous time-temperature recording device and thermocouple. After the ice melted, sampling sites from each head were taken using sterile templates (4x4cm) and scalpels. Sampling sites were the inoculated areas, non-inoculated areas of the top leaf, adjacent leaf on both sides, second leaf down on the top head, first leaf on the second head, bottom leaf on the third head and melted ice (Fig. 3-1). For lettuce at refrigerator, *E. coli* O157:H7 was spot inoculated and held overnight at 4°C.

Inoculated areas were gently rinsed using SDW. Then, three heads of lettuce were stacked in each container and followed by ice lettuce at 4°C. Temperature on the top leaf contacting ice was recorded. After the ice melted, sampling sites from each head were taken using sterile templates (4x4cm) and scalpels. Sampling sites were same as above. Lettuce pieces were gently rinsed with SDW (10 ml) on both sides to remove unattached cells. After rinsing, a 3x3cm template was used to remove edges where cells might attach during the rinsing procedure and lettuce pieces were placed into stomacher bags. From inoculated sites, one piece was used to determine *E. coli* O157:H7 attachment and one to determine recovery after rinsing with chlorinated water (200 µg free chlorine/ml). From non-inoculated areas, three pieces were used to determine attachment and 3 for recovery. The chlorine solutions were prepared each day from a sodium hypochlorite stock solution. The solutions were prepared in SDW stored at 4°C overnight with 0.1 N cirtric acid to adjust pH to 7.0 ± 0.2 and the concentration of free chlorine was verified by Aquachek total/free chlorine test kits (Hach Company). For the recovery test, 100 ml of chlorinated water (200 µg free chlorine/ml) was added to the stomacher bags, and the contents were vigorously shaken by hand for 1 min and immediately neutralized with 30 ml of 0.1 N sodium thiosulfate.

Experiment 2. *E. coli* O157:H7 distribution onto uncontaminated lettuce layers due to melted ice made of contaminated water. Water inoculated with *E. coli* O157:H7 was frozen and used to ice lettuce in shipping containers. For lettuce at room temperature (ca. 20°C), lettuce equilibrated to 20°C was stacked by 3 heads in each container. Lettuce was chilled with ice containing *E. coli* O157:H7 for 1 h at room temperature, followed by storage at 4°C. For lettuce at refrigerator, lettuce stored at 4°C was stacked by 3 heads in each container, followed by ice lettuce at 4°C. After ice completely melted, sampling sites were removed using

a sterile template (4x4cm) and scalpels. The excised pieces were rinsed gently with SDW (10 ml) on both sides. After rinsing, a 3x3cm template was used to remove edges and lettuce pieces were placed into stomacher bags. Thereafter, all procedures were same as described for experiment 1.

Microbiological analysis. Lettuce samples were homogenized with 100 ml of modified EC broth (mEC broth, Becton, Dickinson and Co.) for 1 min in a stomacher at normal speed. Melted ice was diluted with 0.1% peptone water and plated directly. A spiral plater (Autoplate 4000, Spiral Biotech, Bethesda, MD) on TSAN was used for enumeration of *E. coli* O157:H7. TSAN plates and mEC broth were incubated at 37°C for 24 h. The mEC was used in the event that the TSAN plates yielded no typical colonies. Detection limits for attachment and recovery were 1.57 log cfu/cm^2 and 0.87 log cfu/cm^2, respectively. The mEC cultures after inoculation at 37°C for 24 h were streaked onto TSAN plates for presumptive identification. Presumptively identified *E. coli* O157:H7 were done based on colony morphology on TSAN plates, and Gram stain reaction. Randomly selected isolates were confirmed by biochemical testing using Micro ID kits (Remel, Lenexa, KS).

Statistical analyses. All experiments were conducted with three replicates and each replicate consisted of 3 samples. *E. coli* O157:H7 counts for attachment and recovery test were converted to log values and then entered into an analysis of variance (ANOVA) using SAS (SAS Institute, Inc., Ver. 9.1, Cary, NC). When the ANOVA indicated a significant difference at α=0.05 between sampling sites or treatment temperatures, mean separation was achieved using the Duncan's multiple range test. Differences between mean values were considered significant at α=0.05.

RESULTS AND DISCUSSION

We chose two scenarios for introduction of *E. coli* O157:H7 onto romaine lettuce, one for ice contaminated with *E. coli* O157:H7 and one for transfer from contaminated to other uncontaminated lettuce surfaces via melted ice. In addition, ice can be used either in the field to remove field heat or in a refrigeration unit to prevent moisture loss from the surface. Therefore, we performed two experiments (lettuce contamination and ice contamination) at two temperatures (4 and 20°C). There was no detectable nalidixic acid-adapted *E. coli* O157:H7 and *E. coli* detected in the tap water and uninoculated lettuce used in the experiments.

Experiment 1. *E. coli* O157:H7 transfer from contaminated to uncontaminated lettuce surfaces via melted ice. Direct or indirect pathogen contamination of romaine lettuce can occur at many points, such as inadequately treated manure, irrigation water, soil, animals, and infected workers, in the production chain during growth and processing (Beuchat 2002). Ice may play a role in transferring pathogens from contaminated to uncontaminated lettuce via melted ice. In our study, abaxial surface on the top head of lettuce in storage containers were spot inoculated with *E. coli* O157:H7 and chilled with ice prepared from uncontaminated tap water. After icing lettuce at 20°C, temperature on the top leaf was dropped to 2°C within 30 min and was maintained between 3 and 6°C at refrigerator. For icing lettuce at 4°C, temperature dropped to 2°C within 1 h and thereafter was maintained between 3 and 6°C (data not shown). The population of *E. coli* O157:H7 attached to the inoculated site was 5.5 log cfu/cm^2 at 20°C, significantly higher than at 4°C (3.8 log cfu/ cm^2) (Table 3-1). The populations in attachment of *E. coli* O157:H7 to the second and third head of lettuce sites were undetectable, but all showed presumptive positive after enrichment. As ice melted, the water

was able to transfer *E. coli* O157:H7 from the inoculated sites to uncontaminated sites on all of the lettuce heads in the container. The population of *E. coli* O157:H7 in melted ice was 2.8 and 3.3 log cfu/ml for 4°C and 20°C, respectively. These results show *E. coli* O157:H7 can be transferred from contaminated to uncontaminated produce layers in shipping containers due to melted ice.

The level of reduction in populations of *E. coli* O157:H7 on the inoculated site at 20°C after rinsing with chlorinated water was 3.06 log cfu/cm^2 with a large standard deviation (0.89). The cells still remained on the uninoculated lettuce surfaces after rinsing with chlorinated water, while undetectable (Table 3-1). The effect of chlorine as a sanitizer for leaf surfaces has been controversial. Several studies showed rinsing with chlorinated water resulted in a microbial reduction of less than 2 log cfu/g on fruits and vegetables (Beuchat 1999; Brackett 1992; Cherry 1999). Weissinger et al. (2000) showed that sanitizers did not easily remove *Salmonella* Baildon via the spot inoculation of lettuce leaves, but no distinctions were made between outer and inner surfaces of lettuce leaves. On the other hand, other studies reported that pathogens spot inoculated on the outer surface of lettuce leaves were easily killed or removed by treatment with sanitizers including acidified chlorinated water (Park et al. 2001; Singh et al. 2002). Koseki et al. (2003) demonstrated chlorinated water reduced the population of *E. coli* O157:H7 by 4.60 log cfu/g for spot inoculation of the outer surface of the lettuce leaf, whereas 2.76 log reduction for inner surface. In addition, they reported lettuce leaves inoculated with high populations (10^5 to 10^6 cfu/g) of *E. coli* O157:H7 showed larger log reductions than those inoculated with low populations (10^3 to 10^4 cfu/g) with greatest reduction on outer surface.

Many studies have demonstrated negative temperature differential (cold inoculum, warm produce) affected bacterial penetration. Takeuchi and Frank (2000) reported *E. coli* O157:H7

cells showed greater penetration when lettuce was held at 4°C of inoculum compared with 7, 25 or 37°C, resulting in protecting cells from contact with chlorine. Bartz and Showalter (1981) reported that tomatoes submerged in a suspension of *Serratia marcescens* under a negative temperature differential contained the organism more frequently than tomatoes exposed to a positive temperature differential. Buchanan et al. (1999) showed that apples immersed in an *E. coli* O157:H7 suspension had high populations of the pathogen in the outer core region. Burnett et al. (2003) reported similar findings that apples inoculated under a negative temperature differential experienced greater infiltration of *E. coli* O157:H7 cells into the core and surface structures such as stomata, lenticels, and trichomes. In addition, *E. coli* O157:H7 attached better to the cut edge and easily penetrated through openings in the cut edge (Takeuchi and Frank 2000). However, in our study negative temperature differential might result in better attachment but not greater to penetration. Pathogen inoculation on intact abaxial surface as done in our study may not allow penetration of *E. coli* O157:H7. Thus cells attaching on the abaxial surface of the lettuce may be easily accessible to chlorinated water.

Experiment 2. *E. coli* O157:H7 distribution onto uncontaminated lettuce layers due to melted ice made of contaminated water. Several studies showed *E. coli* O157:H7 can survive in water for extended periods (Wang and Doyle 1998; Warburton et al. 1998). Therefore, contamination of ice with *E. coli* O157:H7 could be a hazard. In our study, water inoculated with *E. coli* O157:H7 (approx. 10^7 cfu/ml) was frozen and used to ice lettuce in shipping containers. After water from the melting ice contacted the top leaf on the first head of lettuce, 3.5-3.8 log cfu *E. coli* O157:H7/cm^2 were detected on the leaf surfaces (Fig. 3-2). Populations decreased on the other heads with the lowest populations on the bottom layer. When ice was applied to the top of lettuce for chilling purposes, *E. coli* O157:H7 was distributed

to other lettuce layers due to the water from melting ice contaminated with this microorganism. Populations on the adjacent leaves and second leaf down on the top head of lettuce at 20°C was less than first leaf on the second head of lettuce but slightly higher than the bottom leaf on the third head of lettuce, whereas there was no difference in populations among other leaves on the top head of lettuce and other heads of lettuce at 4°C (Fig. 3-2). This might have been because the ice melting time at 20°C was quicker than 4°C and the run-off to lower levels could have been faster.

After rinsing with chlorinated water, *E. coli* O157:H7 remained on the surface of the top head of lettuce (1.8-2.0 log cfu/cm^2) and was detectable at other sampling areas (Fig. 3-2). There was no difference in numbers of *E. coli* O157:H7 remained on each sampling site at 20 and 4°C. These results quantitate *E. coli* O157:H7 distribution onto other produce layers in shipping containers via the water from melted ice carrying the microorganism.

In conclusion, our present study shows that ice is a possible route for introduction of *E. coli* O157:H7 to romaine lettuce by either directly contacting water from melting ice contaminated with *E. coli* O157:H7 or by transfer from contaminated to uncontaminated lettuce surfaces via melted ice.

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REFERENCES

Ashby, B.H. 1995. Protecting perishable foods during transport by truck. USDA/AMS. Available at http://www.ams.usda.gov/tmd/TSB/Protecting_Perishable_Foods_2006.pdf. Accessed 4 January 2007.

Bartz, J.A. and R.K. Showalter. 1981. Infiltration of tomatoes by aqueous bacterial suspensions. *Phytopathology* 71:515-518.

Beuchat, L.R. 1999. Survival of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces applied to lettuce and the effectiveness of chlorinated water as a disinfectant. *J. Food Prot.* 62:845-849.

Beuchat, L.R. 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microbes Infect.* 4:413-423.

Boyhan, G.E., W.C. Hurst, W.T. Kelly, G.W. Krewer, and K.C. Taylor. 2004. Postharvest handling and transportation of fruits and vegetables. Available at http://pubs.caes.uga.edu/caespubs/pubs/PDF/FS-100.pdf. Accessed 4 January 2007.

Brackett, R.E. 1992. Shelf stability and safety of fresh produce as influenced by sanitation disinfection. *J. Food. Prot.* 55:808-814.

Buchanan, R.L., S.G. Edelson, R.L. Miller, and G.M. Sapers. 1999. Contamination of intact apples after immersion in an aqueous environment containing *Escherichia coli* O157:H7. *J. Food Prot.* 62:444-450.

Burnett, S.L., J. Chen, and L.R. Beuchat. 2003. Attachment of *Escherichia coli* O157:H7 to the surfaces and internal structures of apples as detected by confocal scanning laser microscopy. *Appl. Environ. Microbiol*. 66:4679-4687.

Cherry, J.P. 1999. Improving the safety of fresh produce with antimicrobials. *Food Technol.* 53:54-59.

Deshpande, S.S. and D.K. Salunkhe. 1998. Lettuce, p. 493-509. *In* Handbook of vegetable science and technology: production, composition, storage and processing. Marcel Dekker, Inc., New York. Available at http://www.netlibrary.com/Reader/ Accessed 5 February 2007.

Food and Drug Administration. 2000. Kinetics of microbial inactivation for alternative food processing technologies. Available at: http://vm.cfsan.fda.gov/~comm/ift-glos.html. Accessed 12 December 2006.

Kays, J.S. 1997. Heat, heat transfer, and cooling, p. 457-507. *In* Postharvest Physiology of Perishable Plant Products. Exon Press. GA.

Koseki, S., K. Yoshida, Y. Kamitani, and K. Itoh. 2003. Influence of inoculation method, spot inoculation site, and inoculation size on the efficacy of acidic electrolyzed water against pathogens on lettuce. *J. Food Prot*. 66:2010-2016.

Park, C.M., Y.C. Hung, M.P. Doyle, G.O.I. Ezeike, and C. Kim. 2001. Pathogen reduction and quality of lettuce treated with electrolyzed oxidizing and acidified chlorinated water. *J. Food Sci.* 66:1368-1372.

Singh, N., R.K. Singh, A.K. Bhunia, and R.L. Stroshine. 2002. Effect of inoculation and washing methods on the efficacy of different sanitizers against *Escherichia coli* O157:H7 on lettuce. *Food Microbiol.* 19:183-193.

Takeuchi, K. and J.F. Frank. 2000. Penetration of *Escherichia coli* O157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. *J. Food Prot.* 63:434-440.

USDA/ERS 1998. Leafy greens: foundation of the vegetable industry. Agricultural outlook/ February. Available at http://www.ers.usda.gov/publications/agoutlook/jan1998/ao248b.pdf. Accessed 4 January 2007.

USDA/ERS 2005. U.S. Lettuce statistics. Available at http://usda.mannlib.cornell.edu/MannUsda/viewDocumentInfo.do?documentID=1576. Accessed 3 February 2007.

Wang, G. and M.P. Doyle. 1998. Survival of enterohemorrhargic *Escherichia coli* O157:H7 in water. *J. Food Prot*. 61:662-667.

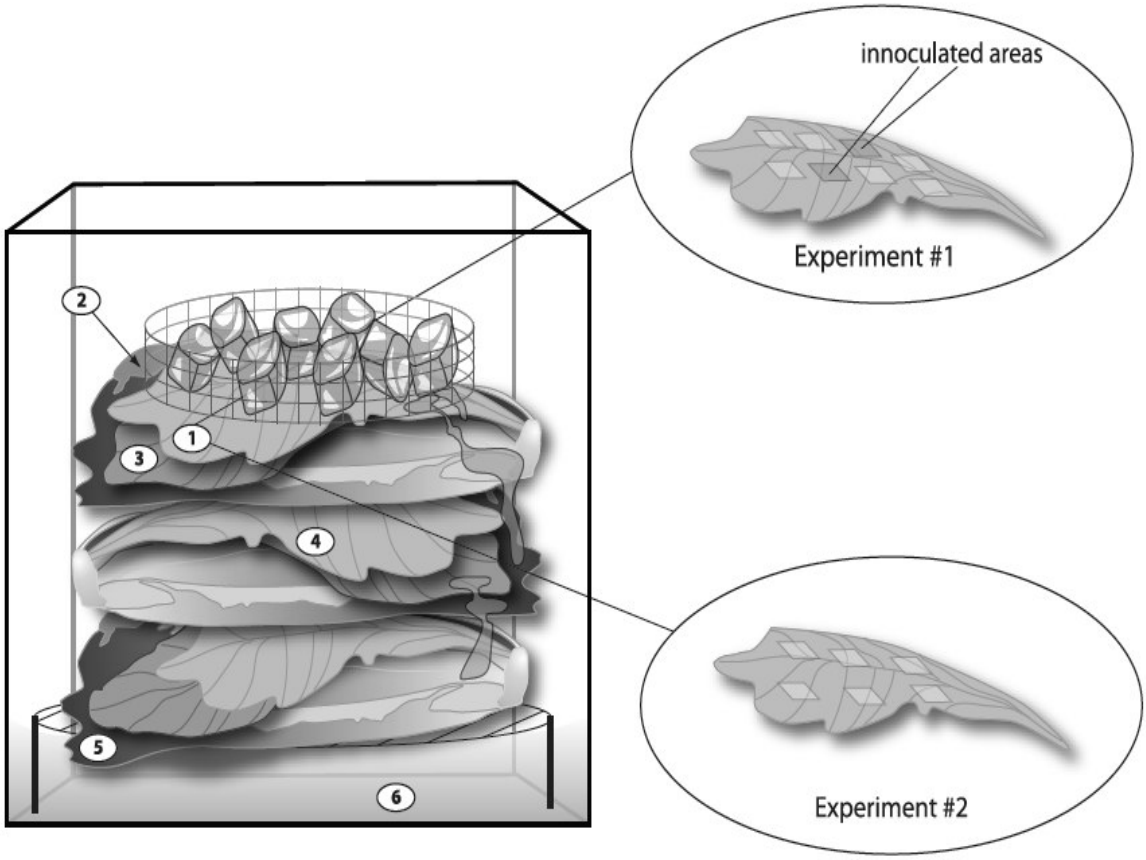
Warburton, D.W., J.W. Austin, B.H. Harrison, and G. Sanders. 1998. Survival and recovery of *Escherichia coli* O157:H7 in inoculated bottled water. *J. Food Prot*. 61:948-952.

Weissinger, W.R., W. Chantarapanont, and L.R. Beuchat. 2000. Survival and growth of *Salmonella* Baildon in shredded lettuce and diced tomatoes, and effectiveness of chlorinated water as a sanitizer. *Int. J. Food Microbiol.* 62:123-131.

Welby, E.M. and B. McGregor. 2004. Maintaining product quality during transportation. p. 53-104. *In* Agricultural Exprot Transportation Handbook. USDA. Available at http://www.ams.usda.gov/tmd/export/maintaing_product_quality.htm. Accesed 10 January 2007.

Fig 3-1. Illustration showing placement of lettuce and ice in a container with sampling sites (1-6). 1: Top leaf on the first head of lettuce. Sampling areas on leaf with darker shading were inoculated, while lighter shaded areas on leaf were uninoculated. 2: adjacent leaves on the first head of lettuce 3: second leaf down on the first head of lettuce 4: first leaf on the second head of lettuce 5: bottom leaf on the third head of lettuce 6: water from melted ice

Fig 3-2. Attachment and recovery of *E. coli* O157:H7 from romaine lettuce contacted with contaminated ice at 20°C (top) and 4°C (bottom) in experiment 2. ■ : attachment □ : recovery 1: top leaf on the first head of lettuce 2: adjacent leaves on the first head of lettuce 3: second leaf down on the first head of lettuce 4: first leaf on the second head of lettuce 5: bottom leaf on the third head of lettuce Means with the different lower case letters in a ■ are significantly different ($p<0.05$). Means with the different upper case letters in a □ are significantly different ($p<0.05$).


innoculated areas
Experiment #1
2
1
3
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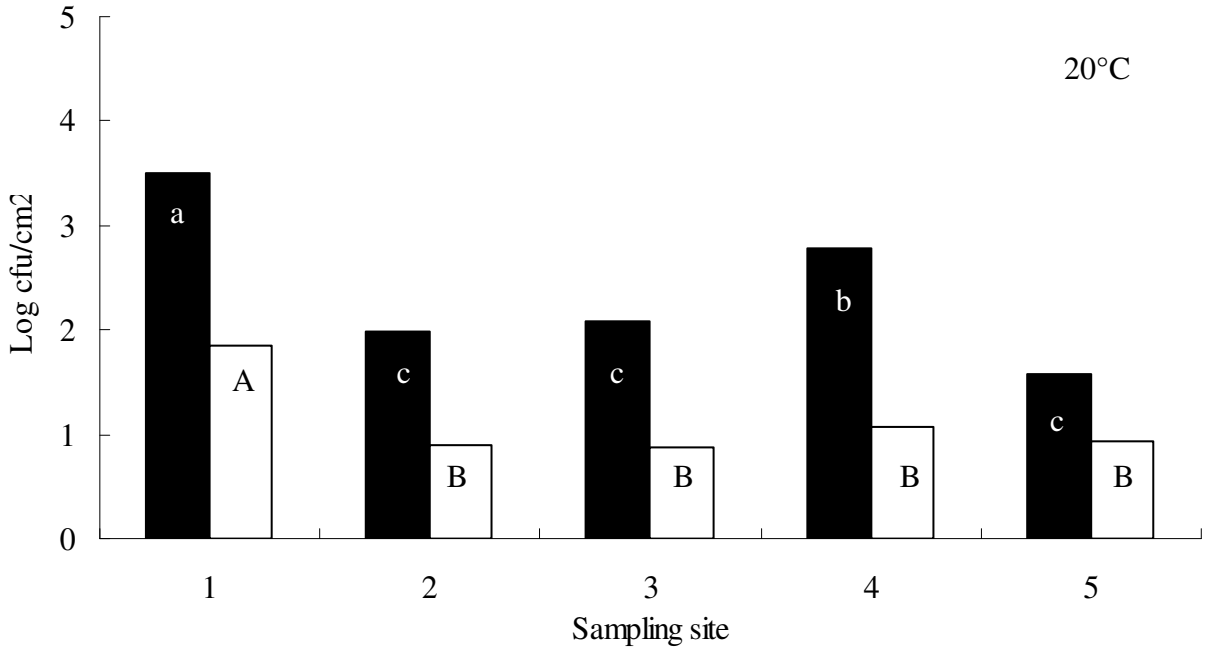
5
6
Experiment #2


20°C
Log cfu/cm2
5
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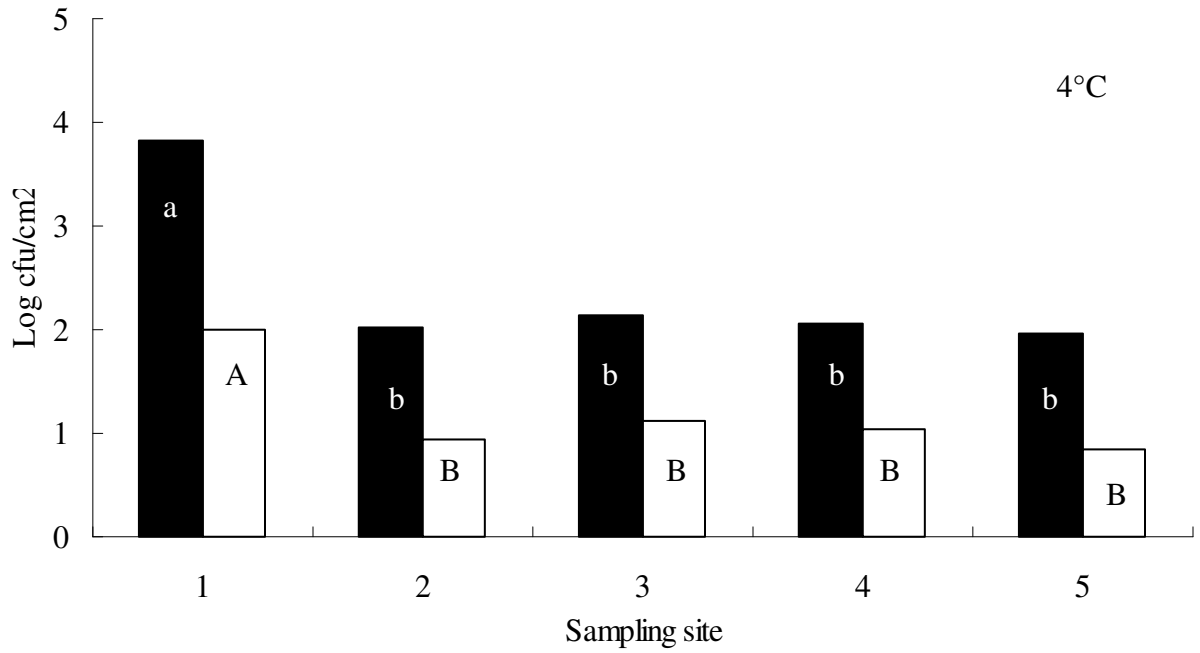
Sampling site


4°C
Log cfu/cm2
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B
1
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5
Sampling site

Table 3-1. Attachment and recovery of *E. coli* O157:H7 from romaine lettuce after spot inoculating on top leaf, held for 1 h at 20°C or overnight at 4°C, and chilled by ice made from uncontaminated water

	Log cfu/cm^2			
	Attachment		Recovery	
Temperature (°C) Sampling sites	20	4	20	4
Inoculated site of top leaf	5.54[a1]	3.78[b]	2.48[a]	1.97[a]
Non-inoculated areas of top leaf	1.73	< 1.57 (3/3)	0.90	< 0.87 (3/3)
Adjacent leaves on 1st head	1.81	< 1.57 (1/3)	0.89	< 0.87 (2/3)
2nd leaf down on 1st head	< 1.57 (2/3)[2]	< 1.57 (2/3)	< 0.87 (2/3)	< 0.87 (1/3)
1st leaf on 2nd head	< 1.57 (3/3)	< 1.57 (3/3)	< 0.87 (2/3)	< 0.87 (2/3)
Bottom leaf on 3rd head	< 1.57 (2/3)	< 1.57 (2/3)	< 0.87 (3/3)	< 0.87 (2/3)

[1]: Within attachment and recovery, means with the different letters in a row are significantly different ($p<0.05$). Comparison of populations on the other sampling sites is inappropriate because the populations are below detectable levels (for attachment <1.57 log cfu/cm^2; for recovery <0.87 log cfu/cm^2)

[2]: Number of presumptive positive *E. coli* O157:H7 in samples after enrichment/number of samples tested when the enumeration count was below detectable levels

CHAPTER 4

SURROGATE SELECTION FOR *ESCHERICHIA COLI* O157:H7 BASED ON CRYOTOLERANCE AND ATTACHMENT TO ROMIANE LETTUCE[1]

[1] Jin Kyung Kim and Mark A. Harrison. To be submitted to *Applied and Environmental Microbiology*, 2007.

Using non-pathogenic surrogates in place of pathogens when evaluating commercial food processing operations offers safety advantages, but may be limited if they do not behave in the same manner in challenge situations. Non-pathogenic *Escherichia coli* strains were compared with *E. coli* O157:H7 based on cryotolerance, cell surface characteristics (hydrophobicity, zeta potential, and morphology) and attachment to lettuce. Populations for all strains were reduced less than 1 log cfu/ml over 7 d storage at –18ºC. After 1 d storage, the survival rate for *E. coli* ATCC 25922 was 44.3%, similar to *E. coli* O157:H7 (49%). No capsule was produced by any of the strains, and *E. coli* O157:H7 expressed curli at both 20 and 37ºC, whereas *E. coli* ATCC 25922 expressed curli only when grown at 20ºC. Hydrophobicity of *E. coli* ATCC 25922 was 53.5%, similar to *E. coli* O157:H7 (56.2%). The zeta potentials of non-pathogenic *E. coli* and *E. coli* O157:H7 cells were –4.95 to –10.92 mV, showing difference among the strains. The zeta potential of *E. coli* ATCC 25922 was –8.3 mV, similar to *E. coli* O157:H7 (–9.0 mV). *E. coli* ATCC 25922 exhibited the greatest attachment to lettuce among surrogates (79% compared to *E. coli* O157:H7). The populations of both *E. coli* ATCC 25922 and *E. coli* O157:H7 on lettuce surfaces decreased 2.2 - 2.4 log cfu/cm^2 after rinsing with 200 µg of free chlorine/ml solution. Based on cryotolerance and cell surface characteristics, *E. coli* ATCC 25922 is a useful surrogate for *E. coli* O157:H7 for studies involving attachment and recovery from chilled produce.

INTRODUCTION

The ability of microorganisms to attach to fresh produce is of great concern since the products are to be sold as raw or minimally processed. Attachment of microorganisms is influenced by the cell surface characteristics (Frank 2001). Cell surface structures (e.g., polysaccharides and fimbriae), hydrophobicity, and surface charge may influence adherence of bacteria to a surface. Many researchers reported curli expression by *E. coli* O157:H7 was rare but when it occurs the organism is more virulent and plays a role in autoaggregation and development of biofilm on inert surfaces (Prigent-Combaret et al. 2000; Ryu et al. 2004; Uhlich et al. 2001, 2002). Capsules can be either adhesive or due to the hydrophilic capsule they can be antiadhesive by masking hydrophobic components of the cell envelope (Ofek and Doyle 1994). Hydrophobic cells are generally more adherent than hydrophilic ones and most bacteria preferentially adhere to hydrophobic surfaces. Surface charges expressed by zeta potential can occur due to the formation of an electrostatic double layer on each surface, depending on the ionic strength and pH of the surrounding medium. Cells also adhere to surface of opposite charge (Frank 2001).

A surrogate is defined as a non-pathogenic species and strain responding to a particular treatment in a manner equivalent to a pathogenic species and strain (FDA 2000). Surrogates play an important role as alternative biological indicators that can mimic survival and growth properties of a pathogen. However, using surrogates has inherent limitations if they do not behave in the same manner to that of the pathogen of interest. Generally, surrogates are selected from the population of well-known organisms that have well-defined characteristics and a long history of being non-pathogenic (Busta et al. 2003). In addition to these characteristics of surrogates, they need to differentiate easily from other microflora. Antibiotic resistance can

be used as selective marker (Peri 2003). Eblen et al. (2005) demonstrated there were considerable differences in survival characteristics among non-pathogenic *E. coli* and *E. coli* O157:H7 and *Salmonella*. Peri et al. (2002) suggested *E. coli* K-12 LMM 1010 is a suitable surrogate for *E. coli* O157:H7 after evaluating their survival at acid/alkaline pH levels and reduced water activities. Among the strains cited in literature *E .coli* ATCC 25922 is one of the most currently used. *E. coli* ATCC 25922 was found to be a suitable surrogate for evaluating the efficacy of hydrogen peroxide in decontaminating apples and cantaloupe melons (Sapers and Sites 2003). Pao and Davies (2001) demonstrated *E. coli* ATCC 25922 showed the resistance to alkaline pH and heat and may be utilized as surrogates to conduct research in fresh fruits. Thayer and Boyd (1993) successfully determined this strain has the same sensitivity to gamma radiation in meats. Duffey et al. (2000) used *E. coli* ATCC 25922 as a surrogate for *E. coli* O157:H7 for validation of UV pasteurization treatments in apple cider processing. However, Mak et al. (2000) demonstrated strain ATCC 25922 was the least thermo-tolerant before and after acid stress and should not be used as a surrogate for *E. coli* O157:H7 on this purpose. The results of these studies suggested that a particular non-pathogenic strain behaves differently under specific process conditions. Thus, a surrogate may not fit all challenged situations to the same degree.

In this research, cryotolerance, cell surface properties such as surface hydrophobicity, cell surface charge, capsule and curli production were examined as related characteristics to attachment studies. The objective of this study was to evaluate non-pathogenic *E. coli* strains that were used in other studies to serve as surrogate for *E. coli* O157:H7 for attachment and recovery studies involving chilled produce.

MATERIALS AND METHODS

Selection of strains. The strains studied were as follows: *E. coli* O157:H7 932 (human isolate), *E. coli* ATCC 8677, 11775, and 25922 from our stock cultures, *E. coli* K-12 LMM 1010, and *E. coli* K-12 MC 4100 from Dr. Sadhana Ravishankar (National Center for Food Safety and Technology, Chicago, IL). These 6 strains were determined to be sensitive to a level of 50 µg/ml nalidixic acid (Sigma Chemical Co., St. Louis, MO). Subcultures of these strains were adapted to nalidixic acid through increasing concentrations of nalidixic acid from 2.5 to 50 µg/ml for 7 days at 20 or 37ºC in tryptic soy broth (Becton, Dickinson and Co., Sparks, MD) supplemented with each concentration of sterile filtered (0.22 µm) nalidixic acid (TSBN). Nalidixic acid-adapted cells were surface plated on tryptic soy agar supplemented with 50 µg of nalidixic acid/ml (TSAN) and incubated at 20ºC for 24 h or 37ºC for 16 to 18 h. Stock cultures were stored at –80ºC in culture broth containing 15% glycerol until used.

Preparation of inocula. To investigate the effect of growth temperature on the behavior of the microorganisms, 20 or 37ºC incubation conditions were used. Frozen stock cultures maintained at –80ºC were activated by two successive transfers in 10 ml of TSBN and incubated at 20 for 24 h or at 37ºC for 16 to 18 h. Cultures were harvested by centrifugation at 9,000 x g for 10 min and the pellets were washed twice with sterile deionized water (SDW) and resuspended in phosphate buffer saline (PBS).

Cryotolerance. Each strain (l ml in 1.5-ml tubes) was frozen in PBS at –18ºC for 1, 2, 4 and 7 days. After storage at –18ºC, each culture was thawed at room temperature (ca. 20ºC) within 15 min and the viable count was estimated by surface plating onto TSAN using a spiral-plater (Autoplate 4000, Spiral Biotech, Bethesda, MD).

Capsule and curli production. To examine the presence of capsular polysaccharide, the cells were stained with India ink (Remel, Lenexa, KS). The preparations were made by mixing a loopful of each strain from TSBN culture with one drop of India ink on a slide. A coverslip was pressed down firmly over the preparation, and it was examined with a phase-contrast microscope at ×1,000 magnification. For curli expression, each strain grown at both growth temperatures was incubated at 28°C for 48 h on Congo red indicator (CRI) agar, which contained 0.1% Bacto tryptone, 0.05% Bacto yeast extract, 1.5% Bacto agar, 40 mg/L of Congo red and 20 mg/L of Coomassie brilliant blue (Sigma Aldrich Co, St Louis, MO). Curli-producing *E. coli* bind Congo red dye and form red colonies on CRI, whereas curli-negative bacteria form white colonies (Pawar et al. 2005). Stability of curli expression was determined by directly reculturing isolates onto CRI and incubating for 48 h at 28°C.

Hydrophobicity. Hydrophobicity was determined as described by Hassan and Frank (2004). One ml of xylene was added to each of three tubes containing 4 ml of cells suspended in PBS. Tubes were vortexed for 2 min and placed in a 37ºC water bath for 30 min. The optical density at 600 nm of the aqueous layer in each tube was determined using Beckman DU-530 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). The results were expressed as percentages calculated by using the following relationship: percent hydrophobicity = 100(1 – final optical density/optical density of initial bacterial cells).

Zeta potential measurements. Zeta potentials were measured for bacteria with a population of ~10^7 cfu/ml in PBS (pH 7.4) using Brookhaven's Zeta Plus instrument (Brookhaven Instruments, Holtsville, NY). Zeta potential measurements for each strain were repeated at least three times.

Procedure of attachment and recovery test. Romaine lettuce (*Lactuca sativa* var. *longifolia*) was purchased at the same day in a local supermarket (Athens, GA). Lettuce pieces (3x3 cm) were prepared using a sterile template and blade. Six pieces of lettuce were spot inoculated with 100 µl of each strain suspension of *E. coli* and held in a biosafety hood to allow the inoculum to dry for 1 h at 20ºC. After 1 h, pieces were gently rinsed with 5 ml of SDW to remove unattached or loosely attached cells and placed into stomacher bags. Three pieces were used to determine attachment and 3 for recovery after rinsing with chlorinated water. The chlorine solutions were prepared each day from a sodium hypochlorite stock solution. The solutions were prepared in SDW stored at 4ºC overnight in advance with 0.1 N cirtric acid to adjust to pH 7.0 ± 0.2 and the concentration of free chlorine was verified by Aquachek total/free chlorine test kits (Hach Company, Loveland, CO). For the recovery test, 100 ml of chlorinated water (200 µg free chlorine/ml) was added to the stomacher bags, and the contents were vigorously shaken by hand for 1 min and immediately neutralized with 30 ml of 0.1 N sodium thiosulfate.

Microbiological analysis. Lettuce samples were homogenized with 100 ml of modified EC broth (Becton, Dickinson and Co.) for 1 min in a stomacher at normal speed. Populations were enumerated on TSAN using a spiral-plater. Uninoculated lettuce pieces were analyzed for the presence of nalidixic acid adapted *E. coli* O157:H7 and *E. coli* using TSAN after incubation at 37ºC for 24 h in enrichment broth.

Statistical analyses. Data were analyzed using analysis of variance (Statistical Analysis Systems Institute, Ver. 9.1, Cary, NC). If differences were significant at α=0.05, populations after storage at –18ºC, cell hydrophobicity, zeta potential, and attachment and recovery for each strain and growth temperature, were compared using the Duncan's multiple

range test and correlation among these characteristics were computed using Pearson coefficient. Differences between mean values were considered significant at α=0.05.

RESULTS AND DISCUSSION

Since there is no killing step to eliminate pathogens on produce but may be a decontamination treatment, surrogates may be used on produce to validate attempts to decontaminate produce during handling. The use of naturally occurring pathogens as indicators for evaluation and validation studies is questionable because they may be present at relatively low concentration in or on the produce and have a wide variations in concentration. Inoculation of surrogates can be experimentally controlled and evaluated. We used 5 non-pathogenic *E .coli* strains to compare with the behavior of *E. coli* O157:H7 for attachment to chilled produce based on cryotolerance and cell surface properties. To evaluate temperature effect to the characteristics of strains, we chose 37 and 20ºC to represent two extremes of produce growing and harvesting temperatures.

Cryotolerance. Initial populations of all strains grown at 37ºC were higher than 20ºC-grown strains by 0.4 to 0.8 log cfu/ml (Data not shown). Cryotolerance of *E. coli* O157:H7 and non-pathogenic *E. coli* strains was determined as the ability to withstand freezing at –18ºC. When the cultures were grown at 20ºC, cryotolerance was significantly higher than 37ºC-grown cultures regardless of strains (Fig. 4-1). For example, with *E. coli* O157:H7 932, 49.0% of the cells survived after freezing for 1 d when they were grown at 37ºC, whereas, after growing at 20ºC , survival rate was 89.1%. The survival rate for non-O157:H7 strains was lower; being between 43.4 and 79.4 % for 20ºC-grown cultures, and between 18.2 and 44.3% for 37ºC-grown cells with *E. coli* K-12 MC 4100 showing the lowest rate regardless of growth temperatures. The populations of the cells were decreased by less than 1 log cfu/ml over 7 d storage, though

populations of the strains were as most reduced after 1 d storage at –18ºC followed by less than 0.1 log reduction for 20ºC-grown cells and 0.3 log reduction for 37ºC-grown cells during storage time (Fig 4-2). Grazdkowska and Griffiths (2001) compared the cryotolerance of *E. coli* O157:H7 strains with non-pathogenic strains of *E. coli* before and after cold shock. They concluded cold shocked (exposed to 10ºC for 6 h) cultures survived better than non-cold shocked (held at 20ºC) cultures at –18ºC. The growth temperature of 20ºC used in this study was not enough to produce cold shock effect, although it allowed the cells to survive at a higher rate. Overall, *E. coli* ATCC 25922 showed similar survival rate to *E. coli* O157:H7.

Capsule production. Capsular polysaccharides are highly hydrated molecules that are over 95% water and their composition is strain dependent. In *E coli*, its composition may be a K antigen or an O antigen (Whitfield and Roberts 1999). In addition, many strains of *E. coli* can produce an exopolysaccharide called colanic acid, major composition of slime polysaccharides. Capsule formation may protect the bacteria against hostile environments such as osmotic shock, low temperatures, and desiccation (Gottesman and Stout 1991; Roberts 1996; Sledjeski and Gottesman 1996; Whitfield and Roberts 1999). Although Hassan and Frank (2004) reported *E. coli* O157:H7 grown in TSB at 37ºC produced capsule, in this study, microscopic examination showed pathogenic *E. coli* O157:H7 and non-pathogenic *E. coli* did not produce capsules at either growth temperatures (data not shown). This is not unexpected because the production of capsule and slime polysaccharides is strain dependent and those are generally not produced under normal laboratory growth conditions (Kelly and Georgopoulos 1997; Yeh and Chen 2004). In addition, if the cells were surrounded by microcapsules, which could be difficult to detect by phase contrast microscopy (Hassan and Frank 2004).

Curli expression. Curli are thin, coiled, aggregative fimbriae that are typically produced under stressful environmental conditions. Many strains of non-pathogenic *E. coli* produce curli. Curli expression by *E. coli* O157:H7 was rare but when it occurs the organism is more virulent (Uhlich et al. 2001, 2002). In *E. coli* K-12, curli production is usually induced when entering the stationary phase when grown at a temperature like 26ºC (Olsén et al. 1993). In this study, we found that *E. coli* O157:H7 strain 932 grown at both 37 and 20ºC revealed curli expression in all replicates, although curli expression at 20ºC was consistent with strong positive indication on CRI agar (all red colonies), with few negative colonies on CRI agar (pink or colorless) at 37ºC. At 37ºC, curli production by *E. coli* ATCC 11775, *E. coli* K-12 LMM 1010 and MC 4100 were positive in some replicates with a few negative colonies on CRI agar, whereas at 20ºC, all these strains produced curli in all replicates. When *E. coli* ATCC 25922 was grown at 20ºC, there was a mix of curli positive and negative colonies, whereas no curli was produced at 37ºC. Neither variant (red or white) altered its phenotype when recultured on CRI agar. These findings indicate that the curli expression in this strain was not inhibited at 37ºC but more consistent when they were grown at 20ºC. In addition, curli expression may be a stable phenotype because repeated culture of white (negative) colonies did not cause them to express the red (positive) colonies, and vice versa. It should be noted, however, that curli expression was observed under ideal growth conditions, and such stability may not be the case on lettuce surface for attachment.

Hydrophobicity. When each strain was compared at between 20 and 37ºC growth temperature, there were no significant differences ($p>0.05$) in hydrophobicity, although all strains grown at 20ºC appeared slightly more hydrophobic than 37ºC-grown cells with exception of *E. coli* ATCC 25922 (Table 4-1). Hydrophobicity of *E. coli* O157:H7 grown at 20 and 37ºC

was 60.9 and 56.2%, respectively, showing most hydrophobic among strains. When *E. coli* ATCC 25922 was grown at 20ºC, hydrophobicity was 51.2%, whereas hydrophobicity was 53.5% at 37ºC growth temperature. When comparing hydrophobicity for those grown at 37ºC, there was significant differences ($p<0.05$) among strains, but *E. coli* O157:H7 and *E. coli* ATCC 25922 were not significantly different. Hydrophobicity of *E. coli* ATCC 25922 at 20ºC was closest to *E. coli* O157:H7. Based on the classification of Li and McLandsborough (1999), *E. coli* O157:H7 and *E. coli* ATCC 25922 exhibited strong hydrophobicity and rest of strains were moderately hydrophobic. No correlation between curli expression and hydrophobicity of the cell surface ($r=0.0976$) was found in this study, although Boonaert and Rouxhet (2000) reported the protein/polysaccharide ratio, and the type and orientation of hydrocarbons and polysaccharides in the cell envelop affect cell surface hydrophobicity.

Zeta potential. The zeta potential of non-pathogenic *E. coli* and *E. coli* O157:H7 cells was –4.95 to –10.92 mV, showing significant differences ($p<0.05$) among strains, but *E. coli* O157:H7 and *E. coli* ATCC 25922 were not significantly different (Table 4-1). The zeta potential of 20ºC-grown cells appeared slightly higher negative surface charge than those of 37ºC-grown cells with exception of *E. coli* ATCC 25922, although there were no significant differences ($p>0.05$). The zeta potential of *E. coli* ATCC 25922 was closest to *E. coli* O157:H7 at both growth temperatures. Bacterial cells have a net negative charge on the cell wall, although the magnitude of this charge varies between species, serotypes or strains, and can change with variation in growth conditions, physiological states of cells, and composition of suspension media (Doyle 1991; Hassan and Frank 2004). Li and McLandsborough (1999) reported dramatic differences of zeta potential within *E. coli* strains (–4.9 to –33.9 mV) and suggested more caution needed when studying bacterial adhesion or developing removal

strategies due to strain variability of surface charge. Boonaert and Rouxhet (2000) revealed the electrokinetic properties of bacteria cells is influenced by carboxyl groups involved in the protein of the S-layer or polysaccharides and the relative exposure of proteins and phosphate groups at the surface. Variance of zeta potential among strains in our study suggested that electrostatic interactions may be responsible for difference in bacterial attachment ability.

Attachment and recovery test. Adhesion of *E. coli* O157:H7 and non-pathogenic *E. coli* grown at 20 and 37ºC to lettuce was determined (Fig 4-3, Table 4-2). The initial population of *E. coli* O157:H7 grown at 20ºC was 6.21 log cfu/cm^2 and 5.12 log cfu/cm^2 attached on the lettuce surface after inoculating for 1 h at 20ºC, showing 8.35 percent of attachment rate. There was no significant difference in the initial population of possible surrogate microorganisms grown at 20ºC (5.90 - 6.45 log cfu/cm^2) except for *E. coli* K-12 MC 4100 (5.90 log cfu/cm^2). However, the attachment of possible *E. coli* surrogates showed significant difference among strains (1.09 - 4.33%), with the greatest rate in *E. coli* ATCC 25922, 52% compared with that of *E. coli* O157:H7. The attachment rate of microorganisms grown at 37ºC was less than that of 20ºC with exception of *E. coli* ATCC 25922. *E. coli* ATCC 25922 grown at 37ºC attached at similar rate (4.38%) on the lettuce as 20ºC of growth temperature (4.33%), indicating 79% attachment rate compared to that of *E. coli* O157:H7. There was approximately 2 log reduction after rinsing with 200 ppm free chlorine solution with range of 1.7 - 2.4 log cfu/cm^2 regardless of strains.

Bacterial attachment is influenced by cell surface charge, hydrophobicity, and structures, including extracellular polysaccharides (Frank 2001). Prigent-Combaret et al. (2000) reported that non-pathogenic curli-producing *E. coli* is likely to attach better than curli-deficient strains on hydrophobic surface of polystyrene. Martino et al. (2003), however, reported a positive

correlation between the expression of type III pili and the adherence by *Klebsiella pneumoniae* to hydrophilic glass as well as hydrophobic polystyrene surfaces. On the other hand, Ryu et al. (2004) founded that curli production by *E. coli* O157:H7 did not affect attachment of cells on stainless steel but enhanced its ability to form biofilms. It was expected that the production of curli would increase hydrophobicity of the cell surface, resulting in an increase of attachment of cells to hydrophobic lettuce surface. However, curli production by *E. coli* strains did not affect hydrophobicity ($r=0.0976$, $p=0.7628$). Only curli expression by *E. coli* O157:H7 increased hydrophobicity and showed better attachment to lettuce. Variation in zeta potential within strains might an important role in attachment with hydrophobicity. We found there was correlation among *E. coli* cell surface charge, hydrophobicity, and adhesion to lettuce. Pearson's correlation coefficient at the 0.05 level for surface charge vs. adhesion, surface charge vs. hydrophobicity, and hydrophobicity vs. adhesion was –0.9873, –0.8631, and 0.8965 respectively. These results differ from the findings of Bouttier et al. (1997) and Li and McLandsborough (1999). On the other hand, other studies demonstrated there were linear relationships between surface charges and adhesion to product (Dickson and Koohmaraie 1989; Ukuku and Fett 2002). Conflicting conclusions between bacterial surface properties and adhesion may be due to the variations of system used and bacterial strains tested. Therefore, caution may be needed to make any generalize conclusions based on limited species and strains. Based on cell surface charge, hydrophobicity and survival rate after storage at –18ºC, *E. coli ATCC* 25922 is a useful surrogate for *E. coli* O157:H7 on attachment studies of chilled produce.

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REFERENCE

Boonaert, C.J.P. and P. Rouxhet. 2000. Surface of lactic acid bacteria: relationships between chemical composition and physicochemical properties. *Appl. Environ. Microbiol*. 66:2548-2554.

Bouttier, S., C. Linxe, C. Ntsama, G. Morgant, M.N. Bellon-Fontaine, and J. Founiat. 1997. Attachment of *Salmonella choleraesuis* to beef muscle and adipose tissues. *J. food Prot*. 60:16-22.

Busta, F.F., T.V. Suslow, M.E. Parish, L.R. Beuchat, J.N. Farber, E.H. Garrett, and L.J. Harris. 2003. Comprehensive reviews in food science and food safety - The use of indicators and surrogate microorganisms for the evaluation of pathogens in fresh and fresh-cut produce. Vol. 2:179-185.

Dickson, J.S. and M. Koohmaraie. 1989. Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces. *Appl. Environ. Microbiol*. 55:832-836.

Doyle, R.J. 1991. Strategies in experimental microbial adhesion research. p. 291-316. *In*: Microbial cell surface analysis – Structural and physicochemical methods. Mozes, N., P.S. Handley, H.J. Busscher, and P.G. Rouxhet (Eds.), VCH Publishers, New York.

Duffy, S., J. Churey, R.W. Worobo, and D.W. Schaffner. 2000. Analysis and modeling of the variability associated with UV inactivation of *Escherichia coli* in apple cider. *J. Food Prot*. 63:1587-1590.

Eblen, D.R., B.A. Annous, and G.M. Sapers. 2005. Studies to select appropriate nonpathogenic surrogate *Escherichia coli* strains for potential use in place of *Escherichia coli* O157:H7 and *Salmonella* in pilot plant studies. *J. Food Prot*. 68:282-291.

Food and Drug Administration. 2000. Kinetics of microbial inactivation for alternative food processing technologies. Available at: http://vm.cfsan.fda.gov/~comm/ift-glos.html. Accessed 12 December 2006.

Frank, J.F., 2001. Microbial attachment to food and food contact surfaces. *Adv. Food Nutr. Res*. 43:320-370.

Gottesman, S. and V. Stout. 1991. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12. *Mol. Microbiol*. 5:1599-1606.

Grazdkowska, D. and M.W. Griffiths. 2001. Cryotolerance of *Escherichia coli* O157:H7 in laboratory media and food. *J. Food Sci.* 66:1169-1173.

Hassan, A.N. and J.F. Frank. 2004. Attachment of *Escherichia coli* O157:H7 grown in tryptic soy broth and nutrient broth to apple and lettuce surfaces as related to cell hydrophobicity, surface charge, and capsule production. *Int. J. Food Microbiol*. 96:103-109.

Kelly, W.L. and C. Georgopoulos. 1997. Positive control of the two component RcsC/B signal transduction network by DjiA: a member of the DnaJ family of molecular chaperons in *Escherichia coli*. *Mol. Microbiol*. 25:913-931.

Li, J. and L.A. McLandsborough. 1999. The effects of the surface charge and hydrophobicity of *Escherichia coli* on its adhesion to beef muscle. *Int. J. Food Microbiol.* 53:185-193.

Mak, P.P., B.H. Ingham, and S.C. Ingham. 2001. Validation of apple cider pasteurization treatments against *Escherichia coli* O157:H7, *Salmonella*, and *L. monocytogenes*. *J. Food Prot*. 64:1679-1689.

Martino, P.D., N. Cafferini, B. Joly, and A. Darfeuille-Michaud. 2003. *Klebsiella pneumoniae* type 3 pili facilitate adherence and biofilm formation on abiotic surfaces. *Res. Microbiol*. 154:9-16.

Ofek, I. and R.J. Doyle. 1994. Bacterial Adhesion to Cells and Tissues. Chapman and Hall, New York.

Olsén, A., A. Arnqvist, M. Hammar, S. Sukupolvi, and S. Normark. 1993. The RpoS sigma factor relieves H-NS-mediated transcriptional repression of *csgA*, the subunit gene of fibronectin-binding curli in *Escherichia coli*. *Mol. Microbiol*. 7:523-536.

Pao, S. and G. Davies. 2001. Comparing attachment, heat tolerance and alkali resistance of pathogenic and non-pathogenic bacterial cultures on orange surfaces. *J. Rapid Methods and Automation in Microbiol.* 9:271-278.

Pawar, D.M., M.L. Rossman, and J. Chen. 2005. Role of curli fimbriae in mediating the cells of enterohaemorrhagic *Escherichia coli* to attach to abiotic surfaces. *J. Appl. Microbiol*. 99:418-425.

Peri, A. 2003. Screening surrogates for food-borne pathogens: pH, water activities and high temperatures. M.S. Thesis. Illinois Institute of Technology. Chicago, IL.

Peri, A., S. Keller, S. Ravishankar, C. Rodriguez, and P.J. Slade. 2002. Bacterial pathogen surrogate evaluation - tolerance of candidates to acid and alkaline pH and reduced water activity. Session 61C-10. IFT Annual Meeting. Anaheim, CA.

Prigent-Combaret, C., G. Prensier, T.T. Le Thi, O. Vidal, P. Lejeune, and C. Dorel. 2000. Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains, role of flagella, curli and colanic acid. *Environ. Microbiol*. 2:450-464.

Roberts, I.S. 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annu. Rev. Microbiol*. 50:285-315.

Ryu, J.-H., H. Kim, J.F. Frank, and L.R. Beuchat. 2004. Attachment and biofilm formation on stainless steel by *Escherichia coli* O157:H7 as affected by curli production. *Lett. Appl. Microbiol*. 39:359-362.

Sapers, G.M. and J.E. Sites. 2003. Efficacy of 1% Hydrogen peroxide wash in decontaminating apples and cantaloupe melons. *J. Food Sci*. 68:1793-1797.

Sledjeski, D.D. and S. Gottesman. 1996. Osmotic shock induction of capsule synthesis in *Escherichia coli* K-12. *J. Bacteriol*. 178:1204-1206.

Thayer, D.W. and G. Boyd. 1993. Elimination of *Escherichia coli* O157:H7 in meats by gamma irradiation. *Appl. Environ. Microbiol.* 59:1030-1034.

Uhlich, G.A., J.E. Keen, and R.O. Elder. 2001. Mutations in the *csgD* promoter associated with variations in curli expression in certain strains of *Escherichia coli* O157:H7. *Appl. Environ. Microbiol.* 67:2367-2370.

Uhlich, G.A., J.E. Keen, and R.O. Elder. 2002. Variations in the *csgD* promoter of *Escherichia coli* O157:H7 associated with increased virulence in mice and increased invasion of HEp-2 Cells. *Infect. Immun.* 70:395-399.

Ukuku, D.O. and W.F. Fett. 2002. Relationship of cell surface charge and hydrophobicity to strength of attachment of bacteria to cantaloupe rind. *J. Food Prot.* 65: 1093-1099.

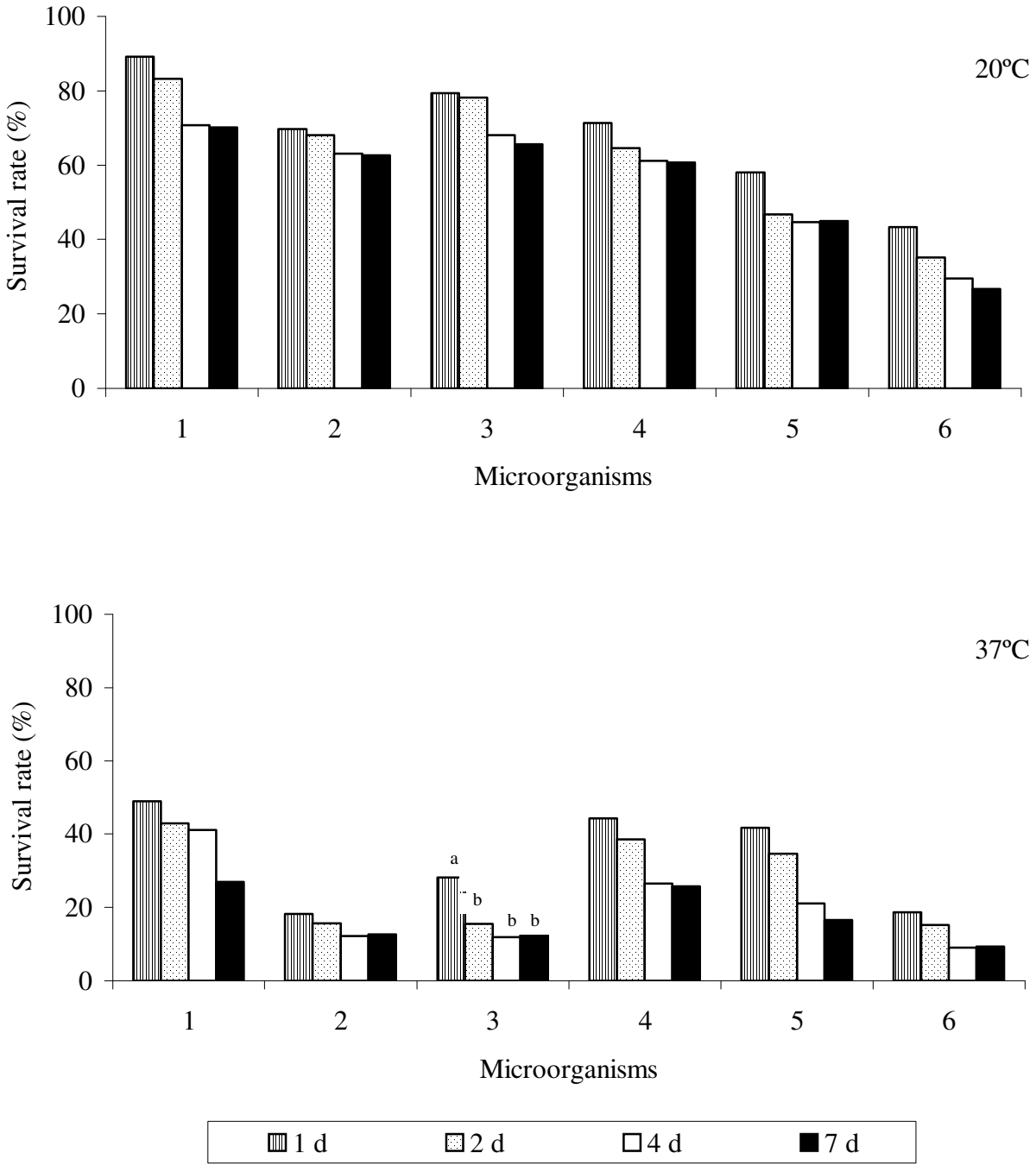
Whitfield, C. and I.S. Roberts. 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. *Mol. Microbiol*. 31:1307-1319.

Yeh, J.Y. and J. Chen. 2004. Production of slime polysaccharide by EHEC and STEC as well as the influence of culture conditions on slime production in *E. coli* O157:H7. *Lett. Appl. Microbiol*. 38:488-492.

Fig 4-1. Cryotolerance of non-pathogenic *E. coli* strains and *E. coli* O157:H7 grown at 20ºC (top) and 37ºC (bottom) during storage at –18ºC for 7 days. Survival (%): count after freezing (cfu)/count before freezing (cfu) x 100. Different letters within strain type indicate significantly differences ($p<0.05$) between means at each storage time.

Fig 4-2. Population reduction of non-pathogenic *E. coli* strains and *E. coli* O157:H7 grown at 20ºC (top) and 37ºC (bottom) during storage at –18ºC for 7 days. Population reduction (log cfu/ml) = count before freezing – count after freezing. Different letters within storage time indicate significantly differences ($p<0.05$) between means among strain types.

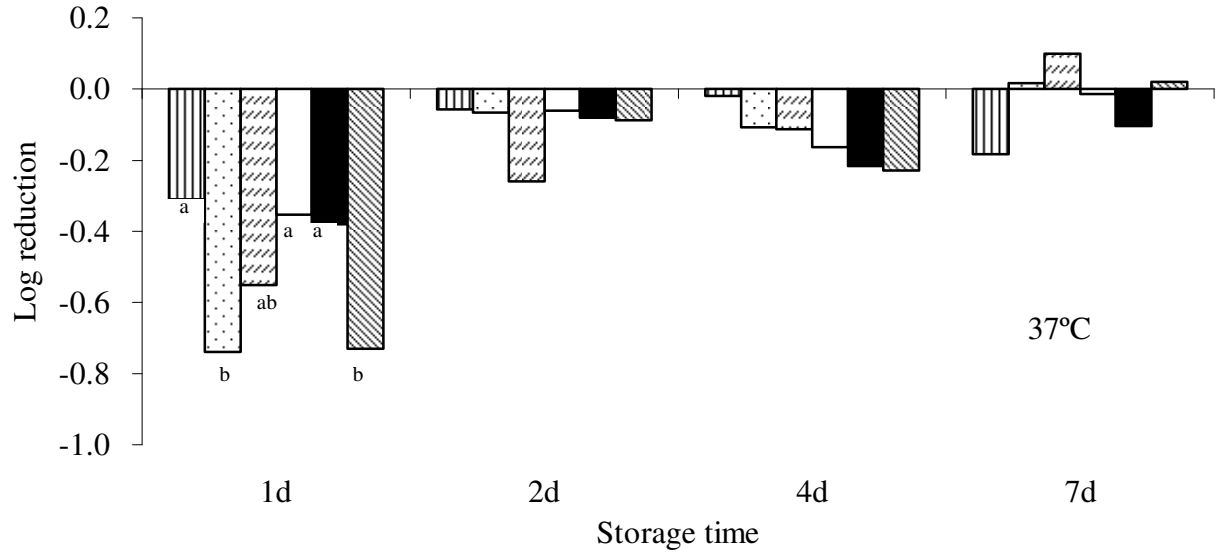
Fig 4-3. Attachment (%) of non-pathogenic *E. coli* strains and *E. coli* O157:H7 grown at 20 and 37°C to romaine lettuce leaf surfaces. Different letters within growth temperature indicate significantly differences ($p<0.05$) between means among strain types. The value in parentheses is relative percentage compared to attachment of *E. coli* O157:H7.



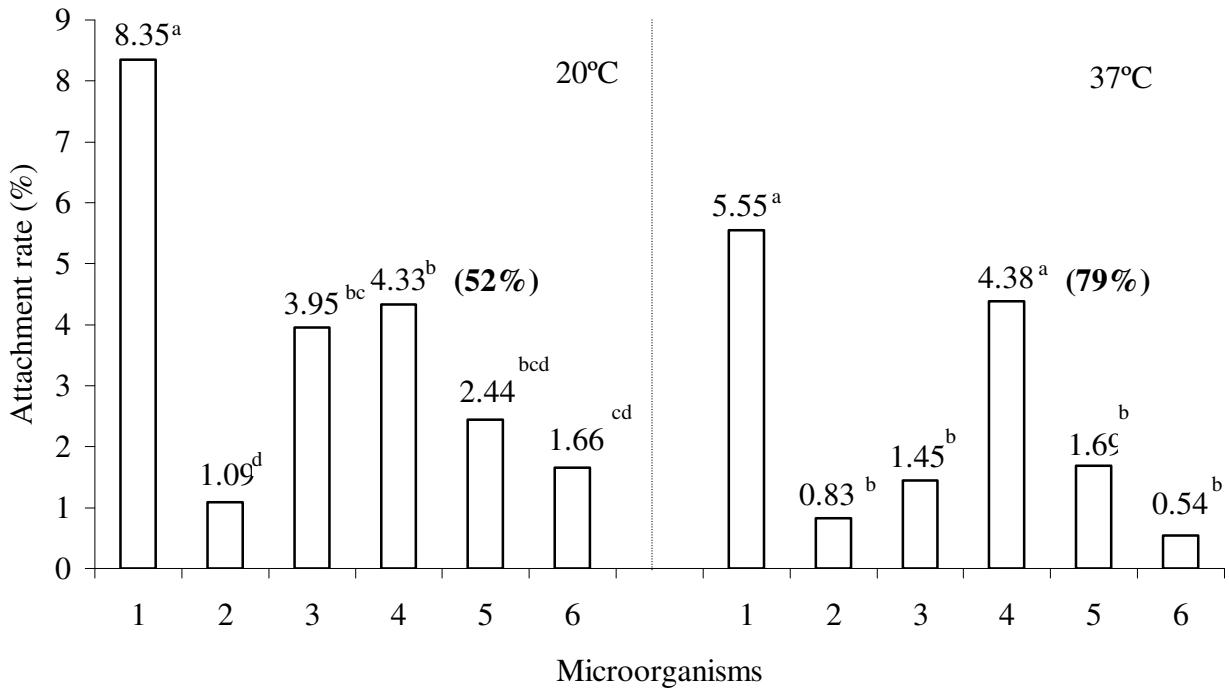
1: *E. coli* O157:H7 932 2: *E. coli* ATCC 8677 3: *E. coli* ATCC 11775

4: *E. coli* ATCC 25922 5: *E. coli* K-12 LMM 1010 6: *E. coli* K-12 MC 4100


0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Log reduction
a a a ab b b
20ºC
1d
2d
4d
7d
Storage time


0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Log reduction
a b ab a a b
37ºC
1d
2d
4d
7d
Storage time


E. coli O157:H7
E. coli ATCC 8677
E. coli ATCC 11775
E. coli ATCC 25922
E. coli K12 LMM 1010
E. coli K12 MC 4100



1: *E. coli* O157:H7 932 2: *E. coli* ATCC 8677 3: *E. coli* ATCC 11775

4: *E. coli* ATCC 25922 5: *E. coli* K-12 LMM 1010 6: *E. coli* K-12 MC 4100

Table 4-1. Hydrophobicity and zeta potential of non-pathogenic *E. coli* strains and *E. coli* O157:H7 grown in TSB at 20 and 37°C

Growth temp (°C)	Strains	Hydrophobicity (%)	Zeta potential (mV)
20	O157:H7 932	60.92[a]	–10.92[a]
	ATCC 8677	45.38[a]	–5.39[d]
	ATCC 11775	46.17[a]	–7.51[bc]
	ATCC 25922	51.22[a]	–8.17[b]
	K-12 LMM 1010	45.09[a]	–7.06[bcd]
	K-12 MC 4100	48.27[a]	–6.02[cd]
37	O157:H7 932	56.17[a]	–8.96[a]
	ATCC 8677	45.54[c]	–5.74[b]
	ATCC 11775	43.67[c]	–6.40[b]
	ATCC 25922	53.53[ab]	–8.27[a]
	K-12 LMM 1010	44.85[c]	–6.47[b]
	K-12 MC 4100	47.40[bc]	–4.95[b]

[abcd] Means with the different letters in a column within growth temperature are significantly different ($p<0.05$)

Table 4-2. Attachment and recovery of non-pathogenic *E. coli* strains with *E. coli* O157:H7 grown in TSB at 20 and 37°C from romaine lettuce leaf surfaces

Growth Temp(°C)	Strains	Log cfu/cm^2			
		Initial Populations	Attachment	Recovery	Pop. Reductions
20	O157:H7 932	6.21^{a}	5.12^{a}	2.77^{ab}	2.35^{a}
	ATCC 8677	6.45^{a}	4.44^{b}	2.63^{b}	1.81^{c}
	ATCC 11775	6.44^{a}	5.00^{a}	3.05^{a}	1.95^{bc}
	ATCC 25922	6.44^{a}	5.08^{a}	2.83^{ab}	2.25^{ab}
	K-12 LMM 1010	6.24^{a}	4.58^{b}	2.69^{b}	1.89^{bc}
	K-12 MC 4100	5.90^{b}	4.09^{c}	2.25^{c}	1.84^{c}
37	O157:H7 932	6.65^{b}	5.38^{a}	3.16^{bc}	2.22^{a}
	ATCC 8677	7.27^{a}	5.13^{b}	3.02^{cd}	2.11^{ab}
	ATCC 11775	7.28^{a}	5.38^{a}	3.45^{a}	1.93^{b}
	ATCC 25922	6.86^{ab}	5.50^{a}	3.31^{ab}	2.19^{a}
	K-12 LMM 1010	6.65^{b}	4.88^{c}	2.96^{d}	1.92^{b}
	K-12 MC 4100	6.50^{b}	4.23^{d}	2.58^{e}	1.65^{c}

^{abcde} Means with the different letters in a column within growth temperature are significantly different ($p<0.05$)

CHAPTER 5

ATTACHMENT PROPERTIES OF *ESCHERICHIA COLI* O157:H7 AND A NON-PATHOGENIC SURROGATE SUBJECTED TO STARVATION[1]

[1] Jin Kyung Kim and Mark A. Harrison. To be submitted to *Journal of Food Protection*, 2007.

***Introduction*:** Water is widely used in fresh produce production. Survival of *E. coli* O157: H7 in water for extended periods can induce starvation. Starvation may enhance *E. coli* O157:H7 survival under subsequent stresses by changing surface morphology which may affect adherence to produce.

Purpose: This study determined the effect of starvation on attachment of *E. coli* O157:H7 and a surrogate, *E. coli* ATCC 25922, to lettuce.

Methods: After starvation (at 37°C for 4 h, 20°C for 24 h, or 4°C for 7 d), cryotolerance, cell surface characteristics (hydrophobicity, zeta potential, morphology, and outer membrane proteins) and attachment to lettuce were investigated.

Results: Overall *E. coli* ATCC 25922 exhibited a greater starvation effect and cryotolerance than *E. coli* O157:H7. For example, starved (4ºC for 7 d) *E. coli* ATCC 25922 showed a 17.6% increase in survival rate, whereas the corresponding increase for *E. coli* O157:H7 was 8.8%. Hydrophobicity of both strains decreased over 7 d storage and zeta potential of *E. coli* ATCC 25922 was more consistent (–8.14 to –6.91 mV) than *E. coli* O157:H7 (–10.30 to –6.21 mV). There were few differences in outer membrane protein (OMPs) patterns of cells exposed to various starvation conditions, whereas intensity of some OMPs of *E. coli* ATCC 25922 was different from *E. coli* O157:H7. Starved cells of both strains appeared elongated, wrinkled and malformed. Starved cells of both strains attached to lettuce less than non-starved cells (49% of *E. coli* O157:H7 and 69% of *E. coli* ATCC 25922 compared to non-starved cells).

Significance: Starvation affects cryotolerance and cell surface properties of *E. coli* O157:H7 and *E. coli* ATCC 25922 at somewhat different degrees. Thus selection of a surrogate should be done under specific environmental conditions. *E. coli* ATCC 25922 is a useful surrogate for *E. coli* O157:H7 for produce attachment and recovery studies.

INTRODUCTION

Sublethal exposure to various stresses may enhance the survival of bacteria under the subsequent stress conditions and this enhancement may offer cross-protection against other stresses (Abee and Wouters 1999). *E. coli* O157:H7 exhibits a stress response to sublethal environmental stresses. For example, acid stress enhanced subsequent acid tolerance, heat tolerance, and freeze-thaw tolerance (Leenanon and Drake 2001; Williams and Ingham 1998). Under stress conditions, *E. coli* O157:H7 may produce several stress proteins and alter membrane lipid composition to survive in these environments (Arneborg et al. 1993; Carty et al. 1999; Gawanda and Griffiths 2005a; Zhang and Griffiths 2003). Sainz et al. (2005) showed survival of pathogenic *E. coli* strains to different acid challenges might be related to the changes in outer membrane protein profiles. Other studies demonstrated that a stress response may affect subsequent virulence (Buncic and Avery 1998; Elhanafi et al. 2004; Yuk and Marshall 2003).

Water is widely used material in fresh produce production and processing. Starvation can occur under the conditions which microorganisms exist in water for extended periods (Harakudo et al. 2000; Rockabrand et al.1995). Several studies showed *E. coli* O157:H7 can survive in water for extended periods under starvation conditions (LeJeune et al. 2001; Maule 2000; Rice and Johnson 2000; Wang and Doyle 1998; Warburton et al. 1998). Previous work has shown that the stress imposed by starvation changed the ability of *E. coli* O157:H7 to survive heat treatments and that this thermal tolerance correlated with induction of the heat shock proteins (Rowe and Kirk 2000). Kim and Kim (2004) showed curli expression of *E. coli* O157:H7 at 37ºC affected adherence to host epithelial cells. Few studies have addressed the impact of starvation (held in water for extended periods) of *E. coli* O157:H7 on cell surface

characteristics including surface structure, hydrophobicity, surface charge, and outer membrane proteins and the relationship between the change of cell surface characteristics and attachment to produce.

Surrogates are preferred instead of pathogenic microorganisms to evaluate the efficacy of intervention strategies in food processing operations without the potential risk posed by pathogens to both the production environment and researchers (FDA 2000). Many studies using *E. coli* ATCC 25922 under different environments have been conducted (alkaline pH, high temperature, heat resistance before and after acid stress, gamma radiation in meats, UV inactivation in apple cider; Duffy et al. 2000; Leenanon and Drake 2001; Pao and Davies 2001; Peri et al. 2002; Thayer and Boyd 1993). The results of these studies suggested that *E .coli* ATCC 25922 behaved differently under specific process conditions; surrogates may not fit all challenged situations to the same degree. A previous study revealed *E. coli* ATCC 25922 was a suitable surrogate for *E. coli* O157:H7 on attachment to produce based on cryotolerance and cell surface properties (Kim and Harrison 2007). Starvation may enhance *E. coli* O157:H7 and surrogate *E. coli* survival under the subsequent stresses by changing surface morphology which may affect adherence to produce. The objective of the present study was to determine the effect of starvation of *E. coli* O157:H7 and *E .coli* ATCC 25922 on the subsequent survival at –18ºC, cell surface properties and relation of the change in these characteristics with attachment and recovery to produce.

MATERIALS AND METHODS

Bacterial Strains. *E. coli* O157:H7 932 (human isolate) and *E. coli* ATCC 25922 were used. They were determined to be sensitive to a level of 50 µg /ml nalidixic acid (Sigma

Chemical Co., St. Louis, MO). They were made nalidixic acid-adapted through the transfers in tryptic soy broth (TSB, Becton, Dickinson and Co., Sparks, MD) supplemented with increasing concentrations of nalidixic acid (TSBN) from 2.5 to 50 µg /ml for 7 days at 20 or 37ºC. Nalidixic acid-adapted cells were surface plated on tryptic soy agar supplemented with 50 µg of nalidixic acid/ml (TSAN) and incubated at 20ºC for 24 h or 37ºC for 16 to 18 h. Stock cultures were stored at –80ºC in culture broth containing 15% glycerol until used.

Starvation. Bacteria were activated by two successive transfers of frozen stock in 10 ml of TSBN and incubated for 16 to 18 h at 37°C or 24 h at 20°C. These active cultures were transferred to 1 L of TSBN and incubated. Cultures were harvested by centrifugation at 9,000 x g for 10 min, the pellets were washed twice with sterile deionized water (SDW), and resuspended in PBS and held for 4 h at 37°C, for 24 h at 20°C, and for 7 days at 4°C.

Cryotolerance. After starvation, *E. coli* O157:H7 and *E. coli* ATCC 25922 in PBS (l ml in 1.5-ml tubes) were frozen at –18ºC for 1, 2, 4 and 7 days. After storage at –18ºC, control and starved cells were thawed at room temperature (ca. 20ºC) within 15 min and the viable count was estimated by surface plating onto TSAN using a spiral-plater (Autoplate 4000, Spiral Biotech, Bethesda, MD). Results were expressed in terms of survival and starvation effect (%).

Starvation effect = % survival of starved cells - % survival of control cells

Capsule and curli production. To examine the presence of capsular polysaccharide, the cells were stained with India ink (Remel, Lenexa, KS). The preparations were made by mixing a loopful of control and starved cells of each strain from PBS after frozen cells thawing at room temperature (ca. 20ºC) within 15 min at each storage time with one drop of India ink on a slide. A coverslip was pressed down firmly over the preparation, and it was examined with a phase-contrast microscope at ×1,000 magnification. For curli expression, control and starved

cells of each strain from PBS after frozen cells thawing at room temperature (ca. 20ºC) within 15 min at each storage time was incubated at 28°C for 48 h on Congo red indicator (CRI) agar, which contained 0.1% Bacto tryptone, 0.05% Bacto yeast extract, 1.5% Bacto agar, 40 mg/L of Congo red and 20 mg/L of Coomassie brilliant blue (Sigma Aldrich Co, St Louis, MO). Curli-producing *E. coli* bind Congo red dye and form red colonies on CRI, whereas curli-negative bacteria form white colonies (Pawar et al. 2005). Stability of curli expression was determined by directly reculturing isolates onto CRI and incubating for 48 h at 28°C.

Hydrophobicity. Hydrophobicity according to storage time was determined as described by Hassan and Frank (2004). After frozen cells thawing at room temperature (ca. 20ºC) within 15 min at each storage time, 1 ml of xylene was added to each of three tubes containing 4 ml of cells suspended in PBS. Tubes were vortexed for 2 min and placed in a 37ºC water bath for 30 min. The optical density at 600 nm of the aqueous layer in each tube was determined using Beckman DU-530 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). The results were expressed as percentages calculated by using the following relationship: percent hydrophobicity = 100(1 – final optical density/optical density of initial bacterial cells).

Zeta potential measurements. After storage at –18ºC, control and starved cells were thawed at room temperature (ca. 20ºC) within 15 min at each storage time. Zeta potentials were measured for bacteria with a population of ~10^7 cfu/ml in PBS (pH 7.4) using Brookhaven's Zeta Plus instrument (Brookhaven Instruments, Holtsville, NY). Zeta potential measurements for each strain were repeated at least three times.

Electron microscopy. The control and starved cells were fixed by immersion in 2% glutaraldehyde in PBS (pH 7.4) for 2 h, washed twice in buffer for 15 min per each and postfixed with 1% osmium tetroxide in PBS for 1 h at 4°C and washed several times in SDW for 10 min

per each. For scanning electron microscopy (SEM), the rinsed samples were filtered onto the membrane substrate using a microanalysis filter holder and a 0.2 µm-pore-size polycarbonate filter (Nuclepore Co., Pleasanton, CA) and dehydrated in a series of ethanol solutions (30 to100%) for 10 min per immersion. During the entire filtration and dehydration, the cells were kept covered with fluid to prevent air drying. The filters were transferred in 100% ethanol to a critical point dryer (SAMDRI 780A, Tousimis Research Co., Rockville, MD) and dried using carbon dioxide as the transition solvent. The filters were mounted on aluminum stubs with carbon adhesive tabs and coated with 15.3 nm gold using a sputter coater (SPI Module, SPI Supplies and Structure Probe Inc.,West Chester, PA), and viewed on LEO 982 field emission scanning electron microscope at 20 kV of accelerating voltage (FE-SEM, LEO Electron Microscopy, Inc., Thornwood, NY).

For transmission electron microscopy (TEM), the dehydrated samples were infiltrated with propylene oxide (PO) and PO/EPON 812 mixtures (33, 66, 100% EPON for 1 h each and 100% EPON overnight) and embedded in pure EPON 812 (Polyscience, Inc., Warrington, PA). The samples were polymerized for 1 d at 65°C and ultrathin sections (70 nm) were cut with glass knives on a Sorvall MT-2 Ultramicrotome, and mounted on 400 mesh copper grids, followed by post staining with uranyl acetate and lead citrate. The samples were searched at an accelerating voltage of 100 kV using the Tecnai 20 (FEI Co., Eindhoven, Netherlands). All specimen samples were prepared in duplicate.

Outer membrane proteins (OMPs) profile. Outer membrane proteins were prepared by a modification of the method of Leyh and Griffith (1992). After measuring OD at 600 nm, the cells grown under different starvation conditions were harvested by centrifugation at 5,000 x g for 20 min and frozen overnight. Ten mM HEPES (pH 7.4) buffer was added to frozen cells

to adjust OD_{600} to 0.2 and the cells were thawed. The cells were lysed by sonication for 1 min and cell debris was removed by centrifugation for 20 min at 5,000 x g. The supernatant was then centrifuged at 100,000 x g for 1 h and the total membrane fraction was harvested from the pellet. The membrane fraction was suspended in 20 ml of 1% (w/v) sodium lauryl sarcosine in 10 mM HEPES (pH 7.4) buffer for 3 h at 4°C. The detergent-insoluble material containing the OMP-enriched fraction was harvested by centrifugation at 100,000 x g for 1 h at 4°C. The final insoluble pellet (outer membrane enriched proteins) was suspended in 10 ml of 10 mM HEPES buffer and used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Forty microliters of total OMPs were mixed with 5 µl of 5 x loading buffer (31 mM Tris [pH 6.8], 2%SDS, 2.5% 2-mercaptoethanol, 10% glycerol), and the proteins were boiled for 10 min. Proteins were resolved by electrophoresis in Criterion Precast Gel System (4-20% gradient Tris-HCl, Bio-RAD Laboratories Inc., Hercules, CA) and the gels run at 200 V until the tracking dye had reached the bottom of the gel. BenchMark™ Pre-Stained Protein Ladder or BenchMark™ Protein Ladder (Invitrogen Co., Carlsbad, CA) were run at the same time. Proteins in the gels were visualized by staining with Coomassie brilliant blue.

Attachment and recovery tests. Romaine lettuce (*Lactuca sativa* var. *longifolia*) was purchased at the same day in a local supermarket (Athens, GA). After storage at –18ºC, control and starved cells were thawed at room temperature (ca. 20ºC) within 15 min at each storage time. Lettuce pieces (3x3 cm) were prepared using a sterile template and blade. Six pieces of lettuce were spot inoculated with 100 µl of each strain suspension of *E. coli* O157:H7 and *E. coli* ATCC 25922 and held in a biosafety hood to allow the inoculum to dry for 1 h at 20ºC. After 1 h, pieces were gently rinsed with 5 ml of SDW to remove unattached or loosely attached cells and placed into stomacher bags. Three pieces were used to determine attachment and 3 for recovery

after rinsing with chlorinated water. The chlorine solutions were prepared each day from a sodium hypochlorite stock solution. The solutions were prepared in SDW stored at 4ºC overnight in advance with 0.1 N cirtric acid to adjust to pH 7.0 ± 0.2 and the concentration of free chlorine was verified by Aquachek total/free chlorine test kits (Hach Company, Loveland, CO). For the recovery test, 100 ml of chlorinated water (200 µg free chlorine/ml) was added to stomacher bag and vigorously shaken by hand for 1 min and immediately neutralized with 30 ml of 0.1 N sodium thiosulfate.

Microbiological analysis. Lettuce samples were homogenized with 100 ml of modified EC broth (Becton, Dickinson and Co.) for 1 min in a stomacher at normal speed. Populations were enumerated on TSAN using a spiral-plater. Uninoculated lettuce pieces were analyzed for the presence of nalidixic acid-adapted *E. coli* O157:H7 and *E. coli* using TSAN after incubation at 37ºC for 24 h in enrichment broth.

Statistical analyses. Data were analyzed using analysis of variance (Statistical Analysis Systems Institute, Ver. 9.1, Cary, NC). If differences were significant at α=0.05, populations during storage at -18ºC, cell hydrophobicity, zeta potential, and attachment and recovery for *E. coli* O157:H7 and *E. coli* ATCC 25922 and two growth temperatures were compared using the Duncan's multiple range test. Differences between mean values were considered significant at α=0.05.

RESULTS AND DISCUSSION

To determine the effect of starvation on *E. coli* O157:H7 and *E. coli* ATCC 25922, three starvation conditions (held in PBS at 37ºC for 4 , 20ºC for 24 h, or 4ºC for 7 d) and two temperatures (20 or 37ºC) were used. Factors evaluated for both microorganisms were

cryotolerence at –18ºC, cell surface characteristics including morphology, hydrophobicity, zeta potential, and outer membrane protein profile, and microbial attachment to and recovery from produce. These factors were measured at the initial day of each starvation condition before freezing, and at 1, 2, 4, and 7 days during storage at –18ºC.

Cryotolerance. Initial populations of *E. coli* O157:H7 and *E. coli* ATCC 25922 grown at 37ºC were higher than 20ºC-grown strains by 0.4 log cfu/ml (Data not shown). Fig. 5-1 showed average survival rate of both strains in each starvation condition during storage at –18ºC. There were significant differences in cryotolerance of both strains grown at 20ºC during frozen storage regardless of starvation condition, whereas the cells grown at 37ºC showed no different cryotolerance with exception of *E. coli* ATCC 25922 starved at 37ºC for 4 h. However, populations of both *E. coli* O157:H7 and *E. coli* ATCC 25922 decreases by less than 1 log cfu/ml over 7 d storage. Growth temperature did not affect cryotolerance within the same starvation condition with exception of *E. coli* O157:H7 starved at 37ºC for 4 h. Within the same temperature and storage time, no significant difference in populations of cryotolerance among starvation conditions was observed. In addition, *E. coli* O157:H7 grown at 20ºC survived better than *E. coli* ATCC 25922, whereas 37ºC-grown *E. coli* ATCC 25922 survived better *E. coli* O157:H7 when they were starved at 37ºC for 4 h. Fig. 5-2 showed the effect of starvation in cryotolerance for *E. coli* ATCC 25922 than *E. coli* O157:H7 grown at 37ºC. For example, starved (4ºC for 7 d) *E. coli* ATCC 25922 showed a 17.6% increase in their ability to survive frozen storage for 1 d at –18ºC, whereas the corresponding increase in survival for *E. coli* O157:H7 was 8.8%. No starvation effect in 20ºC-grown cells was investigated. Very few reports are available on starvation-induced cryotolerance and freeze-thaw tolerance of *E. coli* strains (Leenanon and Drake 2001; Gawande and Griffiths 2005b). Leenanon and Drake

(2001) revealed freeze-thaw resistance of *E. coli* O157:H7 and nonpathogenic *E. coli* were enhanced after starvation and cold stress. Gawande and Griffiths (2005b) investigated the effect of starvation (37ºC for 6 h in water) on *E. coli* O157:H7 grown in different media after subsequent cryotolerance and concluded starved *E. coli* O157:H7 grown in TSB showed greater increase in their ability to survive frozen storage than non-starved cells. In addition, they found that both cryotolerance and starvation effects decreased significantly after 120 h storage at –18ºC. In disagreement with their findings, the present study showed there was no significant difference in cryotolerance and starvation effects for *E. coli* O157:H7 grown at 37ºC during storage over 7 d. This might be due to different strain types and slightly different starvation period for 37ºC used in our study. Overall, *E. coli* ATCC 25922 grown at 37ºC showed similar or better cryotolerance regardless of starvation conditions.

Capsule production. Many *Escherichia coli* strains are covered in a layer of surface-associated polysaccharide called the capsule. In *E. coli*, its composition may be a K antigen or an O antigen (Whitfield and Roberts 1999). Generally capsule formation of *E. coli* strains can occur under stress conditions such as osmotic and oxidative stress, low temperature, and desiccation (Chen et al. 2004; Sledjeski and Gottesman 1996; Whitfield and Roberts 1999). In a pervious study, Kim and Harrison (2007) used two temperatures (20 or 37ºC) for *E. coli* strains to investigate effect of temperature on capsule production, and all strains including *E. coli* O157:H7 did not produce capsules. In the present study, there was no capsule production for either strain when *E. coli* O157:H7 and *E. coli* ATCC 25922 grown at 20 or 37ºC were subjected to three different starvation conditions. This finding suggests starvation may not play a role in capsule production or further more time period may be needed to induce capsule production.

Curli expression. *E. coli* has ability to express thin aggregative fimbriae, known as curli, on the cell surface. Curli expression in serogroup O157:H7 of enterohemorrhagic *E. coli* (EHEC) has been reported (Cookson et al. 2002; Uhlich et al. 2001, 2002; Zogaj et al. 2003). Curli has a high affinity to Congo-red (CR) dye, therefore differentiation of curli expressing from non-curli expressing cells can be accomplished by growth on agar with no salt and the indicator dye after 48 h incubation at 28ºC (Olsén et al. 2002). According to Cookson et al. (2002), only 2 (4%) of 49 isolates among diverse bovine and human *E. coli* O157:H7 strains showed curli production, while 5 (38.5%) of 13 non-O157 STEC elaborated curli. Gophna et al. (2001) reported curli facilitated internalization of *E. coli* by epithelial and HEp-2 cell lines due to fibronectin binding. Other studies demonstrated curli played a role in autoaggregation and development of biofilm on inert surfaces (Prigent-Combaret et al. 2000; Uhlich et al. 2001, 2002). In a previous study, we found that *E. coli* O157:H7 grown at both 37 and 20ºC revealed curli expression, whereas *E. coli* ATCC 25922 produced curli only at 20ºC (Kim and Harrison 2007). Also, curli expression of *E. coli* O157:H7 at 20ºC was strongly positive on CRI agar (all red colonies), with a few negative colonies on CRI agar (pink or colorless) at 37ºC. In addition, red and white colonies retained their parental phenotypes when subcultured on CRI plates. We found curli expression of *E. coli* O157:H7 maintained in all starvation conditions over 7 d storage periods, although 37ºC-grown cells expressed a few negative colonies on CRI agar (data not shown). *E. coli* ATCC 25922 grown at 20ºC expressed curli on CRI agar with negative colonies in all starvation conditions at the initial day before freezing but expression disappeared during storage at –18ºC. *E. coli* ATCC 25922 grown at 37ºC and starved at 4ºC for 7 d expressed curli at the initial day. This may because altering environmental conditions such as temperature may cause curli-producing *E. coli* ATCC 25922 to revert to the white phenotype.

Hydrophobicity and surface charge. Growth temperature had no effect on hydrophobicity of starved cells at the initial day before freezing storage ($p>0.05$) (Fig. 5-3), which is in agreement with our previous study (Kim and Harrison 2007). At the initial day and 1 d after freezing storage, hydrophobicity of *E. coli* O157:H7 was significantly higher than *E. coli* ATCC 25922,whereas there were no differences in hydrophobicity of the strain types grown at both growth temperatures after 1 d storage. Hydrophobicity of both strains changed dramatically during storage with the greatest decrease 1 d after storage at –18ºC. Based on the classification of Li and McLandsborough (1999), *E. coli* O157:H7 and *E. coli* ATCC 25922 exhibited strong hydrophobicity at the initial day of starvation, changed into more hydrophilic and after 4 d of storage both strains were strongly hydrophilic.

Cell surface charge was determined as a zeta potential. In our previous study (Kim and Harrison 2007), the zeta potential of *E. coli* ATCC 25922 was the closest to *E. coli* O157:H7 at both growth temperatures of all the strains evaluated. Starvation conditions did not change zeta potential (Fig. 5-4). Before starvation, zeta potential of *E. coli* O157:H7 was highly negative than that of *E. coli* ATCC 25922 at both growth temperatures (–10.92 vs. –8.17 mV for 20ºC and –8.96 vs. –8.27 mV for 37ºC from our previous study; Kim and Harrison 2007). However, starvation diminished the difference in zeta potential of both strains (–7.91 vs. –7.44 mV for 20ºC and –7.11 vs. –7.51 mV for 37ºC over 7 d storage) with no significant difference regardless of starvation conditions. *E. coli* ATCC 25922 showed more consistent zeta potential (–8.14 to –6.91 mV) than *E. coli* O157:H7 (–10.30 to –6.21 mV) during storage over 7 d. According to Li and McLandsborugh (1999) *E .coli* strains have a net negative charge, although the magnitude of this charge varies from strains. They reported dramatic differences of zeta potential within *E. coli* strains (–4.9 to –33.9 mV) and suggested more caution needed when studying bacterial

adhesion or developing removal strategies due to strain variability of surface charge. Reports have rarely addressed change in hydrophobicity and zeta potential *E. coli* strains during storage time or starvation conditions. Dai et al. (1992) found that hydrophobicity of *Vibrio parahaemolyticus* cells from iron-rich cultures was much higher than from iron-limited culture. Our finding suggested storage time as well as starvation conditions may change hydrophobicity and zeta potential of *E. coli* strains although the degree of variation is different between strains. This might be because starvation may change carboxyl groups, polysaccharides, and the relative exposure of proteins and phosphate groups at the surface of the cells during storage time (Boonaert and Rouxhet 2000). Variance of hydrophobicity and zeta potential after starvation and during storage in this study suggests that electrostatic interactions may be responsible for difference in bacterial attachment ability.

Visualization of starved cells by electron microscopy. The outer membrane (OM) of gram-negative bacteria provides a protective physical barrier to various environments (Raetz and Whitfield 2002). The alteration of membrane lipid composition can occur under stress conditions. For example, Yuk and Marshall (2003) reported heat adaptation altered *E. coli* O157:H7 membrane lipid compositions and verotoxin secretion might increase through the increased membrane fluidity. Membrane changes can be shown by electron microscopy. To investigate the effects of starvation on the morphology of *E. coli* cells, we prepared samples for electron microscopy. Cells of *E. coli* O157:H7 and *E. coli* ATCC 25922 that were exposed to starvation conditions appeared elongated, wrinkled and malformed compared to cells that were not starved, though no difference among starvation conditions was noted (Fig. 5-5, 5-6). In addition, EM images supported curli production of *E. coli* O157:H7 grown at 37ºC and *E. coli* ATCC 25922 grown at 20ºC during starvation which was revealed on Congo-red medium (Fig.

5-5-A, C, D, Fig. 5-6-A) and moreover, these curli were shown to mediate cell-to-cell contacts (Fig. 5-6-A arrow).

In TEM images, non-starved *E. coli* O157:H7 (Fig. 5-6-B) cells had intact membrane structure and cytoplasm closely arranged with outer membrane, whereas bulges in the cell envelop, malformed membranes, more elongated, and empty cells were observed frequently during starvation conditions (Fig. 5-6-C, F). Some of the cytoplasmic membranes of both strains were ruptured, and the cells appeared to be discharging intracellular material (Fig. 5-6-C, E). Others shrunk and separated from the cell wall after starvation but still maintained shape and did not burst (Fig. 5-6-D). The cytoplasmic membrane appeared to bulge against the cell wall but no discharge of intracellular material was observed (Fig. 5-6-F).

These EM images were very similar when cells are exposed to pH stress (Mendoca et al. 1994). Yoon et al. (2005) reported the pO157 *ecf* (*E. coli* attaching and effacing gene-positive conserved fragments) operon and lipid A myristoyl transferase activity are associated with bacterial survival and persistence in bovine gastrointestinal tracts and nutrient-dilute farm water troughs by optimizing bacterial membrane structure and/or integrity. They supported their findings with EM images, which are in agreement with our results. Stretton et al. (1997) reported during multiple nutrient starvations, *Vibrio* sp. lost motility due to shedding of the flagellum from the cell and failure to synthesize a new one during the starvation stress response. *E. coli* also becomes non-motile under high temperature, high salt concentrations, or high concentrations of low molecular mass alcohols due to blocks in flagellin synthesis, possibly as a means of conserving energy (Li et al. 1993). Fine structure of the cell surface including flagella and curli can be better seen through negative staining using grids with a formvar and carbon supporting film or immunogold labeling compared to normal staining procedures for EM.

Although changes in membrane structure or integrity in starvation may be due to sample processing and preparation for SEM or TEM analysis and may not accurately represent the true native structure, these EM images demonstrate relative visual evidence compared with non-starved cells.

Outer membrane proteins profile. *Escherichia coli* possess a number of outer membrane proteins (Omps) some of which play an important role in the specific or nonspecific membrane transport and permeability of nutrients. The major Omps include the porins (OmpC and OmpF) and the OmpA protein in the approximately 35,000-dalton range (Nikaido and Vaara 1985). Darcan et al. (2003) investigated the links between survival of *E. coli* in sea water and the presence of porins in the outer membrane and determined double OmpC-OmpF mutant and the OmpR mutant survived poorly compared with single Omp mutants and the wild-type strain. Zhang and Ferenci (1999) reported OmpF changed when *E. coli* was exposed to prolonged lactose limitation and outer membrane permeability was affected. Other studies also revealed porin proteins have an important role in bacterial survival in the stress environments (Özkanca and Flint 2002; Sato et al. 2000). Sainz et al. (2005) investigated the relationships between acid resistance and changes in outer membrane protein (Omps) profiles of *E. coli* strains isolated from pozol, a fermented food. They found changes in Omp profiles in non-adapted acid challenged cells compared with non-challenged cells that had not been adapted to acid, whereas challenged adapted cells showed no significant changes in these profiles when compared with the acid adapted non-challenged strains. These changes resulted in significantly enhancing their survival in acid conditions. In addition, they found the intensity of the main porins OmpC and OmpA was lower in the acid challenged strains, than in the non-challenged ones. The Omp patterns of *E. coli* O157:H7 and *E. coli* ATCC 25922 during starvation conditions were analyzed

(Fig. 5-7). *E. coli* O157:H7 and *E. coli* ATCC 25922 grown under the different starvation condition expressed major outer membrane proteins of approx. 50, 47, 32, 26, 25, and 20 kDa, respectively, regardless of starvation conditions. No noticeable changes protein profiles were observed for both strains at starvations that had been grown at both 20 and 37ºC. Since both *E. coli* O157:H7 and *E. coli* ATCC 25922 survived well after exposing to starvation conditions and populations decreased by less than 1 log cfu/ml over 7 d storage at –18ºC, it was not surprisingly that there were few variations in the protein profiles regardless of starvation conditions. An Omp (approx. 37 kDa; arrowhead), that was thought to be OmpF, was one of the expressed proteins in *E. coli* ATCC 25922 during starvation, but in *E. coli* O157:H7 its expression was very weak or reduced. In addition, the intensity of approx. 47 kDa Omp (arrow) in *E. coli* O157:H7 at both growth temperatures during starvation was consistent, whereas in *E. coli* ATCC 25922 grown at 37ºC, 4ºC for 7 d-starved cells exhibited stronger band than in other starvation conditions.

Attachment and recovery tests. Adhesion of *E. coli* O157:H7 and *E. coli* ATCC 25922 grown at 20 and 37ºC to lettuce after exposing to starvation condition was determined (Fig 5-8, 5-9). The average attachment rate of *E .coli* O157:H7 was higher than that of *E. coli* ATCC 25922 grown at 20ºC regardless of starvation conditions, but no difference at 37ºC growth temperature. *E. coli* O157:H7 grown at 20ºC attached more than 37ºC-grown cells regardless of starvation conditions, whereas *E. coli* ATCC 25922 showed close rate in attachment among growth temperature as well as starvation conditions. For example, the attachment rate of starved (37ºC for 4 h) *E. coli* O157:H7 grown at 20 and 37ºC after 1 d storage was 7.05 and 3.88%, respectively. On the other hand, *E. coli* ATCC 25922 attached at a 3.02% rate when grown at 20ºC cells and 2.25% when grown at 37ºC cells under the same starvation

condition. The starved cells of *E. coli* ATCC 25922 attached less than non-starved cells regardless of starvation conditions, whereas no significant difference between non-starved and starved cells of *E. coli* O157:H7 was observed (Fig. 5-9).

Starvation affected cryotolerance and cell surface properties of *E. coli* O157:H7 and *E. coli* ATCC 25922 at somewhat different degrees. Storage time after starvation did not significantly change these surface properties, but only hydrophobicity was dramatically reduced over 7 d storage at –18ºC. The change in cell surface properties thereafter affected the attachment rate to lettuce. Despite of significant decrease of hydrophobicity during storage, attachment rates were similar. This might be because other cell surface properties including zeta potential, curli expression, membrane integrity (shown by EM analysis) and outer membrane as well as hydrophobicity may play a role together in attachment to produce. Overall *E. coli* ATCC 25922 is a useful surrogate for *E. coli* O157:H7 for studies involving attachment and recovery of chilled produce.

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REFERENCES

Abee, T. and J.A. Wouters. 1999. Microbial stress response in minimal processing. *Int. J. Food Microbiol.* 50:65-91.

Arneborg, N., A.S. Salskov-Iversen, and T.E. Mathiasen. 1993. The effect of growth rate and other growth conditions on the lipid composition of *Escherichia coli*. *Appl. Microbiol. Biotech*. 39:353-357.

Boonaert, C.J.P. and P. Rouxhet. 2000. Surface of lactic acid bacteria: relationships between chemical composition and physicochemical properties. *Appl. Environ. Microbiol*. 66:2548-2554.

Buncic, S. and S.M. Avery. 1998. Effects of cold storage and heat-acid shocks on growth and verotoxin 2 production of *Escherichia coli* O157:H7. *Food Microbiol.* 15:319-328.

Carty, S.M., K.R. Sreekumar, and C.R. Raetz. 1999. Effect of cold shock on lipid A biosynthesis in *Escherichia coli*: Induction at 12°C of an acyltransferase specific for palmitoleoyl-acyl carrier protein. *J. Biol. Chem*. 274:9677-9685.

Chen, J., S.M. Lee, and Y. Mao. 2004. Protective effect of exopolysaccharide colanic acid of *Escherichia coli* O157:H7 to osmotic and oxidative stress. *Int. J. Food Microbiol*. 93:281-286.

Cookson, A.L., W.A. Cooley, and M.J. Woodward. 2002. The role of type I and curli fimbriae of shiga toxin-producing *Escherichia coli* in adherence to abiotic surfaces. *Int. J. Med. Microbiol*. 292:195-205.

Dai, J.W., Y.S. Lee, and H.C. Wong. 1992. Effects of iron limitation of production of a siderophore, outer membrane proteins, and hemolysin and on hydrophobicity, cell adherence, and lethality for mice of *Vibrio parahaemolyticus. Infect. Immun*. 60:2952-2956.

Darcan, C., R. Özkanca, and K.P. Flint. 2003. Survival of nonspecific porin-deficient mutants of *Escherichia coli* in black sea water. *Lett. Appl. Microbiol*. 37:380-385.

Duffy, S., J. Churey, R.W. Worobo, and D.W. Schaffner. 2000. Analysis and modeling of the variability associated with UV inactivation of *Escherichia coli* in apple cider. *J. Food Prot*. 63:1587-1590.

Elhanafi, D., B. Leenanon, W. Bang, and M.A. Drake. 2004. Impact of cold and cold-acid stress on poststress tolerance and virulence factor expression of *Escherichia coli* O157:H7. *J. Food Prot.* 67:19-26.

Food and Drug Administration. 2000. Kinetics of microbial inactivation for alternative food processing technologies. Available at: http://vm.cfsan.fda.gov/~comm/ift-glos.html. Accessed 12 December 2006.

Gawande, P.V. and M.W. Griffiths. 2005a. Effects of environmental stresses on the activities of the *uspA, grpE* and *rpoS* promoters of *Escherichia coli* O157:H7. *Int. J. Food Microbiol.* 99:91-98.

Gawande, P.V. and M.W. Griffiths. 2005b. Growth history influences starvation-induced expression of *uspA*, *grpE*, and *rpoS* and subsequent cryotolerance in *Escherichia coli* O157:H7. *J. Food Prot.* 68:1154-1158.

Gophna, U., M. Barlev, R. Seijffers, T.A. Oelschlager, J. Hacker, and E.Z. Ron. 2001. Curli fibers mediate internalization of *Escherichia coli* by eukaryotic cells. *Infect. Immun*. 69:2659-2665.

Harakudo, Y., M. Miyaha, and A. Kumadai. 2000. Loss of O157 O antigenicity of verotoxin-producing *Escherichia coli* O157:H7 surviving under starvation conditions. *Appl. Environ. Microbiol.* 66:5540-5543.

Hassan, A.N. and J.F. Frank. 2004. Attachment of *Escherichia coli* O157:H7 grown in tryptic soy broth and nutrient broth to apple and lettuce surfaces as related to cell hydrophobicity, surface charge, and capsule production. *Int. J. Food Microbiol*. 96:103-109.

Kim, J. and M.A. Harrison. 2007. Surrogate selection for *Escherichia coli* O157:H7 based on cryotolerance and attachment to lettuce.

Kim, S.H. and Y.H. Kim. 2004. *Escherichia coli* O157:H7 adherence to HEp-2 cells is implicated with curli expression and outer membrane integrity. *J. Vet. Sci*. 5:119-124.

Leenanon, B. and M.A. Drake. 2001. Acid stress, starvation, and cold stress affect post stress behavior of *Escherichia coli* O157:H7 and nonpathogenic *E. coli. J. Food Prot.* 64:970-974.

LeJeune, J.T., T.E. Besser, and D. Hancock. 2001. Cattle water troughs as reservoirs of *Escherichia coli* O157. *App. Environ. Microbiol*. 67:3053-3057.

Leyh, R. and R.W. Griffith. 1992. Characterization of the outer membrane proteins of *Bordetella avium*. *Infect Immun*. 60:958-964.

Li, C.,C.J. Louise, W. Shi, and J. Adler. 1993. Adverse conditions which cause lack of flagella in *Escherichia coli. J. Bacteriol*. 175:2229-2235.

Li, J. and L.A. McLandsborough. 1999. The effects of the surface charge and hydrophobicity of *Escherichia coli* on its adhesion to beef muscle. *Int. J. Food Microbiol.* 53:185-193.

Maule, A. 2000. Survival of verocytotoxigenic *Escherichia coli* O157 in soil, water and on surfaces. *Symp. Ser. Soc. Appl. Microbiol*. 29:71S-78S.

Mendonca, A.F., T.L. Amoroso, and S.J. Knabel. 1994. Destruction of Gram-negative food-borne pathogens by high pH involves disruption of the cytoplasmic membrane. *Appl. Environ. Microbiol*. 60:4009-4014.

Nikaido, H. and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. *Microbiol. Rev*. 49:1-32.

Olsén, A., H. Herwald, M. Wikstrom, K. Persson, E. Mattsson, and L. Bjorck. 2002. Identification of two protein-binding and functional regions of curli, a surface organelle and virulence determinant *of Escherichia coli*. *J. Bio. Chem*. 277:34568-34572.

Özkanca, R. and K.P. Flint. 2002. The effect of starvation stress on the porin protein expression of *Escherichia coli* in lake water. *Lett. Appl. Microbiol*. 35:533-537.

Pao, S. and G. Davies. 2001. Comparing attachment, heat tolerance and alkali resistance of pathogenic and non-pathogenic bacterial cultures on orange surfaces. *J. Rapid Methods and Automation in Microbiol.* 9:271-278.

Pawar, D.M., M.L. Rossman, and J. Chen. 2005. Role of curli fimbriae in mediating the cells of enterohaemorrhagic *Escherichia coli* to attach to abiotic surfaces. *J. Appl. Microbiol*. 99:418-425.

Peri, A., S. Keller, S. Ravishankar, C. Rodriguez, and P.J. Slade. 2002. Bacterial pathogen surrogate evaluation - tolerance of candidates to acid and alkaline pH and reduced water activity. Session 61C-10. IFT Annual Meeting. Anaheim, CA.

Prigent-Combaret, C., G. Prensier, T.T. Le Thi, O. Vidal, P. Lejeun, and C. Dorel. 2000. Developmental pathway for biofilm formation in curli-producing *Escherichia coli* strains, role of flagella, curli and colanic acid. *Environ. Microbiol*. 2:450-464.

Raetz, C.R. and C. Whitfield. 2002. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem*. 71:635-700.

Rice, E.W. and C.H. Johnson. 2000. Short communication: survival of *Escherichia coli* O157:H7 in dairy cattle drinking water. *J. Dairy Sci*. 83:2021-2023.

Rockabrand, D., T. Arthur, G. Korinek, K. Livers, and P. Blum. 1995. An essential role for the *Escherichia coli* DnaK protein in starvation induced thermotolerance, H_2O_2 resistance, and reductive division. *J. Bacteriol.* 177:3695-3703.

Rowe, M.T. and R.B. Kirk. 2000. Effect of nutrient starvation on the resistance of *Escherichia coli* O157:H7 to subsequent heat stress. *J. Food Prot.* 63:1745-1748.

Sainz, T., J. Pérez, J. Villaseca, U. Hernández, C. Eslava, G.Mendoza, and C. Wacher. 2005. Survival to different acid challenges and outer membrane protein profiles of pathogenic *Escherichia coli* strains isolated from pozol, a Mexican typical maize fermented food. *Int. J. Food Microbiol.* 105:357-367.

Sato, M., K. Machida, E. Arikado, H. Saito, T. Kagekawa, and H. Kobayashi. 2000. Expression of outer membrane proteins in *Escherichia coli* growing at acid pH. *Appl. Environ. Microbiol.* 66:943-947.

Sledjeski, D.D. and S. Gottesman. 1996. Osmotic shock induction of capsule synthesis in *Escherichia coli* K-12. *J. Bacteriol*. 178:1204-1206.

Stretton, S., S.J. Danon, S. Kjelleberg, and A.E. Goodman. 1997. Changes in cell morphology and motility in the marine *Vibrio* sp.strain S14 during conditions of starvation and recovery. FEMS *Microbiol. Lett*. 146:23-29.

Thayer, D.W. and G. Boyd. 1993. Elimination of *Escherichia coli* O157:H7 in meats by gamma irradiation. *Appl. Environ. Microbiol.* 59:1030-1034.

Uhlich, G.A., J.E. Keen, and R.O. Elder. 2001. Mutations in the *csgD* promoter associated with variations in curli expression in certain strains of *Escherichia coli* O157:H7. *Appl. Environ. Microbiol*. 67:2367-2370.

Uhlich, G.A., J.E. Keen, and R.O. Elder. 2002. Variations in the *csgD* promoter of *Escherichia coli* O157:H7 associated with increased virulence in mice and increased invasion of HEp-2 cells. *Infect. Immun*. 70:395-399.

Wang, G. and M.P. Doyle. 1998. Survival of enterohemorrhargic *Escherichia coli* O157:H7 in water. *J. Food Prot*. 61:662-667.

Warburton, D.W., J.W. Austin, B.H. Harrison, and G. Sanders. 1998. Survival and recovery of *Escherichia coli* O157:H7 in inoculated bottled water. *J. Food Prot*. 61:948-952.

Weinstein, D.L., R.K. Holmes, and A.D. O'Brien. 1988. Effect of iron and temperature on shiga-like toxin 1 production by *Escherichia coli*. *Infect. Immun*. 56:106-111.

Whitfield, C. and I.S. Roberts. 1999. Structure, assembly and regulation of expression of capsules in *Escherichia coli*. *Mol. Microbiol*. 31:1307-1319.

Williams, N.C. and S.C. Ingham. 1998. Thermotolerance of *E. coli* O157:H7 ATCC 43895 following exposure to 1.5% acetic acid. *J. Food Prot*. 61:1184-1186.

Yoon, J.W., J.Y. Lim, Y.H. Park, and C.J. Hovde. 2005. Involvement of the *Escherichia coli* O157:H7(pO157) *ecf* operon and lipid A myristoyl transferase activity in bacterial survival in the bovine gastrointestinal tract and bacterial persistence in farm water troughs. *Infect. Immun*. 73:2367-2378.

Yuk, H.G. and D.L. Marshall. 2003. Heat adaptation alters *Escherichia coli* O157:H7 membrane lipid composition and verotoxin production. *Appl. Environ. Microbiol. 6*9:5115-5119.

Zhang, E. and T. Ferenci. 1999. OmpF changes and complexity of *Escherichia coli* adaptation to prolonged lactose limitation. FEMS *Microbiol. Lett*. 176:395-401.

Zhang, Y. and M.W. Griffiths. 2003. Induced expression of the heat shock protein genes, *uspA* and *grpE,* during starvation at low temperature and their influence on thermal resistance of *Escherichia coli* O157:H7. *J. Food Prot.* 66:2045-2050.

Zogaj, X., W. Bokranz, M. Nimtz, and U. Römling. 2003. Production of cellulose and curli fimbriae by members of the Family *Enterobacteriaceae* isolated from the human gastrointestinal tract. *Infect. Immun*. 71:4151-4158.

Fig. 5-1. Survival rate of *E. coli* O157:H7 (top) and a surrogate *E. coli* ATCC 25922 (bottom) grown at 20- or 37°C-starvation conditions during storage at –18°C for 7 days. Different letters within starvation condition indicate significantly differences ($p<0.05$) between means at each storage time.

Fig. 5-2. Starvation effect of *E. coli* O157:H7 and a surrogate *E. coli* ATCC 25922 grown at 37°C-starvation conditions on cryotolerance during storage at –18°C for 7 days. Different letters within starvation condition indicate significantly differences ($p<0.05$) between means at each storage time.

Fig. 5-3. Hydrophobicity changes of *E. coli* O157:H7 (top) and *E. coli* ATCC 25922 (bottom) grown at 20- or 37ºC-starvation conditions during storage at –18ºC for 7 days

Fig. 5-4. Zeta potential changes of *E. coli* O157:H7 (top) and *E. coli* ATCC 25922 (bottom) grown at 20- or 37ºC-starvation conditions during storage at –18ºC for 7 days

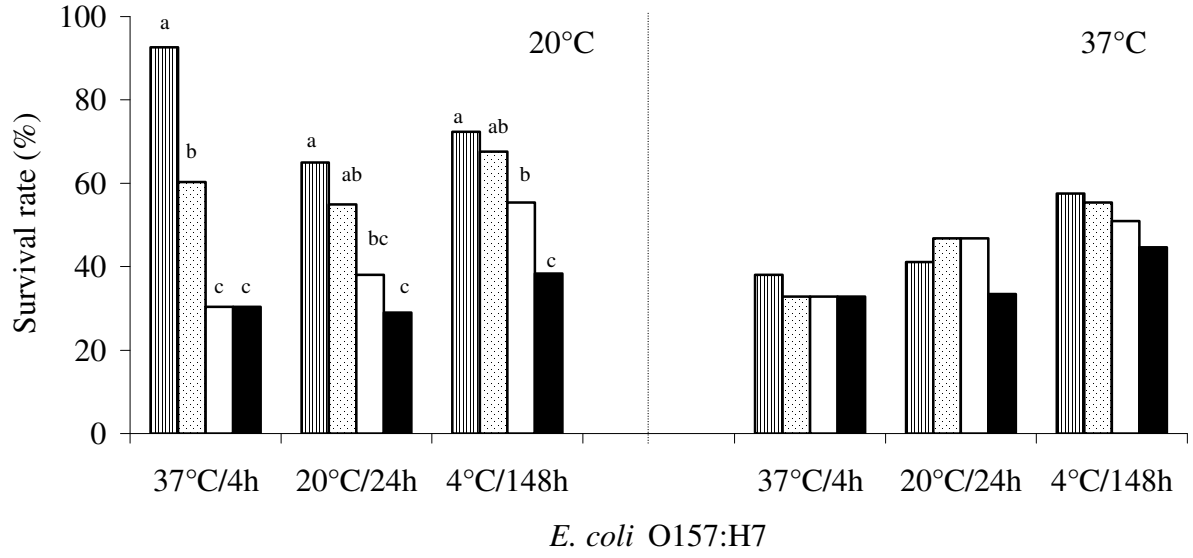
Fig. 5-5. SEM images of starved *E. coli* O157:H7 and *E. coli* ATCC 25922. A: 4°C/7d-starved *E. coli* O157:H7 grown at 37°C, B: 4°C/7d-starved *E. coli* ATCC 25922 grown at 37°C, C: 37°C/4h-starved *E. coli* O157:H7 grown at 20°C, D: 37°C/4h-starved *E. coli* ATCC 25922 grown at 20°C

Fig. 5-6. TEM images of starved and non-starved *E. coli* O157:H7 and *E. coli* ATCC 25922 (A: 20°C/24 h-starved *E. coli* ATCC 25922 B: non-starved *E. coli* O157:H7 C-F: 20°C /24 h-starved *E.coli* O157:H7) Cur: Curli. Bar 500 nm (A, B, D, E, F), 1 µm (C) Arrow shows curli mediates cell-to-cell contacts.

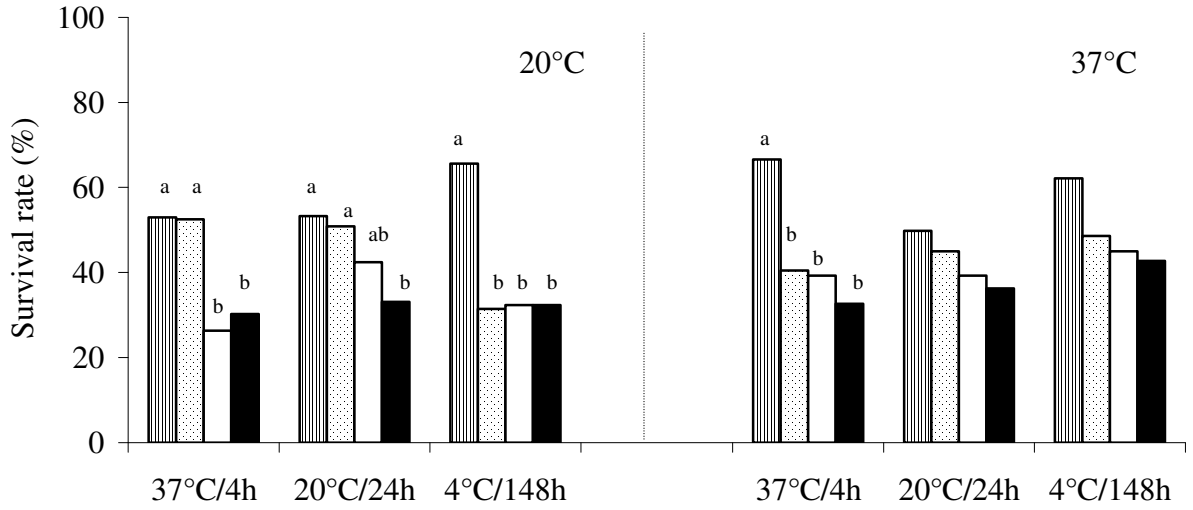
Fig. 5-7. Outer membrane proteins pattern of *E. coli* O157:H7 and *E. coli* ATCC 25922 grown at 20- (top) or 37ºC-starvation conditions (bottom). * represents major outer membrane proteins of approx. 50, 48, 32, 26, 25 and 20 kDa. M: Molecular weight markers a: control b: 37°C/4h c: 20°C/24h d: 4°C/148h

Fig. 5-8. Comparison of attachment rate of *E. coli* O157:H7 (top) and *E. coli* ATCC 25922 (bottom) grown at 20- or 37ºC-starvation conditions during storage at –18ºC for 7 days. Different letters within starvation condition indicate significantly differences ($p<0.05$) between means at each storage time.

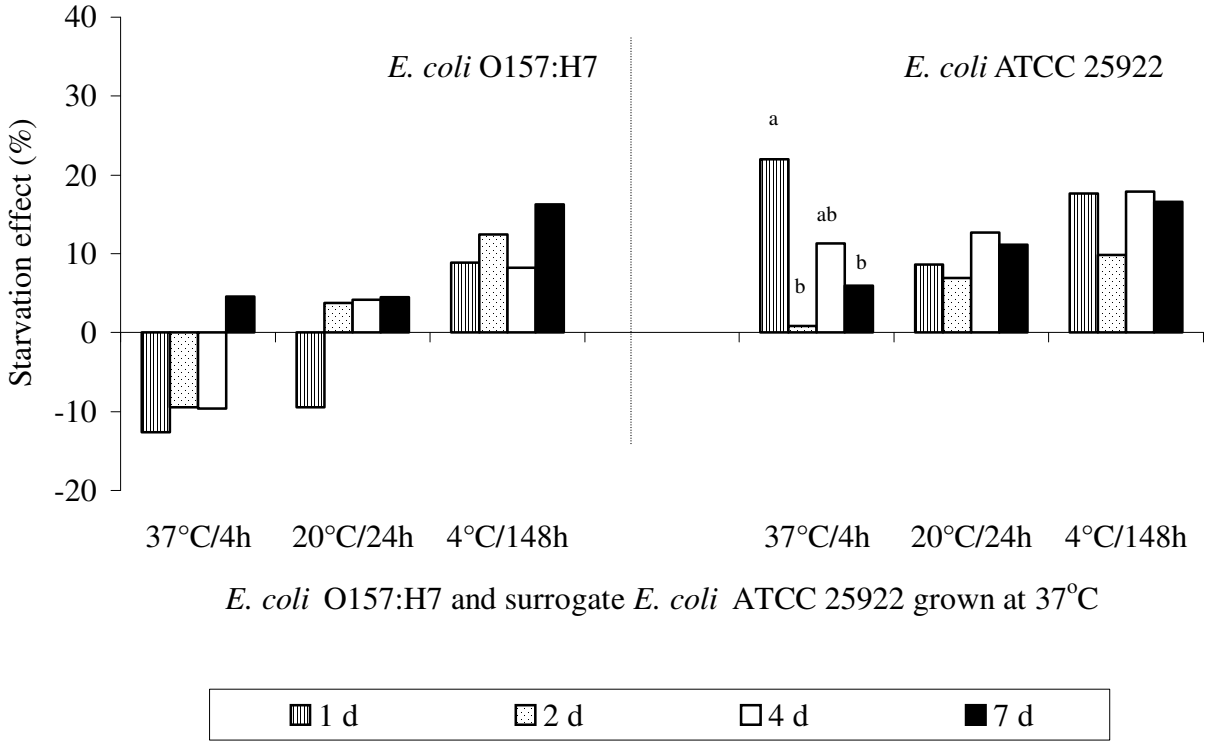
Fig. 5-9. Starvation effect for attachment rate of *E. coli* O157:H7 and *E. coli* ATCC 25922 grown at 37ºC-starvation conditions. Different letters within growth temperature indicate significantly differences ($p<0.05$) between means at each starvation condition



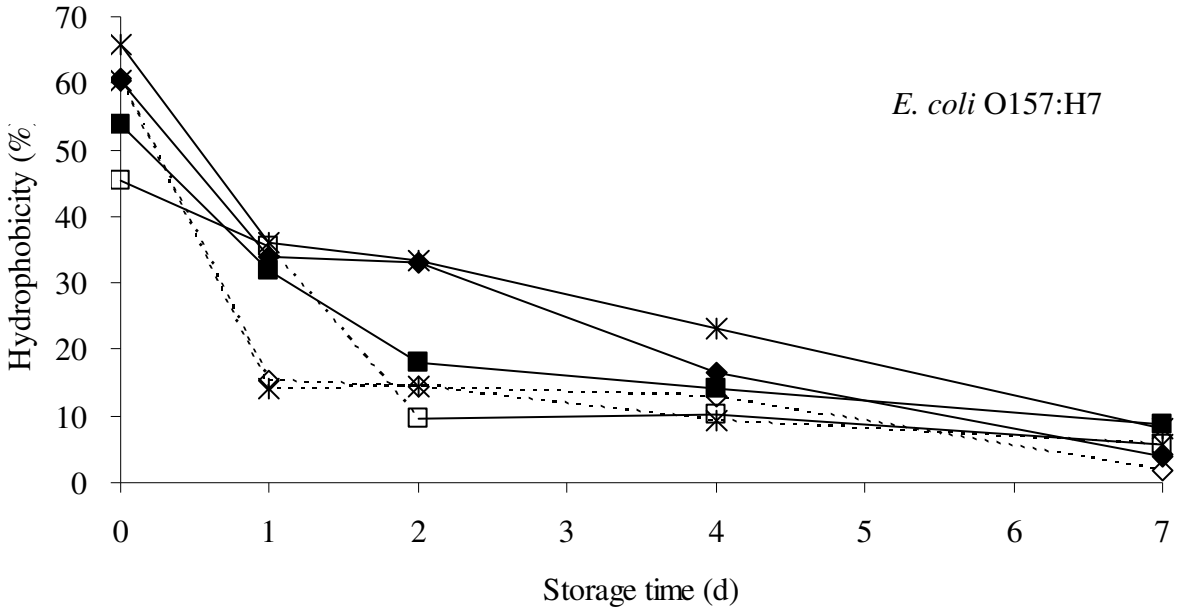
E. coli O157:H7

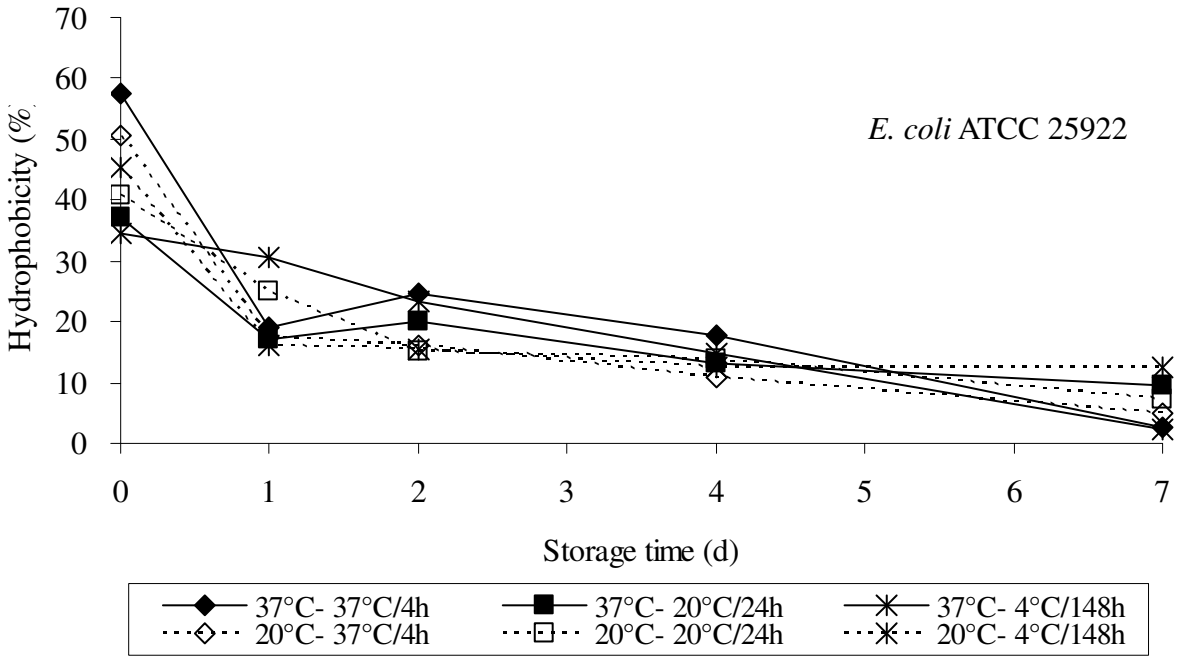


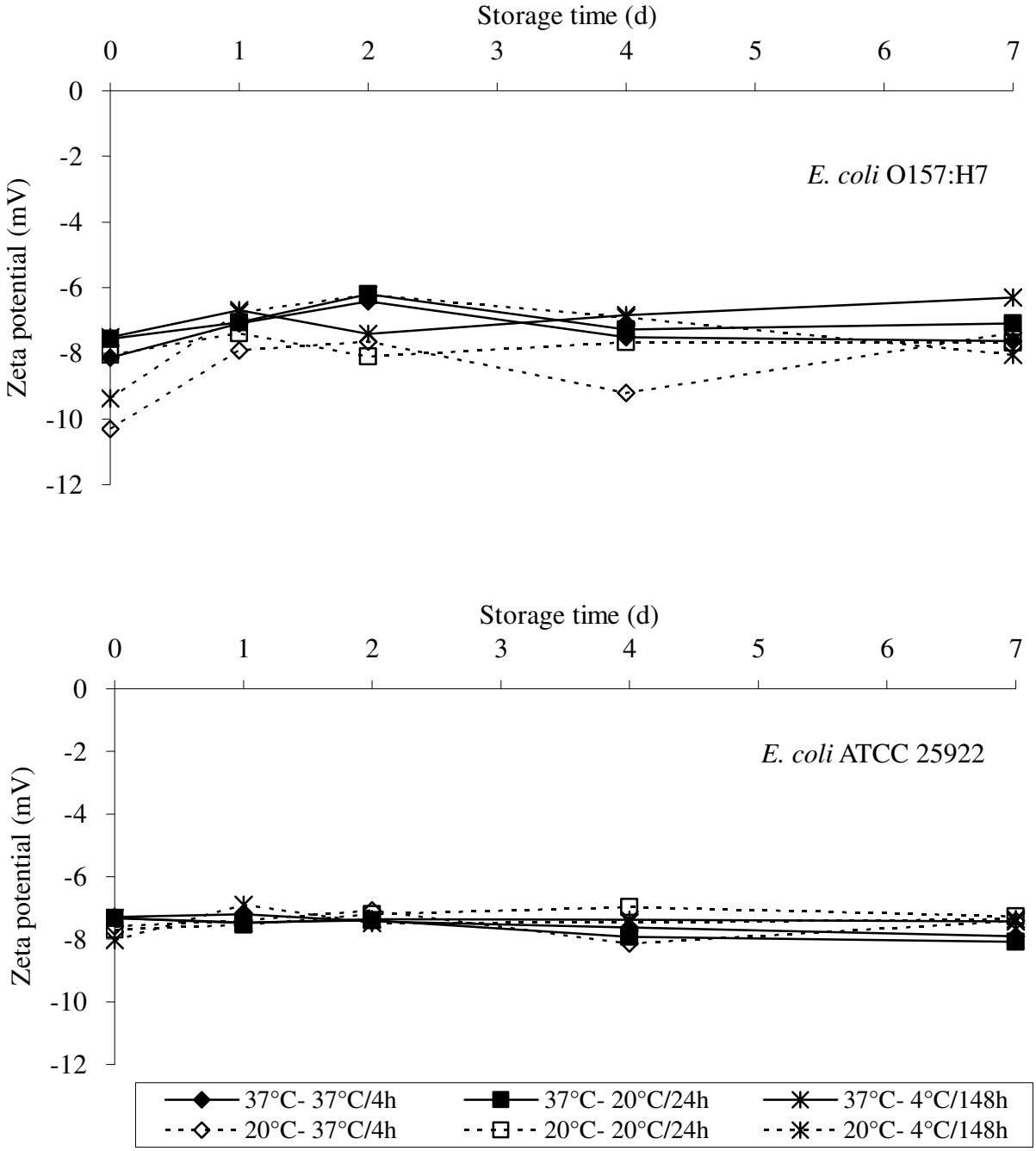
surrogate *E. coli* ATCC 25922

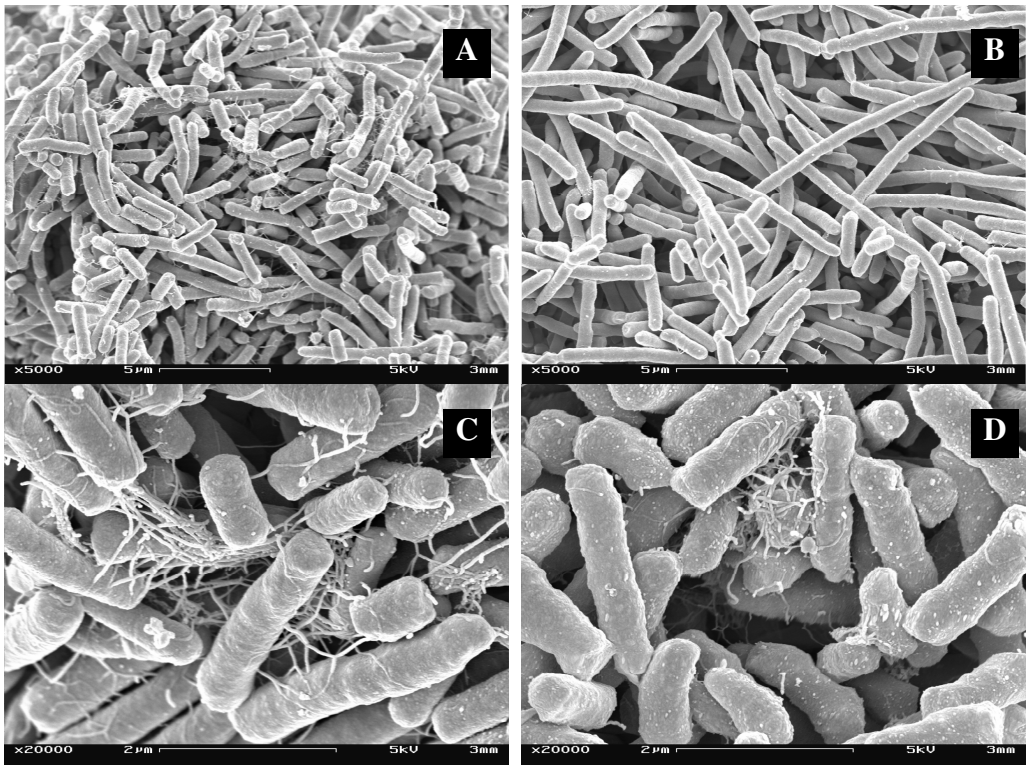


E. coli O157:H7 and surrogate *E. coli* ATCC 25922 grown at 37^{o}C


E. coli O157:H7
Hydrophobicity (%)
Storage time (d)
0
10
20
30
40
50
60
70
0
1
2
3
4
5
6
7


E. coli ATCC 25922
Hydrophobicity (%)
Storage time (d)
0
10
20
30
40
50
60
70
0
1
2
3
4
5
6
7
37°C- 37°C/4h
37°C- 20°C/24h
37°C- 4°C/148h
20°C- 37°C/4h
20°C- 20°C/24h
20°C- 4°C/148h


Storage time (d)
0
1
2
3
4
5
6
7
0
-2
-4
-6
-8
-10
-12
Zeta potential (mV)
E. coli O157:H7
Storage time (d)
E. coli ATCC 25922
37°C- 37°C/4h
37°C- 20°C/24h
37°C- 4°C/148h
20°C- 37°C/4h
20°C- 20°C/24h
20°C- 4°C/148h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B
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C
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D
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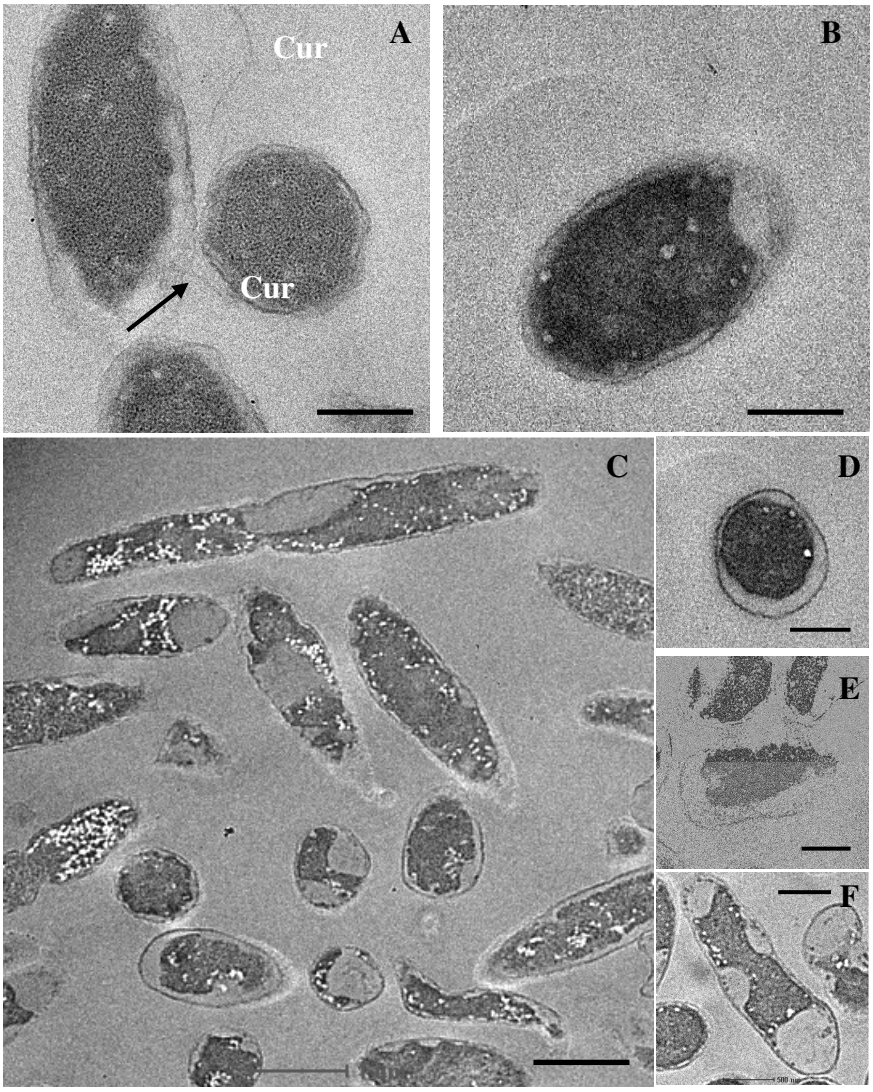
A
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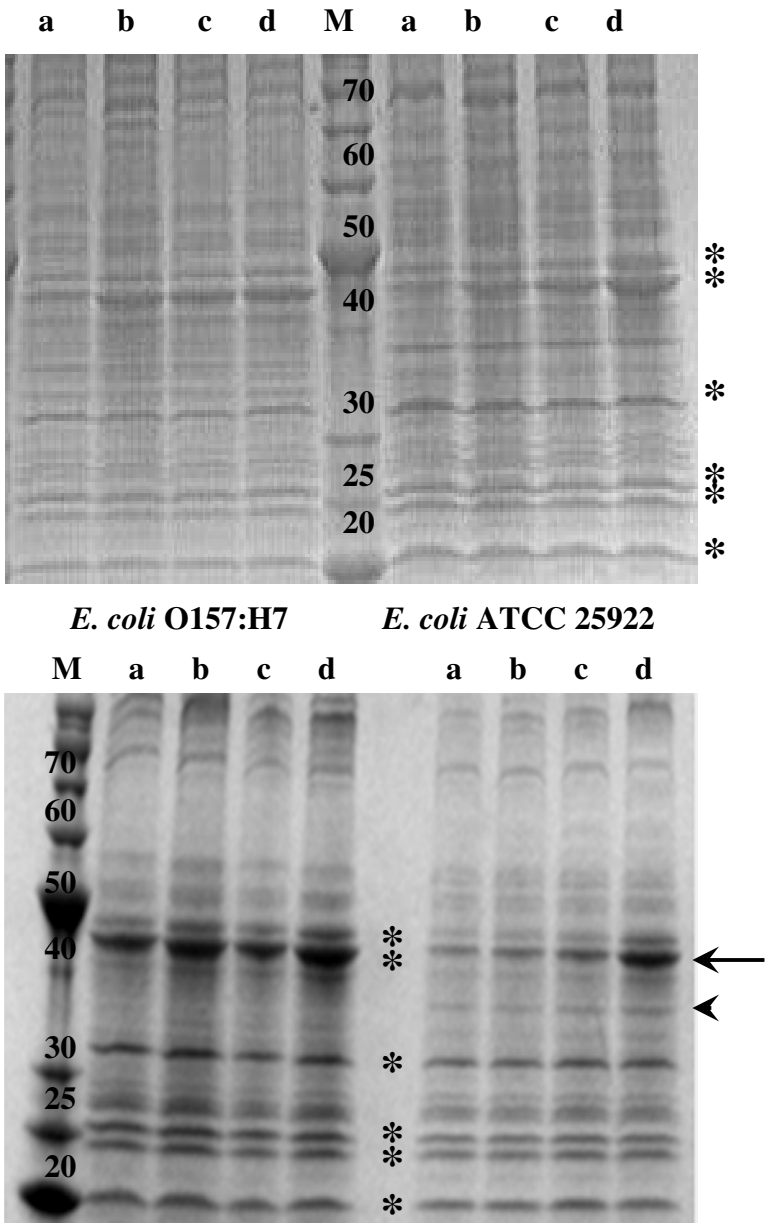
a b c d M a b c d
70
60
50
40
30
25
20
E. coli O157:H7
E. coli ATCC 25922
M a b c d a b c d
70
60
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40
30
25
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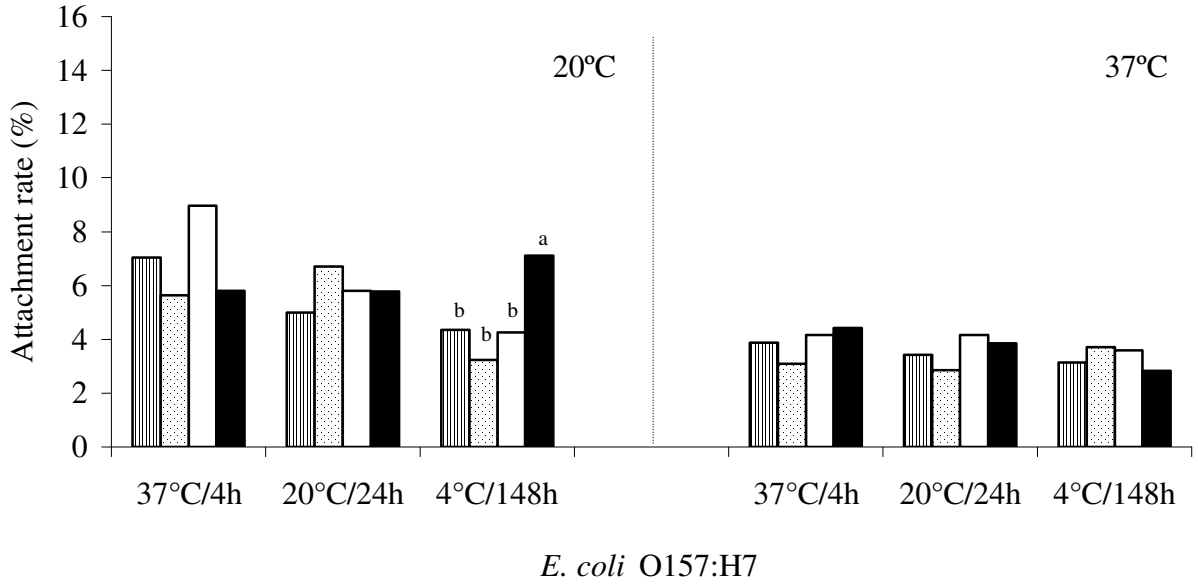
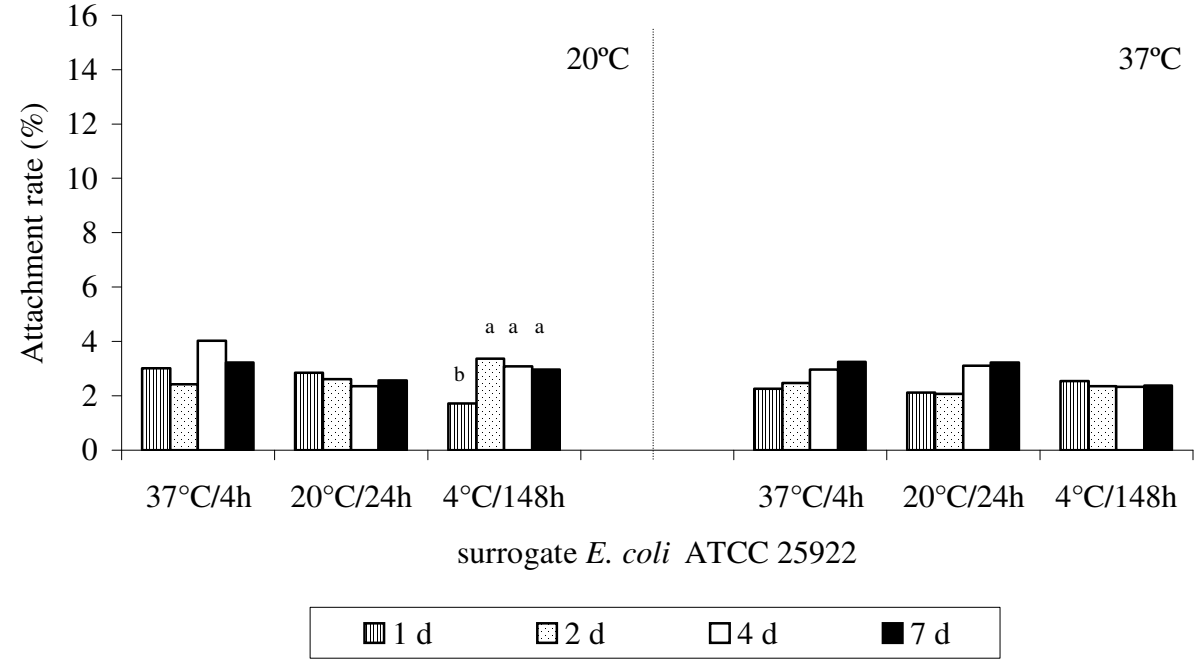
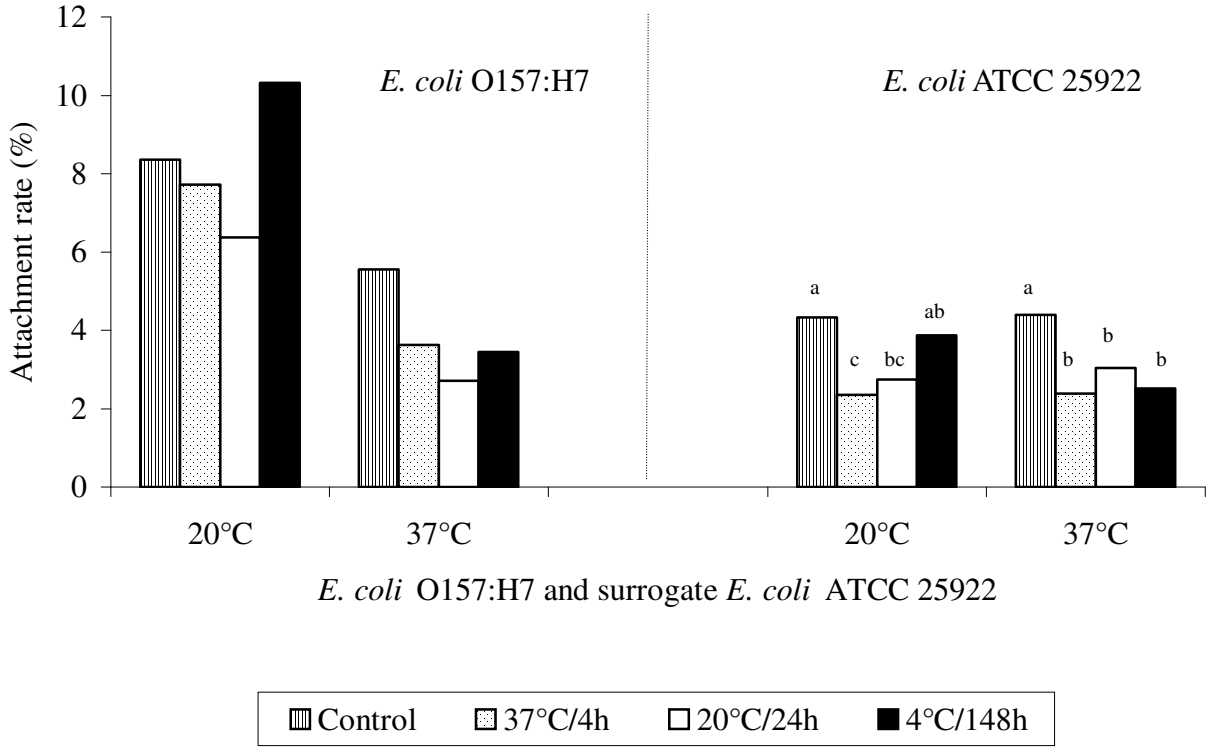
E. coli O157:H7
E. coli ATCC 25922



E. coli O157:H7



surrogate *E. coli* ATCC 25922


12
10
8
6
4
2
0
Attachment rate (%)
E. coli O157:H7
E. coli ATCC 25922
a
c
bc
ab
a
b
b
b
20°C
37°C
20°C
37°C
E. coli O157:H7 and surrogate E. coli ATCC 25922
Control
37°C/4h
20°C/24h
4°C/148h

CHAPTER 6

ATTACHMENT AND RECOVERY OF *ESCHERICHIA COLI* O157:H7 AND A NON-PATHOGENIC SURROGATE FROM ROMAINE LETTUCE AFTER CONTACT WITH CONTAMINATED ICE[1]

[1] Jin Kyung Kim and Mark A. Harrison. To be submitted to *Journal of Food Protection*, 2007.

Romaine lettuce can be top-iced by placement of crushed ice on top of the container to prevent moisture loss from the surface in a refrigeration unit during transportation. Since *Escherichia coli* O157:H7 can survive in water for extended periods, contamination of ice with this microorganism could be a hazard. The objective of this study was to investigate whether *E. coli* ATCC 25922 could serve as a possible surrogate for *E. coli* O157:H7 involving attachment to romaine lettuce after contact with contaminated ice under simulated commercial operation conditions. Recovery from lettuce surfaces for *E. coli* O157:H7 and *E. coli* ATCC 25922 were also compared. Water containing each strain was frozen and used to ice lettuce in shipping containers at refrigeration temperature (4ºC). After the ice melted, samples were taken and attachment and recovery of *E. coli* O157:H7 after rinsing with chlorinated water (200 µg free chlorine/ml) were determined. The population of *E. coli* O157:H7 and *E. coli* ATCC 25922 attached to the top leaf was 4.65 and 4.48 log cfu/cm^2, respectively. There was no significant difference in population. After rinsing lettuce with chlorinated water, the number of *E. coli* ATCC 25922 was reduced by 1.42 log cfu/cm^2, which was similar to *E. coli* O157:H7 (1.79 log reduction). *E. coli* ATCC 25922 showed the same attachment and recovery as *E. coli* O157:H7 from romaine lettuce under icing conditions using simulated commercial operation condition.

INTRODUCTION

Using non-pathogenic surrogates in place of pathogens when evaluating commercial food processing operations offers safety advantages (FDA 2000), but it has inherent limitations if they do not behave in a same manner to that of their pathogenic counterparts in challenged situation. Potential surrogates must exhibit characteristics similar to the target pathogens (Busta et al. 2003). Among the strains cited in literature *E .coli* ATCC 25922 is one of the most currently used. Many studies of *E. coli* ATCC 25922 under different environments such as alkaline pH, high temperature, gamma radiation, UV, and acid stress have been accomplished (Duffy et al. 2000; Leenanon and Drake 2001; Pao and Davies 2001; Sapers and Sites 2003; Thayer and Boyd 1993). The results of these studies suggested that *E. coli* ATCC 25922 behaved differently compared to pathogen of concern under specific process conditions; a surrogate may not fit all challenged situations to the same degree.

The ability of microorganisms to attach to fresh produce is of great concern since the products are to be sold as raw or minimally processed. Beuchat et al. (2001) suggested that standard methods are needed to develop to accurately determine the presence and numbers of pathogenic microorganisms on raw produce and to validate the intervention methods in produce processing operations. They suggested that modifications in these standard protocols will be necessary because raw fruits and vegetables have different surface morphologies and hydrophobicity, and internal tissue compositions and these products may be subjected to different processing conditions. Ideally commercial processing conditions could provide more challenging conditions under which to evaluate attachment and recovery of pathogens on fresh produce. However, due to safety concerns using actual pathogens in commercial settings is not possible.

Recently, several researchers have evaluated the effectiveness of a wide range of chemical disinfectants in killing pathogens on raw fruits and vegetables under simulated processing conditions (Beuchat et al. 2004; Gonzalez et al. 2004). To date, there are no documented studies on the attachment and recovery of *E. coli* O157:H7 on surfaces of romaine lettuce under icing conditions using simulated packing and distribution of romaine lettuce. In our previous study (Kim and Harrison 2007a), we evaluated different icing temperatures and contamination sources of *E. coli* O157:H7 in shipping containers to mimic packing lettuce in the field and distribution of the product. *E. coli* O157:H7 was transferred to previously uncontaminated lettuce in shipping containers due to contact with contaminated water from melted ice. In addition, we revealed *E. coli* ATCC 25922 was a suitable surrogate for *E. coli* O157:H7 on attachment and recovery studies involving chilling produce based on cryotolerance and cell surface properties such as surface hydrophobicity, cell surface charge, capsule and curli production (Kim and Harrison 2007b). The objective of this study was to investigate *E. coli* ATCC 25922 to serve as a surrogate for *E. coli* O157:H7 involving attachment and recovery from romaine lettuce after contact with contaminated ice under simulated commercial operation conditions. Water containing either *E. coli* O157:H7 or the possible surrogate was frozen and used to ice lettuce in shipping containers at refrigeration temperature (4ºC), which represents the condition of lettuce transportation.

MATERIALS AND METHODS

Bacterial strains and inoculum preparation. *E. coli* O157:H7 932 (human isolate), *E. coli* ATCC 25922 were used. They were determined to be sensitive to a level of 50 μg/ml nalidixic acid (Sigma Chemical Co., St. Louis, MO). They were made nalidixic acid-adapted

through the transfers in tryptic soy broth (TSB, Becton, Dickinson and Co., Sparks, MD) supplemented with increasing concentrations of nalidixic acid (TSBN) from 2.5 to 50 µg/ml for 7 days at 37ºC. Nalidixic acid-adapted cells were surface plated on tryptic soy agar supplemented with 50 µg of nalidixic acid/ml (TSAN) and incubated at 37ºC for 24 h. Stock cultures were stored at –80ºC in culture broth containing 15% glycerol until used. Frozen stock cultures maintained at –80ºC were activated by two successive transfers in 10 ml of TSBN and incubated at 37ºC for 24 h. These active cultures were transferred to bulk TSBN and incubated for 24 h at 37°C. Cultures were centrifuged at 4,550 x g for 30 min (Model 5681, Forma Scientific Inc., Milford, MA) at 20ºC and the pellets were resuspended into sterile deionized water (SDW) to yield ca. 10^8 cfu/ml.

Lettuce. Romaine lettuce (*Lactuca sativa* var. *longifolia*) was purchased at a local grocery store (Athens, GA) on the shipping day from the warehouse to keep consistent freshness and all experiments were started at the purchasing day. At the start of the experiment, wilted and blemished outer leaves were removed. Tap water and uninoculated lettuce were tested to confirm the absence of nalidixic acid-adapted *E. coli* O157:H7 and *E. coli* using TSAN after incubation at 37ºC for 24 h in enrichment broth.

Container preparation. To simulate packing, we used plastic containers (20x30x30cm) with ventilation holes. Sterile wire grid racks were used on the bottom of each container to prevent lettuce from touching melted ice that accumulated in the container. Sterile wire crown was used on the top of lettuce to hold ice and keep ice from dropping to the bottom of container without touching lettuce.

Ice preparation. Before making ice, tap water was stored at 4°C overnight. Aquachek total/free chlorine test kits (Hach Company, Loveland, CO) was used to measure the

concentration of free chlorine in tap water. Free chlorine was less than 0.1µg/ml. One hundred milliliter of the inoculum preparation was added to one liter of tap water which was stored overnight at 4°C in advance, to give an inoculum concentration of approximately 10^7cfu/ml and used to make ice. Ice was placed into Ziploc bags (approx. 150g; S.C. Johnson & Son, Inc., Racine, WI) and stored at –20ºC until used. Experiments were performed within 3 d after making ice.

Sampling procedures and bacterial enumeration. Lettuce stored at 4ºC in the shipping container was chilled with ice made of water contaminated with each strain. After ice melted, sampling sites on the top leaf were removed using a sterile template (4x4cm) and scalpel. The excised pieces were rinsed gently with SDW (10 ml) to remove unattached cells. After rinsing, a 3x3cm template was used to remove edges where cells might attach during the rinsing procedure and lettuce pieces were placed into stomacher bags. Three pieces were analyzed for attachment and 3 for recovery after rinsing with chlorinated water (200 µg free chlorine/ml). The chlorine solutions were prepared each day from a sodium hypochlorite stock solution. The solutions were prepared in SDW stored at 4ºC overnight in advance with 0.1 N cirtric acid to adjust to pH 7.0 ± 0.2 and the concentration of free chlorine was verified by Aquachek total/free chlorine test kits (Hach Company). For the recovery test, 100 ml of chlorinated water (200 µg free chlorine/ml) was added to the stomacher bags, and the contents were vigorously shaken by hand for 1 min and immediately neutralized with 30 ml of 0.1 N sodium thiosulfate. Lettuce samples were homogenized with 100 ml of modified EC broth (Becton, Dickinson and Co.) for 1 min in a stomacher at normal speed. A spiral plater (Autoplate 4000, Spiral Biotech, Bethesda, MD) on TSAN was used for enumeration of each strain. TSAN plates were incubated at 37°C for 24 h.

Statistical analysis. All experiments were conducted with five replicates and each replicate consisted of 3 samples. Bacterial counts for attachment and recovery test were converted to log values and then entered into an analysis of variance (ANOVA) using SAS (SAS Institute, Inc., Ver. 9.1, Cary, NC). When the ANOVA indicated a significant difference at α=0.05 between strains, mean separation was achieved using the Duncan's multiple range test. Differences between mean values were considered significant at α=0.05.

RESULTS AND DISCUSSION

Surrogates are preferred instead of pathogenic microorganisms to evaluate the efficacy of intervention strategies in food processing operations without the potential risk posed by pathogens to both the production environment and researchers (FDA 2000). Many studies evaluating *E. coli* ATCC 25922 under different environments have been accomplished (Duffy et al. 2000; Mak et al. 2001; Pao and Davies 2001; Thayer and Boyd 1993). Previously there were no documented studies of surrogate selection on the attachment and recovery of *E. coli* O157:H7 on surfaces of romaine lettuce under icing conditions using simulated packing and distribution of romaine lettuce. Kim and Harrison (2007b) demonstrated *E. coli* ATCC 25922 was a suitable surrogate for *E. coli* O157:H7 on attachment and recovery studies involving chilling produce based on cryotolerance and cell surface properties. However, this study was done under laboratory conditions and not actual commercial conditions. A suitable surrogate would allow for possible evaluation under commercial conditions. Since *E. coli* ATCC 25922 grown at 37°C attached better to lettuce surfaces than at 20°C, 37°C was used for growth temperature in the present study. We revealed the population of *E. coli* O157:H7 attached to the top leaf was significantly higher than other areas and *E. coli* O157:H7 can be transferred from

contaminated to previously uncontaminated surfaces after contaminated water from melting ice contacted them (Kim and Harrison 2007a). In addition, Kim and Harrison (2007c) reported non-starved cells of both strains attached better to lettuce surfaces than starved cells. Therefore, the comparison of the population of each strain grown under non-starvation conditions, attached to the top leaf was conducted in the present study.

There was no detectable nalidixic acid-adapted *E. coli* O157:H7 and *E. coli* detected in the tap water and uninoculated lettuce used in the experiments. The population attached to the top leaf after contact with contaminated ice was 4.65 ± 0.30 and 4.48 ± 0.31 log cfu/cm^2 for *E. coli* O157:H7 and *E. coli* ATCC 25922, respectively. There was no significant difference in populations of attached cells between the 2 strain types. The population of *E. coli* O157:H7 attached to lettuce surfaces in the present study was higher than the populations in our previous study (Kim and Harrison 2007a). This result indicates there will be larger variation in the simulated commercial processing conditions than controlled laboratory environment using pieces of lettuce. After rinsing with chlorinated water, the population of *E. coli* ATCC 25922 was reduced by 1.42 log cfu/cm^2 lettuce, which was similar to *E. coli* O157:H7 (1.79 log reduction). This is in agreement with other studies showed rinsing with chlorinated water resulted in a microbial reduction of less than 2 log cfu/g on fruits and vegetables (Beuchat 1999; Brackett 1992; Cherry 1999).

In conclusion, selected surrogate *E. coli* ATCC 25922 showed the same attachment and recovery from romaine lettuce as *E. coli* O157:H7 under icing conditions using simulated commercial conditions and could prove to be a useful surrogate for *E. coli* O157:H7 for studies under similar conditions.

ACKNOWLEDGEMENT

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REFERENCES

Beuchat, L.R. 1999. Survival of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces applied to lettuce and the effectiveness of chlorinated water as a disinfectant. *J. Food Prot.* 62:845-849.

Beuchat, L.R., B.B. Adler, and M.M. Lang. 2004. Efficacy of chlorine and a peroxyacetic acid sanitizer in killing *Listeria monocytogenes* on iceberg and romaine lettuce using simulated commercial processing conditions. *J. Food Prot.* 67:1238-1242.

Beuchat L.R., J.N. Farber, E.H. Garrett, L.J. Harris, M.E. Parish, T.V. Suslow, and F.F. Busta. 2001. Standardization of a method to determine the efficacy of sanitizers in inactivating human pathogenic microorganisms on raw fruits and vegetables. *J. Food Prot*. 64:1079-1084.

Brackett, R.E. 1992. Shelf stability and safety of fresh produce as influenced by sanitation disinfection. *J. Food. Prot.* 55:808-814.

Busta, F.F., T.V. Suslow, M.E. Parish, L.R. Beuchat, J.N. Farber, E.H. Garrett, and L.J. Harris. 2003. Comprehensive reviews in food science and food safety - The use of indicators and surrogate microorganisms for the evaluation of pathogens in fresh and fresh-cut produce. Vol. 2:179-185.

Cherry, J.P. 1999. Improving the safety of fresh produce with antimicrobials. *Food Technol.* 53:54-59.

Duffy, S., J. Churey, R.W. Worobo, and D.W. Schaffner. 2000. Analysis and modeling of the variability associated with UV inactivation of *Escherichia coli* in apple cider. *J. Food Prot*. 63:1587-1590.

Food and Drug Administration. 2000. Kinetics of microbial inactivation for alternative food processing technologies. Available at: http://vm.cfsan.fda.gov/~comm/ift-glos.html. Accessed 12 December 2006.

Gonzalez, R.J., Y.G. Luo, S. Ruiz-Cruz, and J.L. Mcevoy. 2004. Efficacy of sanitizers to inactive *Escherichia coli* O157:H7 on fresh-cut carrot shreds under simulated process water conditions. *J. Food Prot.* 67:2375-2380.

Kim, J. and M.A. Harrison. 2007a. Transfer of *Escherichia coli* O157:H7 to romaine lettuce due to contact water from melting ice.

Kim, J. and M.A. Harrison. 2007b. Surrogate selection for *Escherichia coli* O157:H7 based on cryotolerance and attachment to lettuce.

Kim, J. and M.A. Harrison. 2007c. Attachment properties of *Escherichia coli* O157:H7 and a non-pathogenic surrogate subjected to starvation.

Leenanon, B. and M.A. Drake. 2001. Acid stress, starvation, and cold stress affect post stress behavior of *Escherichia coli* O157:H7 and nonpathogenic *E. coli. J. Food Prot.* 64:970-974.

Mak, P.P., B.H. Ingham, and S.C. Ingham. 2001. Validation of apple cider pasteurization treatments against *Escherichia coli* O157:H7, *Salmonella*, and *L. monocytogenes*. *J. Food Prot*. 64:1679-1689.

Pao, S. and G. Davies. 2001. Comparing attachment, heat tolerance and alkali resistance of pathogenic and non-pathogenic bacterial cultures on orange surfaces. *J. Rapid Methods and Automation in Microbiol.* 9:271-278.

Sapers, G.M. and J.E. Sites. 2003. Efficacy of 1% Hydrogen peroxide wash in decontaminating apples and cantaloupe melons. *J. Food Sci*. 68:1793-1797.

Thayer, D.W. and G. Boyd. 1993. Elimination of *Escherichia coli* O157:H7 in meats by gamma irradiation. *Appl. Environ. Microbiol.* 59:1030-1034.

CHAPER 7

SUMMARY AND CONCLUSIONS

The following is a summary of results and conclusions drawn from the research reported in Chapter 3-6 of this dissertation:

1. *E. coli* O157:H7 transfer to produce under icing conditions in simulated commercial operations was evaluated.

- The populations of *E. coli* O157:H7 attached to the top leaf contacted with ice made of contaminated water or inoculated with this microorganism was the highest, while undetectable, other sampling sites became contaminated with the pathogens due to ice melt.
- *E. coli* O157:H7 can be transferred onto other produce layers in shipping containers due to melted ice made of contaminated water.
- Water from melted ice can also transfer the pathogen from contaminated to uncontaminated leaf surfaces.

2. Non-pathogenic *E. coli* strains were compared with *E. coli* O157:H7 for attachment and recovery traits involving chilled produce based on survival at –18°C and cell surface properties.

- Based on cryotolerance, cell surface characteristics (hydrophobicity, zeta potential, and morphology) and attachment to lettuce, *E. coli* ATCC 25922 is a useful surrogate for *E. coli* O157:H7 for studies involving attachment and recovery from chilled produce.

3. The effect of starvation on attachment of *E. coli* O157:H7 and a selected surrogate *E .coli* to produce was investigated.

- There were few differences in hydrophobicity, zeta potential, and morphology and outer membrane proteins profile between *E. coli* O157:H7 and a surrogate *E. coli* ATCC 25922 among starvation conditions.
- Compared to *E. coli* O157:H7*, E. coli* ATCC 25922 grown at 37ºC showed similar or better cryotolerance regardless of starvation conditions.
- However, starved cells of both strains attached to lettuce less than non-starved cells.

4. The selected non-pathogenic surrogate *E. coli* was compared with *E. coli* O157:H7 to determine differences and similarities in attachment to and recovery from produce under icing condition.
   - The selected surrogate *E. coli* ATCC 25922 showed the same attachment and recovery as *E. coli* O157:H7 from romaine lettuce after contacting with contaminated water using a simulated commercial operation condition.
   - Overall *E. coli* ATCC 25922 can serve as a useful surrogate for *E. coli* O157:H7 for produce attachment and recovery studies.

Water used in harvest operations should be of appropriate microbial quality because water may contaminate romaine lettuce by means of contacting with edible portions of lettuce. *E. coli* ATCC 25922 in place of *E. coli* O157:H7 can be used in the field to evaluate intervention strategies with safety advantages to facilities and workers.